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RESEARCH PAPERS

pH and CO_2 Effects on *Coelastrella* (*Scotiellopsis*) *rubescens* Growth and Metabolism[1]

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Abstract—We studied effects of pH and CO_2 enrichment on the physiological condition and biochemical composition of a carotenogenic microalga *Coelastrella* (*Scotiellopsis* Vinatzer) *rubescens* Kaufnerová et Eliás (Scenedesmaceae, *Sphaeropleales*, Chlorophyceae), a promising source of natural astaxanthin. The microalga was grown at a constant pH (5, 6, 7 or 8) maintained by direct CO_2 injection. The air-sparged culture served as the control. Cell division rate and size, dry biomass productivity, the rates of nitrogen and phosphorus uptake as well as photosynthetic pigment and total lipid content and fatty acid composition were followed. *C. rubescens* possessed a narrow-range pH tolerance (the optimum pH 6–7). Under these conditions, the highest values of the maximum (1.0–1.1 1/day) and average (0.3–0.35 1/day) specific growth rate, chlorophyll *a* (4.8–4.9%) and total carotenoid dry weight percentages (1.7–1.8%) were recorded. Cell lipid fatty acid unsaturation index (1.851) and polyunsaturated fatty acid percentage (36–39%) and C18:3 ω3/C18:1 ω9 ratio (3.8–4.5) were also the highest under these conditions. A decline of pH to 5 brought about severe stress manifesting itself as a cell division cessation, photosynthetic apparatus reduction, two-fold increase in cell volume, accumulation of dry weight and lipids and a considerable decline in fatty acid unsaturation. Cultivation of *C. rubescens* without CO_2 enrichment resulted in a rapid alkalization of the medium to pH 9.5–10.5 impairing the physiological condition of the cells. Reasons of the deteriorative effects of suboptimal pH values on the physiological condition of *C. rubescens* are discussed.

Keywords: Coelastrella (Scotiellopsis) rubescens, CO_2, cultivation, fatty acids, growth rate, pH, pigments
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INTRODUCTION

Certain species of green microalgae (mainly dwelling in the ephemeral freshwater ponds and various terrestrial habitats) respond to abiotic stresses by massive accumulation of secondary carotenoids which are localized in the cytoplasm being structurally and functionally uncoupled from the photosynthetic apparatus of the cell. This phenomenon known as secondary carotenogenesis, draws considerable attention of researchers as an important mechanisms of stress tolerance in microalgae frequently encountering an abrupt onset of harsh environmental conditions (e.g. drying, an acute nutrient shortage, high solar irradiances, and extreme temperatures) [1–3]. The interest to the growth and metabolism of these microalgae was also fueled by the search for new commercial sources of the value-added ketocarotenoid astaxanthin (Ast), a powerful antioxidant with clinically proven anti-tumor, anti-inflammatory, cardio- and neuroprotective effects [2, 4]. The microalgal producers of Ast are commonly cultivated using a two-stage process developed using the planktonic microalga *Haematococcus pluvialis* Flotow 1844 (Volvocales) as a model. The stage I or "green" stage presumes cultivation under conditions conductive for rapid growth of vegetative cells. At the stage II (the "red" stage) the culture obtained from the stage I is subjected to stressors inducing secondary carotenogenesis accompanied by the transition of the cells to their resting form [5, 6]. The environmental condition effects on the growth and biosynthesis of Ast in *H. pluvialis* as well as stress tolerance of the microalga were extensively studied [2, 5, 6]. At the same, our knowledge of the large group of coccoid aerophilic and soil-dwelling microalgae highly capable of secondary carotenoid accumulation is very fragmented and limited to several representatives of few large genera from Trebouxiophy-

[1] The article was translated by the authors.

Abbreviations: Ast—astaxanthin; Car—carotenoids; Chl *a,* Chl *b*—chlorophylls *a* and *b*; FA—fatty acids.

ceae (Chlorella) and Chlorophyceae (e.g. *Scenedesmus*, *Chlorococcum* etc.) [7, 8].

Recently, a soil chlorophyte *Coelastrella* (*Scotiellopsis*) *rubescens* attracted our attention as a potential source of Ast. This species was shown to be capable of accumulation of biotechnologically significant quantities of Ast (approx. 1 mg/(L day)) under conditions of the two-stage cultivation scheme, it was also tolerant to high temperatures (39–44°C) and irradiances (50–60 klx) and grew well in media containing urea, the cheapest source of nitrogen [9–11]. However, many aspects of its physiology that are important for the development of its industrial cultivation, remain unclear. In particular, the literature lacks the reports on the effects of the important interlinked variables such as pH and CO_2 concentration in the gas-air mixture on *C. rubescens*.

It is commonly accepted that inorganic carbon shortage in the cultivation medium is a major factor limiting photosynthesis and growth of intensively cultivated microalgae. However, it is not always possible to relieve this limitation by enrichment of the cultures with CO_2 gas due to low species-specific tolerance of microalgae to a drop in pH arising when CO_2 is dissolved in the water. The resulting acidic pH affects adversely the structural integrity and functional condition of the membranes and the activity of key enzymes, such as Rubisco [12–15]. A practical way for relieving the inorganic carbon deficiency under conditions of intensive culture is a controlled injection of CO_2 with maintaining of pH in the optimal range [15]. To the best of our knowledge, research on the applicability of this approach to *C. rubescens* is so far lacking. This work aims at filling this gap. Our main goal was to identify the morphometric, physiological, and biochemical indicators of the condition of *C. rubescens* cultivated in pH-stat mode at different pH levels.

MATERIALS AND METHODS

The strain. A strain of *Coelastrella* (Vinatzer) *Scotiellopsis rubescens* Kaufnerová et Eliás (Scenedesmaceae, Sphaeropleales, Chlorophyceae) obtained from the collection of microalgae of the Timiryazev Institute of Plant Physiology, Russian Academy of Sciences in 2006 as *Scotiellopsis rubescens* Vinatzer strain IPPAS H-350 (Vinatzer/Innsbruck V 195 = CCALA 475) [16]. According to the results of electron microscopy and 18S rRNA and ITS2 nucleotide sequence comparison with those of authentic strain Vinatzer/Innsbruck V 195 this species was identified as *Coelastrella rubescens* [17].

Cultivation conditions. The microalga was batch-cultivated in 1 L Erlenmeyer flasks in a modified BBM medium ($NaNO_3$—1.31 g/L, KH_2PO_4—0.99 g/L, K_2HPO_4—0.042 g/L, pH 6.7) [11] at 25 ± 1°C and a unilateral side illumination with daylight fluorescent lamps Feron DL 20W 6400K (Russia), 136 μmol PAR photons/(m^2 s), 15 h light : 9 h dark. Five different conditions were tested, including the control, differing by pH of the medium and CO_2 availability. During the light period the pH was maintained at a constant level (pH 5, 6, 7, 8) with an accuracy of 0.05–0.1 units by means of direct supply of CO_2 from the cylinder controlled by solenoid valves Camozzi A7E (Italy) and a digital pH controller Aqua Medic pH 2001C (Germany). The culture was continuously sparged with air at a rate of 1 L/min using a compressor Resun ACO-9630. A culture started at a pH 7.0 and sparged with atmospheric air only served as the control. The initial cell density and volume of the cultures was (0.8–1.1) · 10^6 cells/mL and 0.45 L, respectively.

Monitoring of the culture growth and cell size. The number of cells (N) and their volumes (V) were analyzed using a flow cytometer FC 500 CytomicsTM λ 488 (Beckman Coulter, United States) [18]. N was evaluated on the log-scaled two-parameter cytograms of forward scattering (FS). Equivalent spherical diameter of cells was determined via a calibration curve constructed using Flow-Check (Beckman Coulter) calibration microspheres.

Average yield (P_{AVG}) and specific growth rate (μ_{AVG}) were calculated for the period of 0–10 days on the cell number and dry weight basis [19] as:

$$P_{AVG} = (N_1 - N_0)/(t_1 - t_0);$$
$$P_1 = (DW_1 - DW_0)/(t_1 - t_0);$$

$$\mu_{AVG} = \ln (N_1/N_0)/(t_1 - t_0);$$
$$\mu_1 = \ln (DW_1/DW_0)/(t_1 - t_0),$$

where P_{AVG} is the average productivity on cell number basis, cell/(mL day); N_0—the initial cell density, cell/mL; N_1—cell density after 10 day of cultivation, cell/mL; t_1–t_0—the duration of cultivation, days; P_1—the average productivity on dry wt basis, mg/(L day); DW_0—initial dry wt, mg/L; DW_1—the final dry wt, mg/L; μ_{AVG}—mean specific growth rate on cell number basis, 1/day; μ_1—mean specific growth rate on dry wt basis, 1/day. Dry weight was determined gravimetrically on nitrocellulose membrane filters "Sartorius" with a pore size of 3 μm [20].

Pigment assay. Pigments were extracted with dimethyl sulfoxide [21] or acetone (Merck, HPLC grade) using the same extraction procedure. Content of Chl *a* and *b* and total carotenoids (Car) in the extracts was determined with a spectrophotometer Agilent Cary 300 (Agilent, United States) [21, 22]. The Car profile was resolved by HPLC as described previously [23].

Total cell lipid quantification. Lipids were extracted using the extraction by Folch. Total cell lipid content was determined by a colorimetric method using the phosphor-vanillin reagent via a calibration curve constructed with olive oil (Extra Virgin ABEA, Greece) purified on Al_2O_3 [24]. Fatty acid (FA) composition of the cell lipids was determined by GC-MS of the FA methyl esters obtained by direct transesterification of cells [23].

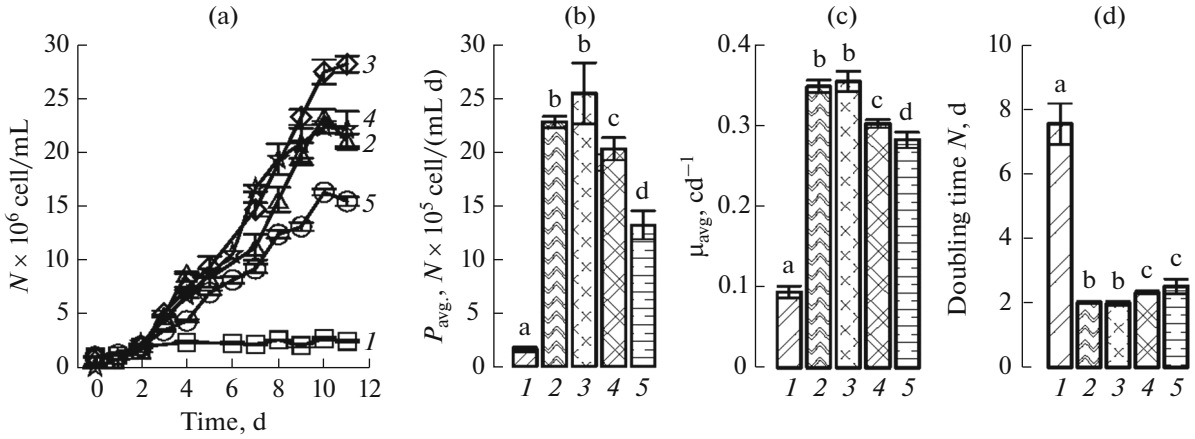

Fig. 1. Growth curves (a) and the rate of growth of cultures of *C. rubescens*: average productivity by the number of cells (b); average specific growth rate (c) and the time of doubling the number of cells (d) depending on pH and availability of CO_2. *1*—pH 5; *2*—pH 6; *3*—7 pH; *4*—pH 8; *5*—control. Panel a indicates the standard error of the mean.

Assay of nutrients in the medium. The concentration of NO_3^- in the medium was monitored using a nitrate-selective electrode 9307BNWP (Thermo Orion, United States), concentration of PO_4^{3-} and NO_2^-—by Murphy-Riley or Bergschneider and Robinson colorimetric methods, respectively [25]. All experiments were carried out in three biological replications with three analytical replications for each. Mean values and their standard deviations are shown in the figures, unless stated otherwise.

RESULTS

The Growth of Cultures at Different Levels of pH and CO_2

Enrichment of the medium with CO_2 significantly accelerated the growth of *C. rubescens* cultures maintained at a pH of 6–8, as compared with the control. The highest average cell density-based growth rate was observed in the cultures grown at a slightly acidic or neutral pH ($P_{AVG} = 22.5 \times 10^5 \pm 0.05 \times 10^5$ and $25.2 \times 10^5 \pm 0.28 \times 10^5$ cells/(mL day), respectively) (Fig. 1).

Injection of CO_2 into the culture to attain pH 5 reduced the average cell density-based productivity 8.3-fold and 12.8–16.0 times as compared with the control and the cultures maintained at pH 6–8, respectively. The doubling time in this case was 7.5 days whereas in the cultures grown at pH 6–7, pH 8 and in the control it was 2.0, 2.3, and 2.5 days, respectively.

The control cultures featured a significant alkalization of the medium typical for green algae batch-cultivated in nitrate-containing media without CO_2 enrichment. After 28 h of cultivation, pH of the medium increased from 7.0 to 9.4, until the end of the experiment it ranged within 9.5–10.5 units. It exhibited a characteristic diurnal change pattern: during the light period pH increased to the maximum of 10.3–10.5 by 2:00–3:00 p.m. Later, pH gradually decreased by 0.1–0.3 till the end of the light period (8:00 p.m.) and continued to decline in the dark (by 0.5–1.0 from the value recorded at the light switch-off). This diurnal pH change pattern was obviously related with the increase in CO_2 concentration in the medium due to the cell respiration. The increase in CO_2 in the second half of the light period might stem from the increased photorespiration due to accumulation of the photogenerated oxygen [26] whereas the rise of CO_2 in the dark was likely due to respiration.

Normally, pH of the control culture medium attained 9.5–10.5 units on the background of a significant decline in the productivity and specific growth rate (in 1.9 and 1.3 times, respectively as compared to the "pH 7" cultures).

Cultivation Condition Effects on the Morphological Parameters of the Cells

A characteristic response to acidification of the medium to pH 5 was comprised by a significant increase in cell size (1.7 times by the end of the cultivation period as compared to the initial cell size), a similar effect (1.8–2.9-fold increase) was revealed in the cell volume ratio in other experimental variants (Fig. 2). By contrast, in the vigorously dividing cultures maintained at pH 6–7 average cell volume decreased 1.3–1.7 times in comparison with the inoculum; the cells of the "pH 7" culture were the smallest (84.84 ± 5.99 μm^3). In the culture with alkaline media ("pH 8" and the control) the average cell volume did not change during the cultivation period but exceeded significantly the cell volumes of the more rapidly dividing culture "pH 7".

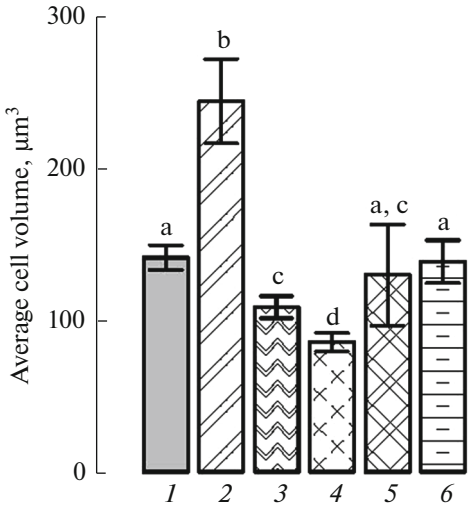

Fig. 2. The average volume Key of cells of *C. rubescens* in the inoculum was (*1*) and on the 11th day of cultivation (*2–6*) depending on pH and availability of CO_2. *2*—pH 5; *3*—pH 6; *4*—pH 7; *5*—pH 8; *6*—control.

Relationships Between C. rubescens Pigment Composition, pH and CO_2 Enrichment

The inhibition of *C. rubescens* growth in the acidic (pH 5) medium was accompanied by a significant decrease in Chl *a* (1.9–2.0 fold), Chl *b* (2.0–2.6 times), and total Car (1.4–1.6 times) dry wt percentages in comparison with the cultures "pH 6–7" (Figs. 3a and 3b) as well as by an increase in Car/Chl mass ratio (up to 0.44 vs. 0.30–0.32 in other cases; Fig. 3d), a characteristic stress response of microalgae. A similar trend was observed in the cultures with alkaline media ("pH 8" and control), but without a similar raise in Car/Chl ratio (Fig. 3d).

According to the chromatographic analysis (Fig. 4), the cultures "pH 5" and "pH 8" exhibited a dramatic decline in the contents of neoxanthin, violaxanthin, lutein and β-carotene as compared with the control. The cultures "pH 6" and "pH 7" also featured a decline in violaxanthin, although the decline in the other carotenoids was significantly less pronounced. No degradation products of the pigments such as phaeophytin *b* were revealed in the HPLC chromatograms.

Dependence of Nutrient Uptake from the Media by C. rubescens Cells on pH and CO_2 Enrichment

The rate of nutrient uptake from the medium by the slow-growing cultures ("pH 5" and control) was significantly lower than in other experimental variants (Figs. 5d and 5e). This was especially evident in the culture "pH 5" which absorbed only 37.1% and 32.9% of the initial amount of nitrogen phosphorus in the medium, respectively, whereas the cultures maintained at pH 7–8 removed 70–89.5% of the nutrients (Figs. 5a and 5b). A characteristic feature of the cultures grown at alkaline pH was accumulation of nitrite in the medium (up to 1.18–1.40 mg N (NO_2^-)/L). In the cultures maintained at pH ⩽ 7 nitrite content did not exceed 0.18 mg N(NO_2^-)/L throughout the cultivation period (Fig. 5b).

Cultivation Conditions Effects on the Dry Weight and Lipid Content in C. rubescens Cultures

The differences in the cultivation conditions exerted a significant effect on the dry weight productivity of the *C. rubescens* cultures. The maximum productivity (147 mg/(L day)) recorded in the cultures grown in a slightly alkaline (pH 8) medium was 11 times higher than that of the "pH 5" culture Figs. 6a and 6b). However, it is unlikely that the large differences in the biomass productivity manifest a

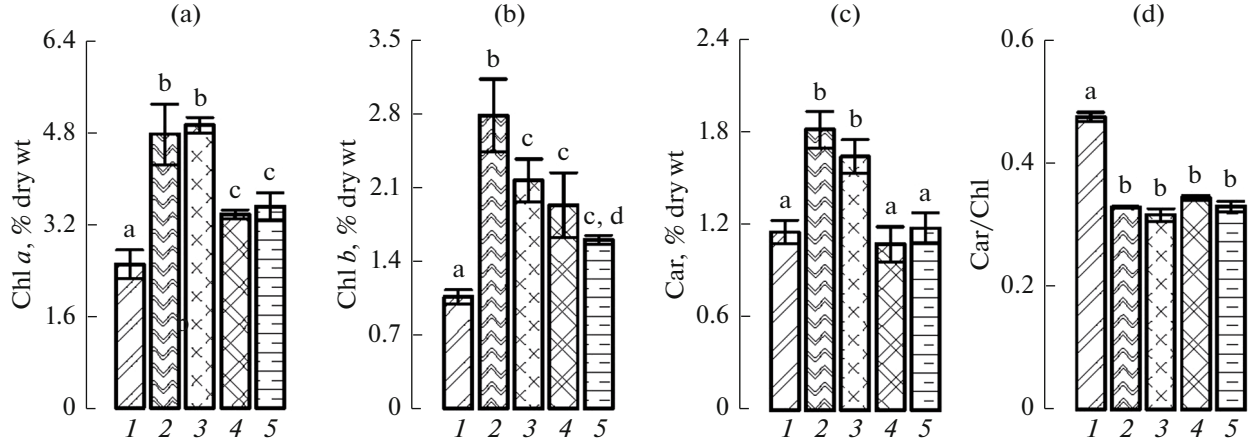

Fig. 3. The content of chlorophyll *a* (a) *b* (b) and amount of carotenoids (c) and the ratio of total carotenoids and chlorophylls (d) dry biomass of *C. rubescens* at different pH levels environment and security cells CO_2. *1*—pH 5; *2*—pH 6; *3*—pH 7; 4—pH 8; *5*—control.

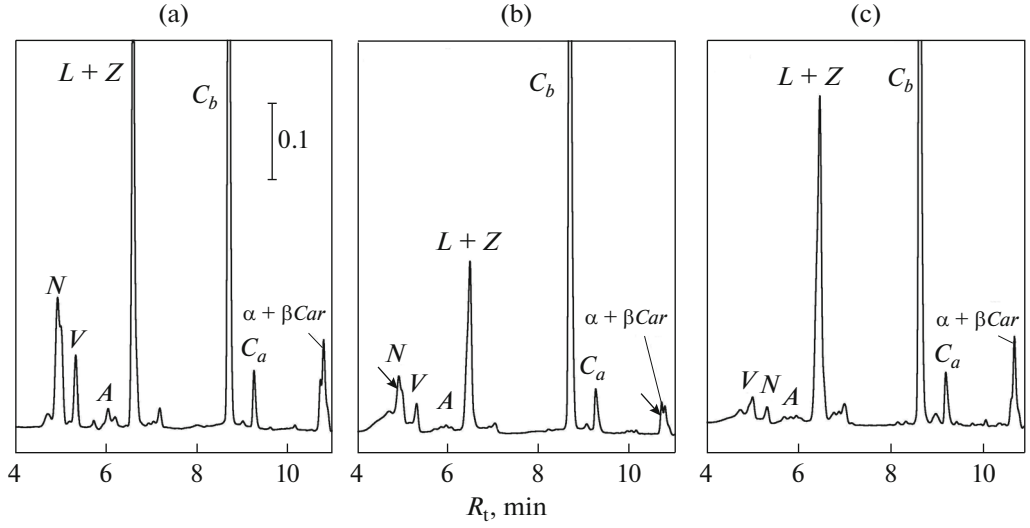

Fig. 4. Changes in the composition of pigments of *C. rubescens* cells grown in the cultures sparged with air (a) or CO_2 to pH 5 (b) and pH 7 (c). Chromatograms for cultures grown at pH 8 and 6, were similar to those in panels b and c, respectively. Are normalized on the peak amplitude of the Chl *b* HPLC chromatograms (detection at λ = 455 nm) of acetone extracts of cells. *N*—neoxanthin, *V*—violaxanthin, *A*—antheraxanthin, *L* + *Z*—lutein + zeaxanthin, C_a, and C_b—Chl *a* and *b*, α + β*Car*—α-carotene + β-carotene.

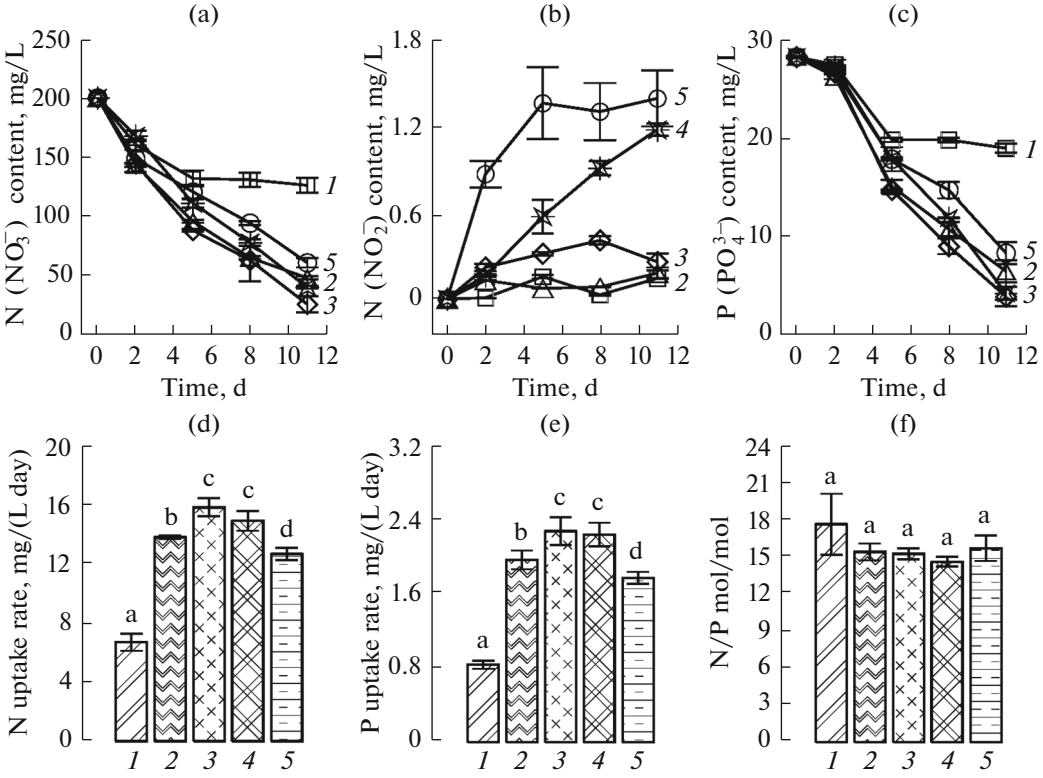

Fig. 5. The kinetics of the absorption of nutrients by crops *C. rubescens* depending on pH and availability of CO_2. Dynamics of biogenic elements in the environment (a–c), average speed (d–e) and the stoichiometry (f) uptake of nitrogen and phosphorus by crops. *1*—pH 5; *2*—pH 6; *3*—pH 7; *4*—pH 8; *5*—control.

sharp decline in metabolic activity of the cells grown in acidified media. On the contrary, the slow-dividing cells accumulated large amounts of reserve compounds and hence increased in size. Indeed, the dry wt and total lipid contents per cell were the highest in this experimental variant (Figs. 6b and 6d).

Special attention is given to the ability of *C. rubescens* to synthesize significant amounts of lipids, even

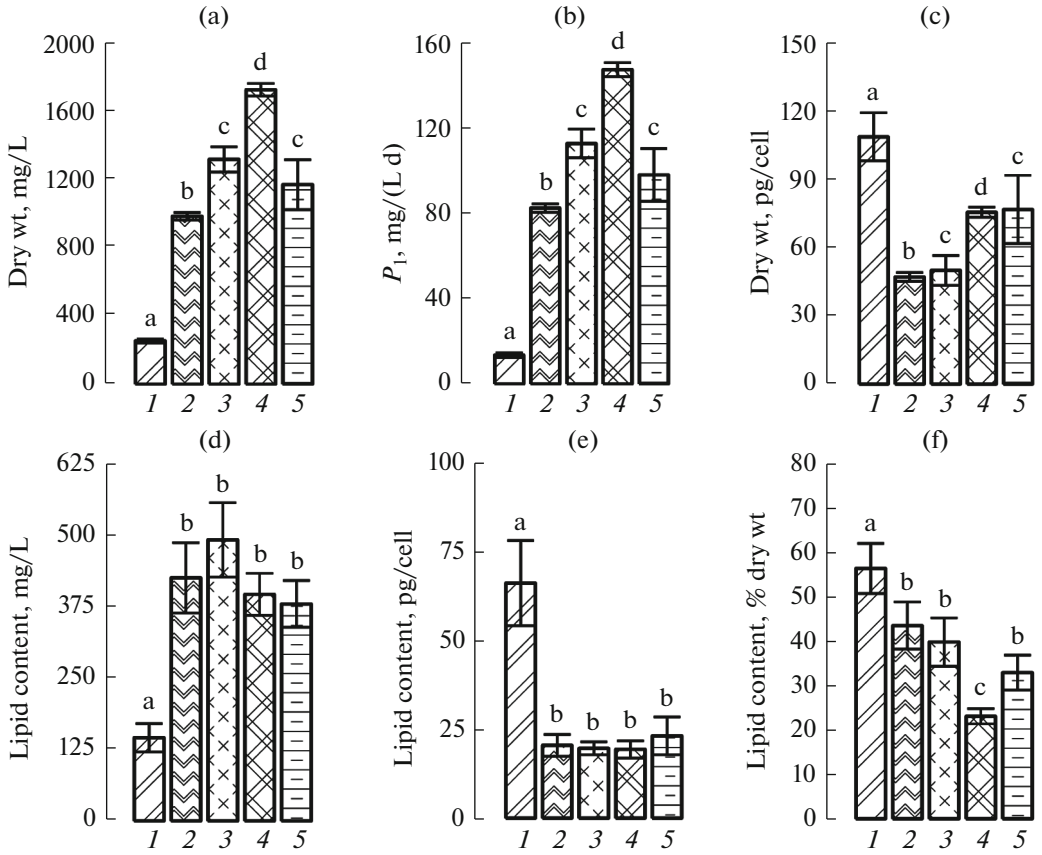

Fig. 6. The dry matter content of (a–b) and lipid (d–f) on the 11th day. the experiment in the cultures of *C. rubescens*, depending on pH and availability of CO_2. 1—pH 5; 2—pH 6; 3—pH 7; 4—pH 8; 5—control.

in the absence of acute shortage of batteries or other abiotic stress factors. Their content in the cultures of variants with pH 6.0–6.8 at the end of the experiment reached 400–500 mg/L (Fig. 6d). Fatty acid composition of total lipids of cells grown in different variants of the experiment are presented in Table. About half of the amount was two fatty acids (FA) essential for human and animal, FA linoleic (C18:2 ω6) and α-linolenic (C18:3 ω3). The main FA was also palmitic acid, oleic acid and hexadecanoate. The content ratio of FA with different carbon chain length and degree of saturation varied significantly depending on the cultivation conditions. When feeding CO_2 into the medium to a pH of 5 we registered a significant reduction (in comparison with the cultures "pH 6" and "pH 7") in a number of coefficients characterizing the physiological state of cells and the degree of unsaturation of its lipid FA. The ratio of C18:3ω3/C18:1ω9 decreased about 3 times, and the ratio of total polyunsaturated FA to the amount of monounsaturated—approximately 2 times. Also decreased the unsaturation index (Table) and the ratio ∑C16/∑C18 (from 0.45 to 0.62–0.63 in the variants, "pH 6" and "pH 7").

A comparison of the relative content of individual FA in the total lipid and the average growth rate of cultures (P_1) at different pH showed that cultures with a higher rate of cell division in lipids increases the total content of C16-acids reduced the content of oleic (significantly) and linoleic acids (as a tendency) and simultaneously, significantly increased the content of α-linolenic acid.

DISCUSSION

When growing microalgae-producers of astaxanthin yield of the target product is determined by three main components: accumulation of biomass at the end of the "green" stage; the survival of cells during stress induction of secondary carotenogenesis and intensity of biosynthesis of astaxanthin in the cells on the "red" stage [5]. Therefore, an important objective of the study the physiology of the production process and carotenogenesis in these organisms is the search for optimal conditions of cultivation for each of the stages. The results of these studies and necessary to create a cost-effective biotechnology of obtaining the Car from microalgae. This primarily relates to ensuring high crop yield at green stage. In autotrophic cultivation of microalgae the productivity of crops rarely exceeds 10–12 g of dry biomass/(m^2 day) due to limitation of

Fatty acid profile of total lipids from 11-day-old *C. rubescens* cells as a function of pH and CO_2 enrichment

Fatty acids**	Fatty acid content, mass. % *				
	pH 5	pH 6	pH 7	pH 8	control
C16:0	17.8	19.8	20.5	20.5	20.7
C16:1 ω7	1.7	2.1	2.2	2.1	2.1
C16:2 ω6	3.9	5.6	5.5	7.0	6.0
C16:3 ω3	5.3	7.9	8.5	7.8	6.3
C18:0	1.7	1.1	0.8	1.0	1.3
C18:1 ω9	16.4	7.4	8.8	6.7	9.0
C18:1 ω7	1.9	***	1.4	1.5	1.7
C18:2 ω6	24.5	22.9	20.8	22.1	23.2
C18:3 ω3	23.0	28.1	30.4	28.5	23.3
Monoenoic	21.8	11.9	11.6	11.4	14.1
Dienoic	28.5	28.6	26.3	29.1	29.2
Trienoic	28.3	35.9	38.9	36.3	32.8
Medium chain length FA (C < 16)	1.2	1.0	0.5	1.0	1.0
Very long chain FA (C ⩾ 20)	1.0	0.8	0.4	0.5	0.6
C18:3 ω3/C18:1 ω9	1.4	3.8	4.5	4.3	3.0
Unsaturation index	1.756	1.805	1.851	1.813	1.732

* The standard deviation was in all cases below 5% of the corresponding average.
** Minor FA were also detected (not shown) including C16:1 ω9, C16:4 ω3, and C16:3 ω6 (⩽1% each).
*** Not detected.

growth by low concentration of CO_2 in atmospheric air (0.04%) and low rate of diffusion of CO_2 in solution. According to the calculations of Lee et al. [27], for increasing the biomass yield to the theoretically possible values of 60–70 g/(m^2 day) you need to place in culture, about 30 g of carbon/(m^2 day) in the form of CO_2 or HCO_3^-. This massive enrichment of the environment with carbon dioxide can withstand only a few CO_2-tolerant species with complex mechanisms maintaining the homeostasis of intracellular pH [15]. In species with low CO_2 tolerance, which include the majority of known objects of biotechnology [14], high CO_2 concentrations are a stressor, causing acidification of the stroma of chloroplasts, inhibition of photosynthesis and other negative deteriorative processes leading to cessation of growth and, often, to the death of crops [15, 28, 29]. In such cases, a more appropriate way to address the shortfall of carbon is autotrophic cultivation of algae in the pH-stat mode with controlled flow of CO_2 into the culture medium to maintain the optimum for this type of algae pH [15, 27]. This approach was used to determine the tolerance of *C. rubescens* to a pH in the enrichment cultures of gaseous CO_2. The results of analysis of physiological-biochemical parameters of *C. rubescens* cells, depending on pH and availability of CO_2 indicate that this alga is stenobiontic in relation to these factors and quickly grows only in a narrow pH range close to neutral. The most optimal combination of pH and levels of CO_2 enrichment observed in the variants "pH 6" and "pH 7". In these conditions, the average productivity, maximum (1.0–1.1 1/day) and average cell number-based specific growth rate (0.3–0.35 1/day) were recorded (Fig. 1), mass fraction of photosynthetic pigments (Fig. 3), the degree of unsaturation of FA in total lipids and the content of polyunsaturated acids, typical for glycolipids of chloroplast membranes [30, 31] were the highest. The results of the analysis of FA composition of total lipids in the control culture are in good agreement with the data of Abe et al. (2007) for another representative of the genus *Coelastrella—C. striolata* var. *multistriata* [32]. Both species in similar conditions (batch culture; BBM medium enriched with nitrogen; sparging atmospheric air), the total proportion of linoleic and α-linolenic acids was about 50%, the second was palmitic acid (18–21%), while the content of oleic acid accounted for 9–13% of the total FA amount. The main difference between closely related species was the lack of hexadecadienoic acid at *C. striolata* var. *multistriata*.

The most complete absorption of nutrients from the medium, is an important condition for the induction of the biosynthesis Ast at the "red" stage of cultivation and reduces overhead on nutrient medium [3, 5]. Culture in the variant, "pH 5" were obviously affected by stress caused by excess CO_2, low pH. This resulted in significant growth inhibition (Fig. 1),

increasing the volume of cells (Fig. 2), the decrease in the rate of absorption of nitrogen and phosphorus from the medium (Fig. 5). It is also possible that at low pH cells microalgae could experience a shortage of inorganic carbon due to the decline in the share of hydrocarbonate-ions, which may be the preferred form of transport of inorganic carbon in *C. rubescens*. The characteristic feature of the stress state cultures, along with the General decrease in the content of Chl and primary Car, was the increase of the ratio Car/Chl compared with the variants "pH 6" abd "pH 7". This observed reduction in the share of Car and performs mainly the function of light harvesting (neoxanthin and lutein). Typical manifestations of stress were the accumulation C18:1 in the cell lipids (Fig. 6) and reduction of the C18:3 ω3/C18:1 ω9 ratio [30–32].

C. rubescens growing without CO_2 enrichment of the culture resulted in alkalization of the medium in the control to pH 9.5–10.5, which also had negative effects on the physiological status of the cells. The average specific growth rate (Fig. 1), the mass fraction of pigments (Fig. 3) and the ratio of C18:3 ω3/C18:1 ω9 here were markedly lower than in more vigorous cultures "pH 6" and "pH 7", but nevertheless significantly higher than in the variant "pH 5". These findings do not uniquely identify the state of the control culture as stressed. The slowdown of growth in this case is rather pointed to the imbalance between carbon supply and the need of the algae in the substrate. In an alkaline medium in the absence of starvation of nitrogen and phosphorus (Fig. 5) inorganic carbon may be the limiting factor since its, availability is significantly decreased due to displacement of the equilibrium in the carbonate system towards the increase concentration of carbonate ions at pH 10.2–10.5 the carbonate ions range from 50–60% of the total inorganic carbon in the medium [12]. Additional factors inhibiting growth may be the reduced availability of micronutrients as a result of their binding to insoluble salts of carbonic acid, and the toxic effects of nitrite (Fig. 5) [33], which can be excreted by cells into the external environment by the incomplete recovery of nitrate nitrogen to NH_4^+ due to insufficient activity of intracellular nitrate reductase [34], and elevated concentrations of OH^- on the structure and functional state of cell membranes and the activity of key enzymes [13, 15].

Collectively, the obtained results suggest that CO_2 enrichment of the "green"-stage culture of *C. rubescens*, a producer of value-added Car, is very beneficial to its growth and productivity provided that there will be no stress from the excessive drop of pH. The maintenance of pH in the optimal range (pH 6–7) is crucial for CO_2-enriched culture of this microalga. Further studies of the influence of CO_2 enrichment on stress-induced secondary carotenogenesis in this microalgae are needed.

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