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ORIGINAL RESEARCH PAPER

Aerobic microbial manufacture of nanoscale selenium: exploiting nature's bio-nanomineralization potential

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Abstract The potential of the environment to yield organisms that can produce functional bionanominerals is demonstrated by selenium-tolerant, aerobic bacteria isolated from a seleniferous rhizosphere soil. An isolate, NS3, was identified as a *Bacillus* species (EU573774.1) based on morphological and 16S rRNA characterization. This strain reduced Se(IV) under aerobic conditions to produce amorphous α Se(0) nanospheres. A room-temperature washing treatment was then employed to remove the biomass and resulted in the production of clusters of hexagonal Se(0) nanorods. The Se(0) nanominerals were analyzed using electron microscopy and X-ray diffraction techniques. This *Bacillus* isolate has the potential to be used both in the neutralizing of toxic Se(IV) anions in the environment and in the environmentally friendly manufacture of nanomaterials.

Keywords *Bacillus* · Nanospheres · Reduction · Rosettes · Selenium

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Introduction

A variety of microorganisms, bacteria, yeast, fungi and algae, can adsorb and accumulate metals but only a few groups can selectively reduce metal ions to produce nano-scale mineral phases (Oremland et al. 2004). These organisms have the unique ability to produce inorganic phases of constant chemical composition and size (Cui and Gao 2003; Pearce et al. 2008). The majority of studies on the biogenesis of nano-Se particles have concentrated on anaerobic systems that have certain limitations, such as culture conditions and isolate characteristics that make optimization and scale-up in bio-manufacturing processes challenging. Selenium-tolerant aerobic organisms, however, provide the opportunity to overcome these limitations in the biosynthetic process. The results presented here show the Se reducing ability of a bacterial isolate, *Bacillus* sp. NS3 (EU573774.1) obtained from a seleniferous rhizospheric soil in the North-Western State of Punjab, India. The isolate tolerates selenium oxyanions and generates selenium

nanoparticles, thus combining the detoxification of oxidized seleniferous environments with the biotechnological production of nanomaterials.

It is the great potential of nano-scale Se(0) phases in electronic, optical, catalytic and medical application that has led to extensive investigations of the production and post-preparative modification of these materials by various inorganic routes (Pearce et al. 2008; Takahashi et al. 2006; Zhou and Zhu 2006). These include, solid-solution-solid transformation from amorphous Se (*a*-Se) colloids to *t*-Se nanowires (Gates et al. 2000, 2002a) and sonochemistry based synthesis and transformation of *a*-Se to *t*-Se nanowires (Gates et al. 2002b). In this study, we report on a solvent-based post-preparative treatment process to convert biomass-associated amorphous Se nanospheres, produced by the aerobic environmental isolate, *Bacillus* sp. (NS3; EU573774.1), into 'clean' crystalline Se nanorods for potential photovoltaic applications.

Materials and methods

Isolation and characterization

The isolate under study was obtained from soil collected from seleniferous belt bordering the Nawanshahr-Hoshiarpur region in North-west India (75°55E; 31°56N) through standard enrichment procedures (Focht 1994). This strain was selected based on its potential to tolerate selenium as selenite (SeO_3^{2-}). Growth experiments were conducted with the test strain to examine the Se tolerance of both aerobic and anaerobic cultures in tryptone soy broth (TSB), supplemented with Na_2SeO_3 (5 mM). Morphological analysis and gram staining were carried out by applying standard protocols (Krieg et al. 1984). Phylogenetic affiliation of this isolate was done by sequencing of the 16S rRNA gene. PCR amplification of the partial 16S rRNA gene was carried out using universal primers (8f and 1492r). The PCR conditions were as follows: Preheating at 94°C for 300 s, 35 cycles of 94°C for 60 s, 55°C for 30 s and 72°C for 30 s and a final extension at 71°C of 300 s. The PCR product was eluted and purified using a gel elution kit (Sigma), ligated to a linearized vector pTZ57 R/T (Fermentas) and transformed in competent *Escherichia coli* DH5-α cells. The cloned gene was then amplified and sequenced at the DNA sequencing facility, University of Delhi, India. The computational tool ClustalW (Thomson et al. 1994) was used to align and compare the 16S rRNA gene sequences of closely related to species of *Bacilli*. Phylogenetic analyses were conducted using the MEGA 4.0.1 software package (Tamura et al. 2007).

Growth profile and Se(IV) reduction under aerobic and anaerobic conditions

Erlenmeyer flasks and serum bottles, containing sterile TSB supplemented with 1 mM Se, were inoculated with a culture grown to log phase (based on optical density) so as to examine the growth profile in aerobic and anaerobic conditions. Additional supplementation of 7.5 g sodium lactate l^{-1} was provided as an electron donor to the anaerobic cultures. H_2 was also passed through the medium in the serum bottles for 5 m in as an additional electron donor. Positive (TSB with inoculum and without selenium) and negative (TSB with selenium and without inoculum) controls were maintained both for aerobic and anaerobic conditions. The negative control was used to assess the potential for chemical reduction of Se by the medium. Growth of the cultures was observed by measuring the OD_{600} over 24 h. The removal of selenite was determined by measuring the concentration of the oxyanion in the cell free supernatant (CFS) using ion chromatography (IC - Dionex DX 600) with 9 mM Na_2CO_3 as the mobile phase and an AS 9-HC column at 16.92 MPa back-pressure. The samples were diluted appropriately and introduced through a GP50 gradient pump to a CD 20 conductivity detector.

Characterization of reduced selenium

The solid fraction of the inocula (reduced selenium phases and biomass) were separated by centrifugation at $4000 \times g$ for 15 min. These samples were then imaged using an environmental scanning electron microscope (ESEM, Phillips XL) employing a GSE detector at 0.4–0.7 Torr. Energy dispersive X-ray (EDX) spectroscopy was performed at 16–20 kV using spot size of 200 nm and a counting for 100 s to provide qualitative chemical characterization of the phases produced. The biomass with the Se-phases was then subjected to sequential washing and

centrifugation (4000×*g* for 5 min) steps in 70, 80, 90 and 99% ethanol/water (90:1 v/v), followed by a final washing step in chloroform/methanol (1:1 v/v). The products were also examined using ESEM/EDX and further examined using transmission electron microscopy (TEM, Philips, CM-200). X-ray diffraction analysis using a Bruker D8 Advance X-ray diffractometer (Bruker AXS Ltd., Coventry, UK) with a Cu $K\alpha 1$ source was used to determine the crystal structure of the selenium phases produced.

Results

Characterization of the isolate

Morphological and phylogenetic analyses suggested that the isolate, NS3, was a *Bacillus* strain (EU573774.1); the isolate stained Gram-positive and was rod-shaped. In addition the 16S rRNA gene of the isolate shares 99% similarity (on 1,513 bp) with the 16S rRNA gene sequences from *Bacillus thuringiensis, B. anthracis,* and *B. cereus* (Fig. 1). Growth of the isolate under aerobic and anaerobic conditions confirmed the isolate to be a facultative anaerobe.

Selenite reduction under aerobic and anaerobic conditions

The potential of NS3 to transform selenite was assessed by challenging both aerobic and anaerobic cultures with sodium selenite (1 mM). IC data showed a decrease in Se(IV) concentration in the cell free supernatant, corresponding with growth of the culture under aerobic conditions, as shown by an increase in OD_{600nm} (Fig. 2). During growth and Se(IV) reduction, production of a red precipitate was observed in the aerobic cultures. Growth and Se(IV) reduction was limited under anaerobic conditions (Fig. 2). A marginal decrease in Se(IV) concentration (~10%) as a result of chemical reduction by the TSB media was observed in the no-cell controls under anaerobic conditions but not under aerobic conditions (data not shown).

Characterization of selenium nanostructures

ESEM examination of the red cell pellet from the Se-supplemented medium inoculated with the NS3 revealed 100–200 nm nanospheres associated with the biomass (Fig. 3a). EDX analysis revealed the nanospheres to be made exclusively of Se (Fig. 3b) indicating the formation of Se(0).

The post-preparative treatment process, involving water/ethanol and chloroform/methanol solvent systems, was employed to isolate the Se nanospheres from the biomass. However, this treatment resulted in the gradual change in the precipitate colour from red to black. ESEM images show that this color change was concurrent with a morphological change from the relatively small (10–200 nm) nanospheres (Fig. 3a) via hexagonal, faceted and increasingly platy nano-structures (Fig. 4a) to larger (up to

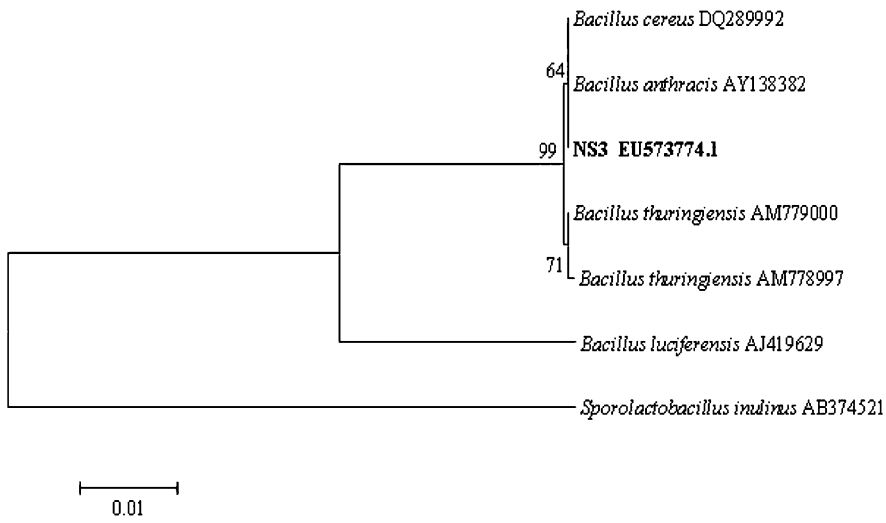

Fig. 1 16S rRNA gene based phylogenetic placement of the isolate as a *Bacillus* species. Scale bars represent substitutions per site

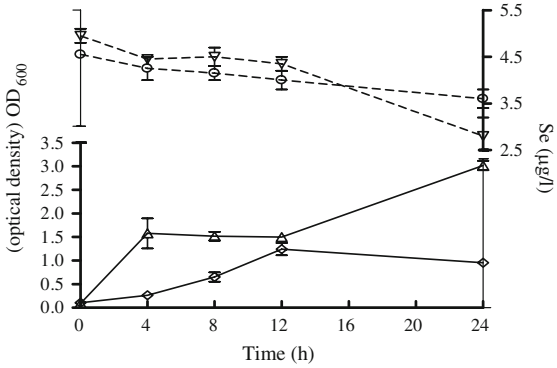

Fig. 2 Change in selenite concentration under aerobic (*inverted triangle*) or anaerobic (*open circle*) conditions in cell free supernatant and growth profile under aerobic (*open triangle*) or anaerobic conditions (*open diamond*) during 24 h exposure of *Bacillus* sp. to 1 mM selenite

10 μm) nano-rosettes comprising several crystalline nano-rods (Fig. 5a). EDX analysis indicated that the intermediate nano-structures and the nano-rosettes were also composed solely of selenium (Figs. 4b, 5b).

TEM examination revealed each nano-rod in the rosette to be ~5 to 10 μm length and 0.5–1 μm wide (Fig. 6a, b). XRD analysis revealed that the red nano-spheres were amorphous Se(0) (Fig. 7a). The diffraction pattern of the black Se nano-rosettes was the same as crystalline synthetic hexagonal Se (powder diffraction file 060362, International Centre for Diffraction Data) with characteristic peaks (2θ) at 23.42, 29.64 and 43.58 (Fig. 7b). The abnormal intensity of the (100) peak (as compared to that of synthetic Se) indicates that the Se nano-rosettes are preferentially orientated along the c axis, the [001] direction.

Discussion

Amorphous Se nanospheres, such as those produced by NS3, comprise both disordered [–Se–$_n$] chains and Se_8-rings and are relatively unstable at ambient temperatures (Peled and Hadziioannou 1991; Kasap and Yannacopoulos 1989). However, the biogenic Se nanospheres produced in this study remained stable until the organic component was removed in the post-preparative washing step, suggesting that the biomass was acting as a stabilizing agent. The amorphous red Se nanospheres were then transformed to crystalline

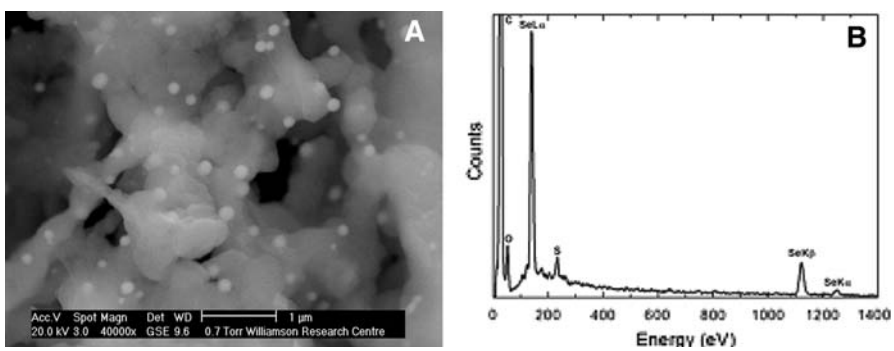

Fig. 3 ESEM image (−2 μm) (**a**) and EDX (**b**) of selenium nanospheres associated with the *Bacillus* sp. biomass

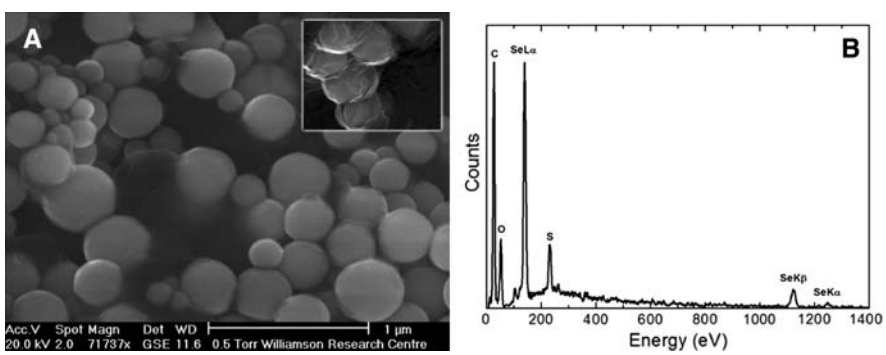

Fig. 4 ESEM image (−1 μm) (**a**) and EDX (**b**) of intermediate selenium nano-structures showing hexagonal facet development. The inset shows the platy nano-structures on 1 μm scale

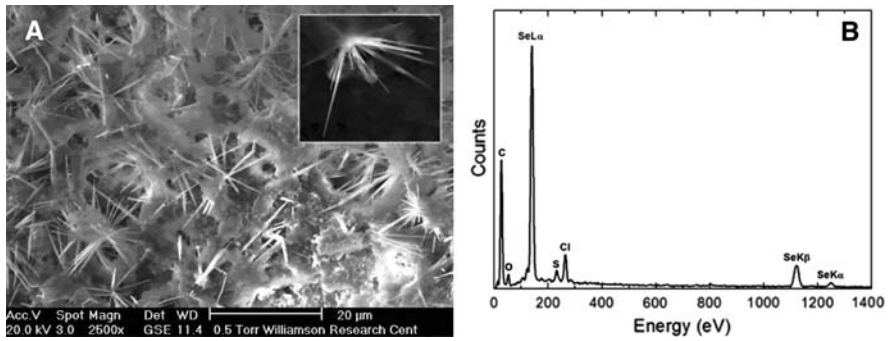

Fig. 5 ESEM image (−20 µm) (**a**) and EDX (**b**) of nanowire/rods formed on biomass in the form of rosettes. Inset shows a closer view of a rosette on 5 µm scale

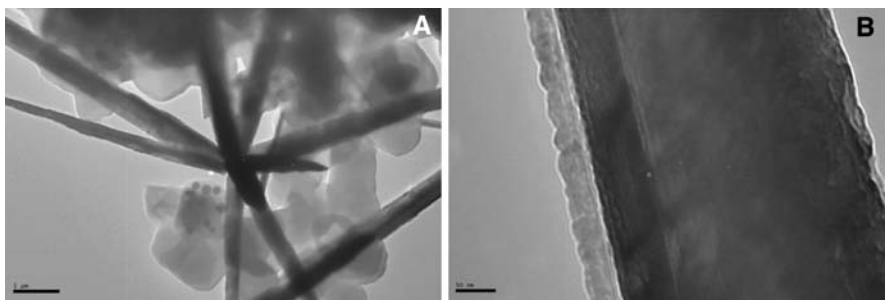

Fig. 6 TEM images of Se nanorods formed as rosettes from the biomass-associated Se nanospheres, showing **a** some precursor nanospheres (−1 µm), and **b** a detail of a nanorod (scale 50 nm)

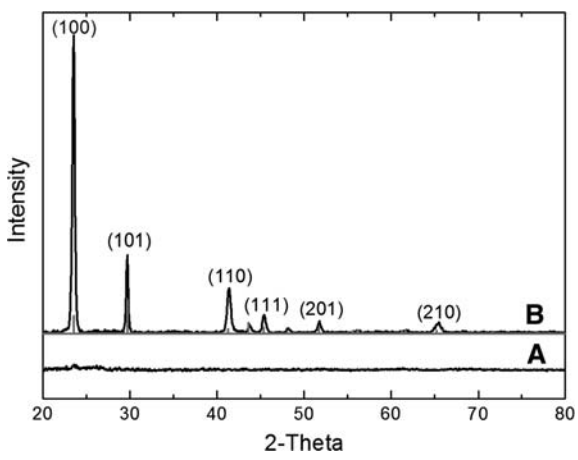

Fig. 7 XRD of amorphous nano-spheres (**a**) and crystalline nano-rosettes (**b**). The peak positions and relative intensities of crystalline *t*-Se are shown in *grey*. The *hkl* indices of *t*-Se are given in *parentheses*

hexagonal selenium (*t*-Se) (Fig. 5a) in the presence of organic solvents, via hexagonal, platy nano-structures (Fig. 4a). The development of Se nanorods in the presence of simple organic solvents has been observed before (Takahashi et al. 2006; Zhou and Zhu 2006; Gates et al. 2002b), although the size of the crystal clusters and the time taken for transformation of red nanospheres to black crystalline nanorods (within 60 min) are exceptional in this case. In previous investigations, the nucleation of the *t*-Se nanorods appeared to occur on hexagonal platelets, which here may have be either neo-formed *t*-Se or an intermediate monoclinic phase (Miyata et al. 1978; Mayers et al. 2001; Gates et al. 2002b; Peled and Hadziioannou 1991). Figures 3a, 4a, 5a in this study show a similar transformation. *t*-Se is very stable and comprises ordered helical [–Se–$_n$] chains linked together by inter-chain Van der Waals forces (Corb et al. 1982; Ren et al. 2004). The nature of the chain structure parallel to the 001 *c*-axis direction in *t*-Se results in one dimensional growth of Se and favors the acicular crystal development seen here. Preferential growth is expected from crystals orientated along the c-axis and the formation of the relatively large radiating rosettes suggests restricted development of *t*-Se nuclei (Zhang et al. 2003; Zhou and Zhu 2006).

Conclusion

Exploring the capabilities of naturally adapted microbial populations present in contaminated habitats offers the potential to isolate bacterial strains that can assimilate metals, transforming them into stable nanoscale mineral phases. The results of this study

reveal the potential of a facultative anaerobic, Se-tolerant environmental isolate (NS3) from seleniferous soil to reduce and transform selenium oxy-anions to elemental selenium. Morphological characterization of the isolate and partial sequencing of its 16S rRNA gene indicated that it belongs to the genus *Bacillus*. Using NS3, it has been demonstrated the biological production of amorphous Se under aerobic conditions offers advantages over chemical processes, in which amorphous Se is produced under environmentally damaging conditions. The effect of the biomass also provides a method of stabilizing this reactive material whereas the washing process provides a facile method for nanorod production. Further investigation of natural environments with anomalous geochemical signatures will provide a range of biomineralizing microbes that can adapt to these chemically hostile environments and have the potential for use in the environmentally friendly manufacture of bionanominerals.

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