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The response reaction of *Dunaliella* cells against the influence of Methylene blue and Norflurazon under the low temperature stress conditions

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Abstract
In this work have been presented the results of investigations of growth indications, pigment formation, catalase activity and the MDA content in Dunaliella cells, grown in photoreactors being supplied by air mixture with the 10^0C and 5^0C temperature (low temperature stress) in control and the treatment with dyes of Methylene blue and Norflurazon herbicide in various concentrations.
It was shown that, the treatment of cells with Methylene blue in low temperature stress conditions decreases the cell bioproductivity. In those conditions on the suppression background of Norflurazon bioproductivity, small herbicide concentration $4*10^{-7}$M stimulates the culture grows.
It has been identified that, the influence of Norflurazon suppresses the carotenoid synthesis and Chlorophyll in low temperature stress conditions.
It was determined that, the treatment of algae by the dyes of Methylene blue and Norflurazon herbicide increases intercellular catalase activity in Dunaliella cells. Methylene blue increases MDA level in Dunaliella cells, but Norflurazon suppresses it in low temperature stress conditions.

Keywords: green microalgae, low temperature stress, bioproductivity, norflurazon, methylene blue, biosynthesis of pigments, catalase activity, the content of MDA

1. Introduction
The effect of temperature impact on plant cells is determined by the guantity and duration of its influence within daily cycle. It is known that, during the long-term 24 – hourly influence under low positive temperature the resistance in plants and in cells to the cold increases [1,2].
The plant reaction under low positive temperature influence resulted in various changes of metabolic and physiological processes, which must lead to the adaptation of plant organisms to changeable conditions. By that, the energy expenses and breathing effectness in cells increase [3]. A certain damage part under low temperature stress conditioned with the impact appearing in cells during the stress of active form of oxygen (AFO) in result of activation of lipid peroxidation processes causing membrane damage. Oxidative stress increases by following catalase inhibition, as the result in cells accumulates H_2O_2 [4]. The antioxidant systems of cells present in AFO neutration which are strong oxidants and their possible accumulation in cells is very dangerous, as they injure membrane structure, protein and DNA [5]. In normal functioning cells exist dynamic balance between the AFO formation and its liguidation. In the liguidation of H_2O_2 present the complex of enzymes, including catalase, peroxidase and others. At the same time, with various responds to stress impact, carotenogenesis is considered adaptative reaction, supplying the microalgae survival in extreme conditions of medium habibat. The accumulation of β - carotene in *Dunaliella* microalgae [6] induced by various stresses, often by UV light [1]. It is known that, the synthesis of carotenoids in those algae can induce artificially in vitro if their cells are treated by methylene blue dyes- generator AFO [7]. There are data that, herbicide norflurazon is the inhibitor of photosynthesis and synthesis of carotenoids [8].
The aim of present study is the investigation of growth indications, pigment formation, catalase activity and the content of MDA in *Dunaliella* cells, grown in photoreactors by scavenging air mass at the temperatures (10^0C) and (5^0C) in control and in the cultivation with methylene blue dyes and herbicide norflurazon of various concentrations.

Materials and Methods
The object of the investigation is the green unicellular algae *Dunaliella salina* IPPAS D-294, taken from the saline lake in Absheron and included into the culture. The algae were grown at 27ºC temperature in photoreactors, volume 250ml, of ordinary glass, in the installation for the growing culture of unicellular algae.

Mineral medium consisted of (g/l): NaCl-87,5 ; KNO_3 - 5,0 ; KH_2PO_4-1,25 ; $MgSO_4$-50 ; $FeSO_4$-0,009 and solution of microelements,1ml/l. The suspension of cells in photoreactors was illuminated by white light (16Wt/m^2) during 24 hours and continuously was scavenged by air mass (air +1,5% CO_2) at temperature 27°C in control and 10°C ; 5°C in experienced suspensions. The growth tempo of culture has been determined by the numbers of cells in the Qoryayeva chamber periodically under the microscope or nephelometrically, measing the optic density of suspension.

Cell suspension, prepared to measure the content of carotenoids, catalase activity and peroxidation of lipids, was retreated to 10^6cells/ml (optic density, OD=0, 8).

The content of carotenoids in cellular extracts (100% acetone) was measured in spectrophotometer and considered on the base of Wetshtain coefficient [9].

For the measuring catalase activity of cells was cooled by centrifugation (3000 rev/min). The sediment has been transported into the mortar with 0,5g $CaCO_3$, added 5ml distillated water and was grinded till homogencaus mass. After that, gained mass was quantitatively transported into the glass with volume 50ml, till the mark and kept periodically shaking 3-4 hours. Within that period the extraction of enzyme from plant material goes on. After keeping, the suspension was filtrated into dry glass. The catalase activity was measured by gasometrical method, which based on the determination of volume after adding into aqueous extract of plants, containing catalase, hydrogen peroxide [10].

The evaluation of peroxidation value in lipids (POL) was carried out by the determination method of MDA content in *Dunaliella salina* cells-by method, based on the reaction with Thiobarbituric acid (TBA).

The cell suspension (35ml) was centrifugated under 3000 rev/min within 10 minutes. Gained sediment homogenized in 20ml 0,1% Trichloracetic acid (TCA). Homogenate was centrifugated under 3000 rev/min within 10 minutes. To 1ml supernatant was added 4ml 20% TCA, containing 0,5% TBA. The mass was heated in aqueous bath under 95°C within 30 minutes and at once cooled under running water. After centrifugation of mass under 3000 rev/min within 10 minutes was determined the optic density of supernatant at 532 nm [11].

Results and Discussion

The growth of control cell suspension under optic conditions (temperature 27°C, light intensity 16Wt/m^2, partial pressure of Carbon dioxide, mineral medium) in 250ml glass photoreactors and scavenge by air mass at 25°C temperature in periodic-accumulative regime of cultivation within 24 hours shows that, the optic density of cellular suspension increases 3,5 - 4 times [1]. The decrease of temperature by air mass till 10°C and 5°C notably suppresses the growth and bioproductivity of control cellular suspension to 10% and 18-20% respectively. In table I have been presented the growth indications, pigment formation, catalase activity, and the content of MDA in *Dunaliella* cells, grown by scavenging air mass into the photoreactors at temperature (10°C) in control and in cultivation treated with dyes of methylene blue of various concentrations. As seen in the tables as result of influence of low positive temperature (10°C) and adding into the nutritional medium of dyes-generator active form oxygen AFO methylene blue in concentrations (2mkM) and (4mkM), growth rate and bioproductivity in experienced suspensions decreased to 3,5-4% and 30-35% respectively. The content of chlorophylle as the result of methylene blue influence hasn't been changed, though one can note the decrease tendency of levels in those "defence" pigments. The carotenoid degradation was followed by the accumulation of MDA in cells. By that, catalase activity in *Dunaliella* cells under the methylene blue influence considerably increased.

Table 1: The indications of growth, pigment formation, catalase activity, and the content of MDA in *Dunaliella* cells, grown by scavenging air mass into photoreactor at the temperature (10°C) in control and in cultivation of methylene blue dyes of various concentrations.

Growth, *OD*			Catalase activity, mkM H_2O_2 $ml^{-1}min^{-1}$				Quantity of enzymes, mg/l			The content of MDA, mol /g untreated weight
			5	10	15	20	Ca	Cb	Ccar	
C	0,25	1,08±0,02	3,0	4,3	5,0	5,7	3,9±0,05	1,9±0,05	1,2±0,05	$0,4*10^{-3}$±0,05
O_1	0.25	1.04±0,02	6,1	8,3	9,7	10,5	3,5±0,05	1,7±0,05	0,6±0,05	$0,5*10^{-3}$±0,05
O_2	0.25	0,70±0,02	6,8	9,2	10,7	12	4,1±0,05	2,0±0,05	0,7±0,05	$0,6*10^{-3}$±0,05

Note: the optic density is OD=0,8; C-control; O_1-the treatment with Methylene blue- 2 mkM; O_2 -the treatment with Methylene blue- 4 mkM

Other picture changes of those indications were watched in *Dunaliella* cells, grown by scavenging air mass into photoreactors under low positive temperature (5°C) in control and in cultivation treated by methylene blue dyes (2mkM) and (4mkM) within 24 hourly cultivation (table 2).

As seen in table 2 the bioproductivity of cell population under the influence of methylene blue dyes (2mkM) decreases till 10-11% and (4mkM) to 20-22% compared to control. It is necessary to note that, under the low temperature stress (5°C) compared to (10°C) has been watched considerably stability of cell population to the dyes, with the indications of bioproductivity. The indications of biosynthesis in chlorophylle authentically haven't changed, the content of carotenoids in concentration (2mkM) considerably increased, but in (4mkM) a little decreased, nevertheless remained in higher level in control cells.

Table 2: The indications of growth, pigment formation, catalase activity, and the content of MDA in *Dunaliella* cells, grown by scavenging air mass into photoreactor at the temperature (5°C) in control and in cultivation of methylene blue dyes of various concentrations.

Growth, *OD*			Catalase activity, mkM H_2O_2 $ml^{-1}min^{-1}$				Quantity of enzymes, mg/l			The content of MDA, mol/g untreated weight
			5	10	15	20	Ca	Cb	Ccar	
C	0,25	0,98±0,02	0,5	0,9	1,3	1,6	3,9±0,05	1,8±0,05	0,6±0,05	$0,5*10^{-3}$±0,05
O_1	0.25	0,88±0,02	1,3	2,4	3,2	3,7	4,3±0,05	1,8±0,05	1,0±0,05	$0,35*10^{-3}$±0,05
O_2	0.25	0,76±0,02	0,7	1,7	2,3	2,8	4,0±0,05	2,0±0,05	0,7±0,05	$0,55*10^{-3}$±0,05

Note: the optic density is OD=0,8; C-control; O_1-the treatment by Methylene blue- 2 mкM; O_2 -the treatment by Methylene blue - 4 mкM

It is also known that in plant cells besides low molecular antioxidants, also present high-active, highmolecular units (catalase, peroxidase and superoxiddismutase), which are able to inhibit the active form of oxygen and free radical processes. By treating the concentration with dyes of methylene blue (2mkM) in experienced cells, the catalase activity increases to 2-2,5 times. Increased concentration of dyes (4mkM) a little suppresses the catalase activity in experienced suspensions, but it increases the catalase activity in control cells. Thus, the treatment of cells with methylene blue, in intensive cultivation in *Dunaliella* cells decreases their bioproductivity, increases the activity of antioxidant system, which effects the carotenoid biosynthesis and catalase activity. The results of investigations of the AFO methylene blue generator influence on the MDA content level in experienced suspension showed that, under the dyes influence (2mkM) MDA level decreases, but under the concentration (4mkM) increases the control cells.

Based on reference data, norflurazon is the inhibitor of photosynthesis and carotenoid synthesis in plant cells. Lack of carotenoids in thylakoid membrane during the plant growth in light leads to chlorophylle protooxidation and to sensitized chlorophyll destruction of many components of plastids [13].

Table 3: The indications of growth, pigmentformation, catalase activity and the content of MDA in *Dunaliella* cells, grown by scavenging air mass into photoreactors at temperature (10ºC) in control and in treatment by herbicide norflurason in various concentrations.

Growth, *OD*			Catalase activity, mkM H_2O_2 $ml^{-1}min^{-1}$				Quantity of enzymes, mg/l			The content of MDA, mol /g untreated weight
			5	10	15	20	Ca	Cb	Ccar	
C	0,25	1,08±0,02	3,0	4,3	5,2	5,7	3,9±0,05	1,9±0,05	0,9±0,05	$0,45*10^{-3}$ ±0,05
O_1	0.25	1,16±0,02	5,8	8,0	9,0	9,6	3,2±0,05	1,6±0,05	0,15±0,05	$0,4*10^{-3}$ ±0,05
O_2	0.25	1,00±0,02	6,0	8,4	9,7	10,7	2,7±0,05	1,5±0,05	0,07±0,05	$0,35*10^{-3}$ ±0,05

Note: the optic density is OD=0,8 C-control; O_1-treatment by Norflurazon ($4*10^{-7}$M); O_2 -treatment by norflurazon ($6*10^{-7}$M)

One of the more important modern tendency to the explanation of slender mechanisms of plant stability to the changes of surrounding medium, particularly concluded in establishing the nature of the interaction between the processes of carotenoid synthesis, and also catalase activity. Due to indications of carotenoid biosynthesis, the MDA level and catalase activity, we can judge on degrees of development of damage processes in *Dunaliella* cells active form of oxygen (AFO) in low temperature stress and in the presence of herbicide norflurazon (HF).

In those conditions the bioproductivity of experienced suspension, under low temperature stress (10ºC)and in treatment of cells by herbicide norflurason in concentration $4 \cdot 10^{-7}$M stimulated the division of experienced cells [12], which outgoes till 6-7% in control suspension, but the concentration $6 \cdot 10^{-7}$M suppressed growth to 7-8% (table 3). The indications of pigment content within 24 hourly cultivation of control and experienced cell suspensions notably differed. As seen in table 3 the treatment of cells with herbicide (HF) in concentration $4 \cdot 10^{-7}$M to 85% and concentration $6 \cdot 10^{-7}$M to 92-93% suppresses the carotenoid synthesis relatively. By that, the biosynthesis of chlorophylle in appropriated concentrations was suppressed to (17-18%) and 27-28%relatively. The estimation of process intensity of peroxidation of lipids (POL), the state of antioxidant systems (catalase activity) have been carried out in herbicide intoxication conditions in *Dunaliella* algae development in intensive-accumulated regime of cultivation. In table have been presented the results of catalase activity increment under herbicide concentrations $4 \cdot 10^{-7}$M to 1,7 times and $6 \cdot 10^{-7}$M to 1,9 times. The intensity indications of peroxidation processes in cell lipids (POL) under the influence on investigating herbicide concentrations decreased. In table 4 have been presented the growth indications, pigmentformation, catalase activity and MDA level in *Dunaliella* cells, grown by scavenging air mass into the photoreactor at temperature (5ºC) in control and in treatment of cells with norflurazon of various concentration.

Table 4: The indications of growth, pigmentformation, catalase activity and the content of MDA in *Dunaliella* cells, grown by scavenging air mass into photoreactors at temperature (5ºC) in control and in treatment by herbicide norflurason in various concentrations.

Growth, *OD*			Catalase activity, mkM H_2O_2 $ml^{-1}min^{-1}$ 1				Quantity of enzymes, mg/l			The content of MDA, mol /g untreated weight
			5	10	15	20	Ca	Cb	Ccar	
C	0,25	0,98±0,03	0,3	0,5	0,7	0,9	3,9±0,05	1,8±0,05	0,75±0,05	$0,45*10^{-3}$ ±0,05
O_1	0.25	1,03±0,03	0,2	0,5	0,9	1,1	3,4±0,05	1,5±0,05	0,6±0,05	$0,4*10^{-3}$ ±0,05
O_2	0.25	0,95±0,03	0,2	0,7	0,9	1,2	2,5±0,05	1,4±0,05	0,04±0,05	$0,35*10^{-3}$ ±0,05

Note: the optic density is OD=0, 8; C-control; O_1- treatment by Norflurazon ($4*10^{-7}$M); O_2 - treatment by Norflurazon ($6*10^{-7}$M)

As seen in table in case of scavenging air mass into photoreactor at temperature (10ºC) the bioproductivity of cells, in treated herbicide concentrations $4 \cdot 10^{-7}$M , leads to the stimulation of growth tempo in culture to (4-5%). The increase of herbicide concentration to $6 \cdot 10^{-7}$M leads to suppression of bioproductivity in algae to 2-3%. It is necessary to note that, the decrease of air mass temperature till (5ºC) leads to some stability in cell population to stressor (HF), compared to the cell population grown by scavenging photoreactor with air mass at temperature (10ºC) (table 3). By that, the general content of chlorophyll and carotenoids decreased. In those conditions antioxidative activity of cells increases, which effects on the increase of catalase activity compared to control cells, also to some decrease of MDA level.

Thus, under intensive cultivation of algae in low temperature stress conditions (10ºC) and (5ºC) decrease the bioproductivity of *Dunaliella* cells to 10-15% and 15-20% relatively compared to cell population in optimal growth conditions (by scavenging air mass into photoreactor at temperature 25ºC). The cell treatment with methylene blue-generator AFO in low temperature stress conditions decreases the cell bioproductivity, but the investigating herbicide concentration of norflurazon in some concentrations $4 \cdot 10^{-7}$M stimulates the growth and bioproductivity in others is suppressed to $6 \cdot 10^{-7}$M .

It is necessary to note that, the decrease at low temperature stress from (10ºC) to (5ºC) leads to the increase of stability in cell population by the influence of herbicide norflurazon. Norflurazon suppresses the synthesis of the general chlorophyll quantity in cells and acutely decreases the carotenoid biosynthesis.

The influence of stressors apart, leads to the increase of intercellular activity in antioxidative systems in *Dunaliella* cells, particularly catalase activity.

The estimation of process of lipid peroxidation (POL) with the MDA content in cells showed that, the influence of generator AFO methylene blue in low temperature stress leads to the MDA accumulation in cells. In herbicide intoxication by norflurazon conditions was noted the decrease of intercellular MDA level.

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