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BIOCHEMICAL CHANGES IN *Nostoc linckia* ASSOCIATED WITH SELENIUM NANOPARTICLES BIOSYNTHESIS

BIOCHEMICZNE ZMIANY W *Nostoc linckia* ZWIĄZANE Z BIOSYNTEZĄ NANOCZĄSTEK SELENU

Abstract: The cyanobacterium *Nostoc linckia* was used to study the biotechnology of selenium nanoparticles synthesis for the first time. The experimental conditions of the nanoparticle production by the studied cyanobacteria in aqueous cobalt selenite solutions were examined. Neutron activation analysis allowed characterization of the dynamics of accumulation of the total selenium quantity by *Nostoc linckia*. Scanning Electron Microscope images demonstrated extracellular formation of amorphous nanoparticles. Released selenium nanoparticles ranged in size from 10 to 80 nm. The changes of essential parameters of biomass (proteins, lipids, carbohydrates, and phycobilin) content during the nanoparticle formation were assessed. During the first 24 h of nanoparticle synthesis, a slight decline of proteins, lipids and carbohydrates content in the biomass was observed. The most extensive was the process of phycobilin degradation. Furthermore, all biochemical component content as well as an antioxidant activity of the biomass extracts significantly decreased. The obtained substance of *Nostoc* biomass with selenium nanoparticles may be used for medical, pharmaceutical and technological purposes.

Keywords: selenium, nanoparticles, *Nostoc linckia*, optical and analytical methods, biochemical analysis

Introduction

The research in the field of nanotechnology has been stimulated by large technological and medical applications of nanoparticles [1]. Selenium possesses excellent photoelectrical and semiconductor properties, which make it extensively used in the production of

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photovoltaic cells, photographic exposure meters, xerography, and the glass industry [2-6]. Beside wide technological uses, selenium is also an essential trace element for living organisms, being a constituent of selenoproteins [7], an amino acid [4], and has a great importance in nourishment and medicine [3, 8]. Selenium can improve the activity of the selenoenzyme, glutathione peroxidase, and prevents formation of free radicals from damaging cells and tissues in vivo [6, 9]. Stable organic selenium compounds are used as antioxidants, enzyme inhibitors, antitumor and anti-infective agents, cytokine inducers and immune-modulators [1]. The selenium at nano size acts as a potential cancer therapeutic agent [10] and chemo-preventive agent with reduced toxicity [1, 2], and has garnered increasing attention in medicine.

Use of toxic chemicals in physical, chemical and hybrid methods for nanoparticle synthesis greatly limits their biomedical applications, in particular in clinical fields [11]. Recently, biogenic systems have emerged as a novel method for the synthesis of a variety of inorganic materials at close to ambient temperatures and pressures and neutral pH [11, 12]. The biological method is clean, nontoxic and environmentally friendly, and most significantly, the synthesized materials are biologically compatible [12]. From a literature search it was found that different types of microorganisms, including cyanobacteria, are able to produce selenium nanoparticles of different sizes and shapes [1, 12-15].

Species of the genus Nostoc are convenient biotechnological objects, since most strains do not produce toxins and grow on simple and inexpensive media [16, 17]. At the same time, the cyanobacterium *Nostoc linckia* has enhanced biosorption and reducing capacity of various chemical elements [18].

In the present work, cyanobacterium *Nostoc linckia* was used for the first time to study the process of selenium nanoparticles (SeNPs) formation. The changes in antioxidant activity of biomass extract, proteins, lipids, phycobilins, and carbohydrates content were determined.

Materials and methods

Materials

Cyanobacterial biomass preparation

To carry out the experiment, the culture of *Nostoc linckia* CNM-CB-03 (from the National Collection of Nonpathogenic Microorganisms, Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova) was cultivated in laboratory conditions on mineral medium Gromov 6 (macroelements [g/dm^3] - KNO_3 - 0.5, K_2HPO_4 - 0.45, $NaHCO_3$ - 0.05, $MgSO_4 \cdot 7H_2O$ - 0.1, $CaCl_2$ - 0.11 and microelements [mg/dm^3] - $ZnSO_4 \cdot 7H_2O$ - 0.05, $MnSO_4$ - 2, H_3BO_3 - 0.85, $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ - 2.25, $FeSO_4 \cdot 7H_2O$ - 4, $Co(NO_3)_2 \cdot H_2O$ - 0.009, EDTA - 4.75) stirring daily, continuous illumination (a light intensity of 2000-3000 lx), at a temperature between 25-30ºC and with an optimum pH range of 6.0-7.0. On stationary growth phase (14th day for *N. linckia*), the cyanobacteria biomass was separated from the culture medium by centrifugation.

Obtained wet biomass (0.5 g), was re-suspended in a 250 cm^3 Erlenmeyer flasks with 100 cm^3 of 100 mg/dm^3 aqueous cobalt selenite solution (analytically pure, Merck, Darmstadt, DE) for synthesis of selenium nanoparticles. The resultant mixtures were put into the shaker repeatedly at between 28-30°C for different periods of time (24-72 h). All samples, prepared in triplicate, were used for further analysis.

For SEM, TEM, and NAA analysis, the Nostoc biomass was harvested from the cultures by centrifugation at 12000 g for 8 min, and then dried at 105ºC. For biochemical studies, the native biomass was used. Biomass was separated from culture medium, washed with ammonium acetate, and then re-suspended in distillate water served as the control. All the experiments were conducted in triplicate and the averages of the measurements for each treatment were used.

Methods

Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy (SEM) was carried out using the Quanta 3D FEG (FEI Company, USA). Operational features of the microscope used in the experiment: magnification 5000-150000x; voltage 1-30 kV.

To estimate the size of the nanoparticles on the obtained SEM micrographs, the points corresponding to the edges of the measured object were determined. For this purpose, the following operations were performed:

(i) The informative signal profile (profile of the color channel intensity distribution) along the straight line segment passing through the object's borders was created.
(ii) The signal, corresponding to the surroundings of each of the edges (the intensity curve of image pixels along a datum line) was fixed. The edge of the measured object corresponds to the half-height of the Gaussian function, used to describe distribution of the signal intensity.
(iii) The size of the nano-object was determined as the distance between two its edges.

Energy-Dispersive Analysis of X-Rays (EDAX)

Microprobe analysis of selenium nanoparticles was conducted with the energy-dispersive X-ray analysis spectrometer (EDAX, USA). The acquisition time ranged from 60 to 100 s, and the accelerating voltage was 20 kV.

Neutron Activation Analysis (NAA)

The selenium content in the biomass was determined using the 103 keV γ-line of ^{81x}Se by irradiation for 60 s under a thermal neutron fluency rate of approximately $1.6 \cdot 10^{13}$ n cm^{-2} s^{-1} at the reactor IBR-2 of (Joint Institute for Nuclear Research, Dubna, Russia). The NAA data processing and determination of elemental concentrations were performed using Genie 2000 and the software developed at FLNP JINR [19]. The experimental equipment and irradiation conditions of samples are described elsewhere [20].

Biochemical analysis of biomass components

For proteins extraction 10 mg of biomass was mixed with 0.9 cm^3 NaOH (concentration 0.1N) for 30 min 0.1 cm^3 of the obtained protein extract was mixed with 1.6 cm^3 Na_2CO_3 (2%) in NaOH 0.1N, 0.4 cm^3 of $CuSO_4$ (0.5%) in $Na_3C_6H_5O_7$ (1%) and 0.2 cm^3 of Folin-Ciocalteu reagent. After 30 min the absorbance was measured at 750 nm. The protein content was determined using a calibration curve for bovine serum albumin. Phycobiliprotein content was determined spectrophotometrically in water extract (10 mg of wet biomass was mixed with 10 cm^3 of distillated water). The absorbance of supernatant separated from the culture medium by centrifugation was measured at 620 and 650 nm. Phycobiliprotein content was calculated using molar absorption coefficient for phycocyanin

and allophycocyanin. To determine carbohydrates content, 0.25 cm^3 of the sample was mixed with 2.5 cm^3 of Antrone reagent (0.5%) in H_2SO_4 (66%). The mixture was incubated at 100ºC for 30 min. The absorbance was measured at 620 nm. Carbohydrates content was calculated using a calibrationn curve for glucose. Lipids content was determined spectrophotometrically on the basis of the lipids degradation using fosfovanilinic reagent. Hydrolysis of the lipids (10 mg biomass) was produced by sulfuric acid at 100ºC. Obtained hydrolysate (0.5 cm^3) was mixed with 1.5 cm^3 fosfovanilinic reagent. After 45 min the absorbance was measured at 560 nm. Lipids content was calculated using calibration curve. The content of aforementioned biomolecules was expressed in % of absolute dry biomass.

Determination of anti-oxidative activity

For antioxidant tests, ethanolic and water extracts were prepared. Water extract contains water-soluble components (phycobilins, vitamins, carbohydrates). Using ethanol, a complex of substances soluble in ethanol (chlorophyll, lipids and carotenoid) were obtained. For each sample, 10 mg of wet biomass was mixed with 10 cm^3 ethanol or distillated water for 60 min. After filtration, the samples were standardized: 1 mg dry active substances in 1 cm^3. The extracts were kept at 0°C.

Determination of antioxidative activity

The total antioxidant activity of extracts was measured by the ABTS (2.2 azinobis 3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization assay [21]. ABTS was dissolved in distillated water to a 7 mM concentration. ABTS radical cation was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration), and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The ABTS stock solution was diluted with ethanol to an absorbance of 0.73 ±0.02 at 734 nm. Then 1 cm^3 of diluted ABTS solution was mixed with 10 mm^3 of the test sample. After 6 min the absorbance was measured at 734 nm. All trials and measurements were performed three times.

Results and discussion

NAA was used to assess the change in the total selenium concentration in the biomass of cyanobacteria after 24, 48, and 72 h of interaction with selenite ions. According to Li and co-authors [12], nanoparticles are biosynthesized when the microorganisms grab SeO_3^{2-} ions from their environment (first step), and then turn them into a nanoparticle form via bioaccumulation and biotransformation (second step).

The data obtained by NAA for silver nanoparticles synthesis by *N. linckia* illustrated the rapid increase of silver concentration in the biomass without significant further changes [22]. In the case of SeNPs formation, the NAA data (Fig. 1) showed a less pronounced division and almost continuous growth of selenium concentration in the biomass. This can be explained by the fact that silver is very toxic for living organisms and leads to damage of the cell surface. Selenium present in the cyanobacteria cultivation medium at some concentrations does not inflict toxic effects on the organic structures [23]. Cyanobacteria affinity for selenium may be the result of its presence in the enzymatic antioxidant system of glutathione reductase. Therefore, selenium is a structural component necessary to maintain the viability of the culture of cyanobacteria, which, in turn, develops the

mechanisms of selenium accumulation from the cultivation medium and its storage in organic structures for further use.

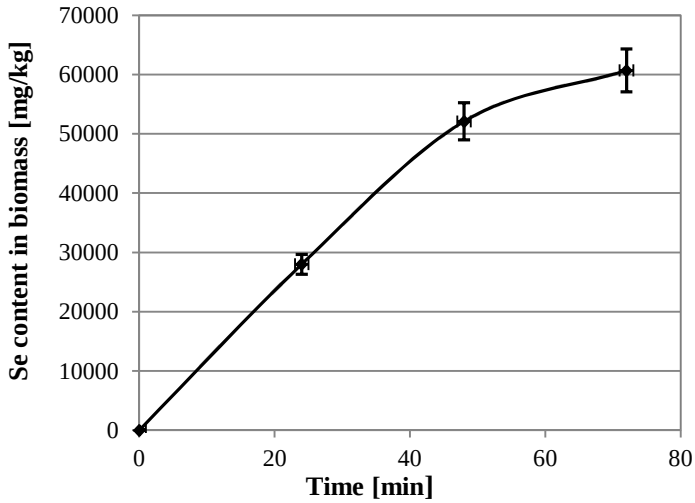

Fig. 1. Total selenium concentrations in biomass of *Nostoc linckia* determined by NAA, $n = 3$

SEM micrographs obtained from the selenite-supplemented biomass revealed the great number of spherical SeNPs formed by the biomass of *N. linckia* both extra- and intracellularly (Fig. 2). It can be suggested that a part of selenite ions was trapped on the cyanobacteria surface and reduced to the elemental form while another part was accumulated inside the cell via sulphate transport channels and reduced to an organic form of selenium through different mechanisms. The nanoparticle size ranged from 10 to 80 nm.

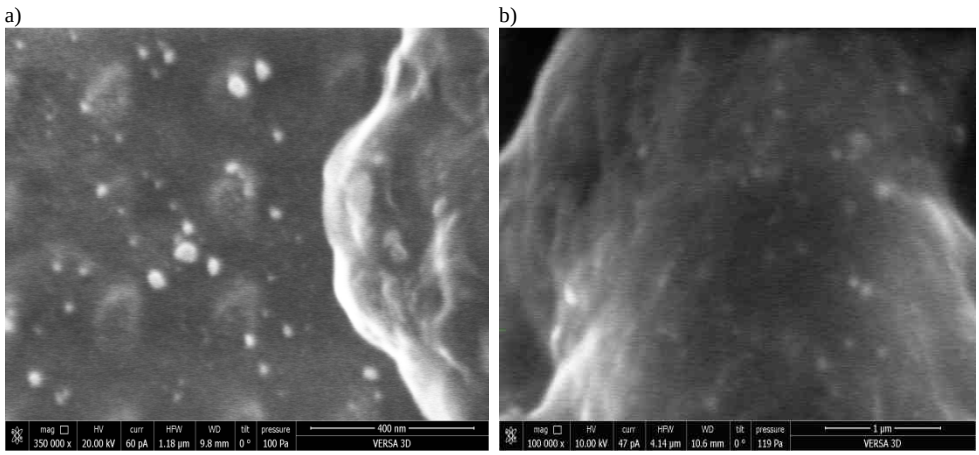

Fig. 2. SEM micrographs of *Nostoc linckia* with selenium nanoparticles taken after: a) 24 h and b) 72 h of incubation

Comparing our results with the results obtained for other biological objects, one can conclude that *N. linckia* can synthesize particles of a smaller size. For example, the extracellular formation of Se(0) nano-spheres by *Sulfurospirillum barnesii*, *Bacillus selenitireducens*, and *Seleni-halanaerobacter shriftii* were observed by Oremland et al [13] to range between 200 to 400 nm. Additionally, nanospheres of diameter ranging from 150-200 nm were also observed to be formed extracellularly by *Bacillus cereus* [14]. Moreover, the transformation of selenite (SeO_3^{2-}) to elemental selenium in the cell cytoplasm and in the extracellular space was showed by *Stenotrophomonas maltophilia* SELTE02 [15]. During biogenic selenite reductions by *Duganella* sp. and *Agrobacterium* sp., large crystals of elemental selenium (100-220 nm) were formed [1]. *Shewanella* sp. strain HN-41 was able to utilize selenite as a sole electron acceptor for respiration in anaerobic condition, resulting in its reduction and precipitation of spherical SeNPs [12].

Beside SEM micrographs, EDAX spectrum of biomass *N. linckia* with selenium nanoparticles was recorded (Fig. 3). In this spectrum, one peak of selenium was observed. The signals from C, K, Si, Ca, S, P, and Mg atoms due to X-ray emission from the cell walls were also recorded.

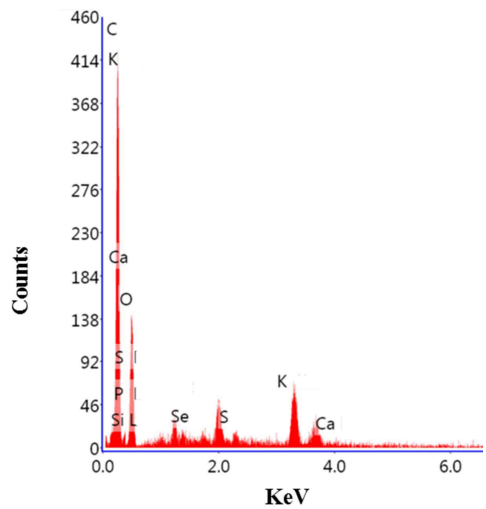

Fig. 3. EDAX spectrum of *Nostoc linckia* with selenium nanoparticles

Extra- or intracellular formation of nanoparticles strongly depends of the localization of the biomolecules involved in the process of metal reduction. Cyanobacterial Nostoc cells are surrounded with an exopolysaccharide layer, which play an important role in metal ions accumulation and reduction [24]. Zhang et al [6] have shown that in the molecular structure of polysaccharides, there are reactive amino, hydroxyl or carboxyl groups that have a great effect in the formation, stabilization and growth of selenium nanoparticles. SEM micrographs obtained in the present study showed that after 24 h, SeNPs were generally located on the cyanobacteria surface but after 72 h they were already covered by a carbohydrates layer (Fig. 2b), which prevents their aggregation. Lenz et al [25] suggests that selenite can be reduced to elemental selenium by reaction with reactive thiol groups of proteins/peptides in the so-called “painter-type” reaction.

Since Nostoc biomass is a valuable product, depending on the field of application, it is not always necessary to extract nanoparticles. Thus, it is very important to maintain the quality of the biomass during nanoparticles formation. The content of the main group of substances in the biomass was determined over the period of the experiment. Formation of selenium nanoparticles by Nostoc biomass leads to modification of its biochemical composition. The most pronounced was the decrease in phycobilin content during the reaction of the biomass with selenite ions (Fig. 4). After 24 h of interaction with selenite ions, the content of phycoerythrin was drastically reduced by 72%. During the next 24 h, the decrease of phycoerythrin content was not observed, and after 72 h of the reaction it was reduced by 80%.

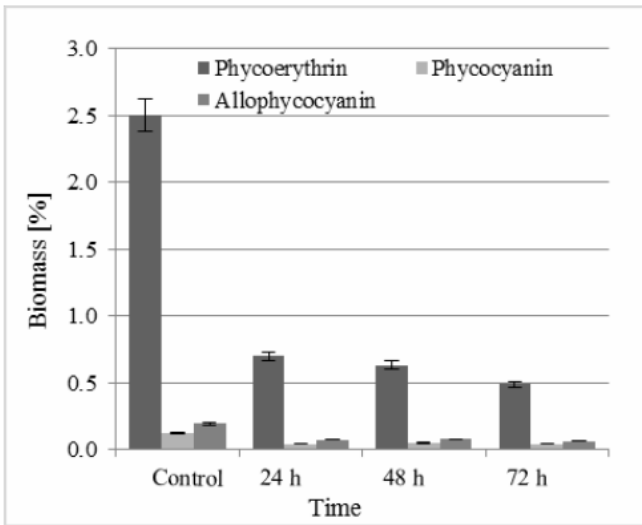

Fig. 4. Change of phycobilin content in *Nostoc linckia* biomass during selenium nanoparticles formation, $n = 3$

The content of phycocyanin was essentially modified and reduced by up to 75% after the first 24 h of contact with the selenium ions. The allophycocyanin content changed in the same manner. The most pronounced reduction of its content was after the first 24 h of contact. Consequently, the Nostoc biomass was exposed to an extensive process of phycobilin degradation. Phycobilins are auxiliary photosynthetic pigments structured in thylakoids, which occupy space near the cell membrane. Thus, the inconspicuous damage of the cell membrane leads to the decrease of the phycobilin content. In the experiment of silver nanoparticle synthesis, Nostoc biomass lost 86% of phycoerythrin content in the first 24 h. After 72 h, phycoerythrin content decreased by 92% [22]. Therefore, Nostoc affinity to selenium is manifested in a slower manner in comparison with that of the silver [22] phycobilin pigment degradation process.

This phenomenon of "finer" Nostoc biomass component degradation is maintained also for proteins, whose content during silver nanoparticle formation was reduced by 45% in the first 48 h of reaction [22]. Protein reaction during the process of selenium nanoparticles formation was rather different (Fig. 5). The content of proteins in biomass was reduced by

16% in the first 24 h, and was followed by a further reduction by 30% after 48 hours. The essential decrease of protein content in biomass is observed after 48 h of contact. After 72 h of contact with selenite ions, the protein content in biomass was 25% of the original content.

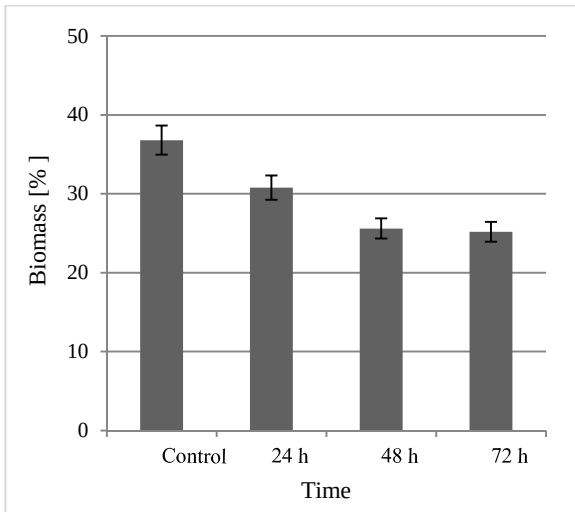

Fig. 5. Change of proteins content in *Nostoc linckia* biomass during selenium nanoparticles formation, $n = 3$

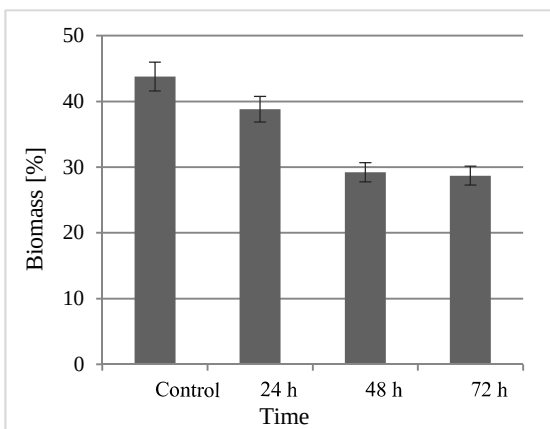

Fig. 6. Change of carbohydrates content in *Nostoc linckia* biomass during selenium nanoparticles formation, $n = 3$

Carbohydrates with protective functions are characteristic for *N. linckia*. As in case of proteins, the essential decrease of carbohydrates content takes place after 48 h of reaction (Fig. 6). During the synthesis of selenium nanoparticles the carbohydrate content in the biomass in the first 24 h was reduced by 6%. After 48 h, the carbohydrate content in Nostoc

biomass was reduced by 11%, without further change, indicating the protective function of exolysaccharide. Similar results were obtained for silver nanoparticles biosynthesis [22].

Lipids from *Nostoc* execute mainly the structural functions of membranes of functional components, thus their quantitative preservation is an essential condition for cellular integrity. The low rate of lipids degradation confirms this fact. Results presented in Figure 7 show that in in the first 48 h of reaction with selenite ions, the lipid content changed unsignificantly by 15%, while after 72 h no decrease was observed. For silver nanoparticles after 72 h, the content of lipids changed only by 9.7% [22].

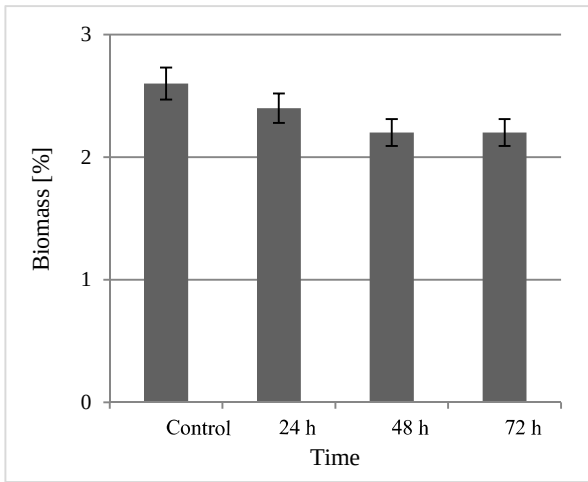

Fig. 7. Change of lipids content in *Nostoc linckia* biomass during selenium nanoparticles formation, $n = 3$

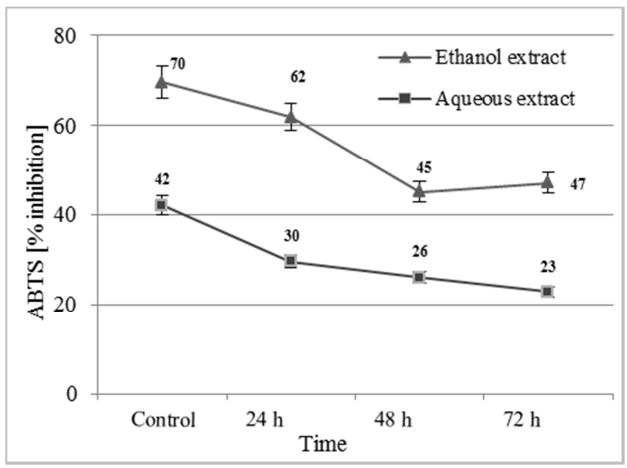

Fig. 8. The antiradical capacity of extracts from *Nostoc linckia* biomass after exposure to selenite ions

To assess the activity of functional components of Nostoc biomass in the process of nanoparticle synthesis, the antioxidant tests of ABTS radical reduction were applied. To determine antioxidant activity of biomass and its modification during the process of nanoparticle formation, the ethanolic and water extracts were obtained from the Nostoc biomass. The results of the ABTS test are presented in Figure 8. By the end of the experiment the antioxidant activity of the water extract decreased by 50%. The ABTS test indicated the reduction of water extract activity by 38% after 48 h and 47% after 72 h of exposure. The relative stability of the antioxidant activity in the period of 24-48 h of nanosynthesis can be mentioned. The same phenomenon was determined for water extract obtained from biomass exposed to silver ions [22].

For ethanolic extract, the picture of antioxidant activity modification is different. In the first 24 h the reduction of antioxidant activity was insignificant (~10%). In the next 48 h, the antioxidant test indicated a decrease of capacity of ethanolic extract to reduce ABTS radical from 35 to 13% for biomass obtained during the formation of selenium nanoparticles. Towards the end of the experiment the antioxidant stability of ethanolic extract was determined.

Therefore, it can be assumed that the indirect impact of the contact time of the interaction on the functional components of the biomass takes place. Water soluble components are the most sensitive, with lysing starting during the first 24 h of contact, and maintaining till the end of the experiment. Destruction of ethanolic extracts becomes visible after 48 h of nanosynthesis without tendency of evolution.

Conclusions

It was shown that *Nostoc linckia* can be used for extracellular and intracellular amorphous selenium nanoparticle production. Biochemical tests revealed the toxic effect of selenite ions on the cyanobacterial cells. The sensitivity of cell components to selenite ions action can be presented in the following order: phycobilins < proteins < carbohydrates < lipids. Time of incubation is the parameter which influences the change of the functional component content and antioxidant activity of biomass. It should be mentioned that during the first 24 h of biomass interaction with selenite ions, the composition of the biomass changed insignificantly (except the phycobiline content), while after 72 h of interaction with selenite ions, *N. linckia* maintained its vital activity.

Antioxidant activity of biomass showed that water-soluble components were more sensitive to selenite ion impact than the ethanolic ones. The results obtained showed that to obtain biomass containing selenium nanoparticles with minimal biomass lysing, the incubation time for experiment should not exceed 24 h.

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