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RESEARCH ARTICLE

Biochemical and antioxidant characteristics of the soil strain *Chlorococcum oleofaciens* (Chlorophyceae, Chlorophyta) grown in light, dark and bicarbonate conditions

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Abstract

This study aimed to examine the biochemical and antioxidant characteristics of a soil strain of *Chlorococcum oleofaciens* CAMU MZ–Ch4 (Chlorophyceae, Chlorophyta) when cultured in light, darkness, and a combination of light-bicarbonate and dark-bicarbonate in an acute 96-hour experiment. The study established that cultivation in the dark is accompanied by an increase in the antioxidant status of *Chlorococcum oleofaciens* CAMU MZ–Ch4 strain cells, which is associated with the accumulation of low-molecular antioxidants and the activation of antioxidant enzymes. Culturing in the dark also causes the accumulation of chlorophyll a, ascorbic acid, astaxanthin, and lipids. But biomass productivity and concentration decreased. Introducing $NaHCO_3$ into the medium inhibits the biosynthesis and accumulation of α-tocopherol, ascorbic acid, phenolic compounds, and chlorophylls both under illuminated and in the dark. The intensity of lipid peroxidation decreases and the activity of antioxidant enzymes increases under light and in the dark conditions with $NaHCO_3$ in the medium.

Keywords
Light, darkening, vitamins, chlorophyll derivatives, bicarbonate, antioxidant enzymes, lipid peroxidation

Introduction

In recent years, microalgae have found increasingly widespread and diverse applications as objects of biotechnology (Posten and Feng Chen 2016). Widespread use is due to several specific qualities, the combination of which is characteristic only of microalgae – it is a high growth rate, biosynthesis, and accumulation of metabolites diverse in structure and biological activity (carotenoids, vitamins, amino acids, lipids; see Rammuni et al. 2019; Maltsev et al. 2019, 2020; Sirohi et al. 2022; Maltseva et al. 2024), high stability, plasticity of metabolism, adaptive mechanisms, and predictability of their direction, which make it possible to finely regulate the metabolic pathways of the cell for targeted synthesis.

Most often, the biotechnological method for obtaining target metabolites from microalgae is stress-induced (Sajjadi et al. 2018; Morales et al. 2021). There is information in the literature and there is an established understanding of what factor of influence provokes the synthesis of a particular product. Many modern studies and natural technologies are built on conditional pairs of 'effect-metabolite'. Stress-induced biosynthesis of valuable metabolites has proven itself quite well, especially with various types of food stress. (Paliwal et al. 2017; Shi et al. 2020; Wang et al. 2023; Maltseva et al. 2024). This is due to the economic effect, since the deprivation of any macronutrient (in the form of salt) from the culture medium reduces the cost of cooking this very medium. Nutritional stress (starvation) has a significant drawback – a decrease in productivity and density of biomass. As a result, the content of the target metabolite decreases in volume terms. In biotechnology, these parameters are among the keys when selecting an object and verifying its stress pattern (Posten and Chen 2016; Mohanta et al. 2024). To solve this problem, it is customary to use two-stage cultivation, the first stage of which involves the accumulation of biomass in a standard medium, the second is the placement of biomass at its peak in stress conditions and further short-term cultivation with the accumulation of the target product (Xia et al. 2013; Nezafatian et al. 2023; Singh et al. 2023). The solution is entirely rational, but the process is phototrophic, so it has a cost factor, it is a light source, which, when scaling the technology to industrial scale, will make energy costs high. To solve this problem, scientists use mixo- or heterotrophic cultivation, which does not require lighting. However, in this case, the medium should contain bioavailable carbon in the form of organic substances (glucose, sodium acetate, and succinates) (Perez-Garcia et al. 2011; Morales-Sanchez et al. 2015; Chen and Jiang 2017; Lopes Da Silva et al. 2019), which often have a higher cost than inorganic carbon sources such as (HCO_3^-). Furthermore, Spalding and Ogren (1982) argued that microalgae are unable to assimilate inorganic carbon in the dark. On the basis

of this information, no one uses two-stage cultivation with alternating phototrophic processes (biomass build-up) and dark stress (obtaining the target product) or uses inorganic carbon sources when cultivating in the dark. However, by analogy with nutritional stresses and the duration of the second stage of cultivation, the loss of biomass productivity in complete darkening will be comparable, even though such an economic factor as the need for lighting will reduce the cost of the target product.

Many scientists widely use inorganic carbon such as HCO_3^- to modify standard cultivation media as a carbon source, to increase biomass productivity and targeted lipid synthesis during long-term cultivation (Pancha et al. 2017; Li et al. 2018; Kim et al. 2019). The heterotrophic cultivation of microalgae in the dark also makes it possible to increase the productivity of biomass and lipids in cells. The factor-metabolite pair is predictable under these conditions (bicarbonate–lipids and darkening+organic carbon–lipids) and does not allow the obtaining of alternative products. Since the system of adaptive restructuring of microalgae is quite subtle, combining these factors can cause a shift in metabolism towards biosynthesis and/or catabolism of other products. Prediction of factor-metabolite pairs is not possible since data from such studies are not available. Namely, the target metabolite is not predictable (darkening+HCO_3^- – ?).

Reactive oxygen species (ROS) and increased lipid peroxidation usually accompany the development of stress (Tripathi et al. 2006; Pereira et al. 2009; Melegari et al. 2012; Kováik et al. 2014; Almeida et al. 2017), which initiates the activation of components of the antioxidant defense system and metabolic rearrangements aimed at increasing the antioxidant resistance of cells. Substances of low molecular weight with antioxidant activity (carotenoids, ascorbic acid, phenolic compounds, alpha-tocopherol, retinol) accumulate more often (Yakoviichuk et al. 2023; Maltseva et al. 2024), or lipids with a low unsaturation coefficient, which are more resistant to radical oxidation. In this regard, the antioxidant system, namely the nature of the interaction and the contribution of low- and macromolecular components to the formation of the antioxidant response, can be a marker of the overall metabolic state and biotechnological potential. The effect of short-term dark stress on the microalgae response of the antioxidant system has not been studied, and the impact of bicarbonate on the antioxidant properties of microalgae under illuminated conditions has only been described only in one study for the *Dunaliella salina* (Dunal) Teodoresco strain V-101 (Srinivasan et al. 2018). Given the unexplored nature of this problem, the poorly understood mechanisms of biological activity of bicarbonate on microalgae in the dark, and the significant economic effect that can be achieved using biotechnology to produce metabolites in the dark, the problem is very relevant. Among other things, Peng et al. (2016) mentioned that bicarbonate has a ROS-binding effect, which positively affects the antioxidant system of the culture.

We selected the species *Chlorococcum oleofaciens* Trainor et H.C. Bold for study as a promising producer of lipids and secondary metabolites (Maltseva et al. 2024). The species is also cosmopolitan and quite resistant to pollution when in culture, which makes maintaining its culture in an algologically pure form a relatively sim-

ple task. On an industrial scale, it is essential. Among other things, some representatives of *Chlorococcum* Meneghini are promising sources of carotenoids, vitamins, and other metabolites, as we described in detail in our previous work (Maltseva et al. 2024).

The aim of the work was to investigate the biochemical and antioxidant characteristics of the soil strain *Chlorococcum oleofaciens* CAMU MZ–Ch4 (Chlorophyceae, Chlorophyta) when grown in light, darkness, and a combination of light–bicarbonate and darkening-bicarbonate in an acute 96-hour experiment.

Materials and methods

Microalgae material

The strain *Chlorococcum oleofaciens* CAMU MZ–Ch4 was isolated from forest litter in the oakwood of the Samara Forest (N 48°39′30.44″, E 35°39′2.17″). The strain was deposited in the Algae Collection of Molecular Systematics of Aquatic Plants at K.A. Timiryazev Institute of Plant Physiology RAS and the Collection of Algae at Melitopol State University CAMU (WDCM1158) as perpetually transferred pure cultures. The strain is stored at 15.0±2.0 °C in vials illuminated by white diodes with a light intensity of 120 lx and a light mode of 16: 8 h (light/dark) in a BBM medium (Yakoviichuk et al. 2023). A culture in the exponential phase was used for the experiments. To do this, 10.0 mL *Chlorococcum oleofaciens* CAMU MZ–Ch4 in 150.0 mL of fresh BBM was inoculated. After 15 days of growth, this culture was used for introduction into experimental media for two-stage cultivation.

Experimental design

As reactors, we used flat bottom flasks with a volume of 250 ml with sealed lids and a system to ensure the consistency of the composition of the gas-air mixture in the flask. The Hailea ACO-308 aquarium compressor (Hailea, China) supplied air to aerate the cell culture with air. The air was passed through a glass tube with an internal diameter of 4 mm at a speed of 0.1 l/min. To prevent bacterial contamination of the culture, we used a bacterial ventilation filter (GSV, Italy) with a diameter of 40 mm; the filter was in the gap between the compressor and the glass tube. This made it possible to maintain the culture in an algologically pure state. To assess the growth and biochemical characteristics of the strain, it was grown in Erlenmeyer flasks with a volume of 250.0 ml and 150.0 mL of BBM at 23.0±2.0 °C. The initial cell concentration was 2.89×105 cells ml-1. Cell concentrations were measured with a C100 Automated Cell Counter (RWD Life Science, China). The light intensity was 5,000 lx (70.0 μmol photons s-1 m-1) with a color temperature of 4,000 K, and the illumination mode was 16:8 (light/dark). The light intensity and color temperature were measured using the Sekonic C-800 spectrometer (Sekonic Corporation, Ja-

pan). Cell cultures were cultured with constant shaking at a frequency of 60 rpm. A two-stage cultivation was used to establish the experiment. Then, for 96 hours, we cultivated the pre-grown biomass according to the experimental scheme. The initial density of the biomass in the experimental groups was 0.25 mg ml. The biochemical parameters were studied during the early logarithmic phase after 96 hours of cultivation in experimental design conditions. The duration of cultivation was 96 hours in accordance with GOST R 57455 – 2017 (Guidelines for the application of criteria for the classification of chemical hazards by their environmental effects, Acute aquatic toxicity).

To study the biochemical parameters of the strain under full shading and exposure to bicarbonate, we formed four experimental groups. A – biomass was cultured in BBM medium under standard illumination; B – biomass was cultured under standard illumination on BBM medium with the addition of $NaHCO_3$ up to a concentration of 6.75 g L^{-1}; C – biomass was cultured on BBM medium at full darkening; D – biomass was cultured under complete darkening on BBM medium with the addition of $NaHCO_3$ concentration of 6.75 g L^{-1}. The bicarbonate concentration corresponded to the medium formulation that is optimal in terms of the ratio of biomass growth and lipid accumulation for *Chlorococcum oleofaciens* CAMU MZ–Ch4 (Pauline and Achary 2019). Group A was the control.

Growth assessment

The growth of microalgae was estimated by measuring dry weight (DW). The measured dry weight is expressed in g L^{-1}.

The biomass productivity (P, g L^{-1} day-1) was estimated using the following equation, where X (g L^{-1}) is the biomass concentration at the end of the cultivation time t and X_0 (g L^{-1}) is the concentration of the biomass at the beginning of the cultivation:

$$P\ (g\ L^{-1}\ day^{-1}) = (X - X_0)\ t^{-1}$$ (Morais and Costa 2007).

Measurement of vitamin C, P (ascorbic acid, phenolic compounds) content

The phenolic compound content of the samples was determined according to the Folin-Chocalteu colorimetric method according to a protocol described by Vazquez et al. (2021). The determination was based on an acidified (chlorophyll-destroying) trichloroacetic acid (TCA) methanol extract of phenolic compounds from microalgae. To achieve this, 30.0 mg of dry microalgae biomass were placed in a sealed vial with a lid and 0.5 mL of 50.0% methanol (LenReaktiv, Russia) was added, containing 1.0% TCA. The vial was blown with argon, sealed with a lid, and extracted at 80.0 °C for 20 min. The vials were cooled and centrifuged at 15 min at 4,000 rpm to precipitate insoluble components. The 0.20 mL aliquot was then placed in a Falcon vial and 100.0 μL of Folin-Chocalteu phenolic reagent (Scharlab, Barcelona, Spain), 1.0 ml of Na_2CO_3 solution (20.0%, w/v) were added. The vials were placed in a dark

place at room temperature for 30 min. The optical density of the solution was then measured at 760.0 nm on the ULab 102 spectrophotometer. The total phenolic compounds content was quantified with a calibration curve obtained using a standard galic acid solution (Rut) (Merck, Germany) in the concentration range of 1.0 to 20.0 mg L^{-1} (R^2 = 0.999). The concentration is expressed in galic acid equivalents mg at 1.0 g of DW (GAE mg/g^{-1}).

The method of Kampfenkel et al. in the Zuffellato-Ribas et al. modification (Zuffellato-Ribas et al. 2010) was used to determine the content of ascorbic acid. For this purpose, 30.0 mg of microalgae biomass had used, which was previously been frozen and defrosting in a centrifugal plastic vial with a lid. 1.0 ml of 10.0% trichloroacetic acid (LenReaktiv, Russia) was added to the biomass and homogenized for 10 min at 4.0 °C. Furthermore, 800.0 μL of 42.0% H_3PO_4, 800.0 μL of 4.0% 2.2'-dipyridyl (Sigma-Aldrich, USA) (in 70.0% ethanol) and 400.0 μL of 3.0% $FeCl_3$ (in H_2O) were introduced into the incubation mixture. The mixture was incubated at 42.0 °C for 45 min and then centrifuged (5 min; 5,000 rpm; 4.0 °C). The optical density of the solution was then measured using a 525.0 nm. A calibration curve was used to quantify the optical density (μg g^{-1} DW). The standard used was a calibration solution made of crystalline ascorbic acid (Supelco, Germany).

Measured vitamin E (α-tocopherol) content

Biomass (10.0 mg) separated from the medium (10 min; 3,000 rpm; 530×g) was alkaline hydrolysis for saponification and conversion of vitamins to alcohol forms. For this purpose, biomass, 5.0 mg of ascorbic acid and 1.0 ml of 10.0% solution of 0.5 M KOH in methanol (Sigma-Aldrich, United States) were introduced into the glass viola. The resulting mixture was then boiling for 30 min at a temperature of 90.0 °C. The resulting hydrolysate was cooled to room temperature, and the hydrophobic fraction was extracted by multistage extraction (4 times by 2.0; 1.0; 1.0; 1.0 ml) using hexane, pre-adding distilled water to the hydrolysate by 1.0 ml. After the hexane and aqueous- methanol phases were settling and separated, the hexane phase was decanted and transferred to a dry vial. The combined hexane extract was washed from KOH with distilled water for neutral reaction on a universal indicator paper. The extract was then dried by freezing water at -18.0 °C. The resulting dry hexane extract was evaporated in a vacuum at 55.0–60.0 °C. The dry residue was dissolved in 100.0 μL methanol (PanReac AppliChem, Spain). We used the resulting extract for the chromatographic separation of *α*-tocopherol.

To determine *α*-tocopherol content high-performance liquid chromatography (HPLC), we used according to the GOST EN 12822-2020 documentation (Determination of vitamin E (*α*-, *β*-, *γ*-, *δ*-tocopherols) by high-performance liquid chromatography, 2022). For the analysis, a Chromatron-1411 HPLC chromatograph (Labtech, Russia) with a spectrophotometric UV detector, a column thermostat, an Inspire C18 column (5 μm 150 x 4.6 mm) and a pre-column Inspire C18 Guard Cartridges 5 μm, 10 x 4.0 mm was used. The volume of the extract used for the determi-

nation was 10 µl. The mobile phase was a mixture in the ratio 97:3 volume:volume, which was methanol–water supplied in isocratic mode to a thermostatically controlled column (30 °C) at a rate of 1.0 ml min^{-1}. The total analysis time was 20 minutes. We used the UV detector at a wavelength of 292 nm for substance detection. The peak of α-tocopherol on the chromatogram sample we identified by the coincidence of the retention time values with the retention time values of α-tocopherol on the chromatogram of the calibration solution. *α*-tocopherol from the Merck catalog T3251 Sigma-Aldrich (Millipore, USA) dissolved in methanol with a final concentration of 1 mg ml^{-1} was used as a standard solution.

Antioxidant enzyme activity measurement

Biomass preparation

The preparation of microalgae biomass for the measurement of antioxidant enzyme activity was carried out according to the protocol described by Yakoviichuk et al. (2023). The homogenate was prepared to determine the activity of antioxidant enzymes. By centrifugation (10 min; 3,000 rpm; 530× g) the biomass was separated from the 0.1 g medium, which was homogenized in 0.9 mL of phosphate buffer (0.1 M; pH 7.5; 4.0 °C). 0.25 ml of 96.0% ethanol and 0.15 mL of chloroform were added to the resulting homogenate and then mixed for 15 min at a temperature of 0.0 to 2.0 ° C to separate lipophilic substances. For further analysis, an upper water-ethanol extract of enzymes (colorless) was used, which was separated from chloroform (lower, green) by centrifugation and decantation. The glutathione peroxidase activity was determined in a homogenate that was not exposed to ethanol and chloroform. The supernatant was produced by centrifuging (10 min; 10,000 rpm; 6,000× g; 4.0 °C) the homogenate in a phosphate buffer with further decanting.

Catalase (CAT) activity determination

CAT activity (EC 1.11.1.6) was determined using the Hamza, Hadwan (2020) method. This method is based on the enzyme's ability to convert H_2O_2 through interaction with hydroquinone/anilinium sulfate/ammonium molybdate reagent into a sustainably colored complex with a maximum absorption of 550.0 nm. Activity was calculated from the residual H_2O_2 content of the final solution.

Glutathione peroxidase (GPx) activity determination

GPx activity (EC 1.11.1.9) has been determined by the Razygraev and Arutiunian method (Razygraev and Arutiunian 2006), which is based on the enzyme's ability to convert glutathione in the peroxide reduction with the further formation of colored conjugates of residual glutathione with Elman reactive (5,5′-Dithiobis-(2-nitrobenzoic acid)), which has maximum absorption at a wavelength of 412.0 nm.

Superoxide dismutase (SOD) activity determination

SOD activity (EC 1.15.1.1) was determined using the Sirota (2012) method. The method is based on the ability of the enzyme to inhibit the reduction of nitroblue tetrasolium dye under the conditions of generation of superoxide anion radical in the autoxidation of adrenaline, in an alkaline medium, initiated by superoxide radicals generated as a result of the reaction of the rearrangement of adrenaline through adrenalinquinone into adrenochrome.

Chlorophyll *a*, *b*, and carotenoids content measured

The determination of chlorophyll content was performed by the Yang et al. (1998) method. The extraction of pigments from microalgae was carried out with 80.0% acetone. For this purpose, 5.0 mg of biomass separated from the medium by centrifugation (10 min; 3,000 rpm; 530× g) was subjected to a triple freezing-defrosting to destroy the cell envelopes. Subsequently, 4.0 mL of acetone was added to create a biomaterial extractant ratio of 1:80 (w:v) and additionally homogenised the biomass by rubbing with quartz sand. The resulting mixture was hermetically sealed and left for 24 hours at a temperature of 25.0 ° C in a dark place for complete extraction of pigments. Furthermore, the acetone extract was separated from the sediment by centrifugation (10 min; 10,000 rpm; 6,000× g; 4.0 °C). The absorption intensity of the pigment extract was analyzed on the spectrophotometer Ulab 102 spectrophotometer (Ulab, China) at wavelengths: 663,4; and 646.6 nm, which corresponds to the absorption maximum for Chl *a* and *b*. The chlorophyll content was expressed in mg g^{-1} of dry biomass, calculations were carried out according to the following formulas:

$$\text{Chl } a = 12.25\ E_{663.6} - 2.55\ E_{646.6} \quad (\mu g\ mL^{-1})$$
$$\text{Chl } b = 20.31\ E_{646.6} - 4.91\ E_{663.6} \quad (\mu g\ mL^{-1})$$
$$\text{Chl } a+b = 17.76\ E_{646.6} + 7.34\ E_{663.6} \quad (\mu g\ mL^{-1})$$

Carotenoids were determined using a method (Yang et al. 1998). The method involves extracting the sum of pigments from the biomass by 80.0% acetone, with further measurement of the maximum absorption of the carotenoid fraction at 440.5 nm and also takes into account the effect of absorption of chlorophylls a and b. The extract was carried out on the Ulab 102 (Ulab, China). Concentrations were expressed in mg g^{-1} dry biomass and calculated using the formula:

$$\text{Car} = 4.69\ E_{440.5} - 0.267\ \text{Chl } a+b \quad (\mu g\ mL^{-1})$$

The pigment extract was obtained by adding 4.0 ml of 80.0% acetone to 5.0 mg of biomass separated from the medium by centrifugation (10 min; 3,000 rpm; 530× g). The biomass was previously subjected to a triple freezing-defrosting and 24-hour

extraction at a temperature of 25.0 ° C in a dark place. The sediment was then separated by centrifugation (10 min; 10,000 rpm; 6,000× g; 4.0 °C).

Astaxanthin content measured

To determine the concentration of astaxanthin, we used the extraction photometric method (Li et al. 2012). For this procedure, 10.0 mg of biomass separated from the medium by centrifugation (10 min; 3,000 rpm; 530× g) was subjected to a triple freezing-defrosting to destroy the cell envelopes. After that, 2.5 mL of dimethylsulfoxide (DMSO) was added. The resulting mixture was hermetically sealed and boiling for 10 min at a temperature of 70.0 ° C for complete extraction of pigment. Furthermore, the DMSO extract was separated from the sediment by centrifugation (10 min; 10,000 rpm; 6,000× g; 4.0 °C). The absorption intensity of the pigment extract was analyzed on the spectrophotometer Ulab 102 spectrophotometer (Ulab, China) at wavelength 530 nm, which corresponds to the maximum absorption of astaxanthin in DMSO. The astaxanthin content was expressed in μg g^{-1} of dry biomass. For the calculation of concentration we used absorption coefficient of astaxanthin in DMSO at 530 nm – A1%1cm = 1556.

Chlorophyll derivatives content measured

For analysis, we selected 5 mg of biomass, which was dried with liquid nitrogen and ground to powder using a pestle and pestle. We extracted the pigments by adding 2.5 ml of 80% acetone and the mixture was centrifuged (10 min; 10,000 rpm; 6,000× g; 4.0 °C). We collected the filler fluid and discarded the sediment. Next, we measured the absorption of the solution at 663.6, 646.6 and 440.5 nm, which corresponds to the absorption maxima of chlorophyll *a*, *b* and carotenoids, respectively. Next, we mixed the above acetone extract with 2.5 ml of hexane, stirred, and left in the dark until phase separation. The upper phase contains fewer polar compounds dissolved in hexane and the lower phase contains more polar compounds dissolved in acetone. The lower fraction was collected separately, and optical density measurements were performed at 440.5, 575, 590, 628, 667 and 650 nm, which corresponds to the maximum absorption of more polar carotenoids (MPCar.), protoporphyrin IX (PPIX), magnesium-protoporphyrin IX (MGPP), protochlorophyllide (Pchlide), chlorophyllide a and b (Chlid *a*, *b*). We evaporated the upper hexane fraction in vacuum and dissolved the dry residue with 80% acetone, destroyed chlorophyll molecules by adding 50 μl of 12.5% HCl solution. Next, we measured the absorption at 470, 665.4 and 653.4 nm, which are the main absorption maxima of less polar carotenoids (LPCar.), pheophytin *a* and *b* (Phe *a*, *b*), respectively. To calculate the concentrations, we used the equations below.

Chlorophyll derivatives:

PPIX = 196.25 E_{575} – 46.6 E_{590} – 58.68 E_{628} (nMole)
MGPP = 61.81 E_{590} – 23.77 E_{575} – 3.55 E_{628} (nMole)
Pchlide = 42.59 E_{628} – 34.22 E_{575} – 7.25 E_{590} (nMole)
Chlide *a* = E_{667}/74.9 (mMole)
Chlide *b* = E_{650}/47.2 (mMole)
MP Car = 4.69 $E_{440.5}$ – 0.267Chl $_{a+b}$ (μg mL^{-1})
LP Car = (1000 E_{470} – 4.28 $E_{665.4}$ – 4.78 $E_{653.4}$)/164 (μg mL^{-1})
Phe *a* = 22.42 $E_{665.4}$ – 6.81 $E_{653.4}$ (μg mL^{-1})
Phe *b* = 40.17 $E_{653.4}$ – 18.58 $E_{665.4}$ (μg mL^{-1})

Measurement of the content of TBA-active products and antioxidant activity coefficient

The content of secondary lipid degradation products (TBA-active products, TBARC) during LPO was determined by Zeb, Ullah (2016). The method is based on the ability of TBA-active products to form a colored complex (maximum absorption of 532.0 nm) with 2-thiobarbituric acid in an acidic medium when heated. The content of the products was determined in the initial homogenate (TBARC) and during LPO induction of LPO by Fe^{2+} ions ($TBARC_i$). The extract of the active products was prepared by homogenizing 50.0 mg of biomass, previously separated from the medium by centrifugation (10 min; 3,000 rpm; 530× g) and decantation of the supernatant liquid. Additionally, 0.45 ml of 1.2% KCl solution was added to the precipitation and the biomass was homogenized with it. The homogenate was then centrifuged (10 min; 10,000 rpm; 6,000× g; 4.0 °C), and a supernatant was used for further research.

The antioxidant activity coefficient (K_{AAC}) was used as a marker of general state of the AOS. The calculation was done by correlating TBARC to $TBARC_i$ (Yakoviichuk et al. 2023):

$$K_{AAC} = TBARC\ TBARC_i^{-1}$$

Measurement of the hydrogen peroxide content of lipids

The method is based on the fact that in dilute aqueous solutions, lipid hydroperoxides (HPx) oxidize Fe^{2+} to Fe^{3+}. Fe^{3+} forms a colored complex with NH_4SCN with an absorption maximum at 480 nm (Gabriël et al. 2000). For this purpose, 5.0 mg of biomass were separated from the medium by centrifugation (10 min; 3,000 rpm; 530× g) and the protein was precipitated by adding 1.0 ml of a 50 % solution of trichloroacetic acid. The resulting sediment was separated from the medium by centrifugation (10 min; 10,000 rpm; 6,000× g; 4.0 °C). 1.0 ml of the supernatant was brought

to 2 ml with 96 % ethanol, 0.2 ml of concentrated hydrochloric acid, and 0.025 ml of a 5 % solution of Mohr salt in 3 % hydrochloric acid were added to equal volumes of 2 ml each, the samples were vigorously shaken. After 30 seconds, we added 1 ml of 20% ammonium thiocyanate solution to the medium. Samples without supernatant were controls. The absorption intensity of the solution was analyzed on the spectrophotometer Ulab 102 spectrophotometer (Ulab, China) at wavelength 480 nm against the control sample. The concentration was expressed in units of optical density (A480) per mg of tissue.

Lipids Content Measurement

The determination of the total lipid content was carried out by modification of the gravimetric method by Maltseva et al. (2024). To achieve this, 50.0 mg of separated biomass was transferred to a pre-weighted sample bottle with a lid. Freeze-defrost and dry in a vacuum at 60.0 ° C until a constant mass is obtained and weighed to determine the mass of the residue. The dry residue was extracted five times with a mixture of hexane-propane-2-ol in a ratio of 3:2 (v/v) in volumes: 2.0; 1.0; 1.0; 0.5; 0.5 ml. Before extraction, 1.0 ml of 0.9% NaCl solution was added to the dry residue for better separation of the lipophilic and hydrophilic phases. To drain the extract, the top hexane layer was transferred by decantation to a 25.0 mL chemical glass, into which the 1.0 mm waterless Na_2SO_4 layer was previously poured. The extract was poured into a preweighted sample bottle, and a glass with precipitation was washed twice with hexane. The extracts were combined and evaporated under vacuum at 60.0 ° C. The dry lipid fraction was weighed and expressed in mg g^{-1} dry mass.

Measurement of protein content

The hydrophilic protein content was used to calculate the activity of enzymes. For this purpose, the Olson protocol (2016), which is based on the bicinchoninic method, was used. To obtain the protein extract of 10.0 mg of biomass separated by centrifugation from BBM medium, 3 times of freezing-defrosting and homogenization in 1.0 mL of ethanol was carried out to remove lipophilic substances. The resulting homogenate was centrifuged (10 min; 10,000 rpm; 6,000× g; 4.0 °C), after which the supernatant liquid was decanted and the residue was resuspended in 1.0 mL of a phosphate buffer (0.1 M; pH 7.5) containing 0.5% sodium dodecyl sulfate by mass. The mixture was left in a hermetically sealed vial for 12.0 hours at 25.0 ° C for complete protein extraction. The homogenate was centrifuged (10 min; 3,000 rpm; 530× g), and a supernatant was used to analyze protein content. The calibration curve was constructed according to the standard solution of bovine serum albumin (Thermo Scientific, United States).

Data analysis

All measurements were performed in three repetitions. The graphs show the average values and errors of the average. Statistical analysis was performed using XLSTAT 2018 software (New York, USA). Statistics obtained in Microsoft Excel ver. 1903 software using single-factor dispersion analysis (ANOVA). The reliability of the differences between the indicators was calculated using the Tukey-Kramer posterior test. Differences at $p < 0.05$ were considered reliable. Calculations and plots were done using Statistica ver. 12.0 software. The relationship between the measured parameters was established by the PCA. The PCA is widely used in cases where algae growth is affected by many physical and chemical factors and its mechanism of action is difficult to describe using traditional statistical approaches. The PCA reduces the size of the data sample, simplifies the model system, and better describes the complex nonlinear system of relationships, as well as provides a dynamic assessment of the system's source variables (De Sá 2007; Gao et al. 2019).

Results

Biomass concentration

The biomass concentration in all groups increased significantly compared to the baseline value after 96 hours of cultivation (Fig. 1). The most significant increases in biomass in groups A (2.04 times) and B (2.72 times). Under aphotic conditions (group C), the concentration increased 1.48 times, in combination with bicarbonate (group D) 1.52 times. Compared to the control, there was an increase only in group B; in other groups, the biomass concentration decreased. In group B, biomass productivity is highest and in aphotic conditions (groups C, D), it is significantly lower relative to group A.

Chlorophyll *a*, *b*, carotenoids and astaxanthin content

The chlorophyll a in conditions of complete darkening conditions (C) is 17.1% higher than in group A (Fig. 2). However, the addition of bicarbonate to the medium under standard lighting (B) and full dark conditions (D) caused a decrease in concentration after 96 hours of exposure by 39.4% and 51% relative to group A. Chl *b* concentration changed similarly ($r=0.990$; $p<0.05$), its concentration decreased significantly relative to the control in groups B (by 2 times) and D (by 2.43 times).

The carotenoids content increased relative to the control only under darkening conditions (group C) by 39.2%. In this case, the addition of bicarbonate to the medium during darkening causes a decrease in the concentration to the control values. The concentration of more polar carotenoids (MP Car; extracted by acetone) did not differ significantly between experiments, while the content of low polar carot-

enoids (LP Car.; the fraction extracted by hexane from acetone extract) increased relative to group A by 58% under darkening conditions (C), in combination with darkening and addition of bicarbonate (group D), the concentration of LP Car decreases to control values. The total concentration of carotenoids varies as a result of low polar derivatives. The astaxanthin content decreased by 40% under standard lighting conditions with bicarbonate in the culture medium (B). Complete darkening without adding bicarbonate causes an increase in astaxanthin concentration by 26.2% compared to the control (A). The pigment content does not change significantly under conditions of complete darkening with bicarbonate in the culture medium. The Car/Chl *a* ratio characterizes the overall metabolic status of the cell. It was highest in group D and lowest in control (A).

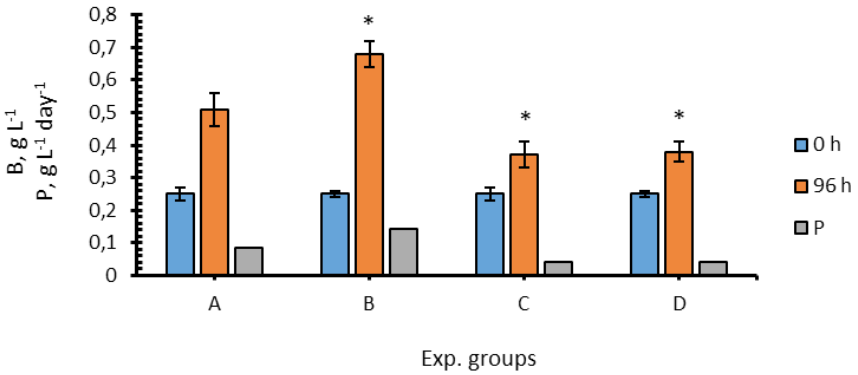

Figure 1. Biomass concentration (Bm) and productivity (P) of *Chlorococcum oleofaciens* CAMU MZ – Ch4 under different cultivation conditions for 96 hours.
Note: * – the difference is significant relative to group A (control) at the level p≤0.05. Here and further in figures 2–7 and table 1 cultivation conditions: A – BBM medium and standard illumination; B – standard illumination in BBM medium with the addition of $NaHCO_3$ (6.75 g L^{-1}); C – BBM medium in full darkening; D – full darkening on BBM medium with the addition of $NaHCO_3$ (6.75 g L^{-1}).

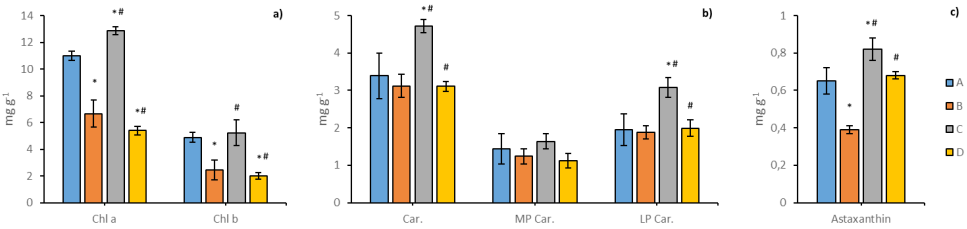

Figure 2. Chlorophyll *a*, *b*, carotenoids and astaxanthin content of *Chlorococcum oleofaciens* CAMU MZ–Ch4 under different cultivation conditions for 96 hours.
Note: Here and in figures 3–7 and table 1 the difference is significant at the level of p <0.05: * – relative to group A (control); # – relative to the previous value.

Chlorophyll derivatives content

The concentration of chlorophyll derivatives varied explicitly depending on the combination of darkening factors and the addition of bicarbonate to the medium. Darkening (C) caused an increase in the concentration of Pchlide alone by 56.5% relative to group A (Fig. 3). Complete darkening in combination with bicarbonate (D) significantly reduces the concentrations of the precursors of PPIX, MGPP and Chlide b precursors by 48, 26.2, and 32.5%, respectively, while the Pchlide content increased 2.44 times under these conditions. Under lighting conditions, with the addition of bicarbonate (B) to the medium, we found a significant decrease in PPIX and chloride b concentrations of 44.2% and 19.6%. The concentration of Pchlide increased by 2.78 times. Under illumination (B) and darkening (D), the content of chlorophyll *a* and *b* degradation products (Phe *a* and Phe *b*) decreased against the background of the addition of bicarbonate to the medium. In the first case, the decrease in the concentration of Phe *a* and Phe *b* was 41.2 and 49.9%, and in the second, 56.3 and 58.9%, respectively. Complete darkening without adding bicarbonate did not cause significant changes (C).

Lipid and proteins content

The lipid content increased under darkening conditions (C) by 56% compared to group A (Fig. 4). Adding bicarbonate to the medium reduced the lipid content under lighting (B) and dark (D) conditions by 35.2% and 47.1%, respectively. Protein concentration did not significantly change with complete darkening. However, the addition of bicarbonate to the culture medium reduced protein concentration by 83.7 and 52.9% in groups B and D, respectively.

Vitamins C, P, E (ascorbic acid, phenolic compounds, *α*-tocopherol) content

During cultivation on BBM under complete darkening, the concentration of ascorbic acid concentration is maximal, at the level of 5.51±0.2 mg g^{-1} ($p<0.05$) (Fig. 5). At the same time, the minimum concentration of ascorbic acid was observed in group (B) under standard lighting with the addition of bicarbonate. The culture of the strain in total darkness (B) and in darkness with bicarbonate (C) provoked significant differences compared to group A, a decrease of 57% and an increase of 18%, respectively.

The content of phenolic compounds decreased significantly in groups B and D by 54% and 19.3%, respectively, compared to the control. In group C, there were no significant differences from group A, but there was a substantial increase in concentration compared with group B (2.4 times). Under dark conditions with the addition of bicarbonate to the medium, the content of phenolic compounds decreased by 25.9% compared to group B. The alpha-tocopherol content decreased when bicarbonate was added to the medium both under dark cultivation conditions and under

standard illumination (Fig. 1a). In particular, the concentration of α-tocopherol in group B was 47.1% lower and in group D, it was 34.4% lower compared to group A. With complete darkening (group C), the concentration of *α*-tocopherol did not change significantly compared to group A. In group D (complete darkening with the addition of bicarbonate to the medium), the concentration was 39.6% lower than in group C.

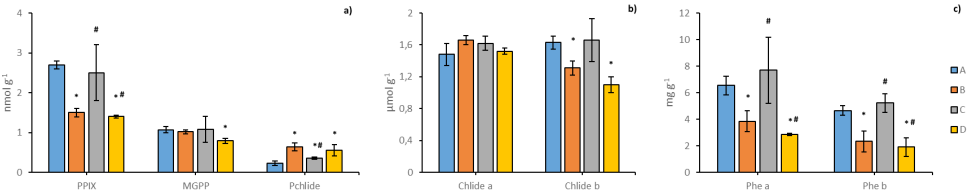

Figure 3. *Chlorococcum oleofaciens* CAMU MZ-Ch4 chlorophyll derivative content under different cultivation conditions for 96 hours.
Note: Protoporphyrin IX (PPIX), magnesium-protoporphyrin IX (MGPP), protochlorophyllide (Pchlide), chlorophyllide *a* and *b* (Chlid *a* and *b*), and pheophytin *a* and *b* (Phe *a*, *b*).

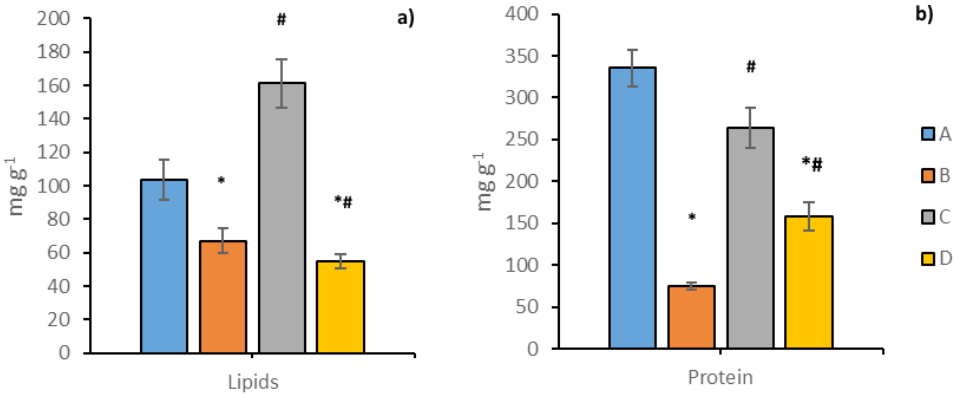

Figure 4. Lipids and protein content of *Chlorococcum oleofaciens* CAMU MZ–Ch4 under different cultivation conditions for 96 hours.

Antioxidant enzyme activity

The change in antioxidant enzyme activity in different groups of the experiment had its distinctive characteristics. SOD activity increased significantly compared to group A, which contains bicarbonate in the medium – 72.2% in group B and by 2.35 times in group D (Fig. 6). CAT activity increased in all experimental groups compared to control (group A). Total darkening with bicarbonate caused the most

significant increase in CAT activity (3.29 times). Standard lighting and bicarbonate (group B) caused a lower effect, while activity increased by 1.87 times. GPx activity also increased in all groups relative to control (A), with a peak in group D. The addition of bicarbonate to the culture medium had the least effect on GPx activity, which caused a 50% increase in activity. It is worth noting that the reaction of CAT and GPx activity to the factors impact of the studied is similar, confirmed by the correlation coefficient ($r=0.943$; $p<0.05$). SOD activity increased significantly in the groups with the addition of bicarbonate, both under illumination and darkening.

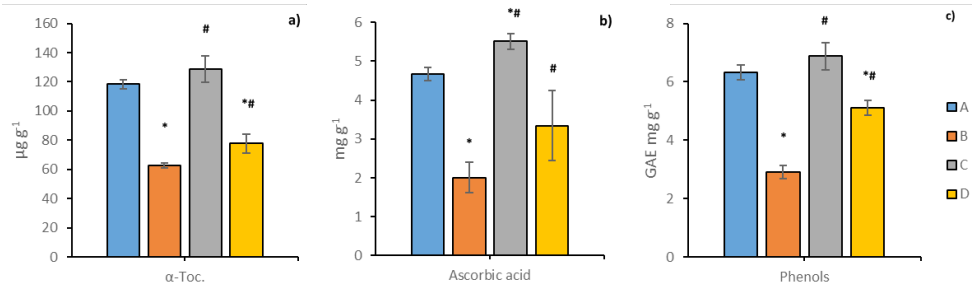

Figure 5. Ascorbic acid, phenolic compounds, and α-tocopherol content of *Chlorococcum oleofaciens* CAMU MZ–Ch4 under different cultivation conditions for 96 hours.

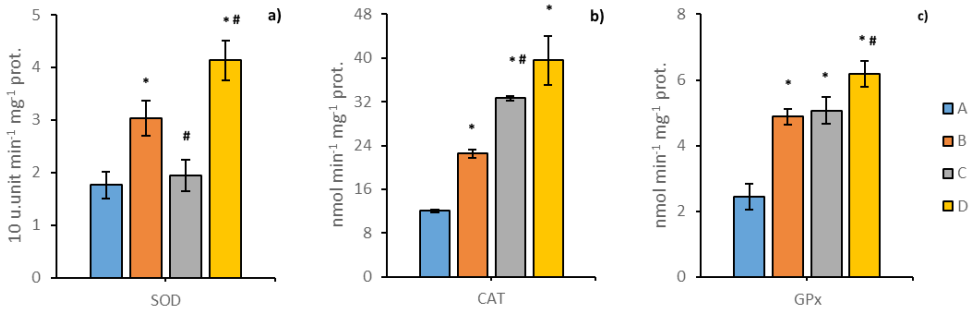

Figure 6. Antioxidant enzyme activity of *Chlorococcum oleofaciens* CAMU MZ–Ch4 under different cultivation conditions for 96 hours.

Hydroperoxide of lipids, content of TBA-active products, and antioxidant activity coefficient

The content of primary products of lipid peroxidation in the form of lipid hydroperoxides decreased significantly in all experimental variants relative to group A, reaching a minimum value under conditions of complete darkening (C) (Fig. 7). The content of TBARC and TBARCi increased significantly at complete darkening (C) by 2.2 and 1.39 times. The addition of bicarbonate to the medium caused a de-

crease in the concentration of secondary decomposition products, regardless of the presence or absence of lighting. In group B, the concentration of TBARC decreased 2.62 times, TBARCi by 2.99 times, and in group D by 2.16 and 1.95 times, respectively. The antioxidant activity increased significantly only under complete darkening (C) by 57.9% and reached a maximum. Kaac did not significantly change in other groups (Fig. 7c).

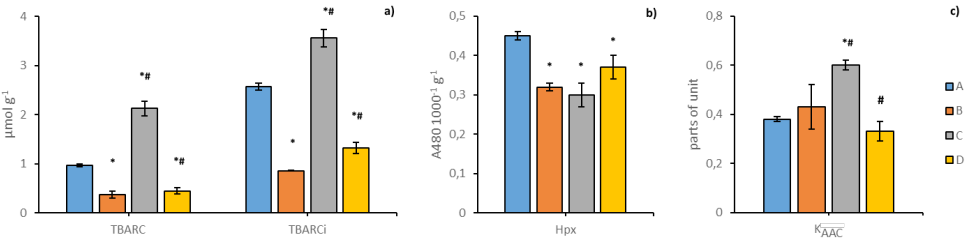

Figure 7. Hydroperoxide lipids, content of TBA active products and antioxidant activity coefficient of *Chlorococcum oleofaciens* CAMU MZ–Ch4 under different cultivation conditions for 96 hours.

Cultivation media parameters

Some parameters of the nutrient medium have changed at the end of cultivation. The pH of the medium in group A did not change significantly after 96 hours of cultivation, while in group C it decreased by 0.7 units (Table 1). In groups B and C, the pH increased by 1 and 0.9 units, respectively, after 96 hours. The dissolved oxygen concentration increased after 96 hours in groups A and D, decreased in group C, and remained unchanged in group B. The dissolved salt content in all groups decreased after 96 hours of cultivation. The bicarbonate concentration decreased after 96 hours by 34.4% and 30.7% in groups B and D, respectively.

Table 1. Medium parameters (M±SE, n = 3)

Parameters	A		B		C		D	
Time, h	0	96	0	96	0	96	0	96
pH	8.4±0.1	8.1±0.1	8.4±0.1	$9.4\pm0.1^{\#*+}$	8.4±0.1$^{\#}$	$7.7\pm0.1^{\#*}$	8.4±0.1$^{\#}$	$9.3\pm0.1^{\#*+}$
O_2 disolv., mg L^{-1}	5.0±0.1	6.0±0.1$^{\#}$	$4.2\pm0.1^{\#*+}$	$4.5\pm0.1^{*+}$	5.1±0.1$^{\#+}$	$4.6\pm0.1^{\#*+}$	$4.0\pm0.1^{*+}$	$5.0\pm0.1^{\#+}$
Salt, g kg^{-1}	0.45± 0.02	0.38± 0.04$^{\#}$	$4.6\pm0.01^{\#+}$	$3.76\pm0.1^{\#*}$	0.43±0.01$^{\#}$	0.37± 0.03*	$4.7\pm0.1^{\#*+}$	3.56± $0.1^{\#*+}$
$NaHCO_3$, g L^{-1}	0	0	6.75± $0.02^{\#*+}$	4.43± $0.1^{\#*+}$	0$^{\#}$	0	6.75± $0.03^{\#*+}$	4.68± $0.1^{\#*+}$

Notes: ANOVA-test. The difference is significant at the level of p≤0.05; * – relative to the control of 0 hours; + –relative to the control of 96 hours; # – relative to the previous value.

PCA-analysis

Analysis of the main components for the entire group of variables showed that the first two components accounted for 79.66% of the total number of changes noted in the PCA (Figs 8, 9).

Variables that reflect both the cultivation conditions and the biochemical and antioxidant characteristics of the strain formed the structure of two main components (PCA 1 and PCA 2). From the point of view of the cultivation conditions, the presence/absence of $NaHCO_3$ in the nutrient medium made the most significant contribution to the structure of PCA 1, and in PCA 2, the presence or absence of lighting during the experiment. PCA1 also reflected strain characteristics such as SOD activity, accumulation of primary and secondary metabolites, and the number of pigments. At the same time, the increase in SOD activity is inversely proportional to the accumulation of primary and secondary metabolites. In general, this indicates the sensitivity of metabolic processes to the presence of $NaHCO_3$ in the nutrient medium.

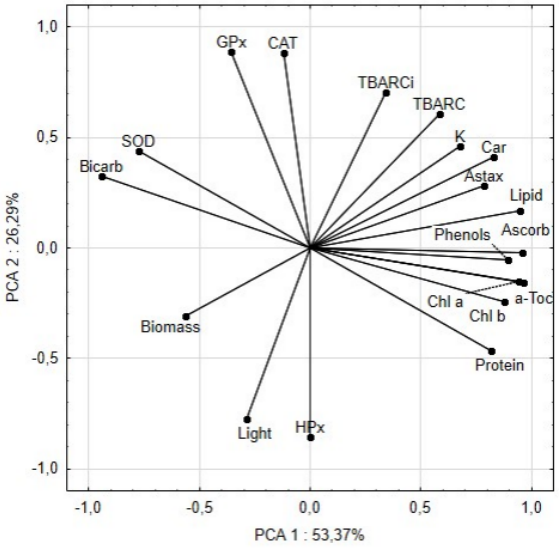

Figure 8. PCA ordination diagram showing the relationship of cultivation conditions, as the well as biochemical and antioxidant characteristics of *Chlorococcum oleofaciens* CAMU MZ–Ch4.

Note: Ch *a* – chlorophyll a content; Ch *b* – chlorophyll *b* content; Car. – carotenoids content; CAT – catalase activity; GPx – glutathione peroxidase activity; SOD – superoxide dismutase activity; TBARC – TBA-active products; TBARCi – TBA-active products after induction of LPO with Fe^{2+}; HC – HCO_3^- in $NaHCO_3$ form; Biomass – biomass concentration; Light – light or dark; Protein – protein content; α-Toc – α-tocopherol content; AscA – ascorbic acid content; Phenols – phenolic compounds content; Lipids – lipids content; Astx – astaxanthin content; Kaac – antioxidant activity coefficient.

PCA2 reflected the influence of lighting conditions on the change in cell metabolic processes. This was especially noticeable at the CAT, GPx activity, and HPx levels. Lighting conditions had a significant impact on the accumulation of TBA-active products. The vectors “Lipid” and “Protein" in the PCA 2 plane had different directions. This indicates that under conditions of variable availability of $NaHCO_3$, the accumulation of lipids or proteins in cells increased, depending on lighting conditions. As evidenced by the PCA graph, which reflected all the observations analyzed (Fig. 2), the presence of $NaHCO_3$ in the nutrient medium both under illumination and in total darkness had a pronounced effect on the biochemical and antioxidant characteristics of the strain.

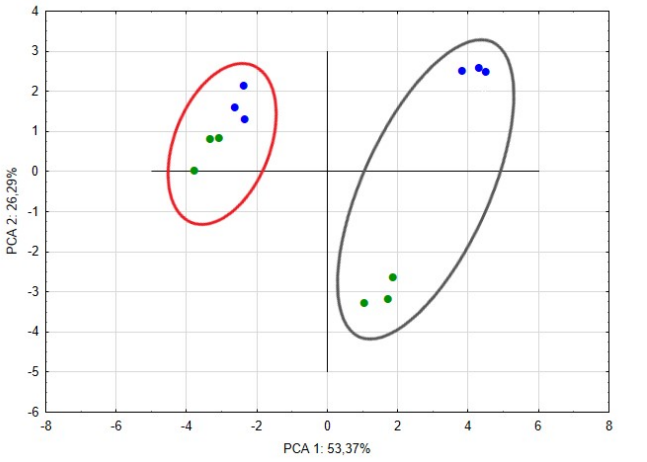

Figure 9. PCA – analysis: distribution of results grouped according to different lighting conditions and presence / absence of $NaHCO_3$ in the nutrient medium. Green color – standard lighting; blue color – full blackout; red color – nutrient medium with $NaHCO_3$; gray color – nutrient medium without $NaHCO_3$.

Discussion

Growth Assessment

The growth rate, productivity, and density of biomass are key parameters for selecting a biotechnological parameter and verifying its stress technology to obtain the target product.

In our case, under aphotic cultivation conditions, the productivity of *Chlorococcum oleofaciens* CAMU MZ–Ch4 biomass decreased relative to the control. However, cell growth and division, as well as an increase in biomass concentration, did not stop completely after 96 hours. We did not find similar information on the nature of microalgae growth in mineral nutrient media under aphotic conditions. However,

Yeh et al. (2010), using the example of *Chlorella vulgaris* Beijerinck ESP–31, found that adding 0.2 g L^{-1} to $NaHCO_3$ at ultralow light intensity (fluorescent lamp, 5 W m^2) provides an increase in biomass at the level of 0.641 mg L^{-1} day^{-1}. In the classical view, dark processes in microalgae cultures are accompanied by a "dark loss of biomass" (Perez-Garcia et al. 2011; Edmundson and Huesemann 2015; Trenkenshu 2016). This phenomenon is closely related to carbohydrate oxidation and dark respiration (Hu et al. 1996; Trenkenshou 2016). The increase in the amount of biomass observed by us for the *Chlorococcum oleofaciens* CAMU MZ–Ch4 culture is due to the use of Calvin cycle products accumulated during illumination, with further assimilation of CO_2 in the C_3 photosynthesis of the dark phase, which is characteristic of microalgae and cyanobacteria. Microalgae use several alternative mechanisms of carbon dioxide absorption – "CO_2 concentration mechanisms", for example, direct "pumping" of inorganic carbon into the cell (Kupriyanova et al. 2023), which can be HCO_3^-. Many authors have well described the presence of these mechanisms for *Chlamydomonas* Ehrenberg species (Jungnickel et al. 2014; Wang et al. 2015; Mackinder 2018). They represent an effective carbon consumption strategy that allows microalgae to survive and multiply when CO_2 concentration limits photosynthesis (Wang et al. 2015). For *Chlorococcum oleofaciens* CAMU MZ–Ch4, the mechanism of direct assimilation of HCO_3 from the cultivation medium under aphotic conditions is probably impossible, as there was no significant differences in biomass concentration between the groups with and without HCO_3 addition. Studies by Spalding and Green (1982) confirm, using the example of *Chlamydomonas reinhardtii* P.A.Dangeard, that the processes of absorption and accumulation of inorganic carbon in the dark are impossible (Spalding and Ogren 1982). In contrast, the rate of absorption of HCO_3^- by the CAMU MZ–Ch4 strain of *Chlorococcum oleofaciens* increased under illumination. A significant increase in the biomass concentration relative to the control group confirmed this and also corresponds to previous evidence of increased HCO_3 absorption under lighting conditions (Kupriyanova et al. 2023).

According to the literature, HCO_3^- positively affects the growth and productivity of microalgae biomass (Yeh et al. 2010; Kim et al. 2019). We found that introducing $NaHCO_3$ into the medium under lighting conditions caused an increase in the biomass concentration for the *Chlorococcum oleofaciens* CAMU MZ–Ch4 strain, reaching a value of 0.68 g L^{-1} after 4 days of cultivation. Kim et al. (2019), in their work for eight strains of green freshwater (*Desmodesmus* sp., *Micractinium inermum* R. Hoshina et Y.Fujiwara (KCTC 12491 BP), *Chlorella sorokiniana* Shihira et R.W. Krauss (KTCT 12171BP), *Chlorella vulgaris*, *Scenedesmus* sp.) and marine (*Microchloropsis gaditana* (L.M.Lubián) M.W.Fawley, I.Jameson et K.P.Fawley, Tetraselmis suecica (Kylin) Butcher (UTEX LB2286), *Tetraselmis chuii* Butcher (UTEX LB0232) microalgae's have established the possibility of the use of $NHCO_3$, which maintained biomass productivity at a high level. However, Peng et al. (2015) for the *Ettlia oleoabundans* strain (S. Chantanachat et Bold) J.Komárek UTEX 1185 and Li et al. (2018) for *Chlorella vulgaris* UTEX 2714 found that the addition of $NaHCO_3$

to the medium caused a dose-dependent decrease in biomass concentration in the logarithmic growth stage.

Chlorophyll *a*, *b*, carotenoids, and astaxanthin content. Chlorophyll derivatives

The photosynthesis system is one of the most sensitive to changes in intensity, frequency of illumination, the effects of various negative factors, and the availability of nutrients. The composition and concentration in pigments of the photosynthetic apparatus (chlorophyll and carotenoids) are a stress indicator in microalgae (Srinivasan et al. 2018). In recent years, algae chlorophyll and carotenoids have gained commercial recognition in the global food and cosmetics market (Da Silva Ferreira and Sant'Anna 2017; Ambati et al. 2019). Due to their biological activity, they are valuable raw materials that humans use in various fields of activity (Posten and Chen 2016; Ambati et al. 2019; Sathasivam et al. 2019; Sirohi et al. 2022). Stress technologies are used to produce carotenoids. Some *Chlorococcum* species are promising sources of carotenoids (Ambati et al. 2019; Sathasivam et al. 2019) on a par with well-known producers such as *Dunaliella salina* (Rammuni et al. 2019; Sirohi et al. 2022).

The culture of *Chlorococcum oleofaciens* CAMU MZ–Ch4 in the dark (group C) caused an increase in Chl *a* relative to cells grown under illumination. The increased concentration of chlorophyll is a classic mechanism of adaptation of microalgae to insufficient lighting (Da Silva Ferreira and Sant'Anna 2016). The concentrations of Chl *a* and Chl *b* obtained in the studied strain when cultured in light and in the dark were higher than the known 5.9 and 4.11 mg g^{-1} for the *Chlorococcum humicola* (Nägeli) Rabenhorst strain TISTR 8461 (Eghbali Babadi et al. 2020). But significantly lower than for *Chlorococcum* sp. MC1, which contains 46.4 mg g^{-1} and 33.7 mg g^{-1} Ch *a* and Chl *b* (León-Vaz et al. 2023) or for *Chlorococcum* sp. (cf. *hypnosporum*) D28Z 56.84 and 22.18 mg g^{-1} (Sassi et al. 2019). Previously, for *Chlorococcum oleofaciens* CAMU MZ–Ch4, we noted that Chl *a* and Chl *b* are in the range of 0.36 – 4.85 and 0.13 – 1.55 mg g^{-1}, and the carotenoid content varies from 0.39 to 2.12 mg g^{-1} depending on the time of cultivation and the availability of nitrogen and phosphorus in the cultivation medium under illumination (Maltseva et al. 2024). In general, the values obtained in the experiment fit the concentration limits for known representatives of *Chlorococcum*, the carotenoid content, which varies in a relatively wide range of 1.57 to 21.02 mg g^{-1} (Sassi et al. 2019; Eghbali Babadi et al. 2020; León-Vaz et al. 2023). The differences with the previous work are due to the stage of culture growth, since in the work of Maltseva et al. (2024), it was on a stationary phase, and in this study, on a logarithmic growth phase. In general, we have established somewhat contradictory data on the effects of bicarbonate and darkening on the pigments content. In experiments with *Chlorella sorokiniana* 211/8k (Salbitani et al. 2020), *Tetraselmis suecica* CCAP 66/38, and *Microchloropsis salina* (D.J.Hibbard) M.W. Fawley, I. Jameson et K.P. Fawley CCAP 849/2 (White et al. 2013), the addition of bicarbonate resulted in an increase in chlorophyll con-

tent. According to the authors, the increase in Chl *a* content in the cell in experimental cultures is due to the greater availability of inorganic carbon in the nutrient medium, which supports pigment synthesis (Salbitani et al. 2020). However, the mechanisms of bicarbonate absorption are not so unambiguous and their effect is dose- and time-dependent. Jayasankar and Valsala (2008) confirmed this using the example of *Chlorella salina* Butcher, in which the concentration of Chl *a* during cultivation in a medium containing 0.005 M $NaHCO_3$ decreases significantly after 4 days of cultivation. However, on day 7, the pigment content is 3 times higher than its concentration in a standard medium. The authors Jayasankar and Valsala (2008) explain this by changing the pH and acclimatizing microalgae to a new enriched medium. Our data are more correlated with the studies by Srinivasan et al. (2018), in which *Dunaliella salina* V–101 showed a decrease in the concentration of Chl *a*, *b* under conditions of addition of $NaHCO_3$ 100 mM with simultaneous deprivation of the medium N, P, and S.

According to Salbitani et al. (2020), the chlorophyll content is a reliable indicator of the physiological state of microalgae. Respectively, the high levels established for cultures under illumination and complete darkening conditions (groups A and C) are a sign of the good condition of *Chlorococcum oleofaciens* CAMU MZ–Ch4 cells. The ratio of chlorophyll degradation products to chlorophyll content for all groups is in the range of 0.53 – 0.60 and 0.95 – 1.0 for the pair Phe *a*/Chl *a* and Phe *b*/Chl *b*, according to Salbitani et al. (2020), this indicates the equivalent content of active chlorophyll in the groups. Since the amount of pheophytin in plant cells usually increases with abiotic stress or with age (Hörtensteiner 2006; Sade et al. 2018; Salbitani et al. 2020), we can conclude that the addition of bicarbonate in the concentrations used did not affect the photosynthetic characteristics of algae cells. Moreover, analysis of the concentration of chlorophyll precursors demonstrates that the decrease in the content in groups B and D is realized by inhibiting biosynthetic reactions. Inhibition at the transformation level of protochlorophyllide (Pchlide), the concentration of which increases relative to the control in the experimental groups. Up to this point, the transformation of the chlorophyll precursors is active, and the reduced concentrations of protoporphyrin (IX) confirm this.

In our study, *Chlorococcum oleofaciens* CAMU MZ–Ch4 showed a significant increase in the total carotenoid content only under aphotic conditions without bicarbonate. Increased concentrations of astaxanthin and LP Car provided an increase in the total concentration of carotenoids. Sources of literature note the accumulation of carotenoids in bicarbonate-based medium for *Dunaliella salina* V–101 (Srinivasan et al. 2018), *Microchloropsis salina* CCAP 849/2, and *Tetraselmis suecica* CCAP 66/38 (White et al. 2013). The absence of significant changes for *Chlorococcum oleofaciens* CAMU MZ–Ch4 may be due to the higher concentration of $NaHCO_3$ that we used in the experiment, since bicarbonate causes dose-dependent changes in growth and biochemical parameters (Peng et al. 2015). Since carotenoids play an important role in neutralizing ROS formed under various types of stress (Ashraf and Harris 2013; Srinivasan et al. 2018), *Chlorococcum oleofaciens* CAMU MZ–Ch4

is under stress only under dark cultivation conditions (group C). At the same time, such a marker of the functional state of microalgae as Car / Chl *a* increased slightly relative to the control in complete darkness. On the contrary, when grown in a medium with $NaHCO_3$, the Car / Chl *a* ratio succeeded in reaching its maximum value under aphotic conditions. According to classical concepts, in stressful situations, the photosynthetic system cannot effectively use the energy of absorbed light, and therefore the mechanism of synthesis of antioxidant carotenoids is activated (Chini Zittelli et al. 2023; Maltseva et al. 2024). Furthermore, when grown in the dark, the astaxanthin content reached a maximum (0.82 mg g^{-1}), when cultivated on a medium with bicarbonate under light conditions (group B), the astaxanthin concentration decreased significantly. The astaxanthin concentration for *Chlorococcum oleofaciens* CAMU MZ -Ch4 was in the range of 0.39 – 0.82 mg g^{-1}. The mass fraction of astaxanthin from the total concentration of carotenoids was 19.2, 12.5, 17.4, and 21.9% for groups A, B, C and D, respectively. The results obtained generally fit into the reference values described for representatives of *Chlorococcum*, the ratio for which is in the range of 3.8 – 23.2%, depending on the presence/absence of a light source, the ecological affiliation of the strain, the duration of cultivation, and the composition of the medium (Zhang et al. 1997; Zhang and Lee 1997; Yuan et al. 2002; Sivathanu and Palaniswamy 2012). Yuan et al. (2002) noted that the astaxanthin content for *Chlorococcum* sp. is 23.2% of total carotenoids when cultured in BBM and on media with an organic carbon source (0.3% glucose), 17.2% in light and 3.8% in the dark (Yuan et al. 2002). The changes in astaxanthin concentration described during cultivation on an organic carbon source are consistent with our observations for bicarbonate under illumination. Astaxanthin exhibits vigorous antioxidant activity, more than one hundred times higher than a-tocopherol (Higuera-Ciapara et al. 2006). Astaxanthin protects the cell from lipid peroxidation of membranes, cells, and tissues (Sathasivam et al. 2019). Consequently, its accumulation by *Chlorococcum oleofaciens* CAMU MZ–Ch4 is a protective mechanism for dark stress during cultivation in standard BBM.

Lipids, protein content

The lipid and protein content may be determined by genetic characteristics, medium parameters, and cultivation conditions (Del Río et al. 2017; Sassi et al. 2019; Maltseva et al. 2024). When grown under aphotic conditions (group C), *Chlorococcum oleofaciens* CAMU MZ–Ch4 accumulated the highest amount of lipids in the biomass, 16.1%. Our previous work noted a lipid concentration of 9.23 – 38.10% for this strain, which depended on the cultivation time, availability and ratio of nitrogen and phosphorus in the medium (Maltseva et al. 2024). The data indicate the lipid content of *Chlorococcum oleofaciens* at the level of 12.0% – 59.18%, which also depends on the cultivation conditions (Adams et al. 2013; Rayati et al. 2020; Maltseva et al. 2024), and for individual representatives of *Chlorococcum* it can even be 5.99% (Sassi et al. 2019). Little information is available about the effect of bicarbo-

nate on the lipid content of representatives of *Chlorococcum*. For example, Pauline and Achary (2019), in their work for a freshwater *Chlorococcum oleofaciens*, found an increase in productivity and lipid concentration to 0.78 g L^{-1} when modifying the culture medium by adding $NaHCO_3$ to a concentration of 6.75 g L^{-1}. Our *Chlorococcum oleofaciens* CAMU MZ–Ch4 results are the opposite, since the lipid concentration during 96-hour culture exposure in a medium with a $NaHCO_3$ content of 6.75 g L^{-1} is 35.2% lower than that of standard BBM. The culture of some species of microalgae on a medium with bicarbonate causes an increase in the lipid content in the biomass. In particular, for the saltwater strain *Chlorella vulgaris* ESP-31 under phototrophic conditions and a $NaHCO_3$ concentration - 1.0 g L^{-1}, the lipid content was 30-40% (Yeh et al. 2010), even though, according to literature data, *Chlorella vulgaris* as a whole contains 14-22% lipids (Spolaore et al. 2006). *Chlorella vulgaris* UTEX 2714 from a culture containing $NaHCO_3$ (160 mM) at pH 9.5, the lipid content in the cells was 49.4%, approximately 20% at pH 8.5, and 15% at pH 7.5 (Li et al. 2018). As follows from the above information, the concentration of lipids in culture depends not only on the content of bicarbonate in the culture medium but also on pH, which in our case is the determining factor, as the pH in group B varied from 8.4 to 9.4 during 96 hours of culture. At high pH values, the solubility of CO_2 decreases, which limits its availability as a carbon source, and HCO_3^- becomes preferable. As a result, the strain will increasingly rely on energy consumed through the 'carbon consumption mechanism" (CCM) to assimilate HCO_3^- as a carbon source, which is bioenergetically disadvantageous compared to CO_2 assimilation of CO_2 by molecular diffusion through the cytoplasmic membrane (Li et al. 2018). As a result, energy-consuming lipid biosynthesis slows down, while catabolism increases. However, in the background of these processes, the productivity of biomass and its concentration in *Chlorococcum oleofaciens* CAMU MZ–Ch4 increases, which may be due to the accumulation of carbohydrates.

Under aphotic conditions without adding $NaHCO_3$ to the medium, *Chlorococcum oleofaciens* CAMU MZ–Ch4 increased the lipid concentration to 16.1%, with the protein concentration at the control group level of 26.4%. At the same time, the growth of the culture did not stop, although biomass productivity was lower. *Chlorella vulgaris* UTEX 259 shows similar performance under heterotrophic conditions (1% glucose), where the lipid content on the sixth day of cultivation is 23% and the protein content reaches 32%. However, biomass productivity increased significantly to 0.151 g L^{-1} day^{-1} (Liang et al. 2009). *Chlorella sorokiniana* AARL G015 also accumulated 11.69% lipids in the dark under heterotrophic conditions (Jareonsin et al. 2023). Adding $NaHCO_3$ to the medium as a carbon source and further cultivation in the dark led to a decrease in the lipid and protein content in *Chlorococcum oleofaciens* CAMU MZ–Ch4, by analogy with cultivation in the light. At the same time, the growth of biomass did not stop. The culture of *Chlorococcum oleofaciens* CAMU MZ–Ch4 in a medium with bicarbonate under illumination resulted in a decrease in protein concentration. The maximum was set in autotrophic culture without bicarbonate (group A) – 33.55%, and the minimum in phototrophic conditions with

the addition of $NaHCO_3$ (group B) – 7.47%. In our previous work, *Chlorococcum oleofaciens* CAMU MZ–Ch4 ranged from 8.78 to 28.36% depending on the age of the culture and the availability and N:P ratio in the medium (Maltseva et al. 2024). We could not find data on the protein content of representatives of the genus *Chlorococcum* when cultivated in media with the addition of bicarbonate under phototrophic and aphotic conditions. However, Yeh et al. (2010), using the example of the saltwater strain *Chlorella vulgaris* ESP–31, presented data on a decrease under protein concentration in phototrophic conditions at a concentration of $NaHCO_3$ -1.0 g L^{-1}, where the protein content was 25-30%, with a well-known value of 51-58% (Spolaore et al. 2006). In general, the protein content for representatives of *Chlorococcum* is in the range of 8.78 – 73.45% (Del Rio et. al. 2017; Sassi et al. 2019; Fatini et al. 2021; Correia et al. 2023; Maltseva et al. 2024).

Vitamin C, P, and E (ascorbic acid, phenolic compounds and α-tocopherol) content

Microalgae have a great potential as a source of vitamins. They can synthesize vitamins in amounts exceeding or comparable to higher plants (Maltseva et al. 2024). Recent studies have shown that some algae contain large amounts of antioxidants, such as vitamin E, pigments, and polyphenols. These compounds (phenols, vitamins, pigments and enzymes) can bind free radicals and can also exhibit antibacterial, antitumor, anticarcinogenic and anti-inflammatory activity in cells. The primary antioxidants in algae are vitamins C, E, and P (phenolic compounds) (Lopez-Hernandez et al. 2020). They provide antioxidant protection to the body by binding free radicals, so their accumulation is usually correlated with an increased antioxidant status of the cell. Similarly, according to these components, highly productive strains are valuable objects of biotechnology.

Chlorococcum oleofaciens CAMU MZ–Ch4 under experimental conditions contained: α-tocopherol, ascorbic acid, and phenolic compounds at a level of 62.6 – 128.6 μg g^{-1}, 2.01 – 5.51 mg g^{-1}, 2.91 – 6.88 GAE mg g^{-1}. The duration of cultivation is one factor determining the concentration of vitamins in biomass. Mudimu et al. (2017) and Durmaz (2007) proved that in the stationary growth phase, cultures contain more alpha-tocopherol than in the logarithmic phase. According to our data for *Chlorococcum oleofaciens* CAMU MZ–Ch4, the introduction of $NaHCO_3$ into the nutrient medium as an additional carbon source had an inhibitory effect on the biosynthesis and accumulation of all vitamins under light conditions, in the dark – only alpha-tocopherol and phenolic compounds. When completely darkened without the addition of $NaHCO_3$, ascorbic acid accumulated. Many scientists have established for microalgae that the concentration of vitamins, using alpha-tocopherol as an example, depends on the availability of nutrients in the cultivation medium (Mudimu et al. 2017; Canelli et al. 2022; Maltseva et al. 2024). Generally, the concentration of α-tocopherol in microalgae ranges from 0.01 to 72.1 mg g^{-1} DW (Del Mondo et al. 2020; Maltseva et al. 2024). The marine strain *Chaetocer-*

os calcitrans (Paulsen) H. Takano has the highest concentration of *α*-tocopherol to date (72.1±1.2 mg g-1) (Goh et al. 2009). Among Chlorophyta representatives, the concentration of α-tocopherol varies from 0.1 to 15 mg g^{-1} DW. *Chlorococcum oleofaciens* CAMU MZ–Ch4, depending on cultivation conditions, had a comparable amount of *α*-tocopherol to other *Chlorophyceae* strains during the logarithmic growth phase. Culture of *Chlorococcum oleofaciens* CAMU MZ–Ch4 in a medium with bicarbonate contained, *α*-tocopherol at a concentration similar to that of the strains *Desmodesmus armatus* (Chodat) EH Hegewald SAG 276-4e (68.02±6.72 μg g^{-1}) and *Scenedesmus obtusus* Meyen SAG 52.80 (74.36 μg g^{-1}). Under standard lighting and total blackout conditions without bicarbonate, the concentration was close to Rostafinski *Haematococcus lacustris* (Girod-Chantrans) (151.18 ± 13.39 μg g^{-1}) (Mudimu et al. 2017).

Ascorbic acid performs several protective and physiological functions in the cell. In particular, it is a cofactor of enzymes, participates in photosynthesis, hormone biosynthesis, and antioxidant regeneration, and plays an essential role in photoprotection and xanthophyll biosynthesis. (Del Mondo et al. 2020). The concentration of ascorbic acid in microalgae varies in the range of 0.06 – 18.79 mg g^{-1} DW (Del Mondo et al. 2020; Maltseva et al. 2024). The content of this metabolite in *Chlorococcum oleofaciens* CAMU MZ–Ch4 under experimental conditions is comparable to other representatives of *Chlorophyta* such as *Chlamydomonas reinhardtii* (2 mg g^{-1}) (Aaronson et al. 1977), *Dunaliella tertiolecta* Butcher, (2.2 mg g^{-1}) (Abalde et al. 1991) and *Picochlorum atomus* (Butcher) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley et K. Fawley CS-183 (5 mg g^{-1}) are in a logarithmic growth stage (Brown et al. 1992). Phenolic compounds are involved in various physiological processes of algae, including antioxidant protection (Kosanić et al. 2015; Maltseva et al. 2024). The concentration of phenolic compounds varies widely, from 0.2 GAE mg g^{-1} to 39.9 GAE mg g^{-1} and 58.15 GAE mg g^{-1} (Maltseva et al. 2024) for *Chlorococcum* sp. C53 strain, the phenol content is 7.5 and 23 GAE mg g^{-1} when extracted with 95% ethanol and water, respectively. When studying *Chlorococcum oleofaciens* CAMU MZ–Ch4, we used a 50% aqueous methanol solution to extract phenolic compounds. In general, the content of phenolic compounds is comparable to the data described by Andriopoulos et al. (2022) for *Mychonastes homosphaera* (Skuja) Kalina et Puncochárová, *Isochrysis galbana* Parke, *Nannochloropsis oculata* (Droop) D.J. Hibberd and *Tisochrysis lutea* El M. Bendif et I. Probert. In general, the content of water and fat-soluble vitamins in *Chlorococcum oleofaciens* CAMU MZ–Ch4 reacts to light and dark conditions and introducing bicarbonate into the medium introduces bicarbonate into the medium, reducing the concentration regardless of lighting. This indirectly confirms the ability of the cells of these microalgae to absorb inorganic carbon in both the light and in the dark. The CCM energy-dependent bicarbonate transport system or physicochemical adsorption processes are probably involved in this process.

Antioxidant enzyme activity, hydroperoxide of lipids, content of TBA-active products and antioxidant activity coefficient

The antioxidant activity (Kaac) is an integral indicator of the state of AOS, including the enzymatic component and the content of low molecular weight antioxidants. It reflects the general ability of cells to neutralize reactive oxygen species and peroxides. Despite the many different indicators used to assess the antioxidant status of microalgae (Safafar et al. 2015; Smerilli et al. 2017; Santiago-Morales et al. 2018; Santhakumaran et al. 2020; Yakoviichuk et al. 2023; Maltseva et al. 2024). This work is the first to evaluate the effect of light and darkening on this coefficient. The analysis of the results indicates the highest antioxidant status for *Chlorococcum oleofaciens* CAMU MZ–Ch4 cells grown under aphotic conditions (group C), which is consistent with the accumulation of low molecular weight antioxidants (carotenoids, astaxanthin, alpha-tocopherol, ascorbic acid) and the activation of AOS enzymes (CAT, GPx). Adding $NaHCO_3$ to the medium with additional cultivation both in light and dark did not cause significant changes in Kaac relative to the control group (A).

The content of secondary and primary lipid degradation products is also a characteristic of AOS. The main factor in the accumulation of secondary lipid degradation products is the overproduction of ROS, which cannot inactivate components of AOS (Srinivasan et al. 2018). TBARC and $TBARC_i$ are products of lipid peroxidation (LPO), particularly the breakdown of unsaturated C18 fatty acids (He and Ding 2020; Coniglio et al. 2023). TBARC is more often the concentration of malondialdehyde (MDA), which researchers commonly used to indicate stress conditions in microalgae (Srinivasan et al., 2018). In *Chlorococcum oleofaciens* CAMU MZ–Ch4, the addition of bicarbonate reduced the concentration of TBARC and $TBARC_i$ in both light and dark conditions, indicating a decrease in the intensity of LPO. These data are consistent with the results of Srinivasan et al. (2018) obtained for *Dunaliella salina* V–101, which demonstrated that adding bicarbonate can reduce the concentration of MDA during starvation of N, P and S – starvation. Peng et al. (2016) established a positive effect of bicarbonate using the example of the freshwater strain *Ettlia oleoabundans* UTEX 1185, for which bicarbonate reduced ROS content in cultures under oxygen stress (400% dO_2), this is associated with activation of CAT and ascorbate peroxidase. *Chlorococcum oleofaciens* CAMU MZ–Ch4 also shows an increased activity of antioxidant enzymes (CAT, SOD, and GPx) when cultured in $NaHCO_3$. This is consistent with the data for *Dunaliella salina* V101, in which the activities of CAT, SOD, and ascorbate peroxidase increase when cultured in $NaHCO_3$-containing medium under nutritional stress (Srinivasan et al. 2018). In *Pachycladella chodatii* (Bern.) Hegewald (SAG 2087), the SOD activity changed dose-dependently. SOD activity increased significantly only at sodium bicarbonate concentrations of 45 mg L^{-1} and 75 mg L^{-1}, the former value causing more pronounced activation and 15 mg L^{-1} did not significantly increasing, while $NaHCO_3$ did not have an effect on CAT activity (Fawzy et al. 2017). An increase

in GPx activity is specific for *Chlorococcum oleofaciens* CAMU MZ–Ch4, which is consistent with a decrease in the content of primary LPO products (HPx) under conditions of $NaHCO_3$ application conditions during cultivation in light and in darkness. The data obtained on the effects of $NaHCO_3$ on *Chlorococcum oleofaciens* CAMU MZ –Ch4 are generally consistent with the results described in the literature. However, the lack and inconsistency of data on the state of the antioxidant system under dark stress and exposure to bicarbonate do not allow us to draw unambiguous conclusions about the mechanisms of its action on the biochemical processes of cells under dark conditions, which requires further study.

Conclusions

Productivity and biomass concentration decreased for *Chlorococcum oleofaciens* CAMU MZ–Ch4 after 96 hours of cultivation in the dark. The addition of bicarbonate causes an increase in the concentration and productivity of the CAMU MZ-Ch4 biomass of *Chlorococcum oleofaciens* under illumination, under aphotic conditions, even with the addition of $NaHCO_3$, cell division and growth slow significantly. Cultivation in the dark increases the concentration of Chl *a* after 96 hours. The introduction of $NaHCO_3$ into the medium and cultivation with and without illumination reduces the concentration of chlorophyll, its decay products and precursors, except protochlorophyllide. The content of carotenoids and astaxanthin separately increased only when the strain in the dark. The illumination of the medium with the addition of $NaHCO_3$ causes a decrease in astaxanthin concentration. Under aphotic conditions, the lipid content in the biomass of the *Chlorococcum oleofaciens* strain CAMU MZ–Ch4 was the highest. The addition of bicarbonate reduced the lipid concentration. The protein concentration decreased when cultured in bicarbonate medium both in light and in the dark. Ascorbic acid accumulated in the cells in complete darkness. Introducing $NaHCO_3$ into the medium inhibits the biosynthesis and accumulation of α-tocopherol, ascorbic acid, phenolic compounds under illumination and α-tocopherol and phenolic compounds in the dark. Cells cultured under aphotic conditions had the highest antioxidant activity, which is consistent with the accumulation of low-molecular-weight antioxidants and activation of antioxidant enzymes. The addition of sodium bicarbonate reduces the intensity of lipid peroxidation, as indicated by a decrease in the concentration of TBARC, TBARCi, and HPx. Cultivation in the dark increases CAT and GPx activity. The addition of $NaHCO_3$ causes an increase in the activity of antioxidant defense enzymes (CAT, SOD and GPx) under illumination and in the dark. In general, the pigment composition of the photosynthetic apparatus, the concentration of primary and secondary metabolites, and the antioxidant system of *Chlorococcum oleofaciens* CAMU MZ–Ch4 react to the addition of bicarbonate to the nutrient medium in a manner different from the reactions described reactions to nutrient stress. This indirectly confirms the ability to absorb this form of carbon even during dark cultivation.

Hydrocarbonate also does not affect the antioxidant activity coefficient during 96 hours of cultivation. However, a decrease in the concentration of lipid peroxidation products is observed, and the activity of antioxidant enzymes increases, which, with a more prolonged effect, will likely lead to a shift in the antioxidant status. The mechanisms of these changes require more detailed study, in particular, the accumulation of biomass in the dark and the initiation of catabolism of primary and secondary metabolites, inhibition of chlorophyll biosynthesis, and activation of the macromolecular branch of the antioxidant defense system in the dark when exposed to bicarbonate.

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