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RESEARCH ARTICLE

Mercury removal and recovery by immobilized *Bacillus megaterium* MB1

Meifang CHIEN, Ryo NAKAHATA, Tetsuya ONO, Keisuke MIYAUCHI, Ginro ENDO (✉)

Faculty of Engineering, Tohoku-Gakuin University, Miyagi 985-8537, Japan

Abstract From several mercury removing microorganisms, we selected *Bacillus megaterium* MB1, which is non-pathogenic, broad-spectrum mercury resistant, mercuric ion reducing, heat tolerant, and spore-forming, as a useful bacterium for bioremediation of mercury pollution. In this study, mercury removal performance of the immobilized *B. megaterium* MB1 was investigated to develop safe, efficient and stable catalytic bio-agent for mercury bioremediation. The results showed that the alginate gel immobilized *B. megaterium* MB1 cells efficiently removed 80% of mercury from the solution containing 10 mg/L mercuric chloride within 24 h. These cells still had high activity of mercury removal even after mercuric ion loading was repeated for nine times. The analysis of mercury contents of the alginate beads with and without immobilized *B. megaterium* MB1 suggested that a large portion of reduced metallic mercury was trapped in the gel beads. It was concluded that the alginate gel immobilized *B. megaterium* MB1 cells have potential to remove and recover mercury from mercury-containing water.

Keywords mercury removal, immobilized bacteria, alginate gel, bioremediation

1 Introduction

Mercury has been used for a variety of purposes such as catalysts of chemical production, fluorescence lamps, thermometers, pesticides, and dental amalgams. However, mercury is harzardous and toxic, and tends to accumulate in the ecosystem through its food chain. Due to the extensive industrial use of mercury in the world, mercury contamination has caused a significant environmental problem. For example, methylmercury chloride is one of the most well known mercury compounds and is identified as a causative agent of the Minamata disease [1]. Therefore, it is needed to develop the efficient and safe remediation technology for mercury pollutions.

Some microorganisms have developed their defense systems to overcome the toxicity of mercury compounds. The most-studied mechanism of bacterial mercury resistance is an enzymatic sequential reaction that degrades organomercurials to the inorganic ionic mercury, Hg^{2+}, and subsequently reduces the Hg^{2+} to the metallic form, Hg^0 [2]. This process depends on the expression of the mercury resistance operon (*mer* operon) and transforms highly toxic water-soluble ionic mercury to insoluble metallic mercury. Reduced metallic mercury is easily volatilized and removed from the bacterial cells. The finding of this microbial function from various environments suggests that this mechanism of microbial mercury resistance is widely distributed and evolved in the microbial world [3,4]. Many researchers have focused their studies on the isolation and identification of mercury resistant bacteria, such as *Staphylococcus* [5], *Pseudomonas* [6], and *Bacillus* [4,7], and their responsible genes. We also isolated and characterized several bacteria that can resist and volatilize mercury compounds [8,9]. These microbes play an important role in mercury detoxification in the environments and therefore offer a valuable possibility for developing new approaches in bioremediation technology for mercury pollution.

The advantages of immobilized microorganisms as biocatalysts are 1) protection of microorganisms from harsh environmental conditions, 2) easy recovery of microbial agents from the reaction solution, 3) provision of higher cell density to increase overall substrate conversion rates, and, 4) reduction of reactor volumes. There are various techniques for immobilization of living microorganisms, including gel bead entrapment, carrier binding, adsorption to solid surface, encapsulation, and biofilm development. These advantages have encouraged researchers to apply immobilized microbial cells in the

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E-mail: gendo@tjcc.tohoku-gakuin.ac.jp

biodegradation of hazardous compounds, such as phenol, carbazole, and mercury compounds [10–12].

Bacillus megaterium MB1 is a mercury resistant bacterium isolated by us from preserved sediment of Minamata Bay. Our previous studies showed that it has a broad-spectrum mercury resistance, effectively degrades organomercurials to ionic mercury (inorganic mercury), reduces the water-soluble inorganic mercury to insoluble metallic mercury, and subsequently volatilizes the metallic mercury from the growth medium solution [8,13].

In this study, we investigated the removal and recovery of mercury from the water samples that contain mercuric chloride by the gel immobilized *B. megaterium* MB1.

2 Experiment

2.1 Bacterial strains and chemicals

B. megaterium MB1 used in this study was a isolated bacterial isolated from preserved sediment of Minamata Bay strain [8]. *Bacillus cereus* RC607 and *Bacillus cereus* VKM684 were kindly presented from Prof. Simon Silver of University of Illinois and Dr. Elena S. Bogdanova of Institute of Molecular Genetics, Russian Academy of Sciences, respectively [4,7]. The mercury sensitive bacterium, *Bacillus subtilis* 168, was used as a negative control for mercury resistance and removal. The bacterial strains were grown in Luria-Bertani (LB) broth amended with or with out 0.25 mg/L of mercuric chloride at 37°C, 160 $r \cdot min^{-1}$[14]. The mercuric chloride was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2 Immobilization of *B. megaterium* MB1 cells

All equipments and materials used for gel immobilization of *B. megaterium* MB1 cells were sterilized and all operations were carried out under biocontamination-free conditions. All optical densities were measured at λ: 600 nm (OD_{600}). Overnight culture of *B. megaterium* MB1 was inoculated into LB broth containing 0.25 mg/L of mercuric chloride and was adjusted to an OD_{600} of 0.1. The inoculated cells were incubated until the optical density reached around 0.5. The culture was then harvested, suspended and adjusted to an OD_{600} of 3.0 in LB broth. The cell-suspension was mixed with equal volume of 4% sodium alginate solution (final concentration of alginate was 2%), extruded as drops using a burette into 1 L of 0.1 mol/L calcium chloride solution. The drops of gel immobilized *B. megaterium* MB1 were stirred for 2 h until those were completely solidified. The solidified gel beads were washed twice with distilled water, sieved to be 2 – 5 mm in diameter and kept at 4°C. All chemicals used were purchased from Wako Pure Chemical Industries, Ltd.

2.3 Mercuric chloride removal assays

Free-living cells of the bacterial strains and immobilized *B. megaterium* MB1 gel beads were used for mercuric chloride removal assays. For free-living cells, the overnight cultures were adjusted to an OD_{600} of 0.1 with the medium, and were inoculated into 10 mL of LB broth containing 10 mg/L mercuric chloride. The mercury concentration of the bacterial culture was measured every 3 h for total 24 h. For the immobilized gel beads, 40 gel beads (the average diameter size is 3.5 mm) were added into 10 mL of distilled water containing 10 mg/L mercuric chloride. The gel beads without *B. megaterium* MB1 cells were used as negative controls. The batch removal assay was performed at 37°C for 24 h. For the repeated mercury removal assay, 40 beads of immobilized *B. megaterium* MB1 and the same amount of free-living *B. megaterium* MB1 cells were applied, and distilled water was used as solvent. The reacted liquor was removed and reloaded with 10 mL of distilled water containing 10 mg/L mercuric chloride at each sampling time. The repeated mercury removal assay was performed at 37°C for 36 h. The mercury concentration of the solution and the mercury content of the gel beads were measured at every sampling time by using a flameless atomic absorption spectrophotometer SP-3D (Nippon Instruments Co., Tokyo, Japan). Each mercury removal experiment was done at least three times.

3 Results and discussion

3.1 Mercury volatilization by mercury resistant *Bacillus* strains

The mercury volatilization by free-living mercury resistant bacteria was investigated first. As shown in Fig. 1(a), total mercury concentrations of the culture solution decreased to less than 20% of the initial concentrations by all mercury-resistant *Bacillus* strains used in this study. Almost no accumulation of mercury was observed in the centrifuged bacterial cell pellet (data not shown). Because ionic mercury and inorganic mercurial compounds are not removed as vapor, these results suggested that the mercury removal observed in this study was due to microbial reduction of mercuric ion and volatilization of produced elemental (metallic) mercury.

Table 1 shows that the minimal inhibitory concentration (MIC) of mercuric chloride to *B. megaterium* MB1 was lower than those to *B. cereus* RC607 and *B. cereus* VKM684. *B. megaterium* MB1 had also lower rate of mercury removal than *B. cereus* RC607 (almost one third) and *B. cereus* VKM684 (almost 0.65 times). However, *B. cereus* strains are known as pathogenic bacteria, but *B. megaterium* strains have not been reported as pathogens. Though *B. megaterium* MB1 does not have the highest

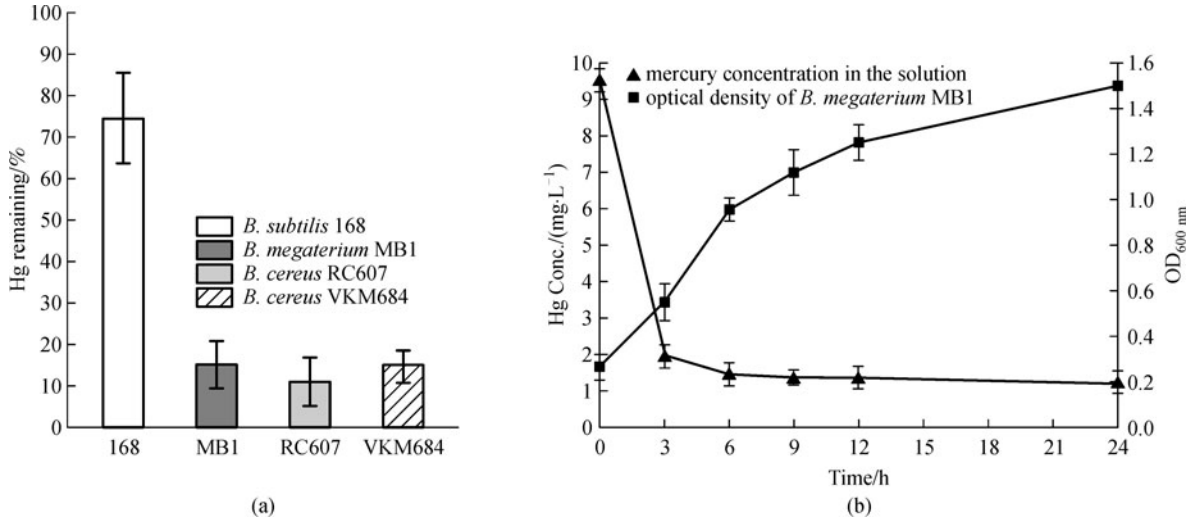

Fig. 1 (a) Mercury volatilization by *Bacillus* strains from mercuric chloride solution. (b) Mercury volatilization by suspended free cells of *B. megaterium* MB1. The initial concentration of mercuric chloride solution was 10 mg/L

activity of mercury removal among the tested *Bacillus* strains, it still has enough activity of mercury removal and biologic safety. Therefore, *B. megaterium* MB1 was selected for the immobilization and the biologic mercury removal.

The relation between the growth phase and mercury volatilization ability of *B. megaterium* MB1 cells was then investigated in order to define the optimal growth phase during that which *B. megaterium* MB1 has the highest ability of mercury volatilization. As shown in Fig. 1(b), most of the mercury was volatilized during the logarithmic phase of the growth of *B. megaterium* MB1, and almost the same ability of mercury volatilization was observed at the temperature range from 20°C to 37°C (data not shown). From these results, *B. megaterium* MB1 cells at the logarithmic growth phase were harvested and applied for cell immobilization.

3.2 Mercury removal by immobilized *B. megaterium* MB1

The mercury removal ability of calcium alginate-immobilized *B. megaterium* MB1 cells was subsequently investigated. As shown in Fig. 2(a), the mercury concentration of the solution, in which the alginate-immobilized *B. megaterium* MB1 was applied, decreased to 20% of the original concentration within 24 h. After the reaction, the mercury content in the alginate gel with immobilized *B. megaterium* MB1 increased to over 1 μg/bead, while the mercury content in the alginate gel without *B. megaterium* MB1 increased slightly (Fig. 2(b)). These results were consistent with previous studies that volatilized metallic mercury was partially trapped and accumulated in the gel [12].

Studies on the mercury removal by immobilized bacteria have been reported. Sinha and Khare reported that an immobilized mercury resistant *Enterobacter* sp. was effective in removal of mercury from 5 mg/L of mercuric chloride within 72 h [15]. Kiyono et al. reported that the immobilized *Escherichia coli* strain engineered with *mer* operon from *Pseudomonas* sp. K62 removed mercury from the solution below 20 μmol/L (around 5 mg/L) of mercuric chloride within 20 h [16]. The concentration of mercuric chloride used in the present study was 10 mg/L, and it was twice of those in the above two studies. The immobilized *B. megaterium* MB1 efficiently removed mercury from the solution within 24 h. These results showed that the alginategel immobilized *B. megaterium* MB1 was very effective in reduction of mercuric chloride to metallic mercury and in removal or deposit mercury in the gel beads.

Besides the selection of bacteria, the selection of optimal immobilization material is also important when applying

Table 1 MICs of mercuric chloride and mercury removal rates of the *Bacillus* strains

	MIC of $HgCl_2$ /(mg·L^{-1})	Mercury removal rate / (mg·L^{-1}·h^{-1})
B. subtilis 168	5.0	–
B. megaterium MB1	20.0	0.35
B. cereus RC607	50.0	1.05
B. cereus VKM684	50.0	0.51

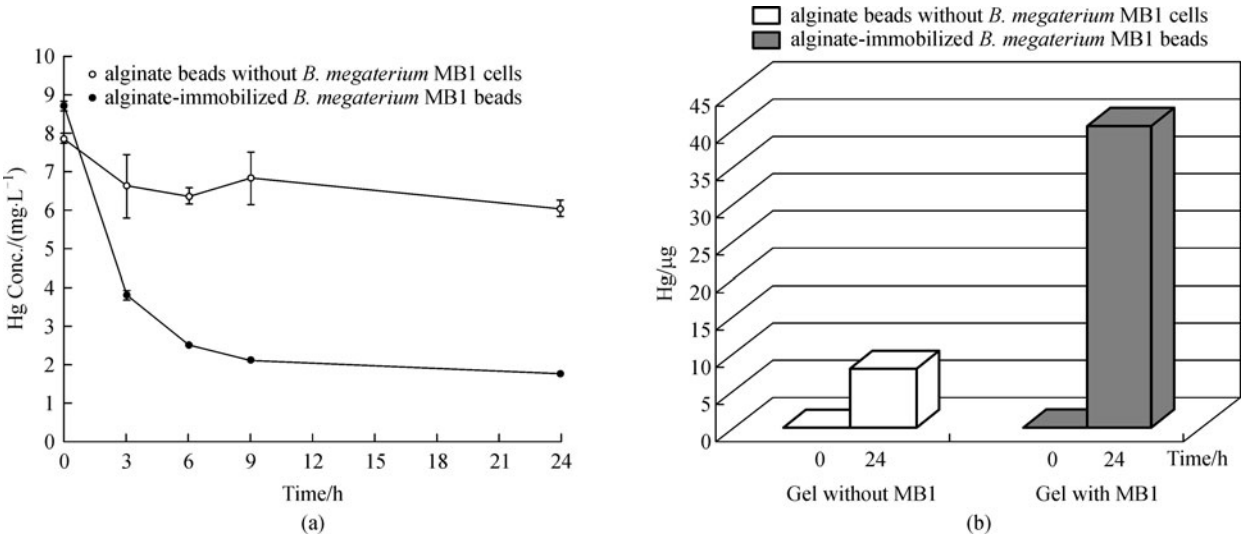

Fig. 2 (a) Mercuric chloride removal by alginate-immobilized *B. megaterium* MB1 beads. (b) The change of mercury content in the alginate gel. The data were averages of triplicate analysis

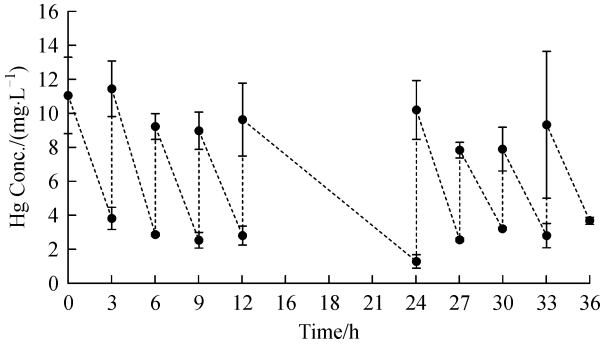

Fig. 3 Repeated mercury removal by alginate-immobilized *B. megaterium* MB1 cells. The data were averages of triplicate experiments

immobilized bacteria to mercury removal from contaminated water. Previous studies on immobilized cells demonstrated that the immobilization materials have different advantageous characteristics, and should be selected in view of superiority in maintaining high activity of bacteria, the property of the contaminated water being treated, and simple gel bead-forming procedure. Okino et al. reported that genetically modified *Pseudomonas putida* cells immobilized by calcium alginate had higher activity than those immobilized by photo-crosslinkable resin and agar [12]. While Wang et al. reported that the gellan gum was the optimal immobilization material for *Sphingomonas* sp. comparing with agar, alginate, and κ-carrageenan [11]. Because alginate-immobilized *B. megaterium* MB1 has high activity for mercury removal, and alginate gel bead is simple to produce, this material is evaluated as a suitable immobilization carrier for *B. megaterium* MB1.

3.3 Performance of repeated mercury removal by immobilized *B. megaterium* MB1 cells

The mercury removal assay was carried out repeatedly to investigate the stability and reusability of immobilized *B. megaterium* MB1 cells. As shown in Fig. 3, these cells efficiently removed mercury from the solution even after mercuric ion loading was repeated for nine times (Fig. 3), which was more than those in the previous reports [12,15,16]. Table 2 showed the mercury volatilization activity of immobilized and free-living *B. megaterium* MB1 cells in the repeated mercury removal assays. Free-living *B. megaterium* MB1 cells lost about 70% of mercury removal ability at the fifth loading, whereas alginate-immobilized MB1 cells did not lose the removal ability until the sixth loading. Furthermore, the immobilized cells maintained about 79% of mercury removal activity even at the ninth loading. In 36 h, immobilized *B. megaterium* MB1 cells removed as 2.2 times of mercury as free-living cells did. In the bioremediation process, the reusability of the biocatalysts is an important factor that determines their effectiveness. Therefore, immobilization has advantages in protection of cells from physical damage and prevention of biologic inactivation. Results of the present study show that the bacterial viability and mercury removal activity of *B. megaterium* MB1 are stabilized by alginate gel immobilization.

3.4 Mercury recovery from the mercury solution by gel immobilized *B. megaterium* MB1

Besides the bulk solution, the mercury retained in the alginate gel beads was also determined to monitor the distribution of reduced mercury in the reaction system.

Table 2 Repeated mercury volatilization of immobilized MB1 cells

Item	Relative mercury volatilization activity in each loading of mercuric ion /% [a)]								
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th
Immobilized *B. megaterium* MB1	100.0	116.5 (0.1)	91.7 (0.1)	84.9 (0.2)	111.7 (2.3)	103.6 (1.2)	64.6 (0.5)	70.6 (0.4)	79.0 (0.8)
Free-living *B. megaterium* MB1	100.0	90.2 (0.5)	65.4 (2.5)	57.3 (3.7)	30.1 (7.5)	7.5 (2.1)	5.1 (2.6)	4.3 (3.1)	3.7 (2.3)

a) Relative mercury volatilization activity is defined as the amount of removed mercury by immobilized and free-living cells, respectively, with the first loading of mercuric ion being 100%. The data were averages of triplicate experiments. The numbers in parenthesis showed standard deviations

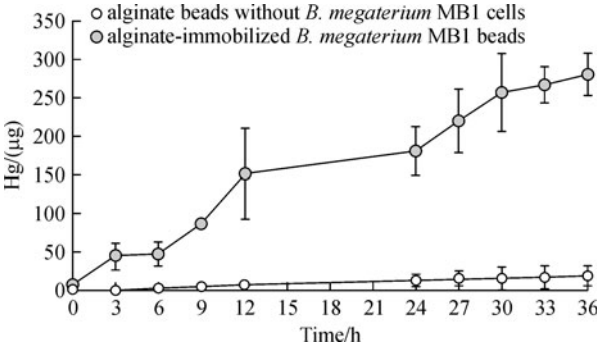

Fig. 4 Accumulation of mercury in alginate-immobilized *B. megaterium* MB1 gel beads during the repeated mercury removal experiment. The data were averages of triplicate experiments

After 24-hour batch reaction experiment, 80% of mercuric ion was removed from the solution by immobilized *B. megaterium* MB1, and 67% of the reduced mercury was entrapped in the gel beads. As shown in Fig. 4, in the 36-hour repeated mercury removal experiment, the mercury accumulated in the gel beads increased with the elapsed time, 73.3% of the calculated total amount of mercuric ion loaded to the system was converted into metallic mercury, and 44.8% of which accumulated in the immobilized gel beads.

Previous study has reported that a part of volatilized metallic mercury accumulated in the alginate-immobilized beads after biologic reduction [12,15,16]. Besides, it has been demonstrated that the polymers for immobilizing microorganisms are able to bind heavy metal. Harel et al. reported the high biosorption of cadmium by κ-carrageenan [17]. Lázaro et al. investigated the biosorption of nickel, copper, lead, and zinc ions by using eight kinds of gel beads, and the gellan gum showed the highest affinity [18]. As the result obtained in this study, the ability of alginate gel to accumulate metallic mercury may find application in mercury remediation process to improve total mercury recovery.

4 Conclusions

B. megaterium MB1 is good for mercury removal and has not been reported as a pathogen. We demonstrated that alginate-immobilization of *B. megaterium* MB1 could be a practical method for mercury removal from mercury-polluted water. Alginate, as an immobilization carrier, was effective for mercury volatilization. Moreover, alginate-immobilized *B. megaterium* MB1 cells exhibited excellent reusability and stability. The ability to accumulate large portion of reduced metallic mercury in the gels would enable the immobilization of *B. megaterium* MB1 to be a potential of recovering mercury from the contaminated water. In conclusion, the gel immobilized *B. megaterium* MB1 is effective to remove and recover mercury from mercury-polluted water.

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