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Research Article

Synthesis of Selenium Nanoparticles by *Geobacillus wiegelii* strain GWE1 Isolated from a Drying Oven

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Abstract

Microorganisms play an important role absorbing and accumulating metals from their environments. In some cases this process leads to the formation of well-defined metallic nanoscale particles. These biologically synthesized nanoparticles differ from the chemically produced in their physical properties and stability, opening additional technological opportunities for their use. In this study, the thermophilic bacterium *Geobacillus wiegelii* strain GWE1 was able to produce selenium nanoparticles. Cultures exposed to Na_2SeO_3 turned from colourless into an intense red colour. This is indicative of salt reduction confirming the presence of elemental selenium. The use of 5% polyvinylpyrrolidone (PVP) in the culture medium reduced the size of the nanoparticles from 250 nm to 120 nm (52% smaller). This method to produce selenium nanoparticles by *Geobacillus wiegelii* strain GWE1 was performed as an approach for biosynthesis of selenium nanoparticles at a pilot-scale process.

Keywords: *Geobacillus*; Nanoparticles; Biosynthesis; Selenium

Introduction

Selenium as part of the amino acid selenocysteine is present in numerous proteins and essential to all living cells [1]. However, at high concentrations, this element, predominantly present in the form of selenate and selenite oxyanions, is toxic and may cause some environmental problems [2]. Microorganisms play an important role absorbing and accumulating metals, leading to the detoxification of some contaminated environments. The microbial capability to oxidize selenium species is attributed to reduction and accumulation of the red amorphous Se^0 formed in the cell [3,4]. In some species like *Thauera selenatis*, *Sulfurospirillum barnesii*, *Bacillus selenitireducens*, or *Bacillus arsenicoselenatis* [5-7], the reduction of selenate is involved in a respiratory pathway. A variety of microorganisms can generate nanoscale structures, called nanoparticles, through the reduction of certain ions, such as selenium, gold, and silver, among others [8-10]. Classically, nanoparticles (NPs) are described as "structures with at least one dimension of 1 to 100 nm" [11]. However, there is another definition of nanoparticles, more related with their implementation and defines them as structures

with at least one dimension between 1-1000 nm [12,13]. Biologically synthesized NPs, are stable and have physical properties that confers them technological opportunities for their industrial use [11]. Such is the case of amorphous selenium nanoparticles (SeNPs). Their characteristics are to have a unique photoelectric, semiconducting and X-ray-sensing properties with multiple applications in medical diagnostics [8], solar cells, rectifiers, photographic exposure meters and xerography [9]. They show biological activity and good adsorptive ability due to their interaction with NH, C=O, COO- and C-N groups of proteins [14]. Even more they can have incorporated this type of functional groups when they are biologically synthezised.

Currently, chemical synthesis of NPs involves conditions such as high temperature, high pressure and the use of toxic chemicals and organic solvents. These conditions are not only cost-intensive, hazardous and contaminant, but also the use of organic solvents over NPs surface restricts their applications. Opposite to this, biological synthesis of NPs has increased scientific and industrial interest. These biological methods have been considered biocompatible, nonhazardous, and ecofriendly. Additionally microorganisms have the natural ability to control the structure, phase, orientation, and nanostructural topography of inorganic crystals [15]. A recurring problem in the synthesis of NPs is the control of their size and shape of them. One way to solve this problem is the use of additives in the process of synthesis, such as polyvinylpyrrolidone PVP [16]. This non-toxic compound is a water soluble polymer formed by multiple chains of vinylpyrrolidones.

Cells of *Geobacillus wiegelii* strain GWE1exposed to Na_2SeO_3 were able to perform selenite reduction and to produce elemental selenium nanoparticles. Microscopic analysis showed the intra and extracellular SeNPs accumulation.

This report is focussed on the synthesis of SeNPs by *Geobacillus wiegelii* strain GWE1 and the effect of PVP on their size modulation. The microorganism used is a thermophile isolated from a drying oven and was characterized by Correa-Llantén *et al*., 2013 [17].

Materials and Methods

Bacterial strains and culture conditions

Geobacillus wiegelii strain GWE1 was isolated as described by Correa *et al*. [17] 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 sodium 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, during 22h at 70°C and pH 5.8 in a micro aerophilic ambient. For nanoparticles biosynthesis a 0,526 g/L of sodium selenite pentahydrate was used.

Biosynthesis and recovery of SeNPs

Geobacillus wiegelii produce intra and extracellular NPs. So, to recover all of them is necessary to use a method to obtain intracellular NPs without the microorganism. Recovery process was modified from Shakibaie *et al*. (2010) [18]. Cells from a culture with OD_{600}~0.5 were inoculated in modified liquid marine medium supplemented with 2 mM Na_2SeO_3 and incubated for 22h. Cells were harvested by centrifugation at 8,200g for 20 min (4°C) and washed with 9 g/L NaCl. The pellet was dried for 3h at 60°C and cells were disrupted with liquid nitrogen and a stem sonicator for 1h. Crude extract was centrifuged at 8,200g for 10 min (4°C) and pellet was washed with 1.5 M Tris-HCl buffer at pH 8.0 containing 10 g/L SDS. NPs were suspended in 4 mL of deionized water and mixed vigorously with 2 mL of 1-octanol. NPs were centrifuged at 2,000g (4°C) for 5 min and kept at 4°C over night. NPs were recovered and the supernatant discarded. Finally, NPs were washed with ethanol and deionized water.

Optimization of pH and temperature for production of SeNPs with a factorial design 2^2

A design of surface response experiments with two levels and two factors (pH, temperature) was performed to determine the optimal pH and temperature for NPs production. Levels for the optimal pH were 4.8 and 6.8 and temperature levels were 60 and 80°C respectively. The core values were 5.8 for pH and 70°C for temperature, which correspond to the optimal values for growth of the microorganism [17]. The matrix of experiment is showed in Table 1. The response of the experimental design for elementary selenium production was determined by measuring absorbance at 500 nm of a recovered NPs solution.

Experiment	1	2	3	4	5	6	7
Temperature (°C)	60	80	60	80	70	70	70

Table1. Optimization of pH and temperature for production of SeNPs by *Geobacillus wiegelii* strain GWE1 with a factorial design 2^2.

NPs formation kinetics

For a large scale production of NPs a kinetic study for the formation of them was performed in a bioreactor of 5 L Biostat B Plus model (Sartorius Stedim). Modified marine medium supplemented with 2 mM of sodium selenite was used as culture medium. Fermentation was performed with 50 rpm stirring

without aeration. A sample was taken every 1h and the optical density at 600 nm was measured with a spectrophotometer to construct the microorganism growth curve. Culture samples were taken at 6, 8, 10, 12, 14h of incubation to recover the NPs using the method previously detailed.

Effect of polyvinylpyrrolidone (PVP) as modulator and stabilizer of NPs size.

Kinetics of NPs production in a bioreactor of 5 L Biostat B Plus model was studied as described above. Similar experiment was performed with the addition of 3% and 5% of PVP (molecular weigth 40,000) to the culture medium, as described by Zhang [16]. The temperature and pH conditions used for these experiments correspond to the optimal conditions, already determined.

SeNPs characterization

Transmission electron microscopy measurements (TEM)

A sample of purified NPs was dropped on a carbon coated copper grid and dried at room temperature. For the size determination of NPs, bacteria were recovered as described by Shakibaie *et al*., [18]. TEM measurements were performed on a Phillips Tecnai 12 Bio Twin TEM operating at 200 kV equipped with digital Olympus Megaview camera. NPs sizes were obtained using the software NIS-Elements D 3.10. Size distribution histograms were constructed with Statgraphics Centurion XV software.

Energy-Dispersive X-Ray Microanalysis (EDX)

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

Results

A culture of *Geobacillus wiegelii* strain GWE1 was inoculated in marine modified medium supplemented with 2 mM of Na_2SeO_3 x 5 H_2O. A change of colour from pale yellow (Figure 1A) to intense red (Figure 1B) was seen after incubation for 22h at 70°C, indicative of selenite reduction. Control experiments, without the addition of biomass to the media with Se salt showed no change in colour of medium. The biosynthesis of NPs was confirmed by transmission electron microscopy (TEM). Figure 1C shows intra and extracellular NPs formation. The recovery of NPs was confirmed by TEM (Figure 1D) and the average size obtained was 250 nm. Selenium elemental composition of NPs was confirmed by energy-dispersive X-ray microanalysis (EDX) (Figure 1E).

To improve selenite reduction in the culture, optimal pH and temperature were determined using a factorial design 2^2. The experimental response was elementary selenium production (mg/mL) in the form of a purified NPs suspension. The results indicate that the optimal temperature and pH for NPs production were 60°C and 6.3, respectively.

The NPs formation kinetic study was performed in a bioreactor of 5 L. The reduction of selenite is associated to *Geobacillus wiegelii* strain GWE1 growth as is shown in Figure 2A, and the final production of selenium is about 0,03 mg/mL. This represents only 20% of Se supplemented reduction.

Histograms in Figure 2B-F show the NPs size obtained at different times along the growth curve of *Geobacillus wiegelii* strain GWE1 in the presence of selenite salt. At 6h of fermentation (Figure 2B), 60% of NPs were in the range of 100-200 nm of size. At 8h all of NPs were in the range of 100-200 nm of fermentation (Figure 2C). After 10h of fermentation (Figure 2D) 45% of NPs were in the range of 200-300 nm and 55% were in the range 100-200 nm. The average size of purified NPs in the final suspension (14h of fermentation) was 250 nm (Figure.2F) and over 90% of NPs obtained were in the range 100-300 nm of size. Furthermore, it was also possible to observe that the microorganism was able to generate spherical NPs and selenium nanorods.

To control NPs size, PVP was added to the fermentation, at 3 and 5% in separated assays. A control ccontaining medium and PVP was carried out and there was no change in color and reduction of selenite observed. At 3% of PVP, selenite reduction was 46% higher (Figure 3A) than the reduction observed without PVP (Figure 2A). Histograms in Figure 3B-F, show the size of NPs in the fermentation process after 6 hours. At this time in the process is where an increase on selenite reduction was observed. In this case, the final average size of NPs (Figure 3F) was 250 nm, similar to the control without PVP (Figure 2F). Over 75% of NPs are in range of 200-400 nm of size.

When 5% of PVP was used, the reduction was 89% higher than when no PVP was added (Figure 4A). Figure 4B-F shows histograms of NPs growth in fermentation containing 5% of PVP after 6 hours of incubation. The final size of nanoparticles was 120 nm, 52% smaller than NPs from fermentation without PVP. For a fermentation using 5% of PVP no significant difference in NPs size was observed between 8h and 14h of incubation (Figure 5).

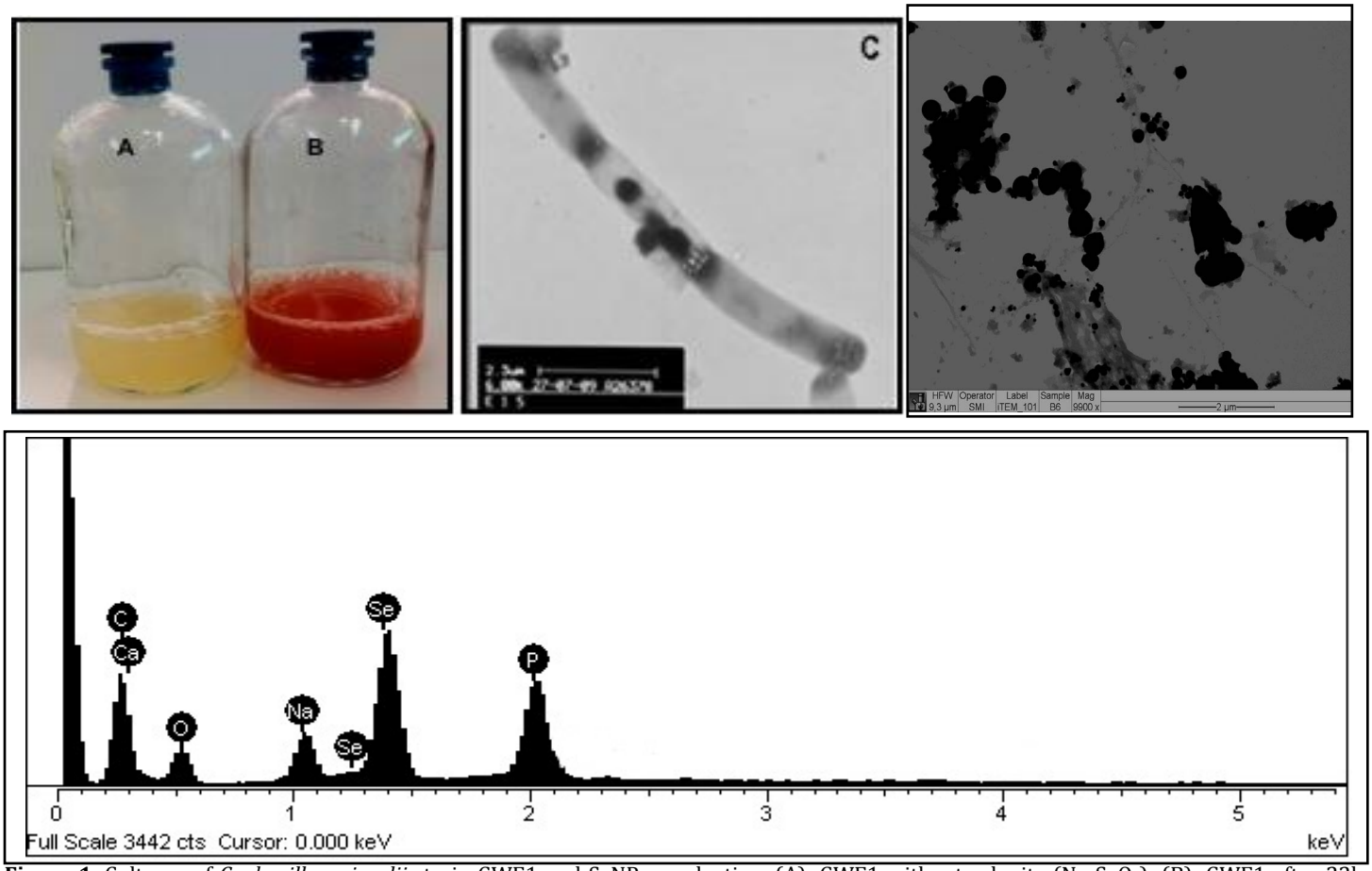
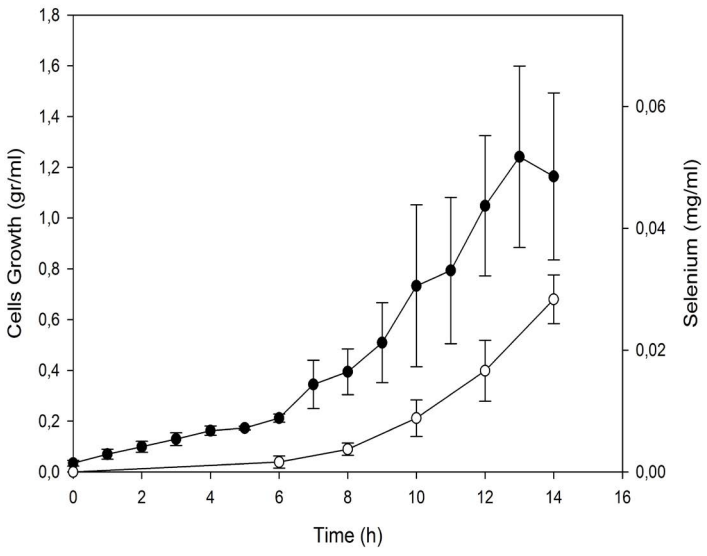
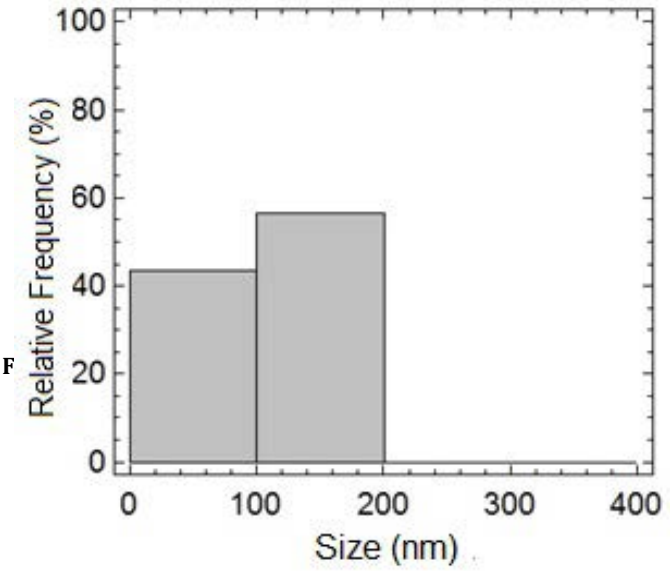


Figure 1. Cultures of *Geobacillus wiegelii* strain GWE1 and SeNPs production. (A): GWE1 without selenite (Na_2SeO_3); (B): GWE1 after 22h incubated with sodium selenite; (C): Transmission electron micrograph. Small black spots correspond to SeNPs; (D): SeNPs produced by the microorganism on a copper grille.





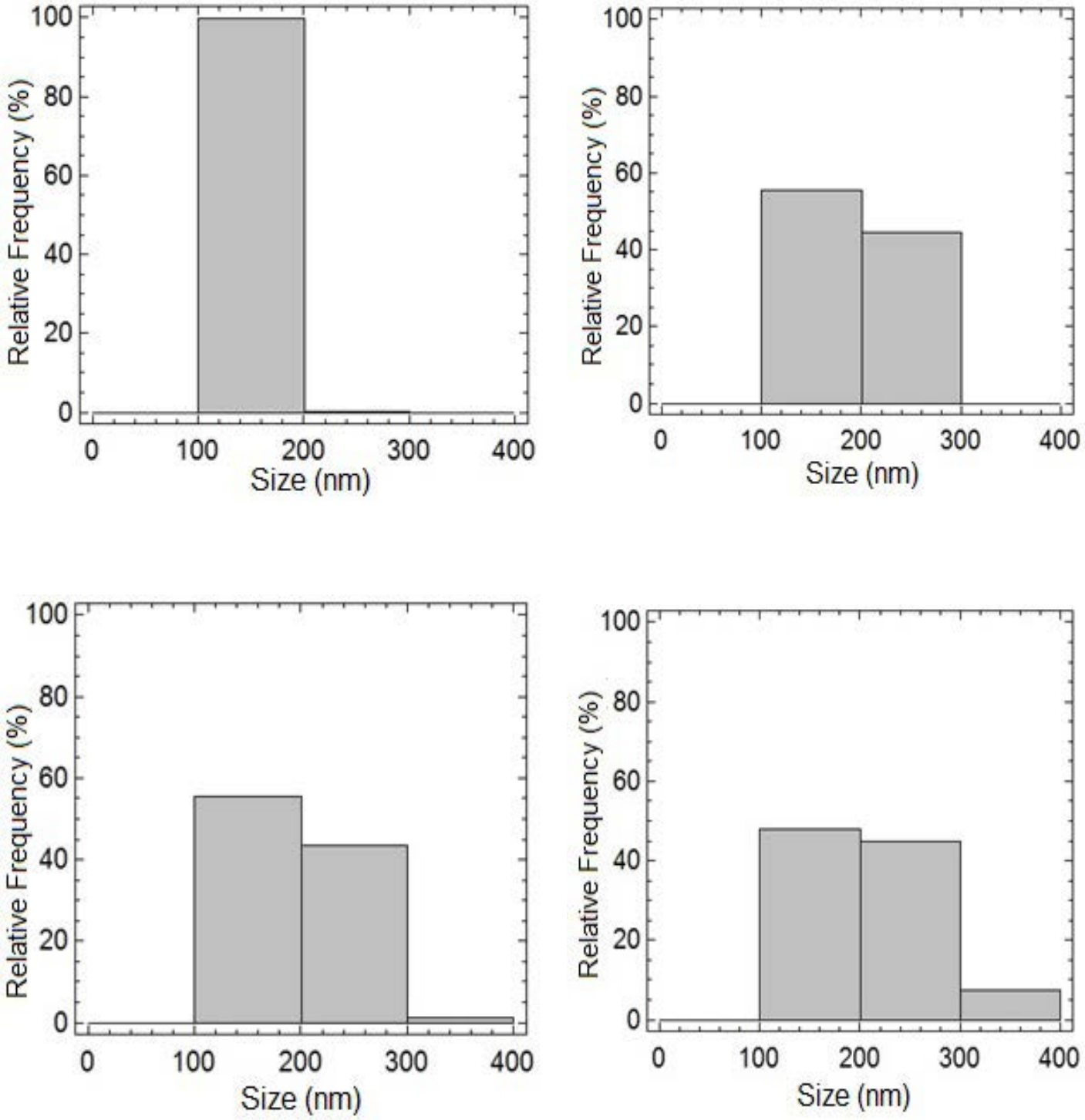
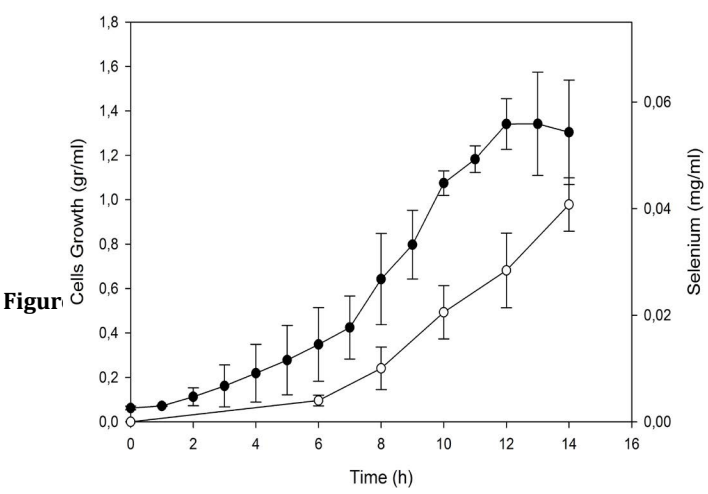
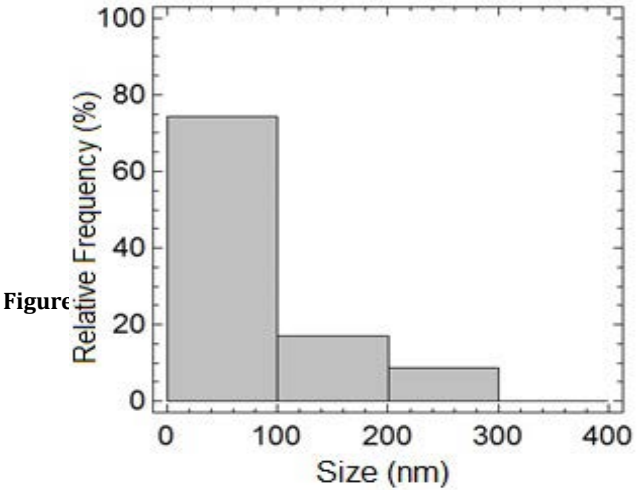
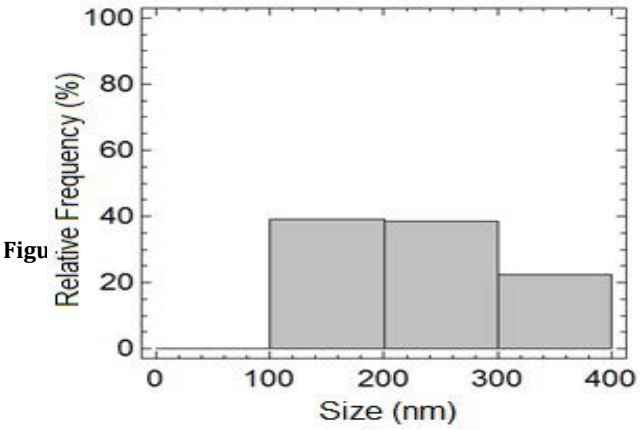
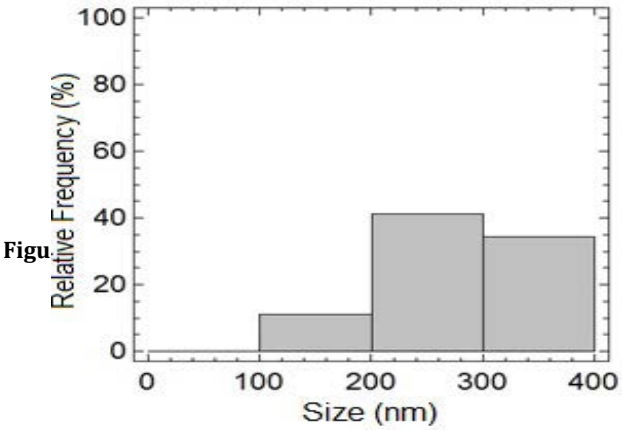


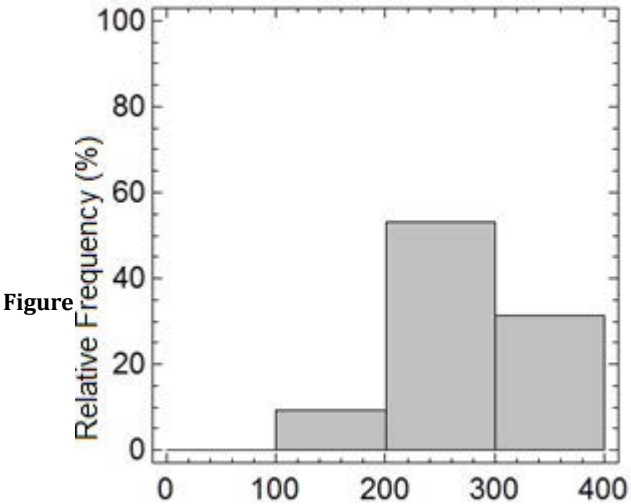
Figure 2. Fermentation process, growth curve of *Geobacillus wiegelii* strain GWE1 and production of SeNPs. (A) Cells growth (g/mL) (•), production curve SeNPs (mg/mL) (○). Histograms with SeNPs size distribution synthesized by GWE1 at different hours of incubation (B): 6h; (C): 8h; (D): 10h; (E): 12h; (F): 14h.











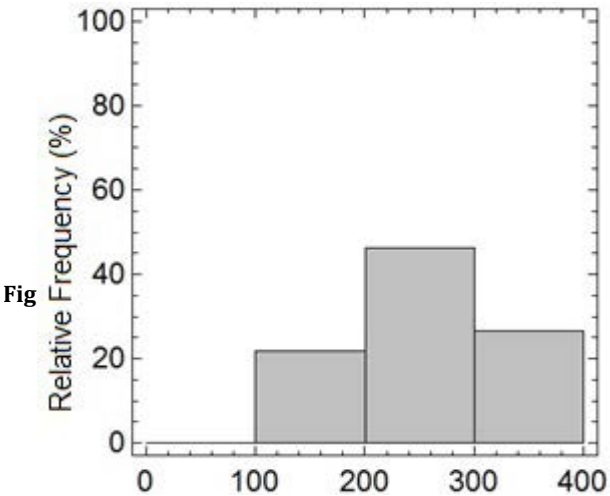


Figure 3. Growth curve and production of SeNPs by *Geobacillus wiegelii* strain GWE1 in modified marine medium supplemented with 2 mM Na_2SeO_3 in the presence of 3% PVP. (A): Cells growth (g/mL) (•), curve of SeNPs production (mg/mL) (○). Histograms with SeNPs size distribution synthesized by GWE1 at different times of incubation using 3% PVP as modulator (B): 6h; (C): 8h; (D): 10h; (E): 12h; (F): 14h.

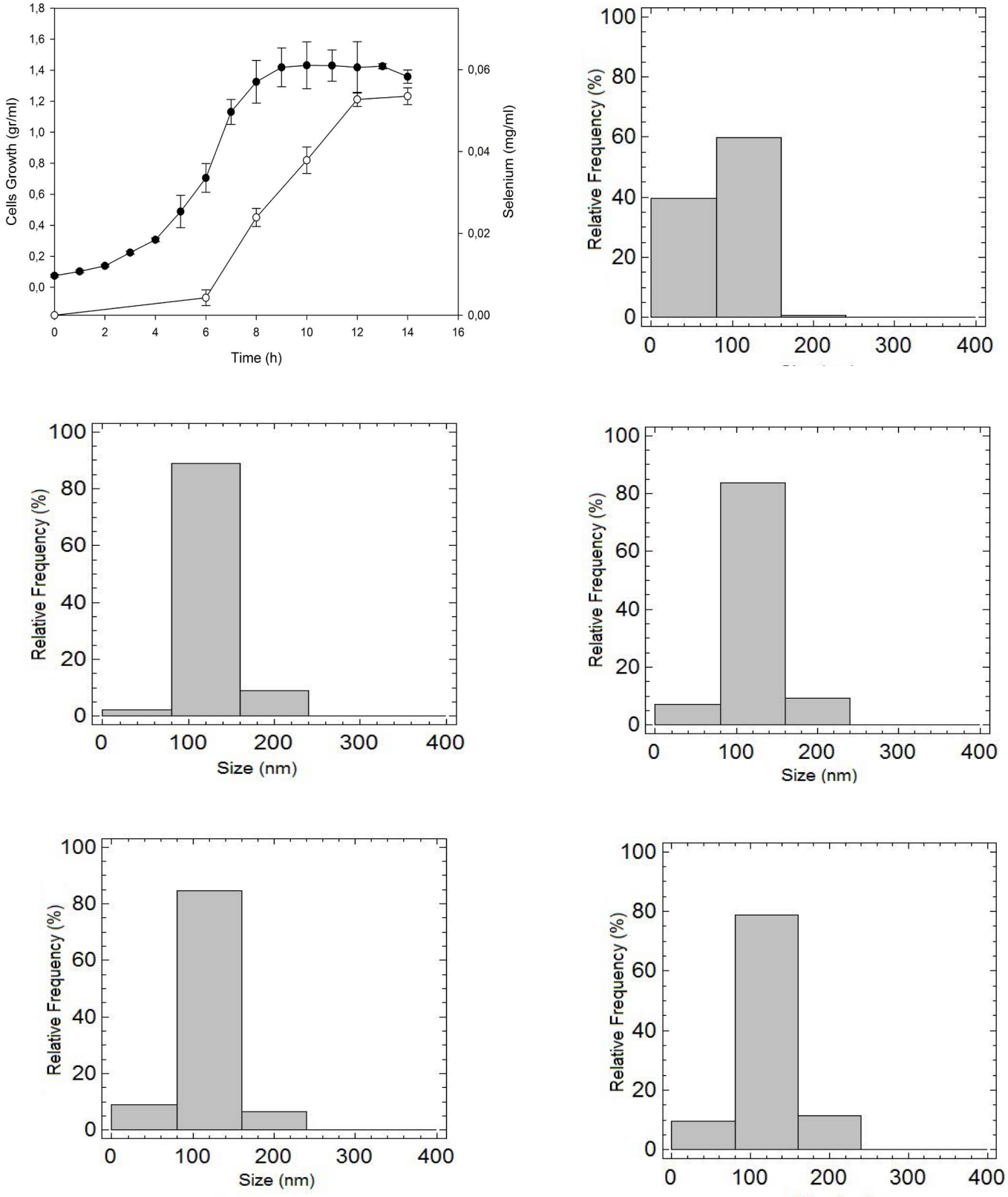


Figure 4. Growth curve and production of SeNPs by *Geobacillus wiegelii* strain GWE1 medium supplemented with 2 mM Na_2SeO_3 and 5% PVP. (A): Cells growth (g/mL) (•), curve of SeNPs production (mg/mL) (○). Histograms with SeNPs size distribution synthesized by GWE1 at different times of incubation, using PVP 5% as modulator (B): 6h; (C): 8h; (D): 10h; (E): 12h; (F): 14h.

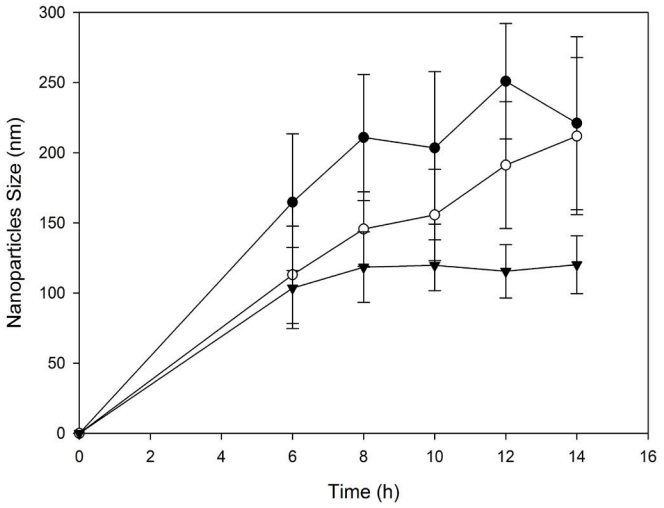


Figure 5. NPs size obtained at different PVP concentrations in time. 0% PVP (•); 3% PVP (○); 5% PVP (▼). Fermentation using 5% of PVP does not show significant difference in NPs size between 8h and 14h of incubation.

Discussion

The biosynthesis of SeNPs by the thermophilic bacterium *Geobacillus wiegelii* strain GWE1 isolated from drying oven [17] was carried out using whole cells.

So far, there are only two other species of *Geobacillus* reported that are capable of generate NPs. *Geobacillus stearothermophilus* is able to generate silver and gold NPs [8] and *Geobacillus* sp. ID17 isolated from Antarctica can produce gold NPs [19].

Geobacillus wiegelii strain GWE1 is able to reduce selenite to elementary selenium with the generation of spherical NPs, with potential applications as biosensors [14] or antioxidant agents [20]. The synthesis of NPs by a microorganism represents advantages over other current chemical synthesis methods, due to the improvement and control of size and shape of NPs in comparison to the physical and chemical methods currently used.

To maximize the amount of NPs obtained with the highest purity and required quality, the recovery of NPs from cells is a critical step in the production process. Recovery process was carried out using a protocol modified from Shakibaie *et al*. (2010) [18]. The liquid nitrogen method leaded to improve the purity of the final product, as seen in TEM images obtained (Figure 1D).

To control the NPs size, PVP was added to the fermentation. PVP acts as modulator of size and shape of NPs improving their quality [Shakibaie *et al*., 2010].

From the two percentages of PVP used in this study as size modulator, size control ability of PVP at 5% was confirmed in this study. Meanwhile concentration of 3% PVP was not always effective on controlling the size of NPs, since the final size of NPs obtained was the similar to the size obtained without using PVP (Figure. 2F and 3F). In contrast when using 5% PVP a 52% decrease in the size of NPs recovered was observed.

Conclusion

SeNPs were biologically synthetized by the microorganism *Geobacillus wiegelii* strain GWE1. Through controlling pH, temperature, and by the addition of 5% of PVP in the production of SeNPs, it is possible to have a homogenous suspension of nanoparticles with a final size of 120 nm. The kinetic of the process suggests that is possible to produce SeNPs of different size and shape with potential industrial application.

Acknowledgment

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