

# Bio-mineralization of Se Nanosphere by *Bacillus Licheniformis*

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**ABSTRACT:** Biological dissimilatory reduction of selenite ( $\text{SeO}_3^{2-}$ ) to elemental selenium ( $\text{Se}^0$ ) is common, but the mineral formation and the biogenic process remain uncertain. In this study, we examined the  $\text{Se}^0$  formation during the selenite bioreduction by *Bacillus licheniformis* SeRB-1 through transmission electron microscope (TEM), energy-dispersive spectrometry (EDS) and X-ray absorption fine structure (XAFS) techniques. Results showed that the reduction process occurred mostly during the exponential phase and early stationary phase, whilst the elemental selenium was produced in these periods. From the TEM images and polyacrylamide gel electrophoresis, it is known that the  $\text{Se}^0$  granule formation is a biologically-induced type, and the cell envelopes are the main bio-mineralization positions, and particles may go through a process from nucleation to crystallization, under the control of microbes. In fact, the minerals are spherical nanoparticles, occurring as a microcrystal or amorphous form. It is vital to recognize which kinds of proteins and/or polysaccharides act as a template to direct nanoparticle nucleation and growth? This should focus for further studies. This study may shed light on the process of formation of  $\text{Se}(0)$  nanosphere.

**KEY WORDS:** bio-mineralization, genesis, selenite reduction, selenium nanosphere.

## 0 INTRODUCTION

Bio-mineralization is a process that forms the minerals under the control of organisms and deposits in the matrix (either cellular or extracellular) of living organisms (Mann, 2001; Boskey, 1998). It combines the disciplines of biological, chemical, and earth sciences together and may shed lights on new materials biosynthesis (Mann, 1993). Although it has long been known that microorganisms play a dynamic role in the biogeochemical cycling of most elements (Ehrlich and Newman, 2008), only the discipline of ‘geomicrobiology’ developed in the last two decades of 20th century (Chen and Yao, 2005; Knoll, 2003), the full extent of the microbial processes that can control mineral formation have gained increased interest (Lloyd et al., 2008). In recent decades, metallic minerals biogenetic is one of the hot spot (Lu, 2007).

Selenium is a redox sensitive element that occurs in four different oxidation states, -2, 0, +4, and +6, in the environment, and selenium oxyanions are easy to be reduced to elemental selenium  $\text{Se}(0)$ .  $\text{Se}(0)$  is the dominant species of Se in anoxic sediments (Stolz and Oremland, 1999) whereas the transformation of Se in nature occurs primarily by biotic processes (Dowdle and Oremland, 1998). Such process is one of the

major sinks for selenium oxyanions, and studies on selenium bio-mineralization have carried out by many researchers (Butler et al., 2012; Lenz et al., 2011; Kaur et al., 2009). But the mineral formation, dissolution or diagenesis processes remain uncertain. The focus of this study is on the microstructure and process of formation of biogenic elemental selenium, in order to elucidate how bacteria generate these novel selenium nanostructures.

## 1 EXPERIMENTAL SECTION

### 1.1 Bacterial Isolate and Culture Conditions

The strain SeRB-1 was previously isolated from high-Se carbonaceous mudstones from Yutangba, Enshi, China, after enrichment with  $\text{Na}_2\text{SeO}_3$  as the electron acceptor and dextrose as the electron donor. The isolate is a kind of facultative anaerobe and was identified as *Bacillus licheniformis* (Genebank accession No. JX512417) (Yuan et al., 2014).

SeRB-1 was routinely cultured in medium YEG (yeast extract and glucose) containing 10 g yeast extract, and 10 g of dextrose per liter of deionized water. The final pH was adjusted to 7.0 using a diluted HCl or NaOH solution. Facultative anaerobic cultures were incubated at 37 °C on a rotary shaker at 200 rpm.

### 1.2 Reduction of Selenium Oxyanions by SeRB-1

To determine the ability of SeRB-1 to reduce  $\text{SeO}_3^{2-}$  during this growth, a growth experiment was conducted in YEG. In a typical procedure, 30 mL aliquots of YEG medium with 865 mg/kg sodium selenite (passing through a 0.22  $\mu\text{m}$  filter) were

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added into 50 mL serum bottles, and about 10% of suspended SeRB-1 culture was inoculated. The bacterium was first pre-grown to the exponential growth phase (~24 h). Cell-free controls where selenite was added to medium were also set up to detect any abiotic transformations. The experiment was done in triplicate.

### 1.3 Analytical Methods

Two mL samples were collected after each interval period, one aliquot was used to measure cell density by viable counting under fluorescence microscope (Olympus, Japan), and another mL was centrifuged for 10 min at 10 000 rpm (TCL-18C-C), the supernatant was used to determine the remaining Se(IV) concentration in the medium by hydride-generation atomic fluorescence spectrometry (HG-AFS), while the precipitates/pellets (including both cells and biominerals) were used for microscopy and fine structure analysis.

The procedures for the preparation of transmission electron micrograph of cultures have been described elsewhere (Yuan et al., 2014). The stained ultrathin section (80–100 nm) was used for ultrastructure analysis by TEM and the unstained one for mineral composition examination by EDS techniques (JEM-2000FX II, Japan). Selected-area electron diffraction was also carried out for crystal type analysis.

Another aliquot of precipitate was added with lysozyme to break the cell structure, and then it was centrifuged to obtain biominerals, and the biominerals were dehydrated and freezing-dried, and finally were measured at 1W1B-XAFS station in Beijing Synchrotron Radiation Facility (BSRF).

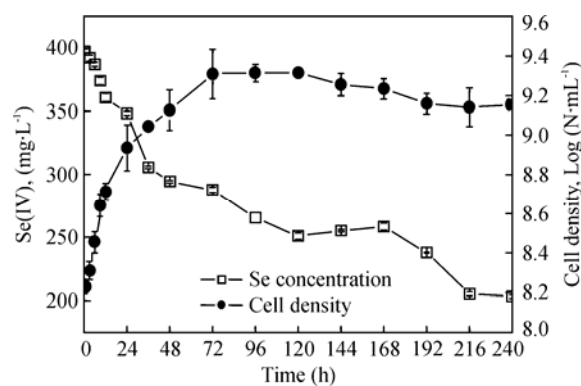
## 2 RESULTS

### 2.1 Selenite Reduction by Strain

Red-colored colonies were present in the cultures grown in the presence of 5 mM sodium selenite in about 6 hours. This indicates that Se(IV) was reduced to elemental selenium, while there was no red color emerging for the cell-free controls. The effect of time on microbial growth and reduction of selenite by SeRB-1 was shown in the Fig. 1. The results demonstrated that the process occurred mainly during the exponential phase. The concentration of Se(IV) decreased with microbial growth (Fig. 1), while there was little change in Se(IV) concentration in medium of the controls, or keeping stable at the initial concentration of 390 mg/L. Approximately 50% of selenite was reduced during the experimental period.

### 2.2 Elemental Analysis of Selenium Precipitates

Electron micrographs of cells grown with selenite were shown in the Fig. 2. Abundant mineral granules were produced on the exterior of the cell envelope during cells of SeRB-1 grown in medium containing selenite for a period of time (~24 h) (Fig. 2a). In addition, TEM images of thin sections also revealed the common presence of intercellular mineral granules (Fig. 2b). These internal biomineral accumulations was formed adjacent to the cell's periphery and deposited to the contour of the cell envelope. The biominerals appeared as a spherical and were approximately 10–200 nm in diameter. The nano-spheres were not present in the TEM images of the cells at the initial time after strains



**Figure 1. Effect of time on microbial growth and reduction of selenite by *Bacillus licheniformis*.**

inoculated to the Se(IV) medium (<6 h) (Fig. 2c). It showed that diffraction rings and weak diffraction spots were present from selected-area electron diffraction (SAD) analysis of the nanoparticles (Fig. 2d, inset).

When the biominerals were analyzed by EDS, the particles produced specific selenium absorption peak at 1.37 keV (peak SeL $\alpha$ ), 11.22 keV (peak SeK $\alpha$ ), and 12.49 keV (peak SeK $\beta$ ) (Fig. 2d). This indicates that it is mostly composed of selenium element, which is consistent with the previous study (Kessi et al., 1999). The Cu peaks were produced by or resulted from the TEM grid, the Ca and S peaks might be the component of the medium and/or the microbes, and the C and O most likely associated with cellular exudates.

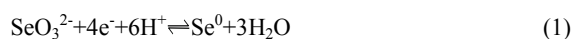
### 2.3 XAFS Analysis

XAFS has very high sensitivity on the absorption of element types and the geometrical structure of the atom, which can reflect the valence state of elements and their compounds (Rehr and Ankudinov, 2001). Figure 2 showed the selenium K-edge XAFS spectra for the biominerals produced by strain SeRB-1 and three reference chemicals of standard elemental Se, sodium selenite and sodium selenate. The spectra exhibited subtle but significant differences between the selenium compounds, and the position of Se K-edge can be used to determine the oxidation state of Se. The XAFS spectra measured for the pellets showed the same peak position of 12.658 keV as Se<sup>0</sup>, indicating the Se particles might primarily be composed of elemental selenium.

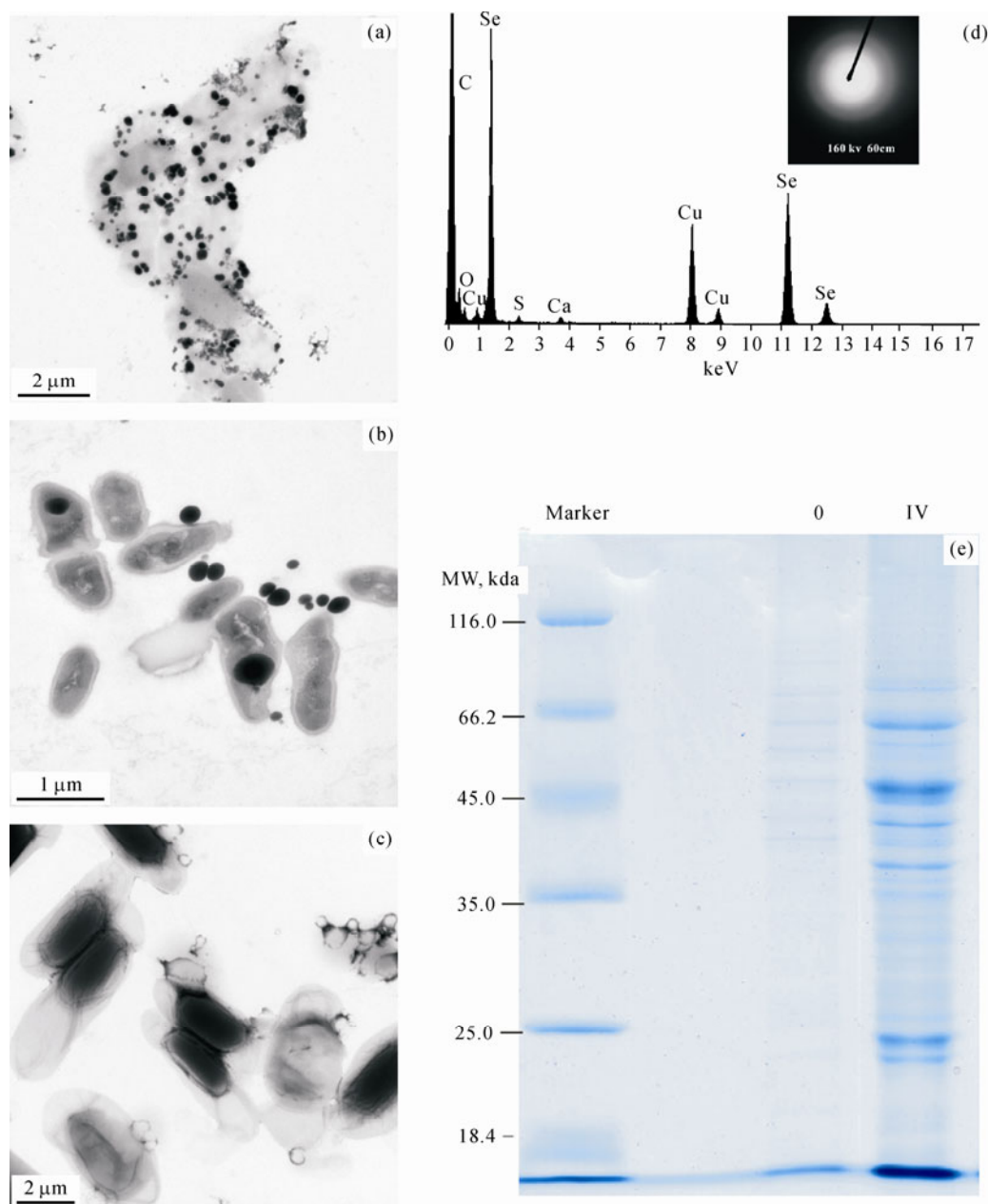
## 3 DISCUSSION

Selenite reduction accompanies microbial growth which is well-known phenomenon. The strain used selenium oxyanions as the sole electron acceptor, and dextrose as the electron donor. Finally, selenium oxyanion was reduced to elemental Se, but the mineral formation and the biogenic process remain uncertain.

The formation of selenium is a kind of redox reaction, which can be described by a simplified equation (1), according to Debieux et al. (2011).



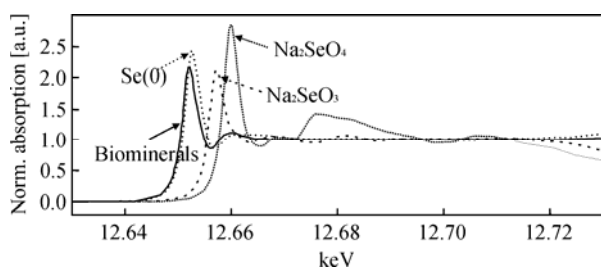
It seems to be a simple electron transfer process for equation



**Figure 2.** Electron micrographs and EDS analysis of the pellets. (a) TEM image of *B. licheniformis* cells grown facultative anaerobically using selenite as the sole electron acceptor after 24 h Se(IV) reduction; (b) thin sections of *B. licheniformis* cells grown facultative anaerobically using selenite as the sole electron acceptor after 24 h Se(IV) reduction; (c) TEM image of *B. licheniformis* cells grown facultative anaerobically using selenite as the sole electron acceptor at the initial time strain inoculated; (d) EDS and SAD (inset) analysis of electron-dense particles formed by *B. licheniformis* grown in facultative anaerobic cultures using selenite as the sole electron acceptor after 24 h Se(IV) reduction; (e) entire protein group SDS-PAGE.

(1), but the mechanism of mineral's biogenesis is a complex process. It depends on both the microbe species involved in the reaction and its conditions including electronic donor and extracellular redox state during biomineralization (Butler et al., 2012; Pearce et al., 2009). In this study, dextrose was used as the carbon and energy source, and  $\text{SeO}_3^{2-}$  was supplied as electronic acceptor, and was reduced to Se(0) through the metabolism of microorganism. Generally, the processes of biominerals formation can be classified as either biologically-controlled

mineralization (BCM), or biologically-induced mineralization (BIM). The differences between them are based on whether the biomineral is used by an organism for a biological function (as in BCM), or is the byproduct of an organism's metabolism (as in BIM) (Weiner and Dove, 2003; Mann, 2001; Lowenstam, 1981). From the comparison TEM images between Fig. 2a and Fig. 2c, it implies that the bacteria are induced to produce a mechanism to cope with Se(IV) stress. Polyacrylamide gel electrophoresis (PAGE) also confirmed this deduction process



**Figure 3. Selenium K-edge X-ray absorption spectra of Se particles precipitated by *B. licheniformis*.**

(Lei, 2010), strain can be induced to produce more selenite reductase in the presence of selenite. From PAGE, it appeared several specific bands for bacterial protein compared with before and after addition of Se(IV) (24 h). Therefore, this result indicate that the process of Se(0) produced by strain SeRB-1 is a BIM type.

A distinct characteristic of BIM is the formation of extracellular minerals along with occurrence of intracellular biominerals (Fig. 1b) (Frankel and Bazylinski, 2003; Weiner and Dove, 2003). It could be a result of metabolic processes of the organism and subsequent chemical reactions involving metabolic byproducts. In the medium, the organisms may secrete a metabolic product(s) that reacts with selenite in the surrounding environment. Usually, the structure/composition of the cell wall and cytomembrane/capsula, such as polypeptide, prion, lipid, and complexes, makes biomineralization to be easier. When cell envelopes are involved in the reductive process of selenium oxyanions, and then it is resulting from the production of mineral particles extracellularly.

Another distinct characteristics of BIM is that the biogenic elemental selenium generally does not form large crystals, or lacking specific crystalline morphologies (seen the SAD image inset in Fig. 2d), but rather spherical nano-particles, having a broad particle size distribution (Lenz and Lens, 2009; Oremland et al., 2004). It is likely to be that the mineralization is an unintended consequence of metabolic activities. A significant discovery is that the longer the biomineralized time, the larger the nano particles. The granules may go through a process from small to big, which is under the control of microbes.

Despite, it is common phenomena for elemental selenium formation during bioreduction, it has remained poorly studied the biological process of selenium nanosphere generation. Recent articles have suggested that proteins/enzymes may play an important role in selenium nanosphere assembly (Debieux et al., 2011; Dobias et al., 2011). For example, four proteins: AdhP, Idh, OmpC and AceA have identified in strain *Escherichia coli*, which are strongly associated with the selenium nanoparticles formed (Dobias et al., 2011). Whether strain *Bacillus licheniformis* has similar to proteins or structures, need further study. In fact, when the cell wall reductive enzymes or soluble secreted enzymes are involved in the reductive process of selenium oxyanions, then it is obvious to find the Se nanoparticles extracellularly. While, for intracellular Se nanoparticles formation, a possible explanation is that selenium

oxyanions reacts with glutathione reductase and generates selenodiglutathione (GS-Se-SG), and finally dismutates into elemental selenium (Kessi and Hanselmann, 2004).

The Se nanoparticles may experience nucleation, growth and cell processing to the phase change process, and finally the nano-selenium mineral generation. But the Se bionanomaterial phases are composite materials, because some biomolecules such as proteins and/or polysaccharides may act as a template to direct nanoparticle nucleation and growth.

#### 4 CONCLUSIONS

The results can be summarized below.

Elemental selenium was produced during bioreduction by *Bacillus licheniformis* SeRB-1. These Se biominerals were in nano-scale level and mainly produced and deposited extracellularly.

From the TEM images and PAGE, it infer that the nano-Se(0) biomineralization is a BIM type.

The biogenic elemental selenium generally does not form large crystals but rather spherical nanoparticles. These biominerals are likely linked to metabolic product(s) secreted by microorganisms, which need further study.

This study represents a further step toward understanding the mechanisms underlying the formation of nano-Se.

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