Selection of Fungi Capable of Removing Toxic Arsenic Compounds from Liquid Medium

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ABSTRACT Thirty eight fungal isolates that grew in 700 mg l^{-1} of either arsenite or arsenate medium were isolated from soil samples collected from arsenic-polluted areas in Ron Phibun District, Nakhon Si Thammarat Province, Thailand. Out of these, the fungal isolate (RRMT2-40I) was the most efficient at removing arsenite/arsenate from potato dextrose broth. This fungus, identified as *Penicillium* sp., grew best in growth medium with a pH of 5.0 or 7.0 at 27° C, reaching the stationary growth phase within 4 days. Its growth was slightly affected by arsenite/arsenate concentrations of 1000 mg l^{-1} in the medium, but lower concentrations (10 and 100 mg l^{-1}) had no effect. Arsenic uptake exhibited a peak and turning point at the stationary growth phase. During this phase, arsenic was excreted from the fungal cells. Arsenic removal depended on culture age and cell viability since there was no arsenite/arsenate removal when the cells were killed by autoclaving.

KEYWORDS: arsenate, arsenite, fungi, removal, transformation, hydride generation.

INTRODUCTION

Mining and mineral processing activities have existed in Ron Phibun district of Nakhon Si Thammarat province in southern Thailand for the past 100 years. These activities are considered to be major causes of arsenic contamination of surface drainage and groundwater systems in Ron Phibun. Hydrochemical analysis of surface water and groundwater have confirmed the presence of dissolved arsenic at concentrations exceeding the current WHO potable water standard of 10 µg l^{-1}.[1] The concentration of arsenic in soil is also high and ranges from 50-5200 mg kg^{-1}.[2] The health problems attributable to arsenic contamination of water supplies in Ron Phibun were realized in 1987, when over 1,000 cases of chronic arsenic toxicity were recorded. Affected persons showed symptoms such as eczema of skin/mucous membranes, hyperkeratosis of palms and soles, warts, leukemia, acute renal failure, confusion, encephalopathy, sensory disorders, neuropathy, and cancers.[3] In addition, more cases are being reported every year.[1] Since the recognition of health hazards related to arsenic contaminated water supplies in Ron Phibun, efforts have been made to reduce exposure of local people to arsenic. In 1994, arsenopyrite mining waste was collected by the Department of Mineral Resources to be placed in a secure landfill. However, the construction of the landfill for containment of these mining wastes was delayed until 1998. Thus, arsenic was released from the collection sites into the soil and the incidence of arsenic poisoning in people living in the area has not decreased. Appropriate technology for arsenic remediation is, therefore, urgently needed.

The application of fungi to remedy the problem of land and water contaminated with toxic compounds has received increasing interest because fungi are ubiquitous in the natural environment and are often the dominant organisms in many soils, particularly those with low pH values. An important component of their role in bioremediation of toxic compounds is the ability to solubilize, transform, and/or uptake metal species. Such processes are integral to fungal growth and metabolism in natural, laboratory and industrial environments.[4-7] Many fungal species are capable of transforming inorganic arsenic compounds, arsenite and arsenate by bio-methylation, into methylated arsenic species such as monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine (TMA), and trimethyl-arsine-oxide (TMAO). Three of these, MMA, DMA and TMAO, are less toxic than arsenate, arsenite and TMA. Chalenger and Higginbottom[8] showed that the fungus *Scopulariopsis brevicaulus* could produce TMA from arsenite. Subsequent work[9,10] confirmed that other fungi, such as *Penicillium* sp., *Gliocladium reseum* and the yeast, *Candida humicola,* were also capable of arsenic biomethylation. Transformation

of arsenic compounds to less toxic forms can help remove toxic arsenic from the environment. To this end, Cerbon[11] reported that the yeast *Saccharomyces calsbergensis* was able to take up ^{74}As-arsenate and bind it exclusively to phosphatidylinositol, thus removing it from the environment.

In this paper, the results of isolating wild fungi from an arsenic-contaminated area in Ron Phibun District and screening them for their ability to efficiently remove arsenic from aqueous solution are reported.

Materials and Methods

Collection and isolation of fungi

Roots of widely dispersed plants, mine tailings and soil samples were collected from an arsenic-contaminated area in Ron Phibun District, Nakhon Si Thammarat province, Thailand. Samples were collected in both the summer and rainy seasons of 1998. The samples were stored in sterile plastic bags and transported to the laboratory in Bangkok within 12 hr. Approximately half of the samples were assigned to media containing arsenate; the rest to media with arsenite. The sodium salts of arsenate and arsenite were used throughout this study. Plant roots were cut into 1-2 mm pieces and laid on potato dextrose agar plates containing 20 μg l^{-1} chloramphenicol (PDAC) and 70 mg l^{-1} of either arsenate or arsenite. For soil and mine tailing samples, 1 g of sample was mixed with 10 ml of sterile distilled water and left for sedimentation. Then 0.1 ml of the supernatant solution was spread on PDAC plates containing 70 μg l^{-1} of either arsenate or arsenite. All plates were incubated at 27°C for 48-72 hour. Fungi growing on these plates were regarded as having at least a low level of arsenic tolerance and were restreaked on potato dextrose agar (PDA) plates for isolation of single colonies. All fungal isolates are currently maintained by P. Visoottiviseth as stock cultures on PDA slants and stored in a refrigerator. Cultures are subcultured every 3-6 months.

Screening for high arsenic tolerance

Preliminary research indicated an average arsenic concentration in soil of approximately 700 mg l^{-1}. The isolated fungi from the previous experiment were, therefore, screened for their abilities to tolerate this level of arsenic. All arsenate tolerant isolates were separately inoculated onto PDAC plates with 700 mg l^{-1} arsenate. The same procedure was followed for the arsenite tolerant isolates. There were 3 replicates per isolate. All plates were incubated at 27°C for 7 days. Isolates that did not grow were discarded whereas ones that grew were regarded as being tolerant to a high level of arsenic and were used for further testing.

Screening for efficient arsenic removal

The fungal isolates that were found to be tolerant of arsenic at 700 mg l^{-1} in the previous experiment were tested for their ability to remove arsenic from liquid medium. Two 8 mm disks were cut with a sterile cork borer from the margin of PDA plates and inoculated into 250 ml Erlenmeyer flasks containing 50 ml potato dextrose broth plus 20 μg l^{-1} chloramphenicol (PDBC) and 10 mg l^{-1} of either arsenate or arsenite. There were 3 replicates per isolate. Uninoculated flasks (no fungus) were used as controls. All flasks were incubated at 27°C on a rotary shaker set at 150 rpm. After 5 days of incubation the medium in each flask was filtered through a 0.45 mm millipore filter. Each filtrate of media with arsenite added was analyzed for arsenite level by hydride generation atomic absorption spectrophotometry (HG-AAS). The samples were digested with nitric acid. For determination of arsenate concentrations, KI (5% w/v) and ascorbic acid (5% w/v) were added to the sample to transform arsenate to arsenite. The arsenite level was then determined by HG-AAS. The arsenate concentration of the filtrate was assumed to equal the arsenite level determined by HG-AAS.

Selection of a fungal isolate for efficient removal of both arsenate and arsenite

The fungal isolates that removed the greatest amount of arsenate or arsenite were further tested to select the best one for removing both these arsenic compounds from liquid medium. To do this, the two most efficient isolates for arsenite removal from the previous experiment were inoculated (8 mm disk) into 50 ml PDBC with 10 mg l^{-1} arsenate. The two most efficient isolates for arsenate removal were treated in a similar manner but with arsenite added to PDBC. There were 3 replicates for each experiment. Controls consisted of flasks with either arsenite or arsenate but with no fungal inocula. 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 mm millipore filter. Filtrates were analyzed for arsenite and arsenate concentration by HG-AAS as described previously.

Identification of selected fungal isolate

Slides of hyphae, conidiophores, and conidia were prepared by wet mounting with lacto-fuchsin

and examined by viewing at 1000X magnification using a compound microscope. Size and color of fungal colonies on PDA were also recorded. The text of Samson and Reenen-Hoekstra[12] was used to identify the fungi.

Temperature and pH effects on arsenic removal

Environmental factors, ie, pH and temperature, were tested to observe their influence on arsenic removal by the selected fungi. The levels of pH and temperature used were those expected to be found in the field. PDBC with 10 mg l^{-1} of arsenate or arsenite was adjusted to pH 5, 7 or 9 with lactic acid and NaOH prior to inoculation with an 8 mm disk of the selected isolate. There were 4 sets of experiments as follows: (1) arsenate with pH 5, 7 and 9 at 27°C, (2) similar to (1) but at 37°C, (3) arsenite with pH 5, 7 and 9 at 27°C and (4) similar to (3) but at 37°C. The low and the high temperatures approximated those of shaded and sunny positions, respectively. There were 3 replicates for each experiment. All flasks were incubated for 5 days on a rotary shaker (150 rpm). After that, the culture media were separately filtered through 0.45 mm millipore filters. Filtrates and fungal biomass were analyzed for arsenite and arsenate by HG-AAS as described previously.

Effect of arsenic concentration on fungal growth

Inoculum (8 mm disk) from a 14-day-old culture of the selected isolate was inoculated on to the center of PDA plates containing different concentrations ie, 0, 10, 100, and 1,000 mg l^{-1}, of arsenate or arsenite. There were 3 replicates for each experiment. All plates were incubated at 27°C for 14 days. Colony diameter was measured every 2 days during the incubation period.

Effect of inoculum source on arsenic removal

Mycelium inoculum of the selected fungus was obtained from 3 sources ie, from a PDA plate (8 mm disk), from a 5 day old 50 ml PDB culture (fungal biomass), and from an autoclaved 5 day old 50 ml PDB culture (fungal biomass). The inoculum from each group was separately inoculated into 50 ml PDBC with 10 mg l^{-1} of either arsenate or arsenite. There were 3 replicates for each treatment. All flasks were incubated at 27°C on a rotary shaker at 150 rpm for 5 days. At the end of the experiment, 20 ml of the medium was withdrawn from each experimental flask. The samples were analyzed for arsenite and arsenate concentrations by HG-AAS as described previously. The biomass was separated from the remaining medium (30 ml) by filtration and washed three times with deionized distilled water. The three washing solutions of each biomass were mixed together and were analysed for arsenite or arsenate concentration by HG-AAS. The washed biomass was then dried for 3 nights at 80°C, digested with concentrated nitric acid, and analysed for arsenic concentration by graphite furnace atomic absorption spectrometry (GF-AAS).

Kinetics of arsenic removal

In this experiment, the effects of arsenic on fungal growth and media pH were monitored and removal and uptake of arsenic from the medium by fungal cells was measured.

Inoculum (8 mm disk) of the selected isolate was added to PDBC containing 10 mg l^{-1} arsenite. The fungus was similarly inoculated to PDBC but with 10 mg l^{-1} arsenate. Each arsenic treatment had 3 replicates. This assay had both positive controls (no arsenic but with fungal inoculum) and negative controls (with arsenic but no fungal inoculum). All flasks were incubated at 27°C on a rotary shaker set at 150 rpm. The pH was measured and a sample was taken from each flask at different exposure times: 0, 2, 4, 6, 8, 10 and 14 days. The samples were filtered through 0.45 mm filters. Filtrates were analyzed for arsenic concentration using HG-AAS. Biomass remaining on filters was dried for 3 days at 80°C, weighed and digested with concentrated nitric acid for determination of arsenic concentration by GF-AAS.

To determine if arsenate was transformed to arsenite by the fungus, the arsenite concentration was first measured in the media of treatments that initially contained only arsenate by HG-AAS. Potassium iodide and ascorbic acid were then added to the media to reduce any remaining arsenate to arsenite and the arsenite concentration was determined again by HG-AAS. The arsenate in the medium was then calculated as the difference in arsenite concentrations before and after KI/ascorbic acid reduction. For the treatments with arsenite-containing media, the filtrates were analyzed for arsenite only.

Results

Fungal isolates

Of the summer samples, 24 isolates could tolerate 70 mg l^{-1} arsenite and 24 could tolerate 70 mg l^{-1} arsenate. Of the rainy season isolates, 72 were arsenite-tolerant and 61 were arsenate-tolerant.

Based on morphological comparisons, fungal isolates from rainy season collections appeared to be more diverse than those from the summer.

High arsenic-tolerant isolates

Of the 24 summer isolates that tolerated arsenite at 70 mg l^{-1}, 22 also tolerated arsenite at 700 mg l^{-1}. Similarly 16 of the 24 summer isolates that tolerated arsenate at 70 mg l^{-1} could also tolerate the same compound at 700 mg l^{-1}. Similar results were obtained for the rainy season isolates with 64 of 72 and 53 of 61 isolates that were tolerant at 70 mg l^{-1} also being tolerant at 700 mg l^{-1}.

Arsenic removal by isolates

The 10 most efficient isolates for removing arsenic from liquid media are shown in Tables 1 and 2. The two isolates that removed the most arsenite (SUM-21I and RRMT2-40I) and the two that removed the most arsenate (RR8-18A and RRMT2-40I) were chosen for further tests to determine their ability to remove the alternate compound (Table 3).

Isolate RRMT2-40I appeared to be the most efficient of all isolates for removing both arsenite and arsenate from liquid medium (ie, approximately 30% of arsenite and 40% of arsenate removed from the liquid media during the 5 day incubation period).

Description and identification of the fungal isolate, RRMT2-40I

This isolate had septate hyphae and grew fast at 27° C, attaining a diameter of 5-6 cm on PDA within 7 days of incubation. The colony consisted of a velvety layer of conidiophores that were initially white but changed progressively to light green and then dark green or grey-green as incubation continued. The color change began at the center of the colony then spread outward. Conidiophores were single (mononematous) consisting of a single stipe terminating in phialides (monoverticillate). Conidiophores were hyaline and smooth-walled. Phialides were flask-shaped. Conidia were arranged in long chains, globose and rough walled. Based on microscopic morphology this isolate was a species of *Penicillium*.

Temperature and pH effects on arsenic removal

Isolate RRMT2-40I grew only at one of the two temperatures tested (ie 27°C). There was no growth at 37°C. Consequently, the concentration of arsenite/arsenate in liquid media (10 mg l^{-1}) at 37° C did not decrease over the 5 day incubation period at any pH value. At 27°C, the highest percent removal of arsenite by the isolate was found at pH 5.0, followed by pH 7.0 (Fig 1a). The lowest percent removal was obtained at pH 9.0. The percent removal of arsenate decreased with increasing pH in a similar manner with arsenite (Fig 1b). For both arsenite and arsenate treatments, percent removal by the selected strain at pH 5.0 was not significantly different to that at pH 7 (Figs 1a and 1b).

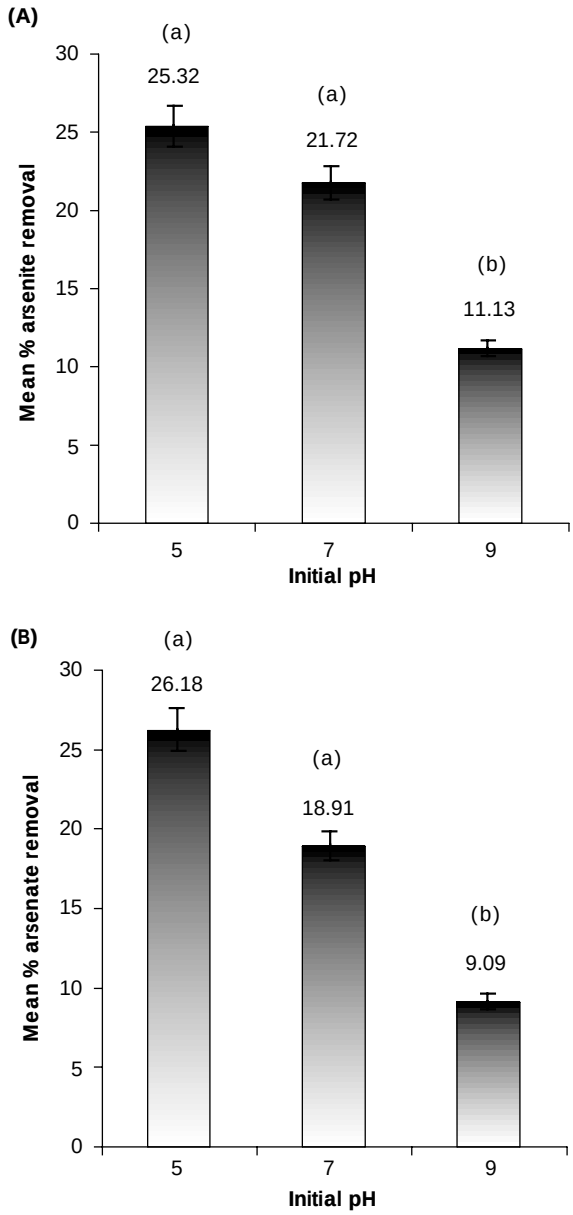

Fig 1. Arsenite (A) and arsenate (B) removal from liquid PDBC media at different initial pH values by the fungal isolate, RRMT2-40I, after 5 days incubation at 27°C. Bars indicate SD (three replicates). Columns with different letters are significantly different (t-test, (α= 0.05).

Table 1. Arsenite removed by the 10 most efficient isolates from PDBC media after 5 day of incubation at 27°C. The two isolates that removed the most arsenite are indicated in both italic and bold font.

Sample	Mean (±SD) concentration of arsenite in medium			
	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***
RR14-30I	9.20±0.18	7.41±0.83	1.79±0.83	19.46±9.06
RR15-36I	9.20±0.18	7.61±0.17	1.59±0.17	17.28±1.83
RRMT2-40I*	***9.20±0.18***	***6.33±1.00***	***2.87±1.00***	***31.27±10.87***
RY3-Y16I	9.20±0.18	8.19±0.24	1.01±0.24	11.02±2.56
RR3-8I	8.70±0.12	7.42±0.35	1.28±0.10	14.71±1.15
RR11-25I	8.70±0.12	6.58±0.33	2.12±0.33	24.37±3.81
RYMT1/1-Y1I	8.70±0.12	7.41±0.26	1.29±0.26	14.83±2.93
RYMT1/2-Y7I	8.70±0.12	7.64±0.39	1.06±0.39	12.18±4.51
RY2-Y15I	8.70±0.12	7.55±0.41	1.15±0.41	13.22±4.70

* *Italic* characters are strains selected for further experiment.

Table 2. Arsenate removed by the 10 most efficient isolates from PDBC media after 5 days of incubation at 27°C. The two isolates that removed the most arsenate are indicated in both italic and bold font.

Sample	Concentration of As(V) in medium			
	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
RR15-32A	8.53±0.10	7.26±0.76	1.27±0.76	14.91±8.93
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
RY1/3-Y7A	8.82±0.85	7.89±0.08	0.93±0.08	10.55±0.91
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***
RRMT2-35A*	***8.82±0.85***	***5.59±1.10***	***3.23±1.10***	***36.62±12.43***

* *Italic* characters are strains selected for further experiments.

Table 3. Arsenite and arsenate removed from PDBC media after 5 days of incubation at 27°C by the most efficient isolates from Tables 1 and 2.

Sample	Concentration of As(III) mg l^{-1}		Concentration of As(V) mg l^{-1}	
	Remaining	% removal	Remaining	% 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***

* *Italic* and bold numbers are results obtained in this experiment while the regular numbers were results taken from Tables 1 & 2.

Table 4. Arsenic removal and uptake from PDBC media of the fungus isolate, RRMT2-40I, by cells from PDA fungal colonies, cells from 5-d old PDB cultures, and autoclave-killed cells from 5 d old PDB cultures. The media were incubated at 27°C for 5 days.

	Mean (±SD) As conc. mg l^{-1} in the filtered medium			Mean (±SD) % As removal from the filtered medium	Mean As weight (±SD) µg in the filtered medium			
(I)	Control (II)	Treatment (III)	Removed (IV)	(V)	Removal * (VI)	In cells (VII)	In washing solution (VIII)	Lost # (IX)
PDA cultured cells								
- 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
- 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
Living 5-d old PDB cultured cells								
- 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
- 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
Autoclave-killed 5-d old PDB cultured 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				

* Calculated from column IV
\# Column IX = column VI - (column VII + column VIII)

By the fifth day of incubation at 27°C, the media containing arsenite and arsenate with an initial pH of 5.0 was more viscous than it was at the start of the experiment and the pH of the medium had decreased to ~ pH 3.0-3.8. Growth of the fungal isolate, based on the observed amount of fungal mycelia, was better at an initial pH of 5 than at other pH values. Our results, therefore, indicate that a pH of 5.0 and a temperature of 27°C was the most suitable for both fungal growth and arsenic removal.

Arsenic concentration effects on fungal growth

There was little effect of 10 mg l^{-1} and 100 mg l^{-1} concentrations of both arsenite and arsenate on fungal colony diameter when compared with that of the controls (Fig 2), but inhibition of growth occurred at the highest concentration of 1,000 mg l^{-1} for both arsenite and arsenate. This was most obvious at day 10 at which time radial colony diameters were approximately 86% those of the controls.

Inoculum source effects on arsenic removal

The percent removal of arsenic compounds from liquid medium was higher with agar culture inoculum than with liquid culture inoculum or with autoclaved inoculum (Table 4). Approximately 25% of arsenate and 22% of arsenite was removed from media inoculated with cells from PDA plate colonies. In contrast, living cells from PDB liquid culture removed approximately 15% and 14%, respectively, of arsenite and arsenate. For autoclaved cells, the percent arsenite and arsenate removal was very low at approximately 4% and 2%, respectively. Cells from the border of fungal colonies cultured on PDA plates were, therefore, used as inoculum in subsequent experiments.

Kinetics of arsenic removal by the fungus RRMT2-40I

Growth of RRMT2-40I was not markedly affected by 10 mg l^{-1} of either arsenite or arsenate during the first 6 days of incubation (Fig 3a, 4a). By the eighth day, however, the dry weight of the fungus was lower in arsenic-containing media than in control media and it remained lower until the end of the experiment. It is most likely that cell lysis occurred in the arsenic-treated media causing the observed differences between the treatments and controls. The pH of the medium decreased to pH 3.0-3.4 by the fourth day of incubation and remained steady until day eight in both arsenic-free and arsenic-containing media (Fig 3b, 4b). However, the pH of the arsenic-

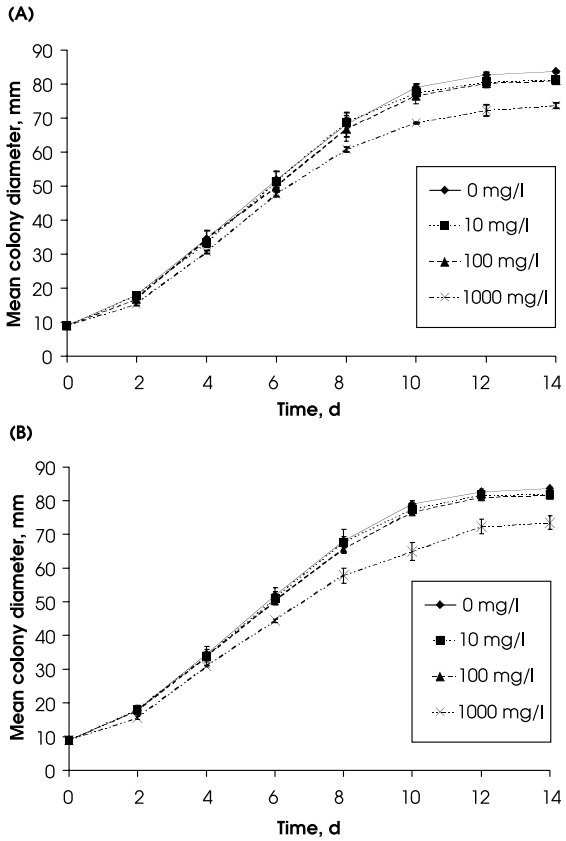

Fig 2. Growth of the fungus isolate RRMT2-40I at different concentrations of arsenite (A) and arsenate (B) on PDA plates over a 14 d incubation period at 27°C. Bars indicate SD (3 replications) and, when not shown, were smaller than the symbol.

Table 5. Reduction of arsenate to arsenite by the fungus RRMT2-40I after 14 days of incubation at 27°C in PDBC media containing 10 mg l^{-1} arsenate.

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 after reduction of arsenate to arsenite with KI & HCl and measured by HG-AAS.

** The amount of arsenite detected in the medium was measured by HG-AAS (no reduction reaction).

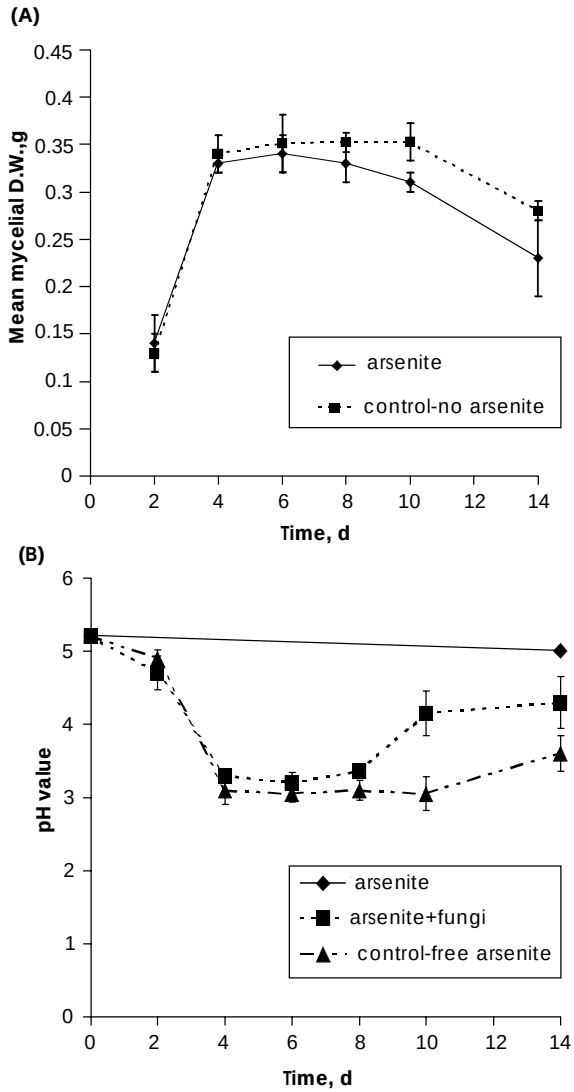

Fig 3. Change in mycelial dry weight (A) and pH (B) during growth of the selected fungus over a 14 d incubation period in PDBC media containing sodium arsenite and two PDBC control media (arsenite but no fungus, fungus but no arsenite). Cultures were incubated on a rotary shaker (150 rpm) at 27°C. Bars indicate SD (three replicates) and, when not shown, were smaller than the symbol.

containing media increased soon afterwards in contrast to the pH of the arsenic-free media. The increase of pH observed in the arsenic-containing media may have resulted from cell lysis and the consequent release of cell contents, which have a pH of about 7.0.

Arsenite accumulation in fungal cells increased sharply between the 2nd and 4th days of incubation, and reached a maximum by the 6th day (Fig 5a). The arsenite concentration in the cells then decreased in the days following incubation. A similar pattern of

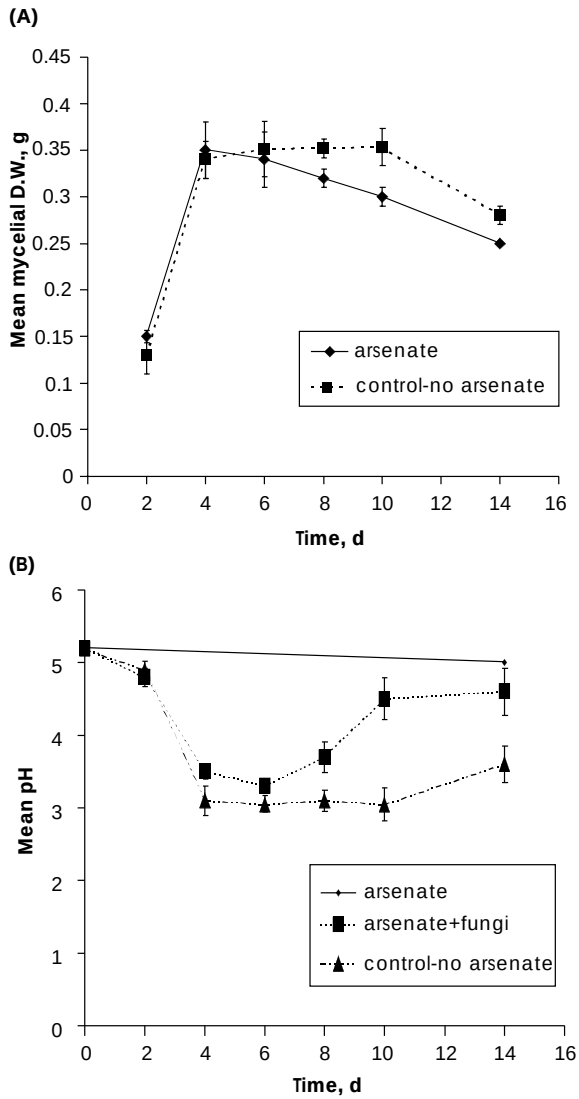

Fig 4. Change in mycelial dry weight (A) and pH (B) during growth of the selected fungus over a 14 d incubation period in PDBC media containing sodium arsenate. Two PDBC control media (arsenate but no fungus, fungus but no arsenate) were included in the experiment. Cultures were incubated on a rotary shaker (150 rpm) at 27°C. Bars indicate S.D. (three replicates) and, when not shown, were smaller than the symbol.

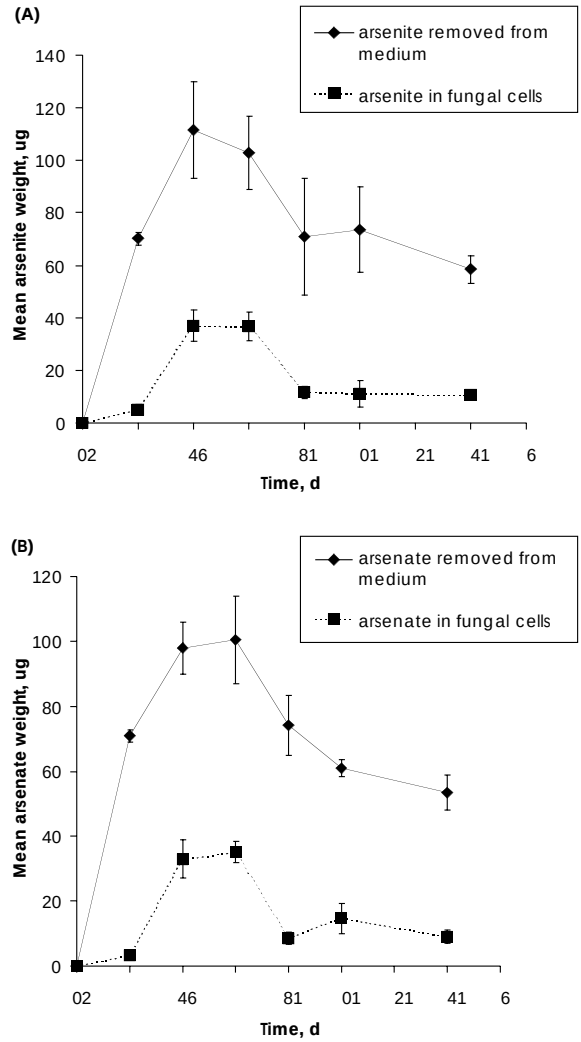

Fig 5. (A) Arsenite and (B) arsenate removal from PDBC medium and accumulation by mycelia of the fungal isolate RRMT2-40I over a 14 d incubation period at 27°C. Bars indicate SD (three replicates).

accumulation occurred for arsenate (Fig. 5b).

Table 5 shows the result of arsenate transformation by the fungus RRMT2-40I. After 2 days of incubation, about 15 % of arsenate was removed from the medium by the fungus. In addition, some arsenite (3 mg l^{-1}) could be detected in the medium that initially had only arsenate. This indicates that some arsenate was transformed to arsenite by the fungus. At day 4, 20.69 % of arsenate had been removed and the level of arsenite in the media had increased. At this time the concentration of arsenite that had been produced was equal to the amount of arsenate present in the medium. This indicates that all arsenate had completely transformed to arsenite by day 4. The results at day 6 were similar to those at

day 4 in that about 21% of arsenate was removed from the medium and almost all the arsenate left in the medium had been transformed to arsenite. By day 8, the concentrations of arsenate and arsenite in the medium had both increased, possibly due to the occurrence of cell lysis (Fig 4). Again all arsenate in the medium had transformed to arsenite. From day 8 until the end of the experiment (day 14), concentrations of arsenate in the medium leveled off, indicating that no more arsenic was released into the medium. In addition, during this period all arsenate in the medium was completely transformed into arsenite by the fungus.

Discussion

The soil isolate RRMT2-40I not only took up and accumulated arsenite and arsenate in its cells but it also transformed arsenate to arsenite, which is the most toxic form of arsenic. The mechanism as well as the resulting products of arsenic transformation by this fungus should be studied in detail.

To date, the most of the attention to biosorption of toxic compounds by fungi has been for remediation of polluted environments.[13-16] The processes contributing to fungal uptake and detoxification of toxic metals depend on an adequate energy source and are limited by toxicity thresholds. Once inside cells, metals may be preferentially stored in the cytosol in association with various metal binding polypeptides, eg, metallothioneins or phytochelatins, or they become localized in organelles, such as vacuoles.[6] Cox and Alexander[17] reported that the mechanism of arsenic uptake by the yeast *Candida humicola* was metabolism-linked and that arsenic competed with phosphate in the medium. Species of *Penicillium*, such as *P. chrysogenum, P. brevicaule* and *P. notatum*, were reported to have the ability to transform arsenic compounds, [18,19] but to the best of our knowledge, the uptake of arsenic compounds by *Penicillium* sp. has never been reported.

Our results on the kinetics of arsenic uptake correspond well with those of others. Maeda et al.[20,21] found no arsenic accumulation by dead cells of the algae, *Noctoc* sp. and *Chorella* sp., when the cells were killed with glutaraldehyde or ethanol. In another study, Maeda et al[22] found that cells of the bacteria, *Klebsiella oxytoca* and *Xanthomonas* sp., that had been killed by 70 % ethanol were unable to accumulate either arsenate or trimethyl arsenic from media.

Cell age has been proposed as an important factor limiting the ability of fungi to accumulate toxic metals. For example, Zajic and Chiu[23] found that *Penicillium* isolated from wastewater exhibited significant growth in media containing salts of uranium, platinum and titanium and that uranium uptake, in particular, was dependent on culture age. Five day-old cultures were twice as effective as 15-day-old cultures. The reasons for the change in toxic metal uptake pattern with culture age are not very clear. A possible explanation is that changes in cell surface chemistry and morphology with age contribute to lower uptake.[24,25] Similarly in this study we also observed that if cultures were stored in arsenate-free media for a long time, the efficiency of the fungus in removing arsenate from media was lower than when the original screening tests were performed.

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References

1. Williams M, Fordyce F, Paijitprapapong A and Charoenchaisri P (1996) Arsenic contamination in surface drainage and groundwater in part of the Southeast Asian tin belt, Nakhon Si Thummarat province, Southern Thailand, *Environ Geol* 27, 16-33.
2. Choprapawon C and Rodcline A (1997) Chronic arsenic poisoning in Ronpibool Nakhon Sri Thammarat, the southern province of Thailand. In: Arsenic: Exposure and health effects (Edited by Abernathy CO, Calderon RL and Chappel WR), pp 68-7. Chapman & Hall.
3. Pollution Control Department and Envisis Ltd Co (1998) Reports on survey and analysis of arsenic contamination at Ron Phibun District, Nakorn Si Thammarat province, for action plan. 10 March 1998.
4. Gadd GM (1993) Interactions of fungi with toxic metals. *New Phytol* 124, 25-60.
5. Gadd GM, Gharieb MM, Ramsay LM, Sayer JA, Whatley AR and White C (1993) Fungal Processes for Bioremediation of Toxic Metal and Radionuclide Pollution. *J Chem Technol Biotechnol* 71, 364-5.
6. Gadd GM and White C (1993) Microbial treatment of metal pollution -a working biotechnology? *Trends Biotechnol* 11, 353-9.
7. Burgstaller W, Strasser H, Wobking H and Schinner F (1992) Solubilization of zinc oxide from filter dust with *Penicillium simplicissimum*: bioreactor leaching and stoichiometry. *Environ Sci Technol* 26, 340-6.
8. Challenger J and Higginbottom C (1935) CCVIII. The production of trimethyl-arsine by *Penicillium brevicaule* (*Scopulariopsis brevicaulis*). *Biochem J* 29, 1758-78.
9. Cox, DP (1975) Microbilogical methylation of arsenic. In: Arsenic Pesticides. American Chemical Society (Edited by Woolson EA), pp 81-95.
10. Cullen WR and Reimer KJ (1989) Arsenic-speciation in the environment. *Chem Rev* 89, 713-63.

11. Cerbon J (1969) Arsenic-lipid complex formation during the active transport of arsenate in yeast. *J Bacteriol* **97**, 658-62.
12. Anonymous (1988) Identification of the comman food-borne fungi. In: Introduction to food-borne fungi (Edited by Samson RA and Reenen-Hoekstra ESV). pp 3-221. Centraalbureau voor Schimmelcultures.
13. Nakajima A and Sakaguchi T (1986) Selective accumulation of heavy metals by microorganisms. *Appl Microbiol Biotechnol* **24**, 59-64.
14. Tobin JM, White C and Gadd G.M (1994) Metal accumulation by fungi: applications in environmental biotechnology. *J Industrial Microbiol* **13**, 126-130.
15. Morley GF and Gadd GM (1995) Sorption of toxic metals by fungi and clay minerals. *Mycol Res* **99**, 1429-39.
16. Volesky B and May-Phillips HA (1995) Biosorption of heavy metals by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **42**, 797-806.
17. Cox DP and Alexander M (1973) Effect of phosphate and other anions on trimethylarsine formation by *Candida humicola*. *Appl Microbiol* **25**, 408-13.
18. Cullen WR and Reimer KJ (1989) Arsenic-speciation in the environment. *Chem Rev* **89**, 713-63.
19. Maeda S. (1994) Biotransformation of arsenic in the freshwater environment. In: Arsenic in environment part I: cycling and characterization (Edited by Nriagu JO), pp 155-87. John Wiley & Sons, New York.
20. Maeda S, Wada H, Kumeda K, Onoue M, Ohki A, Higashi S and Takeshito T (1987) Methylation of inorganic arsenic by arsenic-tolerant freshwater algae. *Appl Organomet Chem* **1**, 465-72.
21. Maeda S, Kusadome K, Arima H, Ohdi A, and Naka K (1992) Biomethylation of arsenic and its excretion by the alga *Chlorella vulgara, Appl Organmet Chem* **6**, 407-13.
22. Maeda S, Ohki A, Miyahara K, Naka K and Higashi S (1992) Metabolism of methylated Arsenic compounds by arsenic-resistant bacteria (*Klebsiella oxytoca* and *Xanthomones* sp.). *Appl Organomet Chem* **6**, 415-20.
23. Zajic JE and Chiu YS (1972) Recovery of heavy metals by microbes. *Dev Indust Microbiol* **13**, 91-100.
24. Kapoor A and Viraraghavan T (1995) Fungal biosorption-an alternative treatment option for heavy metal bearing wastewater: a review. *Bioresource Technol* **53**, 195-206.
25. Silver S and Nakahara H (1983) Bacterial resistance to arsenic compounds. In: Arsenic: Industrial (Edited by Lederer WW and Fensterheim RJ), pp 190-9. Van Nostrand Reinhold Co.