Applied Organometallic Chemistry (1988) 2 353–357

0268-2605/88/02410353/$03.50

Arsenic accumulation by arsenic-tolerant freshwater blue–green alga (*Phormidium* sp.)

Shigeru Maeda, Saori Fujita, Akira Ohki, Isami Yoshifuku,* Shiro Higashi† and Toshio Takeshita

Department of Applied Chemistry (*Department of Chemical Engineering), Faculty of Engineering, (†Department of Biology, Faculty of Science), Kagoshima University, Korimoto, Kagoshima 890, Japan

Received 9 March 1988 *Accepted 26 April 1988*

Accumulation, biomethylation and excretion of arsenic by the arsenic-tolerant freshwater blue–green alga, *Phormidium* sp., which had been isolated from an arsenic-polluted environment, were investigated.

The cellular growth curves were in fair agreement with a 'logistic curve' equation. The growth increased with an increase in the surrounding arsenic concentration up to 100 $\mu g\ g^{-1}$. The cells survived even at 7000 $\mu g\ g^{-1}$. The arsenic concentration of the cells increased with an increase of the surrounding arsenic concentration up to 7000 $\mu g\ g^{-1}$. Phosphorus concentrations in the medium affected the growth and arsenic accumulation. No arsenic was accumulated by cells killed by ethanol.

The arsenic was methylated to the extent of 3.2% of the total arsenic accumulated. When the cells were transferred into an arsenic-free medium, 85% of the arsenic accumulated was excreted; 58% of the excreted arsenic was in methylated form implying extensive methylation in the arsenic-free medium.

Keywords: Arsenic, freshwater algae, *Phormidium* sp., accumulation, methylation, excretion, logistic curve

INTRODUCTION

It is now known that arsenic compounds accumulated in marine organisms are harmless to higher animals and man, the reason for the harmlessness being that the arsenic is highly methylated. Such accumulation and methylation may be carried out most effectively by lower-trophic-level organisms in the marine ecosystem.[1]

Only a few other poisonous elements which are methylated have been found in the natural environment. In most cases the evidence for the methylation pathway or the relationship between the methylation and detoxification have not been fully clarified. The case of arsenic is the only one where the element is actually detoxified by methylation.[2]

In a freshwater ecosystem, the lower-trophic-level organisms might also be expected to have a similar ability to accumulate inorganic arsenic and transform it to harmless methylarsenic compounds. Few papers on the subect, however, have been published.

In previous papers the authors reported some freshwater algae which preferred freshwater containing a high level of arsenic and had a high resistance to arsenic.[1,3–5] One green alga, *Chlorella vulgaris* Beijerinck var. *vulgaris*,[1] and four blue–green algae,[5] *Nostoc* sp.,[4] *Phormidium* sp., *Hydrocoleum* sp., and *Microchaete* sp., were isolated. The present paper reports the experimental results for *Phormidium* sp. The effects of culture conditions on the growth and bioaccumulation of arsenic are discussed.

MATERIALS AND METHODS

Isolation of *Phormidium* sp.

Phormidium sp. was isolated by means of repeated inoculation of the arsenic-tolerant algae[1] on an agar medium containing 100 $\mu g\ g^{-1}$ of arsenic [as elemental arsenic, with appropriate concentration for NA_2HAsO_4, abbreviated as arsenic(V)] and identified by Professor Isamu Umezaki, Kyoto University, Japan.

The culture media used were Gerloff–Fitzgerald–Stoog (GFS) medium,[6] Modified Detmer medium,[1] MA medium,[4] and Modified Chu medium.[7]

The algae were selectively cultured by the following two methods, A and B.

Method A: The culture medium (300 cm^3) containing the algae in an Erlenmeyer flask (500 cm^3) was

aerated by germ-free moisture-saturated air and illuminated for 12 h under fluorescent light (2000–5000 lx) and in the dark for 12 h daily at room temperature (20–30°C).

Method B: The algae were inoculated at a cell concentration of 5 mg dry weight cells dm^{-3} of medium into the culture medium (100 cm^3) in an Erlenmeyer flask (300 cm^3) sealed with a cotton plug and the culture was shaken by a reciprocating shaker (100 strokes min^{-1}) under the same conditions as above.

The whole cells in a culture were harvested by centrifuging (4000 rpm, 15 min) at room temperature, washed twice with water and dewatered by vacuum-heating at room temperature; subsequently, the cells were dried at 105°C for 2 h. The growth of the cells was determined in terms of mg dry weight of cells per dm^3 of medium.

Total arsenic was determined by atomic absorption spectroscopy after ashing the cells in the presence of magnesium nitrate.[3] Methylated arsenic compounds were determined by the method described in the previous paper.[5]

RESULTS AND DISCUSSION

Effect of medium on growth and arsenic accumulation

Phormidium sp. was cultured in four different media containing 100 $\mu g\ g^{-1}$ of arsenic(V) (as Na_2HAsO_4) by method A at room temperature (20–25°C) for two weeks. The experimental results are shown in Table 1.

Phormidium sp. grew well in Modified Detmer and MA media, and arsenic accumulation was the greatest in MA medium (Table 1). MA medium was generally used for the experiments of *Phormidium* sp. reported in this paper.

Table 1 Growth and arsenic bioaccumulation of *Phormidium* sp. in various culture media

Medium[a]	Growth[b] (g dry cells dm^{-3} medium)	Arsenic in cells (μg As g^{-1} dry cells)
Modified Detmer	0.45	1100
MA	0.41	1700
Modified Chu	0.30	310
GFS	0.24	710

[a] containing 100 $\mu g\ g^{-1}$ of elemental arsenic (as Na_2HAsO_4).
[b] Method A, 4000 lx, 20–25°C, 2 weeks.

Growth of *Phormidium* sp.

Fifteen *Phormidium* sp. cells which had been precultured in an MA medium containing 6 $\mu g\ g^{-1}$ of arsenic(V) were inoculated in MA medium with and without 6 $\mu g\ g^{-1}$ arsenic(V), respectively. The cells were cultured by method B and both growth curves were determined at 15 different culture times.

Figure 1 shows the relation between algal growth y (g dry cell dm^{-3} medium) and culture time x (days) together with the theoretical curves (a, b), called the 'logistic curves', calculated from Eqn [1].[8]

$$y = \frac{CM}{1 + (M - 1)\exp(-Kx)} \qquad [1]$$

where C is the initial cell concentration (g dry cell dm^{-3}), M is the multiplication of the cells (the ratio of final cell concentration to initial cell concentration, which are both obtained directly from the experimental data), and K is the growth parameter. The latter parameter K was chosen so as to minimize the deviation between the observed data and the calculated values from Eqn [1].

It was found that algal growth data for arsenic-free (●) and arsenic-containing (○) curves were approximated well by the logistic curve, especially in the former case.

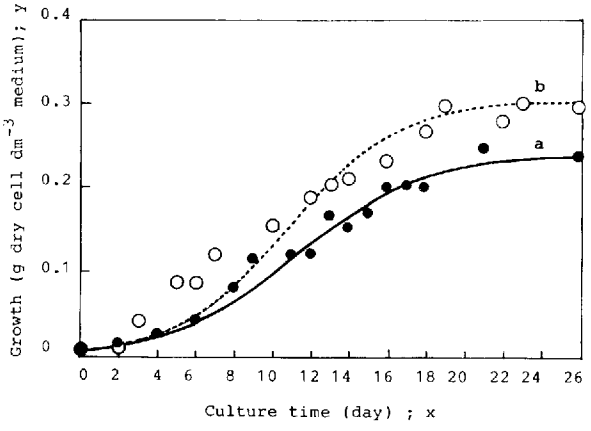

Figure 1 Growth of *Phormidium* sp. in MA medium. Conditions: shaking culture; medium, MA without (●) and containing (○) 6 $\mu g\ g^{-1}$ of arsenic(V). Curves a and b are calculated from a 'logistic curve' $\{y = CM/[1+(M-1)\exp(-Kx)]\}$ for both experiments. Initial algal concentration, C, is 7 mg cells dm^{-3} in dry base. Parameters M, K for curves a and b were 34, 0.31 and 43, 0.35, respectively.

Figure 1 shows that the growth parameter K and the multiplication M of curve b are larger than those of curve a, and these results mean that the growth of *Phormidium* sp. was better in the arsenic-containing medium, as compared with an arsenic-free medium.

Effect of arsenic concentration in the medium on the growth and arsenic accumulation by the alga

Phormidium sp. was cultered in media containing various concentrations of arsenic. The growth and arsenic accumulation were determined in two different media (Table 2).

Table 2 shows that there was a maximal algal growth at 100 μg g^{-1} of arsenic(V) in both culture media with the cells surviving even at 7000 μg g^{-1} of arsenic(V) in the MA medium, and that arsenic accumulation increased with increasing arsenic(V) in both culture media.

Similar results were reported in a previous paper:[3] the growth of *Chlorella vulgaris* increased with an increase in arsenic(V) concentration in the medium up to 2000 μg g^{-1}, with the cells surviving even at 10 000 μg g^{-1}, and arsenic accumulation also rose steadily with an increase in the arsenic level in the medium. The arsenic-tolerance behavior of *Phormidium* sp. and *C. vulgaris* are in striking contrast to the copper-tolerant behavior of *C. vulgaris*. In the latter case, the copper-tolerant cell excluded copper and did not accumulate it.[9] One reason why the algae had a tolerance for higher arsenic levels seems to be that the algae have a function to prevent inorganic arsenic from reacting with the —SH group of an enzyme, thus maintaining the activity of the enzyme, even if inorganic arsenic had entered the cell through the cell membrane. Biomethylation of arsenic by the cell is thought to be one detoxification process for inorganic arsenic in the interior of the cell, as described in the previous paper.[2,5]

Effect of phosphorus on growth and arsenic accumulation by *Phormidium* sp.

MA medium contains 10 μg g^{-1} of elemental phosphorus in the chemical form of β-glycerophosphoric acid monosodium salt ($(HOCH_2)_2CHOPO_3H^-Na^+$). In order to demonstrate the competition between arsenic acid and phosphoric acid for arsenic accumulation, *Phormidium* sp., was cultured in MA medium containing various levels of phosphorus and 100 μg g^{-1} of arsenic, and growth and arsenic accumulation were measured (Table 3).

Table 3 Competition between arsenic acid and phosphoric acid for arsenic bioaccumulation by *Phormidium* sp.[a]

Phosphorus in medium[b] (μg g^{-1})	Growth[c] (g dry cells dm^{-3} medium)	Arsenic in cell (μg as g^{-1} dry cells)
0	0	—
1	0.38	140
10	0.74	1300
100	0.80	470
500	0.56	120

[a] Na_2HAsO_4 and β-glycerophosphoric acid sodium salt.
[b] MA medium containing 100 μg g^{-1} of arsenic(V)
[c] Method B, r.t. (25–30°C), 3500 lx, 2 weeks culture

It was found that growth and arsenic accumulation had peaks at 100 and 10 μg g^{-1} of phosphorus concentrations respectively. This result suggests that

Table 2 Effect of arsenic concentration in the medium on the growth and arsenic accumulation by *Phormidium* sp.

As(V) concn[a] in medium (μg g^{-1})	MA medium[b]		Modified Detmer medium[c]	
	Growth (g dry cells dm^{-3} medium)	Arsenic accumulation (μg As g^{-1} dry cells)	Growth (g dry cells dm^{-3} medium)	Arsenic accumulation (μg As g^{-1} dry cells)
1	0.36	43	0.17	61
10	0.44	160	0.19	230
100	0.74	1300	0.33	440
500	0.72	—[d]	0.27	1200
1000	0.49	1800	0.26	1500
7000	0.24	2800	—	—

[a] As Na_2HAsO_4. [b] Cultured at 25–30°C, [c] Cultured at 20–25°C, [d] Not determined.

arsenic accumulation was inhibited to some degree by phosphorus at phosphorus levels higher than 100 $\mu g\ g^{-1}$.

Methylation and excretion of arsenic by *Phormidium* sp.

Phormidium sp. was inoculated and cultured in MA medium containing 128 $\mu g\ g^{-1}$ of arsenic(V) by method B at room temperature (25–30°C) for 24 days. A portion of the harvested cells was analyzed for total arsenic and for methylated arsenic compounds, and the remainder was transferred into arsenic-free MA medium at the cell concentration of 0.6 g dry cells dm^{-3} and incubated by method B at room temperature (25–30°C) for seven days. The harvested cells and the culture fluid were analyzed for arsenic concentration. The results are summarized in Table 4.

The predominant chemical form of accumulated arsenic from the cell was found to be arsine (AsH_3) after treatment of the cell with 2 mol dm^{-3} NaOH (95°C, 3 h) following hydrogenation. A small percentage (3.2%) of the accumulated arsenic was found to be in methylated form. The non-methylated arsenic detected as arsine is unlikely to be present as free inorganic arsenic compounds in the interior of the cell, as described in the previous paper.[5] In fact, the arsenic is likely to be bound with methylene groups or multidentate thiol compounds of the cellular components.

Table 4 shows that the arsenic concentration in the algal cell decreased from 440 to 68 $\mu g\ g^{-1}$ after the cells were transferred to the arsenic-free medium. This rapid change of arsenic concentration in cells under two different medium conditions means that the cell rapidly excreted arsenic into the arsenic-free medium. The decrease in the amount of arsenic in the cell after excretion, 223 μg, approximately coincided with the amount of the arsenic excreted into the medium, 210 μg.

Table 4 also shows that the excreted arsenic consists of 58% MMA and 42% non-methylated forms. This result means that extensive methylation of arsenic in the cells proceeded during the incubation in the arsenic-free medium, excreting the methylated arsenic as the main component.

Arsenic accumulation by an ethanol-treated *Phormidium* sp. cell

Phormidium sp. which had been killed with 70% ethanol was incubated in an MA medium containing 1.3 $\mu g\ g^{-1}$ of arsenic(V), but no arsenic was detected in the cells. This experimental result shows that arsenic accumulation by *Phormidium* sp. was not caused by a physicochemical adsorption on the cell surface, but that accumulation occurs only under vital conditions in a similar manner to that reported in previous papers for *Chlorella vulgaris*[3] and *Nostoc* sp.[4]

The accumulation mechanism of arsenic by the algal cell is quite different from that for cadmium[10] or uranium[11] by *Chlorella regularis*. These metals were adsorbed by heat-killed *Chlorella* cell surfaces to a greater degree than by living cells, and these accumulations were suggested as not being directly mediated by any metabolic process, but almost completely depended on physicochemical adsoprtion on the cell compounds.

Table 4 Methylation and excretion of arsenic by *Phormidium* sp.

	Before excretion		After excretion	
	Cell (0.6 g)	Medium[b] (1000 cm^3)	Cell (0.6 g)	Medium[b] (1000 cm^3)
Total arsenic				
($\mu g\ g^{-1}$)	440	0	68	0.21
(μg)	264	0	41	210
Non-methylated ($\mu g\ g^{-1}$)	430 (96.8%)	—	—	0.087 (42%)
Methylated arsenic[a] ($\mu g\ g^{-1}$)				
MMA	11 (2.5%)	—	—	0.12 (58%)
DMA	1.6 (0.4%)	—	—	trace
TMA	1.3 (0.3%)	—	—	trace

[a] MMA, monomethylarsenic; DMA, dimethylarsenic; TMA, trimethylarsenic. [b] Incubated by method A in MA medium without arsenic at room temperature (25–30°C) for 7 days.

REFERENCES

1. Maeda, S, Kumamoto, T, Yonemoto, M, Nakajima, S and Takeshita, T *Sep. Sci. Technol.*, 1983, 18:375
2. Maeda, S and Sakaguchi, T Accumulation and detoxification of poisonous metallic elements by algae. In *An Introduction To Applied Phycology*, Akazuka, I (ed), SPB Science Publishing, Amsterdam, in press
3. Maeda, S, Nakashima, S, Takeshita, T and Higashi, S *Sep. Sci. Technol.*, 1985, 20:153
4. Maeda, S, Kumeda, K, Maeda, M, Higashi, S and Takeshita, T *Appl. Organomet. Chem.*, 1987, 1:363
5. Maeda, S, Wada, H, Kumeda, K, Onoue, M, Ohki, A, Higashi, S and Takeshita, T *Appl. Organomet. Chem.*, 1987, 1:465
6. Gerloff, G C, Fitzgerald, G P and Skoog, F *Am. J. Bot.*, 1950, 37:216
7. Eyster, G, Brown, T E, Tanner, H A and Hood, S L *Plant Phycol.*, 1958, 33:235
8. Yamane, T *Bioreaction Engineering*, Sangyo Tosho Publishing, Tokyo, 1982, p 158
9. Foster, P L *Nature (London)*, 1977, 269:322
10. Sakaguchi, T, Tsuji, T, Nakajima, A and Horikoshi, T *Eur. J. Appl. Microbiol.*, 1979, 8:207
11. Horikoshi, T, Nakajima, A and Sakaguchi, T *Agric. Biol. Chem.*, 1979, 43:617