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Determination of size and mass-and number-based concentration of biogenic SeNPs synthesized by lactic acid bacteria by using a multimethod approach

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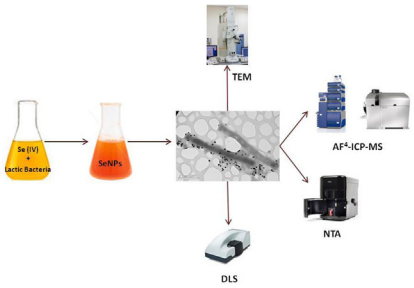
HIGHLIGHTS

- Monodispersed and spherical SeNPs were synthesized by using lactic acid bacteria.
- A procedure based on the use of SDS and NaOH was employed to isolate SeNPs from bacteria cells.
- SeNPs were characterized by TEM, DLS, NTA, AF^4-ICP-MS and AF^4 off line coupled to DLS.
- Number-based concentration data obtained experimentally by NTA and by theoretical calculations were in good agreement.
- Data obtained by independent techniques were in good agreement.

GRAPHICAL ABSTRACT



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ABSTRACT

Selenium nanoparticles (SeNPs) were synthesized by a green technology using lactic acid bacteria (LAB, *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus* and *L. reuteri*). The exposure of aqueous sodium selenite to LAB led to the synthesis of SeNPs. Characterization of SeNPs by transmission electron microscopy with energy dispersive X-ray spectrum (EDXS) analysis revealed the presence of stable, predominantly monodispersed and spherical SeNPs of an average size of 146 ± 71 nm. Additionally, SeNPs hydrodynamic size was determined by dispersive light scattering (DLS) and nanoparticle tracking analysis (NTA). For this purpose, a methodology based on the use of surfactants in basic medium was developed for isolating SeNPs from the bacterial pellet. The hydrodynamic size values provided by DLS and NTA were 258 ± 4 and 187 ± 56 nm, respectively. NTA measurements of number-based concentration reported values of $(4.67 \pm 0.30) \times 10^9$ SeNPs mL^{-1} with a relative standard deviation lower than 5% (n = 3). The quantitative results obtained by NTA were supported by theoretical calculations. Asymmetrical flow field flow fractionation (AF^4) on line coupled to the inductively couple plasma mass spectrometry (ICP-MS) and off-line coupled to DLS was further employed to characterize biogenic SeNPs. The distribution of the particle size for the Se-containing peak provide an average size of (247 ± 14) nm. The data obtained by independent techniques were in good agreement and the developed methodology

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could be implemented for characterizing NPs in complex matrices such as biogenic nanoparticles embedded inside microbial material.

1. Introduction

The synthesis of nanoparticles (NPs) of different sizes, morphologies, compositions, shapes and controlled dispersions is a topic of interest in nanotechnology. Currently, nanoparticles are prepared by means of chemical reactions which imply the use in many cases of hazard chemicals and harsh conditions. During many years, biotechnology has employed microorganisms for the remediation of toxic metals. However, it has been recently when microorganism along with plants and fungi are used for preparing nanoparticles.

The biogenic synthesis of metallic and non-metallic NPs by using biological systems such as microorganisms and plant extracts has gained in acceptance in the last years due to its simplicity, low cost and biocompatibility of the resulting nanostructures with biomedical applications [1–3]. Other advantages over synthetic nanoparticles have been claimed for biogenic ones; for example, it has been reported that biogenic nanoparticles are more stable, do not need the addition of coating agents, and could have an enhanced antimicrobial effect comparing to their synthetic counterparts due to the presence of bacterial membrane components in the nanoparticles [4]. Bacteria has been used for the biogenic synthesis of silver nanoparticles [5], tellurium nanoparticles [6,7] and selenium nanoparticles [4,8], among others. Among bacteria tested for producing biogenic nanoparticles, lactic acid bacteria (LAB) have the advantage of possessing the GRAS (Generally Recognized As Safe) and Qualified presumption of safety (QPS) status for which they are commonly used in food fermentations. Therefore, these bacteria are cheap and easy and safety to handle making them very interesting from producing metallic and non-metallic nanoparticles. It has been reported that some lactobacilli are able to produce titania [9] and silver nanoparticles [10].

In the last few years, selenium nanoparticles (SeNPs) have grown in interest due to their antioxidant and antimicrobial properties [4]. SeNPs can be chemically synthesized by a reaction between a selenium compound and a reducing agent: selenite is usually employed as selenium source, and among reducing agents glutathione peroxidase (GSH) [11] and ascorbic acid are the most widely used [11]. The stability of SeNPs is an important factor to be considered, as their dispersion is normally unstable in absence of stabilizing agents, leading to aggregation. In this regard, proteins such as bovine serum albumin (BSA) [12], monosaccharides (glucose) [13], oligosaccharides (sucrose) [13] and polysaccharides (chitosan,CS) [13] are commonly used to modify the surface of the SeNPs and to control their diameter. As alternative to chemical procedures, SeNPs have been also synthesized using plants, fungi and bacteria. Many bacteria are known to exhibit Se resistance and one of the mechanisms of Se detoxification is the production of elemental selenium as SeNPs [8,14]. Synthesis of these NPs can be extracellular, intracellular, or membrane bound. Characterization of NPs requires multi-method approaches to provide information especially in complex matrices. Such approaches provide information on properties that need to be measured for characterizing nanoparticles. The most commonly methods are based on light scattering measurements such as Dynamic Light Scattering (DLS), Multiangle Light Scattering (MALS) and Nanoparticle Tracking Analysis (NTA), classical electron microscopy (TEM/SEM) and separation techniques such as ultrafiltration and Asymmetrical Flow Field Flow Fractionation (AF^4) [15–22] Characterization of biogenic nanoparticles, especially for those nanoparticles without magnetic properties, is not an easy task and implies a careful separation of biogenic NPs from the bacterial pellet. The presence of microorganisms in the NPs dispersions could alter size measurements when using light scattering techniques.

This work investigated the biogenic synthesis of SeNPs by using LAB and their further characterization in terms of size and morphology, and quantification in terms of number and mass-based NPS by using a multitechnique platform. For this purpose, several sample treatments for isolating biogenic SeNPs from bacteria pellet along with a plethora of techniques including TEM (size and morphology) DLS (hydrodynamic size), NTA (hydrodynamic size and number of nanoparticles) and AF^4 on line coupled to the ICP-MS (particle mass concentration and composition) were applied.

2. Materials and methods

2.1. Reagents and solutions

The strains *Lactobacillus acidophilus* CRL 636, *L. reuteri* CRL 1101, and *L. delbrueckii* subsp. *bulgaricus* CRL 656 used in this work were obtained from the Culture Collection of the Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina). Sodium selenite (Na_2SeO_3) and concentrated nitric acid (HNO_3) were obtained from Merck (Madrid, Spain); 30% (v/v) hydrogen peroxide (H_2O_2) and 99% (w/w) sodium hydroxide (NaOH) were obtained from Panreac (Barcelona, Spain); sodium dodecyl sulfate (SDS) was purchased from Sigma-Aldrich (St Louis, USA) and MRS broth was acquired from Scharlab (Barcelona, Spain). All chemicals were of analytical grade and were used without further purification except MRS-broth and Na_2SeO_3 solutions, which were sterilized with an autoclave and 0.22 μm nylon filters, respectively. All aqueous solutions were prepared with ultrapure water (Milli Q water, 18 MΩ cm^{-1}) from a Milli-Q water purification system unit (Millipore, Bedford, MA, USA).

2.2. Synthesis of selenium nanoparticles using Lactobacillus sp

The *Lactobacillus* sp. strains were previously activated in sterile MRS broth and incubated at 37° C in a thermostated bath. Bacterial cells were transferred twice in fresh MRS and further cultured in the absence (control) and the presence of 25 mg Se L^{-1} as Na_2SeO_3 during 24 h at 37 °C. During incubation, the color medium changes from yellow towards red as a result of the synthesis of SeNPs.

2.3. Determination of total selenium in bacterial pellet

Bacterial cells exposed to selenite were separated from the culture medium by centrifugation (5000 rpm, 5 min) at room temperature. The resulting bacterial pellet was washed twice with Milli-Q water. Then, 100 mg of bacterial pellet were submitted to acid digestion in a 1000 W microwave oven with 1 mL of concentrated HNO_3 and 0.5 ml of 30% (v/v) H_2O_2. The resulting solutions were diluted with Milli-Q water to 10 ml. Finally, selenium concentration was measured by using an Agilent 7700-collision/

reaction cell ICP-MS and employing H_2 as collision gas. The equipment measuring conditions are listed in Table 1.

2.4. Separation of biogenic SeNPs from culture medium

Separation of biogenic SeNPs from culture medium was achieved by applying the procedure developed by Torres et al. [23] with several modifications. Briefly, portions of the bacterial cultures were collected, submitted to ultrasound for 2 min using an ultrasonic bath and finally centrifuged (9449 rpm, 10 min) at room temperature. The resulting bacterial pellet containing nanoparticles was re-suspended in 0.1% SDS (w/v)/1 M NaOH and subjected to ultrasound and centrifugation as mentioned above. Finally, the bacterial pellet was re -suspended in distilled water and the resulting solution was filtered through 0.45 μm and 0.22 μm nylon filters to isolate the biogenic SeNPs.

Mass balances were calculated to evaluate the performance of the process in terms of nanoparticles losses. For this purpose, fractions collected after the first and second filtration step were subjected to an acid digestion following the procedure described in section 2.3. Selenium concentration in the different fractions was measured by ICP-MS following the conditions given in Table 1.

2.5. Characterization of biogenic SeNPs by transmission electron microscopy (TEM) and X-Ray Energy Dispersive Spectroscopy (XEDS) detection

TEM data from both isolated biogenic SeNPs suspensions and bacteria containing-SeNPs were obtained using high resolution Transmission Electron Microscope (TEM) (JEOL JEM 2100, USA) equipped with an X-Ray Energy Dispersive Spectroscopy (XEDS) microanalysis composition system (Oxford Inca, city). Briefly, biogenic SeNPs suspensions were sonicated during 2 min and the solution was dropped into copper grid and dried in clean air for TEM observation operated at an accelerating voltage of 200 kV. More than 1500 SeNPs dispersed in about 20 TEM photos were viewed to measure the size distribution.

2.6. Characterization of biogenic SeNPs by dynamic light scattering (DLS)

The hydrodynamic diameters of the NPs under investigation were measured using a Malver Zetasizer Nano ZS (Malver Instrument LTD, Malvern UK) system equipped with a laser of 633 nm. The scattering angle was 173°, the measuring position was 4.65 mm inside disposable plastic cuvettes, and the mean of ten measurements was taken. The hydrodynamic diameter was given as the average size obtained from a number distribution of NPs. All samples were diluted to a suitable concentration using deionized water prior to analysis.

2.7. Characterization of biogenic SeNPs by nanoparticle tracking analysis (NTA)

The samples were analyzed by a NanoSight NS300 (Iesmat, Spain) using a laser-illuminated microscope, and Brownian motion was recorded in real-time using a CCD (charge-coupled device) camera. Each particle was simultaneously, but separately, visualized and tracked using image-analysis software. Thus, samples were sonicated and diluted again with deionized water to reach a concentration suitable for the analysis. All measurements were taken on an instrument manufactured by Nanosight (Salisbury, UK). The diluted sample was injected into the sample chamber fitted with a 640 nm diode laser. The software used for capturing and analyzing the data was the NTA 2.0 Build 127. The samples were measured for 60 s with manual shutter and gain adjustments. For each sample the measurement was repeated 10 times. After capturing the size, diffusion coefficient and track lengths for the individual particles were determined using the NTA software.

2.8. SeNPs analysis by AF^4-DAD-ICP-MS

An AF2000 system (Postnova Analytics, Landsberg and Lech, Germany) equipped with a regenerated cellulose ultrafiltration membrane of 10 kDa molecular weight mass cut-off and a spacer of 500 μm, was used in this work. A 300 μL injection loop was employed for performing sample injection into the AF^4 system via a Rheodyne valve. The AF^4 system was connected on line to an ICPMS (Agilent HP-7700 Series collision/reaction cell, Santa Clara, CA, USA) and DAD detector set at 369 nm. For accurate characterization of biogenic SeNPs, fraction containing NPs were collected and analyzed by DLS (described above).

3. Results and discussion

3.1. Biogenic synthesis of SeNPs

It has been reported that elemental selenium production by LAB is associated with alcohol dehydrogenase or glutathione reductase

Table 1
ICP-MS and AF4 operating parameters.

ICP MS	
RF-Power (kW)	1.55
Plasma gas flow rate (l min^{-1})	15.0
Carrier gas flow rate (l min^{-1})	0.30
Nebulizer flow rate (l min^{-1})/type	0.75/Conikal
Spray chamber	Scott
Isotopes monitored	^{76}Se, ^{77}Se, ^{78}Se, ^{80}Se
Adquisition mode	Continuous
Reaction gas	H_2
Reaction gas (ml H_2 min^{-1})	6
AF^4	
Carrier	Ultrapure water at pH 5.0
Membrane type	Regenerated cellulose: 10 kDa cut-off filter
Channel thickness (μm)	500
Injection volume (μl)	300
Injection flow (mL min^{-1})	0.1
Focus flow (mL min^{-1})	3.2
Cross flow (mL min^{-1})	3.0
Detector flow (mL min^{-1})	0.3
Gradient mode	Linear
Wavelength	369 nm

activities [24,25]. Glutathione reductase could reduce selenite by glutathione (GSH) oxidation. However, the capacity of LAB to transport GSH inside the cell and reduce it is variable between LAB strains [26]. In addition, the ability of LAB to resist Se is strain-dependent [24,27]. Consequently, and due to the heterogeneity of LAB metabolism and capacity to resist Se, two homofermentative LAB strains (*L. acidophilus* CRL 636 and *L. bulgaricus* CRL 656) and the heterofermentative strain (*L. reuteri* CRL 1101) were selected for this study.

After exposing the *Lactobacillus* strains to Na_2SeO_3 (25 mg Se L^{-1}) and incubating in the culture medium at 37 °C, the reaction solution displayed a time dependent color change as shown in Fig. 1. At the beginning of the reaction, the solution was clear light-yellow, and it became light red after 24 h of reaction. The appearance of the red color indicated the occurrence of the reaction and the formation of elemental selenium in solution. The bacteria pellet collected from the culture media was examined by TEM. Fig. 2 shows the presence of SeNPs with spherical shapes among the LAB. Energy-dispersive X-ray spectroscopy (EDXS) analysis confirmed the presence of Se ((Kα (1.0 keV), Lα (8.7 keV) and Lβ (9.6 keV) in these nanoparticles.

Despite their similarity in morphology, biogenic SeNPs size distribution was different depending on the *Lactobacillus* species involved. Histograms of particle size distribution show a size average of biogenic SeNPs of (176 ± 13 nm), (160 ± 24 nm) and (130 ± 23 nm) when prepared in presence of *L. acidophilus, L. bulgaricus* and *L. reuteri,* respectively. From the experimental data, it was observed that particle size distribution and shape was also dependent on the initial selenite concentration employed for preparing SeNPs. The use of 100 ppm of sodium selenite provides spherical SeNPs with a diameter size of around 300 nm except for those generated by using *L. bulgaricus* which showed a star shape morphology (Fig. 2d).

The TEM micrographs also evidence bacterial cell wall damage either in *L. acidophilus* or *L. bulgaricus*, whereas the presence of selenium produces the excretion of exopolymeric substances (polysaccharides) in *L. reuteri*. It has been reported that some bacteria are able to excrete exopolymers to produce biofilms as a defense mechanisms to enhance their ability to survive in presence of anti-microbial agents or under stress conditions [28]. Regarding the amount of selenium accumulated by the *Lactobacillus* strains after 24 h of incubation, the highest percentages values were found in *L. reuteri* (79 ± 3) followed by *L. acidophilus* (41.2 ± 0.6), and finally the *L. delbrueckii* subsp. *bulgaricus* (28 ± 2) strain. These results are in line with the observed resistance of *L. reuteri* to the presence of selenium by producing an exopolymer. Because of its greatest ability to survive at high levels of selenium and to accumulate the highest amount of selenium, *L. reuteri* was considered as the most suitable *Lactobacillus* specie for producing biogenic SeNPs.

3.2. Determination of size and number and mass-based concentration of biogenic SeNPs by using, TEM, AF^4-ICP-MS, NTA and AF^4 off-line coupled to DLS

The accurate characterization of biogenic SeNPs implies their previous separation from the bacterial pellet. The presence of bacteria with a size much larger than SeNPs could hinder proper NPs characterization when applying techniques such as DLS. Separation of SeNPs from bacteria is not an easy task. First, SeNPs are located inside bacteria therefore a bacteria cell wall lysis step is required, and second the large size of bacteria makes difficult their separation from nanoparticles by filtration. Several approaches were tested to separate SeNPs from bacteria pellet. No positive results were obtained when applied either autoclave sterilization or tip-sonication at pH 7.0 (Tris buffer), since NPs remained occluded inside the bacterial pellet and therefore retained in the filters. Subsequently the use of surfactants (Surfynol and SDS in basic medium) was tested as a way of permeabilizing bacterial cell wall. Best results, in terms of the amount of recovered NPs were attained when the mixture 0.1% SDS/1 M NaOH was employed. The fact that the amount of NPs recovery was higher with SDS than with Surfynol could be attributed to a better bacterial cell wall permeabilization when using SDS, thus allowing the easy release to the extracellular medium of the SeNPs entrapped in the bacterium. Additionally, the use of NaOH favored the release of the NPs to the culture medium by dehydrating the bacteria organelles. This pretreatment (surfactant and NaOH) facilitated the subsequent separation of SeNPs from the bacterial pellet by filtration through 0.45 μm and 0.22 nylon filters.

Separation and filter techniques may lead to SeNPs losses due to membrane filter adsorption. In order to calculate the amount of SeNPs retained in the filter, the total selenium content was determined after acid digestion of the filtrates collected from the 0.45 μm filter. The resulting digests were analyzed by ICP-MS using the conditions given in Table 1. The selenium concentrations in the filtrates evidenced the low yield of the separation process with recovery percentages as low as only a (11.6 ± 0.6) % and (12.8 ± 0.2) % after the first and second filtration step, respectively suggesting that the first filtration through 0.45 μm nylon filters as the limiting step of the process.

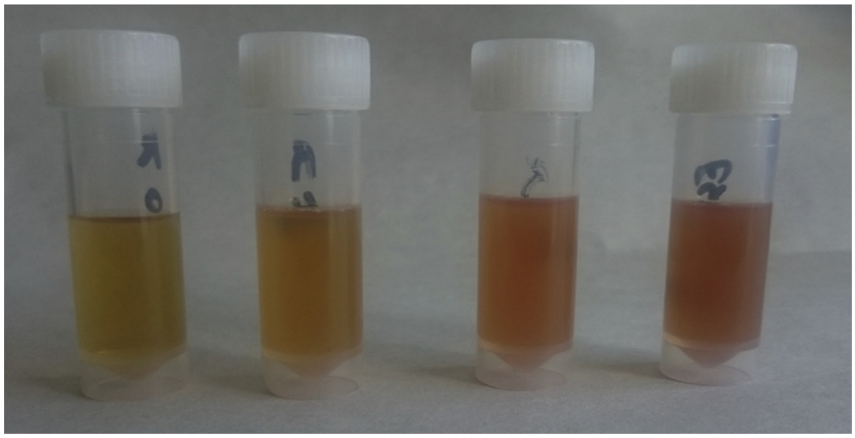

Fig. 1. Color changes in the culture medium as a function of the incubation time of *L. reuteri* CRL 1101 in the presence of selenite. Left to right 0, 4, 6 and 24 h of incubation time. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

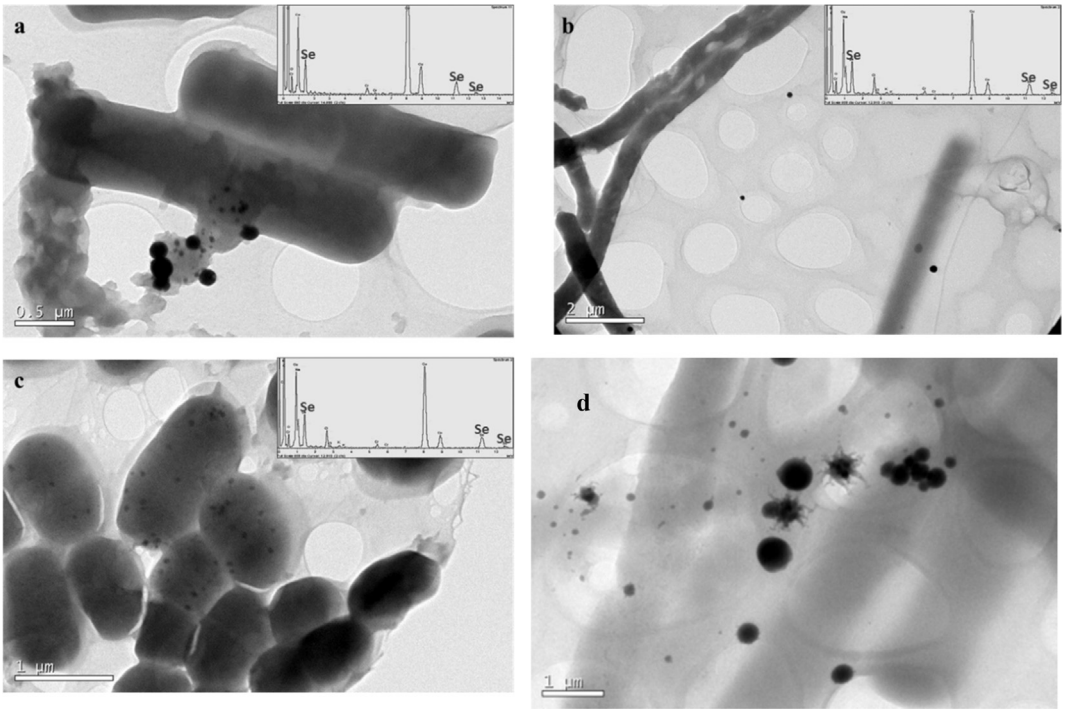

Fig. 2. TEM images and XDS spectra of biogenic SeNPs synthesized by: *L. acidophilus* CRL 636 (a), *L. delbrueckii* subsp. *bulgaricus* CRL 454 (b), and *L. reuteri* CRL 1101 (c) for a concentration of Se of 25 mg L^{-1}, (d) biogenic SeNPs synthesized by *L. delbrueckii* subsp. *bulgaricus* CRL 454 for a concentration of Se of 100 mg L^{-1}.

The isolated SeNPs were characterized in terms of size by using both single particle (transmission electron microscopy) and ensemble methods (DLS and nanoparticle tracking (NTA). The TEM images (Fig. 3a, b, c, d) of the SeNPs synthesized by *L. reuteri* CRL 1101 and released by the above-given procedure showed spherical NPs in the range of 50–200 nm (Fig. 3e) with an average size of (146 ± 71) nm. No significant differences in sizes and morphologies were observed when comparing isolated and non-isolated biogenic SeNPs suggesting the stability of the biogenic SeNPs during the isolation processes. Additionally, the electron diffraction pattern (Fig. 3f) confirmed the non-microcrystalline structure of the synthesized SeNPs.

When using microscopy techniques special care must be focused on selecting the number of particles to be measured.

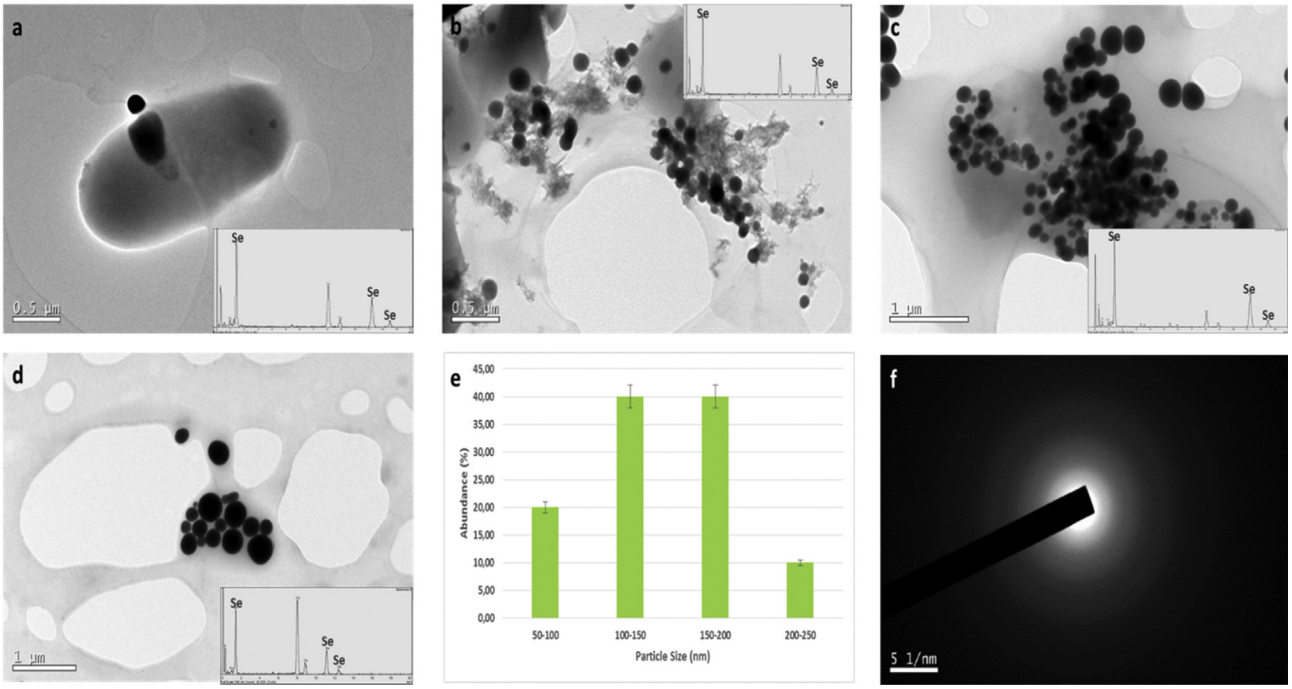

Fig. 3. a) Effect of SDS/NaOH on the bacterial cell wall lysis. b, c,d) TEM images and XDS spectra of biogenic SeNPs synthesized by *L. reuteri* CRL 1101 after isolating from the cell pellet. e) Particle size distribution and f) Diffraction pattern of nanoparticles.

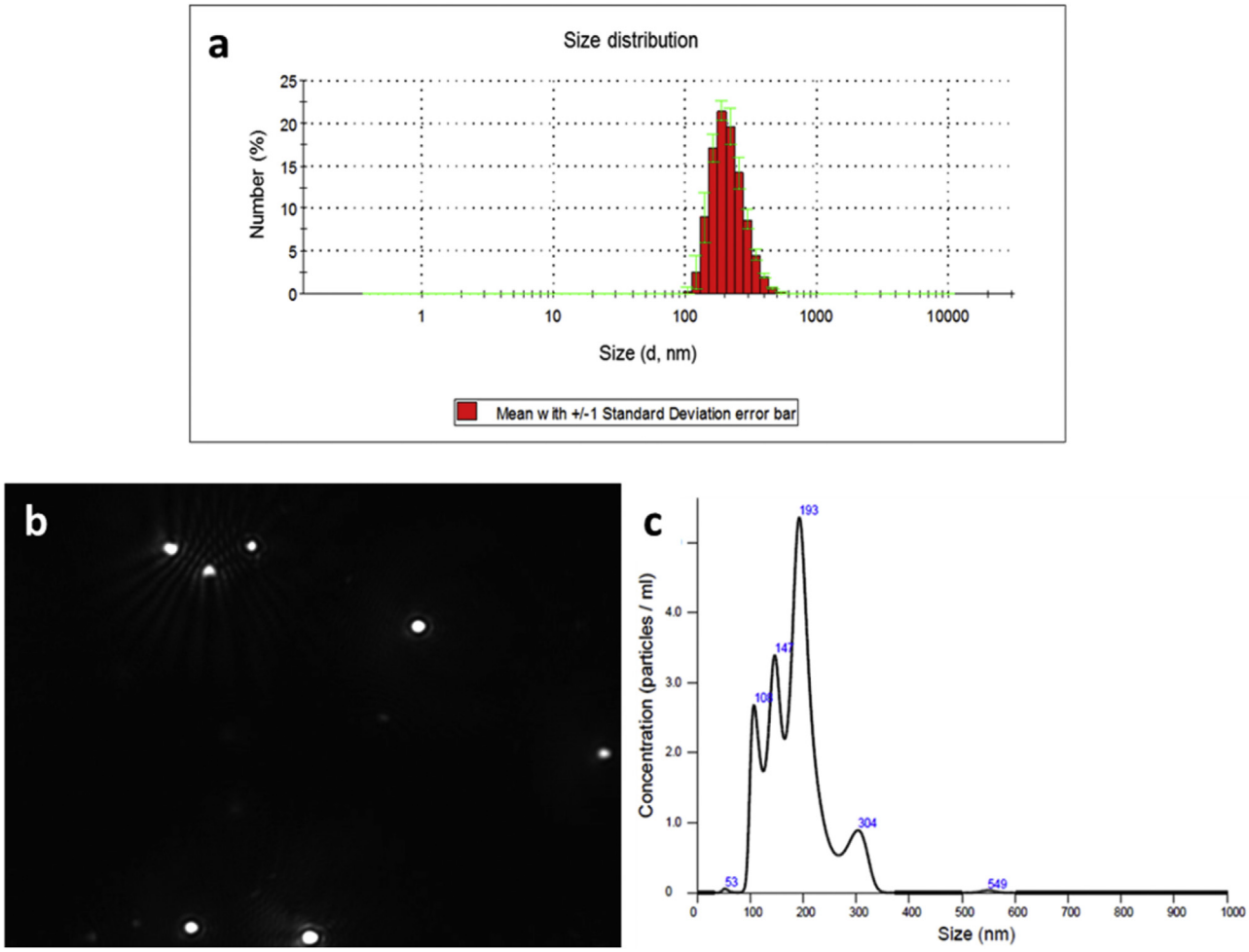

Fig. 4. a) DLS measurements. Size distribution of SeNPs synthesized by *L. reuteri* CRL 1101 with the mean (red) and standard deviation (green). b) NTA measurements. Fragment of the video obtained by NTA for the biogenic SeNPs and size distribution of biogenic SeNPs considering the Stokes-Einstein equation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Typically 200–1000 particles are sampled from a total size of many millions which could lead to inaccurate measurements. Ensemble NPs measurements are techniques where information from many thousands of NPs are obtained. DLS is one of the most frequently used methods to obtain an average diameter of nanoparticles dispersed in liquids providing reasonably accurate results for strictly monodisperse NPs. However, DLS is unable to distinguish between nanoparticles with slight differences in diameter or to precisely resolve polydisperse samples. In the current work the presence of larger NPs as for instance bacterial debris accompanying to SeNPs may cause biased results impairing the validity of the obtained data. Representative diameter distribution graph for SeNPs as obtained by DLS is shown in Fig. 4a. The average hydrodynamic size of nanoparticles was (258 ± 4) nm with a polydispersity index (PdI) of 0.2. The absence of the tail in the distribution obtained along with a low PdI value (PdI must be smaller than 0.6–0.7 to have a reliable measurement, at least for the Zetasizer); indicate that the biogenic NPs are mostly not aggregated suggesting a monodisperse sample with a very narrow width distribution.

Nanoparticle tracking analysis (NTA) which combines single particle and ensemble approach. is based on the ability to detect individual particles using optical methods via scattered laser light. Fig. 4b displays a frame capture from the NTA instrument where the individual SeNPs are highlighted as monodisperse white spheres. Analysis of the tracked movement using the Stokes–Einstein equation provides a size distribution shown in Fig. 4c. The NPs showed an average size of (187 ± 56) nm. The size obtained agreed well with data provided by DLS measurements (258 ± 4) nm. However, the standard deviation was lower when using DLS since the average is obtained from a large number of identical particles that make accurate and repeatable measurements. In case of NTA, the distribution of a smaller particle population (thousands rather than hundreds of thousands as in DLS) may provide results less statistically robust than with DLS.

NTA enables counting nanoparticles directly in solution, therefore it can be used for determining the number of nanoparticles present. In this study, NTA measurements of biogenic SeNPs obtained after the incubation of the strain with 100 mg L^{-1} selenium and subsequent isolation from the culture medium gave us a concentration value of $(4.67 \pm 0.30) \times 10^9$ biogenic SeNPs mL^{-1} with a relative standard deviation lower than 5%. The use of ICP-MS was employed for confirmatory measurements of the quantification data provided by NTA. It is worth noting that ICP-MS is a mass detector, therefore quantification results are provided as mass fraction of NPs. Only for those NPs with spherical morphology, uniform and of known density it is possible to directly transform quantities which are expressed in units of mass into number of nanoparticles units and vice versa. Based on that, the experimental value provides by NTA was compared with the

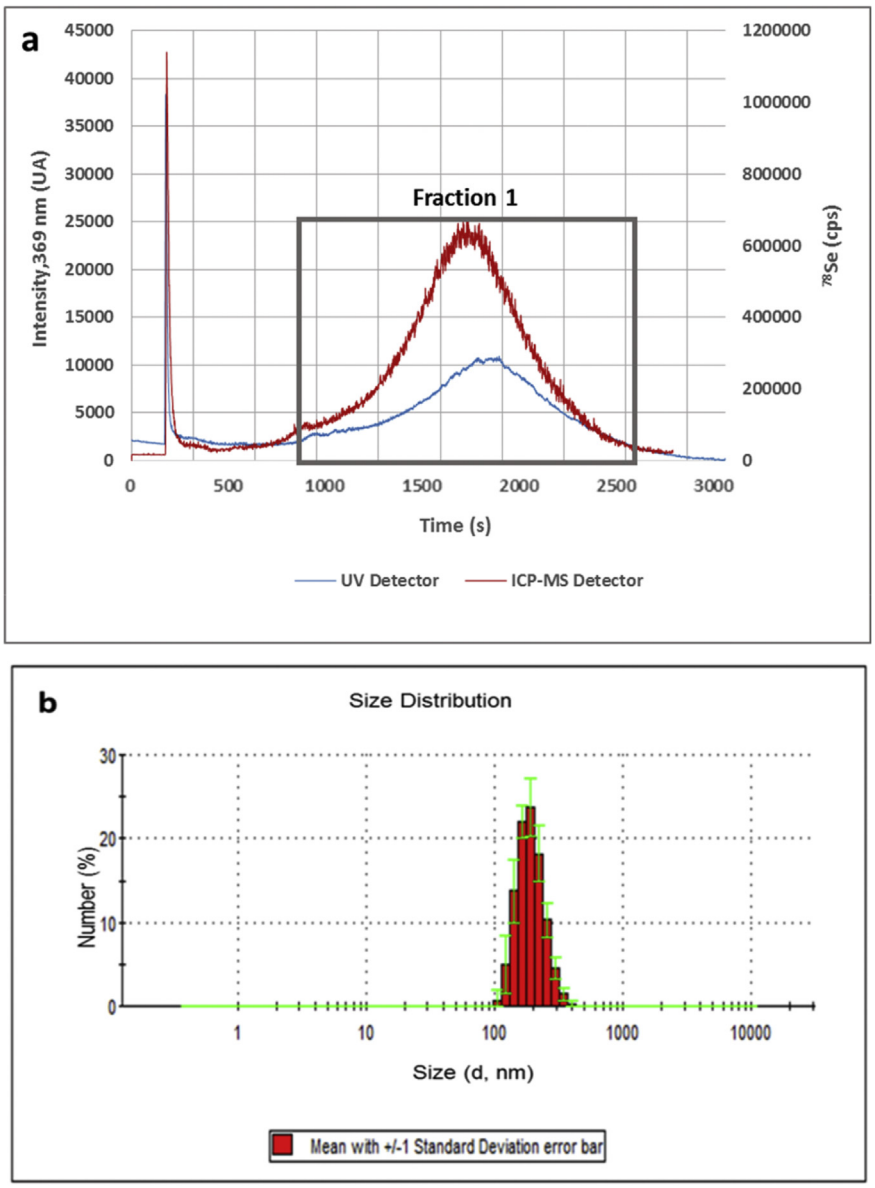

Fig. 5. a) AF^4-DAD-ICP-MS fractograms corresponding to biogenic SeNPs isolated from the bacterial pellet. b) Size distribution found in fraction 1 collected from the fractogram and measured by DLS.

equivalent number of particles in the sample which was theoretically calculated taking into account the selenium amount obtained after digestion and analysis by ICP-MS, the density of this metal (4.79 g cm^{-3}) and the radius of nanoparticles obtained by TEM (146 ± 71 nm). In this way, the theoretical concentration of SeNPs obtained for the initial culture was (5.10 ±0.08)x 10^9 particles mLl^{-1}. These results show a good correlation with the experimental results provided by NTA.

The isolated biogenic SeNPs were also characterized in terms of size, composition and mass fraction by AF^4-DAD-ICP-MS and AF^4 off line coupled to DLS following the optimized conditions listed in Table 1. A selenium -containing peak well separated from the void −volume peak appeared in the fractogram at 29 (Fig. 5). To determine the hydrodynamic size of the fractionated NPs, the fraction was collected and then analyzed by DLS. The distribution of the particle size for the Se-containing peak is shown in Fig. 5b, with an average size and a PdI of (247 ± 14) nm and 0.239, respectively. The results are comparable with those obtained by DLS without using AF^4 where an average size of (258 ± 4) nm and a PdI value of 0.2 was found, however an increase in the standard deviation and PdI values was observed suggesting certain agglomeration of biogenic selenium nanoparticles during AF^4 separation. The off-line coupling AF^4 to ICP-MS was also used for determining the mass concentration fraction of biogenic SeNPs. For this purpose, fractions were collected from the AF^4 system followed by ICP-MS detection. The results obtained were in line with those provided when using ICP-MS suggesting no significant losses of SeNPs in the AF^4-ICP-MS system.

4. Conclusions

A green technology for producing selenium nanospheres homogeneous in form and size has been developed by using LAB. Among LAB species tested, *L. reuteri* was the most suitable one because of its greatest ability to survive at high levels of selenium and to accumulate the highest amount of this metalloid. The

generated SeNPs were characterized in terms of size, morphology and quantified as mass and number-based concentration by using a plethora of techniques. Characterization of SeNPs by light dispersion techniques was challenging since nanoparticles were mainly located inside bacteria cell requiring the development of a separation step that implies lysis and permeabilization of the bacterial cell wall. The size and number-based concentration data obtained by independent techniques were in good agreement The developed methodology could be implemented for characterizing NPs in complex matrices such as biogenic nanoparticles embedded inside microbial material. This paper clearly express the need of using a multiple phasic approach for a full characterization of nanomaterials and shows a technology for producing SeNPs which combines simplicity rapidity along with the safety of the employed strains. The applicability of the proposed methodology could be extended to synthesize other metallic/non metallic nanoparticles. The biogenic SeNPs can be used isolated from the matrix or included in SeNPs enriched probiotics. The effect of SeNPs produced by lactobacilli and SeNPs-enriched probiotics on cancer metastasis prevention have been reported [29], However, separation is always required for SeNPs characterization in order to confirm their applicability or for other SeNPs uses where SeNPs need to be isolated.

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