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Bioaccumulation of arsenic by freshwater algae (*Nostoc* sp.) and the application to the removal of inorganic arsenic from an aqueous phase

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An arsenic-resistant blue–green alga, *Nostoc* sp., was screened from an arsenic-polluted environment. The effects of the culture conditions on the growth and the arsenic bioaccumulation were investigated. In five culture media tested, *Microcystis aeruginosa* medium was found to be optimum for the growth. The effects of the concentration of five nutrients (P, Co, Fe, Mo and N) in the MA medium on arsenic bioaccumulation by the *Nostoc* sp. were also investigated. From the experimental results, the authors proposed a new culture medium which was designed for effective arsenic bioaccumulation by the *Nostoc* sp. The new medium was named the Modified MA medium (abbreviated as MMA).

Removal of arsenic from an aqueous phase by means of arsenic bioaccumulation by the *Nostoc* sp. was investigated. When arsenic-polluted water was enriched with the nutrients of MMA, the arsenic level was found to be effectively lowered by the *Nostoc* sp. to 0.05 ppm.

Keywords: Arsenic, freshwater algae, *Nostoc* sp., bioaccumulation, arsenic removal, effluents, organoarsenic

INTRODUCTION

It is well known that marine organisms generally contain arsenic at very high levels and that the arsenic is in highly alkylated forms harmless to man.[1] It has also been reported that lower members of trophic levels in the marine ecosystem, such as algae, accumulate and alkylate arsenic more efficiently than the higher members.[2–5] There are, however, few papers on the experimental bioaccumulation of arsenic by terrestrial organisms.

The authors screened and isolated several arsenic-resistant freshwater algae from microorganisms which had been sampled at the following arsenic-polluted sites: (1) at a geothermal electric power plant; (2) in certain old heavy-metal mines and (3) at smelters.[6]

In a previous paper it was reported that isolated *Chlorella vulgaris* grew better in a medium containing levels of arsenic up to 2000 $\mu g\,g^{-1}$ and accumulated arsenic at levels of up to 50 000 μg As/g dry cell.[7]

The present paper reports the experimental results for the freshwater alga *Nostoc* sp. The effects of culturing conditions on growth and the bioaccumulation of arsenic, and the application to the removal of inorganic arsenic from arsenic-polluted waters are discussed.

Methylation of arsenic by the algae was observed, but it will be reported in a forthcoming paper which is in preparation.

EXPERIMENTAL

Isolation of arsenic-resistant algae (*Nostoc* sp.)

Some arsenic-resistant algae containing screened green algae and blue–green algae[6] were inoculated into nitrogen-free GFS (Gerloff–Fitzgerald–Stoog) medium[8] with $CaCl_2$ being substituted for $Ca(NO_3)_2$ in the medium. The GFS also contained 10 $\mu g\,g^{-1}$ of arsenic as Na_2HAsO_4 [abbreviated as As(V)]. A part of the suspended culture solution at the stationary phase was inoculated into fresh nitrogen-free

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GFS medium containing successively higher levels of arsenic (up to 200 $\mu g\,g^{-1}$) for further selective screening of arsenic-resistant blue–green algae. Pure *Nostoc* sp. was then finally isolated by means of an agar plate culture which had been prepared with agar powder (2% v/v) and nitrogen-free GFS culture medium containing 100 $\mu g\,g^{-1}$ of arsenic. The reason for using a nitrogen-free medium was that *Nostoc* sp. grows well only in the absence of nitrogen because of its nitrogen-fixing ability. The *Nostoc* sp. was identified by Professor Isamu Umezaki, Kyoto University, Japan.

Pure culture of *Nostoc* sp.

The pure culture was obtained by the two methods shown below.

Method A

Nostoc sp. was cultured by bubbling bacteria-free air saturated with moisture through the *Microcystis aeruginosa* (MA) medium shown in Table 3 with illumination under fluorescent light (7000 lx, 12 h day^{-1}).

Method B

Nostoc sp. which had been precultured in arsenic-free MA medium was inoculated into a pH 7 buffer solution (0.6 g dry cell/dm^3 medium) containing about 2 $\mu g\,g^{-1}$ of As(V) and incubated under germ-free conditions by shaking (100 times per minute) in darkness for 15 days. The cells grew little under these conditions.

Determination of arsenic concentration in algal cells

Algal cells were collected by centrifuging (3000 *g*, 15 min), and were washed twice with water by repeating the centrifuge procedure. The dry cells were ashed with 50% $Mg(NO_3)_2$ (2 cm^3) at 550°C for 6 h. The ash was dissolved with 10 mol dm^{-3} HCl (10 cm^3) added to 40% KI (1 cm^3), the solution was extracted twice with $CHCl_3$ (5 cm^3) and the $CHCl_3$ phase was then back-extracted with water (2 cm^3). The water phase was analysed for arsenic by the diethyldithiocarbamate (DDTC) method (JIS-K-0102, JIS-K-0202): the ashed arsenic was hydrogenated by zinc and HCl, the arsine generated was absorbed in DDTC solution and this solution was determined colorimetrically.

RESULTS AND DISCUSSION

Effects of culture medium and culture conditions on the growth of *Nostoc* sp.

Medium

Five media (GFS, MA Modified Detmer,[9] Allison,[10] and Modified Chu[11]) were tested for the growth of *Nostoc* sp. MA culture medium was selected from the standpoint that *Nostoc* cells grew best in MA media at several temperatures (20°, 30° and 40°C) compared with the other media.

Temperature, illumination intensity and time, and arsenic oxidation state

The following conclusions emerged from the experiments using the medium: (a) culture at 30°C was optimum in the range 20–40°C; (b) growth was greater at 3500 lx than at 900 lx, but nearly equal to that at 7000 lx, and increased with an increase in illumination time; (c) there was little difference in both growth rate and cell concentration between arsenic in oxidation states As(V) and As(III) in the arsenic concentration range 1–10 $\mu g\,g^{-1}$.

Effects of culture conditions on the bioaccumulation of arsenic by *Nostoc* sp.

Arsenic(V) level in medium

The cells (5 mg as dry base) were inoculated into MA medium (300 cm^3) containing variable levels of As(V) and cultured at room temperature for one month by Method A. As shown by the experimental results in Table 1, growth was unaffected by arsenic impact up to an arsenic level of 10 $\mu g\,g^{-1}$. The growth was reduced at arsenic levels higher than 100 $\mu g\,g^{-1}$, and the cell did not survive at 5000 $\mu g\,g^{-1}$. *Chlorella vulgaris* screened by the authors was more resistant to arsenic than the *Nostoc* sp., i.e. the growth of *Chlorolla* increased with an increase in arsenic level up to 2000 $\mu g\,g^{-1}$ and the cell survived at 10 000 $\mu g\,g^{-1}$.[7] The cell concentration of *Nostoc*, however, was higher than that of *Chlorella*, especially at lower arsenic levels.

Table 1 also shows arsenic bioaccumulation by *Nostoc*; it is represented in two different ways. In column A, 'As concentration' means the arsenic concentration in the cell as dry base (μg As/g dry cells). In column B, 'Total As' means the total

Table 1 Effect of arsenic level of culture medium on arsenic bioaccumulation by *Nostoc* sp.

Arsenic level in medium ($\mu g\,g^{-1}$)	Cell growth in MA (g dry cell/dm^3 medium)	Arsenic bioaccumulation	
		(A) As concentration in cell (μg As/g dry cell)	(B) Total As (μg As/dm^3 medium)
0	1.1	0	0
1	1.2	32	38
10	1.1	77	85
100	0.6	1300	780
1000	0.4	3400	1360

Initial cell concentration: 0.005 g dry cell/dm^3 medium.
Culture conditions: Method A at 23°C for 32 days.

amount of arsenic taken up by the cell from a unit volume of medium; i.e. the Total As (μg As/dm^3 medium) is obtained from the As concentration (μg As/g dry cell) multiplied by cell-growth (g dry cell/dm^3 medium). Both As concentration and Total As in the case of *Nostoc* increased with an increase in arsenic level in a similar manner to the case of *Chlorella*.[7]

Figure 1 shows the growth curves and Figure 2 shows arsenic bioaccumulation (As concentration and Total As) at arsenic levels of 1 and 10 $\mu g\,g^{-1}$. As shown in Figure 1, no lag phase was observed and the cell concentration reached about 1 g dry cell/dm^3 medium after 3 weeks' culture in both growth curves.

As shown in Figure 2, the As concentration in the cell was highest at the log phase and then decreased linearly with culture time in the case of an arsenic level of 1 $\mu g\,g^{-1}$. On the other hand, the curve of As concentration at a level of 10 $\mu g\,g^{-1}$ As varied in a different manner from that in the former case. Both Total As plots, however, showed similar curves to each other, i.e. overall uptake of arsenic by the cells completed before the stationary phase in the growth curves was similar.

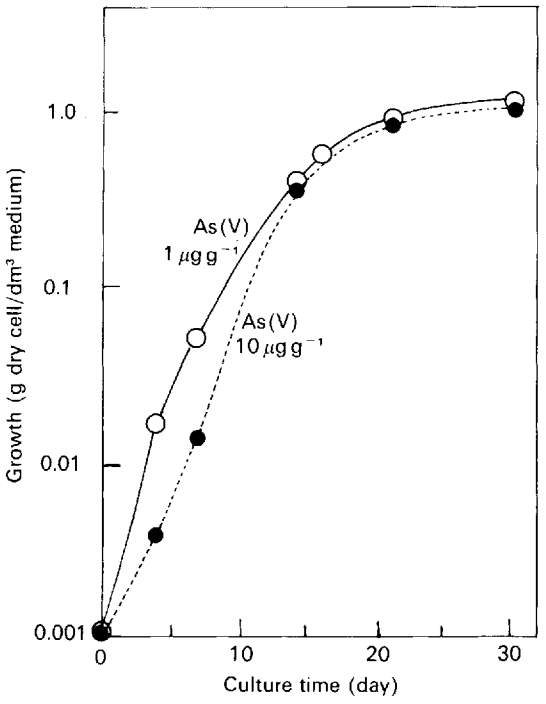

Figure 1 Growth curves of *Nostoc* sp. in MA medium containing 1 and 10 $\mu g\,g^{-1}$ of arsenate. Conditions: Bubbling culture; illumination 7000 lx, 12 h day^{-1}. Medium: MA medium; pH 8; 30°C. Initial cell concentration: 1 mg dry cell/dm^3 medium.

pH of medium and culture temperature

The effects on arsenic bioaccumulation of media pH and culture temperature were investigated by Method B. The media used were the buffer solutions glycine–NaCl (HCl or NaOH) and Bicine–H_3BO_3, respectively [Bicine is *N*,*N*-bis(2-hydroxyethyl)glycine]. The experimental results are shown in Figures 3 and 4, from which the optimum pH and temperature were found to be 8.4 and 23°C, respectively. The optimum temperature slightly deviated from the optimum temperature for growth (30°C).

Phosphorous(V), cobalt(II), iron(III), molybdenum(VI) and nitrogen(V) concentrations in media

The effects on arsenic bioaccumulation of levels

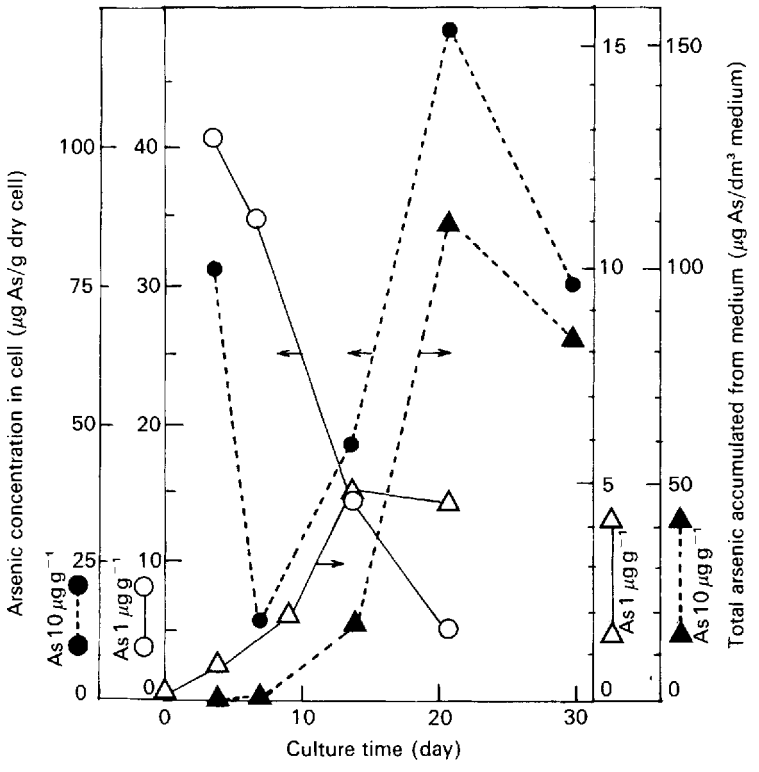

Figure 2 Arsenic bioaccumulation by *Nostoc* sp. in MA medium containing 1 and 10 $\mu g\,g^{-1}$ of arsenate. Conditions: Bubbling culture; illumination 7000 lx, 12 h day^{-1}. Medium: MA medium, pH 8; 30°C. Initial cell concentration: 1 mg dry cell/dm^3 medium.

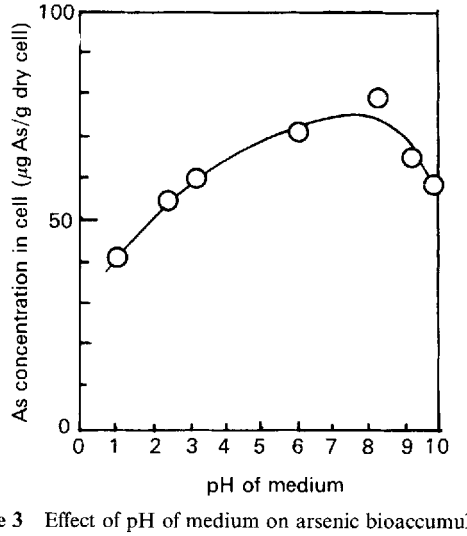

Figure 3 Effect of pH of medium on arsenic bioaccumulation. Conditions: Shake culture; in dark; 20–23°C; 15 days. Medium: pH buffer (glycine + NaCl) system. Initial As(V) concentration: 2.0 $\mu g\,g^{-1}$.

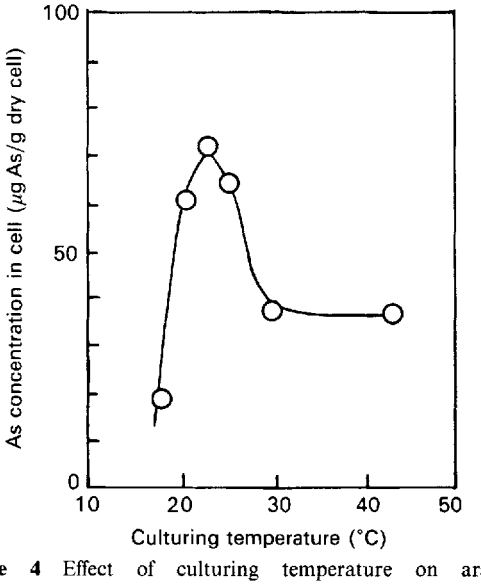

Figure 4 Effect of culturing temperature on arsenic bioaccumulation. Conditions: Shake culture; in dark; 15 days. Medium: pH buffer (Bicine + H_3BO_3) system; pH 7. Initial As(V) concentration: 2.0 $\mu g\,g^{-1}$.

of the above five elements in media were investigated by Method B. The experimental results are shown in Table 2 together with the pH buffer media used in culture temperatures.

(a) Phosphorus There was found to be an optimum phosphorus concentration for arsenic bioaccumulation at about $10\,\mu g\,g^{-1}$. The effect of the concentration, however, was not so significant that a competition phenomenon between phosphate and arsenate on the uptake could not be affirmed.

(b) Cobalt It was found from Table 2 that arsenic bioaccumulation increased with an increase in cobalt concentration up to $50\,\mu g\,g^{-1}$. Cobalt is a constituent of methylcobalamin (CH_3B_{12}) which has been proposed as a methyl-donor to arsenate by McBride.[12]

The above experimental results imply that methylation of arsenic caused by methylcobalamin or the enzyme system catalysed by cobalt participates in the arsenic bioaccumulation.

(c) Iron Iron oxide was precipitated in the preparation of a medium containing $100\,\mu g\,g^{-1}$ of iron, but the precipitate disappeared after a 15-day incubation. No adsorption of iron oxide on the algal cell was observed on cleaning the algal surface and microscopic observation. The disappearance of iron oxide precipitate, therefore, was due to uptake of iron by the algae. The results in Table 2 show that arsenic bioaccumulation increased with an increase in iron concentration up to $100\,\mu g\,g^{-1}$. This implies that arsenic accumulation was catalyzed by an enzyme which was stimulated by iron uptake, or which contained iron as an essential element (e.g. nitrogenase).

(d) Molybdenum It is seen from Table 2 that the existence of molybdenum at a level of $0.5\,\mu g\,g^{-1}$ resulted in a positive arsenic accumulation. Molybdenum is an essential element for blue–green algae such as *Nostoc* sp. and is also essential in nitrogen-fixation ability. The above experimental result suggested that arsenic bioaccumulation was related to a nitrogen-fixing reaction system.

(e) Nitrogen It is seen from Table 2 that arsenic bioaccumulation was at its maximum in the absence of nitrogen and decreased with an

Table 2 Effects of concentrations of five nutrients on arsenic bioaccumulation

Nutrient elemental level ($\mu g\,g^{-1}$)	Arsenic accumulated by *Nostoc* sp. (μg As/g dry cell)				
	Disodium β-glycerophosphate	$CoCl_2.6H_2O$	$FeCl_3.6H_2O$	$Na_2MoO_4.2H_2O$	KNO_3
	Buffer A[a] 18°C	Buffer A 21°C	Buffer B[b] 16–19°C	Buffer B 16–19°C	Buffer B 23°C
0	9	61	2.8	2.8	14
0.1	9	n.d.[c]	n.d.[d]	n.d.	n.d.
0.4	n.d.	80	n.d.	n.d.[d]	n.d.
0.5	n.d.	n.d.	4.2	7.2	n.d.
1	10	n.d.[d]	6.0	n.d.	n.d.
5	11	110	11	5.6	9.4
10	13[d]	n.d.	18	5.4	8.4
50	n.d.	510	430	4.1	7.9[d]
100	10	28	720	n.d.	7.1
500	7	7	n.d.	n.d.	5.5
1000	8	n.d.	n.d.	n.d.	n.d.
5000	7	n.d.	n.d.	n.d.	n.d.

[a]Buffer A: (Bicine + H_3BO_3) system.
[b]Buffer B: ($KH_2PO_4 + Na_2HPO_4$) system.
[c]n.d., not determined.
[d]Elemental level in MA medium.

increase in nitrogen concentration. *Nostoc* sp. possesses a nitrogenase in its heterocyst. The nitrogenase has been reported to be biosynthesized and activated only in the absence of nitrogen compounds in the culture medium.[13] These findings and the experimental results shown in Table 2 suggest that arsenic accumulation was stimulated by the biosynthesis and activation of the nitrogenase.

The above results on cobalt, iron, molybdenum and nitrogen lead to the hypothesis that arsenic bioaccumulation by *Nostoc* sp. is closely related to the enzyme systems participating in a nitrogenase.

Application of arsenic bioaccumulation to the removal of inorganic arsenic compounds from an aqueous phase

Removal of arsenic from MA medium

Five arsenic-free *Nostoc* cell samples were incubated in five Erlenmeyer flasks at 20–30°C for five different incubation times by Method B in MA medium containing 2.4 $\mu g\,g^{-1}$ of As(V). In each case the cells after incubation were separated by centrifuging (5000 *g*, 15 min), washed twice with water by repeating the centrifuge procedure and the resulting three supernatants were combined and analysed for arsenic concentration. The experimental results in Figure 5 show that the arsenic concentration in the medium decreased rapidly in the earlier stages but more slowly with increase in incubation time, reaching 1.1 $\mu g\,g^{-1}$ (about half the initial concentration) after 20 days of incubation. About 90% removal of the arsenic from an aqueous phase containing 2 $\mu g\,g^{-1}$ of arsenic was expected when the cell concentration was its maximum, i.e. 1.0 g dry cell/dm^3 medium.

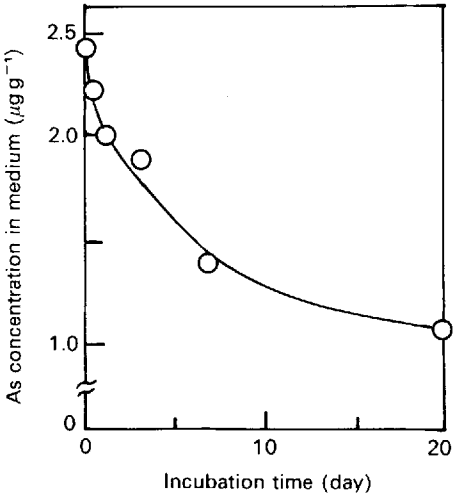

Figure 5 Removal of arsenic from MA medium. Conditions: Shake culture; in dark; 20–23°C. Medium: MA medium; pH 7. Cell concentration: 0.6 g dry cell/dm^3 medium.

Removal of arsenic from a Modified MA medium and an arsenic-polluted subterranean hot water enriched with nutrients of the Modified MA medium

From the experimental results mentioned in the section concerned with the effects of culture conditions (above) we proposed a new culture medium designed for effective arsenic bioaccumulation by *Nostoc* sp. The new culture medium was designated Modified MA (abbreviated as MMA); it is shown in Table 3 together with MA medium.

From the results shown in Table 2, the elemental concentration of phosphorus, cobalt, molybdenum and nitrogen in the medium were modified to 10, 50, 0.5 and 0 $\mu g\,g^{-1}$, respectively. A higher iron concentration would be better for arsenic bioaccumulation but a lower value (10 $\mu g\,g^{-1}$), however, was selected because waste iron concentration is legally controlled below 10 $\mu g\,g^{-1}$ in Japan. The other nutrients are the same as those of MA.

The authors attempted to apply the bioaccumulation of arsenic by *Nostoc* sp. to the removal of arsenic from an arsenic-polluted natural water by use of the MMA medium. Ohtake subterranean hot water (abbreviated as OH in Table 4) is a waste water from the Ohtake geothermal electric power plant (12.5 MW) located at Aso National Park, Ohita, Kyushu Island, and contains 2.6 $\mu g\,g^{-1}$ of arsenic. Ohtake subterranean hot water was enriched with nutrients of MMA (abbreviated as OH-MMA). Arsenic removal by *Nostoc* sp. from OH-MMA was compared with that from OH, MMA, MA, and MA-enriched OH(OH-MA).

Nostoc cells which had been precultured in arsenic-free MA medium by Method A were inoculated into the above medium at a cell concentration of 1.0 g dry cell/dm^3 medium and incubated at room temperature (about 23°C) for 15 days by Method B. The arsenic concentration in each medium after the incubation was determined by the same method as described before.

Table 3 Proposed modified MA (MMA) medium for effective arsenic bioaccumulation by *Nostoc* sp.

Nutrient	Proposed MMA medium		MA medium (pH 8.6)
	Nutrient content ($mg\,dm^{-3}$)	Element content ($mg\,dm^{-3}$)	Nutrient content ($mg\,dm^{-3}$)
$Ca(NO_3)_2$	0	0	50
KNO_3	0	0	100
$NaNO_3$	0	0	50
Na_2EDTA	5	0.5	5
Bicine	500	43	500
Disodium β-glycerophosphate	100	10	100
$CoCl_2.6H_2O$	250	62	5
$FeCl_3.6H_2O$	50	10	0.5
$Na_2MoO_4.2H_2O$	1.2	0.5	0.8
$MgCl_2.6H_2O$	50	0.6	50
$MnCl_2.4H_2O$	5	1.0	5
$ZnCl_2$	0.5	0.2	0.5
H_3BO_3	20	3.5	20
Na_2SO_4	40	–	40

The experimental results are summarized in Table 4.

As can be seen from Table 4, 95% of the arsenic was removed from the MA medium. The arsenic level was further reduced under to $0.01\,\mu g\,g^{-1}$ by use of the MMA medium. The aim of the modification, to promote arsenic bioaccumulation, was clearly achieved.

From pure Ohtake subterranean hot water (OH), only 20% of the arsenic was removed. When OH was enriched with the nutrients of MA and MMA, arsenic removal was elevated to 44% and 98%, respectively. It was found from these results that enrichment by the nutrients was extremely effective for the promotion of removal of arsenic from an aqueous phase by the algal bioaccumulation.

Table 4 Removal of arsenic from arsenic-polluted hot water by means of arsenic bioaccumulation by *Nostoc* sp. by use of Modified MA (MMA) nutrients

Arsenic-polluted water[a]	As(V) concentration $\mu g\,g^{-1}$	
	Initial	After incubation[b]
OH-MMA	2.5	0.05
OH	2.5	2.0
OH-MA	2.5	1.4
MMA	2.0	0.01
MA	2.0	0.1

[a]OH, Ohtake subterranean hot water; OH-MMA, OH enriched with nutrients of MMA medium; OH-MA, OH enriched with nutrients of MA medium.

[b]For 3 days.

CONCLUSION

The *Nostoc* sp. which had been screened from an arsenic-polluted environment was less resistant to arsenic than *Chlorella vulgaris* described in the previous paper;[7] arsenic bioaccumulation, however, was greater in the medium at lower arsenic levels than that for *C. vulgaris*. From investigations on the culture conditions for *Nostoc* sp. for application of its arsenic bioaccumulation properties to removal of arsenic from arsenic-polluted water, the optimum culture conditions under conditions of darkness were found to be 23°C and pH 7–8, with optimum nutrient concentrations as follows: P, $10\,\mu g\,g^{-1}$; Co, $50\,\mu g\,g^{-1}$; Mo, $0.5\,\mu g\,g^{-1}$; N, $0\,\mu g\,g^{-1}$; Fe $> 100\,\mu g\,g^{-1}$.

By use of the Modified MA medium (MMA) proposed from the experimental results and listed in Table 3, arsenic levels in an aqueous phase were lowered to under $0.01\,\mu g\,g^{-1}$. Arsenic in subterranean hot water enriched with the nutrients of MMA was removed nearly completely by the *Nostoc* sp.

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