



POSSIBILITIES OF PHYSICAL METHODS IN DEVELOPMENT OF MICROBIAL NANOTECHNOLOGY

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The results of applied investigations carried out by some groups of Georgian scientists in the field of nanobiotechnology using different physical and chemical methods are presented. A number of new strains of the terrestrial actinomycetes isolated from soils and rocks of Georgia as well as the blue-green alga *Spirulina platensis* were used as the templates for production the noble metal nanoparticles on examples of silver and gold. A variety of spectral and analytical methods was applied to determine optimal conditions of synthesis and characterize the synthesized nanoparticles. The features of the used research methods and some results obtained with them are detail described. The advantages of each method and their ability to characterize the process of synthesis of nanoparticles are shown.

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microorganisms.⁸⁻¹³ In last years the number studies of microbial synthesis of gold and silver nanoparticles was carried out.¹⁴⁻¹⁸ In present paper the experience of use the different physical and chemical methods in these investigations is described and their possibilities are discussed.

Materials and methods

Materials

Georgia is a country with areas characterized by extreme microbial and plant biodiversity. The special interest present the microorganisms have great potential for biotechnology with medical applications. The study of different microorganisms characteristic for Georgia environment was performed in collaboration few groups of Georgian scientists. The distribution of different strains of terrestrial actinomycetes in various types of soils, rocks and rhizosphere of Georgia has been studied. Several groups of new microorganisms isolated in Georgia for the first times were studied as models for developing methods of nanoparticle biosynthesis. The blue-green alga *Spirulina platensis* was also used for obtaining gold and silver nanoparticles for medical and pharmaceutical application. The studied microorganisms are presented in Table 1 below.

The methods of cultivation of studied bacterial culture and production of biomass with gold and silver nanoparticles are described in detail elsewhere [14-18].

Methods

A variety of spectral and analytical methods was used to characterize the synthesized gold and silver nanoparticles.

Introduction

During the last years the use of physical properties and biosynthetic activities of microbial cells for the synthesis of nanosized materials has emerged as a novel biotechnological approach.¹ Numerous bacteria, fungi and yeasts have been exploited for biosynthesis of highly structured metallic nanoparticles. Microbial cells have developed specific mechanisms for surface functional groups (peptides, proteins, nucleic acids) interacting with metal ions in the aqueous solutions which result nanoparticles production.²⁻⁴ Gold and silver nanoparticles have potential applications in electronics, information technology, catalysis, medicine, pharmacology, sensing and photonics. In medicine they have shown therapeutic potential in oncology, cardiology, immunology, neurology and endocrinology.⁵⁻⁷

Currently there is a great need to develop new alternative, easy and eco-friendly methods to search the new effective microbial strains producing gold and silver nanoparticles. The great interests present the microorganisms with potential in medical applications such as the actinomycetes as well as the blue green alga *Spirulina platensis*. The collaboration of Georgian scientists during many years carried out investigations to development of biotechnological methods using different strains of

Table 1. Studied microorganisms

Names of bacteria	Species of bacteria	Site of bacteria isolation
Arthrobacter genera	<i>Arthrobacter globiformis</i> 151B <i>Arthrobacter oxydans</i> 61B	Isolated from the Kazreti region in Georgia
<i>Streptomyces</i> genera	<i>Streptomyces glaucus</i> 71MD <i>Streptomyces sp.</i> 211A	Isolated from the rhizosphere of soybeans in Georgia Isolated from the Cinnamonic calcareous soil of Sagarejo region in Georgia
Extremophile bacteria	<i>Streptosporangium spp.</i> 94A	Isolated from the Black soil of Shiraki Valley in Georgia
Thermophilic actinomycetes	<i>Thermoactinomyce spp.</i> 44Th <i>Thermomonospora spp.</i> 67Th	Isolated from the red soil of Adjara Region Isolated from the cinnamonic calcareous soil of Tetrtskaro region in Georgia
Blue-green alga	<i>Spirulina platensis</i>	Strain IPPAS B-256

The UV-vis (ultraviolet-visible) spectra of the samples were recorded by a spectrophotometer “Cintra 10“ (GBC Scientific Equipment Pty Ltd, Australia) with a wavelength range of 190 – 1100 nm.

X-ray diffraction (XRD) measurements were made with a Dron-2.0 diffractometer. The BCV-23 X-ray tube with the Cu anode ($\text{CuK}\alpha$: $\lambda = 1.54178 \text{ \AA}$) was used as a source of radiation.

Transmission Electron Microscopy (TEM) was performed using the JEOL SX-100 (Japan) equipment operating at 100 kV. The samples were prepared by placing a drop of solution with the gold or silver nanoparticles on carbon-coated TEM grids.

Scanning Electron Microscopy (SEM) was carried out using the Quanta 3D FEG, USA/ Systems for Microscopy and Analysis, (Moscow, Russia). Operational features of the microscope used in the experiment: magnification 100 – 200000 \times ; voltage 1 – 30 kV. Microprobe analysis of gold and silver nanoparticles clusters was conducted with the energy-dispersive X-ray analysis spectrometer (EDAX, USA).¹⁹

Flame atomic absorption spectrometry (AAS) with “Analyst-800” and “Beckman-495” spectrometers was used for gold and silver determination in the experimental samples.

The gold and silver concentrations as well as the elemental content of samples were determined using neutron activation analysis (NAA) at the reactor IBR-2 of the Frank Laboratory of Neutron Physics of the Joint Institute for Nuclear Research (Dubna, Russia). The experimental equipment and irradiation conditions of samples are described elsewhere.²⁰ The NAA data processing and determination of element concentrations were performed using Genie 2000 software.²¹

In addition to study biosorption process on the bacterial cells during nanoparticles production the method of equilibrium dialysis with atomic-absorption analysis were used.

The method sonication of bacterial biomass by ultrasound generator (35 kHz, 10-30 min) was used for intensification of processes nanoparticles production.

Results and discussion

The process of microbial synthesis of metal nanoparticles in a solution of a metal compound can be viewed as a metal ion reduction by the biomolecules, proteins and enzymes of bacterial cells. For example, the aggregation of silver nanoparticles takes place at the reduction of silver ions from Ag (I) to Ag (0) in a reaction of silver nitrate (AgNO_3) water solution with bacterial suspension. Similarly, the gold nanoparticles formation takes place at Au(III) ions reduction to Au(0) in a chloroauric acid (HAuCl_4) aqueous solution.

The ultraviolet-visible UV-vis spectrometry was mainly used for detection nanoparticles and determination experimental conditions of the nanoparticles synthesis by the bacterial cells.

The nanoparticles exhibit localized surface plasmon resonances at visible and near-infrared frequencies leading to sharp peaks in their spectral extinction.²² The extinction is the result of collective excitation of conducting electrons due to strong interaction between the metallic nanoparticles and the incident electromagnetic radiation.²³

In UV-vis surface plasmon resonances absorption spectra the peaks at 530 nm for gold and 425 nm for silver were observed.

In each case for determination the optimal concentrations of aqueous solutions AgNO_3 and HAuCl_4 the dose-dependent formation of nanoparticles was carried out at concentrations 10^{-2} – 10^{-4} M. Figure 1 shows the example of results of such experiment for gold nanoparticle production (a) and absorbance maximums versus the dose of AgNO_3 at silver nanoparticles production (b) in biomass of *Spirulina platensis*. In all cases the optimal concentration for bacterial synthesis of nanoparticles turned out to be 10^{-3} M. It should be noted that the all spectra obtained for the gold nanoparticles are better pronounced that those for the silver nanoparticles.

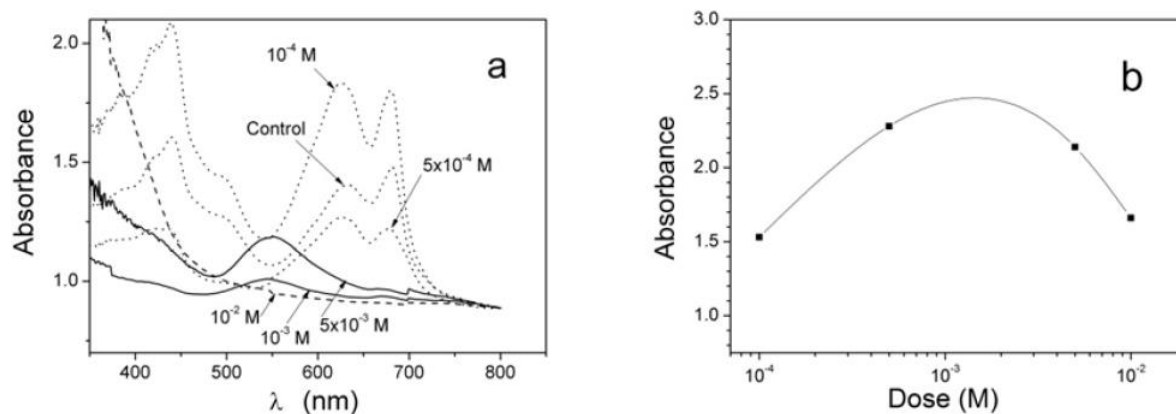


Figure 1. UV-vis spectra of gold nanoparticles in *Spirulina platensis* biomass obtained for different HAuCl_4 doses (a) and silver nanoparticles absorbance maximums versus the dose of AgNO_3 (b).

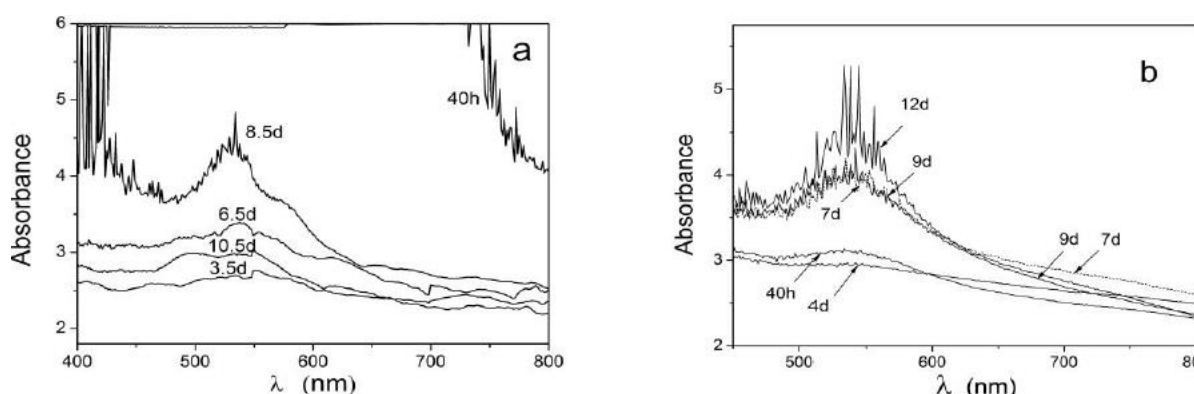


Figure 2. The absorption spectra of Au nanoparticles detected in the suspension of *Streptomyces glaucus* 71MD (a) and *Arthrobacter oxydans* 61B (b) at different time reaction with HAuCl_4 10^{-3} M water solution.

Figure 2 shows the UV-vis spectra recorded from suspension of actinomycetes *Streptomyces glaucus* 71MD (a) and *Arthrobacter oxydans* 61B (b) for different reaction times with HAuCl_4 10^{-3} M water solution. The presented spectra exhibit the appearance of Au absorption peak at 530 nm, which increases in intensity as a function of time of reaction. The shapes of these peaks show that gold nanoparticles have mainly the spherical shapes. The results of these experiments show that the favorable reaction time for Au nanoparticle synthesis using biomass *Streptomyces glaucus* 71MD is hours whereas in case of *Arthrobacter oxydans* 61B it is days.

The example of X-ray diffraction (XRD) spectra obtained for gold nanoparticles in biomass *Arthrobacter oxydans* 61B after reaction with 10^{-3} M HAuCl_4 (chloroauric acid) for 12 days (Figure 3a) and silver nanoparticles in biomass *Spirulina platensis* after reaction with AgNO_3 (silver nitrate) for 1 day (Figure 3b) are shown. As it is seen from Figure 3 a number of Bragg's reflections corresponding to a face centered cubic (fcc) structure of gold (or silver) are seen here: four characteristic peaks (111), (200), (220) and (311). The obtained results clearly show that the gold nanoparticles formed by reduction of Au (III) and Ag (I) ions by the cells of *Arthrobacter oxydans* 61B and *Spirulina platensis* are crystalline in nature.

The Sherrer's formula^{24,25} was applied for evaluating sizes of the gold nanoparticles on the basis one of the peaks in the diffractogram for different samples:

$$d = \frac{K\lambda}{\beta \cos \theta}$$

where K is the dimensionless shape factor, for cubic crystals it is 0.9–1; λ is the X-ray wavelength, for $\text{Cu K}\alpha$: $\lambda = 1.54178$ Å; β is the line width at half the maximum intensity in radians, θ is the Bragg angle, and d is the size of nanoparticles in nm.

The Sherrer's formula is applicable to grains with sizes less than 100 nm. For an approximate assessment of the size of nanoparticles, the (111) interferential maximum was used. In this case $\theta = 38^\circ$. The calculations were carried out taking into account only instrumental broadening of β ($\approx 0.3^\circ$) without evaluation of the influence of crystal defects on the shape of the interferential maximum. The size of gold nanoparticles in the biomass of *Arthrobacter oxydans* 61B determined by this method is ≈ 22 nm. This result coincides with results obtained by other methods.

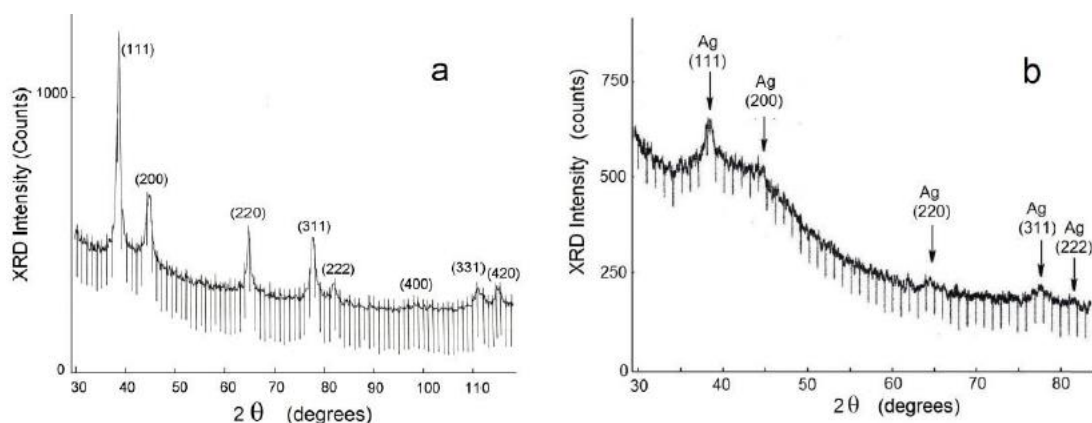


Figure 3. The XRD diffractogram for gold nanoparticles synthesized by *Arthrobacter oxydans* 61B treated with chloroauric acid for 12 days (a) and silver nanoparticles synthesized by *Spirulina platensis* treated with silver nitrate for 1 day (b).

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were carried out for visualization and approximate assessment of sizes of the formed nanoparticles.

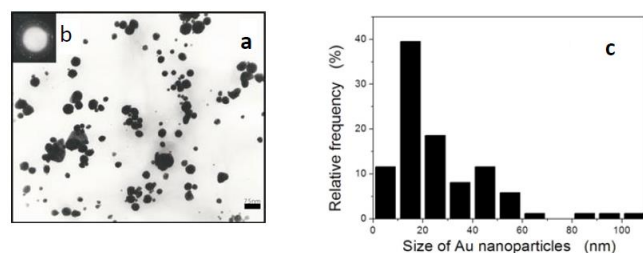


Figure 4. TEM micrographs recorded from drop-cast films of gold nanoparticles formed by the reaction of the HAuCl_4 solution with biomass *Thermoactinomyces* spp. 44Th after 6 days (a) the diffraction pattern of the selected area recorded from the gold nanoparticles (b) and Au nanoparticle sizes distribution (c).

Figure 4a shows the TEM image recorded from the drop-cast film of gold nanoparticles synthesized after reaction of the chloroauric acid solution with *Thermoactinomyces* spp. 44Th biomass for 6 days. The diffraction pattern of a selected area (Fig. 4b) corresponds to the face centered cubic (fcc) structure of gold nanoparticles. The particle size histogram obtained from this image (Figure 4c) shows that the size of the gold nanoparticles ranges from 5 nm to 60 nm with average 20 nm.

In Figure 5 the SEM images of silver nanoparticles formed on the surface of actinomycete *Streptomyces* spp. 211A (a) and gold nanoparticles formed on the surface of actinomycete *Streptosporangium* spp. 94A (b) are shown. These SEM images illustrate that most of the particles are spherical and do not create big agglomerates.

In the EDAX spectra, the energy versus relative counts of the detected X-rays is presented. The spectra shown in Figure 6 proved the presence of silver nanoparticles in *Streptomyces* spp. 211A biomass (a) and gold nanoparticles in *Arthrobacter globiformis* 151B biomass (b). The four peaks of Ag are observed for *Streptomyces* spp. 211A (Figure 6a). The signals from C, O, and P due to X-ray emission from proteins/enzymes existing in biomass are also

recorded. Several peaks of Au and the signals from C, O, K, P, and Ca are observed for *Arthrobacter globiformis* 151B (Figure 6b).

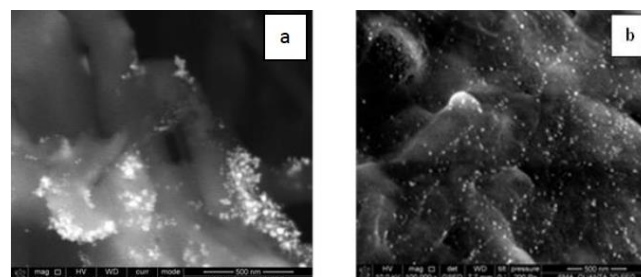


Figure 5. SEM image of silver nanoparticles formed on the surface of actinomycete *Streptomyces* spp. 211A (a) and gold nanoparticles formed on the surface of actinomycete *Streptosporangium* spp. 94A (b).

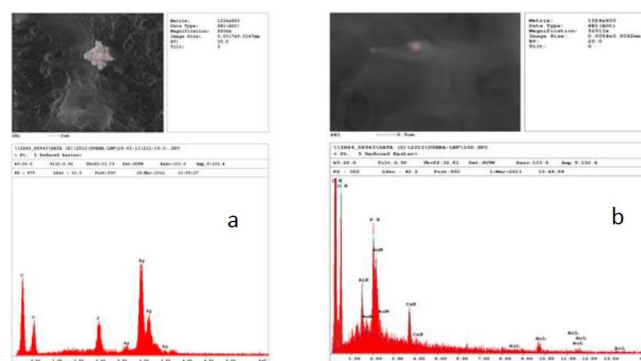


Figure 6. EDAX spectra recorded from *Streptomyces* spp. 211A biomass with silver nanoparticles (a) and from *Arthrobacter globiformis* 151B biomass with gold nanoparticles (b).

To study the biosorption process on the bacterial cells at nanoparticles production the method of equilibrium dialysis and atomic absorption analysis were used. Concentrations of the metal adsorbed by bacteria in the solution at equilibrium dialysis obeyed the Freundlich equation that suggests the presence of heterogeneous sorption sites on bacteria surfaces, since the sorption depends on the nature and the composition of the cell wall.

The capacity of the adsorbent and the equilibrium relationship between adsorbent and adsorbate are described by Freundlich adsorption isotherms:

$$C_b = KC_t^n$$

where C_b is concentration of the metal adsorbed, C_t is equilibrium concentration of the metal ion in the solution, K and n are empirical constants, which may be characterized as the biosorption constant and sorptive capacity, respectively. The Figure 7 shows the Freundlich adsorption linearized isotherms for gold nanoparticles in *Streptomyces spp.* 211A biomass (A – for homogenized and B – for particulate homogenized).

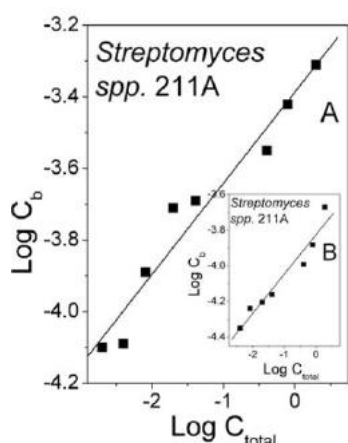


Figure 7. Freundlich adsorption isotherms for Au nanoparticles in *Streptomyces spp.* 211A biomass (A – for homogenized and B – for particulate homogenized).

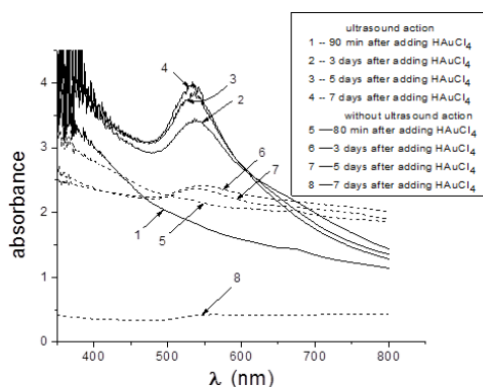


Figure 8. UV-vis spectra recorded as a function of time of reaction aqueous solution of HAuCl₄ with *Spirulina platensis* (after and without sonication).

To study the possibility of intensification of nanoparticle synthesis, the biomass of the blue-green alga *Spirulina platensis* with Au nanoparticles was subjected to sonication for 10 min using ultrasound generator at 35 kHz. The examination under an optical microscope shows that the biomass of *Spirulina platensis* disintegrated into small fragments. Then, for producing Au nanoparticles, an aqueous solution of the HAuCl₄ with a concentration of 10⁻³ M was added to the suspension. The results of measurements of UV-vis spectra for reaction at different time intervals are shown in Figure 8.

As it seen from Figure 8, the absorption peak of Au nanoparticles after the alga has been subjected to sonication is 4 times higher than without sonication. This can be caused by the increase of total surface of small bacterial fragments after sonication that also confirms the assumption about extracellular surface formation of nanoparticles.

In Figure 9a the TEM image of Au nanoparticles obtained upon subjection of the alga to the sonication is shown. The size distribution of these Au nanoparticles, given in Figure 9b shows that mean size of the Au nanoparticles is about 15 nm whereas without sonication this value was 25 nm [17]. Thus an intensification of the nanoparticles formation and reduction of their sizes take place at sonication.

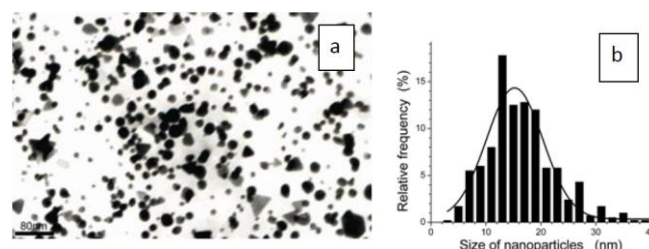


Figure 9. The TEM image of Au nanoparticles formed in the reaction of HAuCl₄ solution with *Spirulina platensis* biomass subjected to sonication (a) and Au nanoparticles size distribution histogram (b).

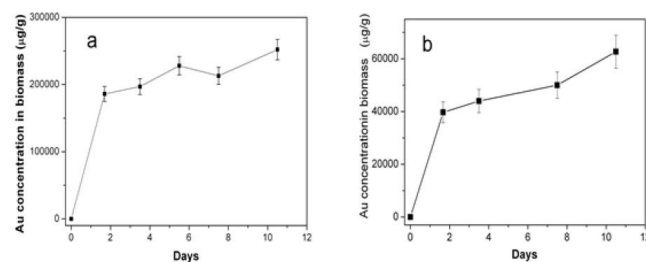


Figure 10. The total gold concentrations in biomass *Streptomyces spp.* 211A determined by NAA (a) and by AAS (b).

The analytical methods of neutron activation analysis (NAA) and atomic absorption spectrometry (AAS) were applied for determining total Au and Ag content in the biomass of studied bacteria. Examples of analytical determination (using NAA (a) and AAS (b)) of gold total concentrations in the bacterial biomass *Streptomyces spp.* 211A are given in Figure 10.

In all cases the analogical dynamic of total metal accumulation was observed: the concentration of metal increases rapidly in the first few hours and then increases slowly. In the first phase, the metal ions were mainly adsorbed onto the surface of bacterial cells extracellular. In the second phase, the metal ions were transported into the cells and accumulated intracellular.

NAA was also used to study multi-elemental content of the bacterial samples taking into account the possible medical application of the synthesized biomass with Au and Ag nanoparticles. The example is shown in Figure 11 for

Streptomyces glaucus 71MD. The NAA results show that the concentrations of some toxic elements in the obtained biomass do not exceed the permissible levels and synthesized materials with Au and Ag nanoparticles may be used for industrial, medical and pharmaceutical purposes.

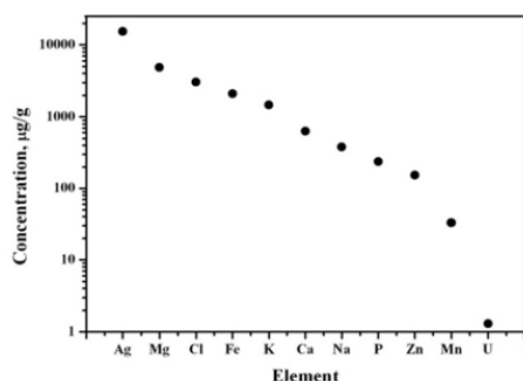


Figure 11. The distribution of elements in *Streptomyces glaucus* 71MD sample.

Conclusions

The performed investigations show that the studied microorganisms – the new strains of actinomycetes: *Arthrobacter* genera (*Arthrobacter globiformis* 151B and *Arthrobacter sp.*61B), extremophiles *Streptomyces spp.* 211A and *Streptomyces glaucus* 71MD, thermophiles *Thermoactinomyces spp.* 44Th and *Thermomonospora spp.* 67Th as well as the blue-green alga *Spirulina platensis* are able to produce gold and silver nanoparticles by interacting with 10^{-3} M aqueous solutions of chloroauric acid (HAuCl₄) and silver nitrate (AgNO₃).

The produced gold and silver nanoparticles formed by bacterial biomass are crystalline in nature and they are mainly produced extracellular. In general, they have the spherical shapes and sizes in the range of 5-60 nm with the average size in the range 15 – 35 nm for different bacterial strains.

The methods of UV-vis and XRD spectrometry, SEM with EDAX and TEM microscopy as well as atomic adsorption spectroscopy (AAS) and neutron activation analysis (NAA) are powerful for examining Au and Ag nanoparticles in bacterial biomass and characterizing mechanisms of their formation. The methods of equilibrium dialysis with AAS were successively used for study of surface biosorption process at formation of nanoparticles by tested microorganisms.

The performed investigations show that the studied microorganisms can be used for development of clean, simple, nontoxic and environmentally acceptable methods of synthesis gold and silver nanoparticles and have great potential in industry and medicine.

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