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Enzyme Involvement in the Biosynthesis of Selenium Nanoparticles by *Geobacillus wiegelii* Strain GWE1 Isolated from a Drying Oven

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***Abstract*—**The biosynthesis of nanoparticles by microorganisms, on the contrary to chemical synthesis, is an environmentally-friendly process which has low energy requirements. In this investigation, we used the microorganism *Geobacillus wiegelii*, strain GWE1, an aerobic thermophile belonging to genus *Geobacillus*, isolated from a drying oven. This microorganism has the ability to reduce selenite evidenced by the change of color from colorless to red in the culture. Elemental analysis and composition of the particles were verified using transmission electron microscopy and energy-dispersive X-ray analysis. The nanoparticles have a defined spherical shape and a selenium elemental state. Previous experiments showed that the presence of the whole microorganism for the reduction of selenite was not necessary. The results strongly suggested that an intracellular NADPH/NADH-dependent reductase mediates selenium nanoparticles synthesis under aerobic conditions. The enzyme was purified and identified by mass spectroscopy MALDI-TOF TOF technique. The enzyme is a 1-pyrroline-5-carboxylate dehydrogenase. Histograms of nanoparticles sizes were obtained. Size distribution ranged from 40-160 nm, where 70% of nanoparticles have less than 100 nm in size. Spectroscopic analysis showed that the nanoparticles are composed of elemental selenium. To analyse the effect of pH in size and morphology of nanoparticles, the synthesis of them was carried out at different pHs (4.0, 5.0, 6.0, 7.0, 8.0). For thermostability studies samples were incubated at different temperatures (60, 80 and 100 ºC) for 1 h and 3 h. The size of all nanoparticles was less than 100 nm at pH 4.0; over 50% of nanoparticles have less than 100 nm at pH 5.0; at pH 6.0 and 8.0 over 90% of nanoparticles have less than 100 nm in size. At neutral pH (7.0) nanoparticles reach a size around 120 nm and only 20% of them were less than 100 nm. When looking at temperature effect, nanoparticles did not show a significant difference in size when they were incubated between 0 and 3 h at 60 ºC. Meanwhile at 80 °C the nanoparticles suspension lost its homogeneity. A change in size was observed from 0 h of incubation at 80ºC, observing a size range between 40-160 nm, with 20% of them over 100 nm. Meanwhile after 3 h of incubation at size range changed to 60-180 nm with 50% of them over 100 nm. At 100 °C the nanoparticles aggregate forming nanorod structures. In conclusion, these results indicate that is possible to modulate size and shape of biologically synthesized nanoparticles by modulating pH and temperature.

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I. Introduction

IN nature selenium occurs in different oxidation states: elemental selenium (S^0), selenide (Se^{2-}), and selenium oxyions (selenate SeO_4^{2-} and selenite SeO_3^{2-}). Its toxicity is related to the degree of water solubility and hence its bioavailability. Among the most soluble (and toxic) forms, selenium oxyions have attracted a great deal of attention for its applications in microbial reduction and nanoparticle synthesis.

The development of new methods for nanoparticles (NPs) synthesis is currently regarded as a cornerstone in nanotechnology.

Studies on the biological function of selenium and its nanoforms revealed that nano-Se particles efficiently increase the antioxidant activity of glutathione peroxidase and thioredoxin reductase [1]. Reference [2] demonstrated the antioxidant properties of hollow spherical Se NPs. In addition to its antioxidant properties, the nanoparticles show reduced risk toxicity [3]. The size of Se NPs plays an important role in their antioxidant activity. It has been shown when size range from 5-200 nm, nano-Se particles can *in vitro* directly scavenge free radicals in a size dependent fashion [4].

The biogenesis of selenium nanostructures was first reported by reference [5]. Se^0 nanoparticles formed by the Se-respiring bacteria *Sulfurospirillum barnesii*, *Bacillus selenitireducens* and *Selenihalanaerobacter shriftii* are structurally unique compared to those selenium nanoparticles formed by chemical synthesis. These three anaerobic microorganisms use toxic selenium oxyions as electron acceptors during anaerobic respiration, resulting in the formation of stable, uniform selenium nanospheres (diameter ~ 300 nm).

Anaerobic bio-synthesis of nano-Se particles implies the maintenance of reducing conditions through the entire process, making bio-manufacturing optimization and scale up processes very challenging [6]. So, selenium-tolerant aerobic microorganisms may provide an opportunity to overcome these limitations in the biosynthetic processes. Very few studies have reported the aerobic formation of selenium NPs: the generation of selenium nanospheres by soil bacteria *Pseudomonas aeruginosa*, *Bacillus* sp. and *Bacillus cereus* under aerobic conditions has being described [6], [7]. In spite of the importance of biological synthesis of NPs in the development of nanotechnology, the molecular basis and

mechanisms of synthesis remain largely untapped. Synthesis of metallic NPs by bacteria is often achieved by a reduction step followed by a precipitation step. The latest is composed of two parts: nucleation and crystal growth. To date, only the reduction step has been extensively studied and the biological process responsible of NPs formation is not fully understood. Several studies [8]-[13], provide evidence that proteins might play a key role in growth of bacteriogenic metal NPs. The understanding of these mechanisms will help to control and modulate the molecular switches that regulate size, shape and stability of NPs [14].

In this context, reported evidence indicates that proteins might play a key role in the nucleation and crystal growth of bacteriogenic metal NPs. These proteins have reductase activity and the reduction process seems to be initiated by an electron transfer from NADH [15]. For selenium reduction and selenium NPs formation in *Bacillus cereus* [6], [7] membrane associated reductases has been described to participate in the process.

Notably, the generation of metallic nanoparticles can take place only in the presence of a specific enzyme, without requiring the presence of the whole microorganism. This indicates that for NPs biogeneration, full metabolic machinery of the microorganism is not needed.

Here, we report the synthesis of Se NPs mediated by NADH-dependent enzymes by, a thermophilic aerobic extremophile isolated from a drying oven [16] capable to reduce Se^{4+} to Se^{0}.

II. Methods

A. Bacterial Strains and Culture Conditions

Geobacillus wiegelii, strain GWE1 was isolated as described in reference [16] from a drying oven. Cells were grown in rich liquid modified marine medium containing: 2.5 g/L yeast extract, 2.5 g/L peptone, 0.0025 g/L citrate, 1.5 g/L maltose, 0.6 g/L NH_4Cl, 17.5 g/L NaCl, 1.75 g/L $MgSO_4$, 0.16 g/L KCl, 0.38 g/L $CaCl_2$, 0.25 g/L KH_2PO_4, 0.025 g/L NaBr, 0.0075 g/L H_3BO_3, 0.0038 g/L $SrCl_2$, 0.025 g/L KI, 0.0055 g/L $FeCl_3$, 0.0025 g/L $MnSO_4$, 0.0015 g/L Na_2WO_4 x $2H_2O$, 0.001 g/L $NiCl_2$, 0.0005 g/L $CoSO_4$, 0.0005 g/L $ZnSO_4$, 0.00005 g/L $CuSO_4$, 0.00005 g/L Na_2MoO_4, by 22 h at 70°C and pH 5.8.

B. Enzyme Purification

The enzyme was purified from *Geobacillus wiegelii,* strain GWE1 cells at 23ºC. 20 grams of cells were resuspended in 50 mM Tris-HCl pH 8.0 buffer containing 15 mM EDTA pH 8.0, lysozyme (1 mg/ml) and DNase I (10 µg/ml) and treated as described in reference [17]. The crude extract was loaded onto a Q-Sephrose Fast Flow column (C 16/20, Pharmacia Biotech) equilibrated with 50 mM Tris-HCl pH 8.0. After the column was washed with the same buffer, the enzyme was eluted with a linear gradient from 0 to 1 M NaCl. Samples with selenite reduction activity started to elute as 0.23 M NaCl was applied to the column. The fractions were subjected to selenite reduction assay (section D). Fractions with activity were combined and applied to a column (C 10/10, Pharmacia) of DEAE-Sepharose Fast Flow (Pharmacia Biotech) equilibrated with 50 mM Tris-HCl pH 8.0 buffer. The elution of the protein loaded in the column was performed with a linear gradient of NaCl 0 to 1 M. Selenite reduction activity started to elute at 0.65 M NaCl. The fractions containing selenite reduction activity were combined and applied to a Q-Hitrap column (Pharmacia Biotech), equilibrated with 50 mM Tris-HCl pH 8.0 buffer. The fractions with selenite reduction activity started to elute at 0.11 M NaCl. The fractions were stored at 4ºC. All columns were controlled by a Pharmacia FPLC system.

C. Enzyme Identification

The enzyme under study was identified by MALDI TOF/TOF in the National Center for Biotechnology, Spain.

D. Selenite Reduction Assay of Obtained Fractions from Purification Process

100 µL of each fraction obtained in the purification process were incubated separately with 10 mM NADH and 100 mM Na_2SeO_3 x $5H_2O$ at 37°C, for 12 h. The presence of a red precipitate was indicative of selenite reduction.

E. Biosynthesis of SeNPs and Enzyme Assay

In order to determine the NADH dependence in selenite reduction by intracellular enzymes involved in the biological transformation, crude extract and/or purified enzyme were incubated with different concentrations of NADH (0-10mM) and 100 mM Na_2SeO_3 x $5H_2O$ at 37°C for 12 h. The reaction was followed by measuring the change in absorbance at 340 nm in a spectrophotometer. The presence of a red precipitate was indicative of the selenite reduction.

F. Effects of pH on SeNPs Size and Morphology

Se NPs were synthesized at 35ºC by the purified enzyme when incubated in buffers at different pH (4.0, 5.0, 6.0, 7.0, 8.0). For this purpose, 25 mM sodium acetate at pH 4.0 and 5.0; 25 mM MES at pH 6.0 and 7.0; 25 mM EPPS at pH 8.0, were used. Se NPs formed were observed by transmission electron microscopy.

G. Effects of Temperature on SeNPs Size and Morphology

Se NPs synthesized by purified enzyme at pH 6 were incubated at 60, 80 and 100ºC for 1 h. Then SeNPs were cooled in ice until room temperature and were observed by transmission electron microscopy.

H. Thermostability of SeNPs

Se NPs synthesized by the purified enzyme at 37 ºC and pH 6.0, were incubated at 60, 80, 100 ºC for 3 h. After this time, Se NPs were cooled in ice until room temperature. The changes experimented by Se NPs were observed by transmission electron microscopy.

I. Transmission Electron Microscopy Measurements (TEM)

The sample was prepared on a carbon coated copper grid and dried at room temperature. For NPs size determination, bacteria were recovered as described in reference [18]. TEM

measurements were performed on a Phillips Tecnai 12 Bio Twin TEM operating at 200 kV. Sizes of nanoparticles were determined using a NIS-Elements D 3.10 software. Histogram size distribution was constructed with Sigma Plot 11.0 software.

J. Energy-Dispersive X-Ray Microanalysis (EDX).

Elemental analyses of NPs were conducted by energy-dispersive X-ray microanalysis**,** using a scanning electron microscope (SEM) Jeol 5410 equipped with an energy dispersive X-ray spectrometer.

III. Results and Discussion

NADH-dependent reductase enzymes have been reported to be involved in the synthesis of NPs. The reduction might be initiated by electron transfer from NADH, reducing selenite to elemental selenium with the concomitant biogenesis of SeNPs by *B. cereus* [6], [7]. The molecular mechanism associated to this reaction is still unknown.

The role of proteins from crude extract of *Geobacillus wiegelii*, GWE1 in the reduction of $Se+^{4}$ was studied. The biocatalytic reaction was assayed incubating crude extract with Na_2SeO_3 x $5H_2O$ and NADH. Crude extract from *Geobacillus wiegelii,* GWE1 was able to catalyze the NADH-dependent Se^{+4} reduction (Figs. 1 (a) and (b)).

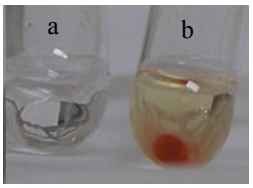

Fig. 1 Enzymatic selenite reduction by crude extract of *Geobacillus wiegelii* GWE1 and NADH. Reddish color in tube (b) indicates SeNPs formation. Tube (a) is a control without NADH

The presence of different enzymes in the crude extract could be mediating the SeNPs synthesis, resulting in the generation of different size nanoparticles. When the whole microorganism is used for the synthesis of NPs the size of the NPs obtained is different than the size obtained when only the crude extract is used for their synthesis. This is probably due to the natural limits imposed by the cell in the first case where the SeNPs obtained were smaller than the ones obtained when the crude extract is used.

Crude extract of *Geobacillus wiegelii* GWE1 was loaded in a column to purify the enzyme involved in SeNPs biosynthesis. Because the enzyme and its specific reaction were unknown we assayed the selenite reduction in each step of purification. The tubes in which a red precipitate was formed were selected (positive reaction) and loaded into the second and third column, respectively. After purification steps, a SDS-PAGE was performed. At the end of purification, six bands were observed (Fig. 2). The band with higher amount of reduced product was sent to identify by mass spectroscopy MALDI-TOF TOF. The results indicate that one of the enzymes implicated in this process of reduction is a 1-pyrroline-5-carboxylate dehydrogenase (P5CDH). This enzyme is the second enzyme involved in proline metabolism, catalyzing the NAD^{+}-dependent oxidation of L-glutamate-γ-semialdehyde to L-glutamate.

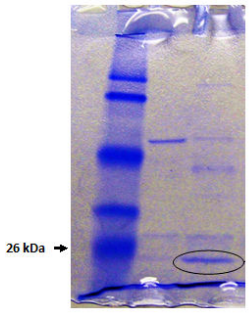

Fig. 2 SDS-PAGE and selenite reduction assay for the fractions (2 and 5) obtained from Q-Hitrap with selenite reduction activity. MW: molecular weight. The circle indicates the band with a protein of 26 kDa sent for identification

SeNPs synthesized by P5CDH purified enzyme from *Geobacillus wiegelii*, GWE1 crude extract were observed by TEM and EDX. Histogram distribution of sizes was constructed using Statgraphics Centurion XV software. NPs sizes were obtained using NIS-Elements D 3.10 software. SeNPs synthesized by P5CDH were spherical (Fig. 3 (a)) of different sizes and smaller than the synthesized using the whole microorganism. SeNPs sizes ranged from 40-160 nm, where 20% of them were of 100 nm (Fig. 3 (b)). EDX analysis confirmed that the elemental composition of NPs was selenium (Fig. 4).

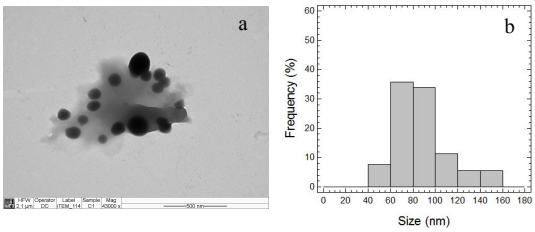

Fig. 3 SeNPs biosynthesized by pyrroline-5-carboxylate dehydrogenase enzyme purified from *Geobacillus wiegelii* GWE1 crude extract at 35 ºC. (A): Transmission electron micrograph on a copper grille. Small black spots correspond to SeNPs; (B): Histogram showing the selenium nanoparticle size distribution

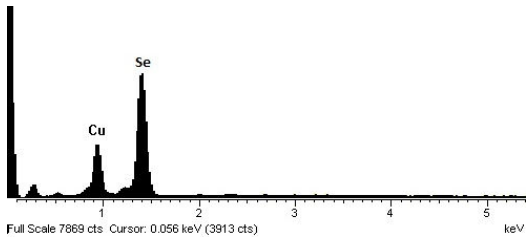

Fig. 4 EDX analysis of SeNPs formed by reduction of Na_2SeO_3 by *Geobacillus wiegelii* GWE1 cells. Strong signals (Black arrows) from the selenium can be observed

With the purpose of controlling size and shape of SeNPs, different pH was used for the synthesis of NPs (Fig. 5) and different temperatures were tested for post-synthesis incubation (Fig. 6). At pH 4.0 (Fig. 5 (a)) 100% of SeNPs were less than 100 nm in size; at pH 5.0 (Fig. 5 (b)) over 50% of their size was less than 100 nm; at pH 6.0 (Fig. 5 (c)) and at pH 8.0 (Fig. 5 (d)) over 90% of SeNPs size was less than 100 nm.

Our experimental observations indicate that acid and basic pH favor the formation of NPs with sizes below 100 nm. At neutral pH (7.0) the NPs were bigger with a size around 120 nm. Only 20% of them were less than 100 nm. These results indicate that it is possible to modulate the size of SeNPs by changing the pH.

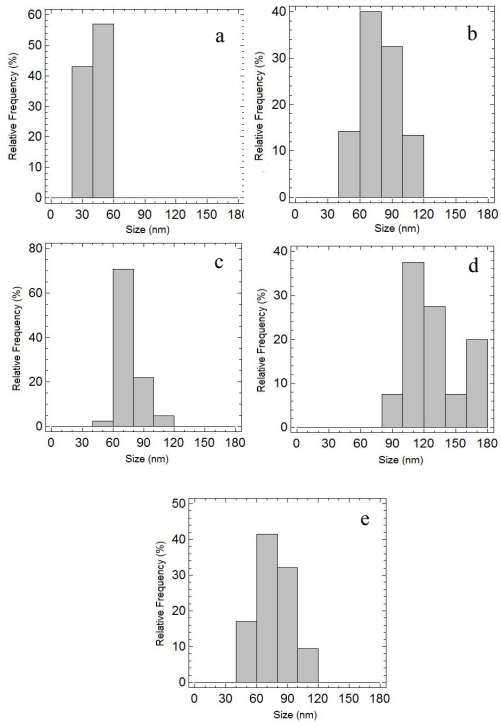

Fig. 5 Histograms of SeNPs synthesized at different pH. Buffer with pH: (a) 4.0, (b) 5.0, (c) 6.0, (d) 7.0 and (e) 8.0.

After the synthesis, SeNPs were incubated at different temperatures for 1 h. When the temperature was 60ºC (Fig. 6 (a)) the NPs did not show a significant difference compared to the control (Fig. 3 (b)). At 80 °C a range size from 20-160 nm was observed, with 35% of them over 100 nm (Fig. 6 (b)). At 100ºC (Fig. 6 (c)), SeNPs had a size between 60-200 nm, with 70% of them over 100 nm.

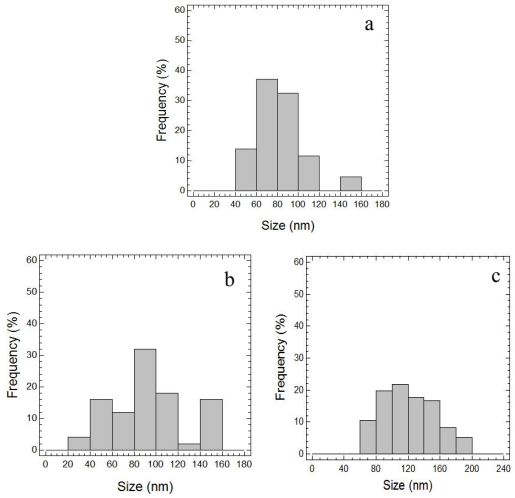

Fig. 6 Effect of temperature on NPs synthesized enzymatically at 35ºC in water. Histogram of NPs size incubated for 1h at: (a) 60°C, (b) 80°C and (c) 100°C

For thermostability measurements, at 60ºC after 3 h of incubation (Fig. 7 (a)) the NPs did not show a significant difference compared with the control (Fig. 3 (b)). At 80°C after 3 h of incubation (Fig. 7 (b)) NPs sizes range from 60-180 nm with 50% of them over 100 nm, showing a significant difference with the control (Fig. 3 (b)), loosing homogeneity.

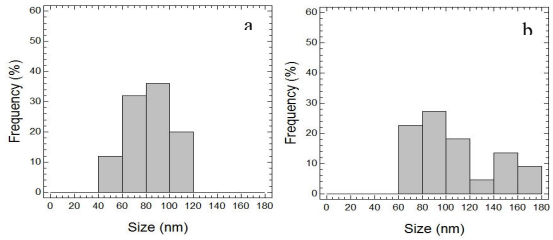

Fig. 7 Effect of temperature on NPs enzymatically synthesized at 35ºC. Histogram of NPs size incubated for 3h at: (a) 60 °C; (b); 80°C

When the temperature was increased to 100ºC, after 2 hours of incubation, the NPs formed nanorods structures (Fig. 8) and large aggregates after 3h of incubation at this temperature. These results demonstrate that at higher temperatures (over 60ºC) NPs tend to form larger aggregates and rod structures. All these transformations occur in the presence of NADH and the purified enzyme. In conclusion, the experimental results presented indicate that biosynthesis of SeNPs is a redox enzymatic process mediated by some enzymes and NADH. Additionally, it is not necessary the presence of the whole microorganism to generate the nanoparticles. Size and shape of SeNPs can be modulated by pH and temperature.

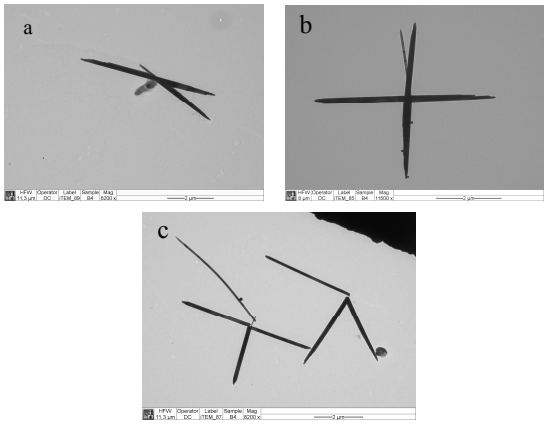

Fig. 8 NPs incubated for 3 h at 100°C. (a), (b) and (c) larger structures formed called nanorods

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