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Reaction of Cells *Desmodesmus armatus* (Chod.) Hegew. on the Induction of Carotynogenesis

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Abstract. Various studies of the components of the antioxidant protection system of microalgae *D. armatus* under the influence of osmotic stress and active forms of oxygen will allow to develop methods for controlling carotenogenesis in a given culture and to obtain carotenoid enriched feed for zooplankton. These studies made it possible to evaluate the activity of catalase, peroxidase enzymes in cells that are cultured under the induction of carotenogenesis by free radical oxidation promoters and osmotic stress on the background of physiological changes. It is established that under these conditions, there is an increase in volumes and aggregation of vegetative cells. At the same time, the amount of biomass remains at the level of the first day of inductors application. Against the background of a decrease in growth activity, a decrease in the number of metabolically active cells in cytochrome oxidase was observed. It is also shown that, when iron sulfate is introduced with hydrogen peroxide and sodium chloride against the background of enhanced carotenogenesis, antioxidant systems are activated by increasing the activity of catalase and peroxidase. Under such conditions, it is possible to achieve increased production of carotenoids in *Desmodesmus armatus* culture.

Introduction

In the industry, carotenoids have a high biological value. They play a significant role in the growth and development of fish. At the same time, the latter are not capable of synthesizing carotenoids and need to get them with feed. Zooplankton, which is a feeder for fish, is also incapable of synthesizing these pigments, but only of their accumulation. Increase the level of carotenoids in such feeder can be under the conditions of application of carotinosynthesizing algae. Obtaining these compounds from algocultures is more economical than from other sources, since they are unpretentious to the composition of the nutrient medium, the conditions of cultivation, and are characterized by a rapid build-up of biomass [1-5].The ability to induce carotenoids and accumulate them in large quantities is a characteristic feature of extremophile microalgae species that experience dramatic changes in environmental conditions (changes in temperature, illumination, salinity, nutritional deficiencies, etc.) [6]. These include representatives of the genus Desmodesmus. In addition, the morphology of their cells and their nutritional value allow to consider them as food objects for zooplankton. This makes them a valuable source of these essential compounds and a promising research object.

Caroteniods are an integral part of the non-enzymatic system of antioxidant protection of cells from damage. Science distinguishes between primary and secondary carotenoids in microalgae cells function as antioxidant protectors. The first of them participate in the absorption of light and photoprotection of the membranes of thylakoids. Others are located in the lipid inclusions of the cytoplasm or on the stroma of plastids, and protect them from the effects of stress factors [7, 8].

For some representatives of microalgae, in particular *Dunaliella salina*, it has been shown that active forms of oxygen are involved in the induction of the synthesis of secondary carotenoids, since when additional generators (sodium hypochlorite, hydrogen peroxide, peroxynitrite) were added to the medium, hypersynthesis of secondary carotenoids was triggered. Most likely, reactive oxygen species act as secondary messengers, activating the biosynthetic pathways of secondary

carotenoids, in particular astaxanthin and its esters through activation of the corresponding enzymes or inducing the expression of genes encoding them [9-11]. Under conditions of osmotic stress or with a decrease in the efficiency of fixing carbon dioxide, an increase in the concentration of reactive oxygen species is also observed [12]. In previous studies, we showed for *Desmodesmus armatus* that the introduction of free radical oxidation and osmotic stress into the medium (NaCl (200 mM) or Fe^{2+} (200 mM)) with H_2O_2 (10^{-4} mM)) allows to increase the yield of carotenoids in *D. armatus* culture in 4 times [13]. Despite the proven fact of induction, its mechanism and regulation have not been essentially studied so far. There is an opinion that the influence of inducers of carotenogenesis can influence the activity of enzymatic mechanisms of antioxidant protection (catalase, peroxidase activity) and the accumulation of carotenoids as a compensatory reaction [14, 15]. As a result of the well-coordinated work of these enzymes due to their different relationship to the substrate, a number of peroxide compounds are inactivated, which provides a flexible regulation of the level of lipid peroxidation in the cell and an adequate response of the body to the action of oxidizing loads. Catalase (EC 1.11.1.6), included in this complex, is a key enzyme [16]. It restores superoxide radicals to hydrogen peroxide. In the complex antiradical protection of cells, along with catalase, peroxidase (EC 1.11.1.7) plays an important role, which is responsible for regulating the concentration of H_2O_2 and organic peroxides in cells. These enzymes are one of the marker enzymes and are practically the first to activate in response to stress [17].

Complex studies of various components of the system of antioxidant protection of *D. armatus* under the influence of osmotic stress and active forms of oxygen will make it possible to clarify the functions of carotenoids in cells, the mechanism of induction of their accumulation and to develop methods for controlling carotenogenesis in culture. The aim of the study was to investigate the changes in the catalase and peroxidase activity of *D. armatus* cells grown under induction of carotenogenesis and the use of free-radical oxidation promoters (Fe^{2+} with H_2O_2) and osmotic stress (NaCl).

Materials and Methods

The studies were carried out using an algologically pure culture of green alga *Desmodesmus armatus* (Chod.) Hegew (IBASH-A), obtained from the collection of the M. Kholodny Institute of Botany, National Academy of Sciences of Ukraine.

As a nutrient medium for the cultivation of *D. armatus*, sterile waste water was used from a recirculating aquaculture system (RAS), standardized for pH and total mineralization [18]. With each intake of water, the control of the composition of biogenic elements was carried out. According to the results obtained in the laboratory of biotechnology of water resources, it is known that waste water from RAS is enriched with various forms of nitrogen (NH_4^+, NO_2^-, NO_3^-) and phosphorus. The material for the study was obtained as a result of two-stage cultivation [19]. The first phase of cultivation of *D. armatus* lasted 16 days until the optimal density of culture was achieved ($5x10^6$ cells/l) (Fig. 1). Biomass of the first phase served as a source of inoculum, which was introduced into the medium for the second phase in a ratio of 1:10. Samples, each 100 ml each, were shaken daily. A corresponding inductor of carotenogenesis was introduced into each of the media: $FeSO_4$ (0.11, 0.22, 0.45mM) with H_2O_2 (10^{-4}mM), NaCl (50, 100, 200mM).

All cultures were manipulated under laminar-box conditions. The cultivation of the algae was carried out in a climatic chamber at a temperature of 21 ± 2 °C, illuminated with 2500 lux fluorescent lamps for a 16-hour photoperiod during 25 days.

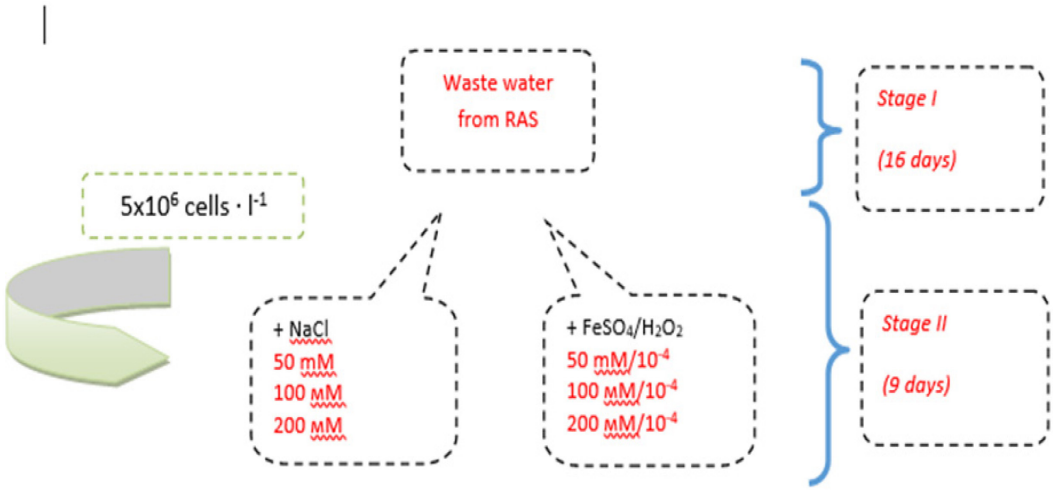

Figure 1. Scheme of two-stage accumulative *D. armatus* cultivation

By the end of the second phase of cultivation, the morpho-physiological parameters of the culture and changes in the enzymatic activity of peroxidase and catalase were evaluated.

The amount of biomass was determined through the culture density using an optical index at 750 nm at CaryWin UV 60 (Agilent, USA). The transition from the units of optical density (D750) to the value of absolutely dry biomass (ADB) was carried out through the empirical coefficient k: ADB = k x D750.

The coefficient k (k = r units of optical density / l) for culture *D. armatus* was determined experimentally in three independent repeats [20].

The proportion of metabolically active cells by cytochrome oxidase test was determined by differential staining. For differential staining, a substrate mixture was used in the composition of ED / MFDA, naphthol, and $NaHCO_3$ [21]. The colored cells were microscopically analyzed using Goryaev's camera, the MicroMed-3300 (×1000) trinocular microscope (Ukraine) and the computer program Micam 2.0.

At the end of the cultivation, the entire biomass was separated at 4°C by centrifugation at 6000 g for 15 minutes and, if necessary, frozen at -20°C. The algae cell walls were disintegrated using ultrasound cell disrupter USDN-2T. To obtain the cytosolic fraction, the cells were homogenized with 0.05 M Tris-HCl buffer (pH = 7.8) containing Triton X-100 (0.25%).

The catalase activity was determined spectrophotometrically by decreasing the optical density at a wavelength of 410 nm as a result of the cleavage of H_2O_2, according to [15].

Extraction and determination of peroxidase activity was carried out according to the method of [14]. The activity was determined spectrophotometrically at a wavelength of 470 nm as a result of oxidation with guaiacol in the presence of hydrogen peroxide. The optical density was determined on CaryWin UV 60 (Agilent, USA).

Simultaneously, the amount of protein was determined by Lowry [22].

Quantitative determinations were carried out in three independent repeats. The data are reported as mean ± standard deviation (**mean ± SD**). Mean values were considered significantly different at P <0.05 according to Student's criterion. The results were analyzed statistically with Microsoft Excel software according to generally accepted methods.

Results and Discussion

In the previous work, we showed that the introduction of compounds - generators of active oxygen species and osmotic stress promoters ($FeSO_4$ / H_2O_2 and NaCl) into the medium led to the preservation of the initial cell number, an increase in the carotenoid yield by 4 times and redistribution of the profile of the main nutrients. At the same time, against the background of a decrease in the activity of cell division, an increase in their volume, aggregation, and a change in

color from green to yellow (aggregated cells actively accumulate carotenoids) were observed, which is characteristic of the activation of carotenogenesis.

When carotenogenesis is activated, it is important to monitor the physiological state and growth activity of the culture. The proper functioning of energy systems and cell cycles ensures the successful formation of a culture survival strategy under stress conditions. One of the indicators of the state of algoculture is its survival in stress conditions, which can be estimated from the accumulation of biomass. Thus, during the entire period of cultivation of *D. armatus* on medium with inductors, there was no mass loss of culture, and at the same time, its low growth activity was observed. During this period, the amount of biomass was at the level of the first day of cultivation in the range of 12-14 g/l (Fig. 2).

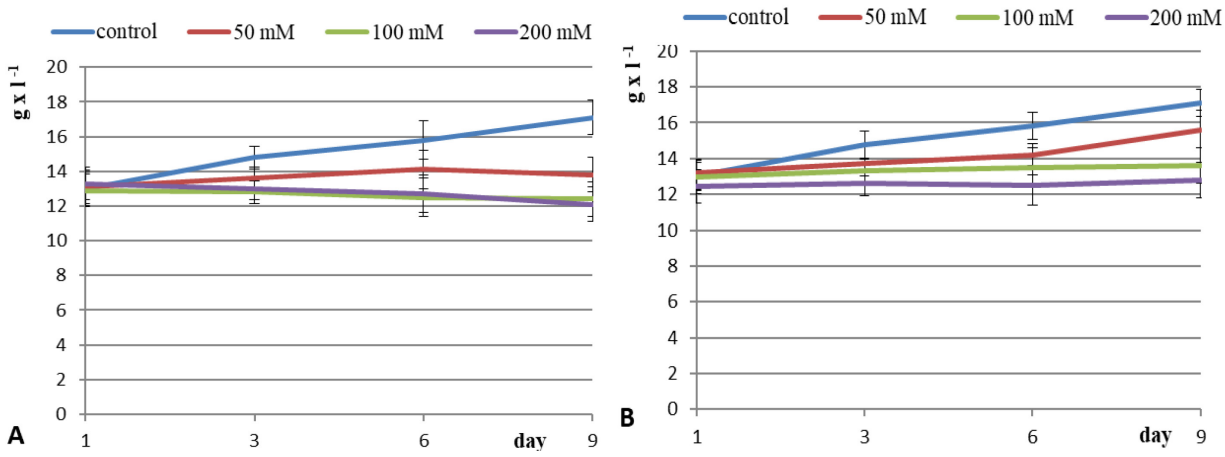

Figure 2. The amount of *D. armatus* biomass in the second stage of cultivation in the presence of $FeSO_4$ / H_2O_2 (A) or NaCl (B)

Another of the most revealing criteria for the survival of a culture under stressful conditions is the efficiency of the functioning of their metabolic systems. One of the key enzymes responsible for the generation of energy in the cell is cytochrome oxidase (EC 1.9.3.1), which catalyzes the final stage of electron transfer to oxygen during oxidative phosphorylation. Its activity is one of the indicators of metabolic activity of culture [21].

Thus, the cytochrome oxidase test proves that against the background of low growth activity, the inhibition of metabolic activity of the culture also occurs (Fig. 3). The effect of $FeSO_4$ with H_2O_2 led to a decrease in the proportion of metabolically active cells by three times to 9 days of cultivation. And the effect of NaCl led to reduction of it by half already on the third day of cultivation. Most likely, the disturbance of osmotic balance has the same effect on biosynthetic processes as the accumulation of reactive oxygen species [23, 24].

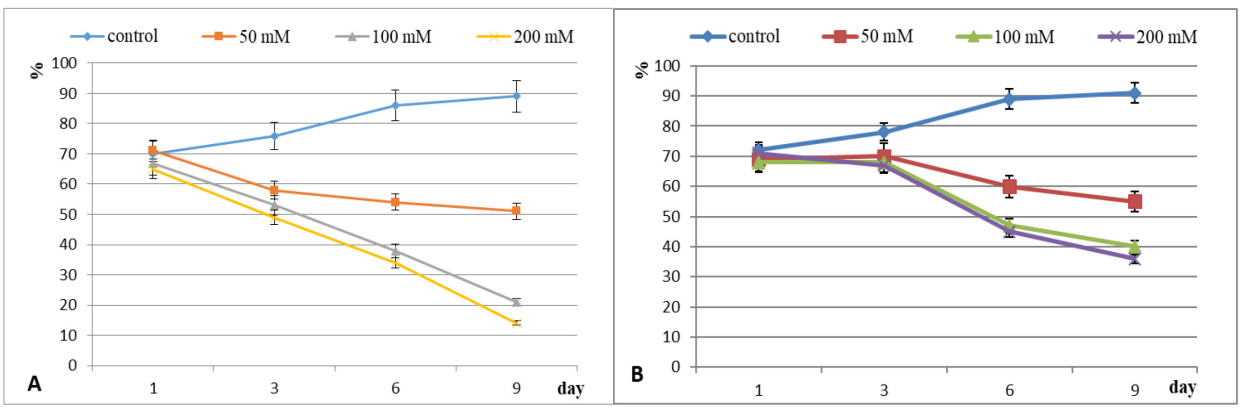

Figure 3. The proportion of metabolically active *D. armatus* cells according to the cytochrome oxidase test in the second stage of cultivation in the presence of $FeSO_4$ / H_2O_2 (A) or NaCl (B)

It is known that the accumulation of carotenoids can be a censor reaction against the background of activation of antioxidant defense processes [12]. Neutralization of ROS in algae and cyanoprokaryotes is effectively provided by a multi-stage antioxidant defense system, in which specific enzymes and low-molecular compounds participate [9]. The most important enzymes for ROS and their products neutralization are superoxide dismutase, catalase and glutathione peroxidase [25]. Usually this is due to an increase in the activity of individual components of this system. One of the marker enzymes, which is practically the first to activate in response to stress, is catalase. We have shown that its activity begins to increase already when 50 mM $FeSO_4$ is introduced into the medium with H_2O_2 and NaCl, and at a concentration of 200 mM of these inducers this activity increases by almost 75% in both cases (Fig. 4).

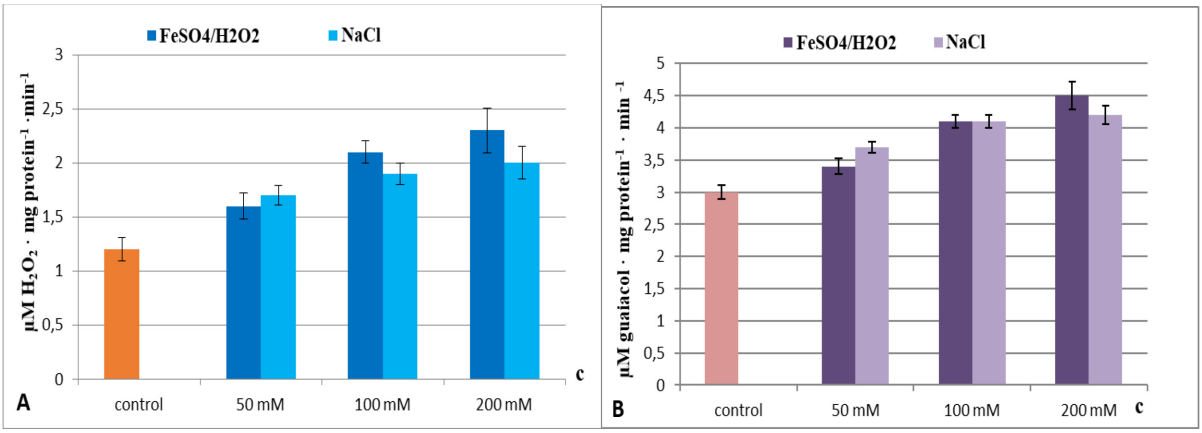

Figure 4. Catalase (A) and peroxidase activity (B) in the cytosolic fraction of *D. armatus* cells in the second stage of cultivation in the presence of NaCl or $FeSO_4$ / H_2O_2

The increase in catalase activity may be due to an increase in the concentration of hydrogen peroxide in the cells. As the concentration of inducing agents increases, the activity of peroxidase also increases.

Since peroxidase is also involved in the metabolism of phenolic compounds, its high activity can be due to its participation in lignification processes during compaction of cell walls and activation of antioxidant defense systems [14]. Their functioning is aimed at reducing the level of oxidative stress, preventing the negative consequences of its action [26]. The level of activity of thes enzymes can be an adequate assessment of the physiological state of hydrobionts under stress conditions. It is known that such peroxide cleavage enzymes, like catalase, can model the homeostasis of the peroxide and, accordingly, its signal capacity. Thus, the increase in catalase activity can be considered as a mechanism for reducing the oxidation-reduction balance and neutralizing the products of reactions of reactive oxygen species, the number of which increases in stressful situations. It has been proved that the activity of catalase in different microalgae species during their growth under culture conditions undergoes certain fluctuations, the amplitude and frequency of which is species-specific. However, in our case, an increase in the activity of the main antioxidant enzymes against the background of suppression of the growth activity of the culture indicates the modeling of stress conditions. It is under such conditions, as known, increased production of carotenoids can be achieved.

Conclusions

This paper considers one of the aspects of the development of technology for the lifetime satiation of feed organisms with carotenoids. It is shown that D. armatus culture can be an industrial producer of these compounds. Though against the background of introducing inducers of carotenogenesis, the growth activity in biomass decreased and the metabolic activity of the culture decreased as was proved, antioxidant systems are activated due to increasing the activity of catalase and peroxidase. Under such conditions the increased production of carotenoids can be reached.

Conflict of Interest

The authors declare that there is no conflict of interest.

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