Biomagnetic Recovery and Bioaccumulation of Selenium Granules in Magnetotactic Bacteria

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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 an account of accumulation and precipitation of amorphous elemental selenium nanoparticles within magnetotactic bacteria alongside and independent of magnetite crystal biomineralization when grown in a medium containing selenium oxyanion (SeO_3^{2-}). Quantitative analysis shows that magnetotactic bacteria accumulate the largest amount of target molecules (Se) per cell compared with any other previously reported nonferrous metal/metalloid. For example, 2.4 and 174 times more Se is accumulated than Te taken up into cells and Cd^{2+} adsorbed onto the cell surface, respectively. Crucially, the bacteria with high levels of Se accumulation were successfully recovered with an external magnetic field. The biomagnetic recovery and the 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 microorganisms 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 an 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 largest 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.

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 reclamation 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 environments 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 nanocrystals 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 magnetot-

actic bacteria offers the most promising system for bioremediation due to the unique advantages of magnetic manipulation with external magnetic field and of effective target molecule accumulation.

Selenium (Se) is a rare element of high use in industry for production of 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 ecological and human health risks [15, 16]. Therefore, the growing demand for Se in industrial technologies and the increased pollution effects of its byproducts into aquatic environments are 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 investigated the MIC of selenium oxyanion (SeO_3^{2-}) for the magnetotactic bacterium *Magnetospirillum magneticum* AMB-1; the effect of this anion on magnetite crystal synthesis; and, if taken up, whether the Se dopes into the magnetite crystals (similar to Co and Mn in previously reported studies) [20, 21] or forms discrete crystals/inclusions within the cells (similar to the Te study) [13]. Finally, we investigated the magnetic recovery of Se using magnetotactic bacteria.

MATERIALS AND METHODS

Determination of the MIC of selenite ion for *M. magneticum* AMB-1 growth. *M. magneticum* AMB-1 (ATCC 700264) [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 of culture. Additionally the optical density at 600 nm (OD_{600}) was recorded.

Transmission electron microscopy and energy-dispersive X-ray 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 transmission electron microscopy (TEM) operated at an accelerating voltage of 100 kV (Philips, CM10). High-resolution TEM imaging and analysis were conducted on an FEI CM200 field emission gun TEM running at 200 kV equipped with an Oxford Instruments energy-dispersive X-ray spectroscopy (EDX) spectrometer and a Gatan imaging filter. EDX analysis was conducted for at least six 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 into and adsorption onto cells by SeO_3^{2-}, 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 three times with HEPES buffer (pH 7.4), dried, and then dissolved with nitric acid solution (0.1 N) with heating in an oil bath. After the supernatant was discarded, the cells were dissolved by the same procedure described above. The dissolved solutions were quantitatively analyzed by
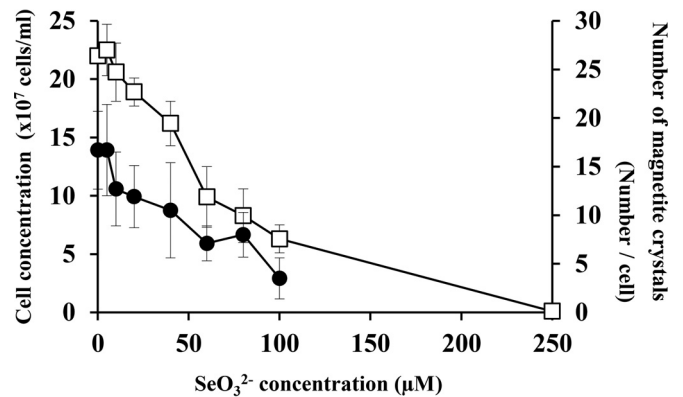

FIG 1 Tolerance of *M. magneticum* AMB-1 to SeO_3^{2-} and magnetite nanoparticle synthesis. The numbers of cells (□) and of magnetite crystals (●) grown in different concentrations (0, 5, 10, 20, 40, 60, 80, 100, and 250 μM) of SeO_3^{2-} were directly counted. To evaluate the number of magnetite crystals within the cells, over 50 cells randomly selected were manually counted. Error bars show standard deviations.

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, and 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 center of each test tube to allow cell recovery to take place. At the designated times (1, 2, 4, 6, 8, 10, 15, and 20 h), 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 amounts of SeO_3^{2-} uptake into and adsorbed SeO_3^{2-} onto magnetically manipulated cells were evaluated using an atomic absorption spectrophotometer (Shimadzu, AA-6600G). In addition, the magnetically collected cells and the Se concentration were measured at the endpoint for further verification.

RESULTS AND DISCUSSION

Effect of SeO_3^{2-} on cell growth and on magnetite biomineralization 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 bacterium compared with the other chalcogen, tellurium oxyanion (e.g., MIC of 60 μM) [13]. As the MIC of SeO_3^{2-} for *Escherichia coli* is 400 mM [24], *M. magneticum* AMB-1 is less resistant to this element. Similar observations have been previously found for other ions, including Co^{2+}, Ni^{2+}, and Cu^{2+}, with *M. magneticum* AMB-1
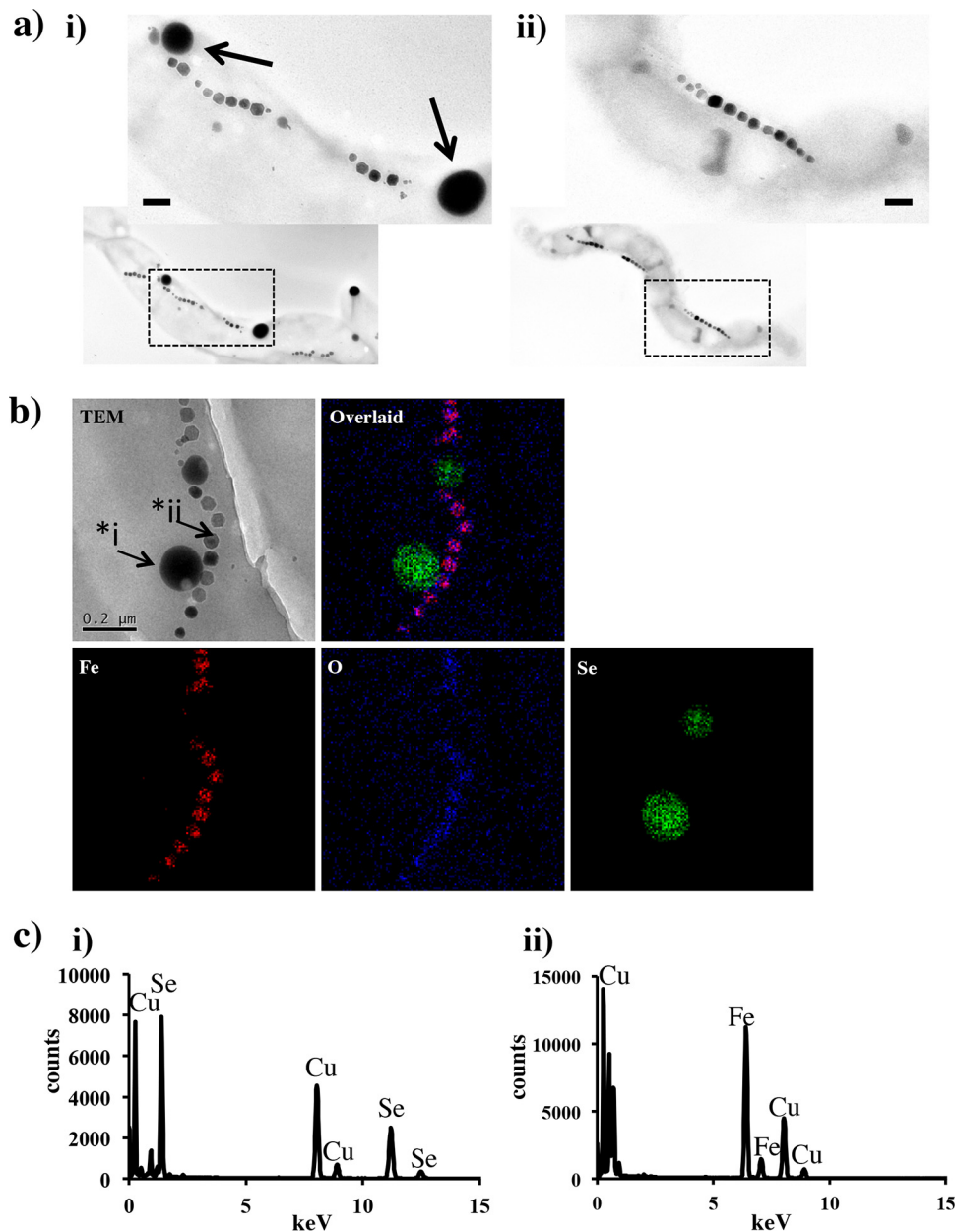
FIG 2 Transmission electron micrographs and STEM-EDX analyses for magnetite and Se within magnetotactic bacteria. (a) TEM micrographs of magnetotactic bacteria grown in the presence of SeO_3^{2-} (100 μM) (i) and in its absence (ii). Characteristic intracellular granules were indicated with arrows. Scale 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 panel b as a representation of Se and magnetite. The Cu signal is from the copper TEM grid.

showing approximately 90% less resistance than *E. coli* (20). It is noteworthy 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 (24, 25). 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 the 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 SeO_3^{2-}, respectively, responded to the external magnetic field.

Observation of discrete formation of magnetite crystals and Se granules in *M. magneticum* AMB-1 grown in the presence of SeO_3^{2-}. Fig. 2a shows representative 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 to 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 observed the doping of some metals (Cu, Mn, and Co) into bacterial magnetite crystal under laboratory-con-
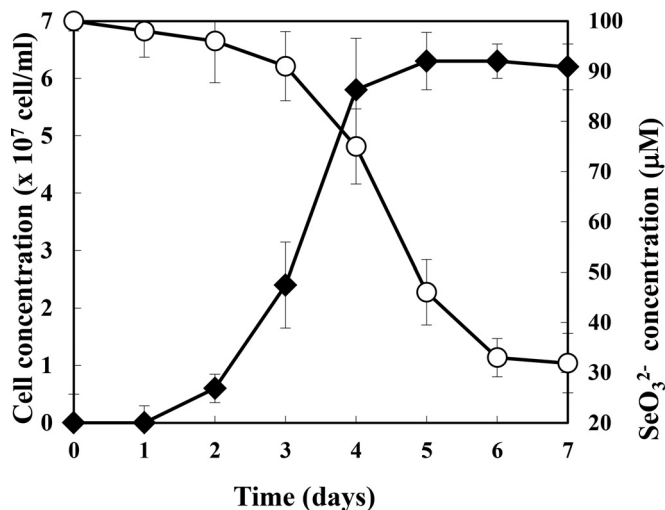
FIG 3 SeO_3^{2-} removal from medium during magnetotactic bacterial cell growth. SeO_3^{2-} concentrations (○) and cell growth (◆) were evaluated for 7 days. The average values from three independent experiments were obtained. Error bars show standard deviations.
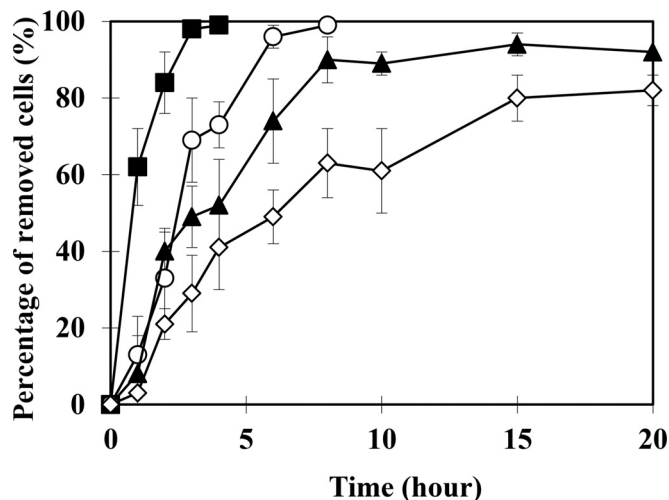
FIG 4 Magnetic recovery assay of Se granule-containing *M. magneticum* AMB-1. The percentage of recovered cells is calculated from the initial cell numbers (1.0×10^8/ml) by counting the number of dispersed cells left within the culture medium. In addition, the number of cells recovered by magnetic force was also verified by counting the cells recovered at the endpoints. *M. magneticum* AMB-1 was cultured and assayed with the following respective SeO_3^{2-} concentrations: 0 μM (control), ■; 25 μM, ○; 50 μM, ▲; and 100 μM, ◇. The average values from three independent experiments were obtained. Error bars show standard deviations.

trolled 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, scanning transmission electron microscope (STEM)-EDX spot spectra were recorded and showed that Se was the only element present (the Cu was from the TEM grid) (Fig. 2b and c). No oxygen was detected, suggesting that 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 nonmetal (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 (24, 26, 27). This is supported by the fact that S globules are present in many microbes, including magnetotactic bacteria (28, 29), and we have also reported the formation of Te nanocrystals in magnetotactic bacteria independent from the magnetosome (13). Here, we showed that magnetotactic bacteria take up, reduce, and intracellularly form discrete Se granules independently of 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 SeO_3^{2-}). Under this condition, 68.1% of the initial Se (100 μM) was accumulated by the cells, which resulted in 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 indicated that 2.4 times more Se was accumulated than Te. Furthermore, surface hexahistidine-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 than Te uptake into the cell

and than Cd^{2+} adsorption onto the cell surface, respectively. These results highlight the greater loading of elemental Se into AMB-1 cells than any other metalloid or nonferrous 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 described. The results shown in Fig. 4 revealed that almost all cells grown in 25 μM SeO_3^{2-} were successfully recovered within 8 h. The time for magnetic recovery of cells gradually increased with increasing concentration of SeO_3^{2-}. This seemed 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 was magnetically recovered within 20 h. 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 showed 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 the 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 spe-

cies 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 similar to those in 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 (30). Therefore, the induction of magnetosome formation within known bacteria showing high resistance to a target element is another promising approach to improve the biomagnetic recovery efficiency.

In conclusion, in this study, we showed an 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 cell magnetosomes. The cells accumulated the largest amount of Se compared to any other foreign elements. For example, 2.4 and 174 times more Se was accumulated than Te into cells and Cd^{2+} adsorption onto cell surfaces, respectively. Importantly, the Se-accumulating bacteria were successfully recovered with an external magnetic field. Therefore, we believe that magnetotactic bacteria have the unique advantage of biomagnetic cell recovery, providing a new effective methodology for bioremediation of polluted water and an additional potential to utilize the pollutant product for further material applications (31).

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