



Title	ENRICHMENT, ISOLATION AND CHARACTERIZATION OF CHEMOLITHOAUTOTROPHIC ARSENITE OXIDIZING BACTERIA FOR REMOVAL OF ARSENIC FROM WATER PHASE
Author(s)	Nguyen, Ai Le
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ENRICHMENT, ISOLATION AND CHARACTERIZATION  
OF CHEMOLITHOAUTOTROPHIC ARSENITE  
OXIDIZING BACTERIA FOR REMOVAL OF ARSENIC  
FROM WATER PHASE

(水相からのヒ素の除去を目指した化学合成独立栄養亜ヒ  
酸酸化細菌の集積、単離、及び特徴付け)

by

NGUYEN AI LE

A thesis submitted in partial fulfillment of the requirements for the  
degree of Philosophy of Doctor in Engineering

Division of Sustainable Energy and Environmental Engineering  
Graduate School of Engineering  
Osaka University

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## TABLE OF CONTENTS

List of Publications and Conferences related to this study.....	i
List of Figures.....	ii
List of Tables .....	iv
<b>Chapter 1: General introduction.....</b>	<b>1</b>
1.1. Arsenic in natural water .....	1
1.1.1. Sources of arsenic .....	1
1.1.2. Chemical properties of arsenic .....	2
1.1.3. Arsenic health effects .....	4
1.1.4. Worldwide groundwater arsenic problems .....	5
1.2. Remediation methods for arsenic contaminated water.....	7
1.3. Oxidation of As (III) to As (V) as pretreatment for enhanced arsenic removal .....	11
1.3.1. Chemical Oxidation of As (III) to As (V) .....	12
1.3.2. Microbial Oxidation of As (III) to As (V) .....	13
1.4. Objectives of the study .....	22
<b>Chapter 2: Enrichment and characterizing of chemolithoautotrophic arsenite oxidizing bacteria (CAOs) for arsenic removal from contaminated water .....</b>	<b>24</b>
2.1. Introduction .....	24
2.2. Materials and methods.....	25
2.2.1. Inoculum source .....	25
2.2.2. Culture media .....	26
2.2.3. Enrichment of CAOs .....	26
2.2.4. Effects of inoculating volume and organic carbon addition on As(III) oxidation...27	
2.2.5. Analytical methods .....	27
2.3. Results and discussion .....	28
2.3.1. Construction of the enrichment culture of CAOs.....	28
2.3.2. Effects of inoculating volume and organic carbon addition on As(III) oxidation...30	
2.3.3. Change in bacterial community during the enrichment process.....	31
2.4. Conclusions .....	32
<b>Chapter 3: Isolation and characterizing of chemolithoautotrophic arsenite</b>	

<b>oxidizing (CAOs) bacteria for arsenic removal from contaminated water .....</b>	<b>34</b>
3.1. Introduction .....	34
3.2. Materials and methods.....	35
3.2.1. Isolation of CAOs from an enrichment culture .....	35
3.2.2. Physiological and phylogenetical characterization of isolated CAOs.....	35
3.2.3. As(III) oxidation studies of isolated CAOs .....	35
3.2.4. Analysis of arsenite oxidase genes of the isolated CAOs.....	36
3.2.5. Analytical methods .....	36
3.3. Results and discussion.....	37
3.3.1. Isolation of CAOs from an enrichment culture .....	37
3.3.2. Physiological and phylogenetical characterization of isolated CAOs.....	37
3.3.3. T-RFLP analysis of isolated CAOs.....	38
3.3.4. As(III) oxidation ability with growing cells of isolated CAOs .....	39
3.3.5. As(III) oxidation ability with whole cells of isolated CAOs.....	40
3.3.6. Analysis of arsenite oxidase genes of the isolated CAOs.....	43
3.4. Conclusions .....	44

<b>Chapter 4: Demonstration of enhanced arsenic adsorption by activated alumina</b>	
<b>with bacterial oxidation of As(III) .....</b>	<b>45</b>
4.1. Introduction .....	45
4.2. Materials and methods.....	46
4.2.1. Bacterial strain.....	46
4.2.2. Arsenic stock solution.....	46
4.2.3. Basal salt medium (BSM) and model contaminated waters.....	46
4.2.4. As(III) oxidation studies with CAO strain B1 in model contaminated waters.....	47
4.2.5. Preparation of activated alumina (AA) adsorbent.....	48
4.2.6. Adsorption of arsenic by AA before and after microbial As(III) oxidation.....	48
4.2.7. Analytical methods .....	48
4.2.8. Adsorption isotherms.....	49
4.3. Results and Discussion .....	50
4.3.1. As(III) oxidation studies with CAO strain B1 in model contaminated waters.....	50
4.3.2. Adsorption isotherms of arsenic by AA in model contaminated waters before and after microbial oxidation.....	51
4.3.3. Arsenic removal efficiency by AA in model contaminated waters before and after microbial oxidation.....	53
4.4. Conclusions .....	55

<b>Chapter 5: Summary and Conclusions.....</b>	<b>56</b>
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<b>References.....</b>	<b>59</b>
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## LIST OF PUBLICATIONS AND CONFERENCES RELATED TO THIS STUDY

### Publications:

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2. Nguyen Ai Le, Akiko Sato, Daisuke Inoue, Kazunari Sei, Satoshi Soda, and Michihiko Ike (2012) Bacterial community succession during the enrichment of chemolithoautotrophic arsenite oxidizing bacteria at high arsenic concentrations. *Journal of Environmental Sciences*, vol. 24, no. 12, pp. 2133–2140.
3. Nguyen Ai Le, Daisuke Inoue, Kazunari Sei, Satoshi Soda, and Michihiko Ike (2012) Arsenic adsorption characteristics of biogenic iron oxides in comparison to chemogenic iron oxides. *Japanese Journal of Water Treatment Biology*, vol. 48, no. 4, pp. 145-156.

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1. Le Nguyen Ai, Akiko Sato, Daisuke Inoue, Kazunari Sei, Satoshi Soda and Michihiko Ike (2011) Enrichment, Isolation and Characterization of Chemolithoautotrophic Arsenic Oxidizing Bacteria for Removal of Arsenic from Groundwater. *Proceedings of the 4<sup>th</sup> IWA-Aspire Conference and Exhibition, Tokyo, Japan*, 266.
2. Nguyen Ai Le, Kazunari Sei, Michihiko Ike (2010) Arsenic adsorption characteristics of biogenic iron sludge in comparison to chemogenic iron sludge. *The 7<sup>th</sup> Scientific Conference of the University of Science, Ho Chi Minh, Viet Nam*, 465.
3. Kazunari Sei, Akiko Sato, Ai Le Nguyen, Daisuke Inoue, Satoshi Soda, Michihiko Ike (2010) Enrichment of chemolithoautotrophic arsenic oxidizers for removal of arsenic from groundwater. *The First International Conference on Environmental Pollution, Restoration and Management (SETAC A/P Joint Conference), Ho Chi Minh, Viet Nam*.

## LIST OF FIGURES

Figure	Title	Page
1.1	Eh - pH diagram for aqueous As species in the system As–O <sub>2</sub> –H <sub>2</sub> O at 25°C and 1 bar total pressure	2
1.2	The schematic flow of this study	23
2.1	Chronological profile of As(III) oxidation by the enrichment culture. The As(V) concentration was monitored from cycle 3	28
2.2	Typical As(III) oxidizing profiles at the 13 <sup>th</sup> (A), 18 <sup>th</sup> (B) and 45 <sup>th</sup> (C) cycles during the enrichment process of CAOs. Symbols: ○, As (III); ●, As (V)	29
2.3	Bacterial growth profile concomitant with oxidation of approximately 12 mM As(III) at the 46 <sup>th</sup> cycle. Symbols: □, As(III) oxidation ratio; ▲, Cell concentration	30
2.4	Effects of inoculating volume (A) and yeast extract addition (B) on As(III) oxidizing capability of the enrichment culture. Symbols: ●, regular (1%) subculturing; ▲, 10% subculturing; ◆, 1% subculturing with 0.05% yeast extract	31
2.5	Typical T-RFLP profiles of the enrichment culture during different enrichment cycles	32
3.1	T-RFs of ten isolated strains	39
3.2	Oxidation of 1 mM (A), 5 mM (B) and 10 mM (C) As(III) by six isolated CAO strains. Symbols: ●, strain B1; ○, strain B2; ■, strain C; □, strain D; ▲, strain E1; △, strain E2. Error bars indicate the standard deviation obtained from three independent experiments	40
3.3	Effects of the initial As(III) concentration on the specific As(III) oxidation rate of six isolated CAO strains. Symbols: ●, strain B1; ○, strain B2; ■, strain C; □, strain D; ▲, strain E1; △, strain E2. Error bars indicate the standard deviation obtained from three independent experiments	41
3.4	Neighbor-joining tree of <i>aoxB</i> genes and two synonyms ( <i>aroA</i> and <i>asoA</i> genes) of CAOs isolated in this study and previously-isolated As(III) oxidizing bacteria ( <sup>1</sup> HAO; <sup>2</sup> CAO; <sup>3</sup> undefined As(III) oxidizing bacteria). Scale bar corresponds to the 0.05 substitutions per nucleotide position. Numbers at nodes show bootstrap values obtained from 1000 replicates	44

4.1	Oxidation of 0.1 mM (A) and 1 mM (B) As(III) by strain B1 in different solutions. Symbols: ●, BSM; ■, model Rokko water; ▲, model Contrex water. Error bars indicate the standard deviation obtained from three independent experiments	50
4.2	Adsorption isotherms of arsenic by AA in BSM (A), model Rokko water (B) and model Contrex water (C). Symbols: ●, As(V); ○, As(III); straight line, Langmuir isotherm; hidden line, Freundlich isotherm. Error bars indicate the standard deviation obtained from three independent experiments	52

## LIST OF TABLES

Table	Title	Page
1.1	Arsenic concentration and potentially exposed populations around the world	6
1.2	Summary of Chemolithoautotrophic arsenite oxidizers	16
1.3	Summary of Heterotrophic arsenite oxidizers	17
1.4	Summary of recent studies using pure culture for arsenic remediation	20
1.5	Summary of recent studies using bacterial consortia for arsenic remediation	21
3.1	Physiological, phylogenetical and As(III) oxidation characteristics of the strains isolated from the enrichment	38
3.2	Kinetic parameters for As(III) oxidation by six isolated CAO strains determined from the Lineweaver-Burk plot based on the experimental data obtained from whole cell assays	42
4.1	Composition of commercial Rokko and Contrex waters	47
4.2	Composition of model Rokko and Contrex waters	47
4.3	Arsenic adsorption constants of AA obtained from the regression by Langmuir and Freundlich isotherms	53
4.4	Removal efficiency of As(V) and As(III) with AA in model contaminated waters	54

## **Chapter 1**

### **General introduction**

#### **1.1 Arsenic in natural water**

##### **1.1.1 Sources of arsenic**

Arsenic (As) is a naturally occurring and ubiquitous element in soils, sediments and natural water, ultimately originating from igneous rock (Cullen and Reimer, 1989). It ranks the 20<sup>th</sup> most abundant element in the earth's crust (Mandal and Suzuki, 2002; Nicholas et al., 2003; Lièvremonet et al., 2009), 4<sup>th</sup> in seawater and 12<sup>th</sup> in the human body (Mandal and Suzuki, 2002). In the environment, arsenic is broadly congregated in sulfide-bearing mineral deposits like arsenopyrite (FeAsS), orpiment (As<sub>2</sub>S<sub>3</sub>), realgar (AsS), loellingite (FeAs<sub>2</sub>) and pyrite (FeS<sub>2</sub>) (Dave et al., 2008; Lièvremonet et al., 2009).

Arsenic and its compounds are mobile in the environment. Natural phenomena such as weathering, biological activities, geochemical reactions and volcanic emissions contribute to the mobilization of arsenic in the environment which, in turn, may cause many arsenic problems in soil and drinking water (Cullen and Reimer, 1989; Mohan and Pittman, 2007). Additionally, anthropogenic sources of arsenic include use of arsenic-based pesticides, mining activities, pigment production for paints and dyes, burning of fossil fuel and use of arsenic additives to livestock feed (Mohan and Pittman, 2007; Nordstrom, 2002). Naturally occurring arsenic is widely dispersed in many subsurface soils, sediments and drinking water aquifers around the world compared to localized points of anthropogenic arsenic pollution, especially caused by mining activities.

Water is one of the major ways of arsenic transport in the environment. Arsenic concentration in groundwater is controlled by the water-rock interactions and the



favorable physical as well as geochemical conditions in aquifers (Smedley and Kinniburgh, 2002).

### 1.1.2 Chemical properties of arsenic

Arsenic, with atomic number 33, is a toxic metalloid belonging to the group VA of the periodic chart. Arsenic occurs in both organic and inorganic forms in several oxidation states (-III, 0, III and V) in natural water. Organoarsenic compounds such as monomethylarsonic acid (MMA (V)), monomethylarsonous acid (MMA(III)), dimethylarsonic acid (DMA (V)), dimethylarsinous acid (DMA (III)), trimethylarsine oxide (TMA (V)), and trimethylarsine (TMA (III)), generally known to be less toxic than inorganic ones, can be found in surface waters more often than in groundwater and contribute less than 10 % of the total arsenic in the terrestrial water except for the isolated instances (Cullen and Reimer, 1989; Anderson and Bruland, 1991; Azcue and Nriagu, 1995). Therefore, organic species are generally considered less significant compared to inorganic arsenic species in drinking water treatment (Edwards, 1994).

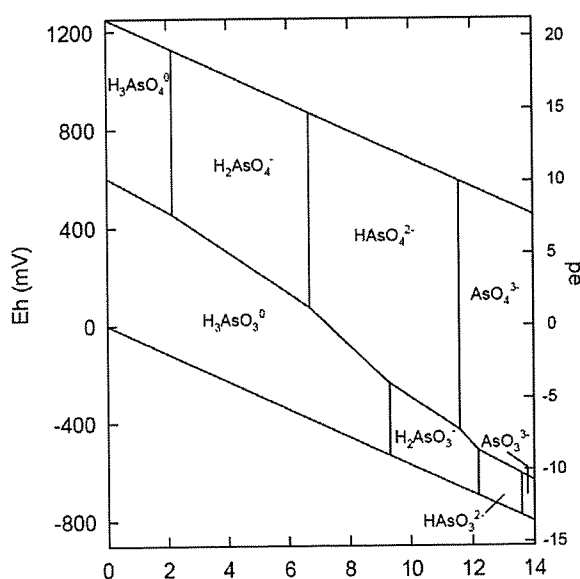


Fig.1.1 Eh - pH diagram for aqueous As species in the system As-O<sub>2</sub>-H<sub>2</sub>O at 25°C and 1bar total pressure (Smedley and Kinniburgh, 2002).

In natural soils and waters, arsenic is mostly found in inorganic forms as oxyanions of trivalent arsenite [As(III)] or pentavalent arsenate [As(V)]. Arsenate presents as  $\text{H}_3\text{AsO}_4$  and its various deprotonated forms including  $\text{H}_2\text{AsO}_4^-$ ,  $\text{HAsO}_4^{2-}$  and  $\text{AsO}_4^{3-}$  depending on the pH of the water, while arsenite presents in aquatic environment as  $\text{H}_3\text{AsO}_3$  and its various protonated and deprotonated forms including  $\text{H}_4\text{AsO}_3^+$ ,  $\text{H}_2\text{AsO}_3^-$ ,  $\text{HAsO}_3^{2-}$  and  $\text{AsO}_3^{3-}$ . Arsenic is a redox-sensitive element and its occurrence, forms, distribution and mobility depend on many factors such as aquatic chemistry, co-existence of other ionic species, microbial activities as well as the pH conditions and oxidation-reduction potential (Eh) (Katsoyiannis and Zouboulis, 2005; Lièvrement et al., 2009). The Eh controls the speciation of arsenic species between the two oxidation states while pH has a role in the distribution of arsenic speciation within a particular oxidation state (Bose and Sharma, 2002). Figure 1.1 illustrates the relationships between Eh, pH and aqueous arsenic species. As(V) is stable in oxygen rich aerobic environments, typically in surface water.  $\text{H}_3\text{AsO}_4$  is a weak acid which has three dissociation constants:  $\text{p}K_a^1 = 2.2$ ,  $\text{p}K_a^2 = 6.9$ , and  $\text{p}K_a^3 = 11.5$ , respectively. Therefore,  $\text{HAsO}_4^{2-}$  is dominant at higher pH,  $\text{H}_2\text{AsO}_4^-$  at pH less than 6.9, and  $\text{H}_3\text{AsO}_4$  in extremely acidic conditions. However, in moderately reducing anaerobic conditions like in groundwater, As(III) which has  $\text{p}K_a^1 = 9.2$ ,  $\text{p}K_a^2 = 12.1$ , and  $\text{p}K_a^3 = 13.4$ , respectively, is stable with  $\text{H}_3\text{AsO}_3$ ,  $\text{H}_2\text{AsO}_3^-$  or  $\text{AsO}_3^{3-}$  forms depending on the pH conditions. Generally, dissolved As(V) and As(III) simultaneously exist in both oxic and anoxic conditions in the environment (Smedley and Kinniburgh, 2002; Oremland and Stolz, 2003). In soils and natural waters with the pH range from 3 to 9, As(III) exists dominantly as the neutral species  $\text{H}_3\text{AsO}_3$ , whereas As(V) can exist as negatively charged  $\text{H}_2\text{AsO}_4^-$  and  $\text{HAsO}_4^{2-}$  that make them easier to interact with most solid

surfaces. As a result, the conventional water treatment processes are usually less effective for removal of As(III) than As(V) (Lièvremonet et al., 2009).

### **1.1.3 Arsenic health effects**

The most common and serious way of arsenic exposure for human is through the ingestion of drinking water containing arsenic compounds. The health effects for arsenic ingested by humans vary based on the dose, duration of exposure and the chemical forms (Edwards, 1994). Inorganic arsenic species are generally considered more toxic than organic ones, and As(III) has higher toxicity than As(V) (Edwards, 1994; Lièvremonet et al., 2009; Wang and Zhao, 2009). The toxicity scale of arsenic can be presented in that decreasing order: arsine > inorganic As(III) > organic As(III) > inorganic As(V) > organic As(V) > arsonium compounds and elemental arsenic (Pontius et al., 1994; Mondal et al., 2006). As(III) can bind strongly to the sulfhydryl groups of enzymes in proteins and inhibits their activities, while As(V) can act as an analog of phosphate ( $\text{PO}_4^{3-}$ ) and cause many injuries at the cellular level (Lièvremonet et al., 2009; Wang and Zhao, 2009).

Arsenic in water supplies causes chronic poisoning rather than acute poisoning. Therefore, chronic toxicity of arsenic is considered as main concern in the evaluation of health significance of arsenic in drinking water (Ferguson and Gavis, 1972). It tends to accumulate in hair and nails of the affected people by the ingestion of arsenic contaminated water. Long-term exposure to arsenic via drinking-water causes cancer of the skin, lungs, urinary bladder and kidney, as well as other skin changes (Pontius et al., 1994; Karim, 1999). Therefore, arsenic has been classified as human carcinogen and is of a great public concern due to its wide natural distribution and widespread usage in

industry (Shih, 2005).

Due to its acute and chronic toxicity and carcinogenic properties, regulatory agencies have established the maximum contaminant level (MCL) for arsenic in drinking water. The objective is to reduce arsenic exposure to a level as close to zero as possible, considering its occurrence, human exposure, feasibility of the treatment technology, availability of analytical techniques to quantify the lower levels of arsenic, and estimated risk for cancer and health effect as a consequence of long term exposure (Viraraghavan et al., 1999).

The World Health Organization (WHO) established an allowable arsenic limit of 0.2 mg/L in 1958, and the recommended arsenic level was lowered to 10 µg/L in 1993 (Ferguson and Gavis, 1972; Ning, 2002; Smedley and Kinniburgh, 2002). This new recommended value was based on the increasing awareness of the toxicity of arsenic, particularly its carcinogenicity, and on the ability to measure it quantitatively in the water (Smedley and Kinniburgh, 2002).

#### **1.1.4 Worldwide groundwater arsenic problems**

Background concentrations of arsenic in groundwater are generally less than 10 µg/L, and sometimes substantially lower. However, its concentration can be much higher than the background values, with levels higher than 100000 µg/L being present in some locations (Nordstrom, 2002). The arsenic contaminated groundwater was first reported in Taiwan in 1968 (Mondal et al., 2006). Recently, the worldwide occurrence of arsenic in drinking water has been recognized as major public health issues in several regions of the world. Arsenic concentration and potentially exposed populations around the world are summarized in Table 1.1 (Mohan and Pittman, 2007; Nordstrom, 2002).

Tab. 1.1 Arsenic concentration and potentially exposed populations around the world

Country/region	Potential exposed population	Concentration (µg/liter)	Environmental conditions
Bangladesh	30000000	< 1 to 2500	Natural; alluvial/deltaic sediments with high phosphate and organics
West Bengal, India	6000000	< 10 to >1000	
Vietnam	> 1000000	1 to 3050	Natural; alluvial sediments
Thailand	15000	1 to > 5000	Anthropogenic; mining and dredged alluvium
Taiwan	100000 to 200000	10 to 1820	Natural; coastal zones, black shale
Inner Mongolia	100000 to 600000	< 1 to 2400	Natural; alluvial and lake sediments; high alkalinity
Xinjiang, Shanxi	> 500	40 to 750	Natural; alluvial sediments
Argentina	2000000	< 1 to 9900	Natural; loess and volcanic rocks, thermal springs; high alkalinity
Chile	400000	100 to 1000	Natural and anthropogenic volcanogenic sediments; closed basin; lakes, thermal springs, mining
Bolivia	50000		Natural; similar to Chile and parts of Argentina
Brazil		0.4 to 350	Gold mining
Mexico	400000	8 to 620	Natural and anthropogenic; volcanic sediments, mining
Germany		< 10 to 150	Natural: mineralized sandstone
Hungary, Romani	400000	< 2 to 176	Natural; alluvial sediments; organics
Spain	> 50000	< 1 to 100	Natural; alluvial sediments
Greece	150000		Natural and anthropogenic; thermal springs and mining
United Kingdom		< 1 to 80	Mining; southwest England
Ghana	< 100000	< 1 to 175	Anthropogenic and natural; gold mining
USA and Canada		< 1 to > 100000	Natural and anthropogenic; mining, pesticides, As <sub>2</sub> O <sub>3</sub> stockpiles, thermal springs, alluvial, closed basin lakes, various rocks

The large-scale natural high arsenic groundwater problem areas can be mostly

found in two types of environment, including inland or closed basins in arid or semi-arid areas, and strongly reducing aquifers often derived from alluvium. These areas are in parts of Argentina, Bangladesh, Chile, China, Hungary, India (West Bengal), Mexico, Romania, Taiwan and Vietnam where arsenic occurring at concentrations exceed 50  $\mu\text{g/L}$  (Smedley and Kinniburgh, 2002). Arsenic associated with geothermal waters has also been reported in several areas, including hot springs from parts of Argentina, Japan, New Zealand, Chile, Kamchatka, Iceland, France, Dominica and the USA. The more localized scale mining related to arsenic problems in water have been identified in many places of the world, including Ghana, Greece, Thailand and the USA (Smedley and Kinniburgh, 2002).

## **1.2 Remediation methods for arsenic contaminated water**

Considering that the natural and anthropogenic occurrence of arsenic in groundwater causes many problems around the world, it is necessary to eliminate arsenic from the contaminated water before distribution to the community for safety drinking water. The most commonly used methods for remediation of arsenic contaminated water can be divided into physical-chemical and biological processes. Most of the physical-chemical techniques usually need pre-oxidation of soluble As(III) to less soluble As(V) followed by separating the As(V) from the solution by various processes including coagulation/co-precipitation, adsorption, ion exchange and membrane technology (Mondal et al., 2006; Mohan and Pittman, 2007; Lièvremon et al., 2009).

### **Coagulation/ co-precipitation and filtration process**

Coagulation/co-precipitation and filtration is one of the most common

treatment processes for removing arsenic from aqueous phase. The most typical chemicals used are aluminum and ferric salts such as ferric chloride and ferric sulfate as well as lime softening agents such as lime stone and calcium hydroxide. The advantage of the coagulation process is that the coagulants are easily available, cheap, less hazardous and easy to handle (Bissen and Frimmel, 2003). During this process, arsenic can be removed through the three main mechanisms: precipitation reactions leading to form insoluble compounds  $\text{Al}(\text{AsO}_4)$  or  $\text{Fe}(\text{AsO}_4)$ , co-precipitation onto the metal hydroxides phase, and adsorption of arsenic onto the external surfaces of the insoluble metal hydroxides (Edwards, 1994). The influent water characteristics including arsenic speciation, pH, and presence of other ions have significant effects on arsenic removal efficiencies (Mondal et al., 2006; Kartinen and Martin, 1995; Ng et al., 2004).

### **Ion exchange**

In this process, the arsenic contaminated water is passed through the “exchange sites” usually saturated with an anion which is exchanged for arsenic in the water (Kartinen and Martin, 1995; Mondal et al., 2006). Since the resin can effectively remove only As(V) in its divalent form ( $\text{HAsO}_4^{2-}$  as opposed to  $\text{H}_2\text{AsO}_4^-$ ), it is necessary to convert arsenic from As(III) to As(V) (Kartinen and Martin, 1995). The media for ion exchange should be replaced or regenerated with a solution of the exchangeable anion before the target ion breaks through in the effluent (Clifford, 1999; Clifford et al., 2003). Some strong base anions have been mainly used for arsenic removal, and they are ranked from the most to the least preferable as following (Clifford, 1999):  $\text{CrO}_4^{2-} \gg \text{SeO}_4^{2-} \gg \text{SO}_4^{2-} \gg \text{HSO}_4^- > \text{NO}_3^- > \text{Br}^- > \text{HAsO}_4^{2-} > \text{SeO}_3^{2-} > \text{HSO}_3^{3-} > \text{NO}_2^- > \text{Cl}^-$ . As with other processes, arsenic removal efficiency with ion exchange depends on the influent water characteristics like pH, arsenic concentration, total

dissolved solids and other competing components such as bicarbonate, sulfate, and particulate iron (Clifford, 1999; Horng and Clifford, 1997; Edwards et al., 1999).

### **Membrane process**

In this technique, arsenic is removed from the aqueous solution by passing it through a semi permeable barrier or membrane. Based on the differences in the driving force for the separation of dissolve species, there are two kinds of membranes: low-pressure membranes (microfiltration (MF) and ultrafiltration (UF)) and high-pressure ones (nanofiltration (NF) and reverse osmosis (RO)). Membrane process, particularly RO and NF systems, shows high arsenic elimination efficiency. The efficiency does not depend on pH and the co-existing of other components including sulfate (Waypa et al., 1997; Ng, 2004).

### **Adsorption process**

Adsorption of arsenic onto solid media is one of the most attractive methods for arsenic removal due to its relatively simple operation, high removal efficiency and low remediation cost (Lièvremonet et al., 2003; Ghosh et al., 2006; Zhang et al., 2004). In this process, the surface of the adsorbents where arsenic is adsorbed by physical or chemical interactive forces is extremely essential. A number of materials ranging from natural materials, conventional and low cost adsorbents, to special synthetic ones have already been examined for the affinity to arsenic. Among them, activated carbon, zeolites, activated alumina, hydrous iron oxides or combination of these materials are the most common effective adsorbents (Daus et al., 2002; Mohan and Pittman, 2007; Ng, 2004). Arsenic adsorption onto the media seem to decrease greatly if the influent water contains many competitive anions such as sulfate, phosphate, chloride, calcium or natural organic matter (Manning and Goldberg, 1997; Wilkie and Hering, 1996;



Swedlund and Webster, 1999; Fuhrman and Tjell, 2003; Xu et al., 1988; Grafe et al., 2001).

The disadvantages of this technique are their relatively high cost of installation and maintenance, the need of using chemicals which may affect water quality and cause another problems in handling, and production of large volume of arsenic contaminated sludge (Mondal et al., 2006; Mohan and Pittman, 2007).

### **Bio-based processes**

Recently, biological techniques, including phytoremediation and bio-sorption/filtration using living microbes, are being concerned as attractive alternatives for removing or reducing the harmful impact of arsenic because of their high efficiency, cost effectiveness and environmentally friendly nature (Katsoyiannis and Zouboulis, 2005; Mondal et al., 2006; Lièvremonet et al., 2009; Wang and Zhao, 2009).

In the phytoremediation, plant or algae is used to extract, immobilizing or remove arsenic from shallow groundwater in which the removal mechanism is similar to that of the adsorption process. Living plants such as several fern species, including *Agrostis tenuis*, *Agrostis stolonifera*, *Pityrogramma calomelanos*, *Pteris cretica*, *Pteris longifolia* and *Pterisumbrosa* (Visoottiviseth et al., 2002), duckweed (*Lemna gibba*) (Mkandawire and Dudel, 2005), water hyacinth (*Eichhornia crassipes*) and lesser duckweed (*Lemna minor*) (Alvarado et al., 2008) have been reported to be able to hyper-accumulate arsenic. Murugesan et al. (2006) also reported that tea fungus, a waste product generating during black tea fermentation, was able to sequester arsenic in groundwater. Plants can be modified to improve arsenic uptake, transport and sequestration to increase arsenic hyper-accumulating capacity (Wang and Zhao, 2009).

On the other hand, in the bio-filtration, microorganisms create ambient conditions that cause adsorption/precipitation of arsenic onto the bio layer formed on the solid support medium (Mondal et al., 2006; Wang and Zhao, 2009). Microorganisms can also catalyze the oxidation of As(III) to As(V) that can enhance the overall removal efficiency of the process. The removal efficiency of this process is often controlled by plasmid gene of the microorganism. Thus, genetic modification of the microorganisms may improve the arsenic tolerance and accumulation capacities. However, these genetically engineered microorganisms may alter the eco system (Mondal et al., 2006). The application of biological processes for the oxidation and removal of dissolved Fe and Mn using iron oxidizing bacteria *Gallionella ferruginea* and *Leptothrix ochracea* (Katsoyiannis and Zouboulis, 2004a, 2004b and 2006) and Mn oxide-depositing fungus, strain KR21-2 (Tani et al., 2004) have been proposed as efficient treatment technologies for arsenic removal from groundwater. Bio-sorption of arsenic onto *Penicillium purpurogenum* (Say et al., 2003) and *Penicillium chrysogenum* (Loukidou et al., 2003) fungal biomass is also considered as a potentially attractive technology to remove heavy metals and arsenic from aqueous solutions.

At present, biological arsenic removal techniques generally exhibit better results than other methods under laboratory and well-defined conditions. However, scale-up experiment, especially focus on various troubles and long time operation, should be further conducted before assessing the application.

### **1.3 Oxidation of As(III) to As(V) as pretreatment for enhanced arsenic removal**

For effective removal of arsenic from water phase containing As(III) as a major component, it is required to oxidize As(III) to As(V), because As(III) cannot be easily

removed by relatively low-cost technologies like coagulation and coprecipitation/adsorption compared with As(V). Oxidation technologies of As(III) can be divided into chemical and biological oxidations.

### 1.3.1 Chemical oxidation of As(III) to As(V)

The chemical oxidation of As(III) to As(V) was first described by Frank and Clifford (1986) utilizing chlorine ( $\text{Cl}_2$ ), monochloramine ( $\text{NH}_2\text{Cl}$ ), and oxygen ( $\text{O}_2$ ). They showed that As(III) oxidation by  $\text{Cl}_2$  was the fastest compared with  $\text{O}_2$  and  $\text{NH}_2\text{Cl}$ ; 100  $\mu\text{g/L}$  of As(III) was oxidized by 1.0 mg/L of free  $\text{Cl}_2$  in less than 5 seconds. Amy et al. (2000) studied As(III) oxidation using  $\text{Cl}_2$ , ozone ( $\text{O}_3$ ), and permanganate ( $\text{KMnO}_4$ ). The results of the study showed that all three oxidants can effective, but achieved less than 100% As(III) oxidation. This was possibly due to the presence of 0.2-2.3 mg/L natural organic matter that caused a competition against oxidant demand (Amy et al., 2000). Many other studies were also done to investigate the oxidation of As(III) using  $\text{O}_2$  (Cherry et al., 1979; Clifford et al., 1983; Bockelen and Nießner, 1992), manganese oxides ( $\text{MnO}_2$ ) (Driehaus et al., 1995),  $\text{KMnO}_4$  and  $\text{MnO}_2$  (Borho and Wilderer, 1996),  $\text{O}_3$  (Bockelen and Nießner, 1992), permanganate ( $\text{MnO}_4$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ )/ $\text{Fe}^{2+}$  (Jekel, 1994),  $\text{Fe}^{3+}$  (Cherry et al., 1979), and  $\text{MnO}_4$  and chlorine dioxide ( $\text{ClO}_2$ ) (Ghurye and Clifford, 2001).

Solid phase oxidants like birnessite ( $\delta\text{-MnO}_2$ ) have been also confirmed to be very useful in the oxidation of As (III) to As (V) by several researchers (Oscarson et al., 1981; Moore et al., 1990; Driehaus et al., 1995; Scott and Morgan, 1995). They all suggested that birnessite directly oxidize As (III) to As(V) through a surface phenomenon and that the adsorption of As (III) onto the oxide surface was the rate

limiting step.

Although chemical oxidants are reported to be effective in As(III) oxidation, they are not always useful in practical applications because of their high cost, inefficient As(III) oxidation in the presence of organic matter etc., and the generation of undesirable by-products because of nonspecific reactions (Ghurye and Clifford, 2001; Dodd et al., 2006).

### **1.3.2 Microbial Oxidation of As (III) to As (V)**

Microbial As(III) oxidation has been regarded as an attractive alternative as pre-treatment of arsenic removal, because of its specific reaction for As(III), which can result in high efficiency, in addition to its cost-effective and environmentally friendly nature (Lièvremonet et al., 2009; Wang and Zhao, 2009).

Microbial As(III) oxidation was first observed by Green in 1918. Recently, various microorganisms capable of oxidizing As(III) to As(V) under both aerobic and anaerobic conditions have been isolated and identified from different environments. Tables 1.2 and 1.3 display reported Chemolithoautotrophic As(III) oxidizing bacteria (CAOs) and Heterotrophic As(III) oxidizing bacteria (HAOs), respectively. CAOs can utilize As(III) as an electron donor and As(III) oxidation can support their growth, while the oxidation of As(III) by HAOs is generally considered as detoxification mechanism (Battaglia-Brunet et al., 2002, 2005; Oremland et al., 2002; Rhine et al., 2006; and Satini et al., 2002). For application to arsenic removal from water phase, the autotrophic As(III) oxidation process may be preferred over heterotrophic one because of its lower nutritional requirement and lower potential for production of any harmful organic metabolites. The first reported HAO named *Bacillus arsenoxidans* was isolated from a

cattle-dipping solution (Green, 1918), whereas an CAO strain *Pseudomonas arsenitoxidans* was first reported in 1981 (Ilyaletdinov and Abdrashitova, 1981).

As for the application of HAOs/CAOs as the pre-treatment for arsenic removal, the use of pure cultures of As(III) oxidizing bacteria has been investigated mainly at the bench scale, while bacterial consortia have been studied at bench-or pilot scale (Lièvremonet et al., 2009). The As(III) oxidation efficiency of isolated pure cultures of As(III) oxidizing bacteria in remediated bioreactors depends on various parameters, such as the initial As(III) concentration, component of influent, operation conditions, and the cell-immobilizing material. The As(V) chemical adsorption needs a careful choice of the adsorbent. Table 1.4 shows some recent studies using the pure cultures for arsenic remediation.

On the other hand, some recent studies has reported the use of bacterial consortia for arsenic remediation, not pure cultures of HAOs/CAOs (Table 1.5). In this approach, there are two ways of use of bacterial consortia for arsenic remediation. In the first way, the consortia of As(III) oxidizers enriched from arsenic certain contaminated environments were inoculated into bioreactors for As(III) oxidation. While in the second approach, natural formation of a biofilm containing As(III) oxidizers from the influents was caused in the bioreactors, thus, the bacterial community could be well-adapted to the given arsenic contaminated water, and therefore they are more resistant to environmental stress, making them more efficient for arsenic bioremediation.

It has been clarified that As(III) bio-oxidation based remediation of arsenic using both pure bacterial cultures and consortia is relatively efficient at various As(III) concentrations, offering an alternative, nonchemical approach to As(III) oxidation and as a safe mean to achieve the efficient arsenic removal. However, these studies have

been done mainly on bench scale systems as the preliminary investigation. Further full scale demonstrations are needed and detailed cost-effectiveness analysis should be investigated to assess the real applicability. Also it is important to understand the characteristics of As(III) oxidizing microorganisms for improving the performance of the remediation processes.

Tab. 1.2 Summary of Chemolithoautotrophic arsenite oxidizers

Microorganism	Culture condition	Source	Phylogeny	References
<i>Agrobacterium/Rhizobium</i> sp. strains NT-25, NT-26	Aerobic	Gold mine	$\alpha$ -Proteobacteria	Santini et al. (2000)
<i>Sinorhizobium</i> sp. strains NT-2, NT-3, NT-4	Aerobic	Gold mine contaminated water	$\alpha$ - Proteobacteria	Santini et al.(2002)
<i>Agrobacterium</i> sp. strain BEN-5	Aerobic	Mine Water	$\alpha$ - Proteobacteria	Santini et al.(2002)
<i>Sinorhizobium</i> sp. strain DAO10	Anaerobic	Contaminated soil and sediment	$\alpha$ - Proteobacteria	Rhine et al. (2006)
<i>Acidicaldus</i> sp. strain AO5	Anaerobic	Geothermal water	$\alpha$ - Proteobacteria	D'Imperio et al. (2007)
<i>Rhizobium/Agrobacterium</i> sp. strain M-14	Aerobic	Gold mining	$\alpha$ -Proteobacteria	Drewniak and Sklodowska (2007)
<i>Bosea thiooxidans</i> sp. strain WAO	Aerobic	Unconsolidated weathered shale	$\alpha$ -Proteobacteria	Rhine et al. (2008)
<i>Ancyclobacter</i> sp. strain OL-1	Aerobic	Polluted soil and sediment	$\alpha$ -Proteobacteria	Garcia-Dominguez et al. (2008)
<i>Thiobacillus</i> sp. strain S-1	Aerobic	Polluted soil and sediment	$\alpha$ -Proteobacteria	Garcia-Dominguez et al. (2008)
<i>Sinorhizobium</i> sp. strain GW3	Aerobic	Polluted sediment	$\alpha$ -Proteobacteria	Fan et al. (2008)
<i>Agrobacterium tumefaciens</i> strain GW4	Aerobic	Polluted sediment	$\alpha$ -Proteobacteria	Fan et al. (2008)
<i>Sinorhizobium Ensifer</i> sp. strain SDB1	Aerobic	Mine tailing	$\alpha$ -Proteobacteria	Lugtu et al. (2009)
<i>Ensifer adhaerens</i> sp. Strain C-1	Aerobic	Activated sludge	$\alpha$ -Proteobacteria	Ito et al. (2012)
<i>Thiomonas arsenivorans</i> sp. strain b6	Aerobic	Disused gold mining site	$\beta$ -Proteobacteria	Battaglia-Brunet et al. (2006)
<i>Azoarcus</i> sp. strain DAO1	Anaerobic	Contaminated soil and sediment	$\beta$ -Proteobacteria	Rhine et al. (2006)
<i>Thiomonas</i> sp. strain 3As	Aerobic	Mine drainage water	$\beta$ -Proteobacteria	Duquesne et al.(2007)
<i>Hydrogenophaga</i> sp. strain CL-3	Aerobic	Polluted soil and sediment	$\beta$ -Proteobacteria	Garcia-Dominguez et al. (2008)
<i>Achromobacter</i> sp. strain GW1	Aerobic	Polluted sediment	$\beta$ -Proteobacteria	Fan et al. (2008)
<i>Acidovorax</i> sp. strain GW2	Aerobic	Polluted sediment	$\beta$ -Proteobacteria	Fan et al. (2008)
<i>Pseudomonas arsenitoxidans</i>	Aerobic	Gold-arsenic deposit water	$\gamma$ -Proteobacteria	Ilialetdinov and Abdrashitova (1981)
<i>Ectothiorhodospira</i> sp. strain MLHE-1	Anaerobic	Lake's anoxic sediment	$\gamma$ -Proteobacteria	Oremland et al. (2002)
<i>Alkalilimnicola ehrlichii</i> sp. strain MLHE-1	Anaerobic	Lake's anoxic sediment	$\gamma$ -Proteobacteria	Hoefl et al. (2007)

Tab. 1.3 Summary of Heterotrophic arsenite oxidizers

Microorganism	Culture condition	Source	Phylogeny	References
<i>Agrobacterium albertimagni</i> strain AOL15	Aerobic	Geothermal water	$\alpha$ -Proteobacteria	Salmassi et al. (2002)
<i>Agrobacterium tumefaciens</i> strain 5A	Aerobic	Irrigated pasture	$\alpha$ -Proteobacteria	Macur et al. (2004)
<i>Agrobacterium</i> sp. strain C13	Aerobic	Mining wastes	$\alpha$ -Proteobacteria	Xiong et al. (2006)
<i>Alcaligenes eutrophus</i> 280		Gold arsenic deposits	$\beta$ -Proteobacteria	Abdrashitova et al. (1981)
<i>Archromobacter arsenoxydans</i>	Aerobic	Cattle-dipping sollution	$\beta$ -Proteobacteria	Turner (1949, 1954)
<i>Thiomonas intermedia</i>			$\beta$ -Proteobacteria	London (1963) ; Moreira and Amils (1997)
<i>Alcaligenes faecalis</i> strain YE56	Anarobic	Raw sewage	$\beta$ -Proteobacteria	Philips and Taylor (1976)
<i>Alcaligenes faecalis</i> strain ANA	Aerobic	Soils	$\beta$ -Proteobacteria	Osborne and Ehrlich (1976)
<i>Thiobacillus cuprinus</i>			$\beta$ -Proteobacteria	Moreira and Amils (1997)
<i>Herminiimonas arsenicoxydans</i> strain ULPAs1	Aerobic	Contaminated soil	$\beta$ -Proteobacteria	Weeger et al. (1999)
<i>Alcaligenes faecalis</i> strain HLE		Soils	$\beta$ -Proteobacteria	Santini et al. (2002)
<i>Achromobacter</i> sp. strain BEN-4	Aerobic	Gold mine contaminated water	$\beta$ -Proteobacteria	Santini et al. (2002)
<i>Hydrogenophaga</i> sp. strains NT-5, NT-6, NT-14	Aerobic	Gold mine contaminated water	$\beta$ -Proteobacteria	Santini et al. (2002)
<i>Achromobacter</i> sp. strain NT-10	Aerobic	Gold mine contaminated water	$\beta$ -Proteobacteria	Santini et al. (2002)
<i>Thiomonas</i> sp. strains B1, B2, B3			$\beta$ -Proteobacteria	Bruneel et al. (2003)
<i>Thiomonas</i> sp. strain WJ68	Aerobic	Mine water	$\beta$ -Proteobacteria	Coupland et al. (2004)



<i>Variovorax paradoxus</i>	Aerobic	Irrigated pasture	$\beta$ -Proteobacteria	Macur et al. (2004)
<i>Hydrogenophaga</i> sp. strains YED6-18, YED6- 4 and YED6-21		Geothermal water	$\beta$ -Proteobacteria	Salmassi et al. (2006)
<i>Thiomonas</i> sp. strain NO115	Aerobic	Disused gold mining site	$\beta$ -Proteobacteria	Battaglia-Brunet et al. (2006)
<i>Leptothrix cholodnii</i> sp. strain S1-1	Aerobic	Mine drainage water	$\beta$ -Proteobacteria	Battaglia-Brunet et al. (2006)
<i>Leptothrix</i> sp. strain S2	Aerobic	Biofilm	$\beta$ -Proteobacteria	Casiot et al. (2006)
<i>Azoarcus</i> sp. strain. V4	Aerobic	Contaminated soil	$\beta$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas putida</i> strain 18		Gold arsenic deposits	$\gamma$ -Proteobacteria	Abdrashitova et al. (1981)
<i>Xanthomonas arsenoxydans</i>	Aerobic	Cattle-dipping solution	$\gamma$ -Proteobacteria	Legge (1954)
<i>Pseudomonas arsenoxydans</i>	Aerobic	Cattle-dipping solution	$\gamma$ -Proteobacteria	Turner and Legge (1954)
<i>Pseudomonas fluorescens</i> strain 1	Aerobic	Irrigated pasture	$\gamma$ -Proteobacteria	Macur et al. (2004)
<i>Pseudomonas putida</i> strain OS-5	Aerobic/ Anaerobic	Mining site	$\gamma$ -Proteobacteria	Chang et al. (2007)
<i>Pseudomonas fulva</i> sp. strains C2 and C6	Aerobic	River water	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas medocina</i> sp. strain C4	Aerobic	River water	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas pseudoalcaligenes</i> sp. strain C5	Aerobic	River water	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas aeruginosa</i> sp. strain C7	Aerobic	River water	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas</i> sp. strain C8	Aerobic	River water	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas stutzeri</i> sp. strain K5	Aerobic	Contaminated soil	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas putida</i> sp. strain M2	Aerobic	Contaminated soil	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas mendocina</i> sp. strain M3	Aerobic	Contaminated soil	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas mendocina</i> sp. strain V1	Aerobic	Contaminated soil	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Pseudomonas stutzeri</i> sp. strain 81D3	Aerobic	Contaminated soil	$\gamma$ -Proteobacteria	Krumova et al. (2008)
<i>Bacillus arsenoxydans</i>	Aerobic	Cattle-dipping solution	<i>Firmicutes</i>	Green (1918)
<i>Bacillus</i> sp. strain ML-SRAO	Anarobic	Lake water samples	<i>Firmicutes</i>	Fisher and Hollibaugh (2008)

<i>Microbacterium lacticum</i>	Aerobic	Municipal sewage	<i>Actinobacteria</i>	Mokashi and Paknikar (2002)
<i>Sulfolobus acidocaldarius</i> strain BC		Mineral leaching	<i>Crenarchaeota</i>	Sehlin and Lindstrom (1992)
<i>Thermus</i> sp. strain HR13	Aerobic	Arsenic-rich terrestrial geothermal environment	<i>Deinococcus-Thermus</i>	Gihring and Banfield (2001)
<i>Thermus aquaticus</i> YT1	Aerobic	Hot spring	<i>Deinococcus-Thermus</i>	Gihring et al. (2001)
<i>Thermus thermophilus</i> HB8	Aerobic	Hot spring	<i>Deinococcus-Thermus</i>	Gihring et al. (2001)
<i>Therms aquaticus</i> strain YT1	Aerobic	Geothermal	<i>Deinococcus-Thermus</i>	Gihring et al. (2001)
<i>Therms thermophilus</i> strain HB8	Aerobic	Geothermal	<i>Deinococcus-Thermus</i>	Gihring et al. (2001)
<i>Hydrogenobaculum</i> sp. strain H55	Anarobic	Hot spring	<i>Aquificae</i>	Donahoe-Christiansen et al. (2004)

Tab. 1.4 Summary of recent studies using pure culture for arsenic remediation

Technical options	Strain	Influent As(III) concentrations	Operation mode	Supported material for immobilizing cells	Arsenic adsorbents	References
Up-flow fixed bed reactors	<i>Ralstonia eutropha</i> MTCC 2487			Granular activated carbon		Mondal et al. (2008)
	<i>Thiomonas arsenivorans</i> B6	10 mg/L	Batch/Continuous	Sieved sand	Zero-valent iron	Wan et al. (2010)
	<i>Ensifer adhaerens</i> sp. strain C-1	1 mg/L	Continuous	Polyvinyl alcohol gel		Ito et al. (2012)
Fluidized bed reactors	<i>Alcaligenes faecalis</i> O1201	75 to 3000 mg/L	Continuous	Carragenan gel beads		Wang et al. (2009)
Stirred tank reactors	<i>Hermiimonas arsenicoxydans</i> ULPA <sub>s</sub> 1		Batch	Aluminosilicate zeolite	Kutnahorite	Lièvremonet et al. (2003)
	<i>Hermiimonas arsenicoxydans</i> ULPA <sub>s</sub> 1	100 mg/L	Batch	Ca-alginate beads		Simeonova et al. (2005)
	<i>Thiomonas arsenivorans</i> B6	2,000–4,000 mg/L	Continuous	Glass beads	Activated alumina	Dastidar and Wang (2010)
	<i>Sinorhizobium Ensifer</i> SDB1	75-600 mg/L	Batch/Continuous	Luminosilicate zeolite		Lugtu et al. (2010)
Small glass columns	<i>Microbacterium lacticum</i>	0.5-10 mg/L	Continuous	Brick pieces	Zero-valent iron, activated charcoal, ferric chloride	Mokashi and Paknikar (2002)

Tab. 1.5 Summary of recent studies using bacterial consortia for arsenic remediation

Technical options	Bacterial consortium	Influent As(III) concentrations	Operation mode	Supported material for immobilizing cells/filtration unit	References
Fixed-bed bioreactors	CAsO1	100 mg/L	Continuous	Pozzolana	Dictor et al. (2003)
	Biofilm of CAsO1	100mg/L	Continuous	Pozzolana	Michel et al. (2007)
	CAsO1	100 µg/L	Continuous	Pozzolana	Michon et al. (2010)
	Natural biofilm of indigenous bacteria contain strains <i>Variovorax paradoxus</i> and <i>Leptothrix cholodnii</i>	100 mg/L	Continuous	Pozzolana	Battaglia-Brunet et al. (2006)
Stirred glass reactors	CAsO1	100 mg/L	Continuous	Pozzolana	Battaglia-Brunet et al. (2002)
Fixed-bed upflow filtration units	Natural biofilm of indigenous bacteria contain strains <i>Gallionella ferruginea</i> and <i>Leptothrix ochracea</i>		Continuous	Polystyrene beads	Katsoyiannis et al. (2004)
	Natural biofilm of indigenous bacteria contain <i>Leptothrix</i> sp. strain S2	6.4 µg/L	Continuous	Polystyrene beads	Casiot et al. (2006)

#### 1.4 Objectives of the study

This study focused on the use of CAOs as the pretreatment of arsenic removal from waters containing As(III) as the major arsenic component. To establish cost-effective pre-treatment technologies for the arsenic removal by adsorption, we have tried to enrich, isolate and characterize CAOs that have high As(III) oxidation potential in this study. Further, it was demonstrated that As(V), which was produced from As(III) by CAOs, can be certainly removed by simple adsorption with activated alumina (AA), a typical adsorbent. That is the confirmation of the role of CAOs in promoting the overall arsenic removal from As(III) contaminated water.

The schematic flow of this study is shown in Figure 1.2

Chapter 1 represents the brief background and the objectives of this study.

Chapter 2 describes a mixed culture of CAOs enriched from arsenic contaminated soils that could effectively oxidize widely ranging concentrations of As(III) to As(V). In addition, the enrichment process and the dynamics of the bacterial community structure in the enrichment culture throughout the enrichment period were also elucidated.

Chapter 3 illuminates the isolation of facultative CAOs bacteria that can effectively oxidize different concentrations of As(III) to As(V). Moreover, the As(III) oxidation characteristics and molecular analysis of As(III) oxidase genes, as well as basic physiological properties and phylogenetic positions of the isolated CAOs were examined.

Chapter 4 experimentally demonstrates the arsenic removal by coupling the biological As(III) oxidation to As(V) and the adsorption of As(V) by AA. As(III) oxidizing ability of isolated CAO strain and subsequent As(V) adsorption using AA in model natural waters (Rokko and Contrex waters) were done to confirm the role of bacterial As(III) oxidation in promoting the overall arsenic removal.

Chapter 5 represents the summary and conclusions from this study. The applicability of CAOs in arsenic removal process and recommendations for further studies are discussed.

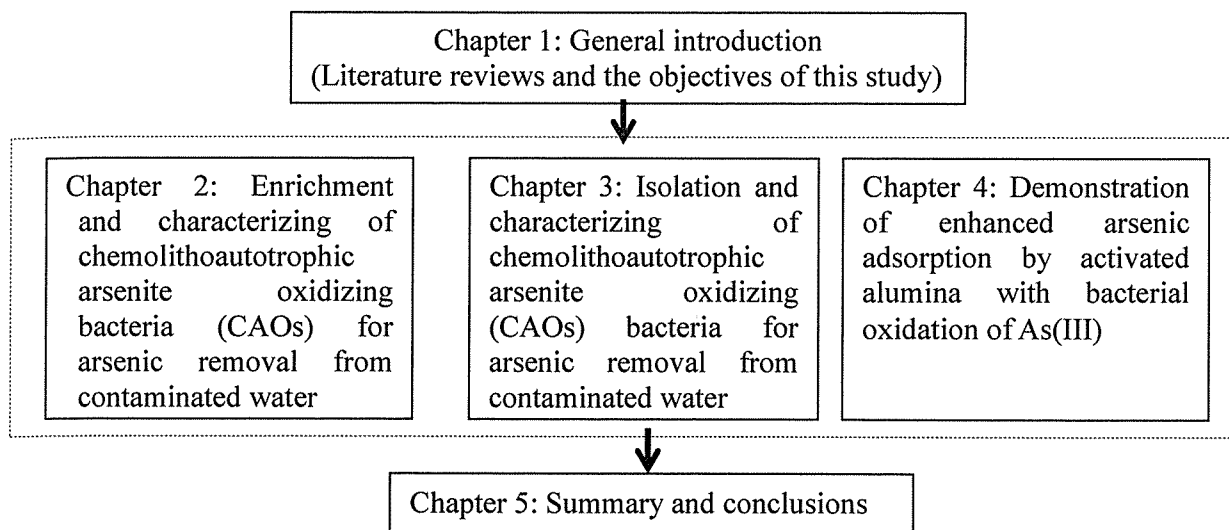


Fig 1.2 The schematic flow of this study.

## Chapter 2

### Enrichment and characterizing of chemolithoautotrophic arsenite oxidizing bacteria (CAOs) for arsenic removal from contaminated water

#### 2.1 Introduction

Arsenic contamination of groundwater has remained as an important public problem in many world regions, particularly in Bangladesh and West Bengal in India, because of its acute and chronic toxicity and carcinogenic properties (Karim, 2000; Nordstrom, 2002). In 1993, The World Health Organization set an arsenic safety level of 10 µg/L in its drinking water guidelines. Cost-effective processes to remove arsenic from groundwater must be developed to make water safe for drinking. Although both inorganic arsenate (As(V)) and arsenite (As(III)) usually coexist in groundwater, the more toxic As(III) often predominates, comprising as much as 74% to 98% of total arsenic (Rasul et al., 2002; Nguyen et al., 2009; Routh and Hjelmquist; 2011). Because As(III) is more difficult to remove than As(V) using conventional treatments such as precipitation, adsorption, ion exchange, and membrane filtration (Battaglia-Brunet et al., 2002; Lièvremonet et al., 2003), preliminary oxidation to As(V) is necessary to enhance overall arsenic removal. Chemical oxidants such as chlorine, permanganate and ozone have been found to be effective for As(III) oxidation (Ghurye and Clifford, 2001, 2004; Dodd et al., 2006). Although chlorine, H<sub>2</sub>O<sub>2</sub> and ozone are effective oxidants, these chemical oxidants are not always useful in practical applications because of their high cost, inefficient As(III) oxidation, and their generation of undesirable by-products because of nonspecific reactions (Ghurye and Clifford, 2001; Dodd et al., 2006). Consequently, chemical oxidation reactions are unfavorable in practical applications. For As(III) oxidation, microbial As(III) oxidation has been regarded as an attractive alternative because of its specific reaction for As(III), which can result in high efficiency, in addition to its cost-effective and environmentally friendly nature (Lièvremonet et al., 2009; Wang and Zhao, 2009).

Recently, various heterotrophic and chemolithoautotrophic As(III) oxidizing bacteria have been isolated from different environments. However, practical use of pure bacterial cultures may not be realistic under certain conditions because they may require well defined feedstock and prior sterilization of water, which results in high remediation

costs. On the other hand, an effective mixed culture of CAOs (enrichment) seems preferable during practical water remediation because they have several advantages over pure cultures. For example, although pure CAOs cultures may exhibit reduced As(III) oxidizing activity if the environmental conditions are not preferable to their growth, the As(III) oxidizing activity of mixed cultures remains stable regardless of minor changes in the environmental/operational conditions (e.g. temperature, pH and nutrient and As(III) concentrations). Despite this, only a few studies have attempted As(III) oxidation via CAOs enrichment cultures for arsenic remediation to date (Rhine et al., 2006; Elizabeth et al., 2008). Furthermore, there have been no studies conducted to monitor the behavior of CAOs during the enrichment process and characterize the obtained enrichment cultures in detail to confirm the stability of the enrichment.

The objectives of this chapter were: (1) to obtain an enrichment culture of CAOs that is useful for the pretreatment step during arsenic removal from water, and (2) to elucidate the enrichment process and characterize the microbial composition of the enrichment culture. As described above, the arsenic concentrations in the environment vary markedly and reach higher than 100,000 µg/L in some cases. Thus, in an effort for effective arsenic bioremediation in such highly contaminated environment, we applied high concentrations (from 1 mM to 10-12 mM) of As(III) with a stepwise increase manner to effectively enrich CAOs from soil samples that had the potential to contain a variety of bacteria including non-As(III) oxidizing bacteria. This enrichment procedure would be useful to obtain an enrichment of As(III) oxidizing bacteria that can exhibit a high As(III) oxidation potential irrespective of the arsenic contamination level. The dynamics of the bacterial community structure in the enrichment culture were monitored by terminal-restriction fragment length polymorphism (T-RFLP) analysis targeting eubacterial 16S rRNA genes throughout the enrichment period.

## **2.2 Materials and methods**

### **2.2.1 Inoculum source**

A soil sample collected from the subsurface zone (a depth of a few centimeters) of an arsenic contaminated area located near a mine in Japan was used as the inoculum for construction of the enrichment culture of CAOs. Analysis of arsenic species after shaking extraction of the soil sample in a tenfold volume of water revealed that it



contained around 0.13 mM of soluble arsenic.

### 2.2.2 Culture media

A slightly modified version of the enrichment medium described by Battaglia-Brunet et al. (2002) was used as a basal salt medium (BSM) for enrichment of CAOs. The medium consisted of  $K_2HPO_4$ , 0.25 g/L;  $KH_2PO_4$ , 0.25 g/L; NaCl, 0.25 g/L;  $(NH_4)_2SO_4$ , 0.1 g/L;  $MgSO_4$ , 0.05 g/L;  $CaCl_2$ , 0.1 g/L; trace element solution (Battaglia-Brunet et al., 2002), 1 mL/L; and vitamin solution, 10 mL/L (Battaglia-Brunet et al., 2002). In addition,  $NaHCO_3$  (0.5 g/L) was added as the carbon source, and the pH of the BSM was adjusted to 6.0 with  $H_2SO_4$ . Aliquots of As(III) stock solution were added to BSM to give appropriate As(III) concentrations. The 500 mM As(III) stock solution was prepared by dissolving  $NaAsO_2$  in ultra pure water, and then sterilizing the solution by filtration through a Dismic-25 cellulose acetate filter (pore size 0.22  $\mu m$ , Advantec, Japan).

CAOs have been reported to grow both autotrophically and heterotrophically (Garcia-Dominguez et al., 2008; Duquesne et al., 2007; Battaglia-Brunet et al., 2005; Santini et al., 2000). Thus, to enable rapid and better growth of CAOs, tryptic soy broth (TSB; Becton-Dickinson, USA) was used for bacterial counts of the enrichment culture. To prepare solid media, 1.8% (*W/V*) agar was added to TSB.

### 2.2.3 Enrichment of CAOs

The enrichment of CAOs was initiated by adding approximately 2 g (wet) of the soil sample to 300 mL Erlenmeyer flasks containing 100 mL of BSM spiked with 1 mM As(III). The flasks were then shaken aerobically at 28°C on a rotary shaker at 120 r/min. Aliquots (1 mL) of the culture were periodically sampled to confirm the oxidation of As(III) to As(V). After the initially added As(III) was completely oxidized to As(V), 1% (*V/V*) of the culture was repeatedly transferred to fresh medium and cultured further. The As(III) concentration in the medium was increased in a stepwise manner as follows: Cycle 1-4, 1 mM; cycle 5-7, 2 mM; cycle 8, 2.6 mM; cycle 9-13, 5 mM; after cycle 14, 10-12 mM. The control experiment was not prepared in our study as the abiotic oxidation of As(III) by dissolved oxygen or air is very slow, which may take several days or even months (Battaglia-Brunet et al., 2005; Rhine et al., 2006).

In addition to periodic measurement of the arsenic concentration, the bacterial growth in the enrichment culture was monitored at certain cycles. Samples were also collected at the end of certain cycles to monitor the bacterial community structure by T-RFLP analysis.

#### **2.2.4 Effects of inoculating volume and organic carbon addition on As(III) oxidation**

The effects of inoculating volume and organic carbon addition on As(III) oxidation by the CAOs enrichment were evaluated at the 22<sup>nd</sup> cycle. After the 21<sup>st</sup> cycle, in addition to the regular subculturing of 1% culture to BSM containing 10 mM As(III), transfer of 10% culture was also performed. At the same subculturing cycle, 1% of the culture was also inoculated into BSM containing 10 mM As(III) and 0.05%(w/v) yeast extract.

#### **2.2.5 Analytical methods**

Bacterial growth was monitored based on changes in the OD<sub>600</sub> using a UV1200 spectrophotometer (Shimadzu, Japan). The dry cell weight was calculated from the linear relationship between the OD<sub>600</sub> and the dry cell weight, which was determined prior to the experiments.

The bacterial community structure of the enrichment culture was monitored by T-RFLP analysis. DNA templates were prepared by the proteinase K method, as previously described (Sei et al., 2000). T-RFLP analysis was carried out as previously described using *Hha*I (Matsuda et al., 2010).

To determine the arsenic concentrations, aqueous samples taken from the culture were centrifuged (20,000 ×g, 4 °C, 10 min) and then filtered through a Dismic-25 cellulose acetate filter (pore size, 0.45 µm, Advantec), after which the filtrates were stored at 4 °C until analysis. As(V) and the total arsenic concentrations were determined by ion chromatography (HIC-20A Super System, Shimadzu) using an HIC-SA3(G) guard column (Shimadzu), an HIC-SA3 analytical column (Shimadzu), and an CDD-10Asp electric conductivity detector (Shimadzu). NaHCO<sub>3</sub> at 5 or 6 mM was applied as the mobile phase at a flow rate of 1.0 mL/min. Prior to measurement of the total arsenic concentration, H<sub>2</sub>O<sub>2</sub> was added to the samples at a final concentration of

3% (V/V) to completely oxidize the remaining As(III) to As(V). The As(III) concentration was calculated based on differences between the total arsenic and As(V) concentrations.

## 2.3 Results and discussion

### 2.3.1 Construction of the enrichment culture of CAOs

We attempted to enrich CAOs from an arsenic contaminated soil sample by repeated subculturing without the addition of any external organic carbon sources (Figure 2.1).

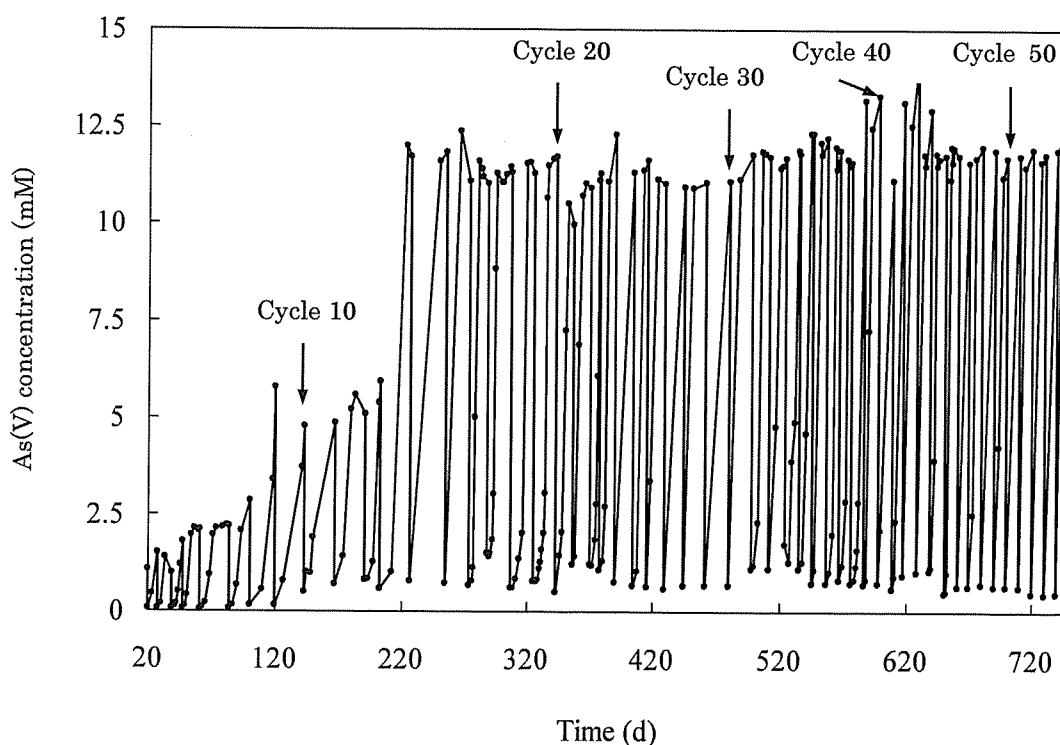


Fig. 2.1 Chronological profile of As(III) oxidation by the enrichment culture. The As(V) concentration was monitored from cycle 3.

During cycle 1 and 2, the arsenic concentration was not measured, and subculturing was carried out after 10 days for each cycle. From cycle 3, the As(III) and As(V) concentrations were measured to evaluate the As(III) oxidation, and subculturing was conducted after almost complete As(III) oxidation. During cycles 3 and 4, 1 mM of As(III) was completely oxidized to the equivalent concentration of As(V) within eight days. Thereafter, the culture was sequentially subcultured with As(III) concentrations

that were increased in a stepwise fashion. During enrichment, As(III) added at each subculturing cycle was almost completely oxidized to As(V), even when the As(III) concentration increased. Furthermore, the As(III) oxidizing capability of the culture was strengthened gradually and apparently during the enrichment process. Figure 2.2 shows some typical As(III) oxidation profiles at different subculturing cycles. At the 13<sup>th</sup> cycle, the enrichment oxidized 5 mM As(III) within 11 days (Figure 2.2A). By contrast, at the 18<sup>th</sup> and 45<sup>th</sup> cycles, the enrichment needed only 7 and 4 days, respectively, to oxidize 10 mM As(III) completely (Figures 2.2B, 2.2C). After more than 60 cycles of subculturing (more than 700 days), enrichment which stably oxidized 12 mM As(III) within 4 days was obtained and maintained.

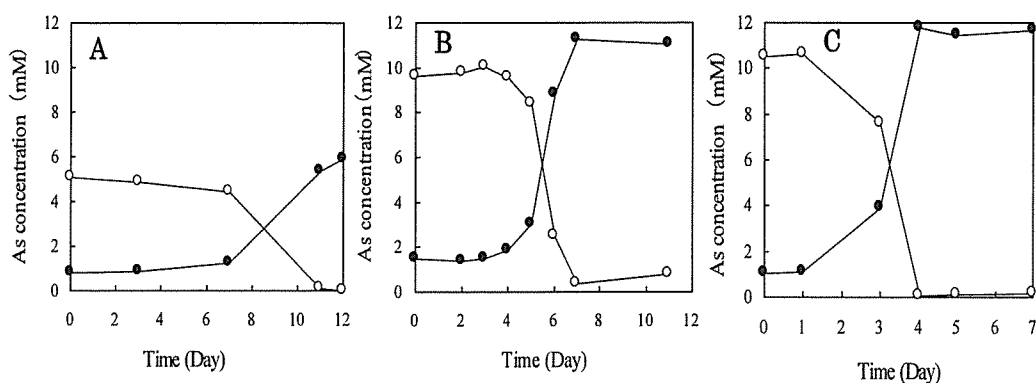


Fig. 2.2 Typical As(III) oxidizing profiles at the 13<sup>th</sup> (A), 18<sup>th</sup> (B) and 45<sup>th</sup> (C) cycles during the enrichment process of CAOs. Symbols: ○, As (III); ●, As (V).

As(III) oxidation activity of the enrichment culture gradually improved as the number of enrichment cycles increased. This indicates that the continuous subculturing with a stepwise increase of As(III) concentration applied in this study would be effective for successful enrichment of CAOs with a high As(III) oxidizing ability from environmental samples that contain divergent bacteria.

During the long-term enrichment process, As(III) oxidation was achieved without supplementation of any organic carbon. Additionally, we examined the relation between As(III) oxidation and bacterial growth at the 46<sup>th</sup> cycle. Figure 2.3 shows that As(III) was oxidized completely to As(V) within 5 days, concomitant with the bacterial growth from  $1.0 \times 10^6$  CFU/mL to  $3.1 \times 10^8$  CFU/mL by 5 days. Consequently, a clear positive correlation was found between As(III) oxidation and bacterial growth in the

enrichment. These results confirmed that CAOs, which use As(III) as the energy source for growth, are (at least) the main constituents in the enrichment. The energy gained by CAOs by As(III) oxidation can be described as following:  $2\text{H}_3\text{AsO}_3 + \text{O}_2 \rightarrow \text{H}_2\text{AsO}_4^- + \text{HAsO}_4^{2-} + 3\text{H}^+$ ,  $\Delta G^\circ = -256 \text{ kJ/Rx}$  (Santini et al., 2000).

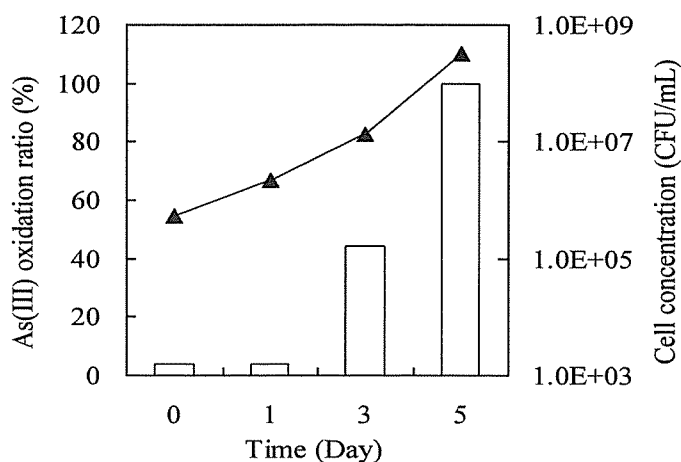


Fig. 2.3 Bacterial growth profile concomitant with oxidation of approximately 12 mM As(III) at the 46<sup>th</sup> cycle. Symbols: □, As(III) oxidation ratio; ▲, Cell concentration.

### 2.3.2 Effects of inoculating volume and organic carbon addition on As(III) oxidation

Effects of subculturing volume and organic carbon addition on As(III) oxidizing capability of the enrichment were assessed at the 22<sup>nd</sup> cycle (Figure 2.4). With regular subculturing, approximately 10 mM As(III) was oxidized completely within 9 days. When 10% of the culture was subcultured, the same concentration of As(III) was oxidized completely within 7 days (Figure 2.4A), suggesting that As(III) oxidation activity was enhanced slightly by the increase of the initial CAOs concentration. By contrast, when 0.05%(w/v) of yeast extract was added to the enrichment, where 1% of the culture was subcultured, As(III) oxidation was accelerated significantly and complete oxidation of approximately 10 mM As(III) required only 3 days (Figure 2.4B). Yeast extract supplementation also enhanced bacterial growth in the enrichment (data not shown). These results demonstrated that the CAOs in our enrichment are not obligatory autotrophs but that they are mixotrophs (facultative CAOs) that can use not only inorganic carbon but also organic carbon. The mixotrophic nature has also been found in some CAOs that had been isolated previously (Santini et al., 2000; Oremland

et al., 2002; Battaglia-Brunet et al., 2005; Duquesne et al., 2007).

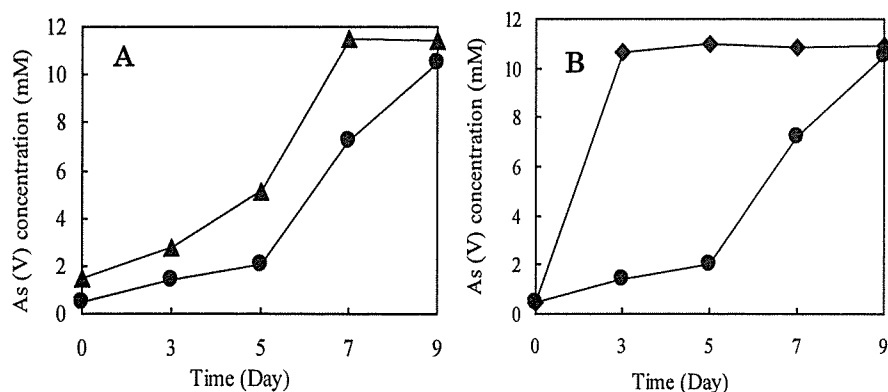


Fig. 2.4 Effects of inoculating volume (A) and yeast extract addition (B) on As(III) oxidizing capability of the enrichment culture. Symbols: ●, regular (1%) subculturing; ▲, 10% subculturing; ◆, 1% subculturing with 0.05% yeast extract.

### 2.3.3 Change in bacterial community during the enrichment process

Variations in the bacterial community during the enrichment process were analyzed by T-RFLP (Figure 2.5). A few dominant and minor T-RFs were detected at the beginning of enrichment (cycle 1). However, the number of T-RFs decreased and specific T-RFs became dominant during the enrichment process. Furthermore, the dominant T-RFs varied depending on the As(III) concentration in the culture. During cycle 4, when 1 mM As(III) was added, a T-RF of 148 bp dominated. After increasing the initial As(III) concentrations to 2.6 and 5 mM, T-RFs of 202-203 bp and 377 bp, respectively, became dominant. After the initial As(III) concentration was increased to 10-12 mM, the enrichment culture had a stable bacterial community in which a T-RF of 141-143 bp always dominated and the minor T-RFs fluctuated temporally.

T-RFLP analysis of the bacterial community in the enrichment culture showed that it varied drastically during the enrichment process. Although the soil sample used here contained various types of bacteria (as indicated by the number of T-RFs), the diversity of the bacterial community decreased greatly after initiation of the enrichment process with 1 mM As(III). This reduction of bacterial diversity was likely due to the selection of specific bacteria, possibly CAOs, by the strong toxicity of As(III) and limited carbon and energy sources. Interestingly, the main T-RFs, which represented the

predominant bacteria, varied with increasing As(III) concentration from 1 to 10-12 mM, and the bacterial community composition became stable with a dominant T-RF of 141-143 bp at 10-12 mM As(III). These results indicated that distinct CAOs that were well-adapted to a given As(III) concentration became dominant at each As(III) concentration. Accordingly, the As(III) concentration was a significant selective pressure for CAOs in the enrichment culture, but CAOs other than the dominant one persisted at various As(III) concentrations as minor populations in the enrichment culture. Thus, some of the CAOs originally present in the soil sample survived even when the selective pressure was unfavorable for their growth, and became dominant when preferable conditions were provided with the enrichment procedure applied here (i.e., the stepwise increase of selective pressure in repeated batch cultivation).

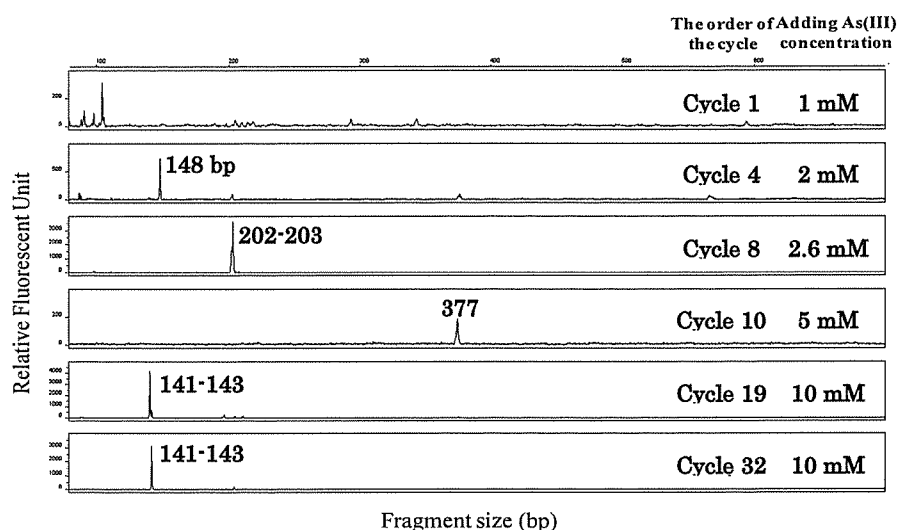


Fig. 2.5 Typical T-RFLP profiles of the enrichment culture during different enrichment cycles.

## 2.4 Conclusions

In this chapter, an enrichment culture of CAOs for As(III) oxidation composed of various bacteria was established and maintained for more than 700 days (more than 60 cycles of subculturing). The results of this chapter demonstrated that specific CAOs that are well-adapted to various As(III) concentrations can be selectively enriched by providing  $\text{NaHCO}_3$  and As(III) as the sole carbon and energy sources, respectively. In contrast, other CAOs that do not play a major role in oxidizing As(III) at certain As(III)

concentrations can survive without complete disappearance in the enrichment culture. This might have occurred as a result of the stepwise increase of the As(III) concentration during repeated batch cultivation. Because CAOs co-existing in the resultant enrichment culture had various As(III) oxidation characteristics, some specific CAOs that exerted higher As(III) oxidation and growth under given environmental conditions could become dominant over other CAOs. Thus, the CAOs enrichment culture demonstrated the high potential of efficient As(III) oxidation activity under various levels of arsenic contaminated water. Considering the application for the pretreatment step in the remediation of arsenic-contaminated water, these characteristics of the enrichment culture are highly desirable since As(III) concentrations are continuously changing in the batch cultivation system. After As(III) oxidation, the obtained As(V) can be effectively removed from water by adsorption and/or co-precipitation process with high capacity adsorbents such as iron hydroxides and activated alumina. Overall, the CAOs enrichment culture developed here has higher flexibility and stability than pure CAO cultures for the preliminary As(III) oxidation step during the removal of arsenic from contaminated water. Further studies are needed to investigate the As(III) oxidation capacity of the enrichment culture in actual environmental conditions.



## **Chapter 3**

### **Isolation and characterizing of chemolithoautotrophic arsenite oxidizing (CAOs) bacteria for arsenic removal from contaminated water**

#### **3.1 Introduction**

As(III) oxidizing bacteria are classified into heterotrophic and chemolithoautotrophic groups (HAOs and CAOs, respectively). In general, HAOs oxidize As(III) for detoxification, while CAOs utilize As(III) as an electron donor for energy acquisition. CAOs are more beneficial as remediating agents because their use can prevent increasing remediation costs and the occurrence of secondary contamination associated with the need to add external organic carbon for HAOs. Several CAOs have been isolated from contaminated aquatic environments, mine residues, and drainage and employed for As(III) remediation studies (Weeger et al., 1999; Santini et al., 2000, 2002; Battaglia-Brunet et al., 2002, 2005; Duquesne et al., 2007; Michel et al. 2007).

Since CAOs generally show higher specific As(III) oxidizing rate than HAOs (Lugtu et al., 2009), they are more attractive in light of practical remediative applications. The evidence that CAOs can also grow under the heterotrophic conditions (Santini et al., 2000; Oremland et al., 2002; Battaglia-Brunet et al., 2005; Duquesne et al., 2007) would be another advantage of the application of CAOs for arsenic bioremediation. However, most As(III) oxidizing bacteria isolated so far were heterotrophs, and only limited numbers of CAOs have been isolated and characterized on their As(III) oxidation ability in detail (Battaglia-Brunet et al., 2002; Santini et al., 2002; Duquesne et al., 2007). Besides, few reports have described arsenic removal using CAOs (Battaglia-Brunet et al., 2002; Lugtu et al., 2009). Consequently, more studies must be undertaken to establish a bioremediation strategy for arsenic-contaminated groundwater using CAOs.

In this Chapter, to obtain effective As(III) oxidizing bacteria for arsenic removal from groundwater, those present in the enrichment culture, constructed from arsenic-contaminated soil under the autotrophic condition in the study shown in Chapter 2, were isolated and characterized on their As(III) oxidation abilities by As(III) oxidation studies. Molecular analysis of As(III) oxidase genes, as well as their basic physiological properties and phylogenetic positions was also performed for their detail

characterization.

## **3.2 Materials and methods**

### **3.2.1 Isolation of CAOs from an enrichment culture**

Aliquots (100 mL) of the enrichment culture after cycle 32 and 46 were plated onto tryptic soy broth (TSB; Becton-Dickinson, Franklin Lakes, NJ, USA) agar containing 1 mM As(III) and then incubated at 28°C. Morphologically distinct colonies were selected and purified by streaking onto the same agar medium, after which they were incubated in BSM shown in 2.2.2 containing 1 mM As(III) and again plated onto TSB agar containing 1 mM As(III). Isolated strains were then subjected to T-RFLP analysis to determine their presence and dominance in the enrichment culture.

### **3.2.2 Physiological and phylogenetical characterization of isolated CAOs**

The isolated bacterial strains were characterized and identified by physiological and phylogenetic analyses. Microscopic observation for cell morphology and motility, gram staining, and catalase, oxidase and oxidation-fermentation (OF) tests were conducted to physiologically characterize the isolated strains. Phylogenetic analyses based on their partial 16S rRNA gene sequences were performed as described previously (Inoue et al., 2008).

### **3.2.3 As(III) oxidation studies of isolated CAOs**

As(III) oxidation studies were conducted using the growing cells and whole cells. All cultivations were carried out on a rotary shaker (120 r/min) at 28 °C. Prior to the As(III) oxidation studies, isolated strains were grown to the late logarithmic phase in TSB supplemented with 1 mM As(III). Next, 1 mL aliquots of the culture were transferred to 50 mL glass vials containing 20 mL of fresh medium and grown again to the late logarithmic phase. The cells were then harvested by centrifugation (15,000 ×g, 4 °C, 10 min) and washed three times with 5 mg/L sodium tripolyphosphate solution. For cell growth assays, the washed cells were inoculated into 50 mL glass vials containing 20 mL BSM supplemented with 1, 5, or 10 mM As(III) to a final cell density of approximately 0.02 (based on the optical density at a wavelength of 600 nm (OD<sub>600</sub>)). For whole cell assays, As(III) was added to BSM at 0.1, 0.5, 1, 5, or 10 mM, and the

bacterial cells were then inoculated at an OD<sub>600</sub> of 0.2. During As(III) oxidation studies, 1 mL of the culture was collected at appropriate intervals to measure the concentrations of As(III) and As(V). The As(III) oxidation kinetic parameters of six CAO strains were determined from Lineweaver-Burk plots of the data.

#### **3.2.4 Analysis of arsenite oxidase genes of the isolated CAOs**

To characterize the arsenite oxidase genes of the isolates, the *aoxB* gene was analyzed. It is equivalent to two synonyms, *aroA* and *asoA* genes, and it encodes the large Mo-pterin subunit of arsenite oxidase. Partial sequences of the *aoxB* genes were amplified using primers *aoxBM1*-2F and *aoxBM3*-2R (Quéméneur et al., 2008). Then PCR products of the expected length were sequenced.

#### **3.2.5 Analytical methods**

Bacterial growth was monitored based on changes in the OD<sub>600</sub> using a UV1200 spectrophotometer (Shimadzu, Japan). The dry cell weight was calculated from the linear relationship between the OD<sub>600</sub> and the dry cell weight, which was determined prior to the experiments.

To determine the sizes of the terminal restriction fragments (T-RFs) of the isolated strains, their colonies on TSB agar were directly subjected to PCR amplification of the 16S rRNA genes. The resultant PCR products were subsequently subjected to T-RFLP analysis as described in Chapter 2.

To determine the arsenic concentrations, aqueous samples taken from the culture were centrifuged (20,000 ×g, 4°C, 10 min) and then filtered through a Dismic-25 cellulose acetate filter (pore size, 0.45 µm, Advantec), after which the filtrates were stored at 4°C until analysis. As(V) and total arsenic were assessed using ion chromatography (HIC-20A Super system; Shimadzu) as described in Chapter 2. The As(III) concentration was calculated based on differences between the total arsenic and As(V) concentrations.

#### **Nucleotide sequence accession numbers**

The sequence data for 16S rRNA genes and *aoxB* genes of isolated strains were registered, respectively, to the DDBJ/EMBL/GenBank under accession numbers of

AB638424–AB638433 and AB638434–AB638439.

### 3.3 Results and discussion

#### 3.3.1 Isolation of CAOs from the enrichment culture

After cycle 32 and 46, during which approximately 10 mM As(III) was stably oxidized and the microbial community composition determined by T-RFLP analysis was unchanged, the bacterial members present in the enrichment culture were isolated. First, we tried to isolate CAOs in the enrichment culture using BSM containing As(III) and inorganic carbon source, but unfortunately failed. Because aforementioned experiments revealed that CAOs in the enrichment were mixotrophs, we next tried to isolate not obligate CAOs but facultative CAOs using TSB agar, a nutrient-rich medium, which was supplemented with 1 mM As(III). Overall, ten morphologically unique bacterial colonies were isolated and designated as strains A, B1, B2, C, D, E1, E2, F, G and H. Preliminary screening of their As(III) oxidizing abilities with 1 mM As(III) indicated that seven strains (B1, B2, C, D, E1, E2 and F) were CAOs, while strains A, G and H did not exhibit significant As(III) oxidizing abilities and were considered to be coexisting bacteria with certain As(III) tolerance (data not shown). As(III) oxidizing ability of strain F was lost its during continuous subculturing.

#### 3.3.2 Physiological and phylogenetical characterization of isolated CAOs

As shown in Table 3.1, physiological analyses revealed that all isolated strains were rod-shaped, gram-negative, catalase-positive, and motile bacteria. Phylogenetic analysis based on partial 16S rRNA gene sequences divided 10 strains into 5 genera belonging to  $\alpha$ -*Proteobacteria* (2 genera),  $\beta$ -*Proteobacteria* (2 genera), and  $\gamma$ -*Proteobacteria* (1 genus). Strain A was a member of the genus *Pseudomonas*. Its closest relative was *Pseudomonas fuscovaginae* ICMP 5940<sup>T</sup> with sequence similarity of 97.9%. Strains B1, B2, and C were all members of the genus *Hydrogenophaga*. They exhibited, respectively, 96.0%, 96.4%, and 98.6% sequence similarity with *Hydrogenophaga defluvii* BSB 9.5<sup>T</sup>. Furthermore, they shared 94.8%, 95.1%, and 97.3% sequence similarity with a previously reported CAO, *Hydrogenophaga* sp. CL3 (Rhine et al., 2007). Strains D, E1, and E2 belonged to the genus *Achromobacter*. They were, respectively, most closely related to *Achromobacter spanius* LMG 5911<sup>T</sup> and an

HAO strain BEN-4 (Santini et al., 2002) with 98.9%, 97.9%, and 98.5% sequence similarity. Strain F was most closely related to *Shinella kummerowiae* CCBAU 25048<sup>T</sup>, with 98.3% sequence similarity. By contrast, although strains G and H appeared to be the members of the genus *Methylobacterium*, they showed only 91.1% sequence similarity with the closest relative *Methylobacterium brachiatum* B0021<sup>T</sup>, suggesting that they might be novel species.

Most previously isolated CAOs belonged to  $\alpha$ -*Proteobacteria*, such as the genera *Rhizobium*, *Sinorhizobium*, *Agrobacterium* (Santini et al., 2002) and *Bosea* (Rhine et al., 2008). It is particularly interesting that 6 facultative CAOs isolated in this study were members of  $\beta$ -*Proteobacteria*, to which only a few CAOs belonged (Battaglia-Brunet et al., 2005; Rhine et al., 2007). Although strains G and H did not exhibit the As(III) oxidizing capability, to our knowledge, this is the first report about *Methylobacterium* that can tolerate high As(III) concentrations.

Tab. 3.1 Physiological, phylogenetical and As(III) oxidation characteristics of the strains isolated from the enrichment

Strain	Gram	Shape	Motility	OF test	Catalase	Oxidase	Assumed genus <sup>a</sup>	As(III) oxidizing ability <sup>b</sup>	<i>aoxB</i> gene <sup>c</sup>
A	—	Short rod	+	Oxidative	+	+	<i>Pseudomonas</i>	—	—
B1	—	Short rod	+	—	+	+	<i>Hydrogenophaga</i>	+	+
B2	—	Short rod	+	—	+	+	<i>Hydrogenophaga</i>	+	+
C	—	Short rod	+	—	+	+	<i>Hydrogenophaga</i>	+	+
D	—	Short rod	+	—	+	+	<i>Achromobacter</i>	+	+
E1	—	Short rod	+	—	+	+	<i>Achromobacter</i>	+	+
E2	—	Short rod	+	—	+	+	<i>Achromobacter</i>	+	+
F	—	Short rod	+	—	+	+	<i>Shinella</i>	—	—
G	—	Short rod	+	—	+	—	<i>Methylobacterium</i>	—	—
H	—	Short rod	+	—	+	—	<i>Methylobacterium</i>	—	—

<sup>a</sup> Assumed genus based on 16S rRNA gene sequence similarity.

<sup>b</sup> As(III) oxidizing ability under the autotrophic condition.

<sup>c</sup> Possession of *aoxB* genes amplifiable by the PCR condition developed by Quéméneur *et al.* (2008).

### 3.3.3 T-RFLP analysis of isolated CAOs

T-RFLP analysis of ten isolated strains revealed that each isolate represented the following sizes of T-RF: strain A, 201-203 bp; strains B1, B2 and C, 141-143 bp; strains D, E1 and E2, 568-569 bp; strain F, 338-340 bp; strains G and H, 342-346 bp (Figure 3.1). The T-RF of 201-203 bp represented by strain A corresponded to the dominant T-RF during cycle 8, while the T-RF for strains B1, B2 and C was identical to

the dominant T-RF during cycle 19 (Figures 2.5 and 3.1). However, T-RFs represented by the other isolates were not dominant in the enrichment culture described in Chapter 2. These results verified that the stable enrichment culture obtained in this study was composed of dominant and non-dominant CAOs, as well as other various bacteria.

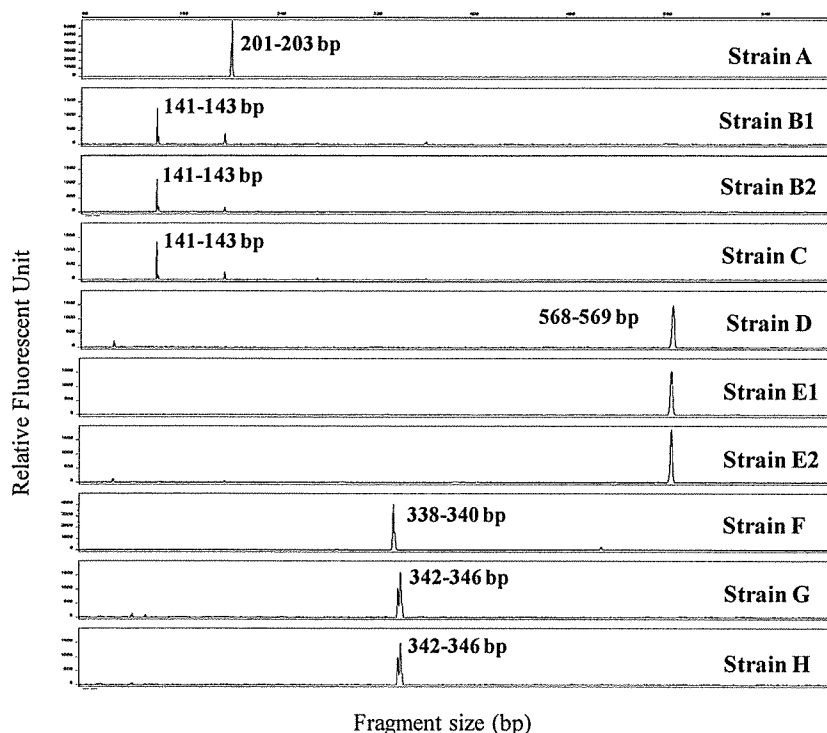


Fig. 3.1 T-RFs of ten isolated strains.

### 3.3.4 As(III) oxidation ability with growing cells of isolated CAOs

Among 7 isolated facultative CAOs, strain F lost its As(III) oxidizing capability during repeated subculturing. There, 6 strains (B1, B2, C, D, E1 and E2) that were confirmed as CAOs and stably exhibited As(III) oxidizing ability were further evaluated for their As(III) oxidizing ability using BSM with 1, 5 and 10 mM As(III).

Although all six strains were able to oxidize 1 to 10 mM As(III), their oxidizing characteristics considerably differed (Figure 3.2). Specifically, strains B1, B2, E1 and E2 showed high As(III) oxidizing ability at all tested As(III) concentrations, and could oxidize 5 and 10 mM As(III) completely within 72 and 120 hr, respectively. Strains B1, B2 and E1 also oxidized 1 mM As(III) completely within 12 hr, while strain E2 required 24 hr. Strain C showed similar As(III) oxidizing ability at 1 mM As(III) to

that of strains B1, B2, E1 and E2. In addition, strain C had the highest ability among the six strains at 5 mM As(III), being able to complete oxidize it within 48 hr. However, when As(III) was added at 10 mM, this strain oxidized only approximately 2 mM within 120 hr. The As(III) oxidizing ability of strain D was lowest among the six strains investigated, requiring 24 and 120 hr for complete oxidation of 1 and 5 mM of As(III), respectively. However, the As(III) oxidizing ability of strain D at 10 mM was slightly higher than that of strain C.

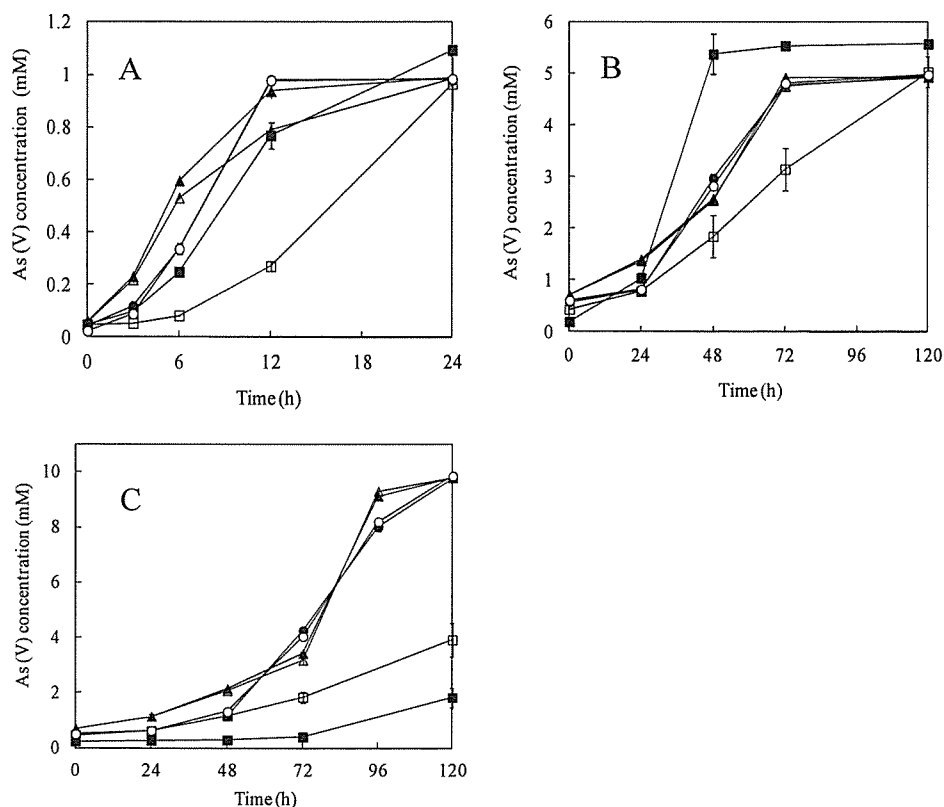


Fig. 3.2 Oxidation of 1 mM (A), 5 mM (B) and 10 mM (C) As(III) by six isolated CAO strains. Symbols: ●, strain B1; ○, strain B2; ■, strain C; □, strain D; ▲, strain E1; △, strain E2. Error bars indicate the standard deviation obtained from three independent experiments.

### 3.3.5 As(III) oxidation ability with whole cells of isolated CAOs

The effects of As(III) concentration (0.1 to 10 mM) on As(III) oxidation ability of the six CAO strains were also examined by whole cell assays. The results are summarized in Figure 3.3. The specific As(III) oxidation rates of strains B1, B2, D, E1 and E2 increased with increasing As(III) concentration from 0.1 to 10 mM, and reached

similar maximum values between 0.22 and 0.28 (mmol-As(III)/(mg cell·hr)) at 10 mM. In contrast, the specific As(III) oxidation rate of strain C reached a maximum of 0.20 (mmol-As(III)/(mg cell·hr)) at 5 mM, and declined to 0.12 (mmol-As(III)/(mg cell·hr)) at 10 mM.

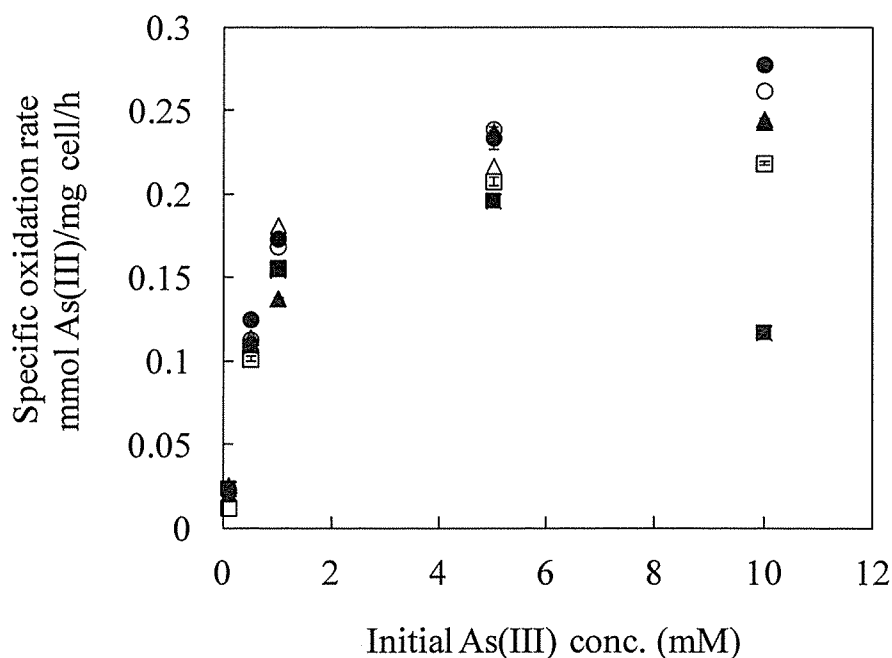


Fig. 3.3 Effects of the initial As(III) concentration on the specific As(III) oxidation rate of six isolated CAO strains. Symbols: ●, strain B1; ○, strain B2; ■, strain C; □, strain D; ▲, strain E1; ◻, strain E2. Error bars indicate the standard deviation obtained from three independent experiments.

The experimental data were further analyzed to determine the oxidation and substrate inhibition kinetics of As(III) oxidation. Oxidation of up to 10 mM of As(III) by strains B1, B2, D, E1 and E2 and up to 5 mM As(III) by strain C can be represented by the Monod equation:

$$V = V_{\max} \cdot S / (K_m + S)$$

where,  $V$  (mmol-As(III)/(mg cell·hr)) and  $V_{\max}$  (mmol As(III)/(mg cell·hr)) are the actual and maximum specific oxidation rate,  $S$  (mM) is the initial As(III) concentration, and  $K_m$  (mM) represents the half saturation constant. The data for the six CAO strains fit the equation well, with correlation coefficients ( $r^2$ ) ranging from 0.96 to 0.99. The As(III) oxidation kinetic parameters ( $V_{\max}$  and  $K_m$ ) determined for the six CAO strains



are shown in Table 3.2.

Tab. 3.2 Kinetic parameters for As(III) oxidation by six isolated CAO strains determined from the Lineweaver-Burk plot based on the experimental data obtained from whole cell assays

Strain	$V_{\max}$ (mmol As(III)/(mg cell·hr))	$K_m$ (mM)
B1	0.28	0.61
B2	0.28	0.73
C	0.22	0.51
D	0.24	0.66
E1	0.25	0.68
E2	0.26	0.60

Although all six CAO strains had similar  $V_{\max}$  values, those of strains B1 and B2 were highest (0.28 mmol As(III)/(mg cell·hr)). The  $K_m$  values of the six CAO strains ranged from 0.51 to 0.73 mM. Strain C had the lowest  $K_m$  value of 0.51 mM, indicating that it has the highest affinity for As(III) among the six CAO strains.

Although strains B1, B2 and C exhibited the same T-RF, which was identical to the dominant T-RF in the enrichment culture adapted to 10-12 mM As(III) (Figures 2.5 and 3.1), their As(III) oxidation activities differed. Strains B1 and B2 were capable of completely oxidizing 10 mM As(III) within five days in the growing cell assays, and no significant inhibition at concentrations of As(III) up to 10 mM was observed in the whole cell assays. In contrast, although strain C could oxidize 1 and 5 mM As(III) rapidly and completely, at 10 mM As(III) its oxidation did not proceed efficiently. Therefore, it was assumed that strains B1 and B2 played a primary role in the oxidation of 10 mM As(III) in the stabilized enrichment culture obtained in Chapter 2, while other CAOs grew slightly via oxidation of As(III) after B1 and B2 oxidized it to some extent. Consequently, a variety of CAOs with distinct As(III) oxidation characteristics appeared to coexist in the enrichment culture described in Chapter 2. Coexistence of multiple bacterial species in CAOs enrichment culture was also reported in a study conducted by Battaglia-Brunet et al. (2002).

Three isolated strains, A, G and H, did not exhibit significant As(III) oxidizing

ability under autotrophic conditions. Those isolates might simply be co-existing bacteria with high As(III) tolerance that are capable of growth by utilizing inorganic carbon in the medium or organic carbon derived from dead CAOs. However, T-RFLP analysis indicated that strain A was dominant in the enrichment culture at 2.6 mM As(III). This suggests that strain A contributed to As(III) oxidation in the enrichment culture. One possible explanation for this finding is that strain A can only oxidize As(III) in mixed cultures via certain interactions with other bacteria; nevertheless, further study of strain A is warranted. Moreover, strain F has lost the ability to oxidize As(III) during repeated subculturing. This may have been due to the lack of some trace (but vital) nutrients produced by other bacteria in the enrichment culture, or the deprivation of plasmids in which the As(III) oxidation genes were located.

### 3.3.6 Analysis of arsenite oxidase genes of the isolated CAOs

PCR amplification of the partial *aoxB* genes was performed against all of the 10 isolated strains. Consequently, PCR products of expected length were detected in 6 strains B1, B2, C, D, E1, and E2 (Table 3.1), which agreed with the As(III) oxidizing capabilities of these strains. This finding corroborates the ideas of Cai *et al.* (2009) that the *aoxB* gene might be specific for most of the aerobic As(III) oxidizing bacteria.

Figure 3.4 shows phylogenetic positions of the *aoxB* genes and two synonyms (*aroA* and *asoA* genes) of 6 isolated CAOs in this study and other As(III) oxidizing bacteria reported previously. The *aoxB* sequences of 6 strains allocated in 2 subclusters of a large  $\beta$ -*Proteobacteria* cluster, which agreed with their phylogenetic positions. The *aoxB* sequences of strains B1, B2, and C had 98.1–99.6% nucleotide identity to one another, and most closely related to the *aroA* sequence of *Hydrogenophaga* sp. WA13, with 89.9%, 89.7%, and 88.3% sequence similarity, respectively. However, the *aoxB* sequences of strains D, E1, and E2 were 100% mutually identical, and were most closely related to the *aoxB* gene of *Achromobacter* sp. SY8 with 96.0% sequence similarity. Although strains D, E1, and E2 had distinct As(III) oxidizing characteristics (Figure 3.2), their *aoxB* gene sequences were identical. The same was true for strains B1, B2, and C. Those might be explained by differences in the structure and arrangement of the respective *aox* clusters, including the presence/absence of the arsenite oxidase Fe–S Rieske subunit (*aoxA*) and other signal transduction systems. These characteristics have

been reported affect the gene expression level (Cai et al., 2009). Another possible reason was the difference in As(III) tolerance or in the regulation system of As(III) oxidizing genes.

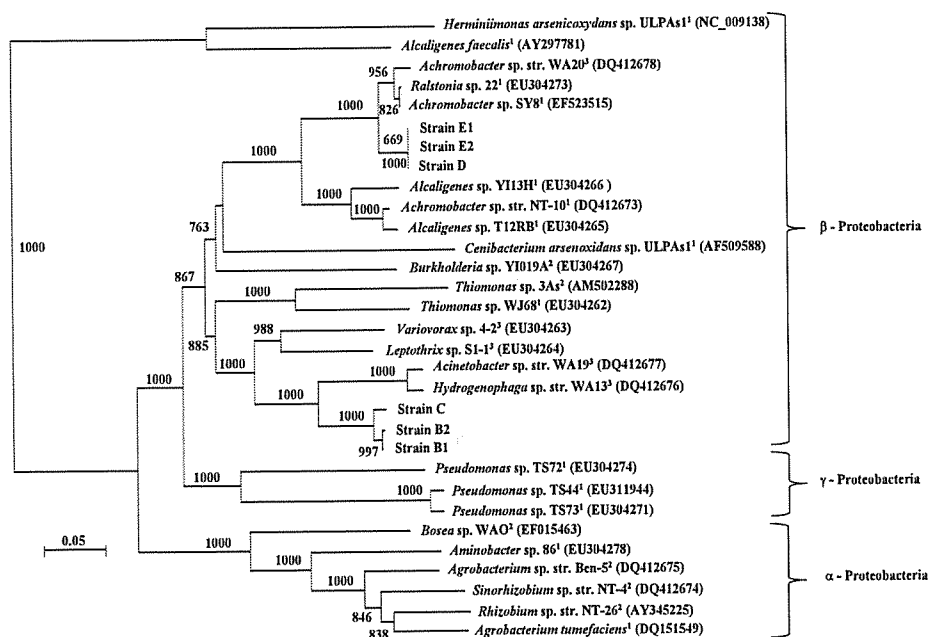


Fig. 3.4 Neighbor-joining tree of *aoxB* genes and two synonyms (*aroA* and *asoA* genes) of CAOs isolated in this study and previously-isolated As(III) oxidizing bacteria (<sup>1</sup>HAO; <sup>2</sup>CAO; <sup>3</sup>undefined As(III) oxidizing bacteria). Scale bar corresponds to the 0.05 substitutions per nucleotide position. Numbers at nodes show bootstrap values obtained from 1000 replicates.

### 3.4. Conclusions

In this chapter, CAOs were successfully isolated from the established enrichment culture that had different As(III) oxidation abilities. Particularly, strains B1, B2, E1, and E2 were effective for oxidation 1–10 mM As(III) under the autotrophic conditions used here. The results show that the isolated strains might be applicable to the bioremediation of arsenic-contaminated groundwater. After As(III) oxidation, the obtained As(V) can be effectively removed from water by adsorption and/or co-precipitation process with high capacity adsorbents such as iron hydroxides and activated alumina. Further studies are needed to investigate the As(III) oxidation capacity of the isolated strains in actual environmental conditions.

## Chapter 4

### **Demonstration of enhanced arsenic adsorption by activated alumina with bacterial oxidation of As(III)**

#### **4.1 Introduction**

Drinking water contaminated with arsenic has caused many health problems to people all over the world, especially in developing countries. In 1993, The World Health Organization set an arsenic safety level of 10 µg/L in its drinking water guidelines. Therefore, the need of high efficiency, low cost and environmentally friendly technologies are essential for As(III) removal of drinking water to make water safe for drinking.

Because As(III) is predominant in groundwater and is more difficult to remove than As(V) is, oxidation of As(III) to As(V) is necessary to improve overall arsenic removal (Battaglia-Brunet et al., 2002; Smedley and Kinniburgh, 2002; Lièvremonet et al., 2003; Oremland and Stolz, 2003). In practical applications, arsenite oxidizing bacteria, which are specific to As(III) oxidation, has gained an increased concern as the oxidation catalyses because they can reduce occurrence of harmful by-products and treatment cost in comparison with chemical oxidation of As(III) (Ike et al., 2008; Lièvremonet et al., 2009; Wang and Zhao, 2009). Moreover, chemolithoautotrophic arsenite oxidizers (CAOs) with very low nutritional requirement are more beneficial as remediating agents (Wan et al., 2010). As described in Chapter 3, the CAO strain B1 isolated from the arsenic contaminated soil allowed efficient As(III) biological oxidation with both the growing cells and whole cells systems. Thus, it seems attractive to use this strain for oxidizing As(III) in arsenic contaminated water (containing As(III) as the dominant species), so as to improve the arsenic removal efficiency by coagulation/co-precipitation or adsorption.

The objectives of this chapter were: (1) to investigate the As(III) oxidizing capacity of the isolated strain B1 with various initial As(III) concentrations in model contaminated waters to confirm that the As(III) oxidation ability of this strain can be exhibited in various environmental conditions, and (2) to experimentally demonstrate that the arsenic removal by adsorption onto activated alumina (AA) in the model contaminated waters can be enhanced with the CAO strain B1 mediated As(III)

oxidation. Laboratory batch arsenic adsorption experiments using AA were conducted to evaluate the arsenic adsorption isotherms before and after the CAO strain B1 mediated oxidation of As(III) in model natural waters with low and high hardness.

## **4.2 Materials and methods**

### **4.2.1 Bacterial strain**

A CAO, strain B1, which can use As(III) as sole energy source for chemolithoautotrophic growth, was isolated from the enrichment of As(III) oxidizing bacteria originated from an arsenic contaminated soil described in Chapters 2 and 3. This strain was used in the experiments of this Chapter.

### **4.2.2 Arsenic stock solution**

The 500 mM As(III) stock solution was prepared by dissolving NaAsO<sub>2</sub> in ultra pure water, and the solution was sterilized by filtration through a Dismic-25 cellulose acetate filter (pore size 0.22 µm, Advantec, Tokyo, Japan).

### **4.2.3 Basal salt medium (BSM) and model contaminated waters**

A slightly modified medium which was described by Battaglia-Brunet et al. (2002) was used as a basal salt medium (BSM) for As(III) oxidation studies. The medium consisted of K<sub>2</sub>HPO<sub>4</sub>, 0.25 g/L; KH<sub>2</sub>PO<sub>4</sub>, 0.25 g/L; NaCl, 0.25 g/L; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.1 g/L; MgSO<sub>4</sub>, 0.05 g/L; CaCl<sub>2</sub>, 0.1 g/L; trace element solution (Battaglia-Brunet et al., 2002), 1 mL/L; and vitamin solution, 10 mL/L (Battaglia-Brunet et al., 2002). Model arsenic contaminated waters were prepared by simulating the composition of two commercial bottled natural waters, Rokko and Contrex waters, respectively. Table 4.1 shows the components of commercial Rokko and Contrex waters which represent low and high hardness natural waters, respectively. The components of model Rokko and Contrex waters are listed in Table 4.2 in which the concentrations of trace element and vitamin solutions were added in the same concentrations as BSM. In addition, 10 mL/L NaHCO<sub>3</sub> (0.5 mg/L) was added as the carbon source for growth of the CAO. The pH of the BSM, model contaminated waters was adjusted to 6.0 with H<sub>2</sub>SO<sub>4</sub>.

Tab. 4.1 Composition of commercial Rokko and Contrex waters

Component (mg/L)	Rokko water	Contrex water
Ca	6.5	468
Mg	3.7	74.5
K	0.8	2.8
Na	29.5	9.4
Hardness	32	1468
pH	7.2	7.3

Tab. 4.2 Composition of model Rokko and Contrex waters

Solution (mg/L)	Rokko water	Contrex water
K <sub>2</sub> HPO <sub>4</sub>	1.1	3.8
KH <sub>2</sub> PO <sub>4</sub>	1.1	3.8
NaCl	74.9	23.9
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	100	100
MgSO <sub>4</sub> ·7H <sub>2</sub> O	37.5	755.5
CaCl <sub>2</sub> ·2H <sub>2</sub> O	23.8	1716.8
NaHCO <sub>3</sub> (Carbon Source)	500	500
Trace element solution (mL/L) <sup>a</sup>	1	1
Vitamin solution (mL/L) <sup>a</sup>	10	10

<sup>a</sup> Described by Battaglia-Brunet et al. (2002)

#### 4.2.4 As(III) oxidation studies with CAO strain B1 in model contaminated waters

As(III) oxidation capability of CAO strain B1 in BSM and model contaminated waters was evaluated with batch cultivation. All cultivation was conducted on a rotary shaker (120 r/min) at 28°C. Before the As(III) oxidation test, cells of strain B1 were grown to the late logarithmic phase in 20 mL glass vials containing trypticase soy broth (TSB; Becton-Dickinson, Franklin Lakes, NJ, USA) supplemented with 1 mM As(III). Next, 1 mL aliquots of the culture were transferred to 50 mL glass vials containing 20 mL of fresh TSB medium with 1mM As(III), and grown again to the late logarithmic phase. The cells were harvested by centrifugation (15000 × g, 4°C, 10 min), washed three times with 5 mg/L sodium tripolyphosphate solution, and inoculated into 20 mL

BSM, model Rokko and Contrex waters supplemented with 0.1 and 1 mM As(III), respectively, to a final cell density (determined by optical density at a 600 nm wavelength [OD<sub>600</sub>]) of ca. 0.02 for starting of As(III) oxidation studies. At appropriate intervals, 1 mL of the culture was collected to measure the concentrations of As(III) and As(V). Control experiments were also done in the same manner without adding strain B1.

#### **4.2.5 Preparation of activated alumina (AA) adsorbent**

AA (300 mesh) (Wako Pure Chemical, Osaka, Japan) was oven-dried (100°C, 24 hr) and stored in a desiccator for use in arsenic adsorption experiments.

#### **4.2.6 Adsorption of arsenic by AA before and after microbial As(III) oxidation**

Batch adsorption experiments were performed to determine the amount of arsenic adsorption by AA in model contaminated waters before and after biological As(III) oxidation. Batch adsorption studies were conducted by shaking BSM or model Rokko and Contrex waters (containing arsenic from 75 to 1000 µg/L) before and after the As(III) oxidation experiments by CAO strain B1 with 0.667 or 10 g/L AA on a mechanical shaker (120 r/min, 28°C). The samples were taken after 24 hr of the reaction time for analyzing the As(III) and As(V) concentrations.

#### **4.2.7 Analytical methods**

Bacterial growth was monitored by measuring the increase in OD<sub>600</sub> using a spectrophotometer (UV1200, Shimadzu, Kyoto, Japan).

To determine the arsenic concentrations in As(III) oxidation study, aqueous samples taken from the culture were centrifuged (20,000 ×g, 4°C, 10 min) and then filtered through a Dismic-25 cellulose acetate filter (pore size, 0.45 µm, Advantec), after which the filtrates were stored at 4°C until analysis. As(V) and total arsenic were assessed using ion chromatography (HIC-20A Super system; Shimadzu) as described in Chapter 2. The As(III) concentration was calculated based on differences between the total arsenic and As(V) concentrations.

To determine the arsenic concentrations in arsenic adsorption studies, aqueous samples taken from the culture were filtered through a Dismic-25 cellulose acetate filter

(pore size, 0.45  $\mu\text{m}$ , Advantec), after which the filtrates were stored at 4°C until analysis. Aqueous As concentrations in collected samples were measured by ICP-AES (SPS 7800, Seiko Instruments, Chiba, Japan) coupled with hydride generation system (THG-1200, Seiko Instruments). Prior to the analysis, 1 mL of 40% KI and 1 mL of 6 M HCl were added to 8 mL of the stored samples, after which the samples were pre-reduced for at least 1 hr.

#### 4.2.8 Adsorption isotherms

In the adsorption process, the distribution of arsenic between the adsorbent surface and the solution at the equilibrium at a given temperature can be expressed by adsorption isotherm models. Among several adsorption isotherm models based on different assumptions, Langmuir and Freundlich isotherms, which have been the most commonly used (Dang et al., 2009), were applied to analyze the data obtained in the adsorption experiments.

Langmuir isotherm, which presents a theoretical adsorption isotherm for ideal sorption of tested compound on the homogenous surface of solid adsorbent with mono layer sorption, is given by the following equation:

$$Q_e = Q_{\max} \cdot C_e \cdot K_L / (1 + K_L \cdot C_e) \quad \text{eq (1)}$$

A linear form of eq (1) can be expressed as follows:

$$1/Q_e = 1/(Q_{\max} \cdot C_e \cdot K_L) + 1/Q_{\max} \quad \text{eq (2)}$$

where  $Q_e$  is the adsorbed arsenic amount per unit mass of total AA at equilibrium ( $\mu\text{g-As/mg-AA}$ );  $Q_{\max}$  is the arsenic adsorption capacity of adsorbent ( $\mu\text{g-As/mg-AA}$ );  $C_e$  is the aqueous arsenic concentration at equilibrium ( $\mu\text{g/L}$ );  $K_L$  is the Langmuir constant related to the binding strength ( $\text{L}/\mu\text{g}$ ). The  $Q_{\max}$  and  $K_L$  values were determined from the slope and intercept of the linear regression of  $1/C_e$  and  $1/Q_e$  plots.

Freundlich isotherm, which presents an empirical adsorption isotherm for non-ideal sorption of tested compound on heterogenous surfaces and multilayer sorption, is given by the following equation:

$$Q_e = K_F \cdot C_e^{1/n} \quad \text{eq (3)}$$

A linear form of eq (3) can be expressed as follows:

$$\log Q_e = \log K_F + 1/n(\log C_e) \quad \text{eq (4)}$$

where  $K_F$  and  $n$  are the Freundlich constants related to the arsenic adsorption capacity



and intensity, respectively. The  $n$  and  $K_F$  values were determined from the slope and intercept of the linear regression of  $\log C_e$  and  $\log Q_e$  plots.

### 4.3 Results and discussion

#### 4.3.1 As(III) oxidation studies with CAO strain B1 in model contaminated waters

In Chapter 3, we elucidated that CAO strain B1 were effective for oxidation of 1–10 mM As(III) under the autotrophic conditions. For better understanding the As(III) oxidizing ability of this strain from the viewpoint of practical application for enhanced arsenic removal, in this Chapter, we applied 0.1 and 1 mM initial As(III) concentration for As(III) oxidation experiments in model contaminated Rokko and Contrex waters which represent low and high hardness waters, respectively.

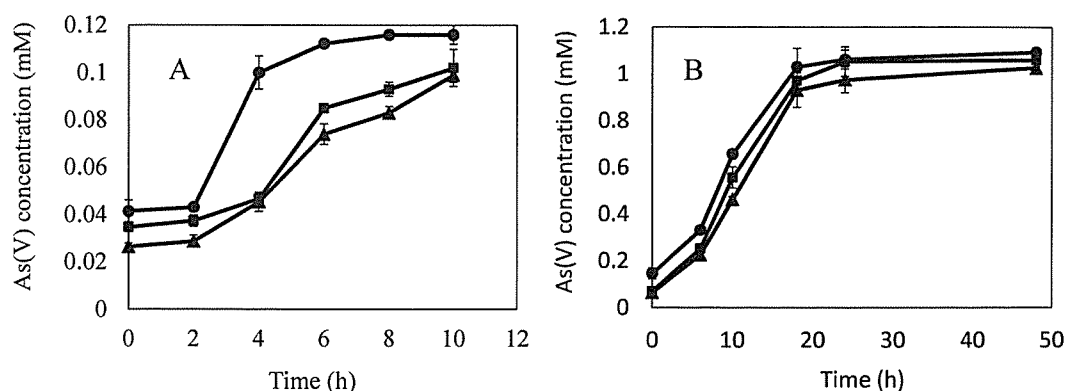


Fig. 4.1 Oxidation of 0.1 mM (A) and 1 mM (B) As(III) by strain B1 in different solutions. Symbols: ●, BSM; ■, model Rokko water; ▲, model Contrex water. Error bars indicate the standard deviation obtained from three independent experiments.

As shown in Figure 4.1, strain B1 could completely oxidize As(III) with both initial concentrations of 0.1 and 1.0 mM in all the tested matrix, i.e., BSM and two model contaminated waters. The As(III) oxidizing ability of strain B1 was slightly higher in BSM solution than that in model contaminated waters at 0.1 mM initial As(III) concentration, while its ability was almost the same at 1 mM initial As(III) concentration in all solutions. Furthermore, although the model Contrex water had higher concentrations of mineral composition than the model Rokko water (Table 4.2), the As(III) oxidation activity in the latter one was a little faster at 0.1 mM As(III) than

that of the former one; it took 8 and 24 hr (in BSM), 10 and 24 hr (in model contaminated Rokko and Contrex waters) for strain B1 to oxidize 0.1 and 1.0 mM As(III), respectively. These results suggested that strain B1 can effectively oxidize As(III) in natural waters independently of the harness, when enough nutrients (nitrogen and phosphorous) are supplemented. Another eminent feature of strain B1 as the biological As(III) oxidizing agent is its ability to oxidize As(III) of a wider concentration range. Although various CAOs have been reported so far, most of them could oxidize As(III) only at certain limited As(III) concentrations (Santini et al., 2000; Oremland et al., 2002; Battaglia-Brunet et al., 2006; Garcia-Dominguez et al., 2008; Lugtu et al., 2009). The results of this Chapter reveal that the CAO strain B1 could effectively oxidize As(III) from low to high concentration (0.1 – 1.0 mM). So it may be conclude that this strain is applicable for As(III) oxidation under a variety of the environmental conditions.

#### **4.3.2 Adsorption isotherms of arsenic by AA in model contaminated waters before and after microbial oxidation**

Equilibrium adsorption studies were carried out to determine the maximum arsenic intake on AA before and after the As(III) oxidation by strain B1. At first the adsorption experiments were done with 0.67 g/L AA in both BSM and model contaminated waters, but this amount of AA was not enough for arsenic adsorption in BSM as it contains relatively high concentrations of competing anions ( $\text{CO}_3^{2-}$ ,  $\text{Cl}^-$ ,  $\text{NO}_4^{3-}$ ,  $\text{PO}_4^{3-}$ ,  $\text{SO}_4^{2-}$ ). These results are in agreement with the study of Tripathy and Raichur (2008) which can be explained by that presence of high  $\text{PO}_4^{3-}$  concentration in the BSM is the strongest competitor of arsenic, specially As(V), for adsorption sites on the AA surface. Thus, higher adsorbent dose of 10 g/L AA was used for arsenic adsorption experiments in BSM, though the original dose (0.67 g/L AA) was used for the experiments in model contaminated waters.

As shown in Figure 4.2, the adsorbed amount of arsenic; As(III) (before bacterial oxidation) and As(V) (after bacterial oxidation); on AA increased with increased initial concentrations in both model contaminated Rokko and Contrex waters. On the other hand, though the adsorbed amount of arsenic in BSM increased with increased initial concentration from 75 to 500  $\mu\text{g/L}$  and 75 to 750  $\mu\text{g/L}$  for As(V) and

As(III), respectively, it decreased with high initial arsenic concentration (750, 1000  $\mu\text{g/L}$  As(V) and 1000  $\mu\text{g/L}$  As(III)).

To better understand the adsorption capacity of applied adsorbents, the Langmuir and Freundlich adsorption isotherms were applied to the equilibrium adsorption data, and isotherm coefficients were calculated for model contaminated water experiments, though they cannot be well applied to BSM experiments. Results of the regression by two different isotherms for As(V) and As(III) adsorption on AA are shown in Figure 4.2, and the calculated parameters are summarized in Table 4.3.

As shown in Figure 4.2 and Table 4.3, within the range of As concentration from 75 to 1000  $\mu\text{g/L}$ , the adsorption of both As(III) and As(V) by AA in model contaminated waters well obeyed the Langmuir and Freundlich isotherms with correlation coefficients ( $r^2$ ) higher than 0.971, and the amount of adsorbed As(V) resulting from As(III) microbial oxidation was always higher than that of As(III) as anticipated.

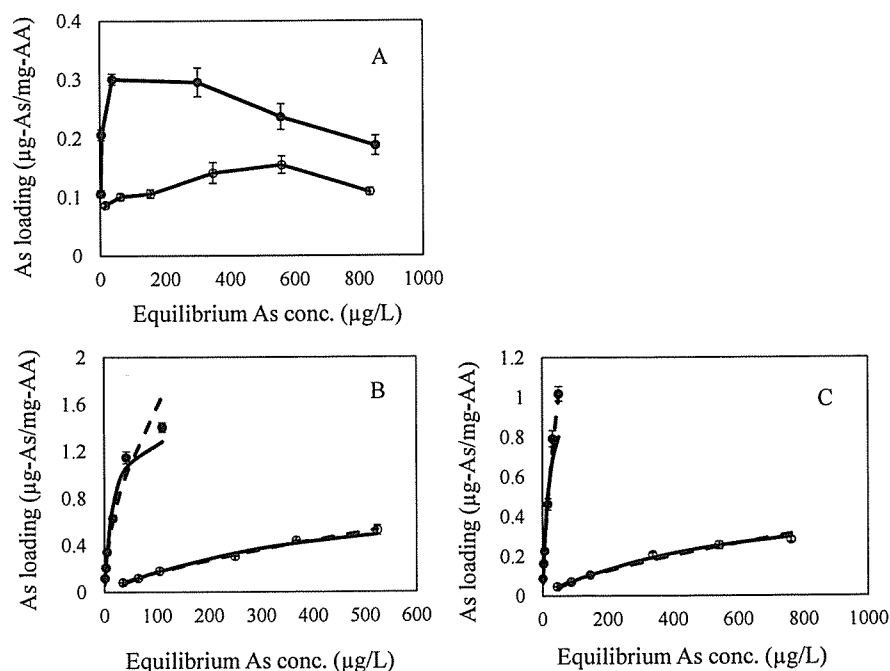


Fig. 4.2 Adsorption isotherms of arsenic by AA in BSM (A), model Rokko water (B) and model Contrex water (C). Symbols:  $\bullet$ , As(V);  $\circ$ , As(III); straight line, Langmuir isotherm; hidden line, Freundlich isotherm. Error bars indicate the standard deviation obtained from three independent experiments.

The regression by the Langmuir equation showed that the  $Q_{max}$  values were higher for As(V) resulting from As(III) microbial oxidation than for As(III) originally added into model contaminated waters. The maximum amount of arsenic adsorbed on AA were 1.48  $\mu\text{g-As(V)}/\text{mg-AA}$ , 0.89  $\mu\text{g-As(III)}/\text{mg-AA}$  and 1.13  $\mu\text{g-As(V)}/\text{mg-AA}$ , 0.53  $\mu\text{g-As(III)}/\text{mg-AA}$  in model contaminated Rokko and Contrex waters, respectively. These results are similar with those reported by Lin and Wu (2000), indicating typical behaviour for arsenic adsorption by AA at neutral pH, where As(III) exists as non-charged form ( $\text{H}_3\text{AsO}_3$ ), while As(V) exists as negative charge form ( $\text{H}_2\text{AsO}_4^-$  or  $\text{HAsO}_4^{2-}$ ). As(V) is adsorbed predominantly by AA through strong inner sphere complexes with a bidentate binuclear configuration. In contrast, both inner-sphere complexes and weaker outer-sphere complexes adsorb As(III) (Ike et al., 2008).

On the other hand, the maximum amount of arsenic adsorbed on AA in model contaminated Rokko water, which represents low hardness natural water, was higher than that in model contaminated Contrex water, which represents high hardness natural water. These results are consistent with those of other study (Rosenblum and Clifford, 1984) which indicated that arsenic adsorption is considerably reduced if the competing ions  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$  present in the solution.

Tab. 4.3 Arsenic adsorption constants of AA obtained from the regression by Langmuir and Freundlich isotherms

	Langmuir parameters			Freundlich parameters		
	$Q_0$	K	$R^2$	K	$1/n$	$R^2$
Model contaminated Rokko water [As(V)]	1.474	0.061	0.974	0.136	1.878	0.982
Model contaminated Rokko water [As(III)]	0.885	0.002	0.996	0.007	1.437	0.997
Model contaminated Contrex water [As(V)]	1.127	0.049	0.971	0.074	1.498	0.996
Model contaminated Contrex water [As(III)]	0.530	0.002	0.998	0.004	1.488	0.989

#### 4.3.3 Arsenic removal efficiency of AA in model contaminated waters before and after microbial oxidation

To clarify the effectiveness of microbial pre-treatment for arsenic removal by

AA, the removal efficiency of various initial concentrations of arsenic before and after microbial oxidation with 0.67 g/L of AA in model contaminated waters was evaluated (Table 4.4). Within the initial arsenic concentration range of 75-1000 µg/L, As(V) resulting from microbial As(III) oxidation was removed from the aqueous phase more efficiently than As(III) by adsorption on AA in both model contaminated waters. From Table 4.4, we can see that almost 90% of arsenic can be removed with all initial arsenic concentration in both model contaminated waters after microbial As(III) oxidation, excepting the case of 1000 µg/L As(V) in model contaminated Rokko water.

The large-scale, natural high arsenic contamination of groundwater can be mostly found in Asian countries (Karim, 2000; Nordstrom, 2002). Kinniburgh and Kosmus (2002) reported that Southern and Eastern areas of Bangladesh are the most contaminated areas, where median arsenic concentration in groundwater of 135 µg/L has been found. Berg et al. (2007) found arsenic concentrations as high as 845 µg/L in groundwater in the Mekong river delta. Arsenic contamination at 250 µg/L or higher has been commonly detected in the groundwater not only in developing Asian countries but also in American and European countries (Nordstrom, 2002). According to our experimental results, 250 µg/L of arsenic in model contaminated waters can be reduced below the WHO drinking water standard (10 µg/L), after 24 hr adsorption treatment with 0.67 g/L of AA after microbial As(III) oxidation.

The excellent removal of As(V) by adsorption using of AA as revealed in this Chapter confirmed that microbial As(III) oxidation as the pre-treatment is an important step in promoting the overall arsenic removal from contaminated water.

Tab. 4.4 Removal efficiency of As(V) and As(III) with AA in model contaminated waters

Initial concentration (µg/L)	Removal ratio (%)					
	75	150	250	500	750	1000
Model contaminated Rokko water [As(V)]	98.97	98.09	97.92	96.22	94.71	89.42
Model contaminated Rokko water [As(III)]	61.03	55.20	53.32	44.89	44.29	40.11
Model contaminated Contrex water [As(V)]	98.00	96.91	96.00	94.94	94.28	93.09
Model contaminated Contrex water [As(III)]	40.70	34.50	32.24	28.92	23.94	19.80

#### **4.4 Conclusions**

In this Chapter, the potential of the CAO strain B1 in the pre-treatment of As(III) containing natural waters for enhanced arsenic removal was experimentally investigated. Strain B1 could efficiently oxidize low concentration of As(III) in model contaminated waters which represent low and high hardness natural waters, indicating that this strain is applicable for waters with a wide range of the hardness as the pre-treatment catalyst, when enough nutrients are supplemented. It was also confirmed that the microbial oxidation can enhance the arsenic removal from low and high hardness model natural waters by adsorption with AA. This combination, microbial As(III) oxidation and AA adsorption, would improve the reliability of the As treatment and maintain As level within the acceptable drinking water standards, making it an essential strategy in the future. Consequently, low-cost, effective, and environmentally friendly treatment of arsenic from groundwater, which is favorable for the application in developing countries, would be possible by using this method. However, the presence of competing ions significantly decreased arsenic removal. Further investigation is needed for the full scale application to achieve complete arsenic in a long operating time.

## Chapter 5

### Summary and Conclusions

The pre-oxidation of As (III) to As (V) is essential for the effective removal of arsenic from water phase containing As(III) as a major component. The necessity of this process stems from the fact that As (III) is more toxic and mobile, thus, cannot be easily removed by relatively low-cost technologies like coagulation and coprecipitation/adsorption compared with As(V). Microbial As(III) oxidation has been regarded as an attractive alternative as pre-treatment of arsenic removal, because of its specific reaction for As(III), which can result in high efficiency, in addition to its cost-effective and environmentally friendly nature compared with chemical oxidation pre-treatment using  $\text{Cl}_2$ ,  $\text{MnO}_4$  and  $\text{O}_3$ . Microbial oxidation of As (III) to As(V) can be performed by CAOs and HAOs. Since CAOs generally possess higher specific As(III) oxidizing rate and need less nutrient addition than HAOs, CAOs are more attractive in light of practical remediative applications. This study focused on the use of CAOs as the pre-treatment agent for arsenic removal from waters containing As(III) as the main arsenic component.

In Chapter 2, an enrichment culture of CAOs that is useful for the pre-oxidation of As(III) was established and maintained for more than 700 days (more than 60 cycles of subculturing) by repeated subculturing with providing  $\text{NaHCO}_3$  and As(III) as the sole carbon and energy sources, respectively. The As(III) oxidizing capability of the enrichment culture was strengthened gradually and apparently during the enrichment process and 10-12 mmol/L As(III) was almost completely oxidized within four days. Terminal restriction fragment length polymorphism (T-RFLP) analysis showed that the dominant bacteria in the enrichment culture varied drastically during the enrichment process with increasing As(III) concentration from 1 to 10-12 mM and the bacterial community composition became stable with a dominant T-RF of 141-143 bp at 10-12 mM As(III). These results indicated that distinct CAOs that were well-adapted to a given As(III) concentration became dominant, and other CAOs that do not play a major role in oxidizing As(III) at certain As(III) concentrations can survive without complete disappearance in the enrichment culture.

In Chapter 3, ten morphologically unique bacterial colonies designated as strains A, B1, B2, C, D, E1, E2, F, G and H were successfully isolated using TSB agar supplemented with 1 mM As(III) from the established enrichment culture. Among them, seven strains (B1, B2, C, D, E1, E2 and F) were confirmed as CAOs, but As(III) oxidizing ability of strain F was lost during continuous subculturing. Six other strains (B1, B2, C, D, E1 and E2) belong to *β-Proteobacteria*, and commonly contained the *aoxB* genes encoding the arsenite oxidase large subunit. These isolated CAOs were considerably different in their As(III) oxidation capabilities. Strains B1, B2, E1 and E2 efficiently oxidized 1, 5, and 10 mM As(III). Although strain C oxidized 1 and 5 mM As(III) efficiently, its oxidizing capability was lowest among 6 strains at 10 mM. Furthermore, although the As(III) oxidizing capability of strain D was similar to those of strains B1, B2, E1 and E2 at 1 mM As(III), this strain was ineffective for 5 and 10 mM As(III). The  $V_{\max}$  and  $K_m$  values of the six CAO strains ranged from 0.22-0.28 mmol As(III)/(mg cell·hr) and 0.51-0.73 mmol/L, respectively.

In Chapter 4, the potential of the CAO strain B1 in the pre-oxidation of As(III) for enhanced arsenic removal in natural waters was experimentally investigated. Strain B1 could efficiently oxidize As(III) at both initial concentrations of 0.1 and 1.0 mM in model contaminated Rokko and Contrex waters which represent low and high hardness natural waters, respectively. It was also confirmed that the microbial oxidation can certainly enhance the arsenic removal from low and high hardness model natural waters by adsorption with AA. The adsorption of 75 to 1000 µg/L As(III) (before bacterial oxidation) and As(V) (after bacterial oxidation) by AA in model contaminated waters well obeyed the Langmuir and Freundlich isotherms with the correlation coefficients ( $r^2$ ) higher than 0.971, and the  $Q_{\max}$  values were higher for As(V) resulting from As(III) microbial oxidation than for As(III) originally added into model contaminated waters. The maximum amount of arsenic adsorbed on AA was 1.48 µg-As(V)/mg-AA, 0.89 µg-As(III)/mg-AA and 1.13 µg-As(V)/mg-AA, 0.53 µg-As(III)/mg-AA in model contaminated Rokko and Contrex waters, respectively. As a result, almost 90% of arsenic can be removed with all initial arsenic concentrations in both model waters after microbial As(III) oxidation, excepting the case of 1000 µg/L As(V) in model Rokko water. Thus, 250 µg/L of arsenic in model contaminated waters after microbial As(III) oxidation can be reduced below the WHO drinking water standard (10 µg/L), after 24 hr



adsorption treatment with 0.67 g/L of AA.

The results of this study suggest that the pre-treatment is an effective step in promoting the overall arsenic removal from contaminated water with a variety of matrices. The combination of microbial As(III) oxidation using CAOs and AA adsorption would improve the reliability of the As treatment and maintain As level within the acceptable drinking water standards, making it an essential strategy in the future. Consequently, low-cost, effective, and environmentally friendly treatment of arsenic from groundwater, which is favorable for the application in developing countries, would be possible by using this method. However, it is possible that the presence of competing ions significantly decreased arsenic removal, which is one of the most important problems to be solved in future. Also further investigation is needed for the full scale application of this system to achieve a long-term operation with sufficient performance.

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