ANTIOXIDANT ACTIVITY IN *HAEMATOCOCCUS PLUVIALIS* CELLS DURING THE VITAL CYCLE

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Abstract. The goal of this work was to determine the changes that occurred in the antioxidant status of microalga *Haematococcus pluvialis* during its life cycle in closed system. The method for determination of antiradicalic activity has been selected concerning its possible use for complex preparations: the $ABTS^{\cdot+}$ radical cation *scavenging* assay (ABTS). The green motile stage is the most representative. Fluctuations in the antioxidant activity reflect physiological changes that occur throughout the microalga life cycle. For this stage, the antioxidant activity determined by the ABTS radical reduction correlated very strong with carotenoid content in algal biomass, $R^2 = 0.93 - 0.99$. The green motile stage with biflagellate cells of *Haematococcus pluvialis* is suitable for studying variations in the antioxidant status of culture under different conditions.

Keywords: life cycle, antioxidant activity, carotenoides, *Haematococcus pluvialis*

The cultures of microalgae in closed systems cross through the same stages that together form the development cycle of culture or life cycle. Life cycle is one of the most primitive expressions of the pattern of species and its genetic components present a chain of evidence of the species survival throughout evolutionary history [1, 2, 3]. The duration of the life cycle is primarily determined by the genetic component and varies from species to species. However, even in the case of the same species, this parameter may vary depending on the age of the inoculum, the physical and chemical conditions of the environment. For biotechnological flows, it is important that any type of final results to be reproducible. For this purpose, as inoculum using a fixed amount of biomass in a particular physiological state and environmental conditions are accurately reproduced from one cycle to another. In such conditions, the qualitative and quantitative changes occurring in culture are reproducible and biological events may be prognosticated and controlled [4].

Oxidative processes that occur during the life cycle in microalgae cells are determined by biochemical processes, cell density, amount of eliminated exometabolites, consumption of nutrients, diurnal variations in light intensity and UV radiation etc. [1, 2]. The level of the antioxidants in biomass varies in dependence on the degree of reactive oxygen species (ROS) accumulation, being subjected to the same biorhythms.

Life cycle of green microalga *Haematococcus pluvialis* include two opposite states: motile (green biflagellate cells) and stationary (green, brown and red cysts). Green motile stage is the phase of cell multiplication and synthesis of photosynthetic pigments. In the brown cysts, the transformation of the carotene into astaxanthin occurs. Finally, they turn into red cysts. At all stages of development, *Haematococcus pluvialis* cells contain antioxidant components. In green vegetative cells prevail chlorophyll, luteine, β-carotene, neoxantine, violaxantine, mostly synthesized lipids are omega-3. The brown cysts contain a complex of carotenoids, which is in permanent fluctuation ratio. Red cysts contain astaxanthin, a superantioxidant.

In terms of biotechnology, it is very important to determine under what conditions and at what stage of development we can get a qualitative biomass for extracting an effective antioxidant complex. Determination of "sensitive moments" during the vital cycle provides opportunities

for automatization of biotechnological processes and obtaining the qualitative products under controlled conditions. Monitoring the changes in the antioxidant activity of algae during the life cycle is possible by daily determining the antioxidant capacity of algal biomass [5].

The purpose of the research, the results of which are presented in this article, was to determine the changes that occur in the antioxidant status of microalga *Haematococcus pluvialis* during its life cycle in closed system.

Materials and methods

As the research object served the strain of green microalga *Haematococcus pluvialis CNM-AV-03*, deposited in the National Collection of Nonpathogenic Microorganisms, Institute of Microbiology and Biotechnology, Academy of Sciences of Moldova.

The cultivation was performed on RD liquid mineral medium with the following composition of macroelements (g/L): $NaNO_3$ - 0,3; KH_2PO_4 - 0,02; K_2HPO_4 - 0,08; $MgSO_4 \cdot 7H_2O$-0,01; $CaCl_2$ - 0,0474, NaCl - 0,02, and microelements (mg/L): $ZnSO_4 \cdot 7H_2O$ - 0,0001; $MnSO_4 \cdot 5H_2O$ - 0,0015; $CuSO_4 \cdot 5H_2O$ - 0,00008; H_3BO_3 - 0,0003; $(NH_4)_6Mo_{24} \cdot 4H_2O$ -0,0003; $FeSO_4 \cdot 7H_2O$ - 9,3; $Co(NO_3)_2 \cdot H_2O$ - 0,0002; EDTA - 0,0075; at 27 ± 1°C; pH 6,8 - 7,0; light intensity - 2500-3500 lx. The synthesis of astaxanthin occurred through the action of high light intensity (10,000 lx). There were used red cysts as inoculum.

Sampling was strictly performed every 24 hours, in order to exclude circadian variations involved in oxidative status of microalgae culture. Assessment of the amount of biomass was carried out spectrophotometrically based on a calibration curve [6].

Preparation of sample extracts. *Haematococcus pluvialis biomass* (green cells) was separated from the culture fluid by centrifugation during 5 min at 1500 g. After centrifugation, the biomass was washed with isotonic solution of ammonium acetate to remove mineral salts. 10 mg of biomass was added to 1 ml of 96% ethanol. The extraction was performed during 60 minutes on an orbital shaker at a speed of 300 revolutions / min. The extract was separated from the cells by centrifugation during 5 min at 2000 g and stored at +4 °C. *Haematococcus pluvialis* biomass (brown and red cysts) was separated from the culture fluid by centrifugation during 5 min at 1500 g. Cysts was subjected to acid hydrolysis [7]. 10 mg of biomass was added to 1 ml of 96% ethanol. The extraction was performed during 60 minutes on an orbital shaker at a speed of 300 revolutions / min. The extract was separated from the cells by centrifugation during 5 min at 2000 g and stored at +4 °C.

Antioxidant activity by the $ABTS^{\cdot+}$ assay (ABTS method).

ABTS radical cation was generated by oxidation of ABTS (2,2 azinobis 3-ethylbenzothiazoline-6-sulfonic acid) with potassium persulphate. The reduction of $\boldsymbol{ABTS^{\cdot+}}$ is carried out by addition electron mechanism. Trolox was used as equivalent for quantitative calculating. Test results can be expressed in % inhibition (for comparing results within the test) and TEAC (Trolox equivalent antioxidant activity) for the comparison with other antioxidants.

ABTS was dissolved in deionized water to a 7mM concentration, and potassium persulphate was added to a concentration of 2.45 mM. The reaction mixture was left at room temperature overnight (12-16 h) in the dark before use. Prior to assay, the $\boldsymbol{ABTS^{\cdot+}}$ stock solution was diluted with ethanol to an absorbance of 0.700 ± 0.020 at 734 nm.

The reaction mixture consisting of 0.3 ml extract and 2.7 ml ABTS work solution. The reduction reaction was conducted at room temperature during 6 minutes. The percent of inhibition was calculated according to the equation:

$$\textbf{\% Inhibition} = (\textbf{Abs}_{t=0} - \textbf{Abs}_{t=6\ min}) / \textbf{Abs}_{t=0} \cdot 100,$$

where $\textbf{Abs}_{t=0min}$ is the $ABTS^{\cdot+}$ solution absorption value in the range 0,700 ± 0,020 at 734 nm, and $\textbf{Abs}_{t=6\ min}$ is the absorption value after incubation.

TEAC value was expressed as mM Trolox/g dry extract, using the calibration curve of Trolox. Linearity range of the calibration curve was 20 to 1000 μM ($R^2 = 0.9976$).

Results and discussions

In the case of compliance with the conditions described above green motile stage is 9 days. Namely, these days form classic life cycle, known for culture of microorganisms in closed systems. The first 48 hours form the lag phase in which the initiation of red cyst germination and the adaptation of green cells occur. The following 48 hours form the acceleration phase, followed by exponential growth phase (5-8 days). 9th day forms slowdown phase. During this phase, the high intensity light is applied to induce the formation of cysts. The 10-16th days form two different stages of stationary phase. The transformation of carotene into astaxanthin and brown cyst formation occur on days 10-13th of life cycle. Red cyst formation lasted 3 days (days 14-16th of life cycle).

Antiradical activity of extracts from *Haematococcus pluvialis* biomass during its life cycle was determined by applying ABTS assay (expressed in TEAC). The results of ABTS assay are shown in Figure 1.

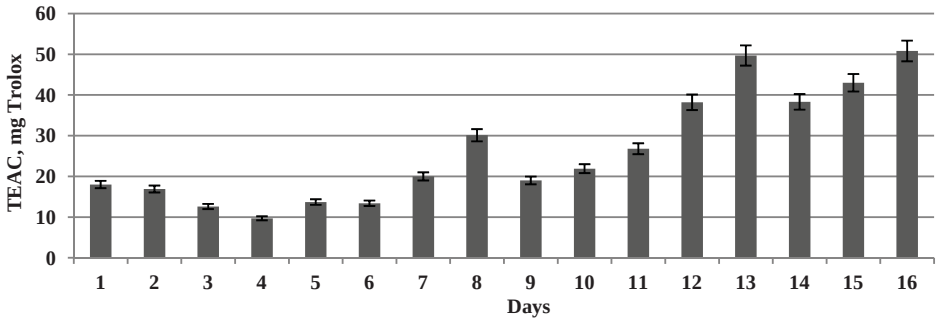

Fig. 1. Antiradicalic activity (ABTS assay, TEAC) of ethanolic extracts from *H. pluvialis* biomass during cultivation (16 days)

A high antiradicalic activity in the first 48 hours after inoculation was due to the fact that culture included a considerable number of cysts which may contain astaxanthin and young green cells pass through a period of adjustment to new conditions. On the fourth day, when the cell division processes started, the astaxanthin reserve was practically exhausted, the green cells became more vulnerable and all their reserves were geared towards increasing the number of cells. The exponential growth phase displayed a slow but steady increase in antioxidant activity of biomass. During this period, the biosynthetic processes were at the maximum rate. Many of the products of biosynthesis have significant antioxidant properties. Sudden decline in the antioxidant activity, which was recorded on 9th day of cultivation, was caused by induced oxidative stress. On the following days (brown cyst stage), a significant increase in antiradical activity of extracts from *H. pluvialis* occurred, determined by the gradual transformation of carotene into astaxanthin. Red cyst stage is characterized by specific processes forming cell coatings, which can decrease the antiradical activity of the extracts. The antiradicalic activity of ethanolic extracts returned to high values on 16th day of the life cycle.

The main modifications in *H. pluvialis* cells during vital cycle consist in qualitative and quantitative changes of carotenoids. Therefore, we supposed the existence of dependencies between the total carotenoid content and reducing activity of ethanolic extract obtained from *H.pluvialis*

biomass. There was a strong correlational dependence between antiradical activity and total content of carotenoids in biomass during germination of red cysts (the inoculum) on the first 4 days of cycle (fig. 2, A). At this stage, the cellular wall suffers certain modifications, astaxanthin lose some functionality and activation of biosynthetic processes occurs due to nutrient-rich growing medium. Cells contain a variable ratio of carotenoids, characterized by gradually decreasing the amount of astaxanthin and increasing the quantity of **β-carotene** and total carotenoids.

The same tendency was maintained the next 5 days (Figure 2, B). For this period, the antioxidant activity determined by the ABTS radical reduction correlated very strong with carotenoid content in algal biomass, $R^2 = 0.99$.

In brown cysts was rather difficult to establish a correlation between the antioxidant activity of ethanolic extracts and their carotenoid content because of lability and reversibility of transformation processes, recorded for the components of this biochemical class (fig. 2, C).

In red cysts occured quantitative and qualitative carotenoid stabilization, which are entirely represented by astaxanthin. In the last days of its life cycle, correlation between antiradical activity of ethanol extract from *H.pluvialis* biomass and carotenoid content recurs (fig. 2, D).

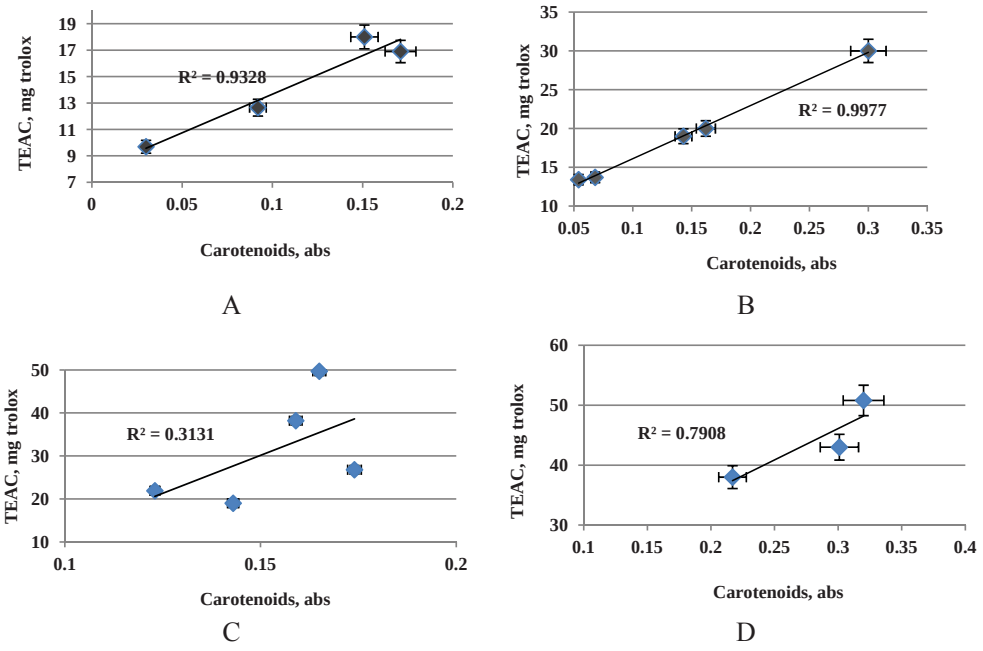

Fig. 2. Correlation between antiradicalic capacity of ethanolic extracts (ABTS assay, TEAC) and carotenoid content (450 nm, abs) in *Haematococcus pluvialis* during its life cycle: (A) - 1-4th days ; (B) - 5-9th days; (C) - 10-13th days; (D) - 14-16th days

The results of this investigation allow us to affirm, that green motile stage of *Haematococcus pluvialis* is suitable for studying changes in antioxidant status of culture. In the case of applying two parameters, which are in close correlation (antiradical activity and carotenoid content), the possibility of errors in the interpretation of results is markedly reduced, especially, when we study the influence of various external factors, including those related to the technological conditions of the industrial cultivation. Elimination of errors is absolutely necessary in the conditions under which we aim to get a maximum safe biomass for human or animal consumption or for subsequent processing.

References:

1. Carvalho A., et al. Circadian protection against oxidative stress in marine algae. In: Hypnos, 1 (suppl 1), 2004, p. 142-157
2. Dufour J., Moreno J., Rodríguez R. Life Cycle Assessment of Biodiesel Production from Microalgae Oil: Effect of Algae Species and Cultivation System. In: M. Finkbeiner (ed) *Towards Life Cycle Sustainability Management.* Springer Science+Business Media B.V. 2011, p. 437- 442
3. Steidinger K.A., Garcces E. Importance of Life Cycles in the Ecologyof Harmful Microalgae. In: Ecological Studies, Vol. 189 , Edna Graneli and Jefferson T.Turner (Eds.). Ecology of Harmful Algae, 2006, Springer-Verlag, Berlin Heidelberg, p. 37-49
4. Rudic, V. ș.a. Ficobiotehnologie –cercetări fundamentale și realizări practice. Chișinău: Elena VI, 2007, p. 362
5. Sadovnic D**.** Monitorizarea activității complexelor antioxidante din *Porphyridium cruentum* prin aplicarea unor metode nespecifice. In: Buletinul Academiei de Științe a Moldovei. Științele vieții, 2012, nr. 1 (316), p. 158-167
6. Schwender J., Gemuenden C., Lichtenthaler H. Chlorophyta exclusively use the 1-deoxyxylulose-5-phosphate/2-C-methylerythritol 4-phosphate pathway for the biosynthesis of isoprenoids. // Planta, 2001, Vol. 212, p. 416-423.
7. Miscu V. Acumularea astaxantinei în biomasa *Haematococcus pluvialis* la cultivare pe mediu cu compuși coordinativi ai Fe(III) cu aminoacizii. // Buletinul AȘM. Științele vieții, 2009, 3(309), p.143-150