APPLICATIONS OF INORGANIC GERMANIUM COMPOUNDS FOR OBTAINING SPIRULINA BIOMASS ENRICHED WITH GERMANIUM AND BIOACTIVE COMPOUNDS

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Abstract. The article deals with the effects of inorganic germanium-containing compounds GeO_2 and $GeSe_2$ with concentrations in the range of 10-30 mg/L on *Arthrospira platensis CNMN-CB-11* (spirulina) productivity, and some valuable components (proteins, phycobiliproteins, lipids and carbohydrates). In addition, germanium accumulation in cyanobacterial biomass was investigated. Data on thiobarbituric acid test showed that inorganic compounds of germanium in the range of tested concentrations did not exert high toxicity to cyanobacterium *Arthrospira platensis CNMN-CB -11*. This study of optimal concentration range revealed, for instance, that addition of 20 mg/L GeO_2 and 10-20 mg/L of $GeSe_2$ induces production of spirulina biomass enriched with germanium and bioactive compounds.

Keywords: *Arthrospira platensis*; germanium-containing compounds; productivity; bioactive substances.

INTRODUCTION

Currently, there is an increasing demand for the use of natural products due to their balanced composition in biologically active substances, known to promote the health of humans and prevent various diseases. Microalgae and cyanobacteria, in particular *Spirulina platensis*, may be used as perspective sources of biologically active components of natural origin for various biotechnologies [4, 9, 38, 43, 45]. Arthrospira (spirulina) biocomponents such as micro- and macroelements, free amino acids, peptides, carotenoids, polyunsaturated fatty acids, vitamins possess some pharmacological properties: anti-cancer, antibacterial, anti-immunosuppressive, anti-tumour, neuroprotective, and antiviral activities [11, 13, 29, 34, 36]. At the same time, spirulina is a very convenient matrix for embedding microelements [5, 7]. It has been shown that germanium embedded in the composition of spirulina biomass is of particular interest. The beneficial effects of spirulina focusing on its antioxidant properties can be significantly enhanced by germanium incorporation to phycobiliproteins in spirulina culture.

Organic germanium has a wide range of biological effects: it stimulates the immune system and protects healthy cells (suppresses the multiplication of microbial cells, activates macrophages and specific immune cells); delays the development of malignant neoplasms and thereby preventing metastatic spread; has strong antioxidant and free radical scavenging activity; provides oxygen transfer to tissues of the body and prevents oxygen deficiency (similar to hemoglobin); regulates all valve systems of the body (in the gastrointestinal tract, cardiovascular system); blocks the movement of electrons in neurons, has analgesic effect, eliminates chronic pain and inflammation; has antimicrobial, antiviral and antifungal effects (facilitating the synthesis of interferon to protect against foreign microorganisms) [1, 24, 28, 39, 44, 50].

Many plants that have long been used in Tibetan medicine and traditional Chinese medicine contain an increased amount of germanium in comparison to other plants [1]. Among plants capable of adsorbing germanium and its compounds from the soil, ginseng is the leader. In addition, high levels (2–9 mg/kg) of the element have been found in garlic, beans, tomato juice, fish and seafood [23, 35]. However, in order to ensure the daily needs of the body in germanium, it is necessary to use very high quantities of tomato juice or salmon.

There is very little information in the literature about the effects of germanium compounds on growth, productivity and biochemical composition with regard to cyanobacterium *Spirulina platensis*. A small number of papers are known on this subject and the influence of GeO_2 on germanium accumulation [14, 19, 47, 51] or some germanium organic compounds [16].

Therefore, it was of interest to compare the effect of two germanium-containing compounds GeO_2 and $GeSe_2$ on germanium uptake. In order to achieve this goal, monitoring of the accumulation of germanium and other bioactive compounds (proteins, phycobiliproteins, carbohydrates and lipids) in spirulina biomass was carried out, since each of these biocomponents plays an important role for the vital functions of cells. Protein content is one of the main biochemical markers of the physiological state of cyanobacteria, and phycobiliproteins are an important part of the photosynthetic apparatus of the cell. Lipids are part of the thylakoid and cell membranes and serve as indicators of the physiological state of the cell, while carbohydrates are vital reserve substances. Whether the content of these cellular components is significantly reduced in the presence of a germanium compound, then it indicates toxicity to culture. The levels of these biochemical components should be investigated to understand the biomass quality changes due to the addition of germanium-containing compounds. This will allow the use of germanium-enriched spirulina biomass in dietary supplements and pharmaceuticals.

The aim of this paper was to study the effect of two inorganic germanium-containing compounds, namely germanium dioxide (GeO_2) and germanium selenide ($GeSe_2$), on the culture productivity, accumulation of germanium and other bioactive components (proteins, phycobiliproteins, carbohydrates and lipids) in *Arthrospira platensis* strain *CNMN-CB-11* (spirulina) biomass.

MATERIAL AND METHODS

The object of study. Cyanobacterium *Arthrospira platensis* strain *CNMN-CB-11(spirulina)* was stored at the National Collection of Nonpathogenic Microorganisms of the Institute of Microbiology and Biotechnology, Republic of Moldova [40].

The cultivation process conditions. The modified Zarrouk medium was used, with a certain ratio of macro- and microelements for the normal development and growth of spirulina culture [41]. In addition, GeO_2 (Sigma-Aldrich) and $GeSe_2$ (Sigma-Aldrich) were introduced into the nutrient medium in three concentrations: 10, 20 and 30 mg/L.

Cultivation was carried out in Erlenmeyer flasks containing 100 mL of culture liquid for 144 hours. During cultivation, the following process parameters have been checked: inoculum – 0.4-0.45 g/L ADB (absolutely dry biomass); temperature of 28-32°C, pH-optimum of medium 8-10, illumination of ~ 37-55 µM photons/m^2/s (throughout the duration of its life cycle under a light regime). The culture was stirred daily for 2 hours on a universal WU-4 laboratory shaker with the oscillation frequency of 2500 Hz.

Methods to determine biochemical composition of spirulina culture.

Spirulina productivity in the experimental samples and control was determined by measuring the optical density at λ - 560 nm with the recalculation of the cell mass to absolute dry biomass (ADB). Productivity was expressed in g/L absolute dry biomass (ADB) and in % relative to control, in order to compare the results [41].

The protein content in biomass was determined spectrophotometrically by the Lowry method using Folin-Ciocalteu reagent [27].

The carbohydrate content was determined based on the dehydration of hexoses in the presence of concentrated sulfuric acid, followed by their condensation with the anthrone reagent [10].

Lipid content was determined spectrophotometrically using the phospho-vanillin reagent in the chloroformic extract of spirulina [49].

Phycobiliprotein content was determined in aqueous extract obtained from spirulina biomass by the method proposed in [3].

The degree of oxidation of lipids was determined based on the degradation products of thiobarbituric acid [37].

The germanium content in spirulina biomass was determined with the photometric reagent phenylfluorone according to the method [31].

The determination of germanium was preceded by biomass mineralization by using H_2O_2 and concentrated HNO_3. Biomass was mineralized for 2-3 hours on a sand bath until a colourless solution was obtained.

All the experimental results were subjected to formal statistical analysis with the application of descriptive statistics tools (calculation of arithmetic means, standard deviation, coefficient of variation and fiducial limits), inferential statistics (tests of statistical significance and validity). Calculation of statistical indicators has been conducted using the possibilities of MS Excel.

RESULTS

The experimental data of the effects of inorganic germanium-containing compounds on the dynamics of germanium accumulation in cyanobacterial biomass and spirulina productivity are shown in Figure 1.

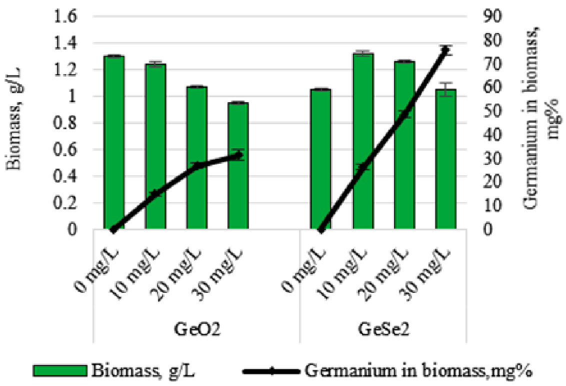

Figure 1. Productivity of *Arthrospira platensis CNMN-CB-11* and germanium uptake under cultivation in the presence of inorganic germanium-containing compounds

The results revealed that GeO_2 had no stimulatory effect on the productivity of cyanobacterium *Arthrospira platensis CNMN-CB-11*. Even adding the GeO_2 at a minimum concentration of 10 mg/L, the productivity decreased with 5% compared to control. Spirulina productivity was reduced to a lower level by adding higher concentrations of this compound to the culture medium. In the case of $GeSe_2$, a stimulating effect on productivity was noted. Maximum biomass production was observed at lower concentrations of this compound, while supplementation in concentration of 30 mg/L adjusted the culture productivity to the control level. Thus, adding $GeSe_2$ in concentration of 10 mg/L, cyanobacterial productivity reached 1.32 g/L (with 25.36% higher than control sample).

Analysis of germanium content revealed a tendency for the accumulation of germanium into biomass with increasing the concentration of compounds in the medium (Fig. 1). Moreover, germanium uptake in cyanobacterial biomass in the presence of $GeSe_2$ was significantly higher than under cultivation conditions with GeO_2 compound. Maximum germanium content of 75.73 mg% in spirulina biomass was achieved in the presence of $GeSe_2$ at a concentration of 30 mg/L, while germanium uptake with the addition of GeO_2 at the same concentration was half less - 35 mg%. Thus, both inorganic germanium compounds enhanced the uptake of Ge in *Arthrospira platensis CNMN-CB-11*.

Subsequent toxicological investigations into germanium-containing compounds revealed significant differences in toxicity profiles between GeO_2 and $GeSe_2$. In order to obtain spirulina biomass enriched with germanium, we studied biochemical changes, in particular, the content of proteins and phycobiliproteins. In spirulina, protein content varies up to 70% [42] and it is one of the main biochemical indicators of the physiological state of cyanobacterium. Phycobiliproteins play an important role in the photosynthetic mechanism of the cell.

Evaluating compound toxicity is of utmost importance, as they may cause a reduction and synthesis of cellular components. The biochemical composition of spirulina biomass should be balanced for prophylactic use in dietary supplements and pharmaceuticals.

The results of study of the effect of inorganic germanium-containing compounds on protein content and phycobiliproteins in spirulina biomass are presented in Figure 2.

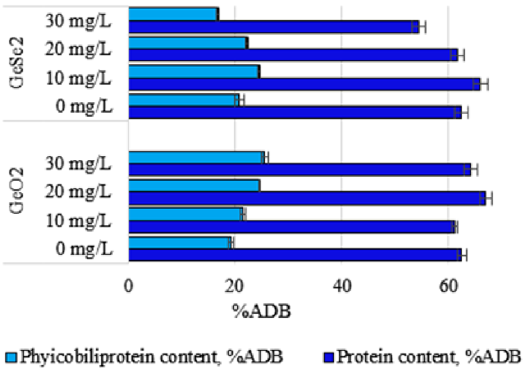

Figure 2. Protein and phycobiliprotein content in *Arthrospira platensis CNMN-CB-11* biomass grown in the presence of inorganic germanium-containing compounds

Both compounds promoted protein synthesis in spirulina biomass. In the case of GeO_2 in concentrations of 20 and 30 mg/L, it was observed a slight increase in protein content of 7.2 and 2.6% compared to control, respectively. The addition of compound $GeSe_2$ in concentration of 10 mg/L enhanced protein content with only 5.7% compared to control. Protein content in spirulina biomass decreased with further increasing the concentration of compound.

The effect of germanium compounds on phycobiliprotein synthesis turned out to be more significant than on the accumulation of total protein in spirulina biomass. GeO_2 in all added concentrations promoted the accumulation of phycobiliproteins in *Arthrospira platensis CNMN-CB-11* biomass. The maximum phycobiliprotein content of 25.5% ADB (1.33 times more than in control sample) was noted with the addition of GeO_2 in concentration of 30 mg/L. $GeSe_2$ in concentrations of 10 and 20 mg/L affected positively the phycobiliprotein content in biomass. The highest accumulation of phycobiliproteins of 24.49% ADB was observed by supplementing $GeSe_2$ in concentration of 10 mg/L. Only, its concentration of 30 mg/L caused a decrease in phycobiliprotein content by 13% compared to control sample.

The results of the influence of inorganic compounds of germanium on the content of carbohydrates and lipids in *Arthrospira platensis CNMN-CB-11* biomass are presented in Figure 3.

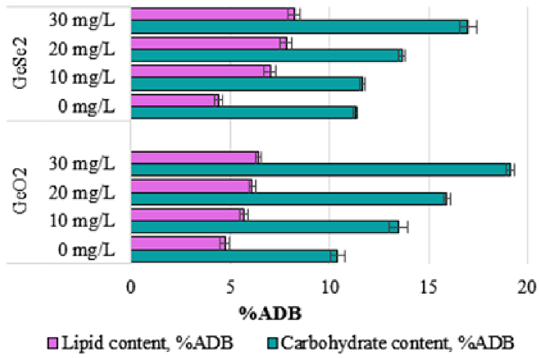

Figure 3. Carbohydrate and lipid content in *Arthrospira platensis CNMN-CB-11* biomass grown in the presence of inorganic germanium-containing compounds

All concentrations of chemical compound GeO_2 contributed to the accumulation of carbohydrates in spirulina biomass. The maximum carbohydrate content of 19.10% ADB (1.83 times more than in control sample) was noted at the addition of germanium dioxide in concentration of 30 mg/L. The compound $GeSe_2$ has also positively affected the accumulation of carbohydrates in spirulina biomass. The data showed that an increase in the carbohydrate content depended on the concentration of the supplied compound. Thus, carbohydrate content increased by 20.9% at a concentration of 20 mg/L and by 50.3% at a concentration of 30 mg/L compared to control sample.

Analyzing the data on the effect of chemical compound GeO_2 on the lipid content in spirulina biomass (Fig. 3), we observed a similar tendency as in the case of carbohydrates. The maximum lipid content of 6.42% ADB (35.9% higher than control sample) was noted at the addition of GeO_2 in concentration of 30 mg/L. According to the obtained results, the compound $GeSe_2$ also enhanced lipid synthesis in spirulina biomass. The lipid content upon addition of $GeSe_2$ increased proportionally with the increase in its concentration. The highest lipid content of 8.21% (1.86 times more compared to control) was noted when $GeSe_2$ was introduced into nutrient medium in concentration of 30 mg/L.

Thus, comparative analysis of the effect of two inorganic germanium compounds on the biochemical composition of spirulina revealed that they increased both the content of carbohydrates and lipids in biomass in concentration of 30 mg/L. Probably, this concentration of compounds exerted a state of stress on *Arthrospira platensis CNMN-CB-11* [6, 30, 33].

One of the markers of spirulina biomass quality was the content of lipid oxidation products. Data on the effect of germanium uptake on malondialdehyde (MDA) as a lipid peroxidation marker in biomass of *Arthrospira platensis CNMN-CB-11* grown in the presence of germanium-containing compounds are shown in Figure 4.

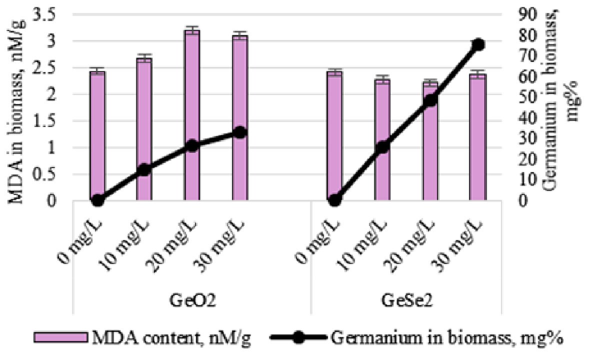

Figure 4. MDA in *Arthrospira platensis CNMN-CB-11* biomass grown in the presence of inorganic germanium-containing compounds and germanium uptake

The content of malondialdehyde (MDA) showed the tendency of increase with increasing the concentration of GeO_2 in nutrient medium, indicating a concentration-dependent free radical generation. In the case of $GeSe_2$, MDA values fluctuated within the control level. MDA production was higher for GeO_2 in comparison to $GeSe_2$ (Fig. 4). Our data on thiobarbituric acid test showed that biomass cultivation with GeO_2 led to an increase in malondialdehyde content of 1.1-1.3 times. Therefore, it can be noted that inorganic compounds of germanium in the range of tested concentrations did not exert high toxicity to cyanobacterium. This result can probably be explained by reduction of germanium-induced stress due to increased lipid content, helping to build lipid rafts within the cell membrane or contributing to other biologically active substances.

Thus, on the basis of assay for measuring malondialdehyde (MDA), it can be concluded that germanium compounds GeO_2 and $GeSe_2$ in added concentrations are acceptable for obtaining spirulina biomass with a high content of germanium and valuable bioactive substances (phycobiliproteins, lipids and carbohydrates).

DISCUSSIONS

The available data on the effect of germanium and its inorganic compounds on microorganisms are contradictory. On the one hand, patent literature reports the germicidal effect of germanium, and on the other hand, numerous cases of absorption and accumulation of hydrated germanium dioxide by microorganisms [12, 22, 46] and algae have been discovered [14, 19, 32, 47, 51].

Over the past decades, various authors have carried out a huge number of studies on the accumulation of germanium and the establishment of its biological role in plants. The germanium content in plants depends on its content in the soil. Germanium enters plants from the soil on which they grow, forming complexes with polyacids, sugars and polyphenols [2]. Germanium plays an essential role in many enzymatic processes and microbial transformation of nutrients, and in the biogeochemical cycling of Ge in soils. In plants, germanium affects the absorption and use of nutrients. It also affects plant photosynthesis by modifying photosynthetic pigments. Germanium enhances the activity of endogenous antioxidant enzymes and non-enzymatic substances due to chelation reaction in some vegetable and grain plants, which leads to an increase in the accumulation of polysaccharides, sugars, soluble proteins and amino acids in some plants. However, the excessive accumulation of germanium by plants disrupts the normal metabolism of the roots and shoots. Germanium is also used in agricultural production for seed germination, seedling growth and plant quality. A number of studies have been conducted on the enrichment of cultivated plants with germanium. The compound was specially introduced into the soil by irrigation. Plants grown in this way were characterized by a high germanium content [2, 8, 17, 20-21, 25, 48].

A number of works describe that germanium is able to replace boron in plants under conditions of boron deficiency. Germanium appears to reduce the lack of boron and even stimulate plant growth [26]. According to other researchers, germanium promotes boron remobilization at low boron supply, delaying its shortage for a period (1-3 weeks) [18].

According to the literature data, different microorganisms exhibit completely different levels of tolerance to germanium. They also accumulate various amounts of germanium either by passive binding or active transport [22, 46]. It was noted the inhibitory effect of germanium on the growth of diatoms, which require silicon. Even at a concentration of 1 mg/L, GeO_2 significantly reduced the growth of these algae [12, 32], and at a concentration of 10 mg/L almost suppressed their growth. This is apparently due to the fact that the biogeochemistry of germanium is closely related to silicon. In many cases, biogeochemical behavior of germanium can be explained by considering it as a very heavy isotope of silicon, with which it is associated in a relatively constant molar ratio of $0.6*10^{-6}$. Deviations from this ratio take place in the environment and are a consequence and indicator of closer complexation of germanium with surface waters rich in organic matter [39].

Spirulina is reported to be more resistant to germanium than diatoms and chlorella [19]. This is possibly explained by the fact that the cultivation medium has a rather high pH and germanium at pH values above 9.5 gradually switches to germanate ions ($HGeO_3^-$) [15]. This altered ionic form of germanium is less toxic and more easily absorbed by cells.

According to our results (Fig. 1), GeO_2 had an inhibitory effect on the productivity of cyanobacterium *Arthrospira platensis.* Even with the addition of germanium compound GeO_2 at a minimum concentration of 10 mg/L, culture productivity decreased with 5% compared to control. The higher the concentration of the compound in the medium, the lower the productivity of spirulina has been established. Although, the author Cao Ji-Xiang in his work [19] indicated higher concentrations of GeO_2 compound, in which experiments were conducted with cyanobacterium *Spirulina platensis* in a wide concentration range from 5 to 100 mg/L. He noted that if the concentration of germanium exceeded 100 mg/L, the culture begins to lose its spiral shape, the filaments become fragmented, and the culture turns yellowish. He recommends an optimal germanium dioxide concentration of 35 mg/L. Another author [14] mentioned that the concentration of germanium oxide should not exceed 25 mg/L, because higher concentrations strongly inhibit the growth of spirulina. This difference in the data appears to be related to different cyanobacterial strains, conditions and duration of cultivation.

In the case of inorganic compound $GeSe_2$, we observed a stimulating effect in some concentrations (10-20 mg/L). The highest productivity of spirulina was noted at lower concentrations of the compound, and the amount of biomass decreased with an increase in its concentration in the nutrient medium. The maximum biomass yield was recorded with the addition of germanium selenide in concentration of 10 mg/L. As a result, the value of cyanobacterial productivity reached 1.32 g/L, which was 25.36% higher than the value of control sample.

The accumulation of germanium by algae, as well as bacteria, depends on many factors: the species, temperature, pH of the medium, contact time with the compound, and its uptake has not yet been fully understood. Thus, the higher the temperature, the accumulation of germanium occurs at a faster rate and the same is true for pH [22].

Therefore, taking into account the accumulation of germanium, the following tendency was observed for both compounds: with increasing the concentration of compound in the medium, its uptake in cyanobacterial biomass also increased. It should be noted that the accumulation of germanium by cyanobacterium in the presence of $GeSe_2$ was significantly higher than when using GeO_2. Thus, the maximum germanium content (75.73 mg%) in spirulina biomass was recorded when it was grown in the presence of 30 mg/L $GeSe_2$, while in the case of adding GeO_2 at the same concentration, the element uptake was half less - 35 mg%. According to other authors, the amount of germanium accumulated by cyanobacterium *Spirulina platensis* did not increase with increasing the concentration of germanium [48], although in our case the opposite results were observed. There is evidence that the accumulation of germanium by spirulina cells also substantially depends on temperature regime. Wang Da-zhi et al. conducted a series of experiments on growing *Spirulina platensis* on Zarrouk medium containing GeO_2 in concentrations of 5.0; 10.0; and 15.0 mg/L under two temperature conditions (25°C and 30°C). The germanium uptake in cyanobacterial cells and its distribution in proteins, lipids and carbohydrates were studied. The results showed that *Spirulina platensis* was able to accumulate germanium. A lower temperature mainly affects the absorption of germanium, and a higher one affects the biochemical formation of macromolecular compounds of germanium with protein, lipids and carbohydrates [47].

Our studies showed that compounds GeO_2 and $GeSe_2$ did not significantly affect the protein content in spirulina biomass compared to control (Fig. 2), but they stimulated the synthesis of phycobiliproteins at some added concentrations. The maximum content of phycobiliproteins of 25.5% ADB (32.7% more than in control sample) was noted with the addition of GeO_2 in concentration of 30 mg/L. On the contrary, supplementing $GeSe_2$ in minimum concentration (10 mg/L) enhanced phycobiliprotein synthesis by 24.49% ADB (17.8% higher than the control). The content of phycobiliproteins decreased with further increase in the concentration of this compound. A positive effect of GeO_2 on the synthesis of phycobiliproteins (up to 35 mg/L) with increasing concentration was also established by another author [19].

In this study, both inorganic germanium-containing compounds have emerged as stimulants of carbohydrate and lipid content in spirulina biomass (Fig. 3). Moreover, a higher level of carbohydrates and lipids in cyanobacterial biomass was recorded with increasing the concentration of compounds in the medium. Despite the enhanced content of carbohydrates and lipids in biomass, grown in the presence of compounds (Fig. 4), malondialdehyde (MDA) content did not indicate that spirulina culture was in a state of oxidative stress.

The level of MDA in cyanobacterial cells was used as biomarker of lipid peroxidation status. Lipid peroxidation indicated to the excess formation of superoxide anion radicals (O_2^-). According to literature data, the presence of high amounts of transition metals such as copper or iron will also lead to an oxidative burst and overgeneration of hydroxyl radicals (OH^-) by both Fenton and Haber-Weiss reactions. In case of germanium compounds, we can conclude that tested concentrations did not exert very high toxicity to cyanobacterium *Arthrospira platensis*. This result indicated a reduction in germanium-induced stress due to increased lipid content that can be used to build cell membranes. Therefore, although *Arthrospira plantensis* growth slightly decreased in the presence of GeO_2, it was able to withstand the stress caused by germanium (IV). To confirm this, it is necessary to conduct additional research on the content of SOD and proline that was not the purpose of this work.

In conclusion, inorganic germanium-containing compounds GeO_2 and $GeSe_2$ did not negativelly affect the production of *Arthrospira platensis* biomass, and the synthesis of some valuable components (proteins, phycobiliproteins, lipids and carbohydrates), but rather allowed the culture to accumulate such amounts of germanium. Thus, GeO_2 (20 mg/L) and $GeSe_2$ (10-20 mg/L) can be used to cultivate cyanobacterium *Arthrospira platensis*, in order to obtain biomass enriched with germanium and bioactive compounds as a valuable source in the production of various nutraceuticals with antimicrobial, antiviral, and antitumor properties.

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