INFLUENCE OF COBALT(II) COMPOUNDS ON THE SYNTHESIS OF β-CAROTENE AND ASTAXANTHIN AT DIFFERENT STAGES OF *Haematococcus pluvialis* GROWTH

Ludmila RUDI[*], Ecaterina PLÎNGĂU[*], Ana VALUȚA[*], Vera MISCU[*]

[*]*Institute of Microbiology and Biotechnology of the Technical University of Moldova, Chisinau, Moldova*
Corresponding author: Ludmila Rudi, Institute of Microbiology and Biotechnology, 1, Academy str., 2028 MD, Chisinau, Republic of Moldova, phone 0037322725306, e-mail: rudiludmila@gmail.com

Abstract. The study aimed to assess the effect of some Co(II) compounds on the production of biomass, β-carotene and astaxanthin by green microalga *Haematococcus pluvialis* at different stages of its life cycle. Cobalt compounds ($Co(OAc)_2$ and $CoCl_2$) were added to the nutrient medium of *Haematococcus pluvialis* on the first day of the cultivation cycle. The biomass of *H. pluvialis* was collected according to some conventional stages of the life cycle: day 3 - green cell stage; day 10 - brown cell stage, and day 14 - aplanospore stage. Changes in the amount of microalgae biomass were determined depending on the stage of the life cycle, as well as the content of β-carotene and astaxanthin pigments. Analysis of the data obtained in the course of a series of experiments indicates that the studied Co(II) compounds did not inhibit cell proliferation within the limits of applied concentrations. Co(II) acetate, depending on the concentration, was involved in the processes of cell multiplication and the synthesis of β-carotene. In concentrations of 1.5 and 2.0 mg/L, $Co(OAc)_2$ stimulated biomass and astaxanthin production. $CoCl_2$ was involved in the proliferation of microalgae cells, the green cell stage being the most sensitive to the action of the compound.

Key words: *Haematococcus pluvialis*; stages of growth; cobalt compounds; biomass; β-carotene; astaxanthin.

INTRODUCTION

Nowadays, microalgae are studied and used as a source of biologically active compounds with a wide range of applications in the pharmaceutical, nutraceutical, cosmetic, and food industries [1, 12, 24]. In addition, microalgae are becoming very popular in promoting a healthy diet due to the complex nutrients they contain [18, 22].

The unique ability of the green alga *Haematococcus pluvialis* to accumulate high concentrations of natural astaxanthin is leveraged for industrial production. *Haematococcus pluvialis* is currently recognized as the richest and most promising source for the production of natural astaxanthin [25]. Astaxanthin is a member of the carotenoid family and it is getting prominence due to its antioxidant superpower [13, 19]. According to the morphological and physiological changes, the life cycle of *H. pluvialis* can be divided into the green stage and red stage [13] or the motile stage and non-motile stage [23]; palmelloid and aplanospore stages [14]. In culture, the alga undergoes its whole vegetative life cycle, starting as green and motile cells during their active growth. When entering palmella stage, the cells start losing flagella, expand their cell size, and become resting vegetative cells. Encystment stage (immature cyst/brown color) is characteristic of astaxanthin-accumulating palmella cells. When stress conditions prevail (nutrient starvation, high light conditions, or high osmotic pressure), palmella transform into asexual aplanospores. Mature aplanospores accumulate large amounts of secondary carotenoids, particularly astaxanthin that is deposited in lipid droplets in the cytoplasm which results in a characteristic bright red color of these cells with increased longevity. When conditions become favorable for growth, the cysts germinate, releasing a large number of new motile cells. Each algal cell stage could be distinguished by the ratio of pigments (carotenoid/chlorophyll) and the intracellular protein content.

Various technological solutions are proposed to stimulate cell multiplication and the accumulation of this carotenoid for each stage separately [3, 14]. Techniques for optimizing and directing biological processes in microalgae cells at different stages of life cycle are proposed. Techniques for monitoring and quantitative regulation of culture medium components, temperature and light intensity were proposed [14, 16, 21]. For the most part, methods to stimulate cell multiplication are most suitable for the vegetative green stage of *Haematococcus pluvialis* [20]. Some of the proposed technologies include the step of stimulating cell proliferation and astaxanthin synthesis with the addition of metals to the culture medium [2, 15]. For example, Fe^{2+} stimulated the accumulation of astaxanthin under conditions of low cell density, and Fe^{3+} promoted the accumulation of biomass during the vegetative stage, while the compound $FeC_6H_5O_7$ increased cell mass by 2 times and astaxanthin production by 7 times [2, 7].

In higher concentrations, cobalt is toxic to aquatic organisms [9]. However, green microalgae *Botryococcus braunii* showed an increase in biomass and carbohydrate content when exposed to cobalt at a concentration of 4.5 mg/L [4]. In the case of microalgae *Monoraphidium minutum* and *Nitzschia perminuta*, collected from the natural environment and subjected to contact with Co^{2+}, depending on the phases of the life cycle, the effects of stimulating the growth of microalgae and reducing carotene synthesis were registered [6]. A difference was noted in the response of microalgae cells depending on the type of cobalt compound [8]. It was shown that some Co(II) complexes with Schiff bases are involved in cell proliferation and β-carotene synthesis at different stages of *H. pluvialis* life cycle [17].

The aim of the study was to assess the effect of some Co(II) compounds on the production of biomass and the synthesis of β-carotene and astaxanthin by green microalga *Haematococcus pluvialis* at different stages of its life cycle.

MATERIAL AND METHODS

Green algae strain *Haematococcus pluvialis* CNMN-AV-05, obtained from the National Collection of Nonpathogenic Microorganisms was used in the study. The microalga was grown on a mineral culture medium with the following composition (in g/L): $NaNO_3$ – 0.3; KH_2PO_4 – 0.02; K_2HPO_4 – 0.08; NaCl – 0.02; $CaCl_2$ – 0.05; $MgSO_4 \cdot 7H_2O$ – 0.01; $ZnSO_4 \cdot 7H_2O$ – 0.0001; $MnSO_4 \cdot 5H_2O$ – 0.0015; $CuSO_4 \cdot 5H_2O$ - 0.00008; H_3BO_3 – 0.0003; $(NH_4)_6MoO_{24} \cdot 4H_2O$ – 0.0003; $FeCl_3 \cdot 6H_2O$ – 0.0175; EDTA – 0.0075, at a temperature of 26°C, under continuous lighting regime with an intensity of 28 μmol $m^{-2}s^{-1}$ and periodic shaking during the first ten days of cultivation. The induction of astaxanthin accumulation was achieved by increasing the the light intensity to 150 μmol m^{-2} s^{-1} over the next 4 days.

Two cobalt compounds $CoCl_2$ and $Co(CH_3CO_2)_2 \cdot 4H_2O$ (hereinafter $Co(OAc)_2$) with a purity ≥98.0% Sigma-Aldrich have been used in the experiments.

The amount of microalgal biomass was determined spectrophotometrically at 680 nm for the green cell stage; at 480 nm for the brown cyst stage and at 565 nm for the aplanospore stage. The quantitative calculation was made on the basis of the calibration curve. The biomass of *H. pluvialis*, consisting of green cells, palmella cells (brown cysts), and aplanospores (red cysts), were separated from the cultural liquid by centrifugation for 5 min at 1500g. Depending on the development stage at which the biomass of *Haematococcus* is collected, it is subjected to a pretreatment procedure. The green cell mass was suspended in distilled water at a final concentration of 10 mg/mL and subjected to repeated freezing and thawing techniques. The biomass of brown and red cysts was processed with microwaves at a power of 450 W for 120 sec. To determine the content of β-carotene and astaxanthin, 1.0 mL of 96% ethyl alcohol was added to 10 mg of pretreated biomass.

Extraction was carried out by stirring at room temperature for 180 min. The alcohol extract was separated from the biomass by centrifugation at 4000 rpm for 10 min. The content of β-carotene in the biomass of green cells and brown cysts was determined spectrophotometrically at 450 nm in alcohol extract with recalculation in mg/L according to the calibration curve. The content of astaxanthin in the biomass of aplanospores was determined spectrophotometrically at 480 nm in the ethanolic extract with recalculation in mg/L, performed on the basis of the calibration curve.

RESULTS

Cobalt compounds were supplemented to the nutrient medium of *Haematococcus pluvialis* from the first day of the cultivation cycle. The biomass of aplanospores was used as an inoculum. In the case of this strain, the transformation of aplanospores into motile green cells lasted 3 days. Next comes the palmella stage with green-brown cells that turn into brown cysts. The biomass of *H. pluvialis* was collected according to some conventional stages of its life cycle: day 3 - green cell stage; day 10 - brown cell stage, and day 14 - aplanospore stage.

Figure 1 shows the amount of *H. pluvialis* biomass at the stage of green cells, produced under the influence of Co(II) compounds.

Thus, under cultivation conditions in the presence of $Co(OAc)_2$ at a concentration of 1.0 mg/L, an increase in the amount of *H. pluvialis* biomass by 24% was found. Concentrations of 1.5 and 2.0 mg/L of the compound stimulated biomass production by 41-43%. The amount of biomass increased by 35% in the experimental variant with the addition of 2.5 mg/L cobalt acetate. While in the variants of the experiment with 3.0 and 3.5 mg/L of cobalt acetate, the amount of biomass decreased slightly, by 8-12%. In the case of cobalt chloride, the values of algal biomass oscillated insignificantly, increasing, and decreasing by about 10% at concentrations of 1.0-1.5 mg/L and 3.5mg/L, respectively. Cobalt chloride concentrations of 2.0-3.0 mg/L did not modify the production of biomass.

Figure 2 shows the content of β-carotene in the biomass - green cell stage (% bm) and in suspension culture (mg/L) of *H. pluvialis* grown under the action of Co(II) compounds.

Higher levels of β-carotene were determined in the culture of *H. pluvialis* (green cell stage) grown on the mineral medium supplemented with $Co(OAc)_2$ in the concentration range of 1.5-3.5 mg/L, microalgae biomass containing 1.64-1.91% of β-carotene. Increased values of β-carotene were also recorded in experiments using $CoCl_2$ in concentrations between 1.5-3.5 mg/L, which favored the accumulation of 1.56-2.3% of β-carotene.

Figure 3 shows the amount of *H. pluvialis* biomass at the stage of brown cysts, accumulated under cultivation conditions in the presence of Co(II) compounds.

When applying $Co(OAc)_2$ in concentrations of 1.0, 1.5 and 2.0 mg/L, the amount of biomass was 1.14-1.22 g/L, the increase being 21-33% compared to control. Concentrations of 3.0 and 3.5 mg/L reduced the amount of algal biomass by 27%. $CoCl_2$ stimulated biomass production at the minimum applied concentrations of 0.5 and 1.0 mg/L. In the concentration range from 1.5 to 3.5 mg/L, a decrease in the amount of biomass by 11-17% was found.

Figure 4 shows the change in β-carotene content in the biomass - brown cyst stage (% bm) and in

suspension culture (mg/L) of *H. pluvialis* grown under the influence of Co(II) compounds.

In the case of $Co(OAc)_2$ compound, concentrations of 1.0, 1.5 and 2.0 mg/L stimulated the accumulation of β-carotene in the algal biomass (brown cyst stage). At these concentrations, the content of β-carotene in the biomass was 3.2-4.11%, which was 27-61% more than in control sample. In the experimental variants with the addition of $CoCl_2$, an increase in the content of β-carotene was achieved in the concentration range of 1.5-3.5 mg/L, and its maximum content in biomass of 4.2% was determined at a concentration of 3.0 mg/L. The same stimulating effect of β-carotene synthesis was also found at higher concentrations of this compound.

Figure 5 shows the change in the amount of *H. pluvialis* biomass at the stage of red cysts (aplanospores), accumulated under cultivation conditions in the presence of Co(II) compounds.

In the variants of the experiment with the addition of $Co(OAc)_2$ at the stage of aplanospores, concentrations of 1.5 mg/L and 2.0 mg/L were identified as stimulants ($p \leq 0.05$). At these concentrations, the amount of biomass was 44-35% higher compared to control. Compound concentrations of 3.0 and 3.5 mg/L reduced more than 20% of the biomass collected at the end of this cultivation stage. In the case of $CoCl_2$, this compound lost its stimulatory activity at the applied concentrations, manifested in the stages of green cells and brown cysts. The amount of aplanospores did not change or was 20-50% lower compared to the control.

Figure 6 shows the astaxanthin content in biomass – aplanospore stage (% bm) and in suspension culture (mg/L) of *H. pluvialis* grown under the influence of Co(II) compounds.

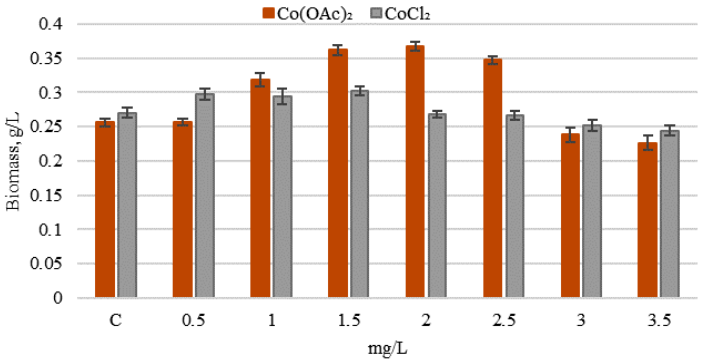

Figure 1. The amount of *H. pluvialis* biomass (green cell stage) produced in the presence of Co(II) compounds

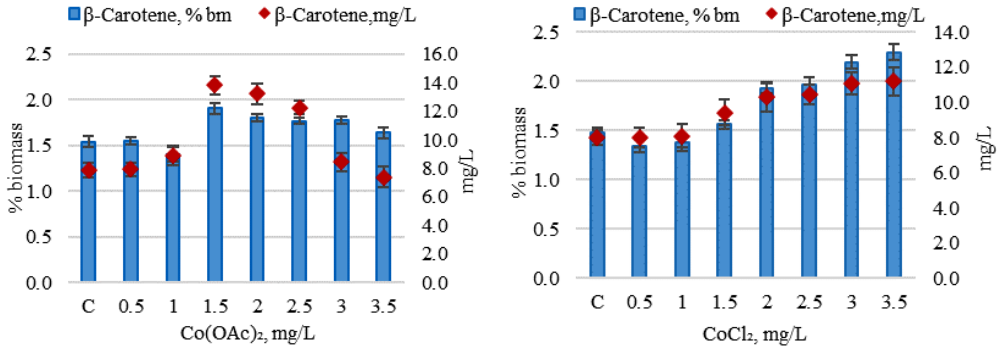

Figure 2. The content of β-carotene in biomass - green cell stage (% bm) and in suspension culture (mg/L) of *H. pluvialis* under the action of Co(II) compounds

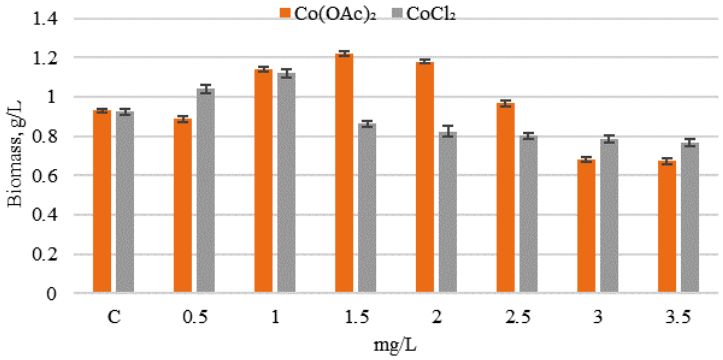

Figure 3. The amount of *H. pluvialis* biomass (brown cyst stage) obtained in the presence of Co(II) compounds

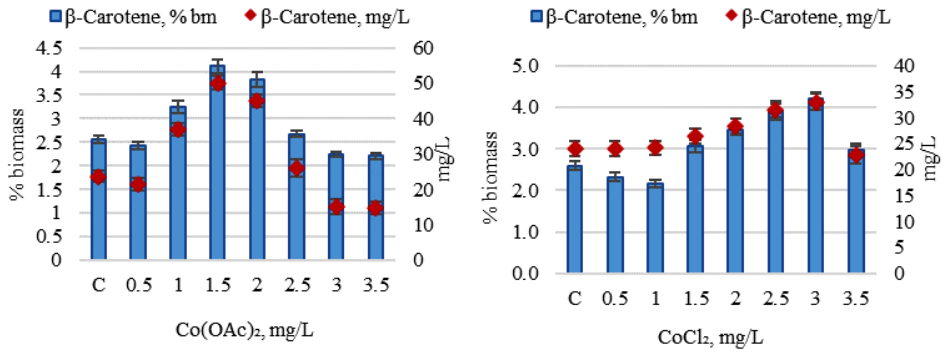

Figure 4. The content of β-carotene in biomass - brown cyst stage (% bm) and in suspension culture (mg/L) of *H. pluvialis* under the action of Co(II) compounds

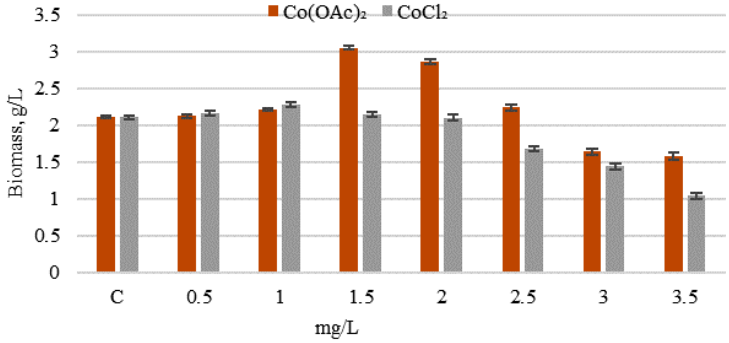

Figure 5. The amount of *H. pluvialis* biomass (aplanospore cyst stage) obtained in the presence of Co(II) compounds

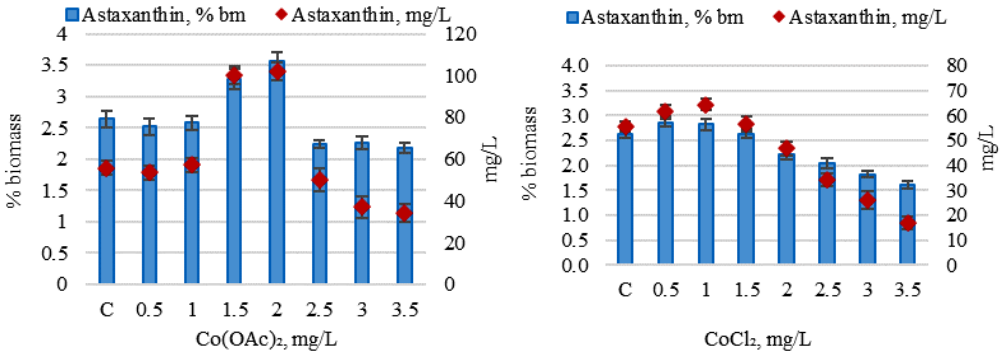

Figure 6. The content of astaxanthin in biomass - aplanospore stage (% bm) and in suspension culture (mg/L) of *H. pluvialis* under the action of Co(II) compounds

The content of astaxanthin in *H. pluvialis* biomass (aplanospore stage) did not change when using $Co(OAc)_2$ concentrations of 0.5 and 1.0 mg/L. Concentrations of 1.5 and 2.0 mg/L stimulated the synthesis of astaxanthin by 24-34% in comparison with control. Compound concentrations of 3.0 and 3.5 mg/L inhibited pigment production by more than 15%. Under the influence of $CoCl_2$ in the applied concentrations, the content of astaxanthin did not change or was 20-30% lower than the control level in the experimental variants with compound concentrations from 2.0 to 3.5 mg/L.

DISCUSSIONS

The life cycle of *H. pluvialis* is characterized by various types of distinguishable cellular morphologies based on microscopic observation: green vegetative biflagellate cell, green vegetative non-motile cell, astaxanthin-accumulating palmella cell, and astaxanthin-rich asexual aplanospore. It is worth mentioning that cell proliferation occurs in both motile and non-motile stages [22].

Mature aplanospore cells (red cysts) were used as inoculum. When aplanospores are transferred to a nutrient medium, they begin to germinate, releasing a large number of new motile cells. The transformation of aplanospores into motile green cells lasted 3 days. At the vegetative stage, proliferation and biosynthetic activity of cells are most intense. The depletion of the components of the nutrient medium is an inducing factor of encystment with the formation of brown cysts, which continue to divide and further accumulate carotene. On the 10th day of the life cycle, most of the

cells in the culture of *H. pluvialis* were brown cysts. In the next days of cultivation, astaxanthin induction was achieved by increasing the light intensity. On the 14th day, astaxanthin-containing aplanospores can be collected.

In the experimental series, *H. pluvialis* biomass was collected corresponding to the stages of intensive accumulation of biomass (green cell stage, brown cyst stage), carotene (green cell stage and brown cyst stage), and astaxanthin (aplanospore stage).

For motile green *H. pluvialis* cells, $Co(OAc)_2$ had a stimulatory effect at concentrations of 1.5, 2.0 and 2.5 mg/L. The use of higher concentrations of the compound revealed a tendency towards inhibition of biomass accumulation (Figure 1). For $CoCl_2$, the dependence of the effect on the applied concentration was obvious: low concentrations of the compound showed a moderate stimulating effect, while higher concentrations caused a delay of cell multiplication process.

Such type of response was established for freshwater microalgae *Monoraphidium minutum* and *Nitzschia perminuta* isolated from their natural habitat for experimental purposes and which, under laboratory conditions, reacted differently to the presence of Co^{2+} in the cultivation medium at concentrations of 0.1-5.0 mg/L [6]. Low concentrations of Co^{2+} led to an increase in the amount of biomass by 8-13% in *M. minutum* and by 5-9% in *N. perminuta*. The concentration of 3 mg/L Co^{2+} reduced the biomass production of *M. minutum* and *N. perminuta* by 42% and 36%, respectively.

In the case of *H. pluvialis* culture in brown cyst stage, the inhibitory effect of high concentrations of 3.0 and 3.5 mg/L $Co(OAc)_2$ was more pronounced, the amount of biomass decreased by 27% below the control level (Figure 3). Microalgae *Pavlova viridis* exposed to Co^{2+} in concentrations higher than 10 µmol/L showed reduction in cell density. In the presence of 200 µmol/L Co^{2+}, algal growth was inhibited by 74.95% compared to the control [11]. Moreover, the microalgae *Chlorella* sp. showed with a significant decrease in biomass production during cultivation for 10 days at concentrations of 10, 50, 100 mg/L Co^{2+} [10].

Concentration of 1.0 mg/L $Co(OAc)_2$ proved to stimulate cell proliferation in *H. pluvialis* with a 21% increase in algal biomass. In the presence of $CoCl_2$ in concentrations of 0.5 and 1.0 mg/L, biomass production increased by 12 and 21%, respectively.

A similar response of microalgale culture depending on the type and concentration of the cobalt compound was reported for microalgae *Phaeodactylum tricornutum*. The effect of stimulating the productivity of microalgae *Phaeodactylum tricornutum* upon contact of the culture with $CoCl_2$ was determined at concentrations between 0.02-0.16 mg/L, and chemical compound $Co(NO_3)_2$ increased biomass production in the concentration range of 0.08-1.25 mg/L [8].

The culture of *H. pluvialis* was relatively tolerant to elevated levels of cobalt, but the effect of the metal on cell multiplication was more evident in the stage of brown cysts. Obviously, microalgae species and strains react specifically to the presence of cobalt ions in the cultivation medium. The response of *H. pluvialis* to cobalt was different for each stage of its life cycle. Microalgae *Phaeodactylum tricornutum* is reporred to exhibit a similar behavior in response to various stimuli when algae cells pass through different growth phases. Thus, when $CoCl_2$ was added to nutrient medium at a concentration of 10 mg/L, the productivity of *Phaeodactylum tricornutum* decreased upon contact of the culture with metal ions in the exponential growth phase, while in the stationary growth phase it was weakly susceptible to contact with cobalt. The concentration of 75 mg/L Co^{2+} significantly reduced productivity when the culture was exposed to cobalt(II) nitrate in both the exponential and stationary phases of growth [8].

The effect of cobalt added to the nutrient medium at the stage of red cysts was investigated. $Co(OAc)_2$ concentrations of 1.5 mg/L and 2.0 mg/L favored the proliferation of microalgae cells (Figure 5). The amount of biomass has increased significantly by 44-35%. Whereas the levels at 3.0 and 3.5 mg/L were inhibitory to the biomass accumulation process.

Analysis of the effect of cobalt compounds on the process of β-carotene synthesis is of great importance for describing the type of response of a microalgae culture to the presence of cobalt ions in the cultivation medium. A high level of β-carotene in the biomass may be the result of induced oxidative stress, since β-carotene modulates intracellular redox status, protecting cells from free radicals.

At green cell stage of *H. pluvialis* life cycle, the level of β-carotene increased with concentrations between 1.5-3.5 mg/L $Co(OAc)_2$ (Figure 2). The content of β-carotene increased by 24% when using at the cobalt acetate concentrations of 1.5 mg/L. The yield of β-carotene in the algal suspension in these variants increased by 75%, 68% and 56%, respectively, and amounted to 12.3-13.8 mg/L. Biomass production was stimulatory at three levels of $Co(OAc)_2$. In conclusion, it beneficial to use this cobalt compound at the stage of vegetative green cells, which can yield algal biomass with high β-carotene content.

The biomass of microalgae *Monoraphidium minutum* and *Nitzschia perminuta* reportedly not change significantly under the influence of Co^{2+}, except for the maximum applied concentration of 3 mg/L Co^{2+}, which reduced the content of β-carotene in *M. minutum* culture by 40%. At the same time, *Nitzschia perminuta* was less sensitive to cobalt, which reduced the level of β-carotene by 30% in the presence of 5 mg/L Co^{2+} [6].

In experiments with $CoCl_2$ supplementation, an increased content of β-carotene in microalgae culture was associated with a decrease in algae growth. The stimulatory effect on the synthesis of β-carotene with a

simultaneous decrease in the biomass of green cells was plausibly due to the production of oxidative stress, while β-carotene was required for antioxidant purposes. In the culture of *Pavlova viridis* grown in the presence of concentrations of 20 and 50 μg/L Co^{2+}, a stimulatory effect on carotene synthesis was found, which increased by 4 times [11].

A different algae responce was identified when *H. pluvialis* passed through the brown cyst stage. $Co(OAc)_2$ concentrations of 1.5 and 2.0 mg/L stimulated the synthesis of β-carotene in the biomass, as well as the yield of β-carotene of microalgae culture, which amounted to 37-50 mg/L (Figure 4). $CoCl_2$ equally induced the increase in the content of β-carotene in both microalgae suspension and algal biomass, which in the experimental variants varied within 10-38% and 18-62%, respectively. In addition, the increase in the content of β-carotene in *H. pluvialis* brown cyst biomass depended on the applied concentration of $CoCl_2$. In the case of microalga *Chlorella pyrenoidosa*, an inverse relationship was reported between the concentration of Co^{2+} and the content of β-carotene in the biomass [5].

It was found that at the end of the cultivation cycle in *Haematococcus*, characterized by the formation of red aplanospores, $Co(OAc)_2$ concentrations enhanced astaxanthin content in both microalgae suspension and algal biomass samples (Figure 6). Thus, concentrations of 1.5 and 2.0 mg/L induced an increase in the content of astaxanthin in biomass by 24-35% and the yield of pigment in it by 80% ($p \leq 0.05$). In these treatments the amount of biomass increased by 35-44%.

It was found that at the end of the cultivation cycle in *Haematococcus*, $Co(OAc)_2$ concentrations enhanced astaxanthin content in both microalgae suspension and algal biomass samples. Thus, concentrations of 1.5 and 2.0 mg/L induced an increase in the content of astaxanthin in biomass by 24-35% and the yield of pigment in it by 80% ($p \leq 0.05$). In these, treatment the amount of biomass increased by 35-44%.

In the case of $CoCl_2$, astaxanthin content decreased in both biomass and culture suspension with an increase in the concentration of the compound. The presence of $CoCl_2$ in the cultivation medium stimulated the biosynthesis of carotene and its accumulation in microalgal biomass at the stages of green cells and brown cysts. Cobalt(II) acetate, depending on the concentration, was involved in the processes of cell multiplication and the synthesis of β-carotene, and accumulation of the astaxanthin at the aplanospore stages. A similar response of *H. pluvialis*, depending on the stage of its life cycle, to the presence of cobalt in the cultivation medium was established when testing some coordination compounds of cobalt with Schiff bases [17]. It was assumed that the change in biomass production during the stages of the vital cycle indicated the adaptability of the culture to the compounds supplemented to the cultivation medium. The synthesis of β-carotene seems to be one of the important factors in maintaining the antioxidant status of microalgae.

The research conducted showed that Co^{2+} plays a role in the biosynthetic activity of *Haematococcus pluvialis*. This was evidenced by the changes observed in the carotene content of the biomass throughout its life cycle.

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