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ACCUMULATION OF TRACE ELEMENTS BY BIOLOGICAL MATRICE OF *Spirulina platensis*

AKUMULACJA PIERWIASTKÓW ŚLADOWYCH W BIOLOGICZNEJ MATRYCY Z *Spirulina platensis*

Abstract: A blue-green alga *Spirulina platensis* biomass is used as a basis for the development of pharmaceutical substances containing such vitally important trace elements, as selenium, chromium and iodine. Using neutron activation analysis the possibility of target-oriented introduction of these elements into the *Spirulina platensis* biocomplexes retaining its protein composition and natural beneficial properties has been proved. The curves of the dependence of the introduced element accumulation in the *Spirulina* biomass on its concentration in a nutrient medium, which make it possible to accurately measure out the required doses of the specified element in a substance, have been obtained. The peculiarities of interaction of various chromium forms (Cr(III) and Cr(VI)) with the *Spirulina platensis* biomass have been studied. It has been found that from a nutrient medium its cells mainly accumulate vitally essential form Cr(III) rather than toxic Cr(VI). Using the EPR technique and colorimetry it has been demonstrated that the *Spirulina platensis* biomass enriched with Cr(III) is free from other toxic chromium forms. The developed technique can be used in pharmaceutical industry for the production of preparations containing Se, Cr, I, etc. on the basis of *Spirulina platensis* biomass with the preservation of its natural beneficial properties and protein composition.

Keywords: *Spirulina platensis,* instrumental neutron activation analysis, essential elements, pharmaceuticals

The investigation of the role of microelements in living systems using modern biochemical and analytical techniques is a promising direction in Life Sciences. The results of these investigations can form a basis for a scientifically grounded approach to the development of new therapeutic and preventive preparations containing such necessary elements as Se, Cr, I, Zn, etc.

According to the well-known Bertrand diagram [1], for each particular micro-element there is a certain concentration range of positive effect on the human organism, with both excess and insufficient concentrations being harmful to the

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organism. Thus, it is evident that a precise choice of required doses depending on the purpose of pharmaceuticals is the most important task in designing their substances.

As a rule, in metabolism and exchange processes, microelements are best assimilated by the organism in a biologically accessible form, ie when they are included into a biological macromolecule. Hence it follows that it is desirable that as a substance base we use biologically active biomass, which is capable to assimilate required elements in prescribed quantities. And, certainly, the choice of biomass should be determined by its own beneficial therapeutic and preventative properties, as well as by the absence of harmful impurities in concentrations exceeding permissible levels.

Taking into account the requirements mentioned above, the Adronikashvili Institute of Physics (Tbilisi, Georgia) in cooperation with the Joint Institute for Nuclear Research (JINR, Dubna, Russia) developed a technique for production of substances for therapeutic and preventative preparations on the basis of blue-green algae *Spirulina platensis* (*S. platensis*) [2-5].

Spirulina is a living microorganism and in the process of cell cultivation it is capable to assimilate certain amounts of some microelements from a nutrient medium and to incorporate them into the composition of its biological macromolecules. The analytical control of this process makes it possible to establish a unique dependence between the element concentration in the nutrient medium and its content in the obtained *S. platensis* biomass. This dependence serves as a basis for substantiation of biotechnology for production of substances for pharmaceutical preparations with required doses of a given element. It is very important that concentrations of compounds added to the nutrient medium as loading, have no influence on the conditions, in which spirulina cells grow normally and retain their beneficial natural properties. That is why, along with the analytical control, the protein composition of the obtained biomass was investigated by the gel-electrophoresis technique.

Biotechnology of target-oriented incorporation of certain elements into *S. platensis* biomass composition in the process of cultivation, was developed using as an example such vitally important elements as Se, Cr and I.

Selenium. The studies of the role of selenium in the human organism over the last 20 years showed it to be such an important element that it was named the element of the century at the 7th International Symposium «Selenium-2000» (Venice, October 2000). Selenium is a normal component of some enzymes, proteins and amino acids. Its low level in the organism raises the risk of such diseases as cardiomyopathy, cancer, endemic osteoarthropathy, anaemia, etc. [6, 7]. The functions of Se are closely related to vitamin E and beta carotene (which are contained in *S. platensis* biomass), therefore in the treatment they are sometimes used in combination. Selenium contributes to the reduction of harmful effects of free radicals and also allows detoxification of the organism from such elements as As, Hg, Cd, Bi, etc. It participates in photochemical reactions related to vision, can influence the immune and endocrine systems, etc. Selenium added to diet in particular doses slows down the ageing processes, favours treatment of cardiological patients and reduces the risk of cancer and AIDS [8, 9].

Iodine. Another equally important element incorporated in the composition of all living organisms and plants is iodine. It is vitally important for their development, growth and functioning. Iodine intake by the organism strongly depends on the state of the environment and its deficiency often has an endemic character. Iodine influences metabolism and enhances reduction-oxidation processes, thus iodine deficiency can affect the physical, mental and emotional state of the organism. Among serious symptoms and results of iodine deficiency are cardiological diseases (atherosclerosis, vessel deformation, etc.), immunodeficiency (susceptibility to infections and colds), emotional disturbance (irritability, sleepiness, etc.), mental disorders (deterioration of memory, low level of intellectual development - low IQ, cretinism and others) [10].

Unfavourable ecological situation and lowering of living standards of population in many countries of the world, as well as studies on the assessment of iodine deficiency at the level of populations have put this problem among UN priorities in the sphere of human health.

Chromium. Chromium interaction with *S. platensis* biomass is of special interest. Cr is known to be a vitally important trace element, which possesses, at the same time, significant toxic properties. It exists in various valence states (from +2 to +6) and forms numerous complex compounds, most stable of which are Cr(III) and Cr(VI). Kinetically stable, non-toxic Cr(III) is most abundant in the environment. Toxic Cr(VI) penetrates cells easier than Cr(III), participates in the reduction-oxidation processes inside them, thus reducing to the stable Cr(III). These processes cause damage of cell genetic material, oxidative injuries, formation of cross-links and thus, have carcinogenic, genotoxic and mutagenic effects [11].

On the other hand, chromium is a necessary chemical element without which normal functioning of the organism, is impossible. The most important function of chromium is control of sugar. It is known to be the key constituent in the so-called glucose tolerance factor. Cr contributes to insulin participation in metabolism of hydrocarbons (proteins, lipids, nucleic acids) and to effective transfer of glucose into tissue cells. It also influences the synthesis of macromolecules, activation of some enzymes and cholesterol metabolism [12, 13].

The deficiency of Cr in the human organism results in the derangement of lipid and fat metabolism, in depressed physical growth and in weight loss, decreased longevity and impaired coordination of movements. Sometimes chromium deficiency is associated with high cholesterol levels, tiredness and fatigue, alcoholic intolerance, etc. The Cr deficiency most often becomes apparent in early childhood and young age through disturbed protein metabolism. In all the above-listed cases the addition of Cr into the diet helps to treat the diseases. Thus, the task to produce chromium-containing pharmaceuticals designed to eliminate Cr deficiency, is an urgent question.

Material and methods

The strain IPPAS B-256 of *S. platensis* from the algeological collection of the Timiryazev Institute of Plant Physiology of RAS was used in the experiments. To stimulate growth, the spirulina culture was previously slightly treated in Potter homogenizer in order to partly shorten its threads. The cultivation of *S. platensis* cells was carried out in the Zaroukh standard water-salt nutrient medium at pH 8.5÷11, at a temperature of 32÷34°C, continuous stirring and illumination with 5000 lx sodium lamp.

The nutrient medium was prepared in two stages: the corresponding chemical compound containing the element to be introduced (Se, Cr, I) was added as a loading into the first part of the solution containing $NaHCO_3$, $K_2HPO_4{\cdot}3H_2O$ and Na_2CO_3 at pH < 10, then this solution was mixed with the second part of the solution, containing all remaining components [4, 5]. This method ensured intensive inclusion of a required element into spirulina biocomplexes with the preservation of its natural beneficial properties.

To study elements accumulation dynamic in the *S. platensis* biomass cultivation was carried out in a nutrient loading with concentrations ranging:

- for selenium - selenious acid H_2SeO_3 - from 0.5 to 15 mg/dm^3
- for iodine - potassium iodine KI - from 10^{-8} to 10^{-4} g/dm^3
- for chromium - Cr(III) - chromium acetate $Cr(CH_3COO)_3$

The investigation of dynamics of *S. platensis* biomass growth in the selected mode demonstrated that the maximal growth occurred in 5-6 days (Fig. 1). In the preliminary experiments, a range of permissible loading concentrations in the nutrient medium was also determined, at which a required dose of a given element in biomass was provided with the retention of its quality.

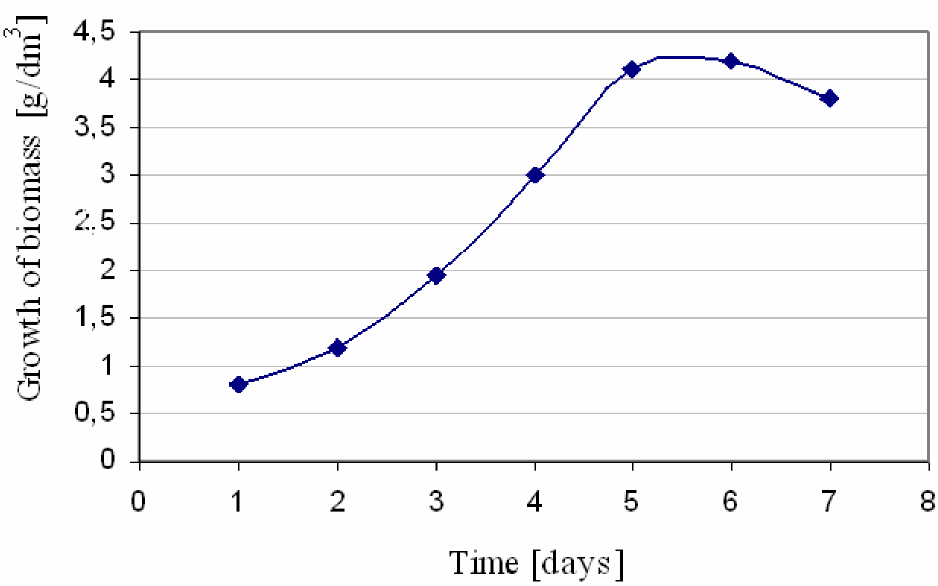

Fig. 1. Curve of growth of *Spirulina platensis* biomass

In each experiment, after cultivating for 5 days, the harvest of *S. platensis* biomass was separated from the nutrient medium by filtering, rinsing and centrifugation. The resulting substance was lyophilically dried in a special adsorption-condensation lyophilizer of an original design [14].

The protein content in the obtained biomass was found (by Lowry technique [15]) to be about 65% and corresponded to a normal level typical for natural *S. platensis* biomass. The quality of biomass was also confirmed by comparison of electrophorograms obtained by gel electrophoresis technique for samples grown with and without loading.

Samples for analysis were prepared in the form of small pellets using a special titanium mould. Neutron activation analysis (NAA) using epithermal neutrons, a well-proven technique for determination of element composition of biological objects, was used as an analytical technique. Due to resonance neutron activation, the technique makes it possible to minimize matrix effects of biological samples and at the same time to determine concentrations of over 30 major, minor and trace elements.

Analytical research was conducted at the IBR-2 fast pulsed reactor of the Frank Laboratory of Neutron Physics JINR (Dubna, Russia). Experimental techniques and analytical information treatment procedure are described in [16, 17].

First, the multielement composition of *S. platensis* biomass was studied by the NAA technique and the concentrations of certain elements were compared with the corresponding permissible level values [2]. The results of the investigations showed that the concentrations of such toxic elements as As, Hg, Cd, Pb, etc. do not exceed those permissible for the human organism, according to the data at the website: http://www.spirulina.com/SPBNutrition.html.

Results and discussion

Selenium. Selenious acid H_2SeO_3 of various concentrations in the range of 0.016÷2770 μg/dm^3 was used as a loading of the nutrient medium to obtain selenium-containing *S. platensis* biomass. The results of determination of Se concentration by the NAA technique are shown in Figure 2. As can be seen from this figure, the curve of Se content in biomass versus its concentration in the nutrient medium is well approximated by a polynomial of the 2nd order $y = -0.00008x^2 + 0.3x - 1$. Starting from Se concentration of 100 μg/dm^3, an intensive growth of its accumulation by cells with a possible maximum in the range of 1100÷1200 μg/dm^3 is observed. For pharmaceutical purposes, the range 100÷1000 μg/dm^3 seems to be the most advantageous: at high degree of Se assimilation the significant steepness of the curve enables rather exact determination of its doses in a substance obtained.

Visual microscopic observation of the state of culture, determination of total protein content in the biomass as well as investigation of its electrophorograms revealed natural properties of the obtained selenium-containing biomass. Thus, by including Se in the composition of its biological macromolecules, *S. platensis* preserves its beneficial properties and compares favourably with other similar preparations, which are either a mechanical mixture of Se compounds with spirulina powder [18] or are obtained at such high Se concentrations in a medium that cells grow on the background of struggle for survival and no normal quality of biomass can be retained [19].

Chromium. Chromium-containing *S. platensis* biomass with vitally essential form Cr(III) was cultivated at the loading of the nutrient medium with chromium acetate $Cr(CH_3COOH)_3$ in concentrations from 0.5 to 15 mg/dm^3 (Fig. 3). The accumulation of toxic Cr(VI) form by spirulina at loading of the nutrient medium with potassium bichromate $K_2Cr_2O_7$ in the similar range of concentrations (Fig. 3) was also investigated. As can be seen from the curves obtained, spirulina assimilates mainly Cr(III), while the degree of binding of Cr(VI) is approximately three times lower. As opposed to other microorganisms, such as, for example, *Arthrobacter oxydans, S. platensis* prefers a non-toxic form of chromium to the toxic one, even if both forms are present in the solution.

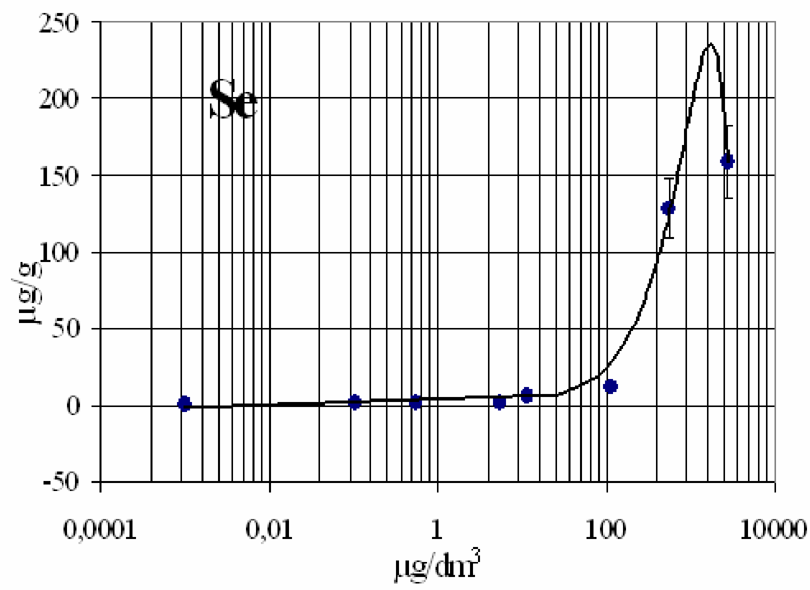

Fig. 2. Selenium in *Spirulina platensis* biomass *versus* its content in a nutrient medium

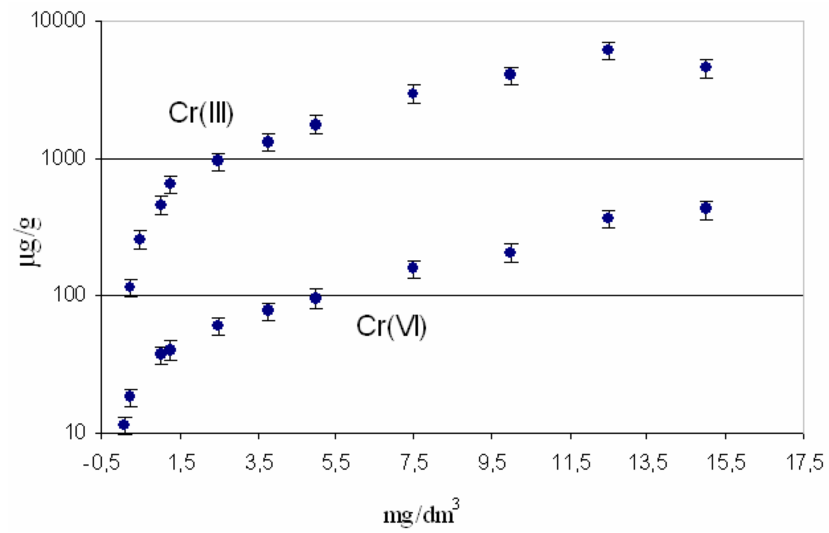

Fig. 3. Observed chromium concentrations in *Spirulina platensis* biomass at different concentration levels of Cr(III) and Cr(VI) in the growth medium respectively

Due to the fact that some microorganisms in the process of metabolism can interact with a number of elements and change their valence, it was necessary to verify whether under loading of the nutrient medium with Cr(III) compounds a toxic form Cr(VI) arises in the possible chain of its valence change Cr(III) → Cr(V) → Cr(VI) or not. For this purpose, the technique of colorimetric determination of Cr(VI) was applied using diphenylcarbohydrazide $(C_6H_5NHNH)_2CO$, which reacts with Cr(VI) in concentrations of 0.1÷10 μg/dm^3 to give a purple-red colour and yields a photometric peak at λ = 540 nm [20]. The investigations showed that Cr(VI) was absent in all cases of *S. platensis* cultivation with Cr(III) loading.

The presence of intermediate form Cr(V) was checked by the electron paramagnetic resonance (EPR) technique with a sensitivity of the order of $5 \cdot 10^{-10}$g of Cr(V). The obtained results showed the absence of a resonance signal, which is typical for Cr(V), in all samples under investigation.

It can be seen from Figure 3 that at loading concentrations within 5÷12 mg/dm^3, the curve of Cr(III) accumulation in *S. platensis* biomass does not reach its saturation level. This makes it possible to obtain optimal chromium doses in the resulting biomass and to recommend 30÷100 μg/dm^3 for food supplement and 200÷250 μg/dm^3 for therapeutic and prevention purposes [5].

Iodine. Iodine-containing *S. platensis* biomass was cultivated in the nutrient medium with loading of potassium iodide KI in the concentration range of 10^{-8}÷10^{-4} g/dm^3. The curve of iodine concentration in biomass versus its concentration in the nutrient medium (Fig. 4) can also be approximated by the 2nd order polynomial $y = 0.00001x^2 - 0.003x + 0.4$.

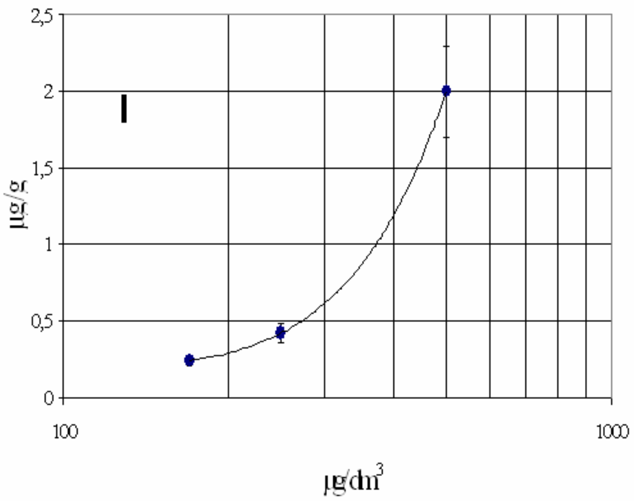

Fig. 4. Iodine in *Spirulina platensis* biomass *versus* its content in a nutrient medium

The biomass enrichment coefficient can be defined as a ratio between the iodine (or other element) concentration in the biomass and the iodine concentration in the nutrient medium in accordance with the curve obtained. This coefficient may serve as an initial technological parameter governing the element dosage in treatment pills and the choice of the pill mass for a given substance. For iodine, it is possible to produce pills 5 mm in diameter, of mass 0.5 g and with iodine content of 100÷200 μg.

The microscopic control of cytological state of the culture as well as of protein content of the obtained biomass demonstrated in all cases that the normal state of *S. platensis* and, consequently, its natural beneficial properties are retained.

Conclusion

The performed investigations demonstrated that the cultivation of *S. platensis* cells under selected conditions allows target-oriented introduction of the required elements (Se, Cr, I, etc.) into the composition of biological macromolecules with preservation of their protein composition and natural properties of biomass. With the application of NAA the curves of concentrations of necessary elements in the resulting *S. platensis* substance versus concentrations of these elements in the nutrient medium were obtained. The possibility of accurate determination of therapeutic and preventative doses of each element according to these curves was demonstrated.

The results of NAA-analysis of multielement composition of *S. platensis* biomass and their comparison with the permissible level demonstrated that the content of such toxic elements as Hg, Cd, As, etc. does not exceed the permissible level accepted in many countries of the world.

The developed technique can also be used for production of substances with introduction of other vitally important elements such as Zn, Cu, Fe and others.

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AKUMULACJA PIERWIASTKÓW ŚLADOWYCH W BIOLOGICZNEJ MATRYCY Z *Spirulina platensis*

Abstrakt: Biomasa niebiesko-zielonych glonów *Spirulina platensis* jest wykorzystywana jako główny składnik opracowywanych preparatów farmaceutycznych zawierających takie niezwykle ważne pierwiastki śladowe, jak selen, chrom i jod. Udowodniono, wykorzystując neutronową analizą aktywacyjną, możliwość sterowanego wprowadzenia tych biopierwiastków do biokompleksu ze *Spiruliną platensis*, zachowując skład jej białka oraz korzystne właściwości fizyczne. Krzywe zależności akumulacji wprowadzanego pierwiastka w biomasie *Spiruliny* do jego stężenie w pożywce, umożliwiają dokładny pomiar obecnie wymaganej dawki określonych pierwiastków w otrzymanym preparacie. Badano wpływ różnych stopni utlenienia chromu (Cr(III) i Cr(VI)) na biomasę *Spirulina platensis*. Stwierdzono, że w komórkach glonów akumulowała się z pożywki głównie niezbędna dla życia forma Cr(III), a nie toksyczna Cr(VI). Korzystając ze spektrometrii EPR oraz kolorymetrii, wykazano, że biomasa *Spiruliny platensis* wzbogacona formą Cr(III) jest wolna od toksycznych form chromu. Opracowana technika może być wykorzystana w przemyśle farmaceutycznym do produkcji preparatów zawierających selen, chrom i jod itp. z biomasy *Spiruliny platensis*, z zachowaniem jej korzystnych właściwości fizycznych i składu białka.

Słowa kluczowe: *Spirulina platensis*, instrumentalna neutronowa analiza aktywacyjna, mikroelementy niezbędne, środki farmaceutyczne