MITIGATION OF METALLIC ION CONCENTRATIONS IN THE ENVIRONMENT USING MICROORGANISMS

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Abstract

Microorganisms, particularly bacteria, have the ability to bind and precipitate metals from different solutions, intracellularly or on the cell surface. An important application field of nanobiotechnologies is the mitigation of certain metals' toxic effects by using microorganisms such as bacteria or yeasts. The product obtained through the microorganism activity is several times less toxic than the initial one. *Bacillus subtilis* is a bacterium that has the capacity of metal bioaccumulation. These bacteria can bioaccumulate Ag and Fe metallic ions from Ag and Fe ions solutions very rapidly. Microorganisms display large silver and iron based nanostructured deposits on the surface and in the immediate vicinity. Biosynthesis of silver and iron nanoparticles with the help of microorganisms is an ecological, viable, low cost, relatively easy and high-efficient method that allows the use of certain inexpensive biotechnologies for the metal absorption from the environment. This method can be tested for the metallic ions mitigation in the environment.

Keywords: Microorganisms, metallic ions, nanoparticles, bacillus subtilis, Ag (silver) and Fe (iron)

1. Introduction

Microorganisms, particularly bacteria, have the ability to bind and precipitate metals from different solutions, intracellularly or on the cell surface (Cohen 2006; Xiangqian Li et al. 2011). This particularity is determined by the small size of the bacterial cell and the great surface/volume ratio which ensure a prolonged contact with the metallic ions of the watery solutions (Warren and Haack 2001; Xiangqian Li et al. 2011).

The existence of two types of bioaccumulation was established:

- metals found in solutions have electropositive charges and they bind to the electronegative structures of the microorganisms' cell surface; these structures correspond to the extracellular polymers of the cell wall (Garbisu and Alkorta 2003);
- metal accumulation in the cells' cytoplasm.

These processes can be detected in both living cells and inactivated ones.

An important application field of nanobiotechnologies is the diminishment of certain metals' toxic effects by using microorganisms such as bacteria or yeasts, which can reduce metallic ions and form metal compounds (Kowshik et al. 2003; Williams et al. 2006).

As their forms and sizes are controllable, the nanoparticles that are obtained through biological methods have special features depending on the microorganism that is being used (Prathna et al. 2010; Sadowski Z. 2010). The product of either the action of microorganisms or even that of parts of certain superior plants (roots, leaves) is several times less toxic than the initial one.

A series of genera of unicellular microorganisms (bacteria, yeasts or algae) accumulate nanoparticles intracellularly or deposit them extracellularly:
- bacteria have been used in order to obtain nanoparticles of Au, Ag, Hg, As, CdS, ZnS, FeS, etc (Williams et al. 2006; Xiangqian Li et al. 2011);
- yeasts have been studied in order to obtain PbS and CdS nanoparticles (Xiangqian Li et al. 2011);
- algae have been used in order to obtain Au nanoparticles (Sadowski 2010).

2. Methodology

2.1. The interaction between *Bacillus subtilis* and metals

Bacillus subtilis is a bacterium that has the capacity of metal bioaccumulation. This bacterium, which naturally lives in the presence of metallic ions, is therefore quite intriguing within the study of metal resistance. Scientific literature provides the results of several research studies regarding the adaptation of the *Bacillus subtilis* bacterium to metals (Bruins et al. 2000; Williams et al. 2006).

Bacterial cells have stick-like shape and are large sized; the polysugars found on the surface of the cell form a capsule; as the spore percentage is determined by the oxygen quantity in the environment, the spore generation process represents an identification criterion. This bacterium is Gram-positive.

In liquid media it does not accentuate turbidity. On solid media it forms large, inconsistent, irregular, opaque, unpigmented colonies. The optimum temperature for multiplication is of 28°C. These bacteria use glucose, fructose, sucrose and maltose as carbon sources. They hydrolyze starch and slowly liquefy gelatine. The reactions of indole, urease and H_2S are negative. The haemolytic activity is weak. They produce catalasis.

2.2. Experimental methods for biomass obtainment

The obtainment of bacterial biomass was achieved through cell cultivation in a minimal culture medium in a discontinuous system, with the following formula:

meat extract	0,30 g %
fish extract	0,30 g %
peptone	2,00 g %
NaCl	0,52 g %
glucose	0,20 g %
the pH of the culture medium	7,2

1 ml of the ATCC 2589 and ATCC 6633 stems of the *Bacillus subtilis* was inoculated into 200 ml of culture medium divided into 250 ml phials. The samples were then incubated at a temperature of 30°C for 48 hours, being stirred every 2 hours for 15 minutes.

2.3. Performed analyses

Samples were taken every 8 hours in order to:
a) assess bacterial purity and fungal sterility;
b) assess the biochemical features of bacterial cells;
c) determine the multiplication dynamics of bacterial cells by tracing growth rate curves for the two *Bacillus subtilis* stems.

2.4. The assessment of bacterial purity and fungal sterility

The purity of the culture was assessed by inoculating 5 test tubes of the culture medium which contained sodium thioglycolate - 3 samples for each stem.

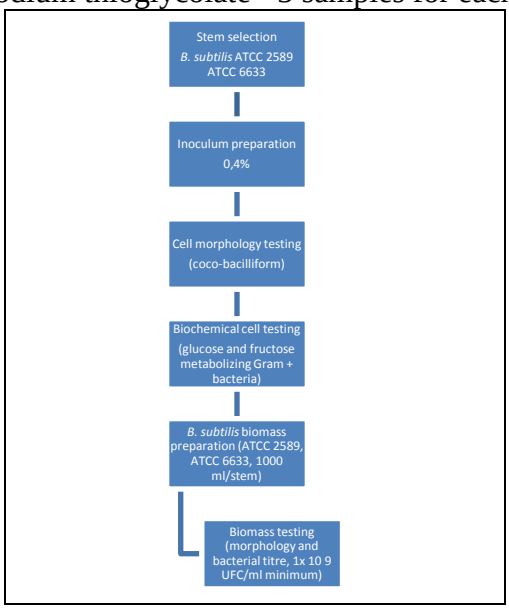

Figure 1. Stages of bacterial biomass obtainment

The samples were incubated at a temperature of 37°C for 7 days.

The fungal sterility was assessed by inoculating 3 samples (for each stem) in a Sabouraud liquid medium.

The samples were incubated at a temperature of 28°C for 14 days.

Results: The samples were bacteriologically pure and fungically sterile.

Conclusion: The cultures were obtained by respecting the appropriate sterility and cultivation requirements.

2.5. The biomass preparation for Ag and Fe ions bioabsorption

The bacterial biomass used in the bioabsorption process was centrifuged at 6 000 rpm for 30 minutes. Each stem was cultivated in a volume of 1000 ml. The deposit was washed once in a $AgNO_3$ solution and once in a $FeSO_4$ 0,001 M solution, by centrifuging at 6 000 rpm. Once the supernatant was removed, the ATCC 2589 şi ATCC 663 cell deposit was placed in $AgNO_3$ 0,05 M, 0,01 M, 0,001 M solutions, at a 1:6 ratio. Equal *B. subtilis* ATCC 2589 and ATCC 6633 biomass quantities were placed in $FeSO_4$ 0,05M, 0,01 M, 0,001 M solutions, at a 1:6 ratio. The resulting samples were incubated for 72 hours, being stirred at 200 rpm every 2 hours; the incubation temperature was of 30 ^{0}C.

Conclusion:

1. The bacterial biomass which was used in the bioabsorption process does not contain traces of the culture medium, the purification of the bacterial cells having been obtained by centrifugation at 6 000 rpm.

2. The dilutions of the salts ($FeSO_4$ and $AgNO_3$) used in the process, were of 0,001 M, 0,01M and 0,05 M.

3. The biomass was incubated for 72 hours, being stirred at 200 rpm; the incubation temperature was of 30 ^{0}C.

4. The dilution at which the best bioabsorption results were obtained - through transmission electron microscopy - was of 0,001 M.

5. The analyzed samples were pure; bacterial cells were not in sporulated form; the microscopic fields showed a high dark cell density due to the metal deposition on their surfaces that followed the action of neutral enzymes - scientific literature confirms the fact

that *Bacillus subtilis* stems can synthesize neutral proteases that are exported by the bacterial cells; these neutral proteases can transport metallic ions and simple molecules and deposit them outside bacterial cells (Williams et al. 2006; Xiangqian Li et al. 2011).

6. The stages of the bioabsorption and nanostructuring of the metals in solutions of varying dilutions are presented in Figure 2.

Bacterial species and stem selection (*Bacillus subtilis,* ATCC 2589, 6633)

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Stem adaptation to a minimal culture medium (*Bacillus subtilis*, ATCC 2589, 6633)

▼

Stem multiplication (*Bacillus subtilis*, ATCC 2589, 6633)

Morphological, biochemical and quantitative analysis of bacterial cells

▼

Biomass obtainment (ATCC 2589, ATCC 6633)

Morphological, biochemical and quantitative analysis of bacterial cells

▼

Bacterial biomass preparation for bioabsorption process (ATCC 2589, ATCC 6633)

Morphological and quantitative analysis of bacterial cells

▼

Placement of bacterial cells in $AgNO_3$ and $FeSO_4$ solutions (0,05 M; 0,01 M; 0,001 M)

Morphological and quantitative analysis of bacterial cells

▼

Sample incubation at 30°C (stir at 200 rpm for 15 min every 2 h; total incubation time 72-96 h)

Figure 2. Scheme of the biotechnological process of bioabsorption (B. subtilis; Ag, Fe)

2.6. Determination of metal concentrations

The determination of metal concentrations was carried out by the use of mass adsorption spectrophotometry, employing lamps of different wavelengths, depending on the characteristics of each metal.

The metal bioabsorption experiments performed on the *Bacillus* species showed that metal ions were very rapidly taken out of the solution (10 minutes). After these first 10 minutes, no other adsorption level augmentations were recorded. The comparison between the biosorbants' adsorption capacities was carried out in identical pH, temperature and stirring speed conditions for each metal.

The maximum adsorption capacity for Ag was of 38%. The reason why Ag's adsorption is lower than that of other metals' is that *Bacillus sp.* is a Gram-positive bacterium and the biosorbants of its cell wall show a high affinity for anions and a lower one for cations.

2.7. Transmission electron microscopy for Ag samples

The transmission electron microscopy was used in order to examine the way adsorption and nanostructured metal formation occur, the way cell division processes occur for the microorganisms which are being used, the transformations the microorganisms go through after having interacted with the culture medium (solutions of high metal ion concentrations).

It was therefore possible to determine the microorganisms' sizes, shapes and internal structures, the disposition and placement of nanostructured particle deposits, as well as their shapes and sizes.

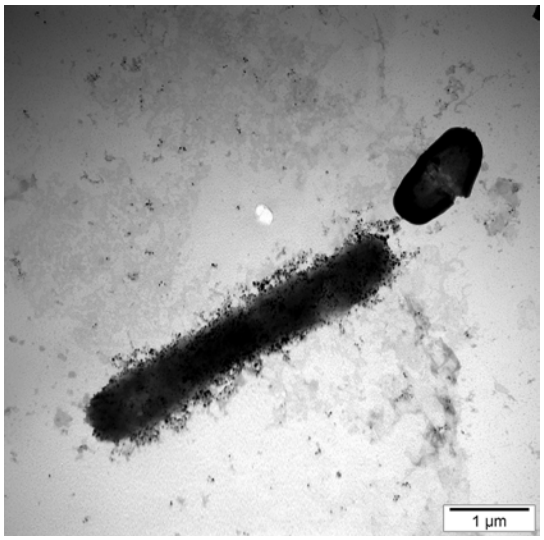

Figure 3. TEM image. Silver-based nanostructured particle deposits found on and inside the microorganism.

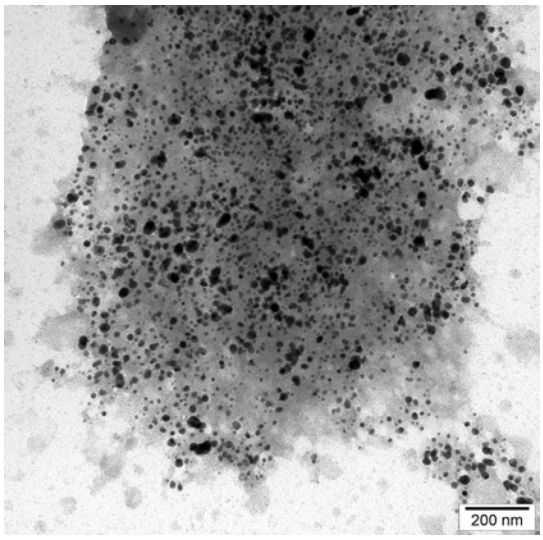

Figure 4. TEM image. A disintegrating microorganism can be seen, as well as a colony with multiple silver-based nanostructured particle deposits.

The resulting particle deposits vary in shape and size. The predominant shape is the spherical one; ellipsoidal, tetrahedral and octagonal deposits were also found. Dimensionally, the nanostructured particle deposits ranged from 4 to 48 nm.

2.8. Electronic microscope scanning of the Ag samples

The scanning electron microscope (SEM) was used in order to obtain information on the morphology and topography of the nanostructured metal deposits.

The microscope is equipped with an EDX detector that helped obtain compositional, qualitative and quantitative analyses, as well as element distribution patterns for each of the analyzed samples.

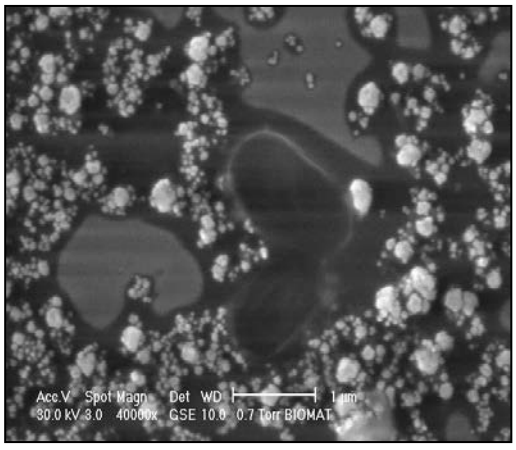

Figure 5. Electronic microscope scanning image. Silver-based nanostructured metal deposits can be seen as light-coloured (bright) points and structures.

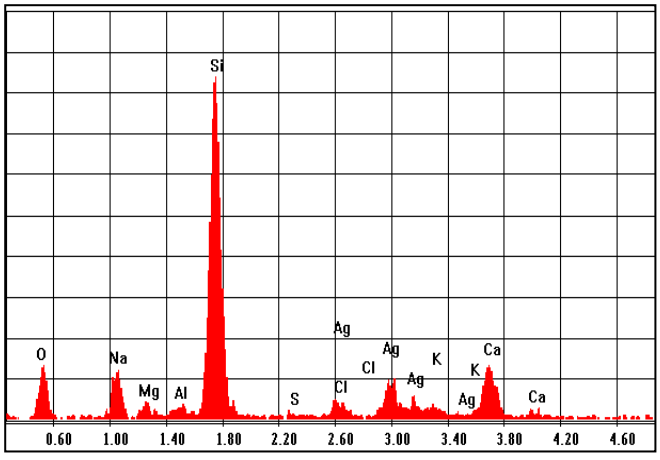

Figure 6. EDX analysis spectrum on a silver-based metal particle. The spots for silicon and other elements correspond to the lines given by the composition of the glass slide

The procedures targeted the highlighting of nanostructured metal deposits through compositional, qualitative and quantitative assessments in the medium, on the microorganisms and on the metal particles of the deposits or those of the microorganisms.

2.9. Transmission electron microscopy for Fe samples

The use of transmission electron microscopy (TEM) revealed that the biosynthesis processes of nanostructured metal particle deposits which use microorganisms in a high iron ion concentration culture medium, differ from those of silver ions. Therefore, particle deposits are less distinguishable than in the case of silver, as the process of deposition is quantitatively lower. The identified deposits comprise less regularly-shaped particles than in the case of silver. Dimentionally, particle sizes range from 5 to 50 nm, thus being similar to silver ones. Although larger particles were also identified, it is quite difficult to say whether the particles were autonomous or agglomerations of smaller particles.

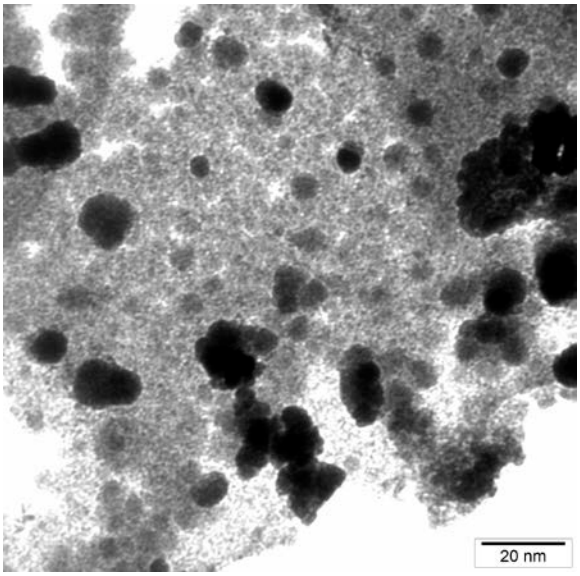

Figure 7. TEM image. Nanostructured particles obtained through biosynthesis, by using a high iron concentration culture medium can be seen.

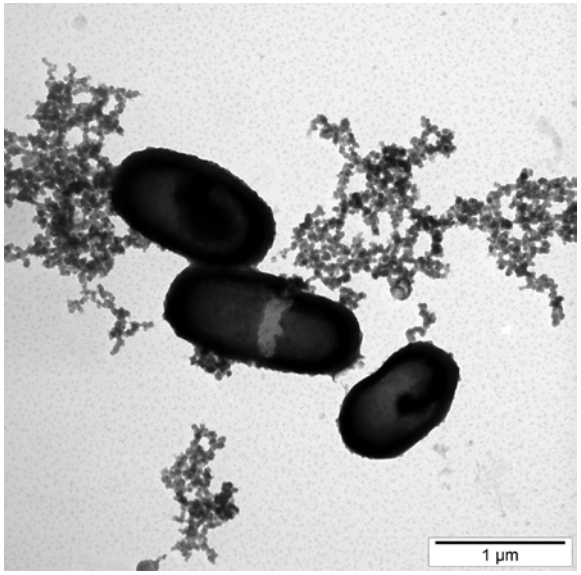

Figure 8. TEM image. Nanostructured particles and microorganisms in various stages can be seen.

2.10. Electronic microscope scanning for Fe samples

Information with regard to the morphology and topography of iron-based nanostructured metal deposits was obtained by using the SEM. The EDX detector provided compositional, qualitative and quantitative analyses, as well as element distribution patterns for the analyzed sample.

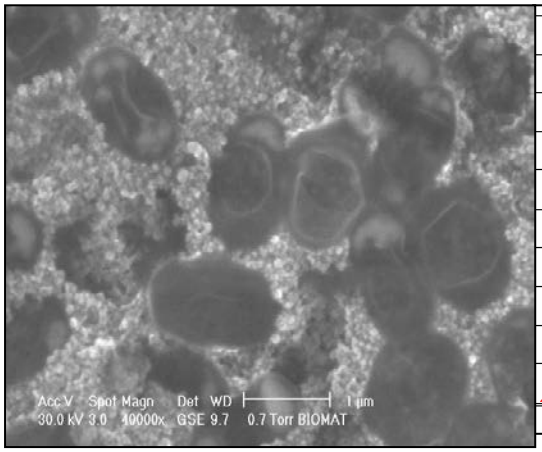

Figure 9. Additionally enlarged SEM image. Microorganism agglomerations and particle deposits due to biosynthesis can be seen.

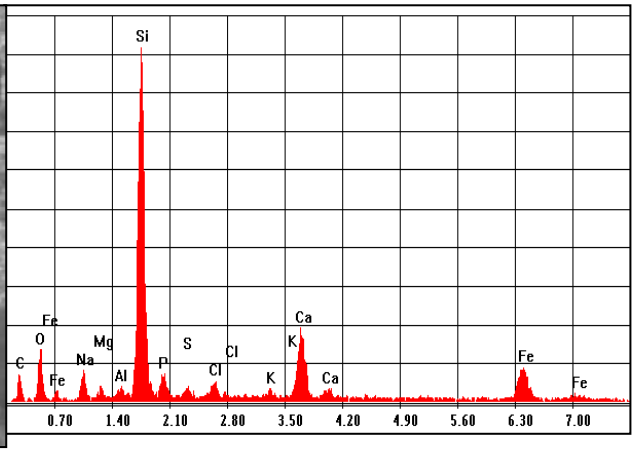

Figure 10. EDX spectrum analysis inside a microorganism on an iron-based metal particle. The spots for silicon and other elements correspond to the

lines given by the composition of the glass slide.

The procedures that were carried out targeted the highlighting of nanostructured metal deposits through compositional, qualitative and quantitative assessments in the medium, on the microorganisms and on the metal particles of the deposits or those of the microorganisms.

3. Discussions and conclusion

3.1. Ag nanoparticles obtainment

From the computations and analysis performed by using TEM and SEM microscopy and EDX methods, the following conclusions could be drawn:

- Silver-based nanostructured particle deposits were highlighted;
- As the particles are separated, the identified deposits do not comprise agglomerations;
- The identified microorganisms display large silver-based nanostructured particle deposits on the surface and in the immediate vicinity;
- Silver-based nanostructured particle deposits show a relatively uniform dimensional distribution, ranging from 4 to 50 nm;
- The TEM diffraction of the silver-based nanostructured particles highlighted their crystallinity;
- With the help of SEM, the metallic nature of nanostructured particle deposits could be confirmed;
- With the help of EDX, compositional, qualitative and quantitative analyses, as well as element distribution patterns for the analyzed samples could be carried out;
- Through EDX analysis, silver concentrations of 8,09 % mass percentage on a medium particle and of 4,32 for a particle inside a microorganism were found (by reducing the background intensity), 1,84 and 1,01 respectively for their atomic percentages.

3.2. Fe nanoparticles obtainment

From the computations and analysis performed by using TEM and SEM microscopy and EDX methods, the following conclusions could be drawn:

- Iron-based nanostructured particle deposits were highlighted;
- The identified deposits comprise agglomerations and separated particles;
- The identified microorganisms display iron-based nanostructured particle deposits on the surface and in the immediate vicinity;
- The iron-based nanostructured particle deposits range dimensionally from 5 to 50 nm;
- By using SEM, the metallic nature of nanostructured particle deposits could be confirmed;
- With the help of EDX, compositional, qualitative and quantitative analyses, as well as element distribution patterns for the analyzed samples could be carried out;
- Through EDX analysis, iron concentrations of 2,64 % mass percentage on a medium particle and of 1,22 for a particle inside a microorganism were found (by reducing the background intensity), 0,76 and 0,36 respectively for their atomic percentages.

3.3. General conclusions

- The biosynthesis of iron and silver nanostructured particles by using microorganisms is a viable, relatively easy and high-efficient method that allows the use of certain inexpensive biotechnologies for the metal absorption from the environment;
- Particles are generally spherically-shaped, with sizes ranging from 4 nm to 50 nm and feature a protein coat;
- The method can be tested for the metallic ions mitigation in the environment.

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