PROCEDURE FOR OBTAINING CHROMIUM FOOD BIOADDITIVE

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Abstract. A study of the $[Cr(Gly)_3]Cl$ effect on the biochemical composition of the cyanobacterium *Spirulina (Arthrospira) platensis* has been carried out at cultivation depending on the lighting regime and the timing of addition of this compound. Its positive effect on protein content (including peptides) was established within the concentration limits of up to 30 mg /l. Chromium accumulation was more efficient in light/dark photoperiod (14/10 hours) in case of supplementation on the 1st and 3rd days in the portions. The technological scheme for obtaining chromium bioadditive was proposed. This procedure is based on using a Cr(III) glicinate as source of Cr(III) for obtaining of spirulina biomass enriched with chromium (0,75%) and valuable bioactive substances.

Keywords: Cyanobacteria cultivation; *Spirulina platensis*; chromium; food bioadditive; insulin activity

INTRODUCTION

Chromium is an essential element in the human body necessary for the formation of glucose tolerance factor and for insulin activity. Recent studies have demonstrated a beneficial effect of chromium (III) on patients with diabetes [2, 10, 14, 18]. The mechanism underlying the treatment of diabetes patients with chromium is based on increasing insulin activity. As a source of chromium, the food supplement Cr(III) picolinate is often used. Its effect on weight loss and muscle gain is known, as well as its ability to alleviate symptoms of type 2 diabetes and to regulate cholesterol levels [19, 22, 24]. On the other hand, CrPic bioabsorption also has some drawbacks, such as a very limited solubility in water (0.6 mM at pH 7) and low stability (in acidic media, the complex hydrolyses to release picolinic acid) − a decisive factor in the low rate of gastro-intestinal absorption (1%) [32] and some negative effect on kidney function [33]. That's why the investigations for finding new alternative sources of chromium are currently of interest.

The research results of some authors have demonstrated the capacity of yeast [15] and cyanobacterium *Spirulina platensis* to accumulate chromium at cultivation in the presence of Cr(III) compounds [4, 6, 7, 13, 20, 23, 28]. The content of chromium in biomass varies from one compound to another and depends on the nature and concentration of the Cr(III) compounds. Some Cr(III) coordinative compounds used in concentrations higher than 30 mg/l manifested a negative influence on the growth and development of cyanobacteriun *Spirulina platensis* [28]. Our recent research has demonstrated the stimulatory effect of the coordination compound $[Cr(Gly)_3]Cl$ (5-30 mg/l) on chromium accumulation in biomass, as well as spirulina productivity [5].

According to the data of the last decades, chromium from organic sources is better assimilated by the body [21]. In this context, the research for developing technologies for obtaining spirulina containing organic chromium remains a relevant issue.

Based on the above, the aim of the present research was to develop a procedure for obtaining a new food bioadditive, using Cr(III) glycinate as the regulator of the content of chromium and other bioactive substances in the cultivation of cyanobacterium *Spirulina platensis*.

MATERIALS AND METHODS

Synthesis of chromium glycinate. The synthesis of chromium glycinate soluble in alcoholic solutions and water was performed according to the method developed by Abdel-Monem and Anderson [1]. To 0.075 mol of $CrCl_3 \cdot 6H_2O$ (18.989 g) were added 135 ml of H_2O and then 0.225 mol of glycine (16.875 g) and the mixture was heated to 90 °C for 30 minutes, stirring the solution until the green color turns into dark blue-green. After the solution cooled down to 30 °C, 2N NaOH solution was added drop-wise until becoming dark purple in color. The solution was evaporated until it became syrupy and 48% alcohol solution was added for $[Cr(Gly)_3]Cl$ solubilization; the resulting NaCl precipitate was removed by filtration. The UV-VIS spectrum of chromium glycinate in diluted solution (0.01ml in 2.5ml of 48% alcohol) showed a maximum absorbance at 225 nm.

Chromium determination by a diphenylcarbazide method. For determination of chromium, spirulina biomass was subjected to mineralization. To 20 mg of dry biomass were added 0.3 ml concentrated solution of $HClO_4$ and 0.15 ml of HNO_3. The samples were placed on the sand bath at 160-180 °C for thermic treatment until the solution was completely discolored. The chromium content was determined by the spectrophotometric method described previously [5]. The chromium content was calculated from the standard curve, using a $K_2Cr_2O_7$ solution as standard. To prepare the stock standard solution, $K_2Cr_2O_7$ was dried at 150 °C for one hour, then placed in a desiccator. A quantity of 8.79 mg of the $K_2Cr_2O_7$ was dissolved in 100 ml water and 80.4 ml of this solution were diluted to 100 ml in a 100 ml flask, producing a 25 mg/l chromium standard stock solution. This was further diluted to create another standard stock solution of 0.25 mg/l. These two stock solutions were used to prepare Cr(VI) standards for the

calibration curves with the following concentrations: 1.5, 1.0, 0.5, 0.25, 0.125, 0.060, 0.040, 0.020, 0.010, 0.0075, 0.0050, 0.0025, 0.0010, and 0.0000 mg/l.

Cultivation of cyanobacterium *Spirulina (Arthrospira) platensis CNM-CB-02* in the presence of [Cr(Gly)$_3$]Cl. Spirulina was cultivated for 10 days (240 hours) in the two lighting modes: light/dark period (14/10 hours) and continuous illumination (2000 lx) with fluorescent light (6500 K). The cyanobacterium was grown in SP-1 medium [29]. Chromium glycinate was supplemented in concentrations of: 1, 5, 10, 20, 30, 40, 45 mg/l in 3 variants: in the first day of cultivation, in the 3rd day and in the first and the 3rd days in two portions. The biomass cultivated during 10 days was separated from the culture liquid, washed with distilled water and suspended in a determined volume of water with a final concentration of 10 mg/ml.

Protein determination was performed by the Lowry method. The content of peptides in the total protein fraction was determined by the Lowry method after extraction with 75% alcoholic solution. The carbohydrate content was determined by reaction with the anthrone-sulfuric reagent.

Testing of the effect of chromium-containing food additive on insulin activity in rats. The research was carried out in the SR Laboratory "Human and Animal Ecophysiology", Insitute of Researh and Innovation, Moldova State University. The experiments were performed on three groups of rats with the mass of 250-300 g: 1 – control group (healthy rats), groups 2 and 3 – the rats with type II diabetes artificially induced by administration of alloxan, a chemical substance that causes the destruction of beta cells of the pancreas, which are responsible for insulin synthesis. To the rats of the third group the chromium food additive was administered for 10 days. The insulin activity has been determined for all three groups by the immuno-fermentative method (biochemical analyzer STAT-FAX 4500).

RESULTS

The results of the research on the biochemical composition of spirulina and chromium content accumulated in the biomass of cyanobacterium *Spirulina platensis* at cultivation in two lighting regimes are presented in Figures 1-5.

The results obtained on the dynamics of the protein content with varying chromium glycinate concentrations revealed an increasing trend with the increase of the concentration (at concentrations of 10-30 mg/l), reaching maximum values (up to 71.7%) in the case of supplementation on the 1st day or on the 1st and 3rd days of cultivation (in two portions) in both lighting regimes. At higher concentrations of chromium glycinate (>30 mg/l), the protein content decreased substantially (Fig. 1).

Upon addition of the compound on the 3rd day of cultivation, the protein content values differ insignificantly from the reference sample value at concentrations not exceeding 30mg/l in the light/dark regime. For concentrations above 30mg/l, the protein content decreased.

An important role in the protein fraction is played by peptides. The dynamics of the peptide content extracted from biomass denoted higher values at the concentration of 30 mg/l in all studied cases, except variant B. Addition of the Cr(III) compound in portions at light/dark regime provided an increase in the peptide fraction synthesis and maximum chromium accumulation in biomass (Fig. 2 and 5).

The carbohydrate content (Fig. 3) registered maximum values of up to 12.6% for [Cr(Gly)$_3$]Cl

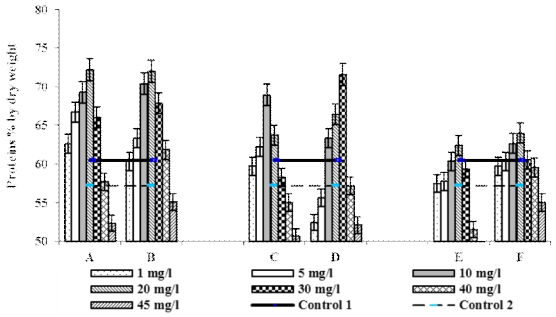

Figure 1. Protein content in spirulina biomass at supplementation of chromium glycinate to culture medium on the 1st day of cultivation (A, B), by portions (C, D) and on the 3rd day of cultivation (E, F) at continuous illumination (A, C, E) and light/dark period (14/10 hours) (B, D, F).

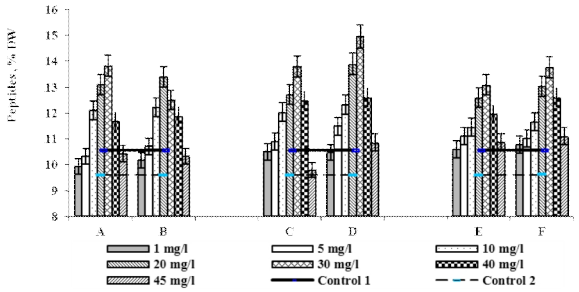

Figure 2. Peptide content in spirulina biomass at supplementation of chromium glycinate to culture medium on the 1st day of cultivation (A, B), by portions (C, D) and on the 3rd day of cultivation (E, F) at continuous illumination (A, C, E) and light/dark period (14/10 hours) (B, D, F).

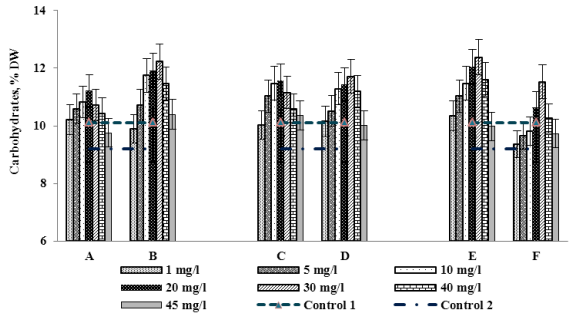

Figure 3. Carbohydrate content in spirulina biomass at supplementation of chromium glycinate to culture medium on the 1st day of cultivation (A, B), by portions (C, D) and on the 3rd day of cultivation (E, F) at continuous illumination (A, C, E) and light/dark period (14/10 hours) (B, D, F).

supplementation on the first day at light/dark regime and on the third day of cultivation for continuous illumination. Upon addition in portions, a 1.1-1.2 fold increase as compared to reference values was also attested.

The lipid content (Fig. 4) reached maximum values within the concentration limits of 10-30 mg/l. Additionally, chromium glycinate in concentrations of up to 30 mg/l demonstrated a positive effect on the accumulation of lipids in biomass in both cultivation regimes. A significant increase in lipid content compared to the reference sample was registered for cultivation under continuous illumination. Thus, the production of lipid-enriched spirulina biomass can be ensured by using $[Cr(Gly)_3]Cl$ supplementation in concentrations of 10-30 mg/l at cultivation under continuous illumination regime regardless of the timing of administration.

The accumulation of chromium in biomass was more efficient in the light/dark period compared to continuous illumination. Chromium accumulation increased with increasing concentrations. At concentrations of 40-45 mg/l $[Cr(Gly)_3]Cl$, the amount of chromium accumulated in the light/dark period (14/10 hours) registered maximum values of 8.98-10.13 mg/g (Fig. 5).

The analysis of the results of $[Cr(Gly)_3]Cl$ use as a chromium source in the cultivation of cyanobacterium *Spirulina platensis* in two lighting regimes for obtaining chromium enriched biomass allowed to propose the technological scheme for obtaining chromium bioadditive (Fig. 6).

The new procedure is based on the application of $[Cr(Gly)_3]Cl$ as a regulator of chromium and bioactive substance content in biomass, as well as the combination of the mode and the term of administration of the compound with the light/dark photoperiod (14/10 hours).

Considering that the Cr-bioadditive (0.75% Cr) is of organic nature and an ecologically pure product, we have proposed to test its efficacy in increasing the insulin activity in rats with experimentally induced type II diabetes.

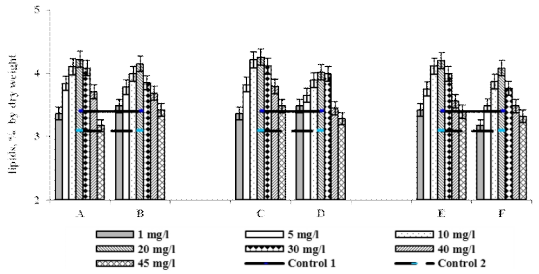

Figure 4. Lipid content in spirulina biomass at supplementation of chromium glycinate in culture medium on the 1st day of cultivation (A, B), by portions (C, D) and on the 3rd day of cultivation (E, F) at continuous illumination (A, C, E) and light/dark period (14/10 hours) (B, D, F).

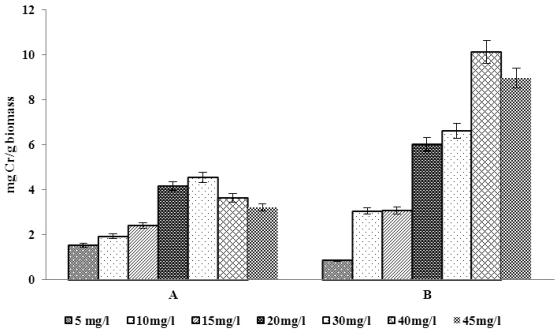

Figure 5. Chromium content in spirulina biomass at supplementation of chromium glycinate to culture medium by portions on the 1st day and the 3rd day of cultivation at continuous illumination (A) and light/dark period (14/10 hours) (B).

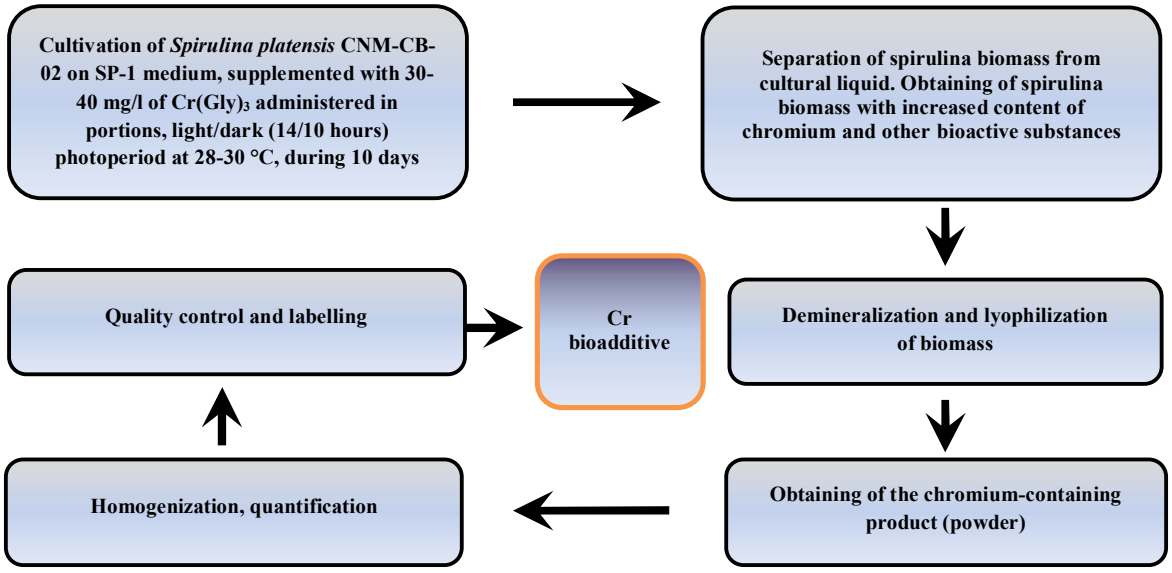

Figure 6. Technological scheme of realization of the procedure for obtaining chromium bioadditive.

Testing the effect of chromium-containing food bioadditive on insulin activity in rats. The experiments were performed on three groups of rats with the mass of 250-300 g. The first − the control group (healthy rats), the second and third groups – the rats with type II diabetes artificially induced by administration of alloxan (a chemical substance that causes the destruction of beta cells of the pancreas − responsible for insulin synthesis). The rats of the third group were administered a chromium food bioadditive for 10 days and the insulin activity was determined. The obtained results are presented in Table 1.

Table 1. Change in insulin activity in rats following administration of the chromium containing preparations

Experimental groups	Insulin activity, µIU/ml*
1. Healthy rats (control)	5.00
2. Rats with alloxanic diabetes	0.27
3. Rats with alloxanic diabetes treated with Cr-bioadditive	7.80

*p≤0,05

The administration of the chromium food bioadditive has induced an increase of insulin activity by 1.56 times in comparison with the control group (healthy rats). We suppose that this fact is due to chromium involvement in the process of increasing insulin activity. Chromium can be part of some oligopeptides – chromodulins – potentiating the effect of insulin by facilitating insulin binding to the cell surface receptors.

DISCUSSION

The elaboration of new technologies for obtaining natural chromium-containing food bioadditives is a very important issue in order to substitute or diversify the variety of antidiabetic products. As an alternative source of organic chromium the biomass of cyanobacterium *Spirulina platensis* can be used. For the enrichment of spirulina with chromium, some inorganic compounds of Cr(III), as well as coordinative compounds of chromium are employed [6, 8, 9, 13, 20, 23, 28].

Previously, some research on the effect of [Cr $(Gly)_3$]Cl on the productivity and chromium content of *Spirulina platensis* biomass has been carried out [5]. These studies confirmed the possibility of application of this compound in some concentrations as a stimulator of biomass production and chromium accumulation. Thus, the obtained results allowed continuing the studies in the development of a new procedure and technology for the production of spirulina biomass enriched with chromium and other bioactive substances.

The results of our investigations revealed that the biochemical composition of the biomass was influenced by the lighting regime, the Cr(III) glycinate concentration, and the timing of its administration. Our research has shown that spirulina cultivation in continuous illumination regime, regardless of the term of chromium glycinate addition, ensures a higher productivity of spirulina (up to 1.05-1.45 g/l), compared to the light/dark regime (0.7-1.1 g/l). The chromium accumulation process in biomass was more pronounced in light/dark cultivation when $[Cr(Gly)_3]Cl$ was administered in the portions.

The application of the light/dark regime was also used by other researchers for the cultivation of some algae and cyanobacteria. The influence of cultivation conditions on algal biomass quantity and biochemical content was investigated using the light/dark cycle in the cultivation of *Chlorella pyrenoidosa* [25]. It was observed that the dark had influenced the amount of chlorella biomass, the carbohydrate and protein content. The authors explained these changes by the fact that in the absence of light energy, deposited intracellular carbohydrates can be metabolized as a source of energy. This energy can be used in part to maintain the cells grown and partially to synthesize proteins.

In the research of the effect of light/dark cycle on the biomass yield of some cyanobacteria, Janssen revealed that if the dark period was less than 33 % of the full cycle duration, the yield was about equal to the one determined under continuous illumination [16]. Maximum growth and protein content in the cultivation of *Oscillatoria agardhii* were observed in alternate light and dark periods (12:12 h) at 27-36 ^oC, while the minimum was in constant lighting at 27-36 ^oC [11].

Chromium glycinate, used in present study in the concentrations 10-30 mg/l, has manifested a stimulating effect on the protein content with increasing concentration, with maximum values (up to 71.7% DW) in the case of supplementation on the 1st day of cultivation or in portions, regardless of the lighting regime; at concentrations >30 mg/l the protein content decreased essentially. Negative effects of heavy metals in high concentrations on protein, carbohydrate and chlorophyll synthesis in *Spirulina platensis* have been reported in more recent research [12, 30].

Similar effects of chromium (III) toxicity in high concentration on the growth and development of cyanobacteria and microalgae, as well as the decrease in protein content with increasing metal concentration have been observed by other authors. Thus, at 5 mg/l Cr(III) concentrations in *Chlorella pyrenoidosa*, a slight increase in algae cells can be observed. But at concentrations of more than 5 mg/l, there was a clear inhibition of growth [27]. The decrease in protein content with increasing Cr(III) concentration could be related to the toxic action of this metal on protein biosynthesis. According to some authors, heavy metals can affect the enzymatic reactions responsible for protein synthesis [3, 26, 31]. An increase in metal concentration caused a decrease in the growth of *Oscillatoria sp*., as well as a decrease in the level of some cellular components like chlorophyll a, carotenoids, C-phycocyanin, allophycocyanin and sugars [17].

Except for the influence of the lighting regime on the accumulation of spirulina biomass and its biochemical composition, the nature of chromium coordinative compound must also be taken into account. In the present study chromium is in complex with the amino acid glycine. This fact diminishes the toxic effect of chromium. In spirulina grown in the presence of Cr(III) glycinate, the maximum chromium content accumulation (1.013%) was observed in the day/night cultivation regime at 40 mg/l $[Cr(Gly)_3]Cl$.

The mechanism of chromium accumulation in spirulina biomass has been partially described by some authors. According to them, chromium accumulation takes place in two stages: rapid adsorption and slow absorption. This process has been shown to be positively influenced by the increase in temperature and light intensity [7, 20].

Although the mechanism of chromium accumulation in cyanobacterium *Spirulina platensis* has been elucidated, some details remain unclear, such as: the storage of chromium in cells, the influence of the nature of the ligand in the Cr(III) coordinative compound on chromium bioaccumulation, as well as other factors.

In previous research the chromium accumulation in biomass was studied in cultivation of cyanobacterium *Spirulina platensis* with the supplementation in nutritive media of some coordinative compounds of Cr(III) in continuous illumination regime. A higher chromium accumulation was found in both alkaline (92.8 and 128.6 mg%) and saline-soluble protein fractions (55.9 and 70.4 mg%) at spirulina cultivation in the presence of coordinative compounds: $[K_2Cr_2(SO_4)_4]\bullet 12H_2O$ and $K_2[Cr(NTA)(C_2O_4)(H_2O)]\bullet 2H_2O$, respectively [6, 9, 28].

The results of the present research confirm the capacity of cyanobacterium *Spirulina platensis* to accumulate chromium at cultivation with $[Cr(Gly)_3]Cl$ supplementation. We suppose that chromium in spirulina biomass can be incorporated in the protein fraction, including peptides, as well as in other components of the biomass (polysaccharides, amino acids, fatty acids). The study of the effect of $[Cr(Gly)_3]Cl$ on the biochemical composition of cyanobacterium *Spirulina platensis* cultivated with variation of the lighting regime and the timing of addition of this compound has been carried out. Its positive effect on protein content (including peptides) was established within limits of the concentration of up to 30 mg/l. Chromium accumulation was more efficient in the light/dark photoperiod (14/10 hours) in case of supplementation on the 1st and 3rd day in the portions. Thus, the results of our investigations demonstrated the possibility of chromium glycinate application in the cultivation of the cyanobacterium *Spirulina platensis* for the production of chromium-rich spirulina biomass with valuable biochemical content for the use as a food bioadditive or for the extraction of bioactive components with high therapeutic qualities.

The technological scheme for obtaining chromium bioadditive was proposed. This procedure is based on using a Cr(III) glycinate as source of Cr(III) to obtain spirulina biomass enriched with chromium (0.75%) and valuable bioactive substances.

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