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Removal of mercuric chloride by immobilized cells of genetically modified *Pseudomonas putida* PpY101/pSR134

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Removal of mercuric chloride was examined by immobilized cells of genetically modified *Pseudomonas putida* PpY101/pSR134 which was endowed with mercury volatilizing activity. The immobilized cells on calcium alginate exhibited the highest mercury volatilization activity in various carriers. Immobilized cells have highly stability of the mercury removal activity against high temperatures and storage than free cells, and maintained mercury removal activity after four cycles of removal the experiments. The experiment using the mercury removal-reactor system demonstrated that about only 80–85% of the added mercury was recovered in the Hg-trapping solution while about 95% of added mercury was removed from reaction mixture. Electron micrographs of the immobilized beads and EDS analyses showed that part of the volatilized mercury was entrapped in gel matrix of immobilized beads.

Key words: mercury, immobilized bacteria, bioremediation

Introduction

Mercury is known to be one of the most toxic metals. Many areas in the world are contaminated by small-scale gold mining and industrial use of mercury, that constituting serious environmental problems[9,13,17]. Removal of mercury from industrial wastewater has been achieved by means of ion-exchange resins or other chemical processes. However, such chemical processes are generally costly and sensitive to environmental conditions, and they require enormous quantities of chemicals. Therefore, new cost-effective, non-sensitive and sustainable technologies for the removal of mercury are needed.

There is currently great interest in bioremediation, a new technology that is known to be cost-effective and clean. Several studies on processes for biological mercury removal mainly investigated sorption[11], accumulation[3,4] and reduction[1,5,14]. In particular, mercuric reduction processes are more efficient because that can transform highly toxic water-soluble ionic mercury to insoluble metallic mercury. It is important that active bacterial cells are maintained at high concentration during mercuric reduction processes. The immobilization of microbial cells stabilizes enzymatic activity and prevents bacterial cell loss, enabling continuous operation. In present study, we examined the removal of mercuric chloride by immobilized cells of mercury-volatilizing bacterium, *Pseudomonas putida* PpY101/pSR134. The mercury removal activity of bacterial cells immobilized on various carriers was investigated. The effects of high temperature, storage and the repeated removal on the stability of mercury removal activity were investigated. Furthermore, we examined the removal of mercuric chloride using a mercury removal-recovery system for the purpose of continuous treatment of wastewater containing mercuric compounds.

Materials and Methods

Microorganism

A genetically engineered mercury-volatilizing bacterium, *P. putida* PpY101/pSR134[6] was used throughout this study. This strain can grow and volatilize mercuric ion to elemental mercury at high concentrations of mercuric chloride[10]. Plasmid pSR134 (18.6 kb) was constructed by inserting two *Eco*RI DNA fragments (4.2 kb and 4.9 kb) encoding the mercury-resistant gene from the *NR1* plasmid, into a broad-host-range vector pSUP104 (9.5 kb) (Fig. 1). Stock culture of *P. putida* PpY101/pSR134 at −80°C was suspended in 50 mM phosphate buffer (pH 7.0) and thawed at 30°C. Cells were washed by centrifugation (6000×*g*, 4°C, 10 min) and suspended in 10 mM phosphate buffer (pH 7.0).

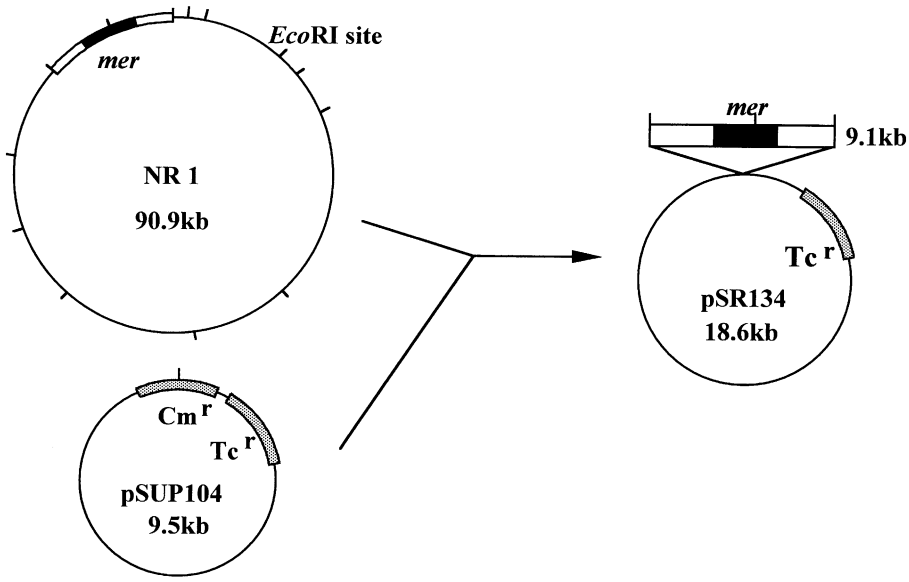

Fig. 1. Construction of plasmid pSR134. *mer*: mercury-resistant gene.

Immobilization of bacterial cells

The cells were immobilized by entrapping in calcium alginate, strontium alginate, agar, or a photo-cross-linkable resin prepolymer. For immobilization on calcium alginate and strontium alginate, 100 ml of cell suspension ($O.D._{600nm}=20$) and 100 ml of 4% sodium alginate were mixed. The mixture was ejected dropwise into either 100 mM calcium chloride or 100 mM strontium chloride at 4°C. To immobilize the cells on the photo-cross-linkable resin prepolymer, 10 ml of 3% sodium alginate, 50 g of photo-crosslinkable resin prepolymer (SPP-HH-13 (BIO), Toyo Gosei Co., Ltd., Chiba, Japan) and 12.5 ml of cell suspension ($O.D._{600nm}=58$) were mixed. The mixture was ejected dropwise into 100 mM calcium chloride at 4°C, and beads were solidified by irradiation. For immobilization on agar beads, 100 ml of 3.3% agar and 10 ml of cell suspension ($O.D._{600nm}=110$) were mixed, and the mixture was ejected dropwise into soy bean oil at 4°C. All beads were washed with distilled water and sieved to be 2–5 mm in diameter.

Mercuric chloride removal assay using serum bottles

Either 1 g of immobilized cells or 1 ml of cell suspension was added to a 69 ml serum bottle with 9 ml of distilled water containing mercuric chloride and sodium thioglycolate. Each bottle was sealed with a Teflon-coated butyl rubber cap and an aluminum ring and incubated at 30°C for 2 hrs on a shaking table (140 rpm). Final concentrations of bacterial cells, sodium thioglycolate and mercuric chloride were 0.5 dry weight g l^{-1}, 1 mM and 5–100 mg l^{-1}, respectively. The Vmax and Km values were determined from Hanes-Woolf plot. The thermal stability of mercury removal activity were examined by calcium alginate, strontium alginate immobilized cells and free cells thermal treated at 40, 50 and 60°C for 20 min. The storage stability was studied by calcium alginate, strontium alginate immobilized cells and free cells stored at 4°C for 14 days in 100 mM calcium chloride, 100 mM strontium chloride and 50 mM sodium phosphate buffer, respectively. Repeated removal of mercury by immobilized cells was examined as follows. Removal of mercury by calcium alginate, strontium alginate immobilized cells and free cells were carried out for 24 hrs. After removal experiments, immobilized cells were collected and washed by distilled water, and free cells were collected by centrifugation ($6000\times g$, 4°C, 10 min) and suspended in 10 mM phosphate buffer (pH 7.0). Removal of mercury by these cells was carried out five times repeatedly. In thermal stability, storage stability and repeated removal experiments, final concentrations of the bacterial cells, sodium thioglycolate and mercuric chloride were 0.5 dry weight g l^{-1}, 1 mM and 10 mg l^{-1}, respectively

Mercuric chloride removal assay using a mercury removal-recovery system

A mercury removal-recovery system was constructed to collect volatilized mercury in the mercury-trapping solution (Fig. 2). This system consisted of six of 500 ml flasks with a working volume of 200 ml. The mercury-trapping solution was 0.25% $KMnO_4$ in 1 M H_2SO_4. After setting flasks with 180 ml of distilled water containing mercuric chloride on the system, 20 g of immobilized cells and 0.2 ml of 1 M sodium thioglycolate were added to the flasks. The flasks were incubated at 30°C for 6 hrs with constant agitation (120 rpm). Final concentrations of cells, sodium thioglycolate and mercuric chloride were 0.5 dry weight g l^{-1}, 1 mM and 10 mg l^{-1}, respectively.

Determination of mercury concentration

The mercury concentrations in the serum bottles, the flasks and mercury-trapping solution were determined with

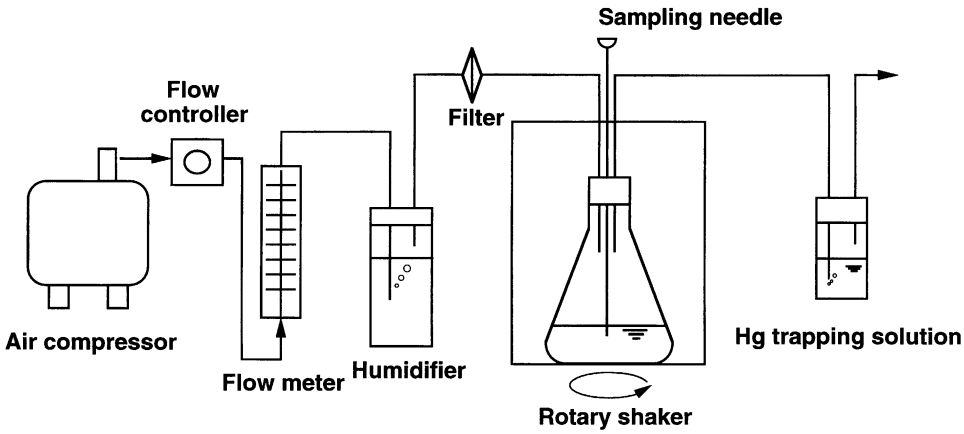

Fig. 2. Schematic flow diagram of a mercury removal-recovery reactor system. This system consisted of 500-ml flask with a working volume of 200 ml. The aeration rate was maintained at 400 ml min^{-1}.

a mercury analyzer (Model 330, Sugiyamagen Co., Ltd., Tokyo, Japan) applying cold vapor atomic absorption photometry. Samples from the serum bottles and the flasks were centrifuged (6000 × *g*, 4°C, 10 min) to remove cells, and the supernatants were used to determine the residual mercury concentration. The mercury-trapping solution was treated with 4.5 ml of 5% $KMnO_4$ between 90 and 95°C for 2 hrs, and 20% $NH_2OH \cdot HCl$ was added after cooling before analysis.

SEM and EDS analysis

Immobilized beads were fixed with 2.5% glutaraldehyde and dehydrated with a graded ethanol series (30%, 50%, 75%, 95% and 100%). Fixed immobilized beads were freeze-dried overnight by a cold trap (Unitrap YT50, Tokyo Rikakiki, Co., Ltd., Tokyo, Japan). Samples were coated with gold by using a model an ion sputtering (JFC-1100, JEOL Ltd., Tokyo, Japan). Electron micrographs were taken using JSM-5800LV (JEOL Ltd. , Tokyo, Japan) operating at 10 kV. Elemental analyses were accomplished using Oxford-Link Isis EDS (Oxford Instruments, Munich, Germany).

Results

Removal of mercuric chloride by various immobilized cells

Figure 3 shows the removal of mercuric chloride (5–100 mg l^{-1}) by various immobilized cells. The calcium alginate immobilized cells and free cells exhibited the almost same mercury removal rate at 5 and 10 mg l^{-1} of mercuric chloride. The free cells removed almost of 20 mg l^{-1} of mercuric chloride for 1 hr, while about 75% and 40% of added mercury were removed by calcium alginate and strontium alginate immobilized cells. Mercury removal rates of the cells immobilized by agar and the photo-cross-linkable resin prepolymer were lower than those of calcium alginate and strontium alginate immobilized cells. The free cells and immobilized cells could remove mercury up to 100 mg l^{-1}. There was no removal or sorption of mercury by the gel beads without cells (data not shown).

Table 1. Maximun mercury removal rates (Vmax) and Km values of various types of immobilized cells.

Samples	Vmax (Hg-mg dry weight cell-$g^{-1}h^{-1}$)	Km (mg^{-1})
Ca-alginate	30.3	20.6
Sr-alginate	29.0	27.2
Photo-crosslinkable resin	27.7	44.8
Agar	20.8	32.4
Free cells	51.8	15.7

The maximum mercury removal rates and Km values of various immobilized cells were shown in Table 1. Immobilization on calcium alginate demonstrated the most effective removal rate in all the carriers. However, the mercury removal rates and the affinity for mercury of free cells were higher than those of all immobilized cells. These results indicated that immobilization decreased the mercury removal rate and affinity for mercury of this mercury-volatilizing bacterium.

Stability of mercury removal activity

Table 2 shows the thermal stability of mercury removal activity of immobilized cells. The immobilized cells and free cells were exposed to 40, 50 and 60°C for 20 min before

Table 2. Thermal stability of mercury removal activity.

Samples	Mercury removal activity (%)[1]		
	40°C[2]	50°C	60°C
Ca-alginate	100.1	41.5	23.0
Sr-alginate	92.8	42.0	32.3
Free cells	46.3	21.2	5.5

[1] The 100% values of mercury removal activity are the amount of removed mercury by cells which were not heat treated.
[2] Immobilized cells and free cells were heat-treated for 20 min. Data were averages based on duplicate analyses.

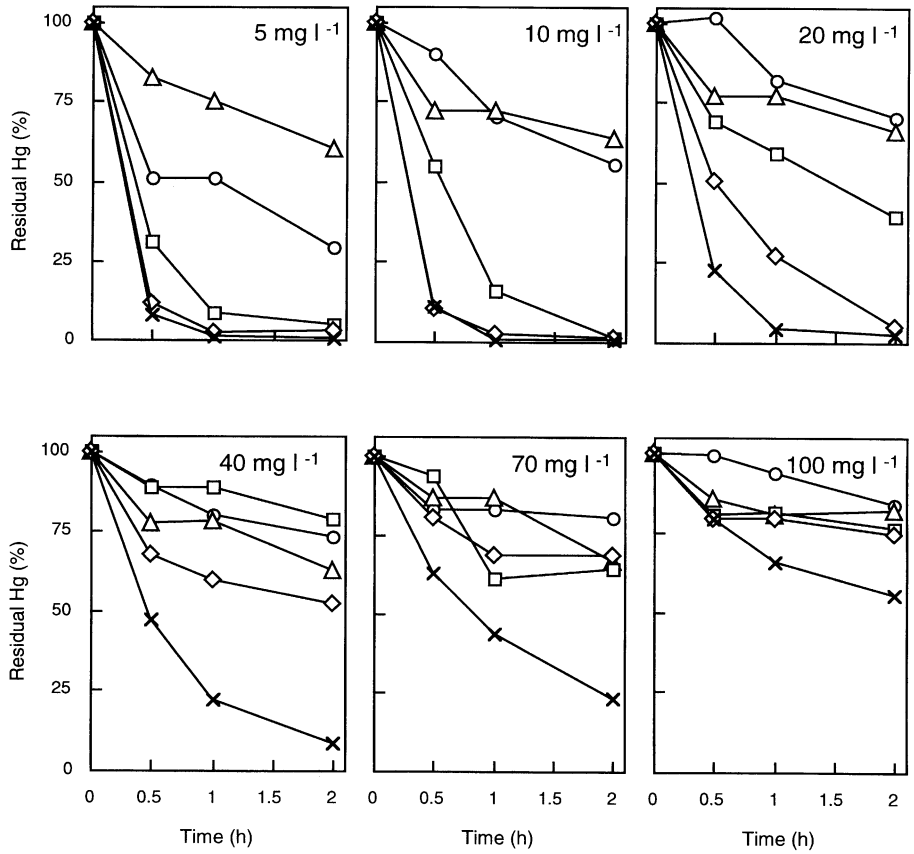

Fig. 3. Time course of removal of mercuric chloride by various immobilized cells. The mercuric chloride concentrations were 5, 10, 20, 40, 70 and 100 mg l^{-1}. Immobilized cells on Ca-alginate (◇), Sr-alginate (□), agar (○) photo-crosslinkable resin prepolymer (△) and free cells (×) were used. Data are averages based on duplicate analyses.

Table 3. Storage stability of mercury removal activity.

Samples	Mercury removal activity (%)[1]
Ca-alginate	97.7
Sr-alginate	93.9
Free cells	78.5

[1] The 100% values of mercury removal activity are the amount of removed mercury by cells which were not stored. Data were averages based on duplicate analyses.

mercury removal assay. The mercury removal activities of calcium alginate and strontium alginate immobilized cells did not decrease at 40°C, in contrast free cells lost about 54% of mercury removal activity. Furthermore, immobilized cells retained higher mercury removal activity than free cells treated at 50°C and 60°C.

Table 3 shows that storage stability of mercury removal activity. Removal of mercury was carried out by free cells and by calcium alginate and strontium alginate immobilized cells that were stored at 4°C for 14 days. Free cells lost about 22% of mercury removal activity after 14 days. In contrast, calcium alginate and strontium alginate immobilized cells lost only 2.3% and 6% of mercury removal activity, respectively.

Furthermore, repeated removal of mercury by immobilized cells were examined (Table 4). All cells removed almost maintained about 100% of mercury removal activity at the second time. Free cells lost about 92% of mercury

Table 4. Repeated removal of mercury by immobilized cells.

Samples	Mercury removal activity (%)[1]					
	1st	2nd	3rd	4th	5th	6th
Ca-alginate	100.0	100.4 (0.1)[2]	99.7 (0.2)	90.6 (3.6)	78.5 (0.4)	5.0 (2.3)
Sr-alginate	100.0	99.8 (0.1)	98.6 (0.2)	93.0 (1.2)	74.5 (2.6)	5.8 (2.6)
Free cells	100.0	100.1 (0.5)	87.6 (4.3)	7.5 (7.5)	4.1 (2.1)	3.5 (2.9)

[1] The 100% values of mercury removal activity are the amount of removed mercury by each cell at first time.
[2] Parenthesis are standard errors.
Data were averages based on triplicate experiments.

removal activity at the fourth time. In contrast, calcium alginate and strontium alginate immobilized cells lost only 9.4% and 7.0% of mercury removal activity, respectively at the fourth time. Furthermore, immobilized cells maintained about 74–78% of mercury removal activity at the fifth time. Amounts of total removed mercury per cells during six times of repeated operation by calcium alginate immobilized cells, strontium alginate immobilized cells and free cells were 93.7, 93.4 and 60.0 Hg-mg dry weight cell-g^{-1}, respectively.

Removal of mercuric chloride by immobilized cells using a mercury removal-recovery system

Removal of mercuric chloride by calcium alginate and strontium alginate immobilized cells was examined using a mercury removal-recovery system can collect bacterial volatilized-mercury. Free cells removed 20 mg l^{-1} of mercuric chloride after 2 hrs (Fig. 4). Calcium alginate and strontium alginate immobilized cells removed all mercuric chloride after 6 hrs, however, there was no mercury removal in the control flask without cells.

Almost all of the added mercury was recovered in the mercury-trapping solution by free cells (Table 5). Although about 95% of the added mercury was removed from the flasks, only approximately 85% and 80% of the added mercury were recovered by calcium alginate and strontium alginate immobilized cells, respectively.

Therefore, electron micrographs of the calcium alginate-immobilized beads after the mercury removal experiment were taken. Figure 5 shows that bacterial cells were clearly visible in the matrix of the calcium alginate. A droplet formed in the beads and elemental analysis of this droplet by the EDS revealed that it was mercury (Fig. 5, insert). It was found that part of the mercury volatilized by bacteria was accumulated in gel matrix of immobilized beads.

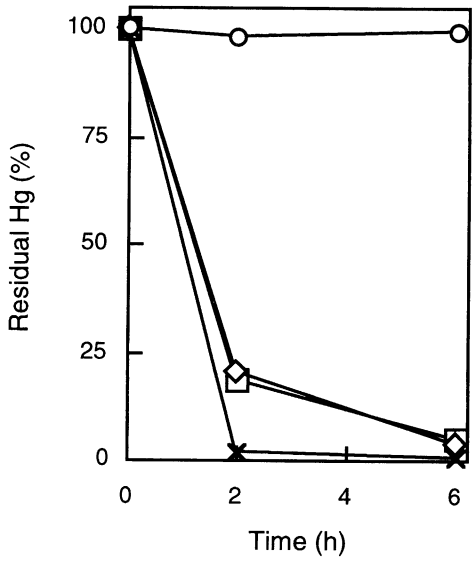

Fig. 4. Time course of removal of mercuric chloride by immobilized cells using the mercury removal-recovery system. Without cell (○), free cells (×), immobilized cells on Ca-alginate (◇) and Sr-alginate (□) were used. The data are averages based on duplicate experiments. Standard errors are within each symbol.

Table 5. Distribution of mercury after mercury removal experiment.

Samples	Amount of mercury (%)[1]	
	Residual in flask[2]	Collected in Hg trap[3]
Ca-alginate	3.9 (1.2)[4]	85.4 (0.2)
Sr-alginate	4.7 (2.4)	80.4 (8.8)
Free cells	1.5 (0.4)	97.3 (0.4)
Without cells	98.3 (1.4)	N.D.

[1] Amount of added mercury was 100%.
[2] The percentage of residual mercury in the flask.
[3] The percentage of collected mercury in Hg trapping solution.
[4] Parenthesis are standard errors.
Data were averages based on duplicate experiments.
N.D.=not detected

Discussion

We examined the removal of mercuric chloride by immobilized cells of genetically modified mercury-volatilizing bacterium, *P. putida* PpY101/pSR134. Immobilization of microbial cells has many advantages including the stabilization of enzymatic activities, prevention of bacterial cell loss and reuse of the bacterial cells. Furthermore, these advantages enable long-term continuous operation. Our study shows that cells of a mercury-volatilizing bacterium immobilized on various carriers are sufficiently able to remove mercuric chloride from wastewater (Fig. 3).

The mercury removal rates of immobilized cells were less than that of free cells (Table 1). A concentration gradient generally occurred between inside and outside of the immobilized beads[12]. It seems that the mercury removal rate of immobilized cells decreased due to limited diffusion of mercuric ions to the inside of immobilized beads. Different immobilizing carriers demonstrated different mercury removal rates. This result is probably due to inhibition of bacterial activity by immobilizing operations and properties of immobilizing carriers.

Several studies on immobilized cells have demonstrated that different immobilizing carriers have various advantages advantageous characteristics. Strontium alginate-immobilized beads are stronger than the calcium alginate immobilized cell[15]. Cells immobilized on agar were not affected by bivalent cations and pH[8]. In the present study, cells immobilized on calcium alginate had higher mercury removal activity than those immobilized on other carriers (Table 1). Immobilizing carriers should thus be selected in view of the properties (e.g., pH; concentration of phosphate, sodium and mercury) of the wastewater being treated.

The thermal and storage stability of mercury removal

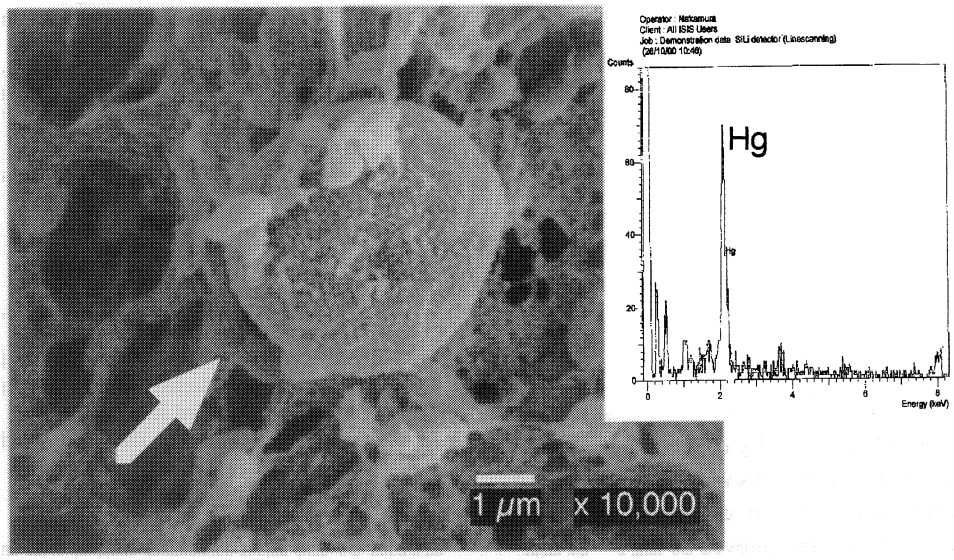

Fig. 5. A SEM micrograph of a Ca-alginate immobilized bead used for mercury removal experiments. A droplet (indicated by the arrow) was formed. (Inset) EDS spectrum obtained from an immobilized bead. Large mercury peaks were generated.

activity was elevated by immobilization of cells (Table 2, 3). Immobilized cells maintained highly mercury removal activity than free cells did after five times repeated removal (Table 4). Immobilizing bacterial cells protects cells against physical damage[7] and prevents interfacial inactivation[2]. Present study shows that the bacterial viability and enzymatic activity appeared be stabilized by immobilization, and immobilized cells removed 1.5 times as much mercury as free cells did in the repeated removal experiment. Such characteristics of immobilized cells would be advantages to long-term operation of biological mercury removal.

Canstein et al.[16] reported the continuous removal of mercury by mercury-reducing biofilms and demonstrated that almost all of removed mercury was retained within the bioreactor. On the other hand, almost all of the mercury was volatilized from the bioreactor and collected in the trapping solution in our present study (Table 5). Accumulation of volatilized-mercury within the bioreactor is undesirable for continuous mercury removal processes. Therefore, the mercury removal-recovery system (Fig. 2) in present study may be more effective for continuous removal of mercury.

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