SCREENING OF FUNGAL ISOLATES WHICH HAVE HIGH CAPABILITY ON ARSENIC REMOVAL

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One hundred and eighty five fungal isolates which could grow in the presence of 70 mg l^{-1} of either arsenite or arsenate were isolated from samples collected from the arsenic-polluted areas in Ron Phibun District, Nakhon Si Thammarat Province and Bannagsta District, Yala Province in southern Thailand. Out of these, one fungal strain (RRMT2-40I) was found to have the best efficiency in removing inorganic arsenic from growth medium (potato dextrose broth). This fungus, identified as *Paecilomyces* sp., removed arsenic and grew well in the growth medium at pH 5.0 and pH 7.0 and at temperature 27° C, reaching the stationary phase in 4 days. The growth of this fungus was slightly affected by inorganic arsenic [both arsenite (III and arsenate (V)] concentration in the medium at 1000 mg l^{-1}. The arsenic uptake exhibited its peak and turning point at the stationary phase. At this phase arsenic was excreted from the fungal cells. Arsenic removal was dependent on the culture age and viability of the cells. When the selected fungus were killed by autoclaving, arsenic was not taken up or removed by the cells.

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ในการทดลองครั้งนี้ได้ทำการคัดเลือกเชื้อราจากตัวอย่างดินและพืชที่เก็บจากบริเวณที่มีการปนเปื้อนสารหนู อำเภอร่อนพิบูลย์ จังหวัดนครศรีธรรมราช และ อำเภอบันนังสตา จังหวัดยะลา ได้เชื้อราทั้งหมด 185 สายพันธุ์ที่สามารถเจริญเติบโตได้ในสภาวะที่มีสารหนู 70 มิลลิกรัมต่อลิตร พบว่า เชื้อราสายพันธุ์ RRMT2-40I มีประสิทธิภาพดีที่สุดในการกำจัดสารหนูออกจากอาหารเลี้ยงเชื้อ (potato dextrose broth) เชื้อราชนิดนี้จัดอยู่ในจีนัส *Paecilomyces* มีความสามารถในการกำจัดสารหนูและเจริญเติบโตในอาหารเลี้ยงเชื้อได้ดีที่ pH 5.0 และ pH 7.0 และที่อุณหภูมิ 27 องศาเซลเซียส การเจริญเติบโตของเชื้อราจะเข้าสู่ระยะ stationary phase ในระยะนี้พบว่าการสะสมของสารหนูภายในเซลจะสูงสุดด้วย หลังจากนั้นเชื้อราจะปลดปล่อยสารหนูออกจากเซล เชื้อราสายพันธุ์นี้สามารถเจริญเติบโตได้ในความเข้มข้นของสารหนูที่สูงถึง 1000 มิลลิกรัมต่อลิตร โดยได้รับผลกระทบเพียงเล็กน้อย ความสามารถในการกำจัดสารหนูขึ้นกับอายุของเซล และการมีชีวิตของเซล เมื่อราสายพันธุ์นี้ถูกฆ่าโดยความร้อนจะไม่พบการสะสมหรือการกำจัดสารหนูโดยเซลที่ตาย

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CHAPTER I

INTRODUCTION

1.1 Problem and Importance

Arsenic (As) is a natural element found in the environment. It is widely distributed and forms 5-6 mg kg^{-1} of the earth's crust (1). Pure arsenic is a gray, metal-like material and often combined with other elements such as oxygen, chlorine and sulfur. In combination with these elements it is called inorganic arsenic. If it combines with both carbon and other elements it is called organic arsenic. Inorganic arsenic is found in many kinds of rock, especially in ores that contain copper or lead. When these ores are heated by smelter to extract the copper or lead, most of arsenic enters the air as fine dust. Smelters collect this dust and purify the arsenic for several uses, such as wood preservation, several types of insecticides and herbicides. All of these activities cause arsenic contamination in the environment. Arsenic does not have good warning properties, meaning no smell and no special taste. Thus, it is not able to tell if arsenic is present in food, water or air. Exposure to high level of arsenic can cause vomiting, diarrhea, blood vessel change, skin cancer ,or death in extreme cases.

In Thailand, the occurrence of human health problems resulting from arsenic contamination of domestic water supplies was first recognized in Ron Phibun district, Nakhon Si Thammarat province. This area has an extensive history of bedrock and alluvial tin mining. Hydrochemical analysis of surface water and groundwater has confirmed the presence of dissolved arsenic at concentrations exceeding the current WHO potable water guideline (10 μg l^{-1}) by a factor of 500. Concentration of arsenic

in soil is also found to be high concentration of arsenic, that is 1.2-4.45 mg l^{-1} (2) and 50-5,200 mg kg^{-1}(3). A preliminary survey about health problems recognized approximately 1,000 case of arsenic induced skin disorders, including several skin cancers and concentration of arsenic in hair and fingernails were found to be elevated in 80% of school-age population, and a clear spatial correlation between human body burdens and arsenic concentrations in drinking water was confirmed (4).

In the management of the above problems, a secure landfill for containment of mining wastes has been constructed by the Department of Mineral Resources in 1998. Although, the high-grade arsenopyrite waste piles, which are considered to be a point source can be managed, arsenic contamination form the presence of the high arsenic background as a non-point source is still existed. Therefore, The area is still contaminated with arsenic and needed appropriate technology for remediation.

There is an increasing interest in the application of microorganisms to remediate contaminated land and water with toxic metals. Fungi are ubiquitous in natural environment and can be dominant organisms in many soils, particularly those of low pH. Biological mechanisms which have been applied to remediation by fungi include: extracellular precipitation, transformation of metal species by oxidation, reduction, methylation and dealkylation, biosorption to cell walls, pigments and extracellular polysaccharide, intracellular compartmentation and precipitation and/or sequestration (5,6).

As aforementioned, this study was carried out to investigate the appropriate fungi which had good efficiency to remove arsenic compounds, observation on the possibility of this fungi to remove arsenic compounds from land/water was also made.

This fungi should be a very promising alternative to remove arsenic compounds from the arsenic contaminated environment.

1.2 Objectives :

1. Selection of fungi which have ability to remove arsenic compound from arsenic contaminated land/water.
2. Study on effects of environmental factors on arsenic removal.
3. Study on mechanism of arsenic removal by the selected fungi.
4. Study on efficiency of the selected fungi on arsenic removal.

1.3 Outcomes :

1. A fungus which has high efficiency on removal of arsenic from contaminated soil/water is selected.
2. Increasing background data for further studies in pilot scale and then *in situ* remediation.
3. Removal of arsenic from soil/water.

1.4 Scope of study :

1. Isolation of fungi which have ability to tolerate arsenic in the contaminated land .
   1.1 Collection of samples
   1.2 Isolation of fungi
2. Screening test on the isolated fungi.
   2.1 Selection of fungi that tolerate 700 mg l^{-1} of arsenic.

2.2 Selection of fungi which have high efficiency on removal of arsenic compound.

3. Study on effects of environmental factors on the activities of the fungi.
   3.1 Effect of pH
   3.2 Effect of temperature
4. Study on effect of the arsenic concentration on growth of the selected fungi.
5. Study on mechanism of arsenic removal in selected fungi
   5.1 Adsorption
   5.2 Absorption
   5.3 Arsine generation
6. Study on kinetic of the arsenic removal by the selected fungi.

CHAPTER II

LITERATURE REVIEWS

2.1 Arsenic in the environment

Arsenic(As) is a ubiquitous element that ranks 20th 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 (7).

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
Olivenite	Cu_2OHAsO_4
Orpiment	As_2S_3
Proustite	Ag_3AsS_2
Realgar	AsS
Scorodite	$FeAsO_4.2H_2O$
Smaltite	$CoAs_2$
Tennantite	$Cu_8As_2S_7$

Source: Kippling (7)

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 (7)

2.1.1 The use 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 (8).

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
Copper aceto-arsenite	$3CuOAs_2O_3Cu$	Paris Green	Larvicide
	$(OOCCH_3)$	Emerald Green	
Orpiment	As_2S_3		Pigment
			Depilatory
Lead arsenate	$PbHAsO_4$		Insecticide , herbicide and
			growth regulator
Sodium arsenate	Na_2HAsO_4	Wolman salts	Wood preservative
	Na_3AsO_4		Calico printing
			Insecticide
			Weed killer
Sodium arsenite	$NaAsO_2$		Herbicides
			Pesticides
			Corrosive inhibitor
			Chemical intermediate
			Fluorescent lamps
Magnesium arsenate	$Mg_3(AsO_4)_2$	Atoxyl	Trypanicide
Sodium arsanilate	$NH_2C_6H_4AsO$		Pharmaceutical
	$(OH)(ONa)$		manufacture

Source : Kippling (7)

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 (8).

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 Forms 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 (mispickel, 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^{5+}) and in the trivalent states as arsenite (As^{3+}) 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^{3+} is much more toxic, more soluble, and more mobile than As^{5+}. 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^{5+}: As^{3+}ratio (1).

2.1.3.3 Gaseous states of arsenic

Extensive use has been made of sodium and amonium salts of monomethyl arsionic acid (MMAA) and dimethylarsinic acid (DMAA) as nonselective, postemergent, foliar-contact herbicites. 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, yeasts, and bacteria can methylate arsenic to MMAA, DMAA and gaseous derivatives of arsine (9).

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 (8).

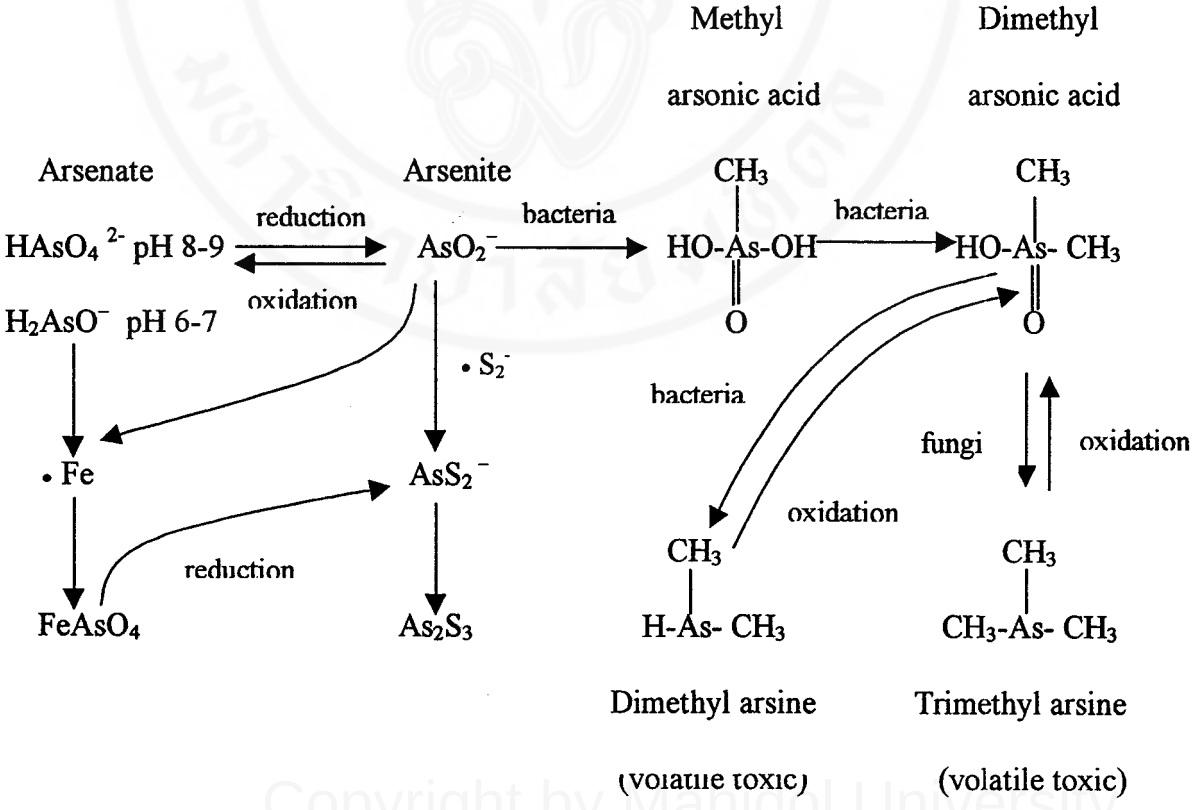


Figure 2-1. Chemical forms of arsenic and their transformations in soils.

Source: Bhumbla and Keefer (8)

2.1.3.5 Speciation of arsenic in environment

Speciation of arsenic in the soil environment:

1. Arsenic (III). Arsenite [As^{3+}], 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^{5+}].
2. Arsenic (V). Arsenate[As^{5+}] 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^{5+} 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 [$(CH_3)_3As$] (10).

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) (8).

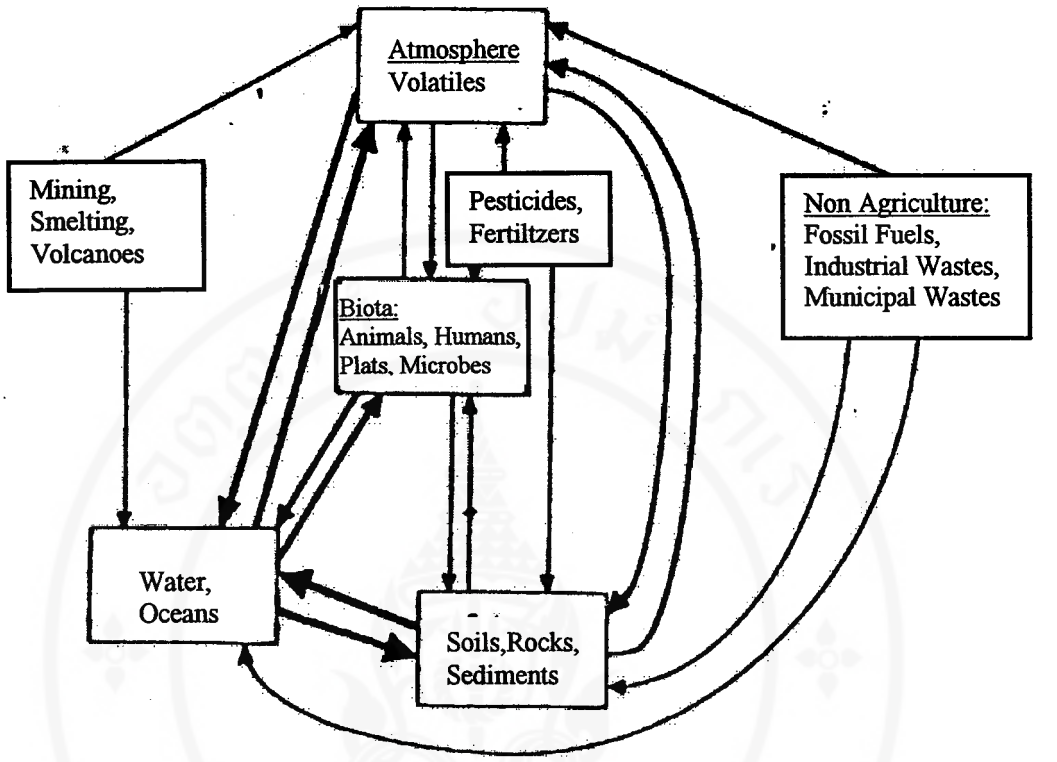


Figure 2-2 A simplified, comprehensive cyclic transfer of arsenic.

Source: Bhumbla and keefer (8)

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 (4), 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 (11).

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) (11). 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 DNA (11) and mutagenicity (11). 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 (11) 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 (11).

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 (11). 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 mgL^{-1} and more by inhalation had immunologic effects (11).

2.3 Arsenic poisoning

2.3.1 Inorganic arsenic poisoning

- **Acute Poisoning**

Symptoms of acute intoxication (12) (Michael S. Gorby; BOOK) 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 vesical 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 (12). 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 (12).

- **Chronic Poisoning**

The most prominent chronic manifestations (12) 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 fraquently punctate 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 fraquently 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 haemoglobin (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 a bright red urine, a bronze color of the skin and jaundice. In less severe cases, anaemia, and sometimes neuritis, prolongs the period before complete recovery (7).

2.4 Ron Phibun District

2.4.1 Physiography

Nakhon Si Thammarat province is one of the biggest provinces in the southern part of Thailand. Administratively, it is divided into 16 districts and 1 branch district. The main occupations are rubber planting, rice farming, fishing and mining. It has been known that this area is affluent with natural resources, so mining is the main income in 10 districts. In 1987, there were 43 mines: 5 tin, 16 wolfram and a few of several minerals (3).

Ron Phibun district is 35 kilometers south-west of the central city in Nakhon Si Thammarat province. It consists of 8 subdistricts and 65 villages. Main incomes of this district are from mining and farming. In the western part of the district is a wide mountainous area from north to south and in the eastern part are agricultural plains. Main water resources are from Ronna canal, and old mining swamp, underground and rain water.

2.4.2 Geology and mining

The geology of this area is characterized by S-type biotite and biotite-muscovite granitoids of Triassic age, with abundant pegmatitic veining. Cassiterite (SnO_2) and wolframite mineralization with high amount of arsenopyrite and pyrite commonly occurs in the pegmatites veining. Mining and mineral processing activities existed in Ron Phibun during the past 100 years. Over 20 bedrock mining concessions and also alluvial mining were held in the Ron Na-Suang Chan mountain range (4).

2.4.3 Hydrology and hydrogeology

Ron Phibun has a high average rainfall of ca. 2100 mm yr^{-1}. Surface drainage systems are orientated predominantly west-east with headwaters in the Ron Na-Suang Chan mountains feeding a series of channels that ultimately enter the Gulf of Thailand. The principal bedrock mining areas of Ron Pahibun District occupy the headwater of the Hai Ron Na, which flows southeastward from the granite massif through areas of alluvial mining to the north of Ron Phibun town. The principal alluvial mining areas of the district are drained by the Klong Sak, Klong Rak Mai, and Konng Nam Khun systems. There are two significant aquifers in the area. A shallow aquifer with a depth of < 10 m and a deeper carbonate-hosted aquifer with a depth of > 15 m. Both surface and groundwater drainage systems are water sources that supply water for domestic use in Ron Phibun (4).

2.4.4 Health effect

Health problems attributable to As-contaminated water supplies in southern Thailand were first highlighted in 1987. A preliminary survey confirmed

approximately 1,000 cases of arsenic-induced skin disorders, including several arsenical skin cancers. Concentrations of arsenic in hair and fingernails were found to be elevated (average 3.1 mg kg^{-1} and 56 mg kg^{-1}, respectively) in 80% pupils, and strong spatial correlation between human body burdens and As concentrations in drinking water was confirmed. A follow-up study of 2,400 school pupils showed 89% have excess blood arsenic concentration, with a 22% incidence of arsenical skin manifestations (4).

2.4.5 Mitigation

Since the recognition of health hazards related to arsenic contaminated water supplies in Ron Phibun District, efforts have been made to reduce exposure through improved public awareness, couple with the provision of alternative water supplies. Many deep boreholes have been sunk with additional water also imported via pipelines from beyond the contaminated area. A secure landfill for the containment of some 3,000 m^3 of high-grade waste has been constructed by the Department of Mineral Resources on the mid-slope of the Ron Na-Suang Chan Range in 1998 .

Despite a concerted effort to improve public appreciation of arsenic toxicity hazards, success in reducing exposure has been limited. A follow surveys have confirmed that many inhabitants continue to utilize shallow wells as these tend to be most conveniently located and yield soft water, which is considered more palatable than that from the underlying carbonate aquifer (4).

Regarding a secure landfill, although, the high-grade arsenopyrite waste piles which are considered to be a point source can be managed, arsenic contamination form

the presence of background arsenic concentration as a non-point source is still existed (4).

Therefore, This area is still contaminated with arsenic and needed appropriate technology for remediation.

2.5 Fungal processes for bioremediation of toxic metal

There is an increasing interest in the application of microbial biotechnology to the remediation of land and water contaminated with toxic metals. Fungi are ubiquitous in the natural environment and can be the dominant organisms in many soils, particularly those of low pH. An important component of their roles in decomposition and nutrient cycling, plant pathogenesis and symbiosis (mycorrhizae) is the ability to transform toxic metals between soluble and insoluble form including volatile derivatives (5). Such processes are integral to fungal growth and metabolism in natural, laboratory and industrial environments, and have an unappreciated but significant role in biogeochemical cycles. The balance between soluble and insoluble metal species in the environment is determined by physio-chemical factors and the physical and biochemical properties of the microorganism present. Some of these processes result in increasing mobility of the metal species, with accompanying implications for increased bioavailability and toxicity, while others lead to immobilization and detoxification. Because such processes may be manipulated under controlled conditions, e.g. in bioreactors, they appear to have potential for bioremediation of metal contamination (6,13).

A number of mechanisms contribute of fungal solubization of metals. The ability to solubilize metal species is a ubiquitous property among natural fungal isolates with the formation of metal-citrate complexes resulting in increased metal mobility and removal from contaminated materials (6), insoluble compounds and metal bearing minerals. For those fungi capable of oxalic acid production, solubilized metal ions/complexes may be precipitated as insoluble metal oxalates which represent a means of metal immobilization and may contribute to tolerance in producing organisms (6). Thus, certain fungi, which include *Aspergillus niger*, are able to transform insoluble inorganic metal species (e.g. oxides, phosphates, minerals) into insoluble organometallic species (oxalate) via a soluble intermediate (organic acid complexes/free cations), the extent and direction of this process being influenced by physicochemical and nutritional factors. Biotechnological applications of these processes are receiveng further attention (6,14).

Other mechanisms of metal immobilization and removal from solution rely on the interactive processes of biosorption (defined as the removal of metal species from solution as a result of physical and chemical attributes of living or dead biomass, excreted and derive components and products), transport and subsequent pathways of intracellular compartmentation and /or sequestration (living biomass). In addition, chemical reduction to elemental or other insoluble forms is also a widespread property, e.g. Ag(I) to Ag(0) and this process is also relevant to the development of fungal treatment methods for waste solutions. To date, the most attention has been given to biosorption (15,16,17,18,19,20,21). Fungal cell walls and their components have the major role in biosorption and can also take up suspended metal particulates and colloids (6,22) while freely suspended biomass has receive attention (6), focus has

also been given to the immobilization of biomass within or on various support matrices. Immobilization support materials have range from natural polysaccharide gels to coal, sand, foams and synthetic ploymers. Such immobilized preparation offer advantages in terms of mechanical strength and durability, handling, solid-liquid separation and ease of scale-up (6).

Transport and intracellular accumulation of toxic metals contribute to metal removal from solution by living organisms, providing an adequate energy source is present and toxicity thresholds are not exceeded (5). Once inside cells, metals such as Cu and Cd may be preferentially stored in the cytosol in association with various metal binding polypeptides, e.g. metallothioneins or phytochelatins, or become located in organelles such as vacuoles in the case of Zn, Co, Ni, and Mn (6). These processes may contribute to fungal survival in the presence of potentially toxic metal concentrations (5,13). The use of purified metal-binding peptides for metal removal is a specialized area of potential application (5).

Metalloids like selenium and tellurium can be transformed into soluble and insoluble forms by fungi. The reduction of metalloid oxyanions, e.g. selenate, selenite, tellurate, tellutite, to elemental selenium or tellurium is a ubiquitous property in unicellular, polymorphic and filamentous fungi (23) with the metalloid element being extensively deposited around and within cellular compartments (23). Methylation of metalloids results in the formation of volatile derivatives, e.g. dimethyl selenide, which may be lost of recovered from a system (24). Again, methylation is a widely found property in fungi and this process has effectively contributed to the detoxification of land and waters contaminated with selenium.

In conclusion, fungi exhibit variety properties (Figure 2-3) which can remove or detoxified the toxic metals in contaminated land and water. Arsenic is considered to be a toxic metal. It causes both large scale environmental pollution and health hazards in several countries. Therefore, This study was carried out to investigate the appropriate fungi that had good efficiency to remove arsenic compounds.

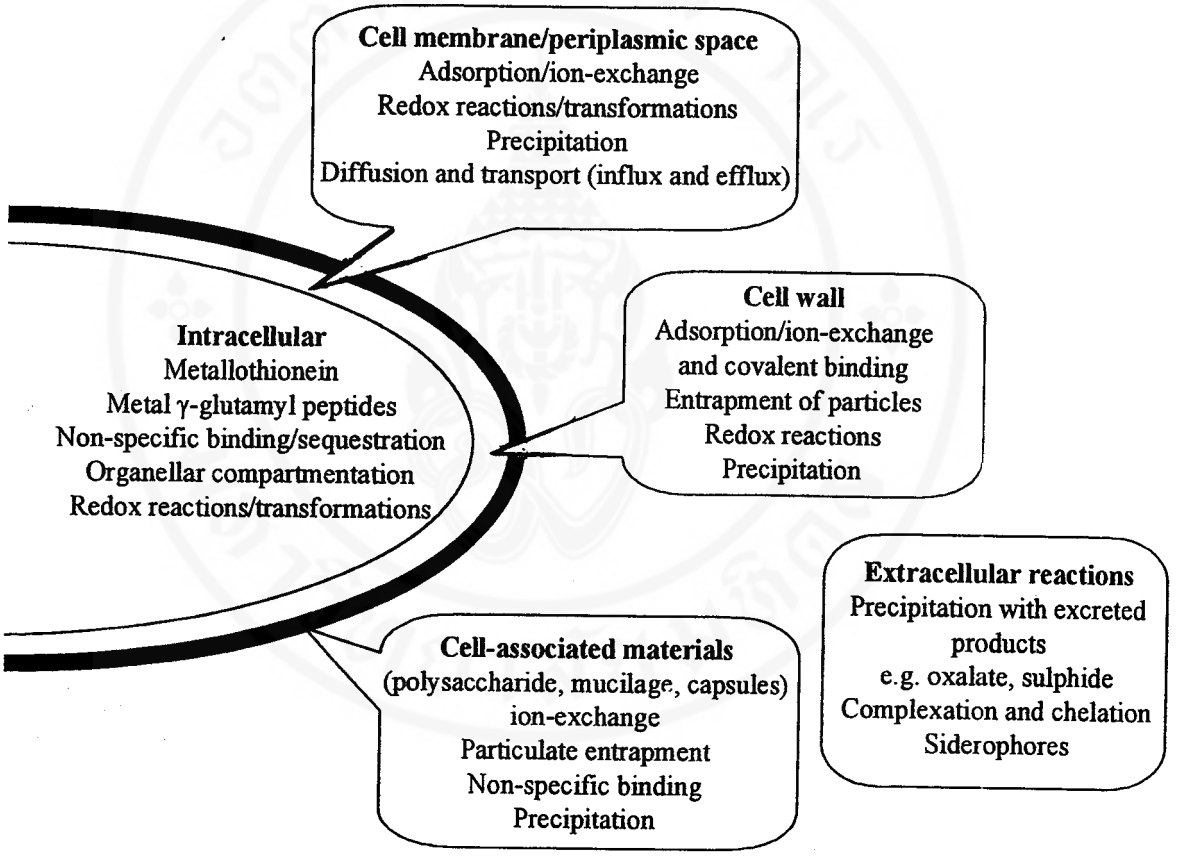


Figure 2-3 The process contributing to fungal uptake and detoxification of toxic metals.

Source: Gadd and White (13).

2.6 Interaction of fungi with arsenic

2.6.1 Arsenic Tolerance

Thom and Raper (25) surveyed a range of fungi with the objective of establishing which of these had the ability to "methylation arsenic", i.e., produce ill-smelling

arsenical gases. In addition to the organisms that did this, a number were found that grew well in Czapek's solution agar augmented with up to 0.2% As_2O_3. These include *Penicillium expansum, P. chrysogenum, P. roqueforti, Aspergillus flavus, A. oryzae,* a species of *Helminthosporium*, and a few *Mucor*. Challenger (26) described how the very common fungus *Cladosporium herbarum* will grow in a solution containing 2% arsenic (but not in 4%); again no garlic odor is produced. In the presence of phosphate, *C. herbarum* will grow in 4.8% solutions of arsenate (26). Some organisms that will tolerate an arsenite concentration of 0.02 M, without obvious effect, have been isolated from sewage (27).

Chromated copper arsenate (CCA) is a commonly used wood preservative against biological decay. The minimum inhibition concentration (MIC) value is the lowest concentration tested at which growth is prevented. The values for particular fungi (obtained from agar plates) indicate that the effectiveness of the CCA preservative is not simply due to the component with the highest MIC value (26). Marine fungi seem to be more tolerant than nonmarine. For example, the MIC values for *Dendryphiella salina* are As_2O_5, 1220 mg l^{-1},CCA, 0.4%. A low concentration of CCA actually stimulates the growth of this fungus. The nonmarine fungus *Botryosporium* sp has the same MIC value of 0.4% for CCA but the value for As_2O_5 is much lower at 224 mg l^{-1}. MIC values for other fungi are available (26), of special interest are the values of 1,722-4,305 mg l^{-1} As_2O_5 for *Lenzites trabea* and > 4,305 mg l^{-1} As_2O_5 for *Poria vaillantii.*

2.6.2 Methylation of Arsenic by fungi

In 1981, Gosio (28) reported the generation of arsenical gas from the action of the mold, *Penicillium brevicule*, on potato mash containing arsenious oxide. He was not able to characterize the gas but later work suggests that it was probably dimethyl or trimethylarsine. Gosio later reported that *Aspergillus gllaucus, A. virens, Mucor mucedo* and *M. ramosus* also produced this gas (Gosio-Gas) ; the exact nature of this gas was debated but was characterized as being pungent and with a garlic odor.

Challenger studied the action of four strains of the mold *Scopulariopsis brevicaulis* on arsenicals (26,28). This mold is identical with one of the "arsine" gas producers earlier identified by Gosio as *Penicillium brevicaule.*

The gallic-like odor of the arsines is intense and unforgettable and is good indicator of reaction. Because of this, a mycological test for arsenical was developed. Smith and Cameron (26,28) studied the use of the mold *Scopulariopsis brevicalulis* (previously *Penicillium brevicule*) to detect arsenic in many kinds of food. Thus *S. brevicaulis* was allowed to act on the suspect substance. If arsenic was present, an odor was detected within 2 h. The test was sensitive to ~1 ppm.

Cullen and Reimer (26) presented that alkylarsonic and diakylarsonic acid afford the appropriate methylarsines, Rme_2As and RR'MeAs, respectively. Rme_2As is produced more easily than RR'MeAs, possibly because RR'AsO(OH) (or its salts) is more toxic than $RAsO(OH)_2$. Allyl groups are not reduced during the methylation process; saturated alkylarsines are not obtained from arsonic acids such as $HOOCCH(CH_3)AsO(OH)_2$ (through a decarboxylation step) or from $HOCH_2CH_2AsO(OH)_2$ (through reduction of C*OH* group). This work was done to investigate possible pathways for the biological methylation. The trace of Me_3As sometimes obtained is

probably the result of hydrolysis of the arsenical and subsequent methylation, and not biological cleavage. Trialkylarsenic (V) derivatives are reduced to trialkylarsines, and even [$PhMe_2AsOH^+$] affords $PhMe_2As$. Arylarsenicals (V) and (III) are not methylated by *S. brevicaulis* (29). Arsine (AsH_3) is not a product of these biological reactions of *S. brevicaulis*. Challenger (26) reports that no intermediates from the proposed metabolic pathway are found in the medium, although no details are available regarding the methodology used to support this statement.

Again, Cullen *et al.* (26) informed methylarsonic oxide ($(MeAsO)_x$ is one of the few organoarsenic (III) derivatives studied. These noteworthy results show that a primary arsine, $MeAsH_2$, is produced as well as Me_3As and that $Me_2AsO(OH)$ can be isolated from the medium.

The arsenic content in the mold *S. brevicaulis* after exposure to As(III) in a liquid culture for 32 days is 0.032% (30).

Other fungi methylate arsenicals. Their names and the transformations studied are listed in Table 2-4.

Table 2-4 Arsenicals as substrates for yeast and fungi

Transformation	Microorganisms
$As_2O_3 \rightarrow Me_3As$	*Scopulariopsis brevicaulis*
	Aspergillus versicolor (trace)
	A. glacus (trace)
	Candida humicola
$As_2O_3 \not\rightarrow Me_3As$	A. *niger, A. fischeri,*
	Penicillium notatum
	P. chrysogenum, Gliocladium roseum,
	Penicillium sp., *Saccharomyces*
	cerevisiae, S. carlsbergensis, S.
	monacensis, "Rasse XII"
$H_3AsO_4 \rightarrow Me_3As$	*S. brevicaulis, C. humicola*
$H_3AsO_4 \not\rightarrow Me_3As$	*G. roseum, Penicillium* sp.
$MeAsO(OH)_2 \rightarrow Me_3As$	*S. brevicaulis, C. humicola, G. roseum*
	Penicillium sp. *P. chrysogenum* , *P.*
	notatum, A. niger, A. ficheri, A. glaucus,
	A. versicolor
$EtAsO(OH)_2 \rightarrow EtMe_2As$	*S. brevicaulis, A. niger*
$ClCH_2CH_2AsO(OH)_2 \rightarrow Me_3Ae$	*P. notatum*
$PrAsO(OH)_2 \rightarrow PrMe_2As$	*S. brevicaulis, P. notatum*
$allyl\text{-}AsO(OH)_2 \rightarrow allyl\text{-}Me_2As$	*S. brevicaulis, P. chrysogenum*
$BuAsO(OH)_2 \rightarrow BuMe_2As$	*C. humicola, S. brevicaulis, G. roseum*

Table 2-4 (continued)

Transformation	Microorganisms
allyl-$AsO(OH)_2$ → allyl-Me_2As	*S. brevicaulis, P. chrysogenum*
$BuAsO(OH)_2$ → $BuMe_2As$	*C. humicola, S. brevicaulis, G. roseum*
$PhAsO(OH)_2$ → $PhMe_2As$	*C. humicola*
$PhAsO(OH)_2$ ↛ $PhMe_2As$	*S. brevicaulis*
4-NH_2-2-$OHC_6H_3AsO(OH)_2$→ Me_3As	*C. humicola*
4-$NH_2C_6H_4AsO(OH)_2$↛ $ArMe_2As$	*C. humicola*
$Me_2AsO(OH)_2$ → Me_3As	*S. brevicaulis, P. chrysogenum, P. notatum* (trace), *A. niger, C. humicola, G. roseum, Penicillium* sp.
EtPrAsO(OH) → EtPrMeAs	*S. brevicaulis, A. niger*
PhMeAsO(OH) → $PhMe_2As$	*C. humicola*
PhMeAsO(OH) ↛ $PhMe_2As$	*S. brevicaulis*
$PhMe_2AsO$→ $PhMe_2As$	*C. humicola ,S. brevicaulis*
Me_3AsO → Me_3As	*C. humicola*
$Me_3As^+CH_2COO^-$ ↛ Me_3As	*C. humicola*
$(MeAsS)_x$ → Me_3As, $MeAsH_2$	*C. humicola*
$(MeAsO)_x$ → Me_3As, $MeAsH_2$, $Me_2AsO(OH)$	*C. humicola, S brevicaulis*
Me_2AsSR → Me_3As, Me_2AsH	*C. humicola*

Source: Cullen and Reimer (26) and Maeda (31)

A number of the results come from Challenger's studies (26,31). More modern studies principally involve three fungi, *Candida humicola* (Dazewska) Diddens and Lodder, *Gilocladium roseum* Bain, and a species of *Penicillium*; all were isolated from raw sewage (26) Cox and Alexander (26) made the first "modern" identification of Me_3As by comparing the gas chromatography retention time of the evolved arsine with that of a known sample and also by using mass spectroscopy. These fungi show a phenomenon of selective methylation first noted by Challenger and co-workers (29). For example, *G. roseum* and *Penicillium notatum* do not methylate inorganic arsenic, but readily metabolize alkylarsenicals. Cox and Alexander (26,28) found that the pH also has an effect on this selectivity: arsenate (0.1% in the medium) is not methylated by *C. humicola* at pH 6 or 7; Me_3As is produced at pH 5, which is the pH for maximum arsine production (26,28). *C. humicola* grows well in 0.01% phosphate. When excess phosphate (≥ 0.1% in the medium) is added to the growing culture, production of Me3As is inhibited from all the substrates but dimethylarsinate (32). They speculate that phosphate may suppress Me3As evolution by blocking the methylation sequence after one methyl group has been added. For further study, they used progressive enrichment techniques to isolate *C. humicola* and the two other arsine-producing fungi from sewage. Preconditioning *C. humicola* with dimethylarsinate enhances the ability of the organism to methylate both arsenate and dimethylarsinate (26), cell preconditioned with arsenate produce less Me_3As from dimethylarsinate. The phenomenon is not due to differences in transport through the wall, as cells preconditioned with dimethylarsinate have the same ability to take up arsenate as cells grown in the absence of dimethylarsinate, as judged from results obtained by using 74As- and 14C-labeled arsenicals (26).

Cullen *et al.* (26) reported that *C. humicola* methylates both $PhAsO(OH)_2$ and PhMeAsO(OH) to $PhMe_2As$, *S. brevicaulis* does not, although it does reduce $PhMe_2AsOH^+$ to this arsine. Arsanilic acid ($4\text{-}NH_2C_6H_4AsO(OH)_2$) is not methylated to a volatile arsine by *C. humicola*, and the aryl group is cleaved from $4\text{-}NH_2\text{-}2\text{-}OHC_6H_3AsO(OH)_2$, the product is Me_3As. A useful technique for trapping volatile arsines was developed for this study (26).

The only arsonium compound studied in pure culture to date, arsenobetaine, The principal arsenical found in marine animals, is not reduced by *C. humicola* to an arsine (26).

The results of Table 2-4 show that arsine oxides R_3AsO are reduced by fungi to arsine R_3As. In particular, the reduction of Me_3AsO by *C. humicola* had been studied in detail by Cullen *et al.* (26). This reduction is rapid, like the others, and in the case of *C. humicola* requires biologically intact cells. Cell-free extracts do not carry out this reduction; thus Me_3AsO can build up in this reaction medium (26). The reduction rate is maximum at pH 5.1-5.2, and the optimum temperature is 40 °c. The reaction follows Michaelis-Meten type kinetics and is inhibited by a number of electron transport inhibitors and uncouplers of oxidative phosphorylation such as azide and oligomycin. Arsenate and arsenite are strong inhibitors, whereas methylarsonate is less so; dimethylarsinate and phosphate have little effect. The enzyme system involved in the reduction, either directly or by producing an agent that reduces the arsine oxide in a chemical reaction, seems to be inducible. Cells grown in the presence of Me_3AsO show a dramatic increase in the rate of reduction. Pickett *et al.* (26) noted that this effect is counteracted by the protein-synthesis inhibitor cycloheximide.

In report by Cullen and Reimer (26), only a few organoarsenic(III) derivatives had been studied as substrates for *C. humicola*. The results from $(MeAsO)_x$ and $(MeAsS)_x$ are particularly interesting, as these, or similar compounds, could be intermediates in the methylation pathway. The oxide is metabolized by *C. humicola* and *S. brevicaulis* to Me_3As; smaller amounts of $MeAsH_2$ are also produced. Moreover, $Me_2AsO(OH)$ is found in the medium (26). As note above, this is the first observation of the production of an arsenic hydride by a pure mold culture, and it is also the first time that a methylated metabolite has been isolated from the culture medium. The same mixture of arsines is produced by *C. humicola* from $(MeAsS)_x$ (26). This is a particularly facile, process and 50% of the available arsenic is volatilized during 3 days. Less than 1% of $Me_2AsO(OH)$, at a similar concentration, would be converted to Me_3As in this time. As note above, The reduction of Me_3AsO to Me_3As by *C. humicola* is also a facile process.

$(MeAsS)_x$ is a potent fungicide and is very toxic to *C. humicola*, where the order, $(MeAsS)_x > (MeAsO)_x >$ As(III), is the reverse of the usual toxicity sequence described above and is an example of methylation resulting in an increase in toxicity (26). An increase in toxicity on methylation is well-known for mercury, where CH_3Hg^+ is much more toxic than Hg_2^+ (26). Oxidative methylation of arsenic results in a decrease in toxicity.

The production of arsines and $Me_2AsO(OH)$ from $(MeAsO)_x$ by *C. humicola* is not greatly affected by the presence of other arsenicals, apart from $(MeAsS)_x$; when the sulfide is present in the medium, the production of $Me_2AsO(OH)$ from $(MeAsO)_x$ over 24 h is doubled (26).

A mixture of arsines, Me_3As and Me_2AsH, is obtained from Me_2AsSR (RSH = cysteine or glutathione) (26). These are reasonably facile reactions, especially the latter example; however, the rate of arsine evolution is lower than from $Me_2AsO(OH)$. Other R_2AsSR compounds ($RSH=HSCH_2CH_2OH$, $HSCH_2COOH$) are not transformed to volaile arsines by *C. humicola* (26).

In addition to the fungi listed in Table 2-4, others have been found to evolve an arsine when growing in contact with inorganic arsenic compounds. An early study by Thom and Raper (25) found that while a number of *Penicillium* species are not arsine producers, 2 of the22 strains of *Aspergillus* studied, *A. fischeri* and *A. sydowi,* are active gas producer and 3 are feeble producers, namely, *A. fumigatus, A. glaucus,* and *A. ochraceus.*

The ability of the wood-rotting fungi to metabolize arsenic compounds is of considerable interest since arsenicals are commonly used as wood preservative. Other detailed was studied by Merrill and French (26), they found "arsine" evolution (presumably Me_3As) from As_2O_3 exposed to two wood-rotting fungi, *Lenzites trabea* and *L. saepiaria* ; 65 species were examined. Cullen *et al.* (26) studied the use of the yeast *C. humicola* in contact with wood treated with the preservative chromated copper arsenate (CCA) was able to generate trimethyarsine also. The simple chemofocusing technique was used to identify the arsine in this case.

The marine yeast *Rhodotorula rubra* reduces arsenate to arsenite, and an unidentified volatile arsine is evolve (26). Extracts of the cell show the presence of $MeAsO(OH)_2$ (first formed) and $Me_2AsO(OH)$. Phosphate competitively prevents arsenate toxicity (33).

2.6.3 Mechanism of Arsenic Methylation by fungi

Challenger (26,34) favored the hypothesis that the methylation of arsenic involved the transfer of a methyl group from some already methylated compound such as betaine, methionine, or a choline derivative. His proposed mechanism, involving alternating oxidation and reduction steps, is outline in figure (Figure 2-4).

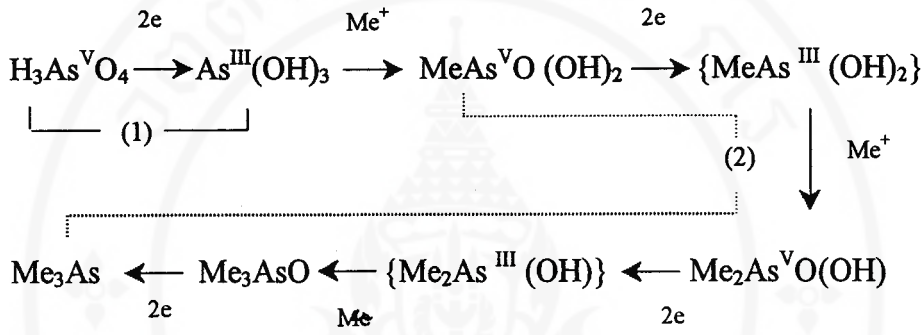


Figure. 2-4 Challenger's mechanism for the methylation of arsenic. The intermediates in {} are unknown as monomeric species. They are formulated as $(CH_3AsO)_n$ and $(CH_3As)_2O$ respectively, when prepared by conventional methods.

(1) reduction of arsenic (v) species to arsenic (III) species, and

(2) subsequent oxidative methylation of arsenic (III) moieties by a methyl donor

Source: Cullen et al. (34).

To investigate this hypothesis, Challenger *et al.* (34) added these compounds, labeled with ^{14}C, to cultures of *S. brevicaulis* growing on breadcrumbs enriched with arsenite. These experiments showed that only ^{14}C-labelled methionine, $^{14}CH_3SCH_2CH_2CH(NH_2)COOH$, was able to transfer its label to arsenite to an appreciable extent. The maximum $^{14}CH_3$ incorporation in the trapped $(CH_3)_3As$ was 28% after five days of incubation.

Cullen *et al.* (34) demonstrated that the CD_3 group in L-methionine-methyl-d_3 was incorporated into the trimethylarsine that was evolved from cultures of *Apiotrichum humicola* (previously identified as *Candida humicola*) and *S. brevicaulis* grown in the presence of arsenite, arsenate, methylarsonate and dimethylarsinate. In these experiments the arsine was collected by cryofocusing in liquid oxygen, and characterized by using mass spectrometry (34). These results strongly indicate that "active methionine", *S*-adenosylmethionine (SAM), [Figure 2-5] is involved in the transfer of the methionine methyl group to arsenic during methylation.

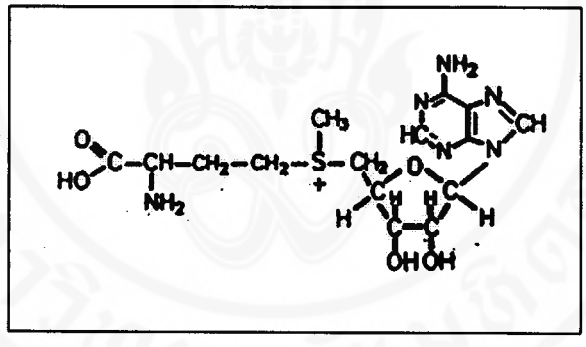


Figure 2-5 SAM

Source: Cullen and Reimer (26)

In order to develop these ideas further, broken cell homogenates of *C. humicula* were examined with respect to their to their ability to methylate arsenicals (26). [74As] Arsenate was incubated with the cell preparation, SAM, and NADPH for 8 h. After the cell debris had been removed by centrifugation, the supernatants was examined their ability to methylate arsenicals (26). The result show that arsenite, arsenate, methylarsonate, and dimethylarsinate are present. When the cell preparation was

replaced by buffer, there was some reduction of the arsenate to arsenite, but no methylation. From this result, Cullen *et al.* (26) suggested that definite interactions of the "simple" arsenical with the cell component are necessary for the methylation and electron-transfer reactions.

This conclusion is reinforced by studies on a model arsenic(III) intermediate, $(MeAsO)_x$ (26). When cell-free extracts of *C. humicola* were incubated with $(MeAsO)_x$, glucose, and NAD, the product is $Me_2AsO(OH)$ (17%); no arsenate is formed. The transformation requires glucose and is pH dependent. The yield of $Me_2AsO(OH)$ is not affected by a range of electron transport inhibitors and methylating agents such as SAM, and a ^{14}C-methyl label on SAM is not transferred to arsenic (26).

2.6.4 Uptake of Arsenic by fungi

Jung and Rothstein (35) reported arsenate competed with phosphate for transport into the yeast cell. The affinity of the two substances for the transport system was about equal, but in mixtures the phosphate was taken up about twice as fast as arsenate. Similar result was obtained by Cox and Alexander (26). They concluded that the mechanism of arsenate uptake in *Candida humicola* was metabolism linked, and there is dramatic reduction in arsenate uptake in a presence of phosphate. The uptake of arsenite, methylarsonate, and dimethylarsinate was much slower; entry of these arsenicals into the cell is probably by passive diffusion (26). In addition to the competitive effect, arsenate caused a continuous and irreversible transport system that can be characterized by first order kinetics. The rate of arsenate inactivation was slower in the presence of phosphate and the amount of arsenate taken up before complete block was established was also decreased (35).

In 1965, Jung and Rothstein (36) studied the kinetic of influx and efflux of arsenate in yeast cells exposed to arsenate plus fermentable sugar. Influx occurred only in the presence of fermentable substrate via a transport mechanism that could be divided by kinetic properties into two components with Michaelis constants of $4x10^{-6}$ and $4x10^{-4}$. During exposure to arsenate the transport system was gradually reduced in capacity and, with sufficient concentrations of arsenate, might be almost completely inactivated. At the same time, previously absorbed arsenate leaked out of the cell with first-order kinetics. The balance of inward transport, inactivation of the transport system, and outward leakage resulted in the arsenate content of the cell reaching a maximum value and then decreasing. Furthermore, arsenate also inhibited the fermentation of the intact cell, but only a maximal extent of 60%, compared to 100% in broken cells. They indicated that only a small fraction of the cellular arsenate was involved in the inhibition of metabolism and of transport and that its distribution and metabolic turnover must be complicated.

The yeast *Saccharomyces calsbergensis* took up ^{74}As-arsenate. The ^{74}As was bound exclusively to the phosphatidylinositol. When arsenate was inhibited by dinitrophenol and sodium azide, the formation of the arsenic-lipid complex was also inhibited (37).

2.6.5 Mechanism of metal uptake

In general, metal uptake by microorganism could be divided into two types of metal sequestering (18,38); **by living cell** which the metal transport and deposition are dependent on the metabolic activities and it is an energy driven process, and **by dead cells** which sequester metals through chemical functional group of the material

comprising the cell and particularly the cell wall, which constitutes a larger percentage of the cellular dry weight.

The cell wall of the fungi tend to be the cellular structure coming first into contact with metal ions in solution, the metals can be deposited on the surface or within the cell wall structure before interacting with the cytoplasmic material of the other cellular parts. For the living cells, intracellular uptake may occur due to the increased permeability as a result of cell wall rupture and subsequent exposure of the metal binding sites. The metal uptake by the cell wall has been broadly based on two mechanism; **uptake directed by functional groups**, these functional groups such as carboxyl, hydroxyl, amine, phosphate and sulfydryl groups. This mechanism is refered to a mass transfer model based on enzyme kinetic. **And the second uptake mechanism**, results from physico-chemical interactions directed by adsorption phenomena. This mechanism is cnsidered in the Langmuir or similar model (38). The removal mechanism for arsenate result from the first process plays an important role.

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and reagents

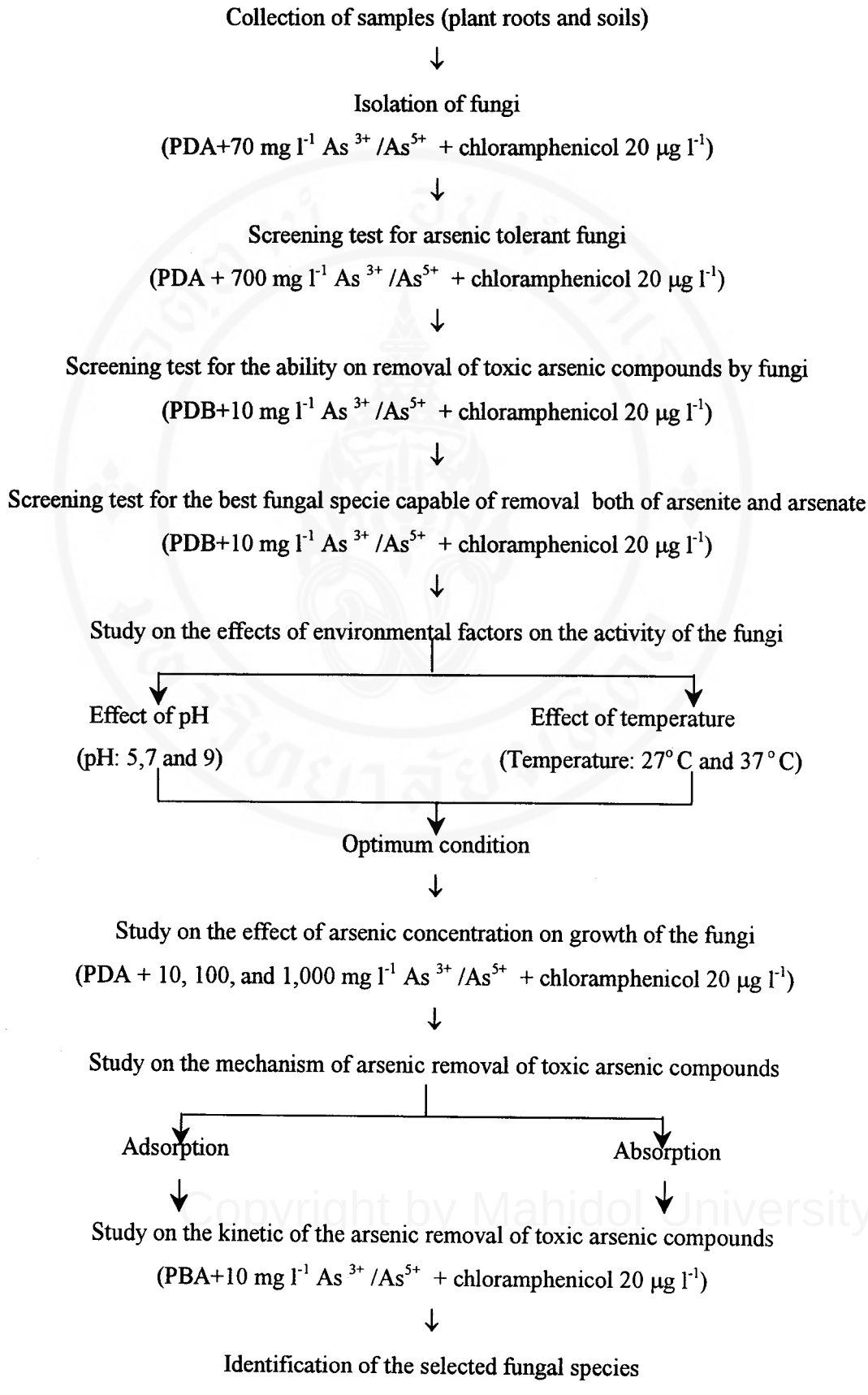
1. Sodium arsenate [Na_2HAsO_4 , F.W.= 312.0 g] and Sodium arsenite [$NaAsO_2$, F.W. = 129.9 g] were analytical grades, obtained from Sigma company.
2. Potato-dextrose agar (PDA) and Potato-dextrose broth (PDB) were obtained from Difco.
3. Chloramphenicol [$C_{11}H_{12}Cl_2N_2O_5$, F.W. = 323.1] for bacterial inhibition was obtained from Sigma company.
4. pH adjustment by using 1 N HCl and 1 N NaOH.
5. Other reagents such as ascobic acid (J.T. BAKER), potassium iodide (J.T. BAKER), and Sodiumborohydride (Merck) were analytical grades. All the reagents were used without further purification.

3.2 Apparatus

1. Sartorius filter paper 0.45 μm ; cellulose acetate membrane.
2. Sartorius filter holder ; polycarbonate housing.
3. Hydride Generation Atomic Absorption Spectrophotometer [HGAAS] model PERKIN ELMER AAS 3100 equipped with a quartz tube was used for determination of arsenic compounds in media.

4. Graphite Furnace Atomic Absorption Spectrophotometer [GFAAS] model PERKIN ELMER Aanalyst 100 equipped with graphite tube was used for determination of the amount of arsenic in fungal cells.
5. The glassware used in the experiments was washed with detergent, soaking in 10% HNO_3 for overnight and followed by rinsing with tap water and deionized distilled water, respectively.

3.3 Frame work of the study



3.4 Methods

3.4.1 Isolation of fungi which have ability to tolerate arsenic in arsenic contaminated land

3.4.1.1 Collection of samples

Root plants, and mine tailing were collected from arsenic contaminated land at Ron phibun district, Nakhon si thammarat province. Root tips of the widely dispersed plants were collected on Ronna mountain. Mine tailings were collected from 3 collected piles of mine tailing at the foot of Ronna mountain. These samples were collected in plastic bags.

Yala was another province which has history of tin mining. There are 2 locations in this province, i.e, Na sua mine and Tham thalu mine which have high concentration of arsenic in soil. Thus soil and root plants samples from these areas were also collected and processed as well as the samples collected from Ron Phibun district.

3.4.1.2 Isolation of fungi

- Isolation fungi from root plants :

The root plants were cut into segments (2 cm) and maintained on PDA plates containing 70 mg l^{-1} arsenite or arsenate and chloramphenicol (20 μg l^{-1}). All plates were incubated at 27° C for 48-72 hours. The colonies developed on those plates were restreaked on PDA plates for isolation of single colonies.

- Isolation of fungi from mine tailing :

Adding 1 g of soil to 10 ml sterile distill water and mixed together. The samples were allowed to sediment, then 0.1 ml of the solution was spreaded on PDA plate containing chloramphenicol (20 μg l^{-1}) and 70 mg l^{-1} arsenite or arsenate. All plates were incubated at 27° C for 48-72 hour.

- Maintaining cultures :

The selected strains have been maintained on PDA slant containing chloramphenicol and store in a refrigerator at 4° C. Cultures should be subcultured every 3-6 months. The selected strains were be identified by morphological characteristics.

3.4.2 Screening test on the isolated fungi.

3.4.2.1 Selection of 700 mg l^{-1}l of arsenic-resistant fungi

Fungi were grown on PDA plates containing 700 mg l^{-1} arsenic and chloramphenicol (20 μg l^{-1}). The arsenic used were either sodium arsenate (Na_2HAsO_4) or sodium arsenite ($NaAsO_2$). All plates were incubated at 27° C for 7 days. After that, fungi which could not grow are discarded as opposed to fungi which have ability to growth were selected for further experiment.

3.4.2.2 Selection of fungi which have high efficiency of removal of arsenic compounds

The selected arsenic-tolerant fungal strain were inoculated with 2 disks (8 mm diameter) cut from the margins of PDA plates into 250 ml Erlenmeyer flasks

containing 50 ml PDB, chloramphenicol (20 μg l^{-1}), and 10 mg l^{-1} of either arsinate or arsenite. There were 3 replicates of each experiment. The uninoculated flasks were treated as control. All of these flasks were incubated at 27° C for 5 days, then filtered through a 0.45 μm filter. Filtrate were analyzed for concentration of arsenic by using hydride generation atomic absorption spectrophotometer (HG-AAS).

3.4.2.3 Selection of the best fungal species which have high efficiency on removal both arsenite and arsenate.

Altogether four strains of fungal species were selected from the previous experiment. Two strains of the best efficient on arsenite removal were inoculated into 50 ml PDB with 10 mg l^{-1} arsenate and chloramphenicol (20 μg l^{-1}). The other two strains of the best efficient on arsenate were inoculated into 50 ml PDB with 10 mg l^{-1} arsenite and chloramphenicol (20 μg l^{-1}). There were 3 replicates for each experiment. The control flasks contained no fungi. All flasks were incubated at 27° C for 5 days on a rotary shaker (150 rpm). The medium in each flask was then filtered through a 0.45 μm filter. Afterward the filtrates were analyzed for concentration of arsenic by using HG-AAS.

3.4.2 Study on the effects of environmental factors on arsenic removal capacity of the selected fungus

3.4.3.1 Effect of pH :

pH influence in arsenic removal was assayed with pH value 5, 7 and 9. pH of the reaction media were adjusted using 1 N HCl and 1 N NaOH. In each experiment, one disk agar (8 mm diameter) taken from PDA plates was placed in 50 ml PDB

containing 10 mg l^{-1} arsenic and chloramphenicol (20 μg l^{-1}). There were 2 set of experiments, i.e., one with arsenate and the other with arsenite. All of these flasks were incubated at 27° C for 5 days on a rotary shaker (150 rpm). After the optimum exposure time, the reaction mixture was filtered through a 0.45 μm filter. Filtrates and biomass are analyzed for arsenic by using HG-AAS. The optimum pH was chosen for further experiment.

3.4.3.2 Effect of temperature :

The temperature values evaluated were 27° C and 37° C . In each assay, one disk agar (8 mm diameter) taken from PDA plates was placed in 50 ml PDB containing 10 mg l^{-1} arsenic and chloramphenicol (20 μg l^{-1}). There were 2 set of experiments, i.e., one with arsenate and the other with arsenite. All of these flasks were incubated at temperature either 27° C or 37° C for 5 days on a rotary shaker (150 rpm). After the optimum exposure time, the reaction mixture was filtered through a 0.45 μm filter. Filtrate was analyzed for arsenic by using HG-AAS. The optimum temperature was chosen for further experiment.

3.4.4 Study on effect of the arsenic concentration on growth in the selected fungus

Each fungal strain from 14-day-old was inoculated at the center of PDA plates containing various concentration of either arsenite or arsenate compounds, i.e., 0, 10, 100, and 1,000 mg l^{-1}. The plates were incubated at 27° C for 14 days. Radial growth measurements are made every 2 days during the inoculation period.

3.4.5 Study on mechanism of arsenic removal in selected fungus

There were 2 sets of experiments, i.e., one with living cells and the other with nonliving cells. Living cells were further divided into 2 groups; one group was inoculated with one disk (8 mm diameter) cut from the margin of PDA plates and the other group was inoculated with mycerial from 5-day-old liquid culture of the selected fungus. The inoculum from each group was separately inoculated into 50 ml PDB containing 10 mg l^{-1} of arsenic in the form of either arsenate or arsenite and chloramphenicol (20 μg l^{-1}). Flasks of nonliving cells were also prepared by adding into 50 ml of PDB containing 10 mg l^{-1} arsenic and chloramphenicol (20 μg l^{-1}) add to a sample of autoclave biomass. These flasks were shaken on rotary shaker at 150 rpm for 5 days. At the end of experiment a 20 ml sample of the medium was withdrawn and the remaining medium and biomass was separated by filtration then media was analyzed for arsenic concentration by HG-AAS. The remaining medium (30 ml) and biomass mixture were autoclaved. The biomass was removed by filtering and then washed 3x with deionized distilled water. The washing solution was determined arsenic concentration by HG-AAS. The fungal cells were dried 3 nights at 80° C and then digested with concentrate HNO_3 at 90° C for 2 hour. After cooling, the digest was the diluted with an appropriate volume of deionized distilled water. The arsenic content of diluted extracts was determined by using graphite furnace atomic absorption spectrophotometer (GF-AAS).

3.4.6 Study on the kinetic of the arsenic removal or detoxification by the selected fungus

Only 1 fungal strain which was the best efficient strain in removing arsenic from both arsenite and arsenate media was selected from the previous experiments. The fungi was then inoculated into PDB with chloramphenicol (20 $\mu g\ l^{-1}$) and 10 mgl^{-1} of arsenite. Another inoculation of this fungi to PDB with chloramphenicol (20 $\mu g\ l^{-1}$) and 10 mgL^{-1} of arsenate was also performed. All flasks were incubated at 27° C on rotary shaker set at 150 rpm. Reaction was finished at different exposure times : 0, 2, 4, 6, 8, 10 and 14 days. This assay had both possitive control (no arsenic in media) and negative control with arsenic but no inoculum. The reaction mixture were filtered through a 0.45 μm filter. Filtrates were analyzed for arsenic concentration by using HG-AAS. The biomass was also harvested, then dried 3 nights at 80° C for measurement growth and finally digested for determination of arsenic concentration by GF-AAS later.

3.4.7 Identification of selected fungus

The selected fungus was identified by observation of morphological characteristic. Slides were prepared by wet mount technique with lacto fuchsin. The observations were concentrated on type of hypha and spores structures.

CHAPTER IV
RESULTS AND DISCUSSION

4.1 Isolation of fungi which have ability to tolerate arsenic

The fungi that can tolerate arsenic contamination were isolated from samples collected in 2 seasons, i.e., summer and rainy season. In summer, 26 fungal strains were isolated which are able to tolerate arsenic (either arsenite or arsenate) at concentration of 70 mg l^{-1}. For rainy season, 133 fungal strains were isolated. Out of these 72 strains were arsenite tolerant fungi and 61 strains were arsenate tolerant fungi. So that there were more diversity of arsenic tolerant strains in rainy season than in summer season. Some of these isolated fungi have similar morphological structures.

4.2 Screening test on the isolated fungi.

4.2.1 Selection of fungi that tolerate 700 mg l^{-1} of arsenic (primary screening)

Since the level of 700 mg l^{-1} was the average concentration of total arsenic found in soil, all fungal strains isolated were thus subjected to testing on the ability to tolerate either arsenite or arsenate at this level. From the fungal strains isolated in summer season, there were 19 arsenite tolerant strains and 15 arsenate tolerant strains while in rainy season, there were 57 arsenite tolerant strains and 52 arsenate tolerant strains.

4.2.2 Selection of fungi which have high efficiency of removal of arsenic compound.

All of the arsenic tolerant fungi obtained in the primary screening test were tested on their efficiencies on removal of arsenic compounds. Each fungal strain was inoculated into 10 mg l^{-1} of either arsenite or arsenate. The result of arsenic removal by all the selected arsenic tolerant fungi are shown in Table 4-1 to Table 4-4.

Table 4-1. Screening test for the ability on removal of **arsenite** by fungal isolates collected in **summer**, 1998.

Sample	Concentration of arsenite in medium ,As(III)			
	Control (no sample)	Remaining As(III)	removal	
	(mg l^{-1})	(mg l^{-1})	(mg l^{-1})	%
MT1-1I	9.10	9.15	-	-
MT2-2I	9.10	8.84	0.26	2.86
MT2-3I	9.10	9.26	-	-
MT3-4I	9.10	9.15	-	-
R5-5I	9.10	8.11	0.99	10.88
R7-6I	9.10	9.05	0.05	0.57
R8-7I	9.10	9.00	0.10	1.15
R8-8I	9.10	8.58	0.52	5.73
R9-9I	9.10	9.13	-	-
R10-11I	9.10	9.93	-	-
R10-12I	9.10	8.84	0.26	2.86
R12-13I	9.10	8.94	0.16	1.72
R12-14I	9.10	8.89	0.21	2.29
R16-16I	9.10	9.00	0.10	1.15
R16-17I	9.10	8.53	0.57	6.30

***Italic* characters are strains that will be selected for further experiment**

Table 4-1. (continued)

Sample	Concentration of arsenite in medium ,As(III)			
	Control (no sample)	Remaining As(III)	removal	
	(mg l^{-1})	(mg l^{-1})	(mg l^{-1})	%
R19-18I	9.10	9.26	-	-
R19-19I	9.10	7.90	1.20	13.17
SUM-20I	9.10	9.31	-	-
***SUM-21I**	***9.10***	***6.78***	2.31	***25.47***

***Italic* characters are strains that will be selected for further experiment**

Table 4-2. Screening test for the ability on removal of **arsenate** by fungal isolates collected in **summer**, 1998.

Sample	Concentration of arsenate in medium ,As(V)			
	Control (no sample)	Remaining As(V)	removal	
	(mg l^{-1})	(mg l^{-1})	(mg l^{-1})	%
MT1-1A	10.34	10.37	-	-
MT2-2A	10.34	10.30	0.04	0.40
MT2-3A	10.34	10.20	0.14	1.34
R8-8A	9.7	9.62	0.08	0.84
R9-9A	9.7	9.42	0.28	2.84
R9-10A	9.70	9.29	0.41	4.18
R10-11A	10.34	10.09	0.25	2.41
R10-12A	10.34	10.20	0.14	1.34
R12-13A	9.70	9.59	0.11	1.17
***R12-14A**	***10.34***	***8.93***	***1.41***	***13.64***
R16-16A	10.34	9.91	0.43	4.15
R16-17A	10.34	9.35	0.99	9.63
R19-18A	10.34	9.98	0.36	3.48
SUM-20A	10.37	10.09	0.28	2.41
SUM-21A	9.70	8.65	1.05	10.86

***Italic* characters are strains that will be selected for further experiment**

Table 4-3. Screening test for the ability on removal of **arsenite** by fungal isolates collected in **rainy season**, 1998.

Sample	Concentration of arsenite in medium ,As(III)			
	Control (no sample)	Remaining As(III)	removal	
	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	%
RRMT2-1I	8.93	7.68	1.25	13.94
RRMT2-2I	8.93	8.67	0.26	2.89
RRMT2-3I	8.93	8.87	0.06	0.66
RR2-4I	8.93	8.69	0.24	2.66
RR2-5I	8.93	8.04	0.89	9.96
RR2-6I	8.93	8.40	0.53	5.98
RR2-7I	8.70	8.56	0.14	1.56
***RR3-8I**	***8.93***	***7.54***	***1.39***	***15.52***
RR5-11I	8.93	8.16	0.77	8.63
RR6-12I	8.93	8.81	0.12	1.33
RR7-13I	8.93	9.16	-	-
RR7-14I	8.93	8.93	0.00	0.00
RR8-15I	8.93	8.16	0.77	8.63
RR8-16I	8.93	8.40	0.53	5.98
RR9-18I	8.93	8.87	0.06	0.66
RR9-19I	8.93	9.11	-	-
RR10-20I	8.93	8.22	0.71	7.97
RR10-21I	8.93	7.98	0.95	10.62
RR10-22I	8.93	7.89	1.04	11.64
RR10-23I	8.93	8.06	0.87	9.70
RR11-24I	8.93	8.87	0.06	0.65
***RR11-25I**	***9.1***	***7.31***	***1.79***	***19.72***
D1-26I	8.70	7.69	1.01	11.66
RR12-27I	8.70	8.02	0.68	7.78
RR13-28I	8.70	8.43	0.27	3.11

***Italic* characters are strains that will be selected for further experiment**

Table 4-3. (continued)

Sample	Concentration of arsenite in medium ,As(III)			
	Control (no sample)	Remaining As(III)	removal	
	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	%
RR13-29I	8.70	8.63	0.07	0.78
***RR14-30I**	***8.70***	***7.28***	***1.42***	***16.33***
RR15-31I	8.70	8.63	0.07	0.78
RR15-32I	8.70	8.02	0.68	7.78
RR15-33I	8.70	8.50	0.20	2.33
RR15-34I	8.70	7.82	0.88	10.11
RR15-35I	8.70	8.43	0.27	3.11
***RR15-36I**	***9.10***	***7.36***	***1.74***	***19.17***
RYMT1-37I	8.70	8.29	0.41	4.67
RYMT1-39I	9.10	9.00	0.10	1.10
***RRMT2-40I**	***8.70***	***7.14***	***1.56***	***25.66***
RRMT3-41I	9.10	8.65	0.45	4.93
***RYMT1/1-Y1I**	***8.93***	***7.31***	***1.62***	***18.11***
RYMT1/1-Y2I	9.10	8.10	1.00	10.95
RYMT1/1-Y4I	8.93	7.77	1.16	12.99
RYMT1/1-Y5I	9.10	9.10	0.00	0.00
RYMT1/2-Y6I	8.93	8.24	0.69	7.62
***RYMT1/2-Y7I**	***8.93***	***7.71***	***1.22***	***13.45***
RYMT1/2-Y8I	8.93	8.67	0.26	2.89
RY1/1-Y10I	8.70	8.56	0.14	1.56
RY1/2-Y11I	8.70	8.50	0.50	2.33
RY1/2-Y12I	8.93	8.47	0.46	5.15
RY1/3-Y14I	8.70	8.50	0.20	2.33
***RY2-Y15I**	***9.10***	***6.98***	***2.12***	***23.26***

***Italic* characters are strains that will be selected for further experiment**

Table 4-3. (continued)

Sample	Concentration of arsenite in medium ,As(III)			
	Control (no sample)	Remaining As(III)	removal	
	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	%
RY3-Y16I	***9.10***	***6.88***	***2.22***	***24.36***
RY3-Y17I	9.10	9.00	0.10	1.11
RYMT1-Y18I	8.70	8.70	0.00	0.00
RYMT2-Y20I	8.70	7.82	0.88	10.11
RY4-Y24I	8.70	8.29	0.41	4.67
RY4-Y25I	8.70	8.02	0.68	7.78
RY5-Y26I	8.70	8.23	0.47	5.44
RY6-Y27I	8.70	8.09	0.61	7.00

***Italic* characters are strains that will be selected for further experiment**

Table 4-4. Screening test for the ability on removal of **arsenate** by fungal isolates collected in **rainy season**, 1998.

Sample	Concentration of arsenate in medium ,As(V)			
	Control (no sample)	Remaining As(V)	removal	
	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	%
RRMT1-1A	9.70	9.68	0.02	0.17
RRMT2-2A	9.70	9.23	0.47	4.83
RR1-4A	10.34	9.32	1.02	9.90
RR1-5A	10.34	9.53	0.81	7.89
***RR2-6A**	***9.70***	***8.49***	***1.21***	***12.48***
RR3-7A	9.70	9.80	-	-
RR3-8A	10.34	9.22	1.12	10.83
RR4-9A	9.70	9.68	0.02	0.20
RR4-10A	10.34	9.68	0.66	6.42
***RR4-11A**	***10.34***	***8.88***	1.46	***14.17***
RR4-12A	10.34	9.75	0.59	5.75
RR6-13A	10.34	9.76	0.58	5.62
RR7-14A	9.70	9.82	-	-
RR7-15A	9.70	9.51	0.19	1.92
RR8-16A	9.70	8.85	0.85	8.73
RR8-17A	9.70	10.09	-	-
***RR8-18A**	***10.34***	***8.46***	***1.88***	***18.19***
RR9-19A	10.34	9.44	0.90	8.69
RR9-20A	9.70	8.71	0.99	10.20
RR10-21A	10.34	9.47	0.87	8.42
RR10-22A	9.70	9.12	0.58	6.00
RR10-23A	9.70	9.68	0.02	0.20
RR11-24A	9.70	9.18	0.52	5.40
RR11-25A	10.34	9.93	0.41	4.01
RR12-26A	9.70	9.66	0.04	0.43
RR12-27A	9.70	9.68	0.02	0.17

***Italic* characters are strains that will be selected for further experiment**

Table 4-4. (continued)

Sample	Concentration of arsenate in medium ,As(V)			
	Control (no sample)	Remaining As(V)	removal	
	($mg\ l^{-1}$)	($mg\ l^{-1}$)	($mg\ l^{-1}$)	%
RR13-28A	9.70	9.47	0.23	2.37
RR13-29A	9.70	9.91	-	-
RR13-30A	9.70	9.87	-	-
RR14-31A	9.70	8.78	0.92	9.47
***RR15-32A**	***9.70***	***8.28***	***1.42***	***14.59***
RR15-33A	10.34	9.57	0.77	7.49
RR15-34A	10.34	10.40	-	-
***RRMT2-35A**	***10.12***	***8.84***	***1.28***	***12.69***
RY1/3-Y6A	10.34	10.32	0.02	0.27
***RY1/3-Y7A**	***10.34***	***8.85***	1.49	***14.44***
RY3-Y8A	10.34	9.79	0.55	5.35
***RY7-Y9A**	***10.34***	***8.63***	***1.71***	***16.58***
RYMT1-Y10A	10.12	9.07	1.05	10.36
RYMT1-I11A	10.12	10.34	-	-
RYMT3-Y13A	10.34	9.22	1.12	10.83
RYMT3-Y14A	10.12	9.34	0.78	7.75
***RY4-Y15A**	***10.12***	***8.71***	***1.41***	***13.96***
RY5-Y16A	10.12	9.73	0.39	3.87
***RY6-Y17A**	***10.12***	***8.22***	1.90	***18.82***
RY6-Y18A	10.12	9.01	1.11	11.00
RY6-Y19A	10.12	9.33	0.79	7.83
RY8-Y20A	10.12	9.52	0.60	5.92
RY2-Y21A	10.12	9.35	0.77	7.60
RY2-Y22A	10.12	9.49	0.63	6.20
RY2-Y23A	10.12	9.51	0.61	6.00
RY2-Y24A	10.12	9.69	0.43	4.20

***Italic* characters are strains that will be selected for further experiment**

There were altogether 20 fungal isolates selected, ten fungal isolates have high efficiencies on removal arsenite and another 10 fungal isolates have high efficiencies on arsenate removal. They were subjected for subsequent testing on their removal abilities by inoculating into the arsenite or arsenate-containing medium at the level of 10 mg l^{-1} for 5 days. The results are shown in Table 4-5 and Table 4-6. Then the best 2 strains from each group were selected for further experiment.

Table 4-5. Screening test for the ability on removal of the **arsenite** by 10 selected fungi.

Sample	Concentration of arsenite in medium ,As(III)			
	Control ®	Remaining	removal	
	(mg l^{-1})	(mg l^{-1})	(mg l^{-1})	%
SUM-21I	***9.20±0.18***	***6.67±0.30***	***2.53±0.30***	***27.51±3.31***
RR3-8I	8.70±0.12	7.42±0.35	1.07±0.10	12.38±1.15
RR11-25I	8.70±0.12	6.58±0.33	6.58±0.33	24.32±3.81
RR14-30I	9.20±0.18	7.41±0.83	1.79±0.83	19.48±9.06
RR15-36I	9.20±0.18	7.61±0.17	1.59±0.17	17.32±1.83
RRMT2-40I	***9.20±0.18***	***6.33±1.00***	***2.87±1.00***	***31.27±10.87***
RYMT1/1-Y1I	8.70±0.12	7.41±0.26	1.29±0.26	14.82±2.93
RYMT1/2-Y7I	8.70±0.12	7.64±0.39	0.82±0.39	12.11±4.51
RY2-Y15I	8.70±0.12	7.55±0.41	1.15±0.41	13.19±4.70
RY3-Y16I	9.20±0.18	8.19±0.24	1.01±0.24	11.02±2.56

***Italic* characters are strains that will be selected for further experiment**

®Control no fungi

Table 4-6. Screening test for the ability on removal of the **arsenate** by 10 selected fungi.

Sample	Concentration of arsenite in medium ,As(V)			
	Control ®	Remaining	removal	
	(mg l^{-1})	(mg l^{-1})	(mg l^{-1})	%
R12-14A	8.53±0.10	7.94±0.10	0.95±0.41	6.93±1.12
RR6-6A	8.82±0.85	7.93±0.27	0.88±0.27	10.04±3.11
RR4-11A	8.82±0.85	7.31±0.08	1.51±0.08	17.14±0.89
***RR8-18A**	***8.82±0.85***	***6.77±0.21***	***2.05±0.21***	***23.22±2.38***
RR15-32A	8.53±0.10	7.26±0.76	1.27±0.76	14.91±8.93
RY1/3-Y7A	8.82±0.85	7.89±0.08	0.93±0.08	10.55±0.91
RY7-Y9A	8.53±0.10	7.67±0.11	0.87±0.11	10.14±1.31
RY4-Y15A	8.53±0.10	7.94±0.08	0.99±0.22	6.89±0.60
RY6-Y17A	8.53±0.10	7.43±0.41	1.10±0.41	12.94±6.42
***RRMT2-35A**	***8.82±0.85***	***5.59±1.10***	***3.23±1.10***	***36.62±12.43***

***Italic* characters are strains that will be selected for further experiment**

®Control no fungi

4.2.3 Selection of the best fungal species which have high efficiency on removal both arsenite and arsenate.

The four fungal isolates selected previously were subjected to testing of their efficiencies on removal of both arsenite and arsenate. The two strains which the best efficiencies on arsenite removal were grown in media containing 10 mg l^{-1} arsenate and ability to remove arsenate from the medium was determined after 5 days of incubation on a rotary shaker set at 150 rpm, and 27° C. (Table 4-7). The other 2 strains which the best efficiencies on arsenate removal were tested on their capabilities to remove arsenite when growing in media containing 10 mg l^{-1} arsenite. The results are shown in Table 4-7. The arsenite/arsenate tolerant fungal strain RRMT2-40I was

selected as it possesses the best efficiency in removing both arsenite and arsenate. This strain could remove approximately 31% of arsenite and 38% of arsenate from the liquid media. The strain RRMT2-35A was not selected because it has similar morphology as the selected fungus, but was less efficient than the isolates RRMT2-40I. Also there is no manpower to carry out 2 isolates for further experiment, thus only isolates RRMT2-40I was chosen in this study.

Table 4-7 Selection of the best fungal species which have high efficiency on removal both arsenite and arsenate.

Sample	Concentration of arsenite in medium		Concentration of arsenate in medium	
	Remaining As(III), mg l^{-1}	% removal	Remaining As(V), mg l^{-1}	% removal
Control-As(V)	***8.78±0.13***		8.82±0.85	
RR8-18A	***6.86±0.31***	***21.97±3.55***	6.77±0.21	23.22±2.38
RRMT2-35A	***6.04±0.54***	***31.23±6.17***	5.59±1.10	36.62±12.43
Control-As(III)	9.20±0.18		***9.22±0.19***	
SUM-21I	6.67±0.30	27.51±3.31	***6.25±0.24***	***27.90±2.57***
♣RRMT2-40I	6.33±1.00	31.27±10.87	***5.67±0.5***	***38.45±5.46***

♣ fungal strain that will be selected for further experiment

4.3 Study on effects of environmental factors

This experiment was designed to determine the suitable environmental factors, i.e., pH and temperature for arsenic removal by the selected fungus. The pH of solution was tested at 3 points, i.e., pH 5.0, 7.0 and 9.0 while the temperature was tested only at 2 points, 27° C and 37° C. These are the soil temperatures in the shadow area and the outdoor area. The highest %removal of arsenite (25.32%) was found at

pH 5.0, followed by pH 7.0 (21.72%). The least %removal (11.13%) was found at pH 9.0 (Figure 4-1a). The pattern of arsenate removal is similar to that of arsenite removal. At pH 5.0, 7.0, and 9.0, the arsenate was removed approximately 26.18%, 18.91% and 9.09%, respectively (Figure 4-1 b).

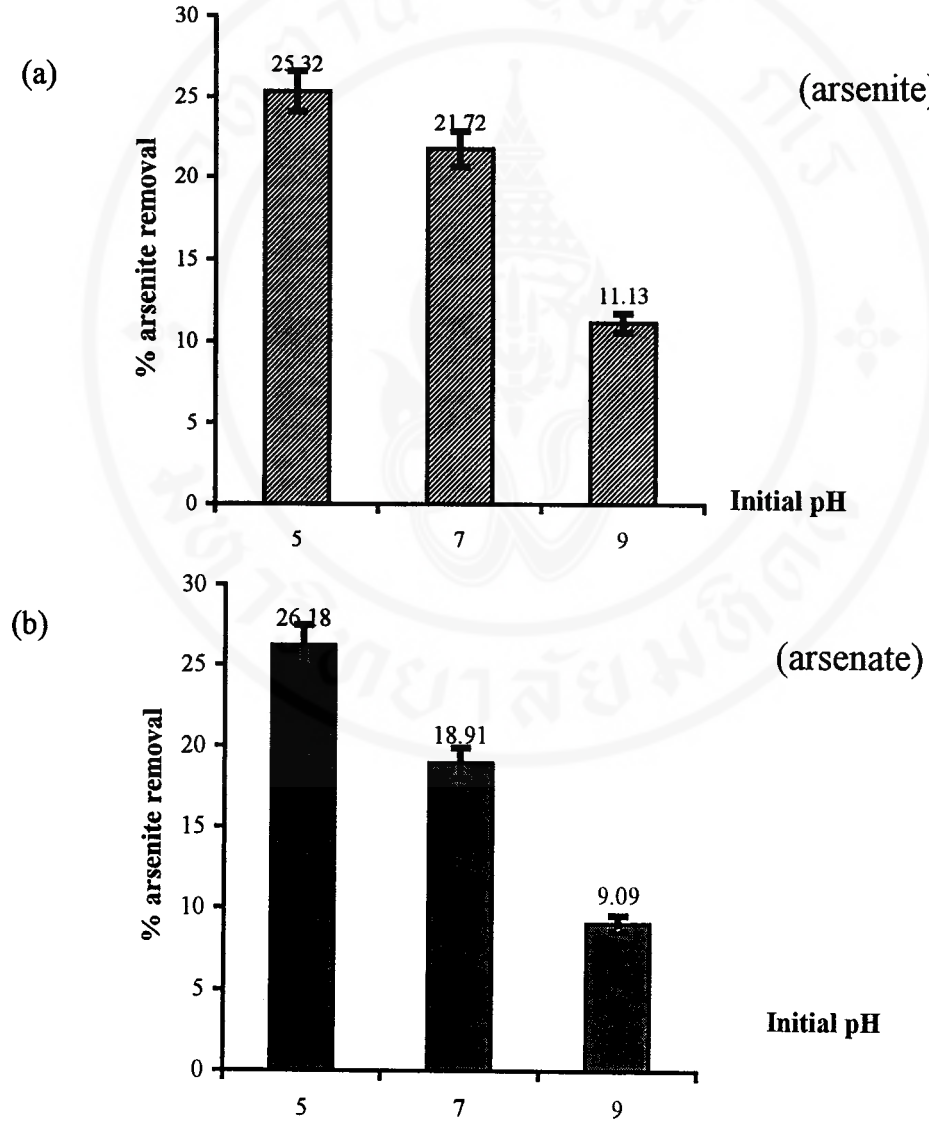


Figure 4-1 Arsenite (a) and arsenate (b) removal from liquid media at different initial pH values by the selected fungus. Cultures were incubated on an rotary shaker (150 rpm) at 27° C as described in the methods. Bars indicate S.D. (three replicates) when not shown were smaller than the symbols.

On the fifth day of incubation, the media was more sticky and thicker and the pH of medium containing arsenite/arsenate decreased to ~ pH 3.0-3.8 (Figure 4-2 a and Figure 4-2 b). It was also observed that at pH 5.0, growth of the fungal isolates was better than at the other pH.

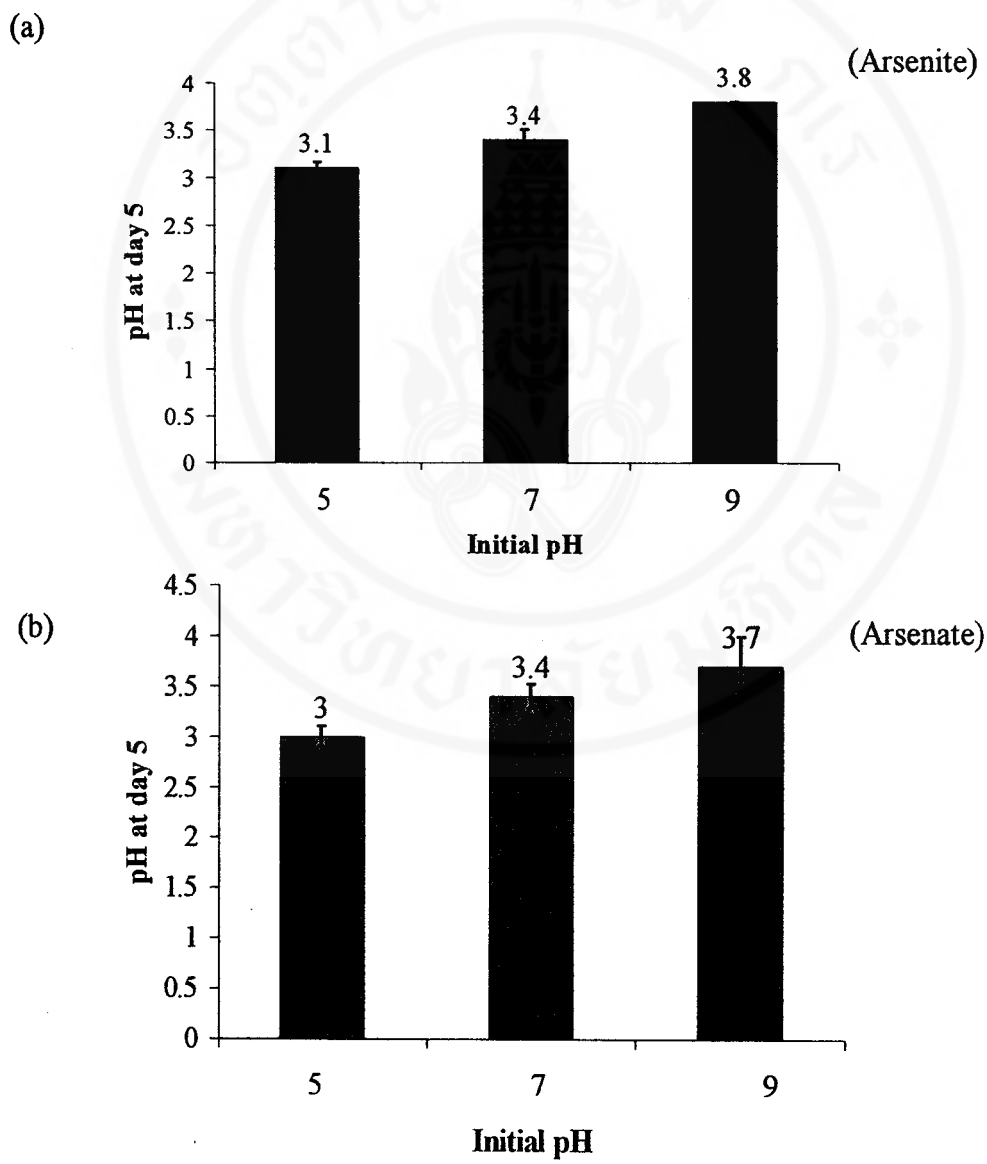


Figure 4-2 pH value of medium on the fifth day of incubation (a) arsenite containing media and (b) arsenate containing media. Cultures were incubated on an rotary shaker (150 rpm) at 27° C as described in the methods. Bars indicate S.D. (three replicates) when not shown were smaller than the symbols.

Regarding the temperature factor, the selected fungus could not grow at 37° C so that the concentration of arsenic compounds in liquid media were remaining constant (10 mg l^{-1}) over the incubation period.

From the result obtained in this study, % removal of both arsenite and arsenate by the selected strain at pH 5.0 and pH 7.0 are not significantly different. But only pH 5.0 was chosen for further experiments because growth of the fungal isolate was best at this pH. The temperature 27° C was selected as the suitable temperature for the selected fungal isolates.

4.4 Study on effect of the high arsenic concentration on growth of the selected fungus.

The effect of either arsenite or arsenate on growth of the selected fungus was tested on solid media. One disks (8 mm diameter) taken from the margins of selected fungus cultures in arsenic-free PDA were inoculated on plates containing either arsenite or arsenate at the level of 10, 100 or 1000 mg l^{-1}. All plates were and incubated at 27° C. Growth of the selected fungus was measured every 2 days as colony diameter during the incubation period. The results are shown in Figure 4.3 a and Figure 4.3 b. At the concentration of 10 mg l^{-1} and 100 mg $l^{-1}$of either arsenite or arsenate, the colony diameters of the selected fungus were reduced somewhat but the colony resumed growth afterwards. Thus after 14 days of incubation the colonies were as big as the control. At higher concentration (1,000 mg l^{-1}) of either sodium arsenite or sodium arsenate, the radial colony diameters of this organism was approximately 86% of the control at day 10 and growth continued to the edge of the plate.

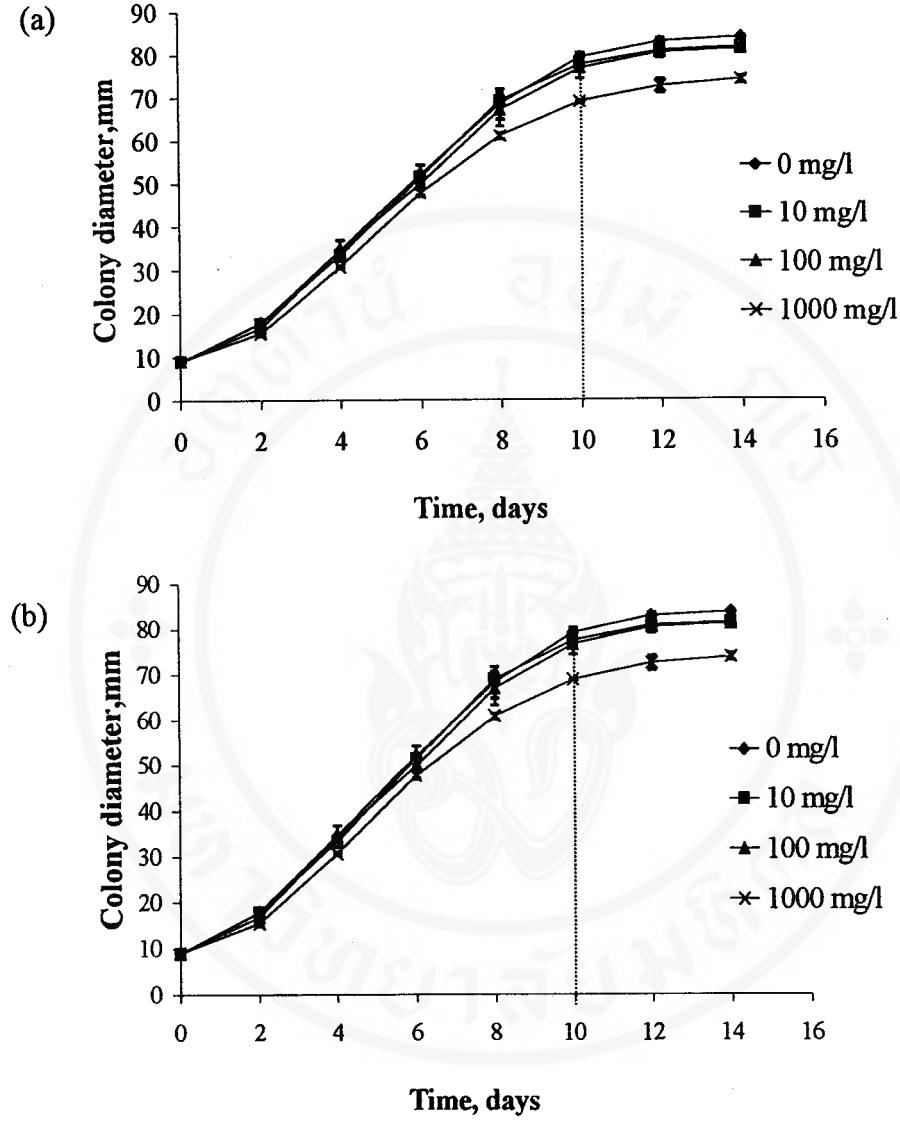


Figure 4-3 Growth of selected fungus on (a) arsenite containing 0, 10, 100, 1,000 mg l^{-1} and (b) arsenate containing 0, 10, 100, 1,000 mg l^{-1}. Disks (8 mm diameter), taken from the margins of selected fungus cultures in arsenic-free PDA, were inoculated on plates containing either arsenite or arsenate and incubated at 27° C. Colony diameters were measured every 2 days during the incubation period. Bars indicate S.D. of the mean (3 measurements) and when not shown were smaller than the symbol dimensions.

4.5 Study on suitable source of inoculum for further experiment.

The suitable source of fungal inoculum was determined in this experiment by comparing of the cells from margin of fungal colony on PDA plates, the cells from 5-day-old liquid culture and the heat-killed cells of the selected fungus. The % removal of arsenic compounds by the cells from margin of fungal colony on PDA plates was higher than % removal obtained by the cells from 5-day-old liquid culture and the heat-killed cells (Table 4-8). Arsenic was removed at 24.70±2.02% (100.50 μg) from 10 mg l^{-1} arsenate and at 21.70±5.01% (101.00 μg) from 10 mg l^{-1} arsenite by the cells from margin of fungal colony on PDA plates. The cells from 5-day-old liquid cultures could remove 17.91±3.69% (92.00 μg) from 10 mg l^{-1} arsenate and 14.36±2.57% (71.00 μg) from 10 mg l^{-1} arsenite. For heat-killed cells, arsenate and arsenite removals were very low approximately 4.34% and 1.96%, respectively.

This corresponds with the results of many studies. Maeda *et al.* reported that no arsenic accumulation in the cells of the algae, *Noctoc* sp. (39) and *Chorella* sp.(40), was found when the cells were killed with glutaraldehyde or ethanol. In another study with bacteria (*Klebsiella oxytoca* and *Xanthomonas* sp.)(41), they found that when the bacteria were killed by 70 % ethanol and then inoculated into the medium containing either arsenate or trimethyl arsenic, neither inorganic nor organic species were observed in the cells.

Zajic and Chiu (38, 42). They isolated fungal strains of *Penicillium* from wastewater, which exhibited significant growth in media containing salts of uranium, platinum and titanium. The uranium uptake was dependent on the culture age. The 5-day-old cultures were twice as effective as 15-day-old cultures. The reasons for the

Table 4-8 Arsenic removal and uptake by fungal cells from margin of fungal colony on PDA plates, the cells from 5-day-old liquid culture , and heat-killed cells.

Condition	Conc. of As in medium * ($mg\ l^{-1}$)			As removal (%)	Amt. of As (μg)				As in cell dry wt. ($\mu g\ g^{-1}$)
	Control-no fungal cells	Treatment	Removal		Removal	In cells	In washing solution	Lost	
The cells from margin of fungal colony on PDA plates									
- As(III)	9.34±0.05	7.32±0.47	2.02±0.47	21.70±5.01	101.00±23.44	38.39±4.67	35.47±4.84	27.14	114.42
- As(V)	8.15±0.09	6.14±0.02	2.01±0.16	24.70±2.02	100.50±15.23	39.97±1.11	34.71±8.49	25.82	121.12
The cells from 5-day-old liquid culture									
- As(III)	9.86±0.06	8.44±0.25	1.42±0.25	14.36±2.57	71.00±12.67	17.46±3.23	21.18±4.05	32.36	40.60
- As(V)	10.30±0.04	8.46±0.38	1.85±0.38	17.91±3.69	92.00±19.01	24.63±5.63	23.43±0.04	43.94	40.89
Heat-killed cells									
- As(III)	9.96±0.03	9.77±0.23	0.20±0.23	1.96±2.34					
- As(V)	10.26±0.01	9.81±0.22	0.45±0.22	4.34±2.14					

* Initial concentration of As in medium ($10\ mg\ l^{-1}$)

change in uptake pattern of toxic metals with culture age are not very clear. A possible explanation is that change in the cell surface chemistry and morphology with age contribute to higher uptake (38, 42).

4.6 Possible mechanism of arsenic removal by the selected fungus

As shown in Table 4-8, the uptake of arsenate was 39.97±1.11 μg and 17.46±3.23 μg by the cells from margin of fungal colony on PDA plates and the cells from 5-day-old liquid cultures, respectively.

Arsenate was emphasized to study the mechanism of arsenic uptake because arsenate (AsO_4^{3-}) is the functional analog for the natural substrate PO_4^{3-}. It can be concluded that arsenate entered the microorganism cells via a transport system that was normally responsible for phosphate uptake (33, 35, 36, 43). Button *et al.* (33) and Rothstein and Donovan (35) reported that arsenate competed with phosphate into the yeast cell. Corresponding reaction occurred in bacterial cell (43). In their study, arsenate was accumulated by highly specific, energy-dependent membrane "pumps" and arsenate was a competitive inhibitor of phosphate transport also. Jung and Rothstein (36) concluded that the uptake of arsenate by yeast cell was specifically associated with the glycolytic pathway of metabolism, either exogenous or endogenous. Because the operation of the transport system depends on energy derived from glycolysis.

For arsenite, 38.39±4.67 μg was uptaken by the cells from margin of fungal colony on PDA plates and 19.63±9.63 μg by 5-day-old cultures.

The arsenite transport mechanism is less well understood. Cullen *et al.* (26) showed that the uptake of arsenite, methylarsonate, and dimethylarsinate was much slower than arsenate. They concluded that entry of these arsenicals into the cell is probably by passive diffusion. The arsenite accumulation was also detected in *Noctoc* sp. (39). The trivalent arsenic [As (III)] was bioaccumulated by *Noctoc* sp. at concentrations about four times larger than pentavalent arsenic [As(V)].

The removal of arsenic compounds by nonliving cells was very low. This indicates that some can be adsorbed on the cell surfaces. But the higher amount of arsenic compounds were uptaken by living cells either the cells from margin of fungal colony on PDA plates or the cells from 5-day-old liquid cultures. This phenomena means the main arsenic removal was caused by absorption. After the experiment, cells from the flasks giving high arsenic removal were harvested and separately washed 3 times with 10 ml deionized distill water. The concentrations of arsenic in the washing solutions were determined by HGAAS. Some amounts of arsenic in washing solution were detected (Table 4-8). Also it can be seen in this table that when the amount of arsenite/arsenate found in cells and in the washing solutions were added together, there were some arsenite/arsenate lost (25-52 μg). However, this amount is in 10% range of technical error that can be accepted. Two of the reasons to the present problem can be speculated. First, the lost of arsenic occur during the experiment procedure, for instance, digestion process. The digestion of arsenic-containing samples might be conducted under conditions that convert all organic compounds to inorganic arsenic or arsenic might be evaporated during heating. Second, arsenic lost in some step during measurement of graphite furnace atomic absorption spectrometry (GFAAS). The samples are injected into the graphite tube as solution having volume

20 µl. The ashing of the injected sample at elevated temperature simplifies the matrix, but may also cause losses of analyte. If the ashing step was performed under the proper conditions, little material should be left that might vaporize during atomization and interfere with the analysis (44).

4.7 Study on kinetic of arsenic removal by the best-selected fungus

4.7.1 pH during growth of the fungus

The growth of the selected fungus was not markedly affected by addition of 10 mg $l^{-1}$of either arsenite or arsenate during the first 6 days of incubation (Figure 4-4 a, Figure 4-5a). On the eight day of the experiment, lysis of the cells could be observed as the dry weight of the fungus decreased. The pH of the medium decreased to pH 3.0-3.4 on the sixth day of incubation in both arsenic free and arsenic-containing media. But the pH increased in the presence of arsenic afterwards as compared to the arsenic-free medium (Figure 4-4 b, 4-5 b). The increased of pH may result from the cell lysis. The pH of the is generally at 7. Thus it will bring up the pH of the medium when the cell lysis.

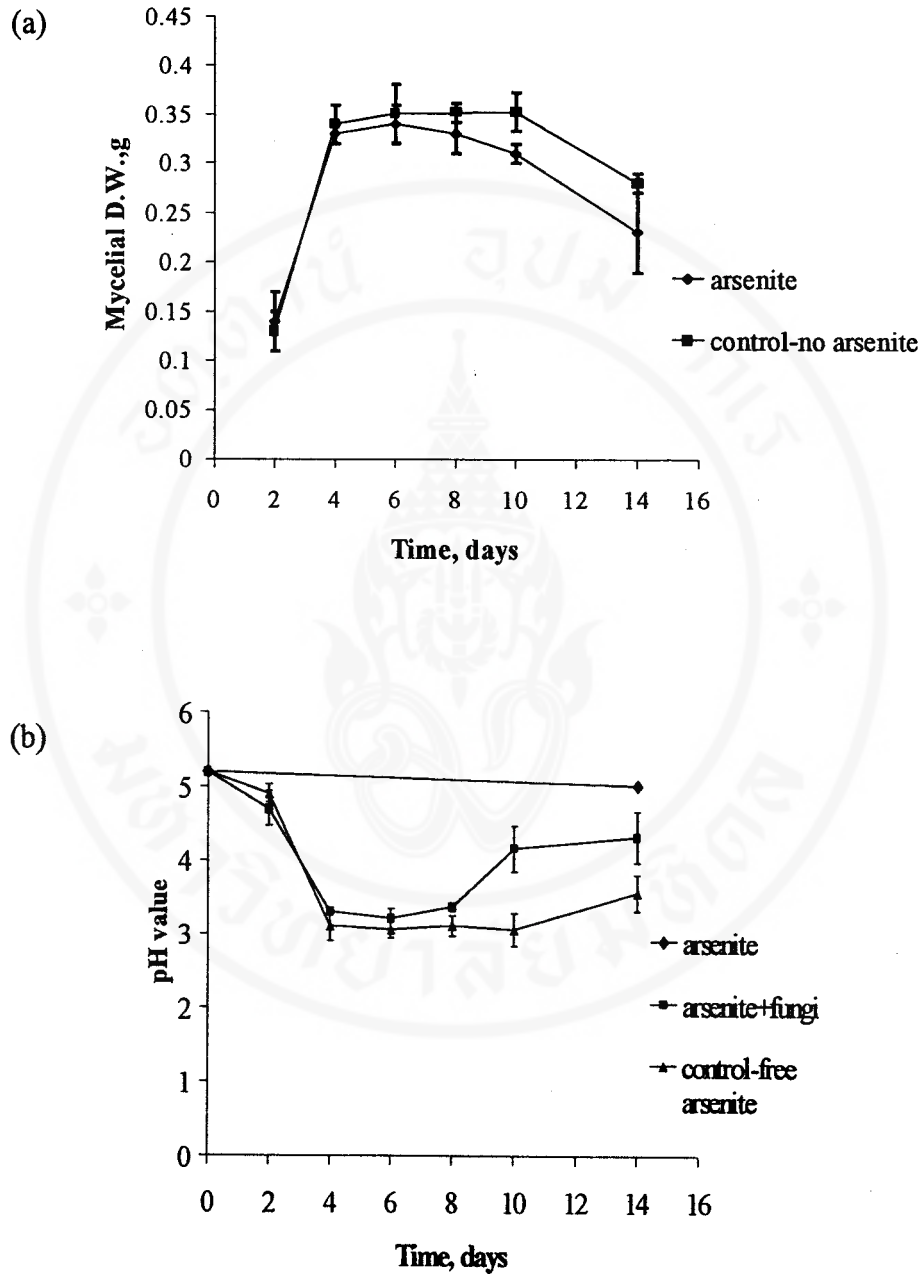


Figure 4-4 Change in (a) mycelial dry wt. and (b) pH during growth of the selected fungus over 14 d incubation in both control and sodium arsenite containing liquid medium. Cultures were incubated on a rotary shaker (150 rpm) at 27° C as described in method. Bars indicate S.D. (three replicates) when not shown were smaller than the symbols.

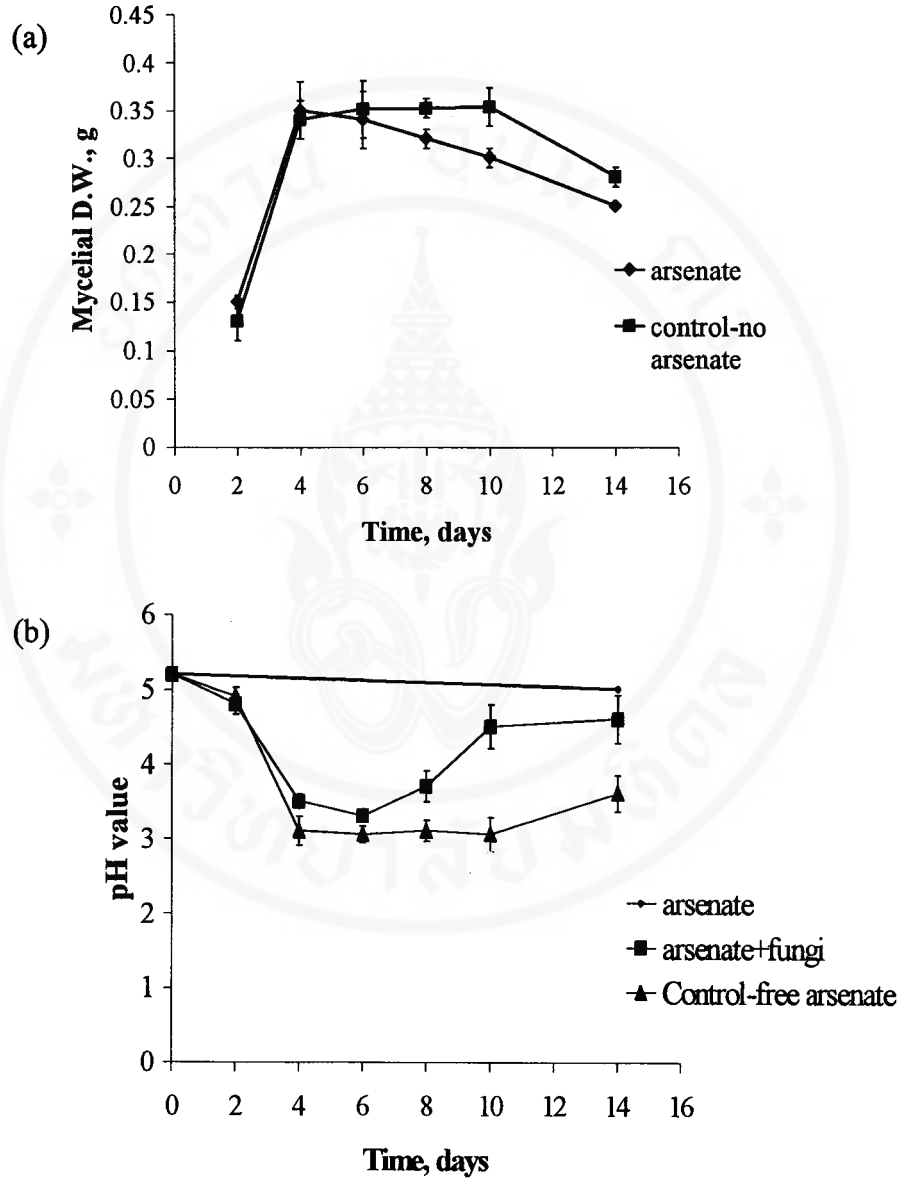


Figure 4-5 Change in (a) mycelial dry wt. and (b) pH during growth of the selected fungus over 14 d incubation in both control and sodium arsenate containing liquid medium. Other details as in figure 4-4. Bars indicate S.D. (three replicates) when not shown were smaller than the symbols.

4.7.2 Amount arsenite/arsenate removal and uptake by the selected fungus

Figure 4-6 a shows that the arsenite accumulation in the cells of isolated fungus was increased significantly during 2^{nd} – 4^{th} day of incubation, and reached the maximum (37.07 ± 5.90 μg). The arsenite removal from the medium was found to be at 110.00 μg (22.74 ± 5.86 %). After the maximal level of arsenite accumulation by the fungus attained, some of the arsenite lost was observed as the amount of the arsenite accumulated in the cells decreased. Meanwhile the arsenite concentration in the medium increased (Table 4-9). This incident can be accounted that the increasing of arsenite concentration in medium was due to the cell lysis.

In the case of arsenate accumulation by the selected fungus, the result was similar to that of the arsenite (Figure 4-6 b). The removal and uptake proceeded at a rapid rate at the beginning of the experiment, but slowed down after day 4. At the maximum level of arsenate accumulation, the amount of arsenate removal from the medium was 102.00 μg (21.23 ± 3.46%) and amount of arsenate accumulated in the fungal cells was 35.12 ± 3.31 μg. When arsenate content in the cells reached a peak, and there after decline, at the same time the concentration of arsenate in medium was increased (Table 4-10). This evident may result form the cell lysis.

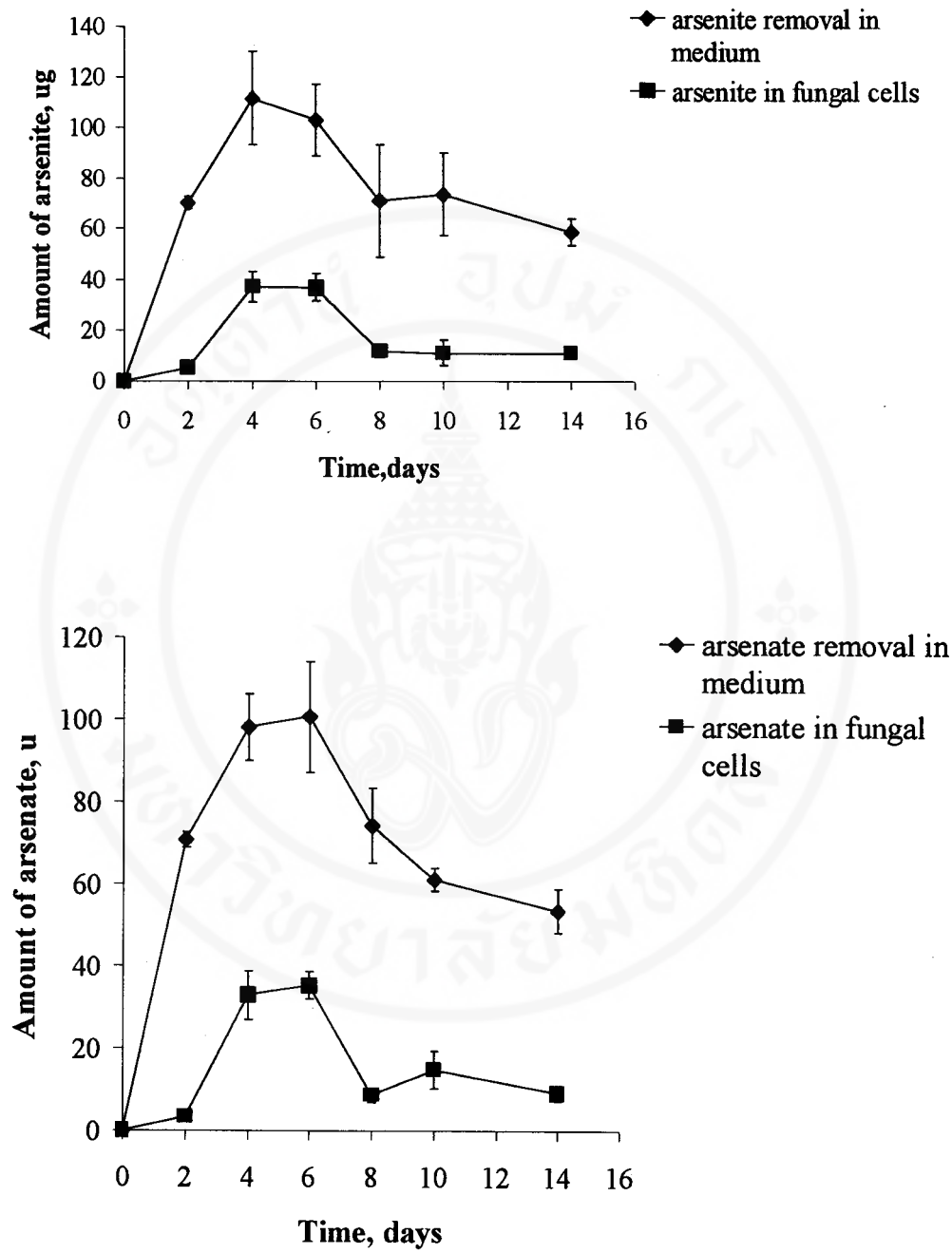


Figure 4-6 Partition of arsenite (a) and (b) accumulation by mycelia of the selected fungus over 14 d incubation. Bars indicate S.D. (three replicates).

Table 4-9 The kinetic of removal and uptake of As(III) by the selected fungus.

Time	Concentration of As(III) @			As(III) @	Amt. of As(III)		Cells dry wt.	Amt. of As(III)
(day)	Control ($mg\ l^{-1}$)	Treatment ($mg\ l^{-1}$)	Removal ($mg\ l^{-1}$)	Removal %	Removal (μg)	In cell (μg)	(g)	g^{-1} dry wt.
0	9.67±0.01*	-						
2	n.d.	8.28±0.48	1.38±0.05	14.32±0.50	69.50±2.40	5.11±1.76	0.14±0.03	36.5
4	n.d.	7.47±0.57	2.19±0.57	22.74±5.86	110.00±28.30	37.07±5.90	0.33±0.01	112.33
6	n.d.	7.64±0.48	2.03±0.48	20.98±4.96	101.50±23.99	36.87±5.36	0.34±0.02	108.44
8	n.d.	8.26±0.64	1.40±0.64	14.48±6.65	70.50±32.13	11.62±2.29	0.33±0.02	35.21
10	n.d.	8.22±0.53	1.45±0.52	15.02±5.46	72.50±26.38	11.05±4.99	0.31±0.01	35.65
14	9.80±0.30 *	8.51±0.11	1.15±0.11	11.93±1.09	58.00±5.28	10.91±1.30	0.23±0.04	47.43

@ Concentration of As(III) in medium and % removal of As(III) from medium

*control-no fungal cells, n.d. = not determined

Table 4-10 The kinetic of removal and uptake of As(V) by the selected fungus.

Time	Concentration of As(V)[@]			As(V)[@]	Amt. of As(V)		Cells dry wt.	Amt. of As(V)
(day)	Control ($mg\ l^{-1}$)	Treatment ($mg\ l^{-1}$)	Removal ($mg\ l^{-1}$)	Removal %	Removal (μg)	In cell (μg)	(g)	g^{-1} dry wt.
0	9.28±0.28*	-						
2	n.d.	8.18±0.04	1.44±0.03	14.95±0.28	72.00±1.92	3.17±0.31	0.15±0.007	21.13
4	n.d.	7.63±0.45	1.99±0.36	20.69±3.74	99.50±18.03	32.90±5.94	0.35±0.031	94.00
6	n.d.	7.58±0.47	2.04±0.47	21.23±3.46	102.00±23.51	35.12±3.31	0.34±0.028	103.29
8	n.d.	8.11±0.38	1.51±0.38	15.66±2.82	75.50±19.19	8.61±1.80	0.32±0.01	26.91
10	n.d.	8.36±0.07	1.24±0.05	12.88±0.57	63.00±2.74	14.66±4.55	0.30±0.01	48.87
14	9.62± 0.21	8.53±0.31	1.08±0.31	13.54±2.26	54.50±15.35	9.02±1.89	0.25±0.002	36.08

[@] Concentration of As(V) in medium and % removal of As(V) from medium

*control-no fungal cells, n.d. = not determined

In order to indicate if the arsenate is reduced to arsenite by the fungus, the culture at day 2 was analyzed for the presence of arsenite in the medium. It was found that some arsenite could be detected in the medium. This indicates that some arsenate was transformed to arsenite by the selected fungus. The level of arsenite detected in media increased according to time of incubation until day 4. At day 4, concentration of arsenite produced was equal to concentration of total arsenate in the medium. This should indicate that at day 4 all arsenate had been transformed to arsenite. Then at day 8, the concentration of arsenite was increased (Table 4-11). This incident could cause from the result of cell lysis at day 8 of the experiment.

Table 4-11 The reduction of arsenate to arsenite by the selected fungus.

Time (day)	% removal	Total arsenate remaining in medium (mg l^{-1})*	Arsenite produced in medium (mg l^{-1}) @
0	0	9.82±0.28	-
2	14.95±0.28	8.18±0.04	3.03±0.04
4	20.69±3.74	7.63±0.45	7.64±0.13
6	21.23±3.46	7.58±0.47	7.47±0.78
8	15.66±2.82	8.11±0.38	8.25±0.17
10	12.88±0.57	8.36±0.07	8.64±0.51
14	13.54±2.26	8.53±0.31	8.73±0.23

* Total arsenate remaining in the medium was measured by reduction of arsenate to arsenite with KI & HCl

@ The amount of Arsenite detected in the medium without reduction reaction.

The reduction of arsenate to arsenite is the first step in methylation pathway of arsenic. Many microorganisms have this ability to reduce arsenate to arsenite but not all of them go on to produce methylarsenical (26). Although, the selected fungi also reduces arsenate to arsenite but the other reduction products were not detected in this study because the limitation of equipment.

4.8 Identification of the selected fungus

The fungi have septate hyphae. Growth of the selected fungus on PDA at 27° C was fast, attaining a diameter of 5-6 cm within 7 days. The colony consist of a velvety layer of conidiophores. The color of colonies was white at the beginning and change to green and dark green or grey-green afterwards. The color change started at the center of the colony and spreaded outward. Conidiophores are single (mononematous): consisting of a single stipe terminating in phialides (monoverticillate) [figure 4-7]. Conidiophores are hyaline, smooth-walled. Phailides are flask-shaped. Conidia are arranging in long chains, globose and rough walled. This selected fungus (figure 4-8) was preliminary identified as *Paecilomyces* sp.

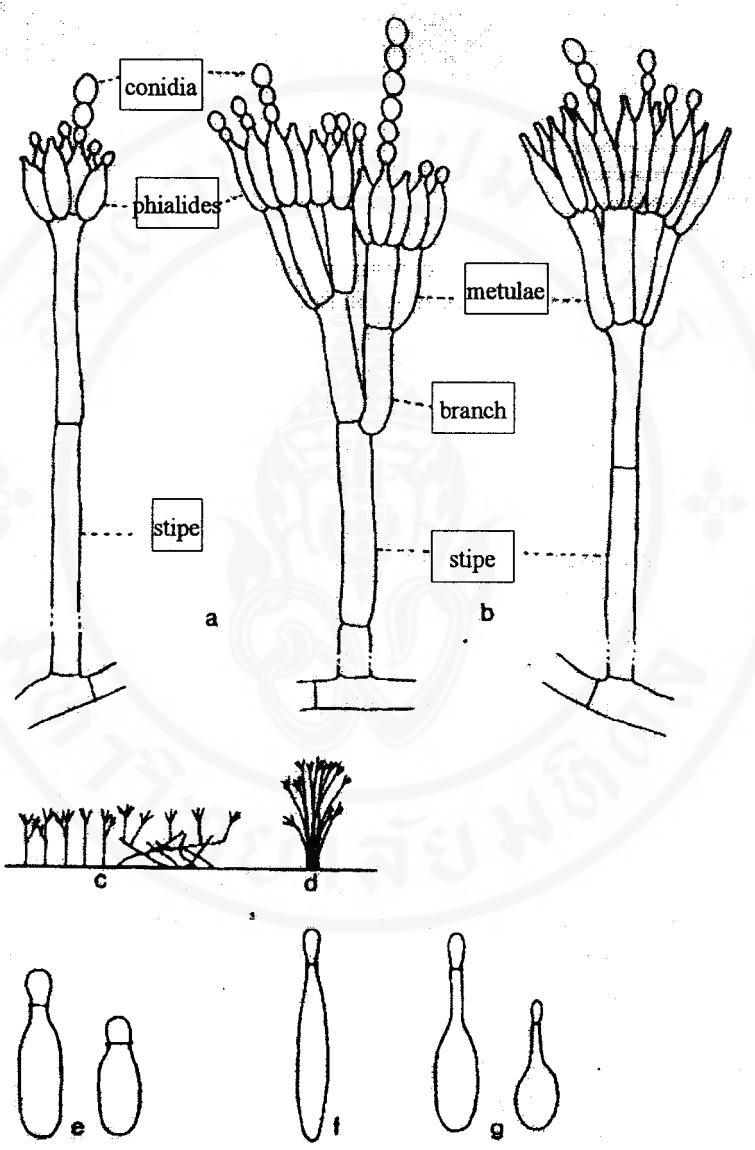


Figure 4-7 Morphological structures in *Penicillium* sp. a-b. Conidiophore structure; c. mononematous; d. synnematous; e. flask-shaped; f. lanceolate (= acerose); g. *Paecilomyces* type.

Source: Samson RA, Reenen-Hoekstra ESV. (45)

(a) (b)

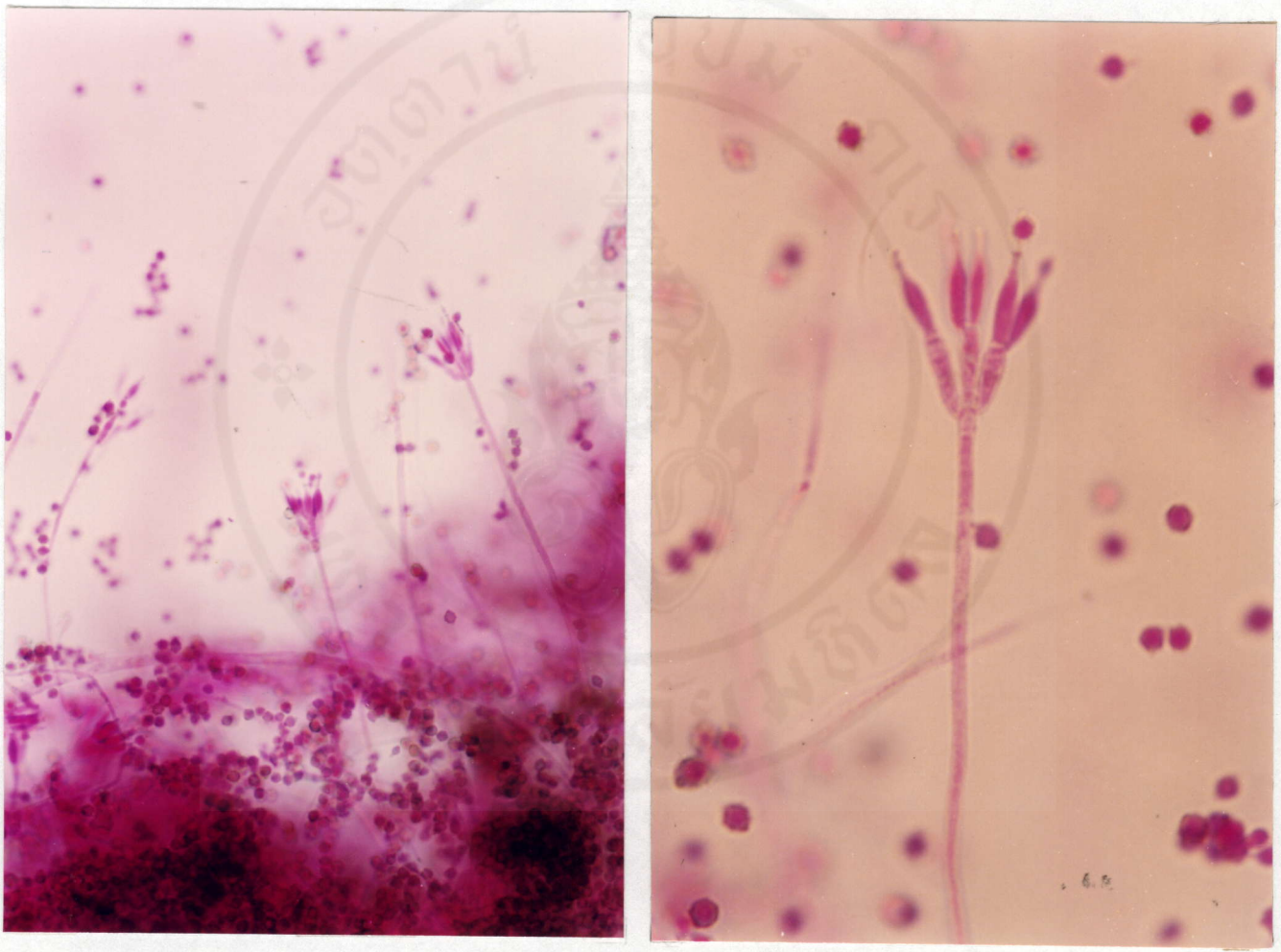
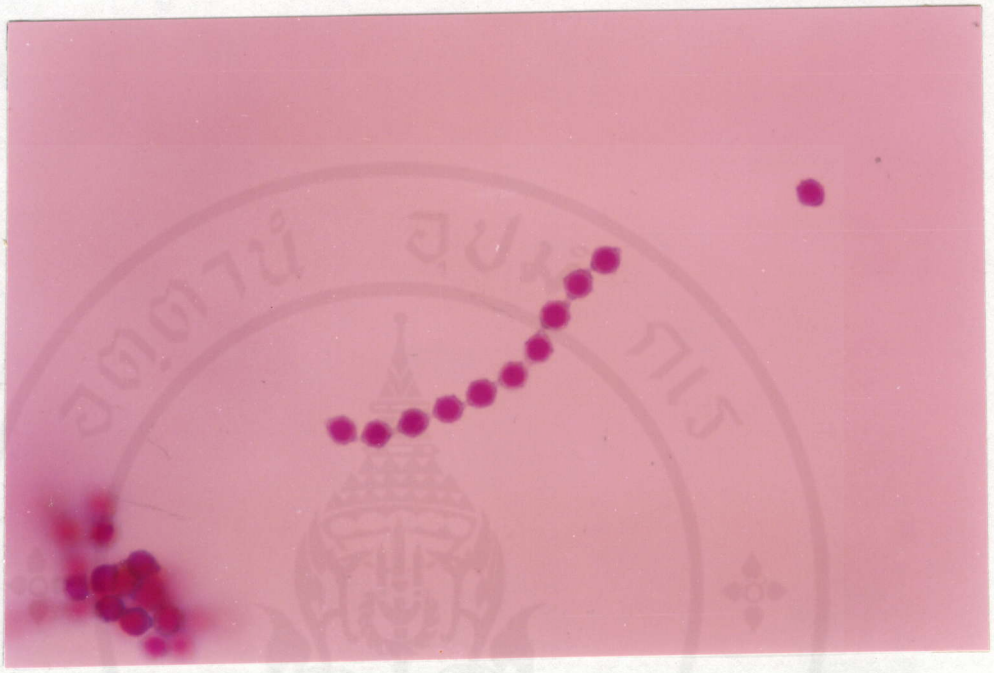


Figure 4-8 Morphology of the selected fungus. (a): general morphology of conidiophore (magnification 400x) and (b): Enlarged morphology of conidiophore (maginfication 1000x)

(c)



(d)

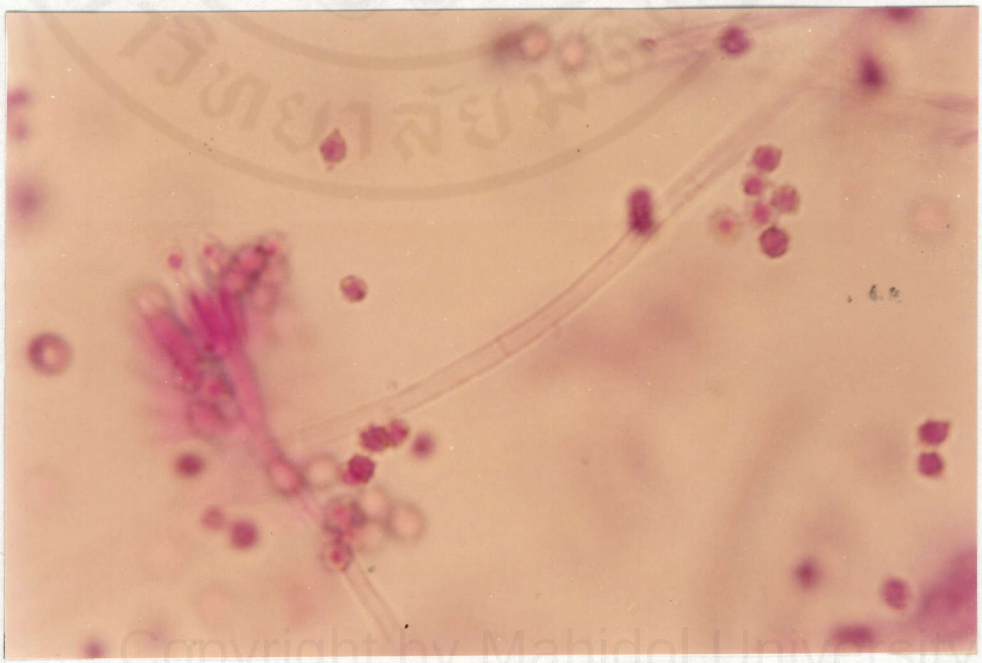


Figure 4-8 (continued) Morphology of the selected fungus. (c) conidia (magnification 1,000x) and (d) septum (magnification 1,000x).

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

From the experimental results and discussions of this study, the conclusion can be drawn as follow:

5.1.1 The fungi which had high efficiency removal either arsenite or arsenate from liquid media (potato dextrose broth) was fungal strain RRMT2-40I.

5.1.2 Arsenic removal was efficient at pH 5.0 and pH 7.0, but less efficient at pH 9.0.

5.1.3 The optimum temperature for growth and removal of arsenic by the selected was 27 °c.

5.1.4 The growth of this fungi was slightly effected by either arsenate or arsenite concentrations in the medium at 1,000 mg l^{-1}.

5.1.5 Arsenic removal in this study was caused from arsenic uptake occurred only in living cells. When the fungi killed by autoclaving, arsenic was not taken up or removed by the cells.

5.1.6 The removal of arsenic was dependent on the cell age because arsenic removal and bioaccumulation by the cells from margin of fungal colony on PDA plates was higher than that obtained by the 5-day-old cultures.

5.1.7 When arsenic content of the cells reached a peak, and there after decline because arsenic was excreted from fungal cells.

5.1.8 The selected fungus was identified as *Paecilomyces* sp.

5.2 Recommendations

The results in this study indicate that the selected fungus had high ability to remove arsenic at early stage of growth. Therefore, before the application of this fungi to remediate the arsenic contaminated land/water, more detail informations about this selected fungus should be studied. The further important studies on arsenic removal are recommended as follow:

5.2.1 More detail informations on methylation of arsenic by this selected fungus. Because methylation pathway of arsenic is viewed as a detoxification of arsenic contaminated on land (46, 47).

5.2.2 The influence of the competition with other metals, e.g. phosphate, in uptake and methylate arsenic by this selected fungus.

5.2.3 The enhancement on ability of uptake and methylate arsenic by this fungi.

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APPENDIX I

Standard preparation

- Standard arsenite preparation

  Standard of arsenite solution at concentration of 1,000 mg l^{-1} was prepared by dissolved 0.174 g sodium arsenite ($NaAsO_2$) with deionized water in a 100 ml volumetric flask, and used as stock solution. Working solutions used in all experiments were prepare from the stock solution.

- Standard arsenate preparation

  Standard of arsenate solution at concentration of 1,000 mg l^{-1} was prepared by dissolved 0.416 g sodium arsenate ($Na_2AsO_4 \bullet 7H_2O$) with deionized water in a 100 ml volumetric flask, and used as stock solution. Working solutions used in all experiments were prepared from the stock solution.

APPENDIX II

Analytical technique for determination of arsenic compounds

- Determination of arsenic concentration in medium by hydride generation atomic absorption spectrophotometer (HG-AAS)

Apparatus :

Initially in the hydride generation methods, atomization was preformed mainly in argon/hydrogen diffusion flames, and later these were replaced by graphite furnace heated quartz tubes. Nowadays, commercial hydride generation systems use almost exclusively either electrically heated or flame heated quartz tubes for atomization. The basic systems are represented in principle in Figure II-1. The burner assembly of the spectrometer is replaced with a quartz tube which is heated electrically by a coiled wire. The hydride vapour is atomized in the quartz tube and the resulting atoms are detected by the radiation beam from the hollow cathode lamp (or EDL) which passes down the axis of the tube. Non-specific background absorption is almost unknown with this technique when heated quartz tubes are used.

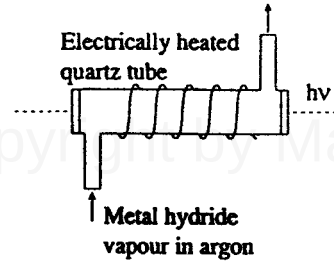

Figure II-1. The basic hydride generation technique systems with electrically heated quartz tubes for atomization

Reagents :

Analytical parameters :

Integration times	:	15 sec
Data Processing	:	Peak Height, Smoothing: 19 points
Lamp	:	HCL or EDL
Slit (nm)	:	0.7 nm (Low or Alt)
Wavelength	:	193.7

Reagents :

Carrier Solution	:	10%(v/v) HCl
Reducing agent	:	0.2% $NaBH_4$ in 0.05% NaOH

Procedures :

1. Prepare a series of standard arsenic solution as 1, 3, 5, 7, 10, and 15 μg/l by using HNO_3 as solvent.
2. Prepare carrier solution and reducing agent .
3. Samples to be analyzed for As^{3+} were diluted with 2% nitric acid to desired concentration and kept in the refrigerator until analysis.
4. The above mentioned (3) sensitivities are for As^{3+}. Therefore, the samples to analyzed for As^{5+} should be reduced to As^{3+} prior to analysis. The reduction rate is faster as the acid concentration increases, to ensure proper reduction the following procedure may be used: To 1 ml 5%(w/v) KI and 5% (w/v)ascorbic acid. Wait 45 minutes at ambient temperature and dilute to 10 ml. If the sample solution contains

other oxidizing agents, e.g. digestion acids, it may be necessary to use more of the KI/ascorbic acid reducing solution. As^{5+} may be measured with higher sensitivity if a longer reaction coil is used.

5. 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.
6. The sample solution to measured the absorbance at 193.7 nm. The arsenic concentration are plotted against the absorbance and determine the value of A (Coefficient) and B(Y-intercept).
7. The value of absorbance can be calculated to the amount of arsenic by this formula,

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

When; X = the amount of arsenic Y = The absorbance value

A= Coefficient B = intercept

- Determination for concentration in fungal cells

Graphite furnace atomic absorption spectrometry (GFAAS) measurements were performed with a Perkin-Elmer Analyst 100 (Norwalk, CT, USA) equipped with a deuterium-arc background corrector and HGA-800 heat graphite atomizer. The sample was introduced by AS-72 autosampler. The cooling system HGA also used to allow the temperature of the atomizer to cool down more rapidly. The atomic signals discharge lamp operated at not over 300 mA, and the 193.7 nm wavelength was monitored. The spectral bandwidth used was 0.7nm. Graphite furnace operating conditions for analysis are shown in Table II-1.

Table II-1 Furnace operating conditions for analysis of sample by GFAAS using aqueous sample introduction.

Step	Temperature (°c)	Ramp/Hold Time (sec)	Argon gas flow (ml/min)
1. Drying	120	10/30	250
2. Pyrolysis	1,200	10/30	250
3. Cooling	20	5/5	250
4. Atomization	2,300	0/5	Stop flow
5. Clean up	2,400	1/5	250

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