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Biomagnetic recovery of selenium: Bioaccumulating of selenium granules in magnetotactic bacteria

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Running title: Selenium granule formation in magnetotactic bacteria

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Abbreviation used are: MSGM, magnetic spirillum growth medium; MIC, minimum inhibitory concentration; M. magneticum AMB-1, Magnetospirillum magneticum AMB-1; TEM, transmission electron microscopy; EDX, energy dispersive X-ray spectrometry.

ABSTRACT

Using microorganisms to remove waste and/or neutralize pollutants from contaminated water is attracting much attention due to the environmentally friendly nature of this methodology. However, cell recovery remains a bottleneck and a considerable challenge for the development of this process. Magnetotactic bacteria are a unique group of organisms that can be manipulated by an external magnetic field due to the presence of biogenic magnetite crystals formed within their cells. In this study, we demonstrated the first account of accumulation and precipitation of amorphous elemental selenium nanoparticles within magnetotactic bacteria alongside and independently to magnetite crystal biomineralisation when grown in a medium containing selenium oxyanion (SeO_3^{2-}). Quantitative analysis shows that magnetotactic bacteria accumulate the highest amount of target molecules (Se) per cell than any other previously reported of non-ferrous metal/metalloid. For example, 2.4 and 174 times more Se is accumulated when compared to Te uptaken into cells and Cd^{2+} adsorption onto the cell surface respectively. Crucially, the bacteria with high levels of Se accumulation were successfully recovered with an external magnetic field. This biomagnetic recovery and effective accumulation of target elements demonstrate the potential for application in bioremediation of polluted water.

IMPORTANCE

The development of a technique for effective environmental water remediation is urgently required across the globe. A biological remediation process of waste removal and/or neutralization of pollutant from contaminated water using microorganism has great potential, but

cell recovery remains a bottleneck. Magnetotactic bacteria synthesize magnetic particles within their cells, which can be recovered by a magnetic field. Herein, we report the first example of accumulation and precipitation of amorphous elemental selenium nanoparticles within magnetotactic bacteria independent of magnetic particle synthesis. The cells were able to accumulate the highest amount of Se compared to other foreign elements. More importantly, the Se accumulating bacteria were successfully recovered with an external magnetic field. We believe magnetotactic bacteria confer unique advantages of biomagnetic cell recovery and of Se accumulation, providing a new and effective methodology for bioremediation of polluted water.

INTRODUCTION

Environmental remediation, a technique of waste removal and/or neutralization of pollutant from a contaminated site, is an attractive field because of the increasing difficulty and importance of pure water acquisition in both developing and industrial countries. Among the various technologies for environmental water remediation, biorecovery of waste using microorganisms has great potential and is an environmentally friendly alternative to conventional techniques such as reclaimation treatment (1–3). Studies of the waste biosorption onto microorganisms and uptake into cells have been well demonstrated, but cell recovery remains a bottleneck in this approach because scale-up of collection methods such as centrifugation and filtration provides a huge logistical and monetary challenge.

Magnetotactic bacteria are unique prokaryotes, recognized by their response to a magnetic field. This is due to the presence of magnetic nanoparticles of Fe_3O_4 or Fe_3S_2 within the cells (4–6).

The particle formation occurs within an organelle, called a magnetosome, which is formed along the intracellular filamentous structure (7–9). The magnetosomes confer a magnetic moment to the cells, allowing them to migrate in aquatic environment under the influence of the Earth's geomagnetic field. We have already investigated the use of magnetotactic bacteria for the biomagnetic recovery of toxic and/or valuable metals and metalloid such as Cd (10, 11), Au (12), and Te (13). In these studies, Cd^{2+} and $AuCl_4^-$ were mainly adsorbed onto the cell surface (10, 12), while the Te oxyanion (TeO_3^{2-}) was reduced and biomineralized as discrete independent elemental Te nano-crystals within the cells with no incorporation into the magnetite crystals (13). The dual crystallization of tellurium and magnetite by magnetotactic bacteria enabled approximately 70 times more bioaccumulation of the pollutant per cell than cell surface adsorption. Therefore intracellular accumulation of target elements within magnetotactic bacteria offers the most promising system for bioremediation due to the unique advantages of both magnetic manipulation with external magnetic field and of effective target molecule accumulation.

Selenium (Se) is a rare element of high use in industry to produce various valuable materials because of its unusual semiconducting and photo-optical physical properties (14). The increased use of Se has led to its rising price and its increase in water contamination, which is in danger of presenting both an ecological and human health risk (15, 16). Therefore, the growing demand for Se in industrial technologies and the increased pollution effects of its byproducts into aquatic environments is rendering the recovery and recycling of this valuable element a very attractive global proposition. In aqueous environments, selenium is generally found as the toxic oxyanions

selenate (SeO_4^{2-}, +VI) and selenite (SeO_3^{2-}, +IV). The selenium oxide ions can adsorb extracellularly to the cell surfaces of microorganisms (1, 17). In addition, some microorganisms in the environment possess various strategies of detoxification such as methylation, assimilation as selenoamino acid, and reduction that could provide the potential to effectively accumulate Se within the cell (18, 19).

In this study we investigate the minimum inhibitory concentration (MIC) of selenium oxyanion (SeO_3^{2-}) for the magnetotactic bacterium M. magneticum AMB-1; the effect of this anion on magnetite crystal synthesis; and if uptaken, whether the Se dopes into the magnetite crystals (similar to the Co and Mn previously reported) (20, 21) or forms discrete crystals/inclusions within the cells (similar to the Te study) (13). Finally, the magnetic recovery of Se using magnetotactic bacteria is investigated.

MATERIALS AND METHODS

Determination of the minimum inhibitory concentration (MIC) of Selenite ion for M. magneticum AMB-1 growth. M. magneticum AMB-1 (ATCC700264) (22) was microaerobically cultured in magnetic spirillum growth medium (MSGM) at 28°C as previously described (23). Microaerobic conditions were established by purging the cultures with argon gas. The MIC of selenium for M. magneticum AMB-1 in MSGM was determined by growing the cells in various initial concentrations of selenite salt (Na_2SeO_3): 0 (control), 5, 10, 20, 40, 60, 80, 100 and 250 μM. The cells were directly counted with a hemacytometer under an optical microscope (Leica DML) after 7 days culture. Additionally the optical density (OD_{600}) was

recorded.

Transmission electron microscopy (TEM) and energy dispersive X-ray (EDX) spectrometry analyses of M. magneticum AMB-1 grown in the presence of SeO_3^{2-}. Cultured bacterial cells harvested from medium were washed with MilliQ three times and spotted onto 300-mesh Formvar/Carbon coated copper grids (Agar Scientific Ltd). The samples were analyzed by TEM operated at an accelerating voltage of 100 kV (Philips, CM10). High resolution TEM imaging and analysis were conducted on a FEI CM200 field emission gun TEM running at 200 kV equipped with an Oxford Instruments EDX spectrometer and a Gatan Imaging Filter. EDX analysis was conducted for at least 6 crystals in different cells under the same experimental conditions with representative spot data shown.

Se accumulation in M. magneticum AMB-1. To evaluate the amount of uptake and adsorbed SeO_3^{2-} in/onto cells, an atomic absorption spectrophotometer (Shimadzu, AA-6600G) was used. After the cells were collected by centrifugation (or in the case of the magnetic recovery assay, collection by magnetic trap in a glass test tube), the precipitates were washed 3 times with HEPES buffer (pH 7.4), dried and then dissolved with nitric acid solution (0.1N) with heating on in oil bath. After discarding the supernatant, the cells were dissolved with same procedure as described above. The dissolved solutions were quantitatively analyzed by atomic absorption spectrophotometry, using a calibration curve derived from standard solutions. All assays were performed three times.

Magnetic recovery assay of magnetotactic bacteria grown in the presence of selenite ions. To verify the ability of biomagnetic recovery of M. magneticum AMB-1 in the presence of

SeO_3^{2-} using magnetic force, a magnetic cell recovery assay was conducted. The M. magneticum AMB-1 wild type strain was harvested at the late logarithmic phase of growth, cells were counted and adjusted to 1.0×10^8 cells/ml of MSGM in the presence of the SeO_3^{2-} at different concentrations (0, 25, 50 and 100 µM). Three milliliters of each sample was then transferred to separate glass test tubes (Diameter: 7 mm, Height: 7.5 cm), each of which was sealed with a rubber cork. Cylindrical neodymium-boron magnets (Diameter: 15 mm, Height: 1 cm) were placed on the exterior of the horizontal centre of each test tube to allow cell recovery to take place. At the designated times (1, 2, 4, 6, 8, 10, 15 and 20h), culture medium was collected by inserting a syringe through the rubber cork and extracting culture medium (20 µl) from around the water surface. A cell count was performed against the extracted culture medium samples. After the magnetic separation, the amount of uptake and adsorbed SeO_3^{2-} in/onto magnetically manipulated cells was evaluated using an atomic absorption spectrophotometer (Shimadzu, AA-6600G). In addition, the magnetically collected cells and Se concentration were measured at the endpoint for further verification.

RESULTS AND DISCUSSION

Effect of SeO_3^{2-} on cell growth and on magnetite biomineralisation in M. magneticum AMB-1. The effect of selenium oxyanion (SeO_3^{2-}) on the growth of M. magneticum AMB-1 was investigated at various concentrations (Fig. 1). Cells cultured in MSGM containing 0 and 5 µM SeO_3^{2-} showed similar growth rates, with stationary-phase cell densities of approximately 2.2×10^8 cells/ml. Cell growth was negatively affected by the increase of SeO_3^{2-} concentration and no

cell growth was found at ≥ 250 μM. The MIC of selenium oxyanion for M. magneticum AMB-1 was determined to be 250 μM under these experimental conditions. The result indicated that SeO_3^{2-} is mildly toxic to this bacteria compared with the other chalcogen, tellurium oxyanion (e.g. MIC = 60 μM) (13). As E. coli has a MIC of 400 mM (SeO_3^{2-}), M. magneticum AMB-1 is less resistant to this element. Similar observations have been previously found with respect to Co^{2+}, Ni^{2+}, and Cu^{2+} showing approximately 90% less resistance than E. coli (20). It is of note that light-orange colors developed during the cell growth in the presence of SeO_3^{2-}. Similar observations were reported in various selenite-reducing bacteria (25, 26). The effect of the chalcogen on magnetite crystal formation in magnetotactic bacteria was also investigated (Fig. 1). The result showed a gradual decrease of magnetosomes with the increase of SeO_3^{2-} concentration but magnetite formation was observed even in the presence of high concentrations (100 μM) of SeO_3^{2-}. In addition, optical microscopy showed that approximately 100% and 70% of cells grown in the presence of 25 μM and 100 μM of SeO_3^{2-} respectively responded to the external magnetic field.

Observation of discrete formation of magnetite crystals and Se granule in M. magneticum AMB-1 grown in the presence of SeO_3^{2-}. Figure 2a shows representative transmission electron microscope (TEM) images of M. magneticum AMB-1 grown in the presence (100 μM) and absence of SeO_3^{2-} in the MSGM medium. Approximately 10 independent spherical granules (30~300 nm diameter) were observed in the cell grown in the presence of SeO_3^{2-} (Fig. 2a), while all cells revealed the presence of the magnetite crystals in a chain structure. The number and size of Se inclusions within the cell increased with increasing initial concentration of SeO_3^{2-} in the

medium. In a previous study, we have observed the doping of some metals (Cu, Mn, and Co) into bacterial magnetite crystal under laboratory-controlled conditions (20). However, in this study the elemental mapping showed no signal from Se in magnetite crystals (Fig. 2b). To verify the elemental components in these Se particles, STEM-EDX spot spectra were recorded and showed Se was the only element present (the Cu was from the TEM grid) (Fig. 2b and c). No oxygen was detected, inferring the inclusions are composed of pure elemental Se (0), which seems to be reduced and precipitated from SeO_3^{2-} in the cell. Selenium is a group 16 non-metal (chalcogens), neighbored by sulfur and the metalloid tellurium. Thiosulfate ($S_2O_3^{2-}$), tellurite (TeO_3^{2-}), and selenite (SeO_3^{2-}) are proposed to be taken up by bacteria and reduced to elemental S, Te, and Se, respectively (25, 27, 28). This is supported by the fact that S-globules are present in many microbes, including magnetotactic bacteria (29, 30), and we have also reported the formation of Te nano-crystals in magnetotactic bacteria independent from the magnetosome (13). Here we show for the first time that magnetotactic bacteria uptake, reduce and intracellularly form discrete Se granules independent to magnetosomes, similar to Te crystal precipitation in the same organism (13). The granules were examined by high-resolution TEM with selected area electron diffraction which showed a diffuse pattern, revealing the amorphous Se structure.

Time course measurements of Se accumulation in M. magneticum AMB-1. The time course of Se accumulation in magnetotactic bacteria was measured (Fig. 3). The cell growth and Se accumulation were saturated within 7 days and the Se uptake in cells mainly occurred in the stationary phase (for cells grown in 100 μM of SeO_3^{2-}). Under this condition, 68.1% of the initial Se (100 μM) was accumulated by the cells, which accounts to 6.6×10^8 Se atoms per cell. In the

case of Te accumulation found in the previous study, the most effective condition revealed that 2.7×10^8 Te atoms were accumulated per cell, which indicates that 2.4 times more Se is accumulated than Te. Furthermore, surface hexa-histidine expressing modified AMB-1 cells have previously been shown to adsorb Cd^{2+} onto these sites on the cell surface, showing the adsorption of 3.8×10^6 metal ions. Therefore, 2.4 and 174 times more Se was accumulated when compared to Te in cell and Cd^{2+} adsorption onto cell surface. These results highlight the greater loading of elemental Se into AMB-1 cells than any other metalloid or non-ferrous metal.

Biomagnetic recovery of SeO_3^{2-} using M. magneticum AMB-1. Magnetotactic bacteria harboring our target element (Se) for recovery can be manipulated and isolated by an external magnetic field, significantly magnifying the bioremediation potential of these cells for targeted recovery from polluted water environments. Herein, biomagnetic recovery of magnetotactic bacteria grown in the presence of SeO_3^{2-} was conducted. The result shown in Fig. 4 revealed that almost all cells grown in 25 μM SeO_3^{2-} were successfully recovered within 8 hours. The time for magnetic recovery of cells gradually increased with increasing concentration of SeO_3^{2-}. This seems to be the result of the decreasing quantities of magnetite under higher Se concentration conditions (Fig. 1). However, even in the presence of 100 μM SeO_3^{2-}, approximately 80% of magnetotactic bacteria were magnetically recovered within 20 hours. To confirm the biomagnetic recovery of Se, the amount of Se from magnetically recovered harvested cells was measured and revealed 3.6×10^8 Se atoms per cell recovery. Though some Se was lost during the recovery process (3.0×10^8 Se atoms after recovery), the result clearly shows that magnetotactic bacteria could be applied in biomagnetic recovery of Se from SeO_3^{2-} containing water. We note

that a more effective recovery could be established by process optimization (e.g. cell number, vessel size and magnetic force enhancement).

Current genetic and environmental microbiological research shows that magnetic particle production within bacteria occurs across a diverse group of bacterial species. In fact, the genetic region corresponding to magnetosome formation, called magnetosome island (MAI), is found within microbes spread across the phylogenetic tree. As M. magneticum AMB-1 does not show strong resistance to SeO_3^{2-} (Fig. 1), a magnetotactic bacterial species with higher tolerance and effective accumulation of target molecule could be found and used to improve the biomagnetic recovery; identified either from environments local to the bioremediation site or through evolving conditions to those similar to the polluted environment for a range of candidate magnetotactic bacteria. In addition, recently, magnetosome formation was enabled in another bacterial species by artificially transferring key genetic regions of the MAI into the host organism (31). Therefore, the induction of magnetosome formation within known bacteria showing high resistance to target element is another promising approach to improve the biomagnetic recovery efficiency.

In conclusion, in this study we showed the first account of amorphous elemental Se particle formation from the reduction of SeO_3^{2-} within the magnetotactic bacterial cell, completely independent of the crystallization of magnetite within the cells' magnetosomes. The cells were accumulated the highest amount of Se compared to any other foreign elements. For example, 2.4 and 174 times more Se was accumulated as compared to Te in cells and Cd^{2+} adsorption onto cell surfaces. Importantly, the Se accumulating bacteria were successfully recovered with an external

magnetic field. Therefore, we believe magnetotactic bacteria have the unique advantage of biomagnetic cell recovery, providing a new effective methodology for bioremediation of polluted water and additional potential to utilize the pollutant product for further material applications.

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M. T., A. A., S. B., S. S., and T. M. conceived and designed the experiments. M. T., W. K., R. B., N. H., and S. S. performed the experiments. All authors analyzed the data. M. T., A. A., S. S., and T. M. wrote the paper. All authors have no conflict of interest directly relevant to the content of this article.

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FIGURES

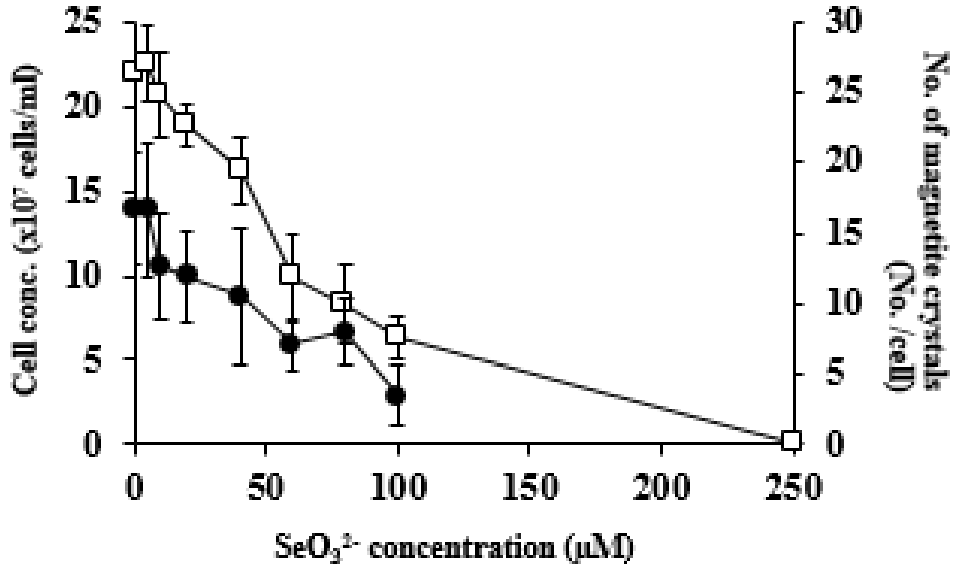

Fig. 1. Tolerance of *M. magneticum* AMB-1 to SeO_3^{2-} and magnetite nano-particle synthesis.

The number of cells (□) and magnetite crystals (●) grown in different concentrations (0, 5, 10, 20, 40, 60, 80, 100, and 250 μM) of SeO_3^{2-} were directly counted. To evaluate the number of magnetite within the cells, over 50 cells randomly selected were manually counted. Error bars show SDs.

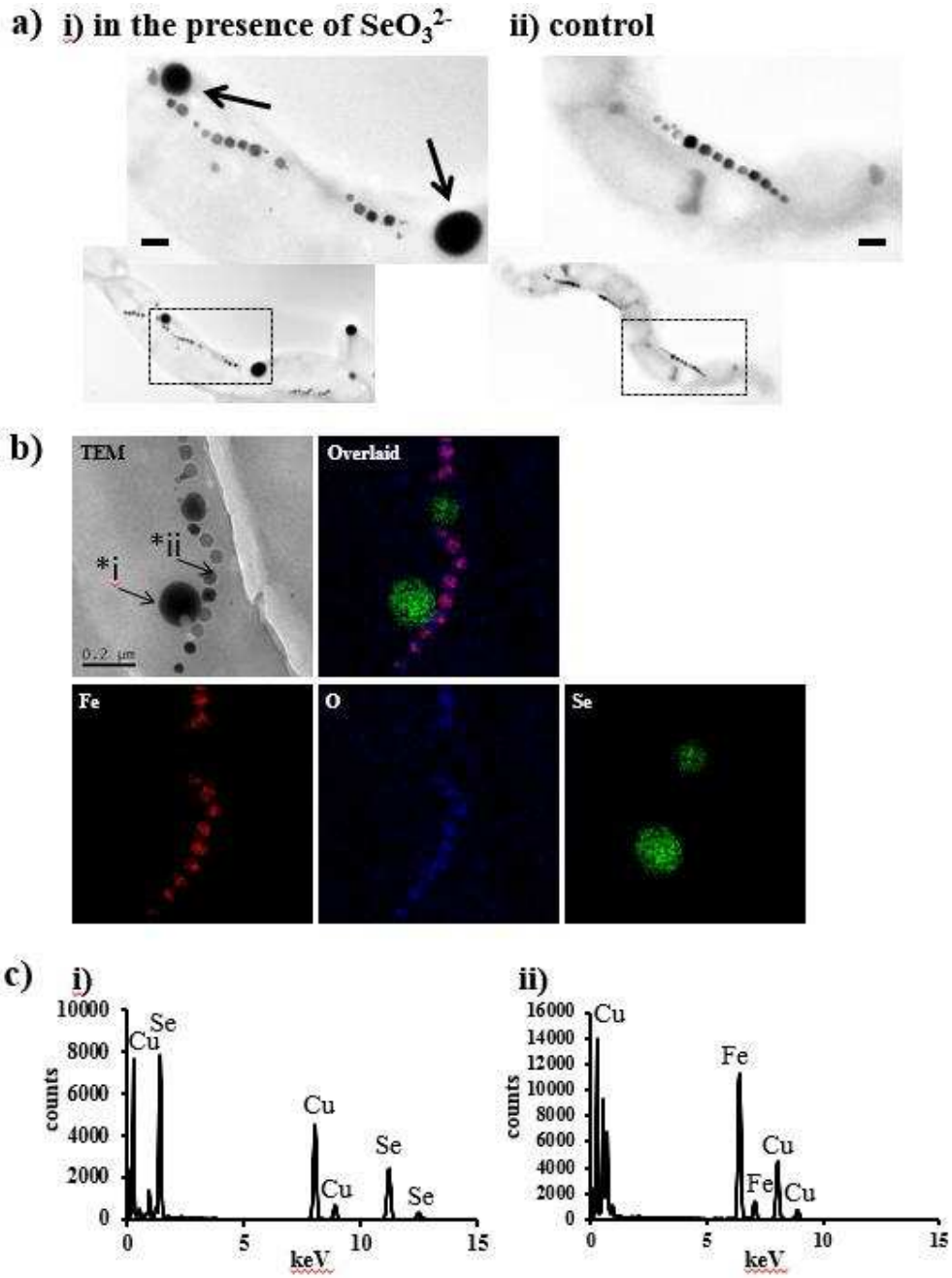

Fig. 2. Transmission electron micrographs, and STEM-EDX analyses for magnetite and Se within magnetotactic bacteria.

(a) TEM micrographs of magnetotactic bacteria grown i) in the presence of SeO_3^{2-} (100 μM) and ii) in its absence. Characteristic intracellular granules were indicated with arrows. Scale bar indicates 100 nm. (b) TEM image and STEM-EDX maps of Se, Fe, and O taken using a probe size of approximately 5 nm. (c) Spot EDX spectra of *i and *ii in b) as a representation of Se and magnetite. The Cu signal is from cupper TEM grid.

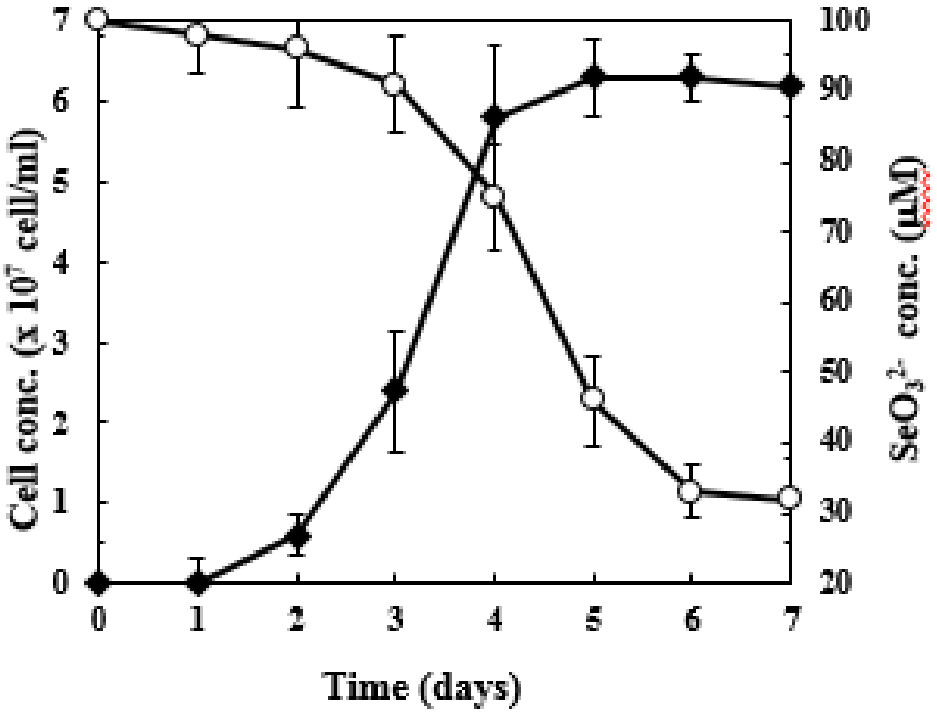

Fig. 3. SeO_3^{2-} removal during magnetotactic bacterial cell growth.

SeO_3^{2-} removal using magnetotactic bacteria (○) and cell growth (◆) was evaluated in the presence of 100 μM SeO_3^{2-} for 7 days. The average values from three independent experiments were obtained. Error bars show standard deviations.

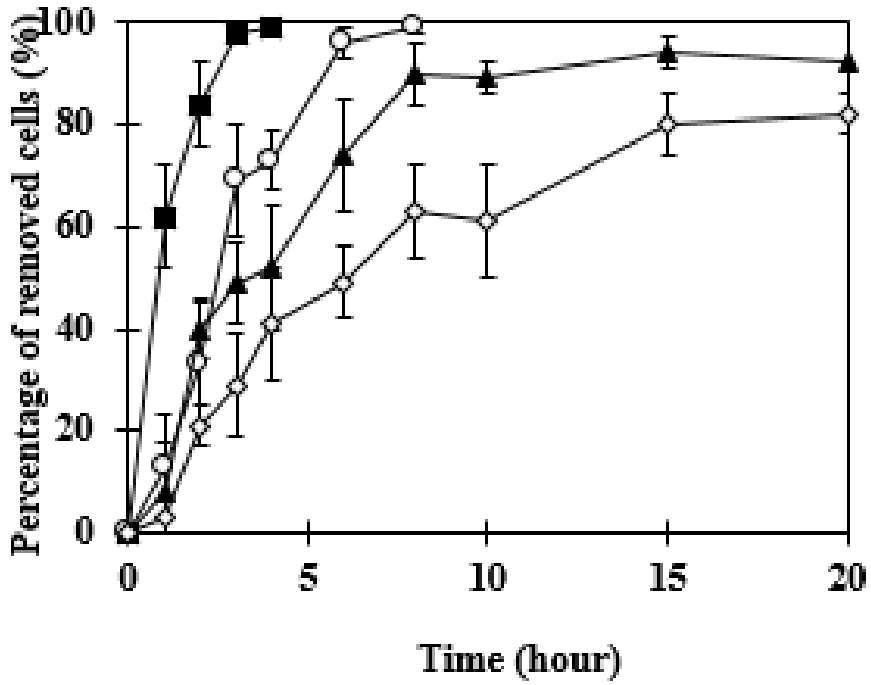

Fig. 4. Magnetic recovery assay of Se granule-containing *M. magneticum* AMB-1. The percentage of recovered cells is calculated from the initial cell numbers (1.0×10^8/ml) by counting the number of dispersed cells left within the culture medium. In addition, the number of cells recovered by magnetic force was also verified by counting the cells recovered at the end points. M. magneticum AMB-1 was cultured and assayed with the respective concentrations of SeO_3^{2-} (SeO_3^{2-} concentration = 0 μM (control) (■), 25 μM (○), 50 μM (▲), and 100 μM (◇)). The average values from three independent experiments were obtained. Error bars show standard deviations.