SCREENING AND CHARACTERIZATION OF BACTERIA CAPABLE OF BIOTRANSFORMATION OF TOXIC ARSENIC COMPOUND IN SOIL

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Out of 188 bacterial strains isolated from soil and mine-tailing samples collected from the arsenic contaminated area in Ron Phibun District, Nakhon Si Thammarat Province, southern Thailand. 113 bacterial strains are resistant to 700 mg l^{-1} arsenic compounds. Within these groups, two of them are found to be highly capable of oxidizing arsenite to arsenate, and were identified as *Alcaligenes xylosoxidans* subsp. *xylosoxidans* and designated as strain no.2/6 and strain no.3/18. Bacterial oxidation occurred in the range of 65% to 90% of added sodium arsenite ($NaAsO_2$) in medium at concentrations 10, 100, and 1,000 mg l^{-1}. At the concentration of 1,000 mg $l^{-1}$of sodium arsenite, there was some inhibitory effect in bacterial oxidation. Optimum arsenite oxidation occurred under aerobic conditions at a pH between 4.0 and 9.0 and at temperature 27 ^{o}c and 37 ^{o}c. Under this optimum condition the strain no.2/6 and strain no.3/18 reached the stationary phase in 24 and 8 hours, respectively. *Alcaligenes* strains were likely to utilize toxic arsenite as an electron donor aerobically because (I) during experiment, while the concentration of arsenite was decreased, the concentration of arsenate was increased and (II) the growth of *Alcaligenes xylosoxidans* accompanied the decrease of toxic arsenite in mineral medium.

These results suggest that the oxidation of arsenite to arsenate at the contaminated area by the isolated bacteria can play an important role in controlling the overall mobility of arsenite and in detoxifying the more toxic arsenite to less toxic arsenic.

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งานวิจัยนี้ได้ทำการแยกเชื้อแบคทีเรียจากตัวอย่างดินและกากขี้แร่จากพื้นที่ ซึ่งมีการปนเปื้อนด้วยสารหนู ในอำเภอร่อนพิบูลย์ จังหวัดนครศรีธรรมราช จากการทดลองสามารถคัดแยกเชื้อแบคทีเรียได้ทั้งหมด 188 สายพันธุ์ จำนวนแบคทีเรียทั้ง 188 สายพันธุ์ที่แยกได้นี้มี 2 สายพันธุ์ที่พบว่า มีประสิทธิภาพดีในการออกซิไดซ์สารประกอบสารหนูที่มีความเป็นพิษสูง (arsenite) ให้เป็นสารประกอบสารหนูที่มีความเป็นพิษน้อยลง (arsenate) แบคทีเรียที่คัดได้ทั้ง 2 สายพันธุ์ ได้วินิจฉัยพบว่า เป็นแบคทีเรียประเภท *Alcaligenes xylosoxidaus* subsp. *xylosoxidans* ตั้งชื่อเป็นสายพันธุ์ 2/6 และสายพันธุ์ 3/18 กระบวนการออกซิเดชั่นของแบคทีเรียที่คัดเลือกไว้ทั้งสองสายพันธุ์นี้ พบว่าสามารถออกซิไดส์อาร์ซิไนต์ ได้ 65-90 เปอร์เซ็นต์ เมื่อเลี้ยงในอาหารเลี้ยงเชื้อที่มีความเข้มข้นของโซเดียมอาร์ซิไนต์ 10-1,000 มิลลิกรัมต่อลิตร ความเข้มข้นของโซเดียมอาร์ซิไนต์ที่ 1,000 มิลลิกรัมต่อลิตรมีผลยับยั้งกระบวนการออกซิเดชันเป็นบางส่วน กระบวนการออกซิไดซ์สารประกอบอาร์ซิไนท์ที่มีประสิทธิภาพสามารถเกิดขึ้นได้ภายใต้สภาพความเป็นกรดด่างระหว่าง 4-9 และที่อุณหภูมิ 27-37 องศาเซลเซียส ภายใต้สภาวะนี้แบคทีเรียสายพันธุ์ 2/6 และสายพันธุ์ 3/18 เข้าสู่ระยะ stationary phase ที่ 24 และ 8 ชั่วโมง ตามลำดับ จากการศึกษาพบว่าแบคทีเรียทั้ง 2 สายพันธุ์อาจจะใช้สารประกอบอาร์ซิไนต์เป็นแหล่งให้อิเลคตรอนภายใต้สภาวะแอโรบิค เนื่องจากในระหว่างการทดลองในขณะที่ความเข้มข้นของสารประกอบอาร์ซิไนต์ลดลงพบว่า ความเข้มข้นของสารประกอบอาร์ซิเนตเพิ่มขึ้นและยังพบว่าการเจริญเติบโตของแบคทีเรียทั้งสองสายพันธุ์นี้เจริญไปพร้อมกับการลดลงของสารประกอบอาร์ซิไนต์ในอาหารเลี้ยงเชื้อ

จากผลการทดลองการออกซิเดชันของสารประกอบอาร์ซิไนต์ให้เป็นสารประกอบอาซิเนตโดยแบคทีเรียที่คัดเลือกไว้นี้สามารถลดความเป็นพิษของสารหนูให้อยู่ในรูปที่เป็นพิษน้อยลงและช่วยลดการกระจายสารหนูไม่ให้เข้าสู่สิ่งแวดล้อม

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CHAPTER I

INTRODUCTION

1.1 Problems and Importance

Arsenic, a metalloid, is present in ores and crusts rock at average concentrations of 5-6 mg kg^{-1} pyrites (1). The most naturally-occurring arsenic compound is combined with iron or nickel and with sulfur, for example, as mispickel, an arsenic pyrite (2). Arsenic is a commonly occurring toxic metal in natural ecosystem. Naturally-occurring arsenic in the environment is augmented by that extracted from the earth by man such as mining operation, and often converted from the pentavalent to the more active valent state. It is also present in the neighborhood of smelters and industrial processes in which arsenic is used (2). Arsenic compounds have been used extensively as pesticides, herbicides and fungicides. It can cause toxic effects to plants or may accumulate in plants and thereby enter the animal and human food chain. The total amount of arsenic in soil and its chemical forms have an important influence on plant growth and animal and human health (1).

Arsenic compounds are composed of various derivatives with different degree of toxicity toward organisms. It causes both large-scale environmental pollution and health hazards in several countries e.g. Taiwan, Thailand, Spain, etc. The occurrence of human health problem resulting from arsenic contamination of domestic water supplied was recognized in Ron Phibun district, Nakhon Si Thammarat province, southern Thailand. This area has an extensive history of bedrock and alluvial tin mining, the waste from which is typically rich in arsenopyrite and related alteration products. Hydrochemical analyses of surface-and groundwater have confirmed the presence of dissolved arsenic at concentrations exceeding WHO potable water guideline by up to a factor of 500 (10 $\mu g\ l^{-1}$)(3).

Bioremediation of heavy metal by microorganisms has received much attention recently due to its' potential use in toxic metal treatment processes in involving removal or detoxification of heavy metal pollutants from contaminated environment. The removal or detoxification of such toxic heavy metal from environment by microbes-based technologies may provide an alternative or additional means of metal removal or detoxification for economic reasons and/or environmental protection. Many bacteria are known to be capable of accumulation of heavy metal or reduction of them from contaminated environment. These bacteria are effectively useful for metals detoxification/removal application (4). Detoxification and/or removal of toxic arsenic compound from arsenic contaminated land by using bacteria has potential to provide significant improvements in dealing with the problems of arsenic contamination of environment.

In this study, attempts were made to isolate bacteria from arsenic contaminated area on Ronna Mountain, Ron Phibun District, Nakhon Si Thammarat Province, southern Thailand. The isolated bacterial strains were selected for high ability to transformation arsenite to less toxic arsenic. The application in field, the optimum condition was determined for ability of cell. Data from this study may be useful in further development for remediation of arsenic contaminated area using biological method.

1.2 Objectives

1.2.1 Isolation of bacteria capable of biotransformation of arsenite in soil.

1.2.2 Screening of the best efficient isolated bacterial strains which could transform arsenite to less toxic arsenic compound. Study on the optimum environmental factors on the activities of the selected bacterial strains.

1.2.3 Study on the effects of arsenite concentrations on growth and transformation ability of the selected bacterial strains.

1.2.4 Kinetic studies of the selected bacterial strains.

1.3 Outcomes

1.3.1 The bacteria which have high efficiency in biotransformation of arsenite in the contaminated areas will be selected.

1.3.2 The environmental impacts of arsenite in arsenic contaminated areas will be minimized when application of the selected bacterial strains is performed.

1.3.3 The optimum environmental factors for performing the best activities of the selected bacterial strains will be determined.

1.3.4 The reaction time for highest percentage of transformation of arsenite to less toxic arsenic by the selected bacterial strains will be determined.

1.4 Scope of study

1.4.1 Isolation of bacteria which capable of transformation of more toxic arsenic to less toxic arsenic in contaminated area.

1.4.1.1Collection of soil and mine tailing

1.4.1.2 Isolation of arsenate/arsenite tolerance bacteria from samples

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1.4.2.2 Selection of bacterial strain which have high efficiency in arsenate/arsenite transformation.

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1.4.4.1 Effect of temperature.

1.4.4.2 Effect of pH.

1.4.5 Study on the effect of the arsenite concentrations on growth of the selected bacterial strain.

1.4.6 Study on the effect of the arsenite concentrations in arsenite transformation.

1.4.7 Kinetic studies on the arsenite transformation of the selected bacterial strain.

1.4.8 Identification of the selected bacterial strain.

CHAPTER II

LITERATURE REVIEWS

2.1 Arsenic in environment

Arsenic(As) is a ubiquitous element that ranks 20^{th} in abundance in the earth's crust. It is widely distributed and forms 5-6 mg kg^{-1} of the earth's crust (1). It is present in varying concentrations for example, 1 mg kg^{-1} in limestone and siliceous deposit, 2 mg kg^{-1} in igneous rocks, and up to 20 mg kg^{-1} in volcanic rocks. In virgin soil and forest humus, an arsenic content of 3-5 mg kg^{-1} has been found (2).

Table 2-1 Some naturally-occurring compounds of arsenic.

Mineral name	Formula
Arsenolite	As_2O_3
Arsenopyrite	FeAsS
Cobalite	CoAsS
Enargite	Cu_3AsS_4
Kupfernickle	NiAsS
Lollingite	$FeAs_2$
Mimetite	$Pb_5Cl(AsO_4)_3$
Mispickel	FeSAs
Nicolite	NiAs
Olivenite	Cu_2OHAsO_4
Orpiment	As_2S_3

Table 2-1 (continued)

Mineral name	Formula
Proustite	Ag_3AsS_2
Realgar	AsS
Scorodite	$FeAsO_4.2H_2O$
Smaltite	$CoAs_2$
Tennantite	$Cu_8As_2S_7$

From: (Kippling, 1977)(2)

The commonest naturally occurring arsenical compound is mispickel, an arsenical pyrites, which occurs together with other metallic ores. Less widely distributed are the two brightly colored sulphide pigments, realgar, which is orange red, and orpiment, which is of a yellow lemon colour. Arsenical sulphides are also combined with copper (enargite) and silver (proustite), and arsenic also occurs in some nickel, cobalt, silver and lead ores (2).

2.1.1 The used of arsenic

It is certain that arsenic compounds were observed by man from the earliest times. Arsenic has been used in medicine, cosmetics, and as a deadly poison. In the current technological age, it is increasingly being used as a doping agent in solid-state devices such as transistors. Gallium arsenides are being used as laser material to convert electricity directly into coherent light. Arsenic is also used in bronzing, pyrotechnics, and for hardening and improving the sphericity of gunshot. All these industrial used contributed to addition of arsenic-containing compounds to the environment. In agriculture, arsenic was used as an insecticide, and it still finds uses a desiccant, rodenticide, and herbicide (5).

Table 2-2 Arsenic compounds used in industry, agriculture and medicine

Arsenic compound	Formula	Known as	Uses
Arsenic	As		Alloying additive Electronic devices i.e. transistor. etc.
Arsenic pentoxide	As_2O_5	Arsenic oxide Boliden salts	Chemical intermediate Defoliant Wood preservative
Arsenic trioxide	As_2O_3	Arsenic Arsenolite White arsenic Arsenious oxide	Insecticides and Fungicides Glass Chemicals Anti-fouling paints Taxidermy Timber preservation
Arsenic trichloride	$AsCl_3$	Butter of arsenic	Pharmaceuticals and Chemicals
Arsine	AsH_3		Stabilizing selenium in transistors
Calcium arsenate	$Ca_3(AsO_4)_2$		Insecticide , herbicide and larvicide
Copper arsenite	$CuHAsO_3$	Scheele's green	
Copper aceto-arsenite	$3CuOAs_2O_3Cu(OOCCH_3)$	Paris Green Emerald Green	Larvicide
Orpiment	As_2S_3		Pigment Depilatory

Table 2-2 (continued)

Arsenic compound	**Formula**	**Known as**	**Uses**
Lead arsenate	$PbHAsO_4$		Insecticide , herbicide and growth regulator
Sodium arsenate	Na_2HAsO_4 Na_3AsO_4	Wolman salts	Wood preservative Calico printing Insecticide Weedkiller
Sodium arsenite	$NaAsO_2$		Herbicides Pesticides Corrosive inhibitor Chemical intermediate Fluorescent lamps
Magnesium arsenate	$Mg_3(AsO_4)_2$	Atoxyl	Trypanicide
Sodium arsanilate	$NH_2C_6H_4AsO$ $(OH)(ONa)$		Pharmaceutical manufacture

From : (Kippling, 1997)(2).

2.1.2 Sources of arsenic in environment

2.1.2.1 Sources of arsenic from parent materials

The sources of arsenic in soil are mainly the parent (or rock) materials from which it is derived. Arsenic is concentrated in magmatic sulfides and iron ores. The most important ores of arsenic are arsenic pyrites or mispickel, realgar, and orpiment. These minerals are often present in the sulfide ores of other metals. The arsenic levels in soil enriched in these ores often higher than in normal soil. In regions of contemporary or recent volcanism (e.g. Colorado, Mexico, Italy and Japan), arsenic concentrations also is higher than in normal soil. There are differences in the arsenic content of soils derived from various igneous rock types. The parent materials of soils are usually sedimentary rocks. During the formation of these rocks, arsenic is carried down by precipitation of iron hydroxides and sulfides. Therefore, iron deposits and sedimentary irons ores are rich in arsenic. The arsenic levels in soil derived from sedimentary rock may attain a value of 20 to 30 mg l^{-1} (1).

2.1.2.2 Anthropogenic sources of arsenic

Another source of arsenic in soils is human activities. It has been added to soils by modern industry, mining operation, agriculture, forestry, and manufacturing. The mining operation, arsenic is a natural contaminant in lead, zinc, gold, and copper ores and can be released during the smelting process. The stack dust and flue gases from smelters often contaminated soil with downwind from the operation. The agriculture, inorganic arsenicals, usually as Pb, Ca, Mg, and zinc arsenate, zinc arsenite were used extensively as pesticides in orchard. Sodium arsenite was used as a herbicide and nonselective soil sterilant, while arsenic acid was used as a cotton desiccant. Organic arsenicals have also been used as silvicides, herbicides and desiccants. Various chemical combinations of arsenic were widely applied for a long time as fungicides, herbicides, and insecticides, some of which are still used today (5)

The anthropogenic influence on the level of arsenic in soil depends on the intensity of human activities, the distance from the pollution sources, and the pollutant dispersion pattern (1).

2.1.3 Form of arsenic in soil

2.1.3.1 Inorganic and organic form

Arsenic occurs mainly as inorganic species but also can be bound to organic material in soils. Arsenic may accumulate in soils through the use of arsenical pesticides. Inorganic arsenic may be converted to organoarsenic compounds by soil microorganisms. Arsenic is widely distributed in nature in the combined state. Minerals of arsenic include sulfides (realgar, AsS; orpiment,As_2S_3), oxides (claudetite or arsenlite, As_2O_3), arsenites (mispikel, FeAsS; nickel glance, NiAsS) and arsenates (pharmacolite, $CaHAsO_4.2H_2O$). The distribution of arsenic in the different size fractions of the soil can be related to the stability of the primary minerals in which it is found and the extend to which weathering has taken place (1).

The forms of arsenic in soils depend on the type and amounts of sorbing components of the soil, the pH, and the redox potential. The percentage of water soluble arsenic is proportional to arsenic added to the soil, and inversely proportional to the iron and aluminum content.

2.1.3.2 Redox states of arsenic

Arsenic occurs frequently in the pentavalent states as arsenic acid (As(V)) and in the trivalent states as arsenite (As(III)) in soil solution, and oxidation states can be subjected to chemically and microbiologically mediated oxidation, reduction, and methylation reactions. The biological availability and physiological and toxicological effects of arsenic depend on its chemical from. As(III) is much more toxic, more soluble, and more mobile than As(V). Under the influence of oxidizing factors, the H_3AsO_3 in soil can be converted to H_3AsO_4. The theoretical oxidation-reduction potential of this system is 0.557 V at 20 °C. Because the redox potential of soils depends on the redox potentials of all the reducing and oxidizing systems occurring in the soils these relationships are very complex, and the redox value for soil and is not directly proportional to As(V): As(III) ratio (1).

2.1.3.3 Gaseous states of arsenic

Extensive use has been made of sodium and ammonium salts of monomethyl arsionic acid (MMAA) and dimethylarsinic acid (DMAA) as nonselective, postemergent, foliar-contact herbicides. There is evidence concerning the bioaccumulation of these arsenicals, as well as their reduction by soil microorganisms to the corresponding toxic and highly volatile arsine (1).

Researchers have reported that common fungi, yeast, and bacteria can methylate arsenic to MMAA, DMAA and gaseous derivatives of arsine (6).

2.1.3.4 Transformation of arsenic in soil

Chemical forms of arsenic and their transformation in soil can be illustrated (Figure 2-1). Oxidation, reduction, dissolution, precipitation and volatilization of arsenic reactions commonly occur. Some soil reactions are associated with bacterial and fungal microorganisms. The volatile organic arsines are extremely toxic.

Decomposition of any organic material added to soils (e.g., sewage sludge, sawdust, compost, manure, and crop residues) yields organic substances that can adsorb arsenic. Also, substances containing Al, Fe, or Ca, such as fluidized-bed waste, may form sparingly soluble compounds that arsenic renders unavailable for plant uptake (5).

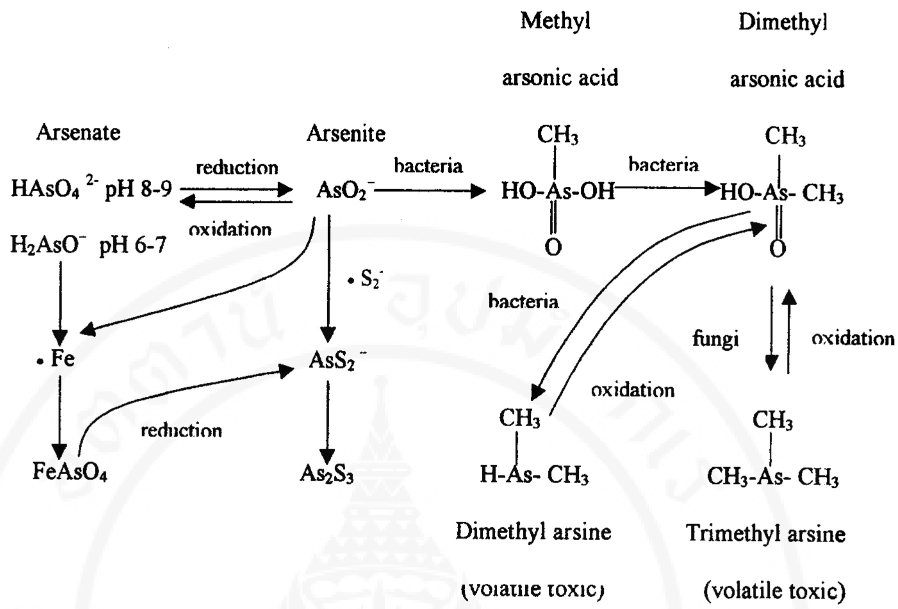

Figure 2-1. Chemical forms of arsenic and their transformations in soils.

2.1.3.5 Speciation of arsenic in environment

Speciation of arsenic in the soil environment:

1. Arsenic (III). Arsenite [As(III)], the reduced state of inorganic arsenic, is a toxic pollutant in natural environments. It is much more toxic and more soluble and mobile than the oxidized state of inorganic arsenic, arsenate [As(III)].
2. Arsenic (V). Arsenate[As(V)] can be sorbed onto clays, especially kaolinite and montmorillonite. In a montmorillonitic, calcareous clay, arsenate was strongly adsorbed onto koalinite and montmorillonite at low pH with a maximum near pH 5.0, and became less adsorbed at high pH . Adsorption of As(V) by calcite increased from pH 6 to10, peaked at pH10 to 12, and decreased above pH 12.
3. Organic arsenic. A ubiquitous, volatile, arsenic compound, dimethyl-arsenic acid (cacodylic acid) seems to be present in all soils and may dominate in many. The arsenic compounds are reduced and methylated by microorganisms in

environment. Some important methylated species are methanearsonate [$CH_3AsO^3{}_{2-}$], dimethylarsonate [$(CH_3)_2AsO^{2-}$], dimethylarsine [$(CH_3)_2AsH$], trimethylarsine [$(CH_3)_3As$] (7).

2.1.3.6 The Arsenic Cycle

Numerous cycles for arsenic have been proposed. A simplified, comprehensive cycle has been diagrammed (Figure 2-2), with the main transfers shown by bold arrows. The main components of this cycle are air (volatile); mining and smelting; biota (animals, man, plants and microbes); pesticides and fertilizers; water and oceans; soils, rocks, and sediments; and nonagricultural materials (fossil fuels, industrial and municipal wastes) (5).

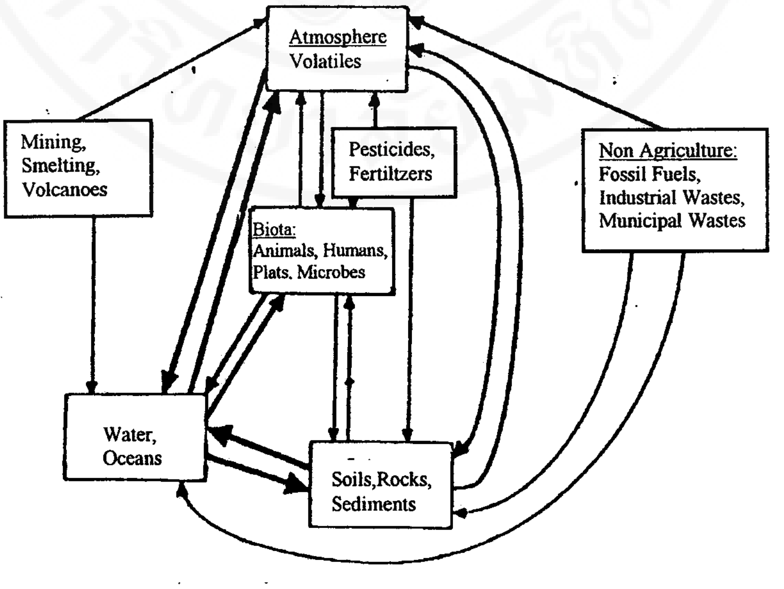

Figure 2-2 A simplified, comprehensive cyclic transfer of arsenic.

2.2 Toxicity of arsenic compounds

Toxicity of arsenic depend on the type of arsenical involved and on the time-dose relationship of exposure.

2.2.1 Inorganic arsenic

The incidence patterns of chronic inorganic arsenic poisoning in the past and in the present show that arsenic contamination of drinking water is the most frequent cause. Arsenic poisoning has occurred in Taiwan, Chile, Mexico, and Thailand (3), through consumption of contaminated well water. It has generally been accepted that among the chemical species of inorganic arsenic, arsenite is more toxic than arsenate. However, this conclusion is derived from the median (50%) lethal doses in animals (Table 2-3), and there is, in reality, no great difference in toxicity between the chemical species of inorganic arsenic. However, both inorganic species are still more toxic than other arsenic compounds.

Table 2-3 A Comparison of Arsenic Compounds of LD_{50} in Animals

Arsenic Compound	LD_{50} (mg kg^{-1})	Animal/Mode of Administration
Arsenite: arsenic trioxide[a]	34.5	Mouse/oral
Arsenite: sodium arsenite[b]	4.5	Rat/intraperitioneal
Arsenate: sodium arsenate[b]	14-18	Rat/intraperitioneal
MA: monomethylarsonic acid[c]	1,800	Mouse/oral
DMA: dimethylarsinic acid[c]	1,200	Mouse/oral
TMA: arsenobetaine[a]	10,000	Mouse/oral
Trimethylarsine oxide[c]	10,600	Mouse/oral
Trimethylarsine[d]	8,000	Mouse/subcutaneous
Trisdimethylaminoarsine[d]	15	Mouse/subcutaneous

[a]Kaise et al.(1985)

[b]Franke and Moxon, (1936)

[c] Kaise et al.(1989).

[d]Yamamura et al.(1993).

Source : Yamauchi and Fowler (8).

2.2.2 Methylated Arsenic Compounds

The methylated arsenic compounds are far less acutely toxic than the inorganic arsenic compounds. The trimethylated compounds appear to be least toxic.

MA (monomethylarsonic acid) and DMA (dimethylarsinic acid) are derived from inorganic arsenic compounds *in vivo*, and DMA tends to be the second most abundant biological form in relation to inorganic arsenic compounds detected in human tissues (but not in the urine) (8). A conclusion has been drawn that the methylation of inorganic arsenic in mammals is a detoxification mechanism. On the other hand, there are reports on toxicological problems with DMA, such as damage to and mutagenicity (8). In this regard, these genetic studies indicate that the major metabolites of inorganic arsenic are not innocuous.

The main chemical species of arsenic that human beings ingest from foods, especially people who eat large quantities of fish and shellfish, is arsenobetaine. Arsenobetaine is practically nontoxic (Table 2-3), and this may be the reason why there is no arsenic poisoning reported in such people. Among the trimethylated arsenic compounds, trimethylarsine oxide is rarely detected in fish. However, it is not ingested in such amounts as to cause concern over its toxic effect on the body, and it is as nontoxic as arsenobetaine (Table 2-3).

2.2.3 Alkylarsine (TMAs, TMAO and TDAA)

TMAs (trimethylarsine) is metabolized to form TMAO (trimethylarsine oxide), and as shown in Table 2-3, the acute toxicity of the metabolite is very low. However, because TDAAs (Trisdimethylaminoarsine) is hydrolyzed *in vivo* to produce inorganic arsenic, the toxicity of inorganic arsenic should also be considered. The median lethal dose of TDAAs is comparable to that of inorganic arsenic; hence, the acute toxicity of TDAAs (LD_{50} = 15 mg kg^{-1}) is higher than that of TMAs (LD_{50} = 8,000 mg kg^{-1}). Because both TMAs and TDAAs belong to the arsine class, their hemolytic potential should be considered. In a study utilizing a single-dose, subcutaneous administration of these compounds in hamsters, Yamauchi and Fowler (8) observed the occurrence of mild, transient hemolysis, whereas in a study by subacute exposure (to half of the median lethal dose for 10 days), neither of the compounds was found to be hemolytic. TMAs tends to be slightly more hemolytic than TDAAs. Hematologic studies of these compounds showed that acute exposure to either compound tended to increase hemoglobin concentrations, but that subacute exposure to either compound was not associated with any appreciable changes. The toxicity of alkylarsines is chiefly manifested as hemolysis, and tends to be far lower than that of arsine gas. From the toxicologic point of view, alkylarsines are desirable substitutes for arsine gas (8).

2.2.4 Arsine gas

Very useful information on the acute toxicity of arsine gas is available from poisoning cases in the past and from animal experiments. It is known that its main toxic effect is hemolysis, and that renal failure is a secondary effect (Yamauchi and Fowler, 1994). In a report on the toxicity of arsine gas by the National Institute of Environmental Health Sciences, it was shown that exposure to arsine gas at concentrations of 2.5 mg l^{-1} and more by inhalation had immunologic effects (8).

2.3 Arsenic poisoning

2.3.1 Inorganic arsenic poisoning

2.3.1.1 Acute Poisoning

Symptoms of acute intoxication (9) usually occur within 30 minutes of ingestion but may be delayed if arsenic is taken with food. Initially, a patient may have a metallic taste or notice a slight garlicky odor to the breath associated with a dry mouth and difficulty swallowing. Severe nausea and vomiting, colicky abdominal pain, and profuse diarrhea with rice-water stools abruptly ensue. In acute arsenic poisoning of massive proportions, almost always as an attempt at suicide, the fundamental lesion of endothelial cellular toxicity can be considered to account for the predominant clinical features. Capillary damage leads to generalized vasodilaion, transudation of plasma, and shock. Arsenic's effect on the mucosal vascular supply, not a direct corrosive action, leads to transudation of fluid in the bowel lumen, mucosal vesicle formation, and sloughing of tissue fragments. The patient may complain of muscle cramps and intense thirst. In severe poisoning, the skin becomes cold and clammy, and some degree of circulatory collapse usually occurs along with kidney damage and decreased urine output. Drowsiness and confusion are often seen along with the development of a psychosis associated with paranoid delusions, hallucinations, and delirium. Finally, seizures, coma, and death, usually due to shock, may ensue.

Following the gastrointestinal phase, multisystem organ damage may occur. If death does not occur in the first 24 hr from irreversible circulatory insufficiency, it may result from hepatic or renal failure over the next several days. Cardiac manifestations include acute cardiomyopathy, subendocardial hemorrhages, and electrocardiographic changes. A case of an atypical ventricular fibrillation resembling *torsades de pointes* has been reported (9). The pathological lesions described in patients with rapidly fatal arsenic intoxication are fatty degeneration of the liver, hyperemia and hemorrhages of the gastrointestinal tract, renal tubular necrosis, and demyelination of peripheral nerves (9).

2.3.1.2 Chronic Poisoning

The most prominent chronic manifestations (9) involve the skin, blood, and neurologic systems. The cutaneous changes are characteristic yet nonspecific. An initial persistent erythematous flush slowly, over time, leads to melanosis, hyperkeratosis, and desquamation. The skin pigmentation is patchy and has been given the poetic description of "raindrops on a dusty road." The hyperkeratosis is frequently punctuate and occurs on the distal extremities. A diffuse desquamation of the palms and soles is also seen. Long-term cutaneous complications include the development of multicentric basal cell and squamous cell carcinomas.

Anemia and leukopenia are almost universal with chronic arsenic exposure; thrombocytopenia frequently occurs. The anemia is usually normochronic and normocytic and caused at least partially by hemolysis. Interference with folate metabolism and DNA synthesis may result in megaloblastic changes. Karyorrhexis, an accelerated pyknosis of the normoblast nucleus, is characteristic of arsenic poisoning. Aplastic anemia progressing to acute myelogenous leukemia has been reported. A peripheral neuropathy is the hallmark of chronic arsenic poisoning.

2.3.2 Arsine poisoning

Poisoning by the inhalation of arsine is a dramatic event. The inhaled arsine liberates hemoglobin (from the red blood cells) which blocks the kidneys and the liver. The symptoms are pain in the loins and general collapse. The released blood pigments produce bright red urine, a bronze color of the skin and jaundice. In less severe cases, anemia, and sometimes neuritis, prolongs the period before complete recovery (2).

2.4 Ron Phibun District

Ron Phibun District is located in Nakhon Si Thammarat Province, southern part of Thailand. This area lies at the eastern foot of the Ron Na-Suang Chan mountain subrange. Ron Phibun is part of the South-East Asian Tin Belt, a zone of granite-related Sn minerization, characterized by S-Type biotite and biotite-muscovite granitoids of Triassic age, with abundant pegmatitic veining. Cassiterite (SnO_2) and wolframite minerization with high amount of arsenopyrite and pyrite commonly occurs in the pegmatites vein margins. Mining and mineral processing activities existed in Ron Phibun during the past 100 years. Over 20 bedrock mining concessions and also alluvial mining were hold in the Ron Na-Suang Chan mountain range (3).

Potential sources of As contamination in the Ron Phibun mining area have been classified by the the Department of Mineral Resource as (i) high-grade arsenopyrite waste piles in bedrock mining localities, (ii) sub-ore grade waste rock piles, (iii) sulphide wastes from ore-dressing plant, (iv) disseminated sulphide waste from small scale prospecting and flotation activities, and (v) alluvial tin workings (3).

Several agencies had examined soil and food grown in this area and found that the range of arsenic level was 22-250 mg l^{-1}, respectively in the villages of high incidence of chronic arsenic poisoning. Plants and vegetables that are grown in the sandy soil have higher arsenic content than those grown in clay soil. Factors that influence arsenic level in the soil are soil texture, content of humus and other minerals, e.g. calcium, iron and aluminum (10). Suwanmanee examined 143 soil samples in 12 km^2 in the Ron Phibun District and found arsenic levels of 50-5200 mg kg^{-1}. She also found that the arsenic level depended on the iron content in soil and good solubility in low pH. Intarasut tested the solubility of contaminated soil from the level of 5-40 feet depth, and found that an arsenic level of 40 mg kg^{-1} in soil could make rain water have arsenic high over accepted level (10).

Health problems attributable to As-contaminated water supplies in Southern Thailand were first highlighted in 1987, following the diagnosis of a case of arsenical skin cancer in Ron Phibun District, Nakhon Si Thammarat Province. Research into the extent distribution and epidemiology of arsenism in the provinces was initiated by the Ministry of Public Health in 1988, and a preliminary survey confirmed approximately 1000 cases of As-induced skin disorders, including 20 arsenical melanomas. In the school-age population, As concentrations in hair and fingernails were found to be elevated (up to 3.1 mg kg^{-1} and 56 mg kg^{-1}, respectively) in 80% of the pupils examined, and a strong spatial correlation between human burden and As concentrations in drinking water was confirmed. A follow-up study of 2400 school pupil in 1992 showed 89% to have excess blood As concentration, with a 22% incidence of arsenical skin manifestations (3).

2.5 Interaction of bacteria with arsenic compounds

2.5.1 Arsenic tolerance bacteria

Bacteria appear to have mechanisms of coping with all toxic heavy metals. The microbial community consisted of two populations, either resistant or sensitive to heavy metals. However, a metal-resistant isolate was isolated from a soil with no known previous exposure to heavy metal, suggesting widespread of heavy metal resistance (11).

Many bacteria have since been isolated, often from hospital sources that show a resistance to arsenite and/or arsenate. This resistance is coded for by an inducible operon-like system in both *Staphlococcus aureus* and *Esherichia coli*. This system is also turned on by Sb(III) and Bi(III) (12,13).

In the reviewed by Cullen (14), these strains of bacteria *Alcaligenes faecalis*, *Psuedomonas aeruginosa, P. fluorescense*, and *P. putida* could be accustomed to grow in 0.15 M arsenite without oxidizing it to arsenate. These bacteria and other fungi had a greatly increased tolerance to arsenate in the presence of phosphate.

Silver (15) reviewed that many bacterial species had variants that showed resistance to arsenite and/or arsenate. The determination of arsenic resistance could be governed by genes on the bacterial chromosome or on extrachromosomal plasmids, which also contained genes governing resistance to antibiotics. *Escherichia coli* strains isolated from a Tokyo hospital showed a 61% frequency of As(V) resistance.

Maeda (16) isolated two bacteria exhibiting resistance to toxic arsenic. The two bacteria were identified as *Klebsiella oxytoca* and *Xanthomonas sp.* .The growth of the bacteria was not affected by arsenate concentrations in the medium as high as 1000 mg l^{-1}.

There are two reports of isolation of soil or sewage isolates of *Alcaligenes* that were arsenite resistant, but it is not known whether the genes governing this resistance are chromosomal or plasmid-borne (17,18).

Krafft (19) reported that arsenic resistance appeared to be widespread among bacteria. The mechanism for arsenate and arsenite resistance has been investigated in depth only in organisms where resistance is conferred by proteins encoded by similar *ars* operons. Diorio (20) described that these operons (*ars*) isolated from both gram-positive and gram-negative bacterial species had been found to be very homologous and generally consist of either three or five genes that have been organized into single transcriptional unit. These operons are located on the chromosome of *Escherichia coli*, on plasmid R773 of *E. coli,* on the IncN plasmid R46 found originally in S*amonella typhimurium*, on plasmid pI258 of *Staphylococcus aureus* and on plasmid pSX267 of *Staphylococcus xylosus*. The arsenate reductase of these arsenic resistance systems does not appear to be involved in energy conservation when catalyzing the reduction of arsenate to arsenite.

In addition, Cullen (14) discussed that chromosomally determined arsenate resistance appeared to result in reduced accumulation of arsenate; i.e., the cell switched on a phosphate transport system that was more selective for phosphate. The usual transport system was not selective for phosphate. Thus phosphate could act as a protecting agent against arsenate toxicity (but not arsenite). Plasmid-mediated resistance to arsenate is due to the synthesis of a highly specific arsenate efflux pump that eliminates intracellular arsenate. Arsenite oxidation by an inducible enzyme system is one mechanism of resistance to arsenite. Another plasmid-mediated mechanism seems to exist, but it is not known how this functions, apart from not involving extracellular detoxification.

Many organisms have ability to methylate inorganic arsenic. Biomethylation seems to be a mechanism of arsenic detoxification (21)

2.5.2 Oxidation of Arsenite to Arsenate

Oxidation of arsenite is one of the protective mechanisms. Arsenite is more toxic than most other forms of arsenic. The "spontaneous'" oxidation of arsenite to arsenate in cattle dipping fluids was first noted in 1909. The suggested relationship of the oxidation to bacterial growth was confirmed in 1918 by isolation of a bacterium from arsenical cattle dips in South Africa which grew in 1% arsenic trioxide medium and oxidized the arsenite to arsenate. The bacterium, provisionally named *Bacillus arsenoxydans*, was eventually lost. The next observation of this phenomenon was not recorded until 1954, Turner (22,23) isolated 15 strains (5 species) of arsenite-oxidizing bacteria from cattle dips in Australia. These were provisionally characterized as three *Pseudomonas*, one *Xanthomonas*, and one *Achromobacter*. His main effort was to characterize the " arsenite dehydrogenase" and its associated cytochromes. Growth of bacteria could take places in the pH range 6.1 – 9.4 (the pH dropped during growth as a result of the formation of arsenate). There was no evidence that the energy of arsenate oxidation was used for growth. A moderately stable, cell free, arsenite dehydrogenase was liberated from cell of "*Pseudomonas arsenoxydans quinque*" after grinding with powdered glass (24,25). The electron acceptor used in the assay was 2,6-dihloroindophenol. Arsenate is an inhibitor, as is Hg(II). Studies on these cell-free preparation suggest that arsenite oxidation in the intact cell involved a loose association between the enzyme and the cytochromes.

In other studies, 18 different strains of arsenite-oxidizing bacteria were isolated from sewage (18) and classified as *Alcaligenes faecalis*. No methylarsenicals were found in the medium.

Extended studies on one organism, *Alcaligenes faecalis* YE56, have been described (18). It is a strict aerobe, but under anaerobic conditions the organism can use nitrate for the oxidation of various carbon sources; arsenite is not oxidized under these conditions. Sonicated cells are unable to oxidize arsenite in the presence of air; the same preparation is able to couple oxidation of arsenite to arsenate, with the reduction of 2,6-dichloroindophenol. The oxidation of arsenite by the whole organism seems to be associated with the appearance at the stationary phase, of an enzyme and/or component of the electron transport system; the optimum pH is 6.6. Phillips has speculated that an acquired tolerance to ingested arsenite, the more toxic state, might be due to the enrichment of arsenite-oxidizing population of *Alcaligenes* in the intestine.

Osborne (17) also isolated a strain of *Alcaligenes* from soil and grown in nutrient broth in the presence of arsenite, possed the ability to oxidize arsenite to arsenate. They discussed that the arsenite-oxidizing enzyme system was induced by growth in arsenite. Response of the arsenite-oxidizing enzyme system to respiratory inhibitors suggested that electrons resulting from arsenite oxidation by oxido-reductase with a bound flavin were transferred *via* cytochrome c and cytochrome oxidase to oxygen.

Hambsch (26) investigated that the microbial process influenced the oxidation of As(III) to As(V) in natural water. The result showed that at 4 °C, no As(III)-oxidation was observed within 14 days. At room temperature, however, in the bacteria-containing samples, an As(III) was left over. In contrast, in the sterile samples, no As(III)-oxidation could be observed within 14 days.

2.5.3 Reduction of Arsenate to Arsenite

The reduction of arsenate to arsenite, a more toxic form, has been described. Cullen (14) reviewed that *Pseudomonas flurescens*, a common aquatic bacterium, carried out this reduction under aerobic condition; activated sewage sludge did so under anaerobic conditioned. Further transformations are possible in sludge. Another study (27) used as aerated mixed culture of bacteria from seawater. The phosphate concentration steadily decreased during the experiment, but the total arsenic concentration remained constant, indicating no accumulation by the bacteria.

Forsberg (28) reported that rumen bacteria reduce arsenate to arsenite. In 1994, Ahmann & Roberts (29) isolated MIT-13 from Aberjona watershed in eastern Massachusetts. They demonstrated that the strain MIT-13 reduced arsenate to arsenite and gained energy for growth from this reduction. In addition, the selenate-respiring bacterial strain SES-3 also showed that it was able to use arsenate as electron acceptor to sustain growth (30).

An interesting example of arsenate to arsenite reduction by *Chrysiogenes arsenatis* has been described. Kraff (19) studied that *Chrysiogenes arsenatis* was the bacterium known that respired anaerobically using arsenate as the electron acceptor and the respiratory substrate acetate as the electron donor.

In addition, a newly discover bacterium, *Desulfotomaculum auripigmentum*, could precipitate arsenic trisulfide (As_2S_3). Precipitation of As_2S_3 by this organism resulted from its reduction of As(V) to As(III) and S(VI) to S(II) (31).

2.5.4 Reduction of Arsenate to Arsine

Macbride & Wolfe (6) described that Methanobacterium strain M.o.H. reduced and methylated arsenate to dimethylarsine under anaerobic conditions. Wood (1974) pointed out that, in the reduced environment such as flooded soils, arsenate was reduced to arsine and then was methylated to form methylarsine acid forms. These arsenic compounds might further be reduced to methylarsines that volatilize to the atmosphere.

The addition of sodium arsenate to soils enriched with glucose and urea resulted in the production of arsine (AsH_3) as determined by GC/mass spectrometry (Carbowax 1000 column). Only traces of arsine were produced from unenriched cultures. Two bacteria, *Pseudomonas* and *Alcaligenes*, were isolated from soil and shown to be arsine producers under anaerobic conditions (32)

2.5.5 Conversion of Arenite to Arsine

Cheng and Focht (32) studied the production of arsine from arsenite. The report showed that the isolated soil bacteria, *Pseudomonas* and *Alcaligenes*, released arsine from arsenite under anaerobic conditions.

2.5.6 Methylation of Arsenic

Both fungi and bacteria are reported to be involved in the biotransformation of arsenic in soils. Methylated arsenic compounds were detected in a culture in which sediments from various locations (lake, river, and pond) in Ontario, Canada were incubated with or without the addition of extraneous arsenic (33). MMAA (Monomethyl arsonate acid) and DMAA (Dimethyl arsenic acid) were found in lake and river sediments. Trimethylarsine oxide was found only in pond sediment. These pure bacteria culture (*Aeromonas* sp., *E. coli,* and *Flavobacterium* sp.) grown in a chemically defined medium could also methylated arsenic compounds.

McBride and Wolfe (6) found that cell extracts and whole cells of the *Methanobacterium* strain M.o.H. reduced and methylated arsenate to dimethylarsine under anaerobic conditions. Methylcobalamin is the methyl donor of choice. Adenosine triphosphate and hydrogen are essential for the formation of dimethylarsine by cell extracts.

According to a review by Cullen and Remer (14), arsenate was converted to monomethylarsine and dimethylarsine by *Achromobacter* sp. and *Enterobacter* sp., and to monomethylarsine, dimethylarsine and trimethylarsine by *Aeromonas* sp. and *Nocardia* sp. The rate of methylation and demethylation of monosodium methylarsonate at 10 and 100 μg As/cm^3 of normal culture media by *Aeromonas. Nocardia, Enterobacter, Flavobacterium, Achromobacter, Pseudomonas,* and *Alcaligenes* species followed first order composite kinetics. The rate constants were independent of species and the cell concentrations for both monomethylarsine levels were similar to each other. *Nocardia* being the only bacteria that formed all four of the metabolites.

Maeda (16) reviewed that Baker *et al.* (1983) collected sediment samples from Plastic Lake, Ontario, and examined the effect of pH on the biomethylation of mercury and arsenic in the sediment amended with nutrients. Methyl mercury from inorganic mercuric chloride was formed only in the pH range 5.5 to 6.5. Dimethyl mercury was not produced. Methylarsonic acid and dimethylarsinic acid were formed over the pH range 3.5 to 7.5. In contrast to the mercury methylation experiments, the arsenicmethylating microorganisms were not so sensitive to changes in pH, and different microorganism might have been responsible for methylating arsenic over this pH range.

Maeda *et al.* (34) isolated two arsenic-tolerant freshwater bacteria (*Klebsiella oxytoca* and *Xanthomonas* sp.) from a contaminated culture of the algae *Chlorella* sp. Growth was not impaired by arsenic concentrations as high as 1000 As(V) mg dm^{-3}, but it decreased drastically at higher concentrations. Most of the arsenic in the cell was inorganic and 3% of the arsenic was in the trimenthylated form. During the stationary phase, the bacteria excreted arsenic largely in the inorganic form, but also as monomethyl-, dimethyl-, and trimethylarsenic compounds (MMA, DMA, and TMA). The relative content of methylated arsenic in the excrement was greater than that in the bacteria cell. The adaptation exposure to inorganic arsenic caused an increase in the bioaccumulation of methylated arsenic, and the demethylation of these species was also observed. When the bacteria were killed by ethanol, uptake of arsenic compounds did not occur by the dead cell.

Five bacteria (*Proteus* sp., *Escherichia coli, Flavobacterium* sp., *Corynebacterium* sp., and *Pseudomonas* sp.) capable of biotransforming sodium arsenate were grown in the presence of the arsenate to determine arsenic uptake and distribution in the microbial calls. Species grown in the presence of arsenate showed an average accumulation of 25 and 30 μg g^{-1} total arsenic following 48-hour exposure to 100 and 10 mg dm^{-3} arsenic, respectively. A trichloroacetic acid fractionation procedure indicated that most of the accumulated arsenic was confined to the residual protein, with smaller amounts found in the lipid-protein and nucleic pools (16).

CHAPTER III

MATERIALS AND METHODS

3.1 Isolation of bacteria which capable of transformation of more toxic arsenic to less toxic arsenic in arsenic contaminated area.

3.1.1 Collection of samples

The samples were collected from various locations on the Ronna mountain in Ron Phibun district, Nakhon Si Thammarat province where there was a history of contamination from arsenic. These samples were mine tailings and soils on the Ronna mountain. Mine tailings were taken from the collection site 1, 2, and 3. Soil samples were taken from surface soil along the trail on the Ronna mountain. Each soil samples was taken at 50 m apart, up to Dong Mai Hom area at the height of 400 m in above sea level. Each sample was separately packed in the plastic bag.

3.1.2 Media and Growth condition.

The arsenite stock solution (1000 mg l^{-1}) was prepared by dissolving 1000 mg of $NaAsO_2$ per liter in distilled and deionized water (ddH_2O), while the arsenate stock solution was prepared from 1000 mg of $Na_2HAsO_4.7H_2O$.

The mineral medium contained (g l^{-1}): NH_4Cl 0.5 g; $MgCl_2.6H_2O$ 0.25 g; KH_2PO_4 0.5 g; Beef extract 0.5 g; Glucose 0.3 g; Distilled water 1 l.

The enriched medium contained (g l^{-1}): Nutrient broth (Difco) 8 g; Yeast extract 0.5 g; Distilled water 1 l.

In preparing these media, all others ingredients were dissolved in distilled water, then an aliquot of either arsenate or arsenite stock solution were added to make up the required concentration of arsenate/arsenite in the media. After that the volume was brought up to 1 l with distilled water, and adjusted the pH by the addition of 1N HCl or 1N NaOH. All media were autoclaved at 121 °C (15 psi) for 15 min before use. All glasswares for the experiments were routinely washed with 10% HNO_3 and rinsed extensively with ddH_2O to prevent interference by contaminants.

3.1.3 Isolation of bacteria

The bacteria were isolated from soil samples collected on the Ronna mountain. Enrichment methods were used for isolating bacteria with arsenate/arsenite tolerate ability from environmental samples. Each soil sample of 5 g was suspended in 20 ml of 0.85% NaCl solution. The soil suspension was allowed to precipitate for about 30 minute to get rid of big soil particles. Each 1 ml of suspension was added to 50 ml of enrichment medium in a 250 ml flask and incubated at 30 °C on a shaker set at 200 rpm for 7 days. After that 1 ml of sample was transferred into 100 ml mineral medium containing 70 mg l^{-1} of arsenate/arsenite concentration (pH adjusted to 5.6 by HCl). The cultures were subcultured every 2 weeks into a fresh medium and incubated under the same conditions. After three subsequent transfers of the culture to a fresh medium, the samples from flasks which showed high turbidity were subsequently streaked on nutrient agar plate (Difco) containing arsenate/arsenite (pH 5.6) for isolation of single colonies. Plates were incubated at 30 °C for 24-48 h. The isolated colonies was purified by restreaking on the nutrient agar plate and stored on agar slant at 4 °C in a refrigerator.

3.2 Screening test of the isolated bacterial strains

3.2.1 Selection of 700 mg l^{-1} of arsenate/arsenite tolerant bacterial strain.

Selection of the bacterial strains which can grow on nutrient agar containing 700 mg l^{-1} of arsenate/arsenite was performed. The level of arsenate/arsenite in the experiment was set at 700 mg l^{-1} as the average concentration of total arsenic in soil samples at the collection sites of the present study. Each the isolated bacterial strain from a stock culture was streaked on nutrient agar plate containing 700 mg l^{-1} of arsenate/arsenite. Plates were incubated in an incubator at 30 °C for 24-48 h. A colony of bacteria, which could grow on plate, was selected for further study. These selected bacterial strains were restreaking on nutrient agar slant and stored at 4 °C in a refrigerator.

3.2.2 Selection of bacterial strains which have high efficiency in arsenate/arsenite transformation.

Prior to each experiment, the selected bacteria were cultured overnight on a nutrient agar plate containing no arsenate/arsenite. Then they were transferred (with a loop) to nutrient broth and incubated for 24 h to obtain a standard inoculum for each experiment. Experiments were carried out in 250 ml Erlenmeyer flasks containing 50 ml of mineral medium. The arsenate/arsenite compound was used as a substrate, at the initial arsenate/arsenite concentration of 5 mg l^{-1}. 1 ml inoculum of bacteria was added to each flask. The flasks were incubated at 30 °C for 3 days.The initial pH of the medium was adjusted to 5.6. At the end of the incubation period, each sample solution was centrifuged at 12,000 Xg (Sorvall RC 28S) at 25°C for 15 minutes to remove the bacterial cells and particulate matters. The supernatant was then collected and analyzed for the amount of the remaining arsenate/arsenite by Hydride Generation

Atomic Absorption Spectrophotometer (HGAAS). 2% HNO_3 was added into each flask to preserve arsenate/arsenite before the determination on the amount of arsenic was performed. The percentage of arsenate/arsenite transformation obtained from these strains was determined and the bacterial strains that have high arsenate/arsenite - transformation ability were chosen for further study. In this work, arsenate/arsenite transformation percentage was calculated as:

$$\% \text{ Arsenate/Arsenite transformation} = 100\,(C_0 - C_f) / C_0$$

where C_0 is the initial arsenate/arsenite concentration (mg l^{-1}), C_f is the final arsenate/arsenite concentration (mg l^{-1}) at the end of the incubation period.

3.3 Effect of adaptation of bacterial cells in improving arsenate/arsenite transformation ability

It becomes important to determine whether the arsenate/arsenite transformation of the selected bacterial strain are acquired through adaptaion of bacterial cells. The experiment was set up as follows: a flask of nutrient broth was inoculated with a loop of bacterial strain. Adapted cells were pre-grown in nutrient broth containing 100 µg l^{-1} of arsenate/arsenite concentrations, while unadapted cells were pre-grown in nutrient broth without arsenic. After overnight incubation at 30 °C, 1 ml of each turbid broth cultured was separately inoculated into each of two a 250 ml Erlenmeyer flask containing 100 ml mineral medium with 5 mg l^{-1} of the arsenate/arsenite concentrations. The flasks were incubated overnight at 30 °C for 2 days. Each sample had 3 replications. After incubation, each sample was centrifuged at 12,000 Xg for 15 min. The supernatant was analyzes for the amount of remaining arsenate/arsenite concentrations and total aresenic by HGAAS.

3.4 Study on the effects of environmental factors on the activities of the selected bacterial strains in arsenite transformation.

The effects of environmental factors such as pH and temperature on the arsenite transformation were studied. The selected bacterial strains were grown in mineral media containing 10 mg l^{-1} of arsenite concentrations. These cultures were subjected to testing on the effects of low and high temperature (27°C and 37°C) and various pH (at pH 4, 6 and 9). The cultures were incubated in triplicate at 30 °C for 2 days and then analyzed for the amount of remaining arsenite by HGAAS. The pH at the beginning and at the end of the experiment was recorded.

3.5 Effect of the arsenite concentration on growth and on the ability of arsenite transformation.

The selected bacterial strains were inoculated into 100 ml mineral media in which arsenite a level varies from 0 to 10, 100 and 1000 mg l^{-1}. The samples were withdrawn from each flask at 1, 2, 3 and 5 days. Each samples was centrifuged at 12,000 Xg for 15 minute and the supernatant was analyzes for the amount of arsenite and total arsenic by HGAAS. There were 3 replication for each arsenite concentration. Culture solution was withdrawn everyday at the constant time intervals to be measured the absorbance at 680 nm by using spectrophotometer. The generation time (G) for the growth of bacterial population was calculated as:

$$G = (t \log 2) / (\log OD_b - \log OD_a) \text{ min.}$$

where G is the time (minutes) for each generation, t is the time (minutes) for n generation, OD_a and OD_b are optical density at the beginning and at the end of the experiment, respectively.

In this study, the μ value was calculated for comparing the growth rate among the selected bacterial strains. The μ value is the instantaneous growth rate constant, it was calculated as :

$$\mu = \ln 2/G \quad min^{-1}$$

3.6 Kinetic study of the arsenite transformation.

The selected bacterial strains were grown in 500 ml Erlenmeyer flasks containing 200 ml mineral medium with 10 mg l^{-1} of arsenite. The flasks were incubated at the optimum conditions obtained in the earlier experiments. The sample of homogeneous suspension were withdrawn by a 5 ml syringe from each flask at 0, 2, 4, 6, 8, 10, 12, 24 and 48 h after inoculation. Each sample were filtered through a 0.45 μm filter. The filtrates were analyzes for the amount of the remaining arsenite and total inorganic arsenic (As(III) + As(V)) by HGAAS. During the experiment, growth of the culture is also monitored by measuring the absorbance at 680 nm using spectrophotometer.

The substrate control flask received similar treatment but without any inoculum. Each sample had 3 replicates.

3.7 Identification of the selected bacterial strain with possess capability to arsenite transformation.

The selected bacterial strains to be identified were examined for morphological appearances and biochemical properties. These tests include Gram staining, cell shape, motility, catalase, and triple sugar iron (TSI). The API system was also use to identify the selected bacteria. About 20 standard biochemical tests was performed including oxidase, denitrification, indole production, acidification from glucose, arginine dihydrolase, urease, esculin hydrolysis, fermentation of PNPG, and carbon source for growth such as glucose, arabinose, mannose, manitol, N-acetyl-glucosamine, maltose, gluconate, caprate, adipate, malate, citrate and phenyl-acetate. The detailed procedures of these tests are described in Appendix I. The scheme used for identification followed the methods outlined in Bergey's Manual of systematic Bacteriology(35).

3.8 Analyses of arsenic

Dissolved arsenate/arsenite in solution was determined by Hydride Generation Atomic Absorption Spectrophotometer (HGAAS). The hydride generation techniques is commonly used for the determination of trace amounts of arsenic. In this experiment, hydride generation can be coupled with atomic absorption spectrometry. The samples containing arsenic, often acidified, react with the reducing reagent, usually sodiumborohydride ($NaBH_4$), to generate hydrides. They are then transported by an inert gas N_2 or Ar into a quartz tube, where (air/H_2 or air/acetylene) atomizers the hydrides. The arsenic sample was modified when necessary by diluting the arsenic solution sample. The procedures and analytical parameters for arsenic determination described in Appendix II.

CHAPTER IV

RESULTS

4.1 Sample collection and isolation of bacterial strains capable of transformation of more toxic arsenic to less toxic arsenic compounds in soil

Of 63 soil samples collected from various locations on the Ronna mountain, Nakhon Si Thummarat Province in Thailand, all of them showed growth on mineral medium containing 70 mg l^{-1} of arsenate/arsenite compound. The bacteria from these cultures were isolated and purified. 198 strains could be isolated from these soil samples, 110 strains could tolerate 70 mg l^{-1} of arsenate and 88 strains could tolerate 70 mg l^{-1} of arsenite. When these bacteria were cultured in medium containing arsenate/arsenite up to concentration of 700 mg l^{-1} (The average arsenic concentration of soil samples at collection site), it was found that 57 strains could be grown in the arsenate medium and 56 strains could be grown in arsenite medium. In Appendix III, the bacterial strains that could tolerate 700 mg l^{-1} of arsenate/arsenite compound were shown and they were chosen for preliminary screening.

4.2 Screening of the best efficient bacterial strains for arsenate/arsenite transformation

Preliminary screening of the ability of the isolated bacterial strains that could tolerate 700 mg l^{-1} arsenite and arsenate compound were performed. Altogether 113 bacterial strains, each 57 of arsenate tolerance strains and 56 of arsenite tolerance strains, was cultured in the medium containing 2 mg l^{-1} of arsenate/arsenite for 2 days. The percentages of arsenate/arsenite transformation obtained from these strains are shown in Table 4-1 and 4-2. The ability of arsenate/arsenite transformation by bacterial cells differs markedly in different species of bacteria. The results show that the high arsenate-transformation ability were obtained for the strains no.1/4, 2/10, 2/25, 2/34, 2/49, 3/9, 4/31 and the high arsenite-transformation ability were obtained for the strains no. 2/6, 2/26, 2/45, 3/2, 3/18, 3/25, 4/36. Secondary screening tests were carried out among the bacterial strains exhibited the high arsenate/arsenite–transformation ability obtained from the preliminary screening tests. The selected bacterial strains were cultured in medium containing up to 5 of mg l^{-1} arsenate/arsenite for 2 days. The experiments were repeated twice in different times. The results of secondary screening (Table 4-3 and Table 4-4) show that the bacterial strains no. 2/25 and no. 4/31 have high efficiency to transformed arsenate and the bacterial strains no. 2/6 and no. 3/18 have high efficiency to transformed arsenite from the mineral medium. Thus, the strain no.. 2/25, 4/31, 2/6 and 3/18 were chosen for further investigation.

In addition, the percentage of arsenate/arsenite transformation (Table 4-3 and Table 4-4) of the experiment 2 was less than that of the experiment 1. A possible explanation, the arsenate/arsenite transformation ability may be decreased when the bacteria stored on the arsenic-free agar medium for long time. After 2 weeks of the experiment 1, the experiment 2 was performed. In order to the bacteria inocula used in the experiment 1 and 2 were taken from the arsenic-free stock culture. The ability of arsenate/arsenite transformation might be lost.

Table 4-1. Preliminary screening of 700 mg l^{-1} arsenate tolerance bacterial strains for their ability on arsenate transformation at concentration of 2 mg l^{-1} for 2 days.

Strains no.	% Arsenate transformation
1/1	0
1/2	0
***1/4**	**53.79**
1/5	14.20
1/8	0
1/9	12.57
1/11	0
1/15	0
1/16	8.87
1/23	0
1/26	0
2/1	0
***2/10**	**25.98**
2/11	6.20
2/13	7.58
2/23	12.41
2/24	19.99
***2/25**	**50.34**
2/27	14.48
2/28	8.96
2/31	13.10
***2/34**	**68.53**
2/39	13.85
2/40	19.30
2/43	0
***2/49**	**33.10**
3/8	0
***3/9**	**37.93**
3/17	0
3/21	28.96

Table 4-1 (continued)

Strains no.	% Arsenate transformation
3/22	0
3/26	14.48
3/31	6.20
3/34	0
3/37	7.53
3/42	25.51
3/46	0
3/47	19.30
3/49	0
3/53	0
3/54	0
3/56	6.20
3/71	0
3/77	0
3/80	0
4/4	0
4/6	11.24
4/10	11.29
4/11	0
4/19	0
4/21	9.03
4/22	11.29
4/27	2.39
***4/31**	**30.13**
4/33	0
4/35	18.56
4/37	0

* the bold numbers indicated that the bacterial strains were selected for further testing

Table 4-2 Preliminary screening of 700 mg l^{-1} arsenite tolerance bacterial strains for their ability on arsenite transformation at concentration of 2 mg l^{-1} for 2 days.

Strains no.	% Arsenite transformation
1/6	6.45
1/7	3.41
1/10	3.69
1/14	8.28
1/19	0
1/20	4.92
1/24	0
2/3	0
2/5	6.59
***2/6**	**94.46**
2/12	0
2/20	4.43
***2/26**	**52.62**
2/30	0
2/36	0
2/38	6.00
2/41	3.80
2/42	0
2/44	3.39
***2/45**	**50.77**
2/47	6.31
2/50	7.10
***3/2**	**55.75**
3/3	5.22
3/10	0
3/11	6.75
3/14	10.73
***3/18**	**94.02**
3/19	18.48
3/20	5.02
3/24	12.94

Table 4-2 (continued)

Strains no.	% Arsenite transformation
***3/25**	**40.01**
3/28	3.48
3/29	0
3/38	21.87
3/41	7.06
3/45	3.69
3/52	5.56
3/59	22.35
3/74	6.49
3/75	5.22
3/79	9.14
4/3	7.36
4/5	0
4/7	0
4/9	0
4/12	6.14
4/15	4.02
4/20	6.94
4/23	11.66
4/26	4.95
4/30	6.45
4/32	4.31
4/34	2.49
***4/36**	**47.11**
4/38	0

* the bold numbers indicated that the bacterial strains were selected for further testing

Table 4-3 Secondary screening of bacterial strains with high ability on arsenate transformation at concentration of 5 mg l^{-1} for 2 days. The experiments were repeated twice in different times.

Strains no.	% Arsenate Transformation	
	Experiment 1	Experiment 2
1/4	5.77 ± 2 .45	2.80 ± 2.21
2/10	8.70 ± 0.57	6.10 ± 0.75
***2/25**	**11.74 ± 1.05**	**6.40 ± 0.45**
2/34	6.86 ± 2.52	3.90 ± 1.04
2/49	4.77 ± 0.86	1.60 ± 4.16
3/9	8.03 ± 0.48	2.80 ± 0.17
***4/31**	**15.44 ± 1.51**	**7.10 ± 0.34**

* the bold numbers indicated that the bacterial strains were selected for further testing

Table 4-4 Secondary screening of bacterial strains with high ability on arsenite transformation at concentration of 5 mg l^{-1} for 2 days. The experiments were repeated twice in different times.

Strains no.	% Arsenite Transformation	
	Experiment 1	Experiment 2
***2/6**	**94.53 ± 0.81**	**89.72 ± 0.46**
2/26	7.98 ± 1.66	2.02 ± 1.42
2/45	7.07 ± 1.97	2.49 ± 1.17
3/2	7.07 ± 1.97	5.14 ± 2.03
***3/18**	**94.89 ± 0.15**	**91.75 ± 0.26**
3/25	63.56 ± 8.71	68.22 ± 1.61
4/36	4.34 ± 2.18	4.36 ± 1.07

* the bold numbers indicated that the bacterial strains were selected for further testing

4.3 Effect of adaptation of bacterial cells in improving arsenate/arsenite transformation.

Adaptation of bacterial cell for arsenate/arsenite transformation ability are shown by comparing the percentage of arsenite transformation of the adapted cells and the unadapted cells growing in mineral medium containing 5 mg l^{-1} arsenate/arsenite compound for 2 days in Table 4-5. The bacteria inocula used in this experiment were called "adapted cell" as they were pre-grown in medium containing arsenate/arsenite. Those called "unadapted cell" as they were pre-grown in arsenic free medium. The ability of arsenate transformation in adapted cell was increased when compared with unadapted cell. However, the ability of arsenite transformation in adapted cell showed no different. In the experiment, total arsenic concentrations (As(III) + As(V)) in the medium remained unchanged. This indicated that none of the arsenic was removed from the solution. When the arsenate tolerance strains from arsenate medium were cultured in the arsenate medium, arsenite was detected in the solution. These cells thus exhibited the ability to reduce arsenate to arsenite. It can also found that the cell which was cultured in arsenite, it could decrease arsenite concentration in the medium and at the same time arsenate could be detect in the medium. These cells, thus, exhibited the ability to oxidize arsenite to arsenate. As it has been shown that arsenite were more toxic than arsenate, the transformation of arsenite, under this experimental condition, to arsenate was interested in this study. Among the 5 selected strains from the secondary screening test, the strain no. 2/6 and no. 3/18 showed the highest efficiency in arsenite transformation and, thus, they were selected as the lead candidates for further study.

Table 4-5 Comparison of capability of the selected bacterial strains in arsenate/arsenite transformation ability between adaptation and unadaptation.

Strains no.	% Arsenate transformation		% Arsenite Transformation	
	[a]Adaptation	[b] Unadaptation	[a]Adaptation	[b] Unadaptation
***2/6**	-	-	**89.62 ± 0.76**	92.19 ± 0.04
***3/18**	-	-	**90.77 ± 1.85**	87.69 ± 3.33
2/25	19.74 ± 2.68	3.22 ± 1.64	-	-
3/25	68.05 ± 1.53	36.48 ± 0.0	-	-
4/31	8.61 ± 1.71	1.24± 1.08	-	-

[a] The bacterial strain was pre-grown in arsenate/arsenite containing medium

[b] The bacterial strain was pre-grown in arsenate/arsenite free medium

*The bold numbers indicated that the bacterial strains were selected for further testing

4.4 Effect of environmental factors in arsenite transformation

Environmental factors were tested to see their influences on arsenite transformation by the selected bacterial strains. The effects of temperature and pH on arsenite transformation were evaluated for appropriate environmental conditions. The effect of pH on the transformation of arsenite by the strains no. 2/6 and no. 3/18 was determined at pH 4, 6 and 9 and at temperature 27 °C and 37 °C. As shown in Table 4-6, after 2 days of incubation, transformation capabilities were similar at all pH and temperatures tested values. The percentage of arsenite transformation reached 90 %. Thus the strains no. 2/6 and no. 3/18 exhibited the high arsenite transformation ability after 2 days of incubation, approximately 90 % with the optimum temperature range between 27 °C and 37 °C and the optimum pH range between pH 4 and 9.

Table 4-7 presents the initial and the final pH of culture medium after 2 days of incubation. After inoculation with the selected bacterial strains the pH of medium was slightly decreased. The pH dropped during growth as a result of the formation of arsenate.

Table 4-6 Effect of environmental factors in arsenite transformation by strains no. 2/6 and strains no. 3/18, culture for 2 days.

Strains no.	Environmental Factors		% Arsenite transformation	Absorbance (680 nm)
	PH	Temp. (°C)		
2/6	4	27	90.40 ± 0.42	0.179
		37	90.08 ± 0.00	0.229
	6	27	91.24 ± 0.48	0.198
		37	89.98 ± 0.29	0.271
	9	27	90.26 ± 0.37	0.183
		37	89.97 ± 0.67	0.247
3/18	4	27	91.26 ± 0.45	0.088
		37	91.03 ± 0.37	0.085
	6	27	90.80 ± 0.18	0.090
		37	90.91 ± 0.23	0.081
	9	27	90.78 ± 0.83	0.085
		37	91.55 ± 0.19	0.080

Table 4-7. Changes of pH in mineral medium before, and after incubation, in the experiment on transformation of arsenite. Cultures were grown in mineral medium with 5 mg l^{-1} of arsenic at 30 °C for 2 days.

Initial pH	Final pH					
	Control		Strains no. 2/6		Strains no. 3/18	
	27°C	37°C	27°C	37°C	27°C	37°C
4.0	4.3	4.2	3.6	3.7	3.7	3.9
6.0	6.0	6.0	5.2	5.7	5.8	6.0
9.0	8.0	8.0	6.0	7.8	7.5	7.7

4.5 Effect of arsenite concentration on growth of the selected bacterial strains.

The growth curve of bacterial strain no. 2/6 growing in the media containing 0, 10, 100 and 1000 mg l^{-1} of arsenite concentration are shown in Figure 4-1 (A). After 2 days of incubation, strain no. 2/6 grew equally well in medium containing 10, 100 mg l^{-1} of arsenite and as well as the control (without arsenite). But its growth was considerably inhibited at 1000 mg l^{-1} arsenite concentration. Figure 4-1(B) shows the effect of arsenite concentration on growth of bacterial strain no. 3/18. The strain no. 3/18 shows higher growth rate in the medium containing at 1000 mg l^{-1} of arsenite than other arsenite concentration. However, growth of strain no. 3/18 was poor comparing with that of strain no. 2/6.

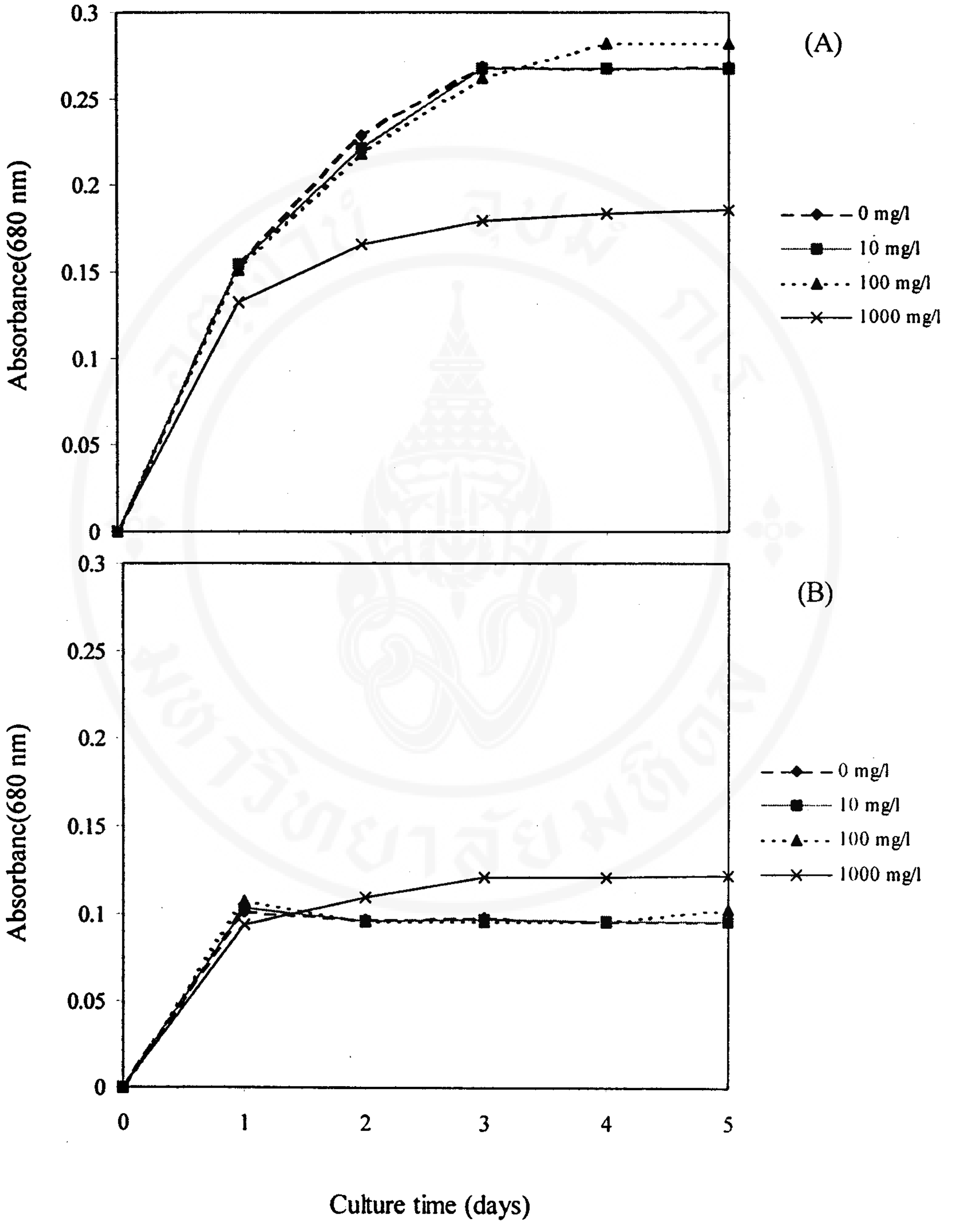

Figure 4-1 Growth curve of the bacterial strain no. 2/6 (A) and strain no. 3/18 (B) in mineral medium containing various concentrations of arsenite.

4.6 Effect of arsenite concentration in arsenite transformation ability.

Figure 4-2 (A) shows the transformation of arsenite from solution by strain no. 2/6 at the initial concentration of 10, 100 and 1000 mg l^{-1} arsenite. The transformation was inversely proportional to the initial concentration of arsenite. Figure 4-2 (B), the strain no. 3/18 showed similar behavior as the strain no. 2/6, but the ability of arsenite transformation showed no different in arsenite concentration of 10 and 100 mg l^{-1}. Table 4-8 summarizes the effect of the arsenite concentration in the arsenite transformation ability over time by strain no. 2/6 and strain no. 3/18. The purpose of this experiment was to determine the percentages of arsenite transformation at various arsenite concentrations. The results indicate that both strains are able to effectively transformed arsenite over a concentration range of 10 to 1000 mg l^{-1}. The greatest percentage transformation (approximately 90 %) was found at 10 and 100 mgl^{-1} arsenite within 5 days. The percentage of arsenite transformation at 1000 mgl^{-1} was somehow inhibited. Table 4-8 also shows that the strain no. 3/18 had better transformation rate (75.24 %) in medium containing 1000 mg l^{-1} of arsenite than the strain no. 2/6 (65.81 %). In the uninoculated controls, no decrease of arsenite was observed over 5 days of incubation.

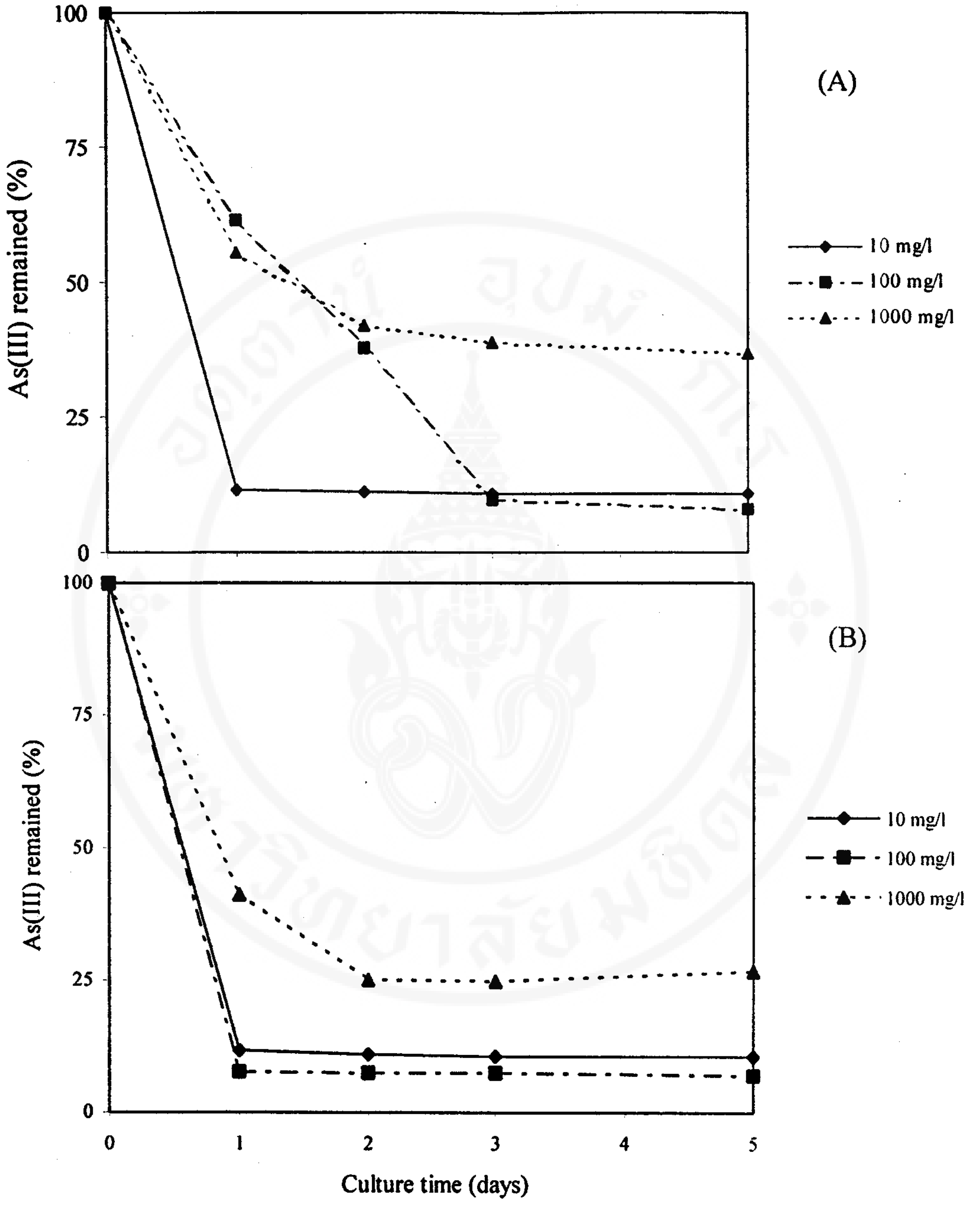

Figure 4-2 Transformation of arsenite over time by the bacterial strain no. 2/6 (A) and strain no. 3/18 (B) growing in various concentration of arsenite in the mineral medium.

Table 4-8 Effect of arsenite concentration in the arsenite transformation ability in the strains no.2/6 and no.3/18

Test System	Initial arsenite Conc. (mg l^{-1})	Concentration of remaining arsenite (mg l^{-1})				% Arsenite transformation
		Day 1	Day 2	Day 3	Day 5	
Control	10.08 ± 0.09	*nd	*nd	*nd	10.01 ± 0.04	0.79
	101.80 ± 0.10	*nd	*nd	*nd	98.91± 1.30	2.83
	1080.95 ± 12.83	*nd	*nd	*nd	1024.83 ± 14.94	5.19
No. 2/6	10.08 ± 0.09	1.14 ± 0.10	1.09 ± 0.09	1.08 ± 0.02	1.06 ± 0.01	89.41
	101.80 ± 0.10	61.37 ± 0.74	37.76 ± 4.91	9.82 ± 0.24	7.69 ± 0.27	92.44
	1080.95 ± 12.83	556.85 ± 63.52	419.17 ± 19.95	389.69 ± 19.71	369.56 ± 20.27	65.81
No. 3/18	10.08 ± 0.09	1.16 ± 0.01	1.09 ± 0.02	1.06 ± 0.01	1.05 ± 0.02	89.52
	101.80 ± 0.10	7.63 ± 0.32	7.42 ± 0.50	7.45 ± 0.17	7.03 ± 0.13	93.09
	1080.95 ± 12.83	411.92 ± 23.43	250.83 ± 24.97	247.49 ± 28.52	267.55 ± 11.70	75.24

* nd = not determined.

4.7 Kinetic study of the arsenite transformation in the strain no. 2/6 and no. 3/18

The growth pattern of strain no. 2/6 and its ability to transformed arsenite are shown in Figure 4-3 (A). Figure 4-3 (B) indicates similar plots for strain no. 3/18. The strain no. 2/6 and strain no. 3/18 reached the stationary phase after 24 and 8 h, respectively of growth. The values of μ (instantaneous growth rate constant) were 0.190 and 0.171 min^{-1}, respectively. Thus the strain no. 2/6 showed growth better than the strain no. 3/18. Biotransformation of arsenite by strain no. 2/6 and strain no. 3/18 exhibited from the log phase to the stationary phase, without a lag phase. The concentration of arsenite in the medium was found to be gradually decrease, with the concurrent increase in the turbidity of the arsenite culture. Comparison of strain no. 2/6 to strain no. 3/18 in transformation of arsenite from mineral medium containing 10 mg l^{-1}, the strain no. 3/18 could transformed approximately 90 % within 8 h, while the strain no. 2/6 could oxidized approximately 90 % within 24 h. Thus, strain no. 3/18 showed better efficiency in transformation of arsenite than strain no. 2/6.

The decrease of the arsenite concentration and the formation of arsenate during a kinetic run are shown in Figure 4-4. The total arsenic (As(III) and As(V)) concentrations remained constant after incubation, while arsenite was further transformed and arsenate was released into the solution. In this experiment, arsenite transformation only occurred in the presence of bacteria but not in the controls (without bacterial inoculum). After 24 h of incubation, the bacteria could transform 90 % of dissolved arsenite in the experiment system. This result indicated that the strain no. 2/6 and strain no. 3/18 showed the ability of transformation of arsenite to arsenate.

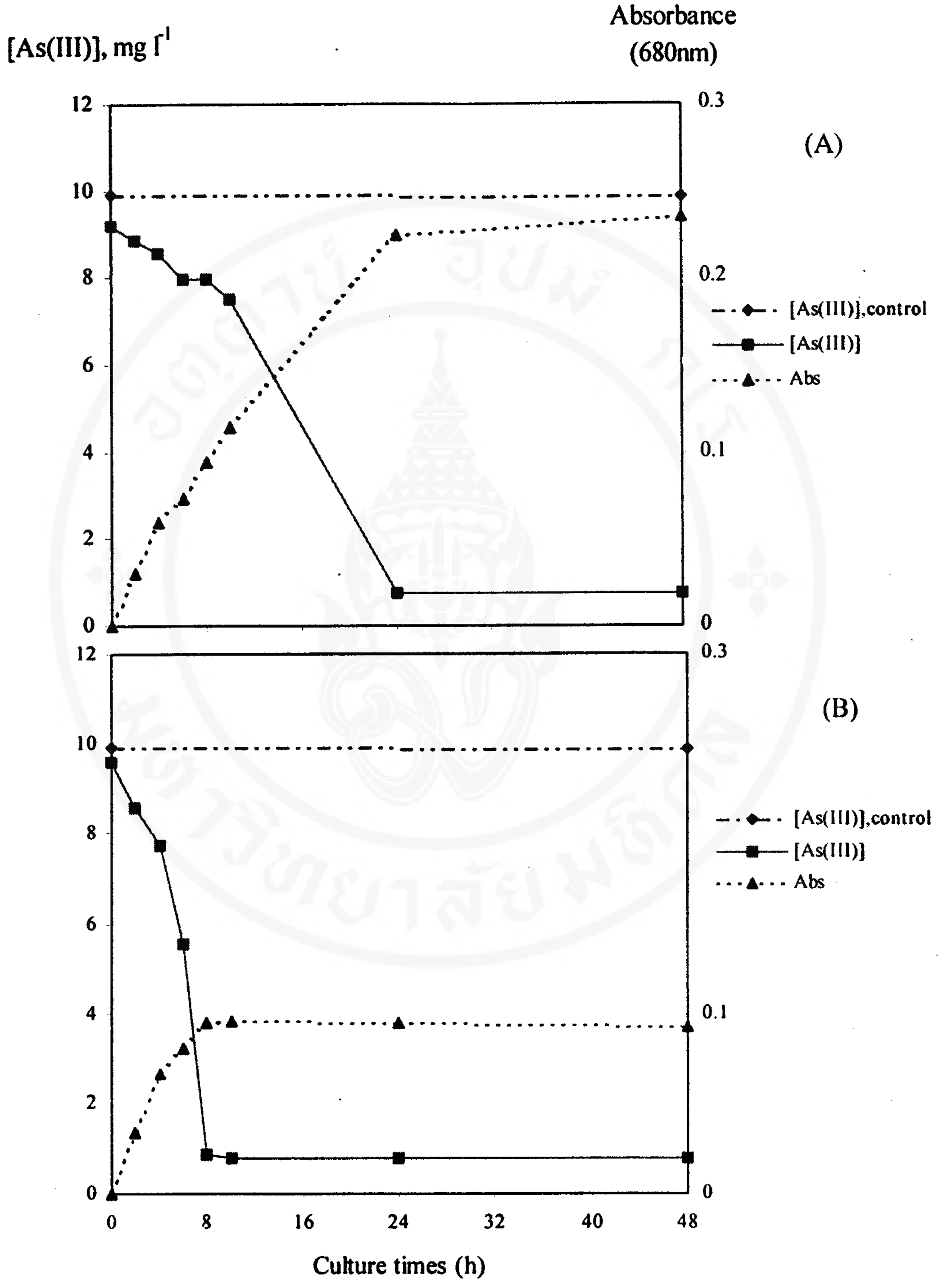

Figure 4-3 Kinetics for growth and transformation of arsenite by the strain no. 2/6 (A)and strain no. 3/18 (B). Growth of the bacterial strain (Abs) and the remaining concentration of arsenite [As(III)] were plotted against at various time intervals for the experiment (with inoculum) and the control (without inoculum).

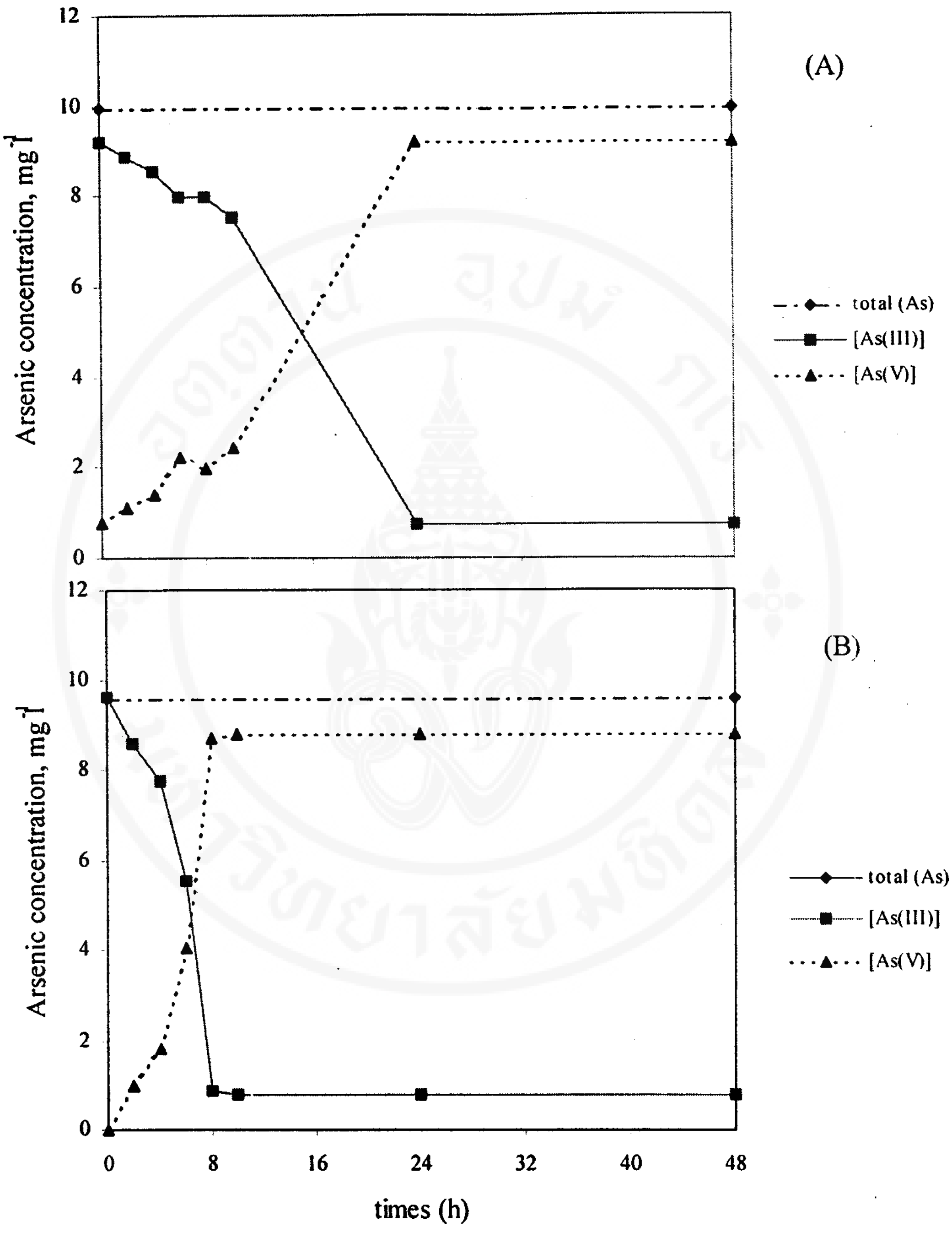

Figure 4-4 Decrease of arsenite [As(III)] and formation of arsenate [As(V)] during a kinetic run by the bacterial strain no. 2/6 and strain no. 3/18.

4.8 Identification of bacterial strain no. 2/6 and strain no. 3/18 with possess capability to arsenite transformation

After obtaining pure cultures of the two bacterial strains capable to transformed arsenite (As(III)) to arsenate (As(V)), the isolates were identified based on the procedure given in the Bergey's Manual of Systematic Bacteriology. The isolates were found to be Gram negative, aerobic and, appeared microscopically as short rods. On nutrient agar plate, These isolates formed circular, smooth, buff, translucent colonies of 2-3 mm. in diameter when grown at 30 °C for 48 hr. Further biochemical tests were performed and the results are shown in Table 4-9.

Base on the data illustrated in Table 4-9, and the classification scheme outlined in Bergey's Manual of Systematic Bacteriology, these bacteria were identified as *Alcaligenes xylosoxydans* subsp. *xylosoxydans* at a confidence level of 99 %. One strain was designated as *Alcaligenes xylosoxydans* subsp. *xylosoxydans* strain 2/6 (Figure 4-5A) and the other one was designated as *Alcaligenes xylosoxydans* subsp. *xylosoxydans* strain 3/18 (Figure 4-5B) these designation were used throughout this study.

Table 4-9 The biochemical characteristic of the selected bacterial isolate strains no. 2/ 6 and no. 3/18*

Biochemical Tests	Isolated strain no. 2/6	Isolated strain no. 3/18	*Alc. xylosoxidans* subsp. *xylosoxidans*
Gram reaction	-	-	-
Cell shape	rod	rod	rod
Motility	+	+	+
Oxidase	+	+	+
Catalase	+	+	+
Denitrification	+	+	+
Indole production	-	-	-
Acidification from glucose	-	-	-
Arginine dihydrolase	-	-	-
Urease	-	-	-
Esculin hydrolysis	-	-	-
Gelatin hydrolysis	-	-	-
Fermentation of PNPG	-	-	-
H_2S production	-	-	-
Carbon source for growth:	+	+	+
Glucose	+	+	+
Arabinose	-	-	-
Mannose	+	+	+
Manitol	+	+	+
N-acetyl-glucosamine	-	-	-

Table 4-9 (continued)

Biochemical Tests	Isolated strain no. 2/6	Isolated strain no. 3/18	*Alc. xylosoxidans* subsp. *xylosoxidans*
Maltose	-	-	-
Gluconate	+	+	+
Caprate	+	+	+
Adipate	+	+	+
Malate	+	+	+
Citrate	+	+	+
Phenyl-acetate	+	+	+

* Details for all procedure appeared in appendix I

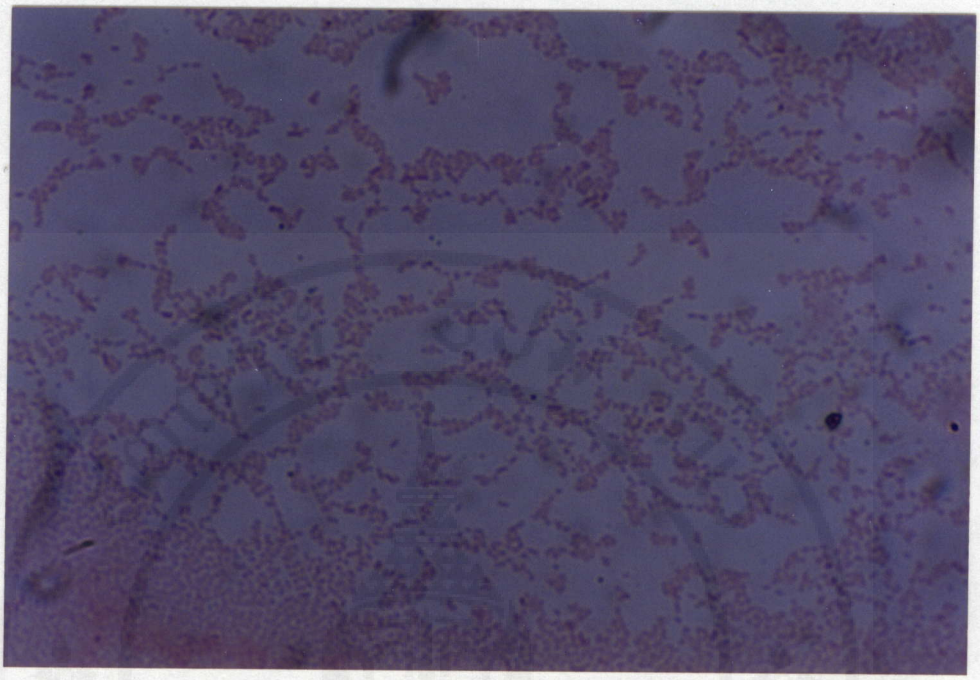

Figure 4-5A : *Alcaligenes xylosoxidans* subsp. *xylosoxidans* strain no. 2/6 (x1000)

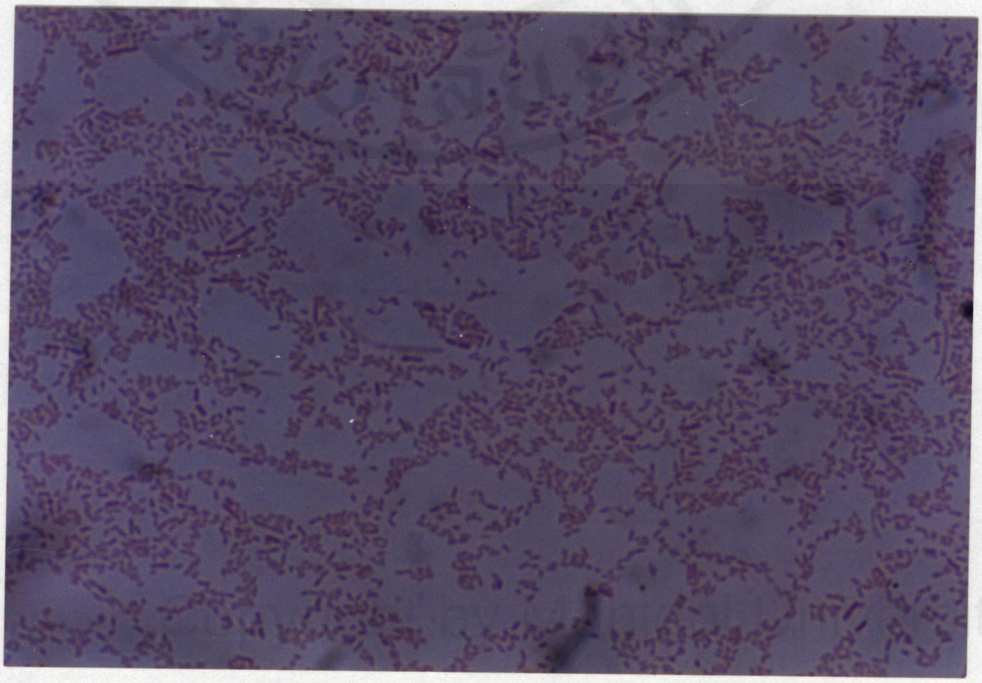

Figure 4-5B : *Alcaligenes xylosoxidans* subsp. *xylosoxidans* strain no. 3/18(x1000)

CHAPTER V

DISCUSSION

In the course of our study on the transformation of arsenic from arsenic contaminated area by bacteria. By screening of 188 bacterial strains isolated from soils and mine tailings collected from the Ronna Mountain, Ron Phibun District, Nakhon Si Thummarat Province, southern Thailand, it was found that two bacterial strains, showed the highest efficiency on transformation of toxic arsenic (As(III)) compound under the experimental condition. These strains are Gram-negative, aerobic rod and are identified as *Alcaligenes xylosoxydans* subsp. *xylosoxydans*. One was designated as *Alcaligenes xylosoxydans* subsp. *xylosoxydans* strain no. 2/6 and the other one was designated as *Alcaligenes xylosoxydans* subsp. *xylosoxydans* strain no. 3/18. Both isolated bacterial strain no. 2/6 and strain no. 3/18 were obtained from soil sample. This result suggested that the high capability to transformed arsenite was not widespread among bacteria, which were commonly found in the environment. In this study it was found that the concentration of arsenite was decreased during the experiment, but the total arsenic concentration in flask remained constant. It also was found that while the concentration of arsenite was decreased, the concentration of arsenate was increased. Growth of bacteria accompanied by decrease of arsenite and increase of arsenate in the cultures. It is known that the normal inorganic form of arsenic are the trivalent (As(III)) and pentavalent (As(V)). Trivalent arsenic (As(III)) is much more toxic and mobile than the pentavalent form (As(V)). This indicates that these selected bacterial strains are able to transformed the more toxic arsenic As(III) to the less toxic form As(V) by oxidation. Oxidation of arsenite by bacteria has been previously reported by the genera *Bacillus* (36) and *Pseudomonas* (22,23). In 1976, Phillips (18) and Taylor could isolated *Alcaligenes faecalis* from sewage, and also shown to be capable of oxidizing arsenite to arsenate.

The bacterial strains were able to grow and transformed arsenite in the medium into arsenate. From this result, there are two possible explanations. First, the oxidation of arsenite to arsenate occurred by respiratory metabolism (arsenite acted as electron donors). Osborne and Ehrlich (17), reported on arsenite oxidation by a strain of *Alcaligenes faecalis*. They suggested that bacterial growth in arsenite induced arsenite-oxidizing system response to respiratory metabolism. Phillips and Taylor (18), also studied the arsenite oxidation by *Alcaligenes faecalis.* They reported that the arsenite oxidation was due to oxygen-sensitive, inducible enzyme and/or component of the electron transport system when the bacteria were grown in nutrient broth-yeast extract medium, the enzyme appeared in the late exponential phase of growth. Secondly, the bacteria might directly oxidized arsenite by extracellular enzymes. The results obtained from this study suggested that the mechanism of transformation of arsenite to arsenate by the isolated bacteria *Alcaligenes xylosoxidans* subsp. *xylosoxidans* occurred via the respiratory metabolism. According to Figure 4-3 and Figure 4-4, it can be seen that the arsenite was decreased while the bacteria were in the exponential growth. When the bacteria reached the stationary phase, the concentration of arsenite did not changed.

In theory of the respiratory metabolism (37), the rapid and complete oxidation of substrate with the subsequent removal of electrons takes place. The electrons are passed through an electron transport chain to terminal electron acceptor. If the terminal electron acceptor is oxygen, the process is called aerobic respiration. However, If the terminal electron acceptor is any other type of in organic compound, the process is referred to as anaerobic respiration. Respiratory catabolism uses enzymes that rapidly oxidize compounds. In these oxygenative pathways, electrons are passed from one cytochrome to another in the electron transport chain. With each step the electrons transfer some of their energy to the phosphorylation of adenosine diphosphate (ADP) to from adenosine triphosphate (ATP). This process is called oxidative phosphorylation. Oxidase and catalase, two enzymes involved in the utilization of oxygen by aerobically respiring bacteria.

Turner and Legge (24,25) have shown that the isolate of *Alcaligenes faecalis*, when arsenite induced, is able to oxidize arsenite to arsenate stoichometrically with oxygen as terminal acceptor. The results of their experiment suggest that the following electron transport chain may involve in arsenite oxidation by the organism.

$$AsO_2^- \xrightarrow{\text{arsenite dehydrogenase}} \text{cyt. c} \longrightarrow \text{cytrochrome oxidase} \longrightarrow O_2$$

When comparing the oxidation of arsenite by *Alcaligenes faecalis* with that of *Pseudomonas arsenoxydans-quinque* (24,25), it is found that the oxidoreductase enzymes of *Alcaligenes faecalis* are capable of transfering electrons from arsenite to O_2. The *Alcaligenes faecalis* also appears to utilize sulphydryl groups and cytochrome c, while according to Turner & Legge (24,25), *Pseudomonas arsenoxydans-quinque* does not. In this study the results (Figure 4-3 and Figure 4-4) suggested that the process of arsenite oxidation might be dependent upon energy conserved during the oxidation of arsenite to arsenate. The use of arsenite as the electron donor excludes the possibility that ATP is formed via any substrate-level phosphorylation reactions. The *Alcaligenes xylosoxydans* subsp. *xylosoxydans* may also gain energy from arsenite respiration. Presumably, the energy is conserved via electron-transport phosphorylation, with the arsenite oxidase functioning.

Results from this study also showed that *Alcaligenes xylosoxydans* subsp. *xylosoxydans* has much the high capability to remove arsenite almost to completion (approximately 90 %). From the literature reviewed by Silver (38), it was well document that the tolerance function of arsenic by bacteria might be controlled by plasmids of which most were found in soil strains. Many bacteria species have variants that show tolerance to arsenite and/or arsenate. The determination of arsenic tolerance can be governed by gene on bacterial chromosome or on extrachromosomal plasmids. Silver and Nakara (15) quoted that "the mechanism of chromosomally governed arsenate resistance is as alteration in the properties of the phosphate transport system which is responsible for cellular accumulation of arsenate. Plasmid mediated arsenate resistance is due to a separate highly specific arsenate efflux "pump". The mechanism of plasmid-determined arsenite resistance is distinct from that for arsenate. The mechanism of plasmid-determined arsenite resistance is not understood, but does not involve extracellular chelation by thiol compounds or detoxification. There appears to be two mechanisms of chromosomally governed arsenite resistance in different bacterial species: one involves oxidation of arsenite to arsenate by an inducible enzyme system. The second, non-oxidizing mechanism is not known. The mechanism of plasmid-mediated arsenite resistance does not involve extracellular detoxification, but it also is not known at this time. Presumably chromosomally determined arsenite resistance in *Alcaligenes* strains is due to oxidation of arsenite to arsenate by an inducible enzyme system.

The development of enzyme systems for the oxidation of arsenite to the less toxic arsenic suggests itself as one mechanism for protecting vulnerable thiol groups from inactivation by arsenite. However, this property is not essential to high tolerance towards arsenite. As it can be seen from Turner (23,24) that certain arsenite-resisting fluorescent and non-fluorescent pseudomonads isolated from cattle-dipping fluids could be trained to tolerate 0.1 M arsenite or more, without showing any evidence of arsenite oxidation.

All of the arsenite-oxidizing bacteria discovered so far are only gram negative bacteria. They have been shown to possess higher ability to remove arsenite. This might be due to differences in structural and functional differences in the cell envelopes of Gram positive and Gram negative bacteria.

Adaptation of bacterial cells could play an important role in improving biomass properties for metal removal. Maeda (34) considered that the ability to detoxify arsenic is enhanced by adaptation of the cell to arsenic, leading to an increase in the uptake of arsenic. In addition Osborn (17) reported that, a strain of *Alcaligenes faecalis* isolated from soil could also oxidize arsenite to arsenate. The optimum pH for arsenite oxidation is 7.0 and the enzyme was induced in about three-cell generation. From the result of this study (Table 4-5), adaptation of bacterial cells resulted in increasing of arsenate transformation ability but no different in arsenite transformation ability. The strain no. 2/6 and no. 3/18 were the arsenite-oxidizing bacteria, which possess higher ability to oxidize arsenite to arsenate than others isolated bacteria at low arsenite (5 mg l^{-1}) concentration.

Because arsenite is both more toxic and more mobile in natural environments than arsenate, efforts to minimize arsenical hazards have concentrated on maintaining aerobic condition in the arsenic contaminated areas (39). The arsenite (As(III)) oxidation process results in less toxic and more strongly adsorbed arsenate (As(V)), which would cause a decrease in the mobility of As in the environment. This transformation shown, in this study, mediated by the bacteria *Alcaligenes xylosoxidans* subsp. *xylosoxidans.*

Environmental factors were tested to see their influences on arsenite oxidation by 2 strains of the isolated bacteria. The effects of temperature and pH on arsenite oxidation were evaluated for appropriate environmental conditions. The result shows that the suitable condition of arsenite oxidation occurred at pH 4-9 and at temperature 27 °C to 37 °C (Table 4-6). This indicates that strains of *Alcaligenes* could tolerate a wide pH and temperature range. It can be seen that these two factors have influenced on cell growth. At temperature 27 °C, the strain no. 2/6 showed lower growth than at temperature 37 °C. Also at this temperature, pH 6 gave the best growth. Thus although the strain no. 2/6 can grow in a wide pH and temperature range but the suitable condition of this bacterial strain is temperature 37 °C and pH 6. For strain no. 3/16 neither temperature nor pH has any influence on cell growth (Table 4-6). So this bacteria could perform their activities at temperature 27 °C - 37 °C and pH 4 – 9. This experiment also shows a small pH decreased, while the oxidation is in progressed. It is probably the decrease in pH occurred as a result of the bacterial action (Table 4-7). It demonstrates that if the electron transfer is the rate-determining step, as enhanced rate constant with decreasing pH would be expected. Turner (23,24) studied growth of *Pseudomonas arsenoxydans-quinque* in KA3 medium. He showed that growth of this bacterial strain could take place in the pH range 6.1-9.4. Because arsenic acid (As(III)) can dissociate much better than arsenious acid (As(V)), the pH dropped during growth as a result of the formation of arsenate. However, in this experiment two strains of *Alcaligenes* were cultured in mineral medium and the pH shows only a small decrease when compared with Turner's experiment. Thus the buffering system used in this study may be highly efficient.

Studies on growth of the two selected bacterial strains of *Alcaligenes xylosoxydans* subsp. *xylosoxydans* in various initial concentrations of arsenite indicated (Figure 4-1) that the strain no. 2/6 exhibited the inhibition effect of As(III) becomes obvious at 1000 mg l^{-1} of arsenite as the growth of the bacterial strain decreased. This may be due to toxicity of the higher arsenite concentration. However, growth of strain no. 3/18 was better at 1000 mg l^{-1} than at the lower arsenite concentration, i.e., 10 and 100 mg l^{-1}. It might be explained that the bacteria strain no. 3/18 obtained energy to support growth also from arsenite oxidation. Nevertheless, the turbidity of the culture of strain no. 3/18 was lower than that achieved with strain no. 2/6. It suggested that the strain no. 2/6 had higher growth rate than the strain no. 3/18. Thus the mineral medium for culture these strains was more suitable for growth of strain no. 2/6 than that of strain no. 3/18.

Experiments were conducted to investigate the effect of arsenite concentration on arsenite oxidation. Results of this study (Figure 4-2) indicates that the two strains of *Alcaligenes* are capable of oxidizing arsenite at high concentrations up to 1000 mg l^{-1} to less toxic form. However, the strain no. 2/6 (Figure 4-2A) the oxidation rate decreased with increasing arsenite concentrations. This may be due to the toxicity of arsenite. The strain no. 3/18 (Figure 4-2B) shows high ability on arsenite oxidation when compare with strain no. 2/6, while the strain no. 2/6 shows growth better than the strain no. 3/18 at higher concentration of arsenite (Figure 4-1). This suggested that the ability to oxidize arsenite by the selected bacterial strain does not depend on the tolerance ability. The study using other bacterial strain by Phillips & Taylor (18) produced similar results. They reported that several bacteria were exhibited that tolerated 0.02 M arsenite but could not oxidized or reduced arsenite or convert it to organic arsenicals.

Figure 4-3 indicates that the rate of disappearance of arsenite compound corresponds with the rate of increase in turbidity of bacterial culture. There was no lag phase occurred in growth curve because the bacteria were pre-grown in nutrient broth overnight until to the midexponential growth phase. Arsenite oxidation occurred concurrently with the exponential growth phase. This result suggests that the bacterial grew with decreasing arsenite. The higher oxidizing rate of arsenite during the 1^{st} day of the experiment occurs as the bacteria are in the log phase. For the strain no. 2/6 (Figure 4-3A), between 0 h and 24 h after incubation, the cell number of bacteria was rapidly increased, while the removal rate was also higher. However, the strain no. 3/18 (Figure 4-3B) showed the similar behavior, but the difference in time of log phase. The time required for the almost to complete oxidation of arsenite (approximately 90 %) were 24 and 8 hours by strain no. 2/6 and no. 3/18, respectively. During the exponential phase, the strain no. 3/18 could oxidize arsenite higher rate than strain no. 2/6. From the value of μ (instantaneous growth rate constant) of both strain no. 2/6 and strain no. 3/18 being 0.190 min^{-1} and 0.171 min^{-1}, respectively. It suggested that the strain no. 2/6 had higher growth rate than the strain no. 3/18, while the rate of arsenite oxidation of the bacterial cell strains no. 3/18 higher than that of strain no. 2/6. The results also show the decrease of the arsenite concentration and the formation of arsenate during a kinetic run (Figure 4-4). This result confirmed that the bacterial oxidized the more toxic arsenite to less toxic arsenate.

Data from this study indicates that the oxidation of arsenite to arsenate by the isolated *Alcaligenes xylosoxidans* subsp. *xylosoxidans* strain no. 2/6 and strain no. 3/18 may be useful in further development for detoxification of arsenic compounds on contaminated area using biological method.

CHAPTER VI

CONCLUSIONS AND RECOMENDATIONS

6.1 Conclusions

Soil samples collected from Ronna mountain, Ron Phibun District, Nakhon Si Thammarat Province, were screened for bacteria having arsenite transformation capability. The following conclusions can be drawn from the foregoing study:

6.1.1 Of the 63 samples collected, 188 strains can be isolated from soil samples that could resist to 700 $mg.l^{-1}$ of arsenite in medium.

6.1.2 Two bacteria showed the highest arsenite transformation capability (approximately 90 %) were purified, identified and designated as *Alcaligenes xylosoxydans* subsp. *xylosoxydans* strain no. 2/6 and no. 3/18

6.1.3 The two selected strains of *Alcaligenes* transformed arsenite from solution by oxidation process, they possessed the ability to oxidize arsenite in the mineral medium to arsenate.

6.1.4 Adaptation treatment bacterial cell caused increasing in arsenate transformation capability but no difference in arsenite transformation.

6.1.5 The arsenite oxidation occurred under aerobic conditions, at pH between 4 and 9 and at temperatures of 27 °C to 37 °C. A small pH decreased at the end of the experiment resulted from the formation of arsenate.

6.1.5 The two selected strains oxidized arsenite to arsenate at initial concentrations up to 1000 $mg.l^{-1}$ arsenite. The arsenite oxidation was almost to complete in medium at arsenite concentration of 10 and 100 mg l^{-1} (approximately 90 %). The concentration of 1000 mg l^{-1} arsenite inhibited both growth and arsenite oxidation of the strain no. 2/6. However, this concentration had no effected on growth but had considerable inhibitory effect on arsenite oxidation of strain 3/18.

6.1.7 The strain no. 2/6 and no. 3/18 reached the stationary phase after 24 and 8 h of growth, respectively. Their values of μ were 0.190 and 0.171, respectively. The strain no. 3/18 could oxidize arsenite more rapidly than the strain no. 2/6 while strain no. 2/6 could grow higher than strain no. 3/18.

6.1.8 The two strains of *Alcaligenes* possessed their potentials for use in bioremediation of arsenic contaminated area.

6.2 Recommendations

The results in this study indicate that the selected bacterial strains had high ability to transformation arsenite to arsenate. Therefore, before the application of these bacterial strains to remediate the arsenic contaminated area, more detail informations about this selected bacterial stain should be studied. The further important studies on arsenite transformation are recommended as follow:

6.2.1 More information on transformation of arsenite is recommended. This is to achieve a more understanding of the arsenite to bacterial cell interaction.

6.2.2 The influence of related factors and the rate of arsenite transformation should be further studied in more details.

6.2.3 The enhancement of arsenite transformation ability of the selected bacterial strains should be studied.

6.2.4 The application of the selected bacterial strain in arsenite transformation should be studied.

REFERENCES

1. Chu HY. Arsenic distribution in soils. In: Nriagu JO, editor. Arsenic in environment part I: cycling and characterization. New York: John Wiley & Sons; 1994.p. 17-49.
2. Kipling MD. Arsenic. In: Lenihan and Flecher, editors. The chemical environment: environment and man volume 6. Glasgow: Blackie; 1977. p. 93- 120.
3. Williams M, Fordyce F, Paijitprapapong A, Charoenchaisri P. Arsenic contamination in surface drainage and groundwater in part of the Southeast Asian tin belt, Nakhon Si Thummarat province, Southern Thailand. Environ Geol 1996; 27:16-33.
4. Gadd GM. Heavy metals accumulation by bacteria and other microorganisms. Experentia 1990; 46: 834-840.
5. Bhumbla DK, Keefer RF. Arsenic mobilization and bioavailability in soils. In: Nriagu JO, editor. Arsenic in environment part I: cycling and characterization. New York: John Wiley & Sons; 1994.p. 51-81.
6. McBride BC, Wolfe RS, Biosynthesis of dimethylarsine by methanobacterium. Biochemistry 1971; 10: 4312-4317.
7. Sadiq M. Arsenic chemistry in soils: an overview of thermodynamic predictions and field observations. Water, Air, and Soil Pollut 1997; 93: 117-136.
8. Yamauchi H, Fowler BA. Toxicity and metabolism of inorganic and methylated arsenicals. In: Nriagu JO, editor. Arsenic in environment part II: human health and ecosystem effectes. New York: John Wiley & Sons; 1994. p. 35-53.

9. Goeby MS. Arsenic in human medicine. In: Nriagu JO, editor. Arsenic in the environment Part II human health and ecosystem effectes. New York: John Wiley & Sons; 1994. P. 1-16.

10. Choprapawon C, Rodcline A. Chronic arsenic poisoning in Ronpibool Nakhon Sri Thammarat, the southern province of Thailand. In: Abernathy CO, Calderon and Chappel WR, editors. Arsenic: Exposure and health effects. Chapman & Hall; 1997. p. 68-77.

11. Roane TM , Kellog ST. Characterization of bacterial communities heavy metal contaminated soils. Can. J. Microbiol 1996; 42:593-603.

12. Willsky GR , Malamy MH. Effect of arsenate on inorganic phosphate transport in *Escherichia coli*. J. Bacteriol 1980; 144: 366-374.

13. Smith HW. Arsenic in enterobacteria: its transmission by conjugation and by phage. J. Gen. Microbiol 1978; 109: 49-56.

14. Cullen WR , Remer KJ. Arsenic-speciation in the environment. Chem. Rev 1989; 89: 713-764.

15. Silver S, Nakahara H. Bacterial resistance to arsenic compounds. In: Leaderer WH and Fensterheim, editors. Arsenic: Industrial, biomedical, environmental perspectives. Van Nostrand Reinhold Co; 1983. p. 190-199

16. Maeda S, Ohki A, Miyahara K, Naka K, Higashi S. Metabolism of methylated arsenic compounds by arsenic-resistant bacteria (*Klebsiella oxytoca* and *Xanthomonas* sp.). Appl Organomet Chem 1992; 6: 415-420.

17. Osborne FH, Ehrlich HL. Oxidation of arsenite by a soil isolates of *Alcaligenes*. Journal of Applied Bacteriology 1976; 41: 295-305.

18. Phillips SE, Taylor ML. Oxidation of arsenite to arsenate by *Alcaligenes faecalis* Appl. Envir. Microbiol 1976; 32:392-399.

19. Krafft T, Macy JM. Purification and characterization of the respiratory arsenate reductase of *Chrysiogenes arsenatis*. Eur. J. Biochem 1998; 255: 647-653.

20. Diorio C, Cai J, Marmor J, Shinder R , DuBOW MS. An *Escherichia coli* chromosomal *ars* operon homolog is functional in arsenic detoxification and is conserved in Gram-negative bacteria. J. Bacteriol 1995; 177: 2050-2056.

21. Mann H. Biosorption of heavy metals by bacterial biomass. In: Volesky B, editor. Biosortion of heavy metals. Boca Raton (FL): CRC Press; 1990. P94-118.

22. Turner AW. Bacterial oxidation of arsenite. I. Description of bacteria isolated from arsenical cattle-dipping fluids. Australian Journal of Biological Sciences 1954; 7: 452-478.

23. Turner AW, Legge JW. Bacterial oxidation of arsenite. II. The activity of washed suspensions. Australian Journal of Biological Sciences 1954; 7: 479-495.

24. Legge IW. Bacterial oxidation of arsenite. III. Cell-free arsenite dehydrogenase. Australian Journal of Biological Sciences 1954; 7: 496-503.

25. Legge IW. Bacterial oxidation of arsenite. IV. Some properties of the bacterial cytochromes. Australian Journal of Biological Sciences 1954; 7: 504-514.

26. Hambsch B, Raue B, Brauch HJ. Determination of arsenic (III) for the investigation of the microbial oxidation of arsenic (III) to arsenic. Acta hydrochim. Hydrobiol 1995; 23: 166-172.

27. Johnson DL. Bacterial reduction of arsenate in sea water. Nature 1972; 240: 44-45.

28 Forsberge CW. Some effects of arsenic on the rumen microflora; an in vitro study. Can. J. Microbiol 1978; 24: 36-44.

29 Ahmann D, Roberts AL, Krumholz LR, Morel FMM. Microbe grow by reducing arsenic. Nature 1994;.371: 750.

30 Laverman AM, Blum JS, Schaefer JK, Phillips EJP, Lovley DR, Oremland RS. Growth of strain SES-3 with arsenate and other diverse electron acceptors. Appl. envir. Microbiol 1995; 61: 3556-3561.

31 Newman DK, Beveridge TJ, Morel FMM. Precipitation of arsenic trisulfide by desulfotomaculum auripigmentum. Appl. envir. Microbiol 1997; 63: 2022-2028.

32 Cheng CN, Focht DD. Production of arsine and methylarsines in soil and in culture. Appl. envir. Microbiol 1979; 38: 494-498.

33 Wong PTS, Chau YK, Luxon L, Bengert GA. Methylation of arsenic in the aquatic environment. Trace subst. Environ Healt 1977; 11: 100-106.

34 Maeda S. Biotransformation of arsenic in the freshwater environment. In: Nriagu JO, editor. Arsenic in environment part I: cycling and characterization. New York: John Wiley & Sons; 1994.p. 155-187.

35 Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST, editor. Bergey's Manual of Determinative Bacteriology 9th .Baltimore : Williams & Wilkins Co; 1994.

36 Green HH. Description of bacterium which oxidizes arsenite to arsenate, and of one which reduces arsenate to arsenite, isolated from a cattle-dipping tank. South African Journal of Science 1918; 14: 465-467.

37 Wistreich GA, editor. Microbiology laboratory fundamental and applications. Prentice-Hall, In C :1997.

38 Silver S, Budd K, Leahy KM, Shaw WV, Hammond D, Novick RP, Willsky MH, Malamy MH, Rosenberge H. Inducible plasmided resistance to arsenate, arsenite, and antimony (III) in Escherichia coli and Staphylococcus aureus. J. Bacteriol 1981; 146: 983-996.

39 Ahmann D, Krumholz LR, Hemond HF, Lovely DR, Morel FMM. Microbial mobilization of arsenic from sediments of the Aberjona Watershed. Environ. Sci. Technol 1997; 31: 2923-2930.

APPENDIX I

Biochemical tests for identification of *Alcaligenes xylosoxidans* subsp. *xylosoxidans* strains no 2/6 and no. 3/18

1. Gram- staining (Hucker's method)

Procedure:

1.1 Dry the material on the slide by gently warming the slide to remove all water from the material.

1.2 Stain I min with crystal violet solution (primary dye). Wash in tap water.

1.3 Apply the iodine solution (mordant) for I min . Wash in Tap water.

1.4 Decolorize with alcohol, adding dropwise on the tilted slide until all free color (violet)has been removed (20 to30 sec) . Wash in tap water.

1.5 Flood the slide with safranin (counterstain) for I min . Wash in tap water and air-dry.

1.6 Examine the stained smears under the oil-immersion objective to determine which organisms are Gram-positive (those retaining the blue color and which are Gram-negative (those decolorized and counterstained red)

2. Motility test

A semi-solid motility medium was inoculated by stabbing into the medium with culture of the isolated to be tested. The tube was incubated at 37 C for 24 hr. Non motile organisms grew only along the stab line, whereas motile organisms spread out into the medium, and resulted in a brushlike appearance.

3. Oxidase

Moisture a piece of filter paper with a few drops of 1% solution of tetramethyl-p-phenylenediamine dichloride, prepared on the same day as it is to be used. With a platinum loop, remove growth from the surface of an agar medium and smear it on the moistured paper. The test was determined as positive by the development of a violet or purple color in 10 seconds.

4. Catalase

1 ml of 3% hydrogen peroxide was added to a slant culture of A. xylosoxidans subsp. ylosoxidans grown in nutrient agar medium. Then, examined immediately after 5 min for the presence of gas bubles, which indicates a positive test.

5. The API 20 NE

The API 20 NE strip consists of 20 microtubes containing dehydrated media and substrates. The conventional tests are inoculated with a saline bacterial suspension which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The assimilation tests are inoculated with a minimal medium and the bacteria grow if they are capable of utilizing the corresponding substrate. The reactions are read according to the reading Table and the identification is obtained by referring to the Analytical Profile Index or using the identification software.

Reading Table

Test	Substrated	Reactions/enzymes	Results	
			Negative	Positive
NO_3	Potassium nitrate	Reduction of nitrates to nitrites	NIT 1+NIT 2 / 5 min Colorless	NIT 1+NIT 2 / 5 min Pink-red
		Reduction of nitrates to nitrogen	Zn / 5 min Pink	Zn / 5 min Colorless
TRP	Tryptophane	Indole production	JAMES / immediate Colorless Pale green-yellow	JAMES / immediate Pink
GLU	Glucose	Acidification	Blue to green	Yellow
ADH	Arginine	Arginine dihydrolase	Yellow	Orange/pink/red
URE	Urea	Urease	Yellow	Orange/pink/red
ESC	Esculin	Hydrolysis (β-glucosidase)	Yellow	Grey/brown/black
GEL	Gelatine (with India ink)	Hydrolysis (protease)	No pigment Diffusion	Diffusion of black pigment
PNPG	p-nitro-phenyl-β-D-galactopyranoside	β-galactosidase	Colorless	Yellow
GLU	Glucose	Assimilation	Transparent	Opaque
ARA	Arabinose	Assimilation	Transparent	Opaque
MNE	Mannose	Assimilation	Transparent	Opaque
MAN	Mannitol	Assimilation	Transparent	Opaque
NAG	N-acetyl-glucosamine	Assimilation	Transparent	Opaque
MAL	Maltose	Assimilation	Transparent	Opaque
GNT	Gluconate	Assimilation	Transparent	Opaque
CAP	Caprate	Assimilation	Transparent	Opaque
ADI	Adipate	Assimilation	Transparent	Opaque
MLT	Malate	Assimilation	Transparent	Opaque
CIT	Citrate	Assimilation	Transparent	Opaque
PAC	Phenyl-acetate	Assimilation	Transparent	Opaque
OX	Tetramethyl-p-Phenylene diamine	Cytochrome oxidase	OX / 1-2 min Colorless	OX / 1-2 min violet

The characterization of Genus *Alcaligenes*

Rod, coccal rods, or cocci, 0.5-1.0 x 0.5-2.6, usually occur singly. Resting stages are not known. **Cells stain Gram negative. Motility occurs** with 1 to 8 (occasionally up to 12) **peritrichous** flagella. **Obligately aerobic,** possessing a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor. Some strains are capable of anaerobic respiration in the presence of nitrate or nitrite. Optimum temperature is 20-37^{O}C. Colonies on nutrient agar are **nonpigmented. Oxidase positive** and catalase positive. Indole is not produced. Cellulose, esculin, gelatin, and DNA usually are not hydrolyzed. **Chemoorganotrophic, using a variety of organic acids and amino acids as carbon sources. Alkali is produced** from several organic salts and amides. Carbohydrates are usually not utilized. Some strains produce acid from D-glucose and D-xylose and utilize both carbohydrates as carbon source. **Occur in water and soil.** Some are common, apparently saprophytic, inhabitants of the intestinal tract of vertebrates. Numerous strains have been **isolated from clinical material such as blood, urine, feces, purulent ear discharges, spinal fluid, wounds,** etc. Occasionally, cause opportunistic infections in humans.

APPENDIX II

Procedures for arsenic determination

1. Prepare a standard arsenic solution by dissolve 0.416 g of Na_2HAsO_4 (Sodium arsenate) and 0.173 g of $NaAsO_2$ (Sodium arsenite) in distilled water 1 L as a stock of 1000 mg l^{-1}.
2. Prepare a series of standard arsenic solution as 1, 3, 5, 7, 10 μ g.l^{-1} by using HNO_3 as solvent.
3. Prepare reducing solution containing 5% (w/v) KI and 5% (w/v) ascorbic acid.
4. Prepare carrier solution containing 10% (v/v) HCl.
5. Prepare reducing agent containing 0.2% $NaBH_4$ in 0.05% NaOH.
6. Sample solution and standard solution should be reduced to As^{3+} prior to analysis. To 1 ml of sample or reference solution add 1 ml conc. HCl and 1 ml 5% (w/v) and 5% ascorbic acid. Let the samples stand for 45 minute at ambient temperature, then dilute to 10 ml.
7. After that, sample solution should be reacted with reducing agent and carrier solution in Hydride Generation and generated arsine gas (AsH_3). The arsine gas is swept by as inert gas (argon) into the quartz cell heated to 900 °C.
8. The sample solution is measured the absorbance at 193.7 nm. The arsenic concentration are plotted against the absorbance to determine the value of A (Coefficient) and B(Y-intercept).

9. The value of absorbance can be calculated to the amount of arsenic by this equation,

$$X = (Y-B)/A$$

when X = the amount of arsenic

Y = the absorbance value

A = coefficient

B = intercept

Analytical parameters for arsenic determination

Table IA Spectrometer

Technique	AA
Integration time (s)	15
Data Processing	Peak Height, Smoothing: 0.5 sec or 19 points
Lamp	HCl or EDL
Slit (nm)	0.7 nm (Low or Alt)
Wavelength (nm)	193.7

Reagents

Carrier Solution: 10% (v/v) HCl

Reducing Agent: 0.2% $NaBH_4$ in 0.05% NaOH

Sample Solution: As^{3+} in 10%(v/v) HCl

Sensitivity Check

Analysis of 500 µL of a 10 µg/L arsenic solution should provide a signal of about 0.45 A. Data obtained using an EDL. With a HCl about 0.300 A.

APPENDIX III-A

Table III-A Screening of 70 mg l^{-1} arsenic by bacterial strains isolated from samples collected during March, 1998 for their survival on 700 mg l^{-1} arsenic medium.

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Mine tailing 1	+	-	1/1	A	++
Mine tailing 2	+	-	1/2	B	++
Mine tailing 2	+	-	1/3	K	--
Mine tailing 2	+	-	1/4	D	++
Mine tailing 3	+	-	1/5	A	++
Mine tailing 3	-	+	1/6	G	++
Mine tailing 3	-	+	1/7	D	++
Soil 3.1	+	-	1/8	A	++
Soil 3.3	+	-	1/9	A	++
Soil 3.3	-	+	1/10	A	++
Soil 3.6A	+	-	1/11	D	++
Soil 3.6A	+	-	1/12	F	--
Soil 3.6A	+	-	1/13	A	--
Soil 3.6A	-	+	1/14	A	++
Soil 3.6B	+	-	1/15	F	++
Soil 3.6B	+	-	1/16	A	++
Soil 3.6C	+	-	1/17	A	--
Soil 3.6C	-	+	1/18	A	--
Soil 3.6C	-	+	1/19	G	++
Soil 3.7	-	+	1/20	A	++
Soil 3.7	-	+	1/21	N	--
Soil 3.8B	+	-	1/22	A	--
Soil 3.8B	+	-	1/23	A	++
Soil 3.8B	-	+	1/24	A	++
Stream 3.9	+	-	1/25	A	--
Stream 3.9	+	-	1/26	P	++

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

APPENDIX III-B

Table III-B Screening of 70 mg l^{-1} arsenic by bacterial strains isolated from samples collected during May, 1998 for their survival on 700 mg l^{-1} arsenic medium.

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Mine tailing 2	+	-	2/1	D	++
Mine tailing 2	-	+	2/2	G	--
Mine tailing 2	-	+	2/3	D	++
Soil A.	+	-	2/4	A	--
Soil A.	-	+	2/5	N	++
Soil B.	-	+	2/6	D	++
Waterfall C.	+	-	2/7	A	--
Waterfall C.	+	-	2/8	I	--
Waterfall C.	-	+	2/9	H	--
Waterfall D.	+	-	2/10	G	++
Soil 3.1	+	-	2/11	A	++
Soil 3.1	-	+	2/12	D	++
Soil 3.2	+	-	2/13	A	++
Soil 3.2	-	+	2/14	A	--
Stream 3.3-1	+	-	2/15	H	--
Stream 3.3-1	+	-	2/16	E	--
Stream 3.3-1	-	+	2/17	D	--
Soil 3.4	+	-	2/18	A	--
Soil 3.4	+	-	2/19	B	--
Soil 3.4	-	+	2/20	A	++
Soil 3.5	+	-	2/21	J	--
Soil 3.5	+	-	2/22	I	--
Soil 3.5	+	-	2/23	A	++
Soil 3.6	+	-	2/24	A	++
Soil 3.6	+	-	2/25	D	++
Soil 3.6	-	+	2/26	A	++

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

Table III-B (continued)

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 ppm
Soil 3.7	+	-	2/27	D	++
Soil 3.7	+	-	2/28	G	++
Soil 3.7	-	+	2/29	K	--
Soil 3.7	-	+	2/30	D	++
Soil 3.8	+	-	2/31	D	++
Soil 3.8	-	+	2/32	A	--
Soil 3.9	+	-	2/33	D	--
Soil 3.10	+	-	2/34	D	++
Soil 3.10	+	-	2/35	A	--
Soil 3.10	-	+	2/36	A	++
Soil 3.11	+	-	2/37	A	--
Soil 3.11	-	+	2/38	A	++
Soil 3.12	+	-	2/39	A	++
Soil 3.12	+	-	2/40	Q	++
Soil 3.12	-	+	2/41	A	++
Soil 3.12	-	+	2/42	S	++
Soil 3.13	+	-	2/43	A	++
Soil 3.13	-	+	2/44	A	++
Soil 3.13	-	+	2/45	G	++
Soil 3.14	+	-	2/46	G	--
Soil 3.14	-	+	2/47	G	++
Soil 4	+	-	2/48	S	--
Soil 4	+	-	2/49	Q	++
Soil 4	-	+	2/50	A	++

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

APPENDIX III-C

Table III-C Screening of 70 mg l^{-1} arsenic by bacterial strains isolated from samples collected during October, 1998 for their survival on 700 mg l^{-1} arsenic medium.

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Nakhon Si Thummarat					
Soil 1	+	-	3/1	J	--
Soil 1	-	+	3/2	C	--
Soil 1	-	+	3/3	F	--
Soil 1	-	+	3/4	L	--
Soil 2	+	-	3/5	D	--
Soil 2	-	+	3/6	D	--
Soil 2	-	+	3/7	J	--
Soil 2	+	-	3/8	G	++
Soil 3.1	+	-	3/9	A	++
Soil 3.1	-	+	3/10	G	--
Soil 3.1	-	+	3/11	A	--
Soil 3.2	+	-	3/12	M	--
Soil 3.2	-	+	3/13	M	--
Soil 3.2	-	+	3/14	A	--
Soil 3.2	-	+	3/15	M	--
Soil 3.3	+	-	3/16	P	--
Soil 3.3	+	-	3/17	D	++
Soil 3.3	-	+	3/18	D	++
Soil 3.3	-	+	3/19	D	++
Soil 3.3	-	+	3/20	A	++
Soil 3.4	+	-	3/21	G	++
Soil 3.4	+	-	3/22	D	++
Soil 3.4	+	-	3/23	N	--
Soil 3.4	-	+	3/24	A	++
Soil 3.4	-	+	3/25	G	++

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

Table III-C (continued)

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Soil 3.4	+	-	3/26	G	++
Soil 3.5	+	-	3/27	A	--
Soil 3.5	-	+	3/28	A	++
Soil 3.5	-	+	3/29	A	++
Soil 3.6	+	-	3/30	Q	--
Soil 3.6	+	-	3/31	A	++
Soil 3.6	-	+	3/32	R	--
Soil 3.7	+	-	3/33	D	--
Soil 3.7	+	-	3/34	G	++
Soil 3.7	-	+	3/35	F	--
Soil 3.7	-	+	3/36	B	--
Soil 3.8	+	-	3/37	G	++
Soil 3.8	-	+	3/38	N	++
Soil 3.8	-	+	3/39	G	--
Soil 3.9	+	-	3/40	G	--
Soil 3.9	-	+	3/41	D	++
Soil 3.10	+	-	3/42	G	++
Soil 3.10	+	-	3/43	D	--
Soil 3.10	-	+	3/44	Q	--
Soil 3.10	-	+	3/45	A	++
Soil 3.11	+	-	3/46	G	++
Soil 3.11	+	-	3/47	D	++
Soil 3.11	-	+	3/48	D	--
Soil 3.12	+	-	3/49	A	++
Soil 3.12	+	-	3/50	D	--
Soil 3.12	-	+	3/51	D	--
Soil 3.12	-	+	3/52	A	++
Soil 4.	+	-	3/53	G	++

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

Table III-C (continued)

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Soil 4.	+	-	3/54	D	++
Soil 4.	+	-	3/55	Q	--
Soil 4.	+	-	3/56	A	++
Soil 5.1	+	-	3/57	G	--
Soil 5.1	+	-	3/58	D	--
Soil 5.1	-	+	3/59	G	++
Soil 5.2	+	-	3/60	Q	--
Soil 5.2	+	-	3/61	D	--
Soil 5.2	-	+	3/62	A	--
Waterfall 6.1	+	-	3/63	D	--
Waterfall 6.1	+	-	3/64	A	--
Waterfall 6.1	+	-	3/65	D	--
Waterfall 6.1	+	-	3/66	G	--
Waterfall 6.1	-	+	3/67	G	--
Waterfall 6.1	-	+	3/68	D	--
Waterfall 6.2	-	+	3/69	D	--
Waterfall 6.2	-	+	3/70	A	--
Yala					
Mine tailing 7	+	-	3/71	A	++
Soil 8	+	-	3/72	G	--
Soil 8	+	-	3/73	A	--
Soil 8	-	+	3/74	A	++
Soil 8	-	+	3/75	F	++
Soil 9	+	-	3/76	G	--
Soil 9	+	-	3/77	A	++
Soil 9	-	+	3/78	J	--
Soil 9	-	+	3/79	A	--

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

Table III-C (continued)

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Soil 9	+	-	3/80	R	++
Stream 13	+	-	3/81	A	--
Stream 13	+	-	3/82	R	--
Stream 13	-	+	3/83	G	--
Stream 13	-	+	3/84	Q	--

+ = growth on 70 mg l^{-1} arsenic medium
- = no growth on 70 mg l^{-1} arsenic medium
++ = growth on 700 mg l^{-1} arsenic medium
-- = no growth on 700 mg l^{-1} arsenic medium

APPENDIX III-D

Table III-D Screening of 70 mg l^{-1} arsenic by bacterial strains isolated from samples collected during February, 1999 for their survival on 700 mg l^{-1} arsenic medium.

Samples	Arsenate	Arsenite	designation	Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Nakhon Si Thummarat					
Soil 1	+	-	4/1	E	--
Soil 1	+	-	4/2	E	--
Soil 1	-	+	4/3	E	++
Soil 2	+	-	4/4	E	++
Soil 2	-	+	4/5	E	++
Soil 9	+	-	4/6	A	++
Soil 9	-	+	4/7	B	++
Soil 9	-	+	4/8	C	--
Soil 9	-	+	4/9	N	++
Soil 3	+	-	4/10	A	++
Soil 3	+	-	4/11	N	++
Soil 3	-	+	4/12	B	++
Soil 4	+	-	4/13	B	--
Soil 4	+	-	4/14	I	--
Soil 4	-	+	4/15	A	++
Soil 5	+	-	4/16	J	--
Soil 5	+	-	4/17	Q	--
Soil 5	-	+	4/18	D	--
Soil 6	+	-	4/19	L	++
Soil 6	-	+	4/20	L	++
Mine tailing 10	+	-	4/21	N	++
Mine tailing 10	+	-	4/22	B	++
Mine tailing 10	-	+	4/23	C	++
Mine tailing 10	-	+	4/24	L	--
Soil 7	+	-	4/25	M	--

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

Table III-D (continued)

Samples	Arsenate	Arsenite	designation	*Colonial morphology	Arsenic resistance of 700 mg l^{-1}
Soil 7	-	+	4/26	A	++
Soil 8	+	-	4/27	Q	++
Soil 8	+	-	4/58	O	--
Soil 8	-	+	4/29	O	--
Soil 8	-	+	4/30	Q	++
Yala					
Mine tailing 11	+	-	4/31	L	++
Mine tailing 11	-	+	4/32	F	++
Soil 13	+	-	4/33	B	++
Soil 13	-	+	4/34	B	++
Soil 15	+	-	4/35	A	++
Soil 15	-	+	4/36	B	++
Soil 14	+	-	4/37	B	++
Soil 14	-	+	4/38	B	++

+ = growth on 70 mg l^{-1} arsenic medium

- = no growth on 70 mg l^{-1} arsenic medium

++ = growth on 700 mg l^{-1} arsenic medium

-- = no growth on 700 mg l^{-1} arsenic medium

***Symbols** : Colonial morphology(size/color/margin/density/elevation)

A= Large/White/ Filamentous/ Opaque/ Raised
B= Large/ White/ Irregular/ Opaque/ Raised
C= Large/ Buff/ Irregular/ Transparent/ Flat
D= Medium/Buff/Smooth/Translucent/ Convex
E= Medium/ White/ Smooth/ Opaque/ Convex
F= Medium/ Orange + White/ Rough/ Opaque/ Convex
G= Medium/ Buff/ Smooth/ Opaque/ Convex
H= Medium/ White/ Smooth/ Opaque/ Flat
I= Medium/ White/ Rough/ Opaque/ Raised
J= Medium/White/Rough/Translucent/ Convex
K= Medium/ Yellow/ Rough/ Translucent/ Flat
L= Medium/ Orange/ Rough/ Opaque/ Convex
M= Medium/ Yellow/ Rough/ Opaque/ Convex
N= Small/ White/ Rough/ Opaque/ Convex
O= Medium/ Violet/ Smooth/ Opaque/ Convex
P= Small/Yellow/Smooth/Translucent/ Convex
Q= Small/ Buff/ Rough/ Translucent/ Convex
R= Small/Green +Yellow/ Rough/ Translucent/ Flat
S= Pinpoint/ White/ Smooth/ Translucent/ Convex

BIOGRAPHY

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