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A NEW STRAIN OF A CAROTENOGENIC GREEN MICROALGA *COELASTRELLA RUBESCENS*, SUITABLE FOR CULTIVATION IN THE CLIMATIC CONDITIONS OF SOUTHERN RUSSIA

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A new carotenogenic strain of a green microalga *Coelastrella* Chodat (Scenedesmaceae, Sphaeropleales) was isolated in unialgal culture from the thallus of a Crimean epilithic lichen sampled in Sevastopol in March 2024. The strain is characterized by a pronounced ability to accumulate secondary carotenoids under acute abiotic stress conditions. We describe the methods we used to isolate phycobionts from small lichen samples, purify algae, establish axenic culture, adapt cells to laboratory conditions, and maintain strains in long-term culture collection. Species identification was performed using a combination of morphological (light and scanning electron microscopy) and molecular genetic approaches (analysis of the ITS1–5.8S–ITS2 fragment of the 18S rRNA gene cluster). The novel strain was identified as *Coelastrella rubescens* (Vinatzer) Kaufnerová & Eliás, 2013 and deposited into the collection of living cultures of carotenogenic microalgae at IBSS (https://algae.ibss-ras.ru), designated as IBSS-156. The growth characteristics of the IBSS-156 strain were evaluated under two-stage batch culture conditions. The data indicate high specific growth rates of the alga at the vegetative stage: up to (0.62 ± 0.11) day^{-1}. The dynamics of the algal pigment composition that we received reflects the massive accumulation of secondary carotenoids under stressful conditions. We found that measuring the absorption spectra of living microalgal cultures in the visible region allows for a rapid assessment of their physiological state and the rate of ketocarotenoid accumulation in biomass during two-stage cultivation. The obtained results highlight the potential of Crimean lichens as a source of promising astaxanthin-producing strains suitable for mass cultivation in arid and hot climates, such as those found in the Crimea and adjacent regions of southern Russia.

Keywords: carotenogenic microalgae, phycobionts, *Coelastrella*, two-stage cultivation

The term “carotenogenic microalgae” refers to a taxonomically and ecologically heterogeneous group of unicellular algae with a pronounced capacity for secondary carotenogenesis. This trait constitutes a key adaptation mechanism in extremophiles enabling survival during abrupt environmental deterioration through the massive accumulation of ketocarotenoids in cytoplasmic lipid inclusions (oleosomes) [Cheng, Shah, 2023; Debnath et al., 2024; Patel et al., 2022; Solovchenko, Minyuk, 2021].

The main biological features of carotenogenic microalgae include ephemerality of vegetation, the presence of a resting stage in their life cycle, the induction of secondary carotenoid and neutral lipid biosynthesis during the transition to the resting stage, and the ability to rapidly shift between rest and active growth.

The rising research interest in this algal group is rooted in both fundamental and applied aspects. On the one hand, it aims to advance our understanding of the general patterns and specific features of adaptation mechanisms in diverse ketocarotenoid producers. On the other hand, it seeks to reveal new commercially promising sources of C_{40} ketocarotenoids of the astaxanthin group and neutral lipids suitable for the production of high-quality biodiesel [Islam et al., 2013; Karpagam et al., 2015; Patel et al., 2022].

The most extensively studied and still the only astaxanthin producer successfully introduced into mass cultivation is a freshwater microalga *Haematococcus lacustris* (Girod-Chantrans) Rostafinski, 1875 (Chlorophyceae, Chlamydomonadales, Haematococcaceae). With a two-stage cultivation protocol, astaxanthin in its biomass can reach 5 % of dry weight [Han et al., 2013; Mota et al., 2022]. However, the low ecological plasticity of this species restricts its large-scale cultivation to areas with a subtropical maritime climate, where temperature does not exceed +28 °C during the hottest part of the day [Mohanasundaram et al., 2023]. Within the Russian Federation, such areas are localized zones on the Crimean and Caucasian coasts, which are of significant recreational value. Consequently, Russian companies interested in astaxanthin are completely dependent on import. Solving this problem requires searching for and introducing into cultivation new, high-yielding strains of astaxanthin producers that are easily adaptable to local conditions, resistant to contamination, and tolerant to extreme environmental factors [Patel et al., 2022; Solovchenko, Minyuk, 2021]. In literature, over 130 such species are reported [Minyuk, 2020]. Many of them occur in the algal flora of both the Crimean subtropics and the steppe and foothill zones of the peninsula. These are chiefly representatives of two orders of green microalgae: Chlamydomonadales and Sphaeropleales [Dantsyuk et al., 2021]. Their growth characteristics in culture and the specific mechanisms regulating astaxanthin biosynthesis remain poorly understood. Nevertheless, several studies conducted at IBSS suggest that some of these species may be undemanding and highly productive sources of astaxanthin [Chelebieva et al., 2018; Dantsyuk et al., 2021]. These considerations underscore the need for a comprehensive analysis of the Crimean carotenogenic microalgal flora, an assessment of its bioresource potential, and a search for novel strains with high potential for industrial cultivation across diverse natural and climatic zones of the peninsula and adjacent areas of southern Russia.

A poorly characterized group of carotenogenic microalgae includes phycobionts of Crimean crustose and foliose epilithic lichens adapted to extreme conditions: high daytime temperatures, low humidity, and intense solar radiation. These lichens form dense, bright orange-red crusts on rock surfaces [Czeczuga, Osorio, 1989; Voitsekhovich, 2013; Voytsekhovich, Beck, 2016]. Those are promising candidates for open-air mass cultivation in Crimean regions unsuitable for conventional agriculture.

The aim of this work was to isolate astaxanthin-producing microalgae from Crimean lichens, with a focus on strains adapted to local conditions. The following tasks were solved:

1) to isolate and obtain algologically pure cultures of phycobionts from Crimean lichens;
2) to reveal a strain with high ketocarotenoid accumulation potential and identify it taxonomically using an integrative approach;
3) to assess the biotechnological potential of the selected strain by evaluating its growth performance during the vegetative stage and pigment dynamics under combined abiotic stress conditions.

MATERIAL AND METHODS

Crustose lichens – bright orange, tightly attached crusts on rock surfaces, tree bark, and limestone boulders – were sampled in foothills of the Western Crimea (Sevastopol area) in spring and summer (Fig. 1). Sampling sites were as follows:

- A, the Victory Park (Sevastopol, 44°36′10″N, 33°27′23″E, isolated by I. Drobetskaya in March 2024);
- B, the Chelebi Yaurn Beli mount (Baydar Gate mountain pass, Orlinoye settlement, Sevastopol, 44°24′18″N, 33°46′49″E, isolated by N. Dantsyuk in April 2024);
- C, the Saint George monastery (Cape Fiolent, Sevastopol, 44°30′29″N, 33°30′29″E, isolated by N. Dantsyuk in April 2024);
- D, the Toropova Dacha forest park (Balaklava municipal district, Sevastopol, 44°30′34″N, 33°41′2″E, isolated by I. Mansurova in May 2024).

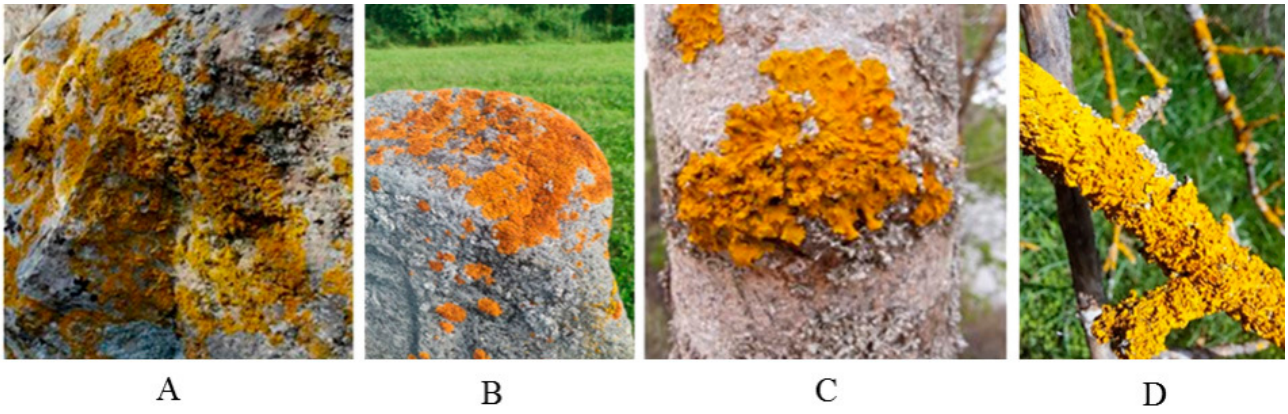

Fig. 1. Crustose epilithic (A, B) and foliose epiphytic (C, D) lichens

Material was separated from a substrate with a sharp spatula and placed in labeled polyethylene bags, each marked with sample number, sampling date, GPS coordinates, and sampler's surname. In a laboratory, samples were air-dried at room temperature for 24 h and then stored at +20 °C in labeled paper envelopes in a dark and dry place until analysis.

Isolation of algologically pure phycobiont cultures. To isolate microalgal cells into unialgal culture, we used a modified method of density gradient centrifugation proposed by [Fontaniella et al., 2000]. The technique involves step-by-step centrifugation of macerated lichen thalli in a density gradient of solutions: 0.25 M sucrose – 80% potassium iodide solution – 10 mM phosphate buffer. The core of our modification was the optimization of sample-to-solution ratios for biomass inputs of 0.1–0.3 g.

A sample of a dry lichen thallus was washed with distilled water to remove debris and ground in a mortar with 5 mL of distilled H_2O. A homogenate was filtered through six layers of gauze into a polypropylene Falcon tube and centrifuged at 200 g for 10 min. The supernatant was discarded, and the pellet was resuspended in 4 mL of 0.25 M sucrose. Into a centrifuge tube, 3 mL of 80% KI solution were placed. Along the inner wall of the tube, 2 mL of the resulting cell suspension in sucrose was carefully layered on top of the solution. The tube was centrifuged at 50 g for 1 min. Large fragments of lichen thalli precipitated and concentrated in the lower KI layer, while microalgal cells remained suspended in the upper sucrose layer. This layer was carefully transferred with a micropipette into a new tube containing 3 mL of 80% KI solution. Then, 2 mL of 10 mM phosphate buffer was added, and the samples were centrifuged

at 130 g for 2 min. Microalgal cells formed an intermediate layer (an interphase) between the phosphate buffer and KI solution, while fungal hyphae fragments passed into the KI layer as a dense pellet. The interphase containing algal cells was carefully sampled with a micropipette and transferred into 3 mL of 80% KI solution. Along the inner wall of the tube, 3 mL of the phosphate buffer was carefully layered on top of the KI solution and centrifuged at 200 g for 3 min. If necessary, this step was repeated. Subsequently, 1 mL of distilled H_2O was added to the microalgal cell suspension sampled from the interphase layer, and the sample was centrifuged at 290 g for 5 min. The resulting cell pellet was resuspended in 0.5–1.0 mL of twice diluted BBM (Bold's Basal Medium) [Bischoff, Bold, 1963]. The resulting "primary inoculum" was transferred into a glass tube with a cotton plug and stored at room temperature under illumination with a photosynthetically active radiation (hereinafter PAR) flux density not exceeding 35 μmol photons·m^{-2}·s^{-1} until antiseptic treatment.

Antiseptic treatment. To combat microbial contamination, antibiotics with a broad spectrum of antimicrobial activity were used: cefotaxime ("Biokhimik") and ampicillin ("Belmedpreparaty"). A mixture was prepared by combining 500 μg·mL^{-1} of ampicillin and 100 μg·mL^{-1} of cefotaxime [Kan, Pan, 2010]. As a fungicide, "Fundazol" was used ("Zelenaya Apteka Sadovoda"): when penetrating plant tissue, it blocks the reproductive function of fungi. Its solution (40 μg·mL^{-1}) was added to suppress micromycete contamination [Mokrosnop, Zolotareva, 2012].

Isolation of unialgal cultures. After antiseptic treatment, an alga was acclimated to laboratory conditions by maintaining the cell culture on liquid BBM under optimal temperature for lichen phycobionts, +18…+20 °C, and PAR flux density of < 35 μmol·m^{-2}·s^{-1} [Voitsekhovich, 2013]. To obtain unialgal cultures, we applied the standard streak plate method [Gaisina et al., 2008; Temraleeva et al., 2014]. Microalgae were inoculated on agar-enriched (1.5–2%) BBM in Petri dishes. The dishes with microalgae were incubated in a refrigerated cabinet at +14…+16 °C and a PAR flux density of 20–26 μmol photons·m^{-2}·s^{-1} in a 15 h light : 9 h dark mode. Germinated colonies were transferred with an inoculation loop into separate test tubes containing approximately 0.3 mL of liquid BBM to obtain an actively growing cell culture. Within one week, the alga was examined under a microscope to assess algological purity. If necessary, the procedure was repeated until obtaining a monoclonal culture of phycobiont cells. Once a unialgal culture was obtained, it was inoculated into agar slants for long-term storage in the collection of living cultures of carotenogenic microalgae at IBSS.

Conditions for two-stage culturing of phycobiont cells. Phycobiont cell were cultured in a two-stage batch culture mode [Minyuk et al., 2016]. *Prior* to cultivation, a synchronized cell culture in an actively growing state was obtained: an algal inoculum was prepared and grown on BBM for 7 days under unilateral side illumination (JazzWay DL 8W 6500K LED lamps, China) with continuous air bubbling. PAR photon flux density incident on the culture surface was 120 μmol·m^{-2}·s^{-1}. The photoperiod was 15 h light : 9 h dark (close to natural mode and recommended for carotenogenic microalgae cultivation). Temperature was maintained at the level of (+23 ± 1) °C.

During the I stage (the "green" one), which lasted 11 days, the alga was cultivated in 0.5-L glass conical flasks. Cultivation conditions did not differ from those of the preliminary phase. The cultures were continuously aerated with air at a rate of 1 L·min^{-1} using an air compressor Hailea ACO-9620 (China). During the light period, the medium pH was maintained at 7 by pulsed addition of carbon dioxide (CO_2 volume fraction of 99.8% according to [GOST 8050-85, 2006]) with a solenoid valve Camozzi A7E (Italy) and a digital pH controller Aqua Medic pH 2001 C (Germany). The initial cell abundance in flasks was $1.5 \cdot 10^6$ cells·mL^{-1}, and the culture volume was 0.45 L.

To proceed to the II stage (the "red" one), the culture was concentrated by centrifugation at 290 g for 5 min. The supernatant was discarded, and the cell pellet was resuspended in BBM with a 10-fold reduction in nitrogen and phosphorus content. The initial cell abundance at the "red" stage was approximately $1.5 \cdot 10^6$ cells·mL^{-1}, with a culture volume of 0.45 L in each flask. The mode of lighting was switched to continuous bilateral (140 μmol photons photons·m^{-2}·s^{-1} from each side). This configuration, combined with the 10-fold reduction in cell abundance, resulted in a 20-fold positive gradient of cell irradiation [Minyuk et al., 2017]. The supply of CO_2 was adjusted to a level that maintained the medium pH close to 7 around the clock.

Monitoring of culture growth and cell sizes. Cell abundance (n) was analyzed using a MACSQuant Analyzer flow cytometer ("Miltenyi Biotec," Germany), equipped with three lasers (405, 488, and 635 nm), at "Spectrometry and Chromatography" core facility at IBSS. Data were processed applying FCS Express 7 Research Edition software.

The average specific growth rate (μ) of cultures at the "green" stage was calculated from the daily increase in n [Wood et al., 2005]:

$$\mu_n = \frac{\ln \frac{n_i}{n_0}}{(t_i - t_0)},$$

where μ_n is specific growth rate for Δn, day^{-1};

n_0 is initial cell abundance, cells·mL^{-1};

n_i is cell abundance on day i, cells·mL^{-1};

$t_i - t_0$ is duration of the cultivation period, days.

Light microscopy and scanning electron microscopy. For light microscopy, we used an inverted microscope Nikon Eclipse Ts2R. Cell sizes were determined from micrographs taken with an Infinity 3 Lumenera camera applying ImageJ software (NIH, the USA).

For scanning electron microscopy (hereinafter SEM), 2.5 mL of culture of vegetative microalgal cells was sampled during the exponential growth phase (day 4 of the experiment) and fixed by adding 25% glutaraldehyde solution to a final concentration of 0.5%. Fixation requires a neutral, slightly alkaline environment [Chubchikova et al., 2022], and to ensure this, pH in the medium was adjusted to 7.3 right before fixation by regulating the supply of CO_2. Fixed samples were immediately mixed with a microvortex (Microspin FV-2400, "Biosan," Latvia), placed in a refrigerator at +3 °C, and mixed every 10–15 min for 1 h. If necessary, samples fixed according to this protocol can be stored refrigerated for two weeks [Rylkova et al., 2024]. At the next stage, microalgal suspension was concentrated on polycarbonate track-etched membranes with a pore size of 2 μm (manufacture by Joint Institute for Nuclear Research, Russia) placed on moist filter paper supports [Rylkova et al., 2024]. Filtration was carried out under a vacuum of no more than 0.2 atm. Fixative was washed off from the suspension on the filters treating them twice with 1 mL of Na/K phosphate buffer (chloride-free), the use of which is preferred for Scenedesmales [Chubchikova et al., 2022], and then rinsed once with 1 mL of deionized H_2O. Then, the filters with the alga were placed together with the supports into a 16-well plate and dehydrated in increasing concentrations of ethanol: 20, 30, and 50%, for 5 min; 57 and 96%, for 10 min; and 100%, twice for 10 min. Solutions were changed using a dispenser. Critical point drying was performed in a Leica EM CPD300 (Germany) in a soft mode for 2.5 h. Sputter coating (Au/Pd, 0.5–1.0 min) was carried out with a Leica EM ACE200 system. SEM images of cells were obtained under a Hitachi SU3500 scanning electron microscope (Japan) in the Microscopy Laboratory at IBSS.

Molecular genetic analysis. Genomic DNA was extracted from concentrated algal cultures with a "DNA-Extran-3" kit ("Syntol," Russia) following the manufacturer's protocol. DNA quality was assessed by electrophoresis in a 1.5% agarose gel stained with ethidium bromide (0.2 μg·mL^{-1}). Amplification of a DNA fragment encoding the 5.8S region and the adjacent ITS1 and ITS2 regions of the nuclear ribosomal gene cluster was performed by polymerase chain reaction (PCR) using the ready-to-use ScreenMix kit ("Evrogen," Russia) and ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) primers [White et al., 1990]. The protocol was as follows: initial denaturation at +94 °C for 3 min; 34 amplification cycles (+94 °C for 30 s; +60 °C for 30 s; and +72 °C for 60 s); and a final extension at +72 °C for 10 min. PCR product was purified with Cleanup Standard reagents ("Evrogen") according to the manufacturer's protocol and sequenced with GenSeq reagents ("Syntol") on a genetic analyzer Nanophore 5 (Institute of Analytical Instrumentation of RAS, Russia).

The search for similarity between the obtained sequence and that available in the GenBank database (National Center for Biotechnology Information, NCBI) was carried out applying the BLASTN algorithm [Morgulis et al., 2008; Zhang et al., 2000]. Alignment was performed using MAFFT v. 7.48 [Katoh, Toh, 2010] with L-INS-I option; ambiguously aligned 5' and 3' ends were trimmed. Genetic distances between nucleotide sequences were calculated using the Kimura's two parameter model (K2P model) with MEGA 11 software [Kimura, 1980; Tamura et al., 2021]. Phylogenetic analysis and tree construction involving the maximum likelihood (ML) algorithms were performed in IQ-TREE 1.6.12 [Trifinopoulos et al., 2016] with the SYM + G4 nucleotide substitution model automatically selected by ModelFinder based on the Bayesian information criterion (BIC). Branch support was assessed using an SH-aLRT test and ultrafast bootstrap with 10,000 replicates, and also an approximate Bayes test with search stopping after 1,000 iterations and perturbation strength set to 0.1. Tree topology accuracy was evaluated by bootstrap analysis with 1,000 replicates. Trees were visualized and edited using the service https://phylo.io/index.html.

Spectrophotometry. Absorption spectra of cell suspensions of living microalgal cultures at the "green" and "red" stages were recorded on a Shimadzu UV-2600i spectrophotometer ("Spectrometry and Chromatography" core facility at IBSS) over the wavelength range of 380–800 nm in a 1-cm quartz cuvette. Right before measurement, each sample was thoroughly mixed to minimize sedimentation effects; the measurement time *per* sample was within 30 s. As the reference, BBM was used.

Graphs were plotted in Golden Software Grapher 17.3.454. Measurements were performed in three biological replicates and three analytical replicates. Data in figures and text are presented as mean values and standard error of the mean.

RESULTS AND DISCUSSION

Cells of green algae, lichen photobionts, were isolated from specimens of epilithic and epiphytic lichens sampled in foothills of the Western Crimea. Isolation involved a method of density gradient centrifugation adapted for small samples. Microscopic analysis revealed the absence of thallus debris and foreign impurities in resulting cell suspensions (Fig. 2A). During one week of acclimation to laboratory conditions, isolated cells acquired characteristics typical of free-living populations: more uniform cell sizes, shape, and intracellular content (Fig. 2B).

During the isolation of algologically pure cultures on agar-enriched nutrient media in Petri dishes, the first colonies appeared within 2–4 weeks. Similar germination times for photobionts on agar media have been reported by other researchers [Bačkor et al., 1998; Gasulla et al., 2010].

The colonies varied in morphology and consistency, appearing as powdery, smooth, or granular, with various shades of green (Fig. 2C–E). Such features of cell colonies may serve as secondary taxonomic criteria.

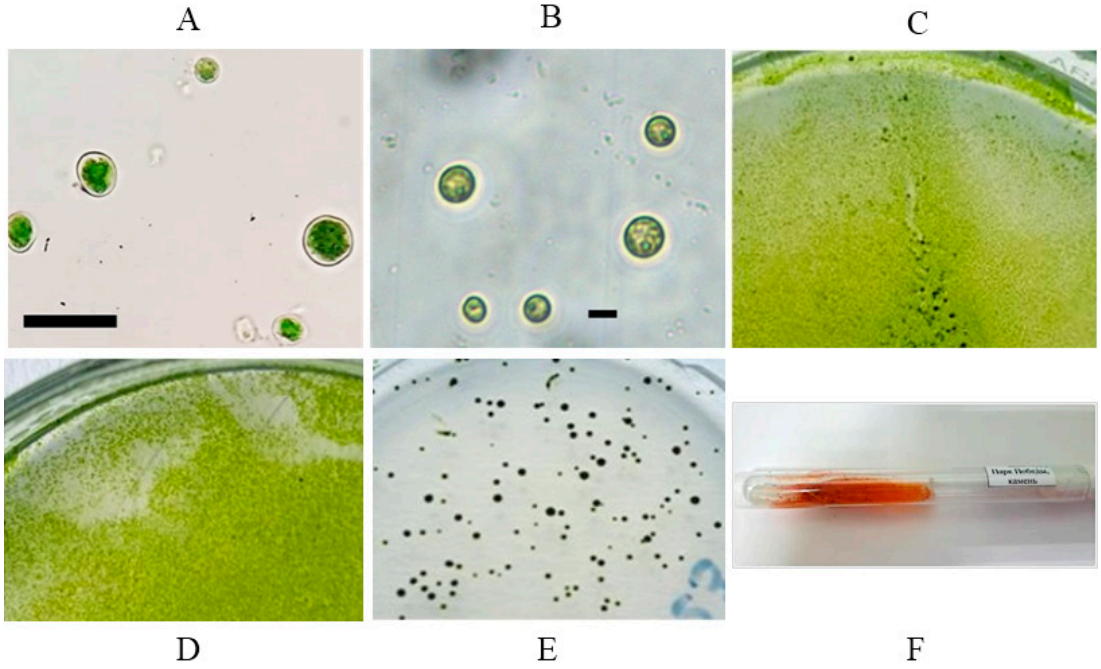

Fig. 2. The stages of isolating phycobionts into a pure culture: cells under a light microscope immediately after the isolation (A) and after one week of culturing (B); solid media cultures of phycobionts isolated from epilithic (C, D) and epiphytic (E) lichens; aging culture of the IBSS-156 strain on agar (F). Scale bars are 10 μm

Repeated subculturing allowed obtaining axenic cultures suitable for long-term preservation. After a month of incubation on agar slants in a refrigerated cabinet, the aging culture of a strain isolated from a thallus of a crustose epilithic lichen sampled in the Victory Park in Sevastopol (see Fig. 1A) changed its color from green to red-orange (Fig. 2F). This color change indicates the accumulation of secondary carotenoids in the microalga: a characteristic trait of extremophiles under abiotic stress.

Taxonomic identification of a strain using morphological and molecular approaches. Cell morphology and the life cycle of the isolated strain were examined *via* light microscopy and SEM. In actively growing cultures, light microscopy revealed single vegetative cells of oval or lemon shape (Fig. 3A). Mean cell length was 5–7 μm, and width was 3.5–6 μm. The chloroplast was parietal, with one pyrenoid. Asexual reproduction occurred *via* autospore formation. Those are enclosed in a spherical sporangium (typically 2 or 4 *per* sporangium) and released after rupture of a sporangial wall (Fig. 3D). Sexual reproduction with gamete formation was not observed. Mature cells at the stage of secondary carotenogenesis turned orange while retaining an oval shape. Mean cell length was 8 μm, and width was 6.5 μm (Fig. 3B).

SEM images of vegetative cells at magnification ×7000 (Fig. 3C) reveal 8 meridional ribs converging at the cell poles and forming a thickening. The cell wall architecture, in conjunction with distinctive cell sizes and other morphological traits, enables unambiguous classification of the isolated strain within the genus *Coelastrella* Chodat (Scenedesmaceae, Sphaeropleales) [Krivina et al., 2024;

Wang et al., 2019]. Algae of this genus are widely distributed, and many species are cosmopolitan ones, exhibiting tolerance to a broad temperature range. *Coelastrella* representatives are typically soil-associated species inhabiting various types of soils and water bodies: from wetlands to hot springs. Many species are aerophytes; those colonize natural and artificial solid substrates and contribute to biofilm formation [Kawasaki et al., 2020; Mikhailyuk et al., 2019; Wang et al., 2019]. To date, documented report of *Coelastrella* sp. as a lichen photobiont is restricted to one publication [Loureiro et al., 2024]. It describes the morphological and molecular genetic traits of three *Coelastrella* sp. strains isolated from lichens in northern Portugal.

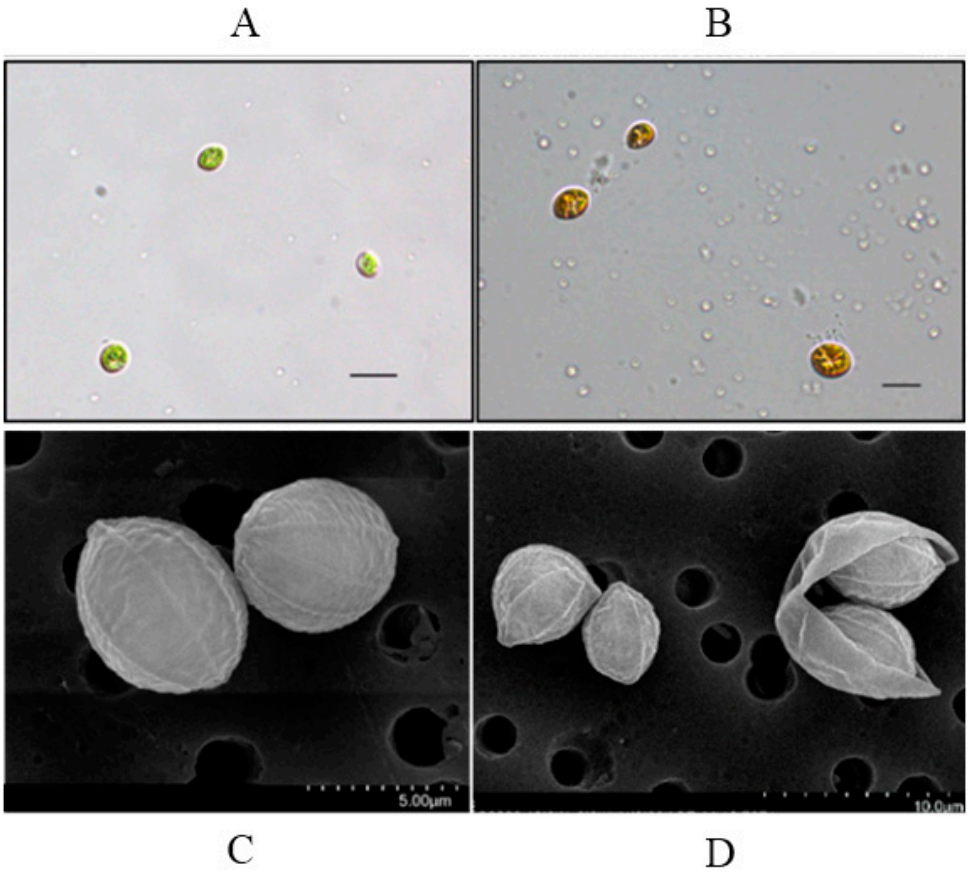

Fig. 3. Cells of the IBSS-156 strain under a light (A, B) and electron (C, D) microscope. Scale bars are 10 µm (A, B, D) and 5 µm (C)

As known, analysis of even ultrastructural cell features does not allow unambiguous identification of certain *Coelastrella* species [Krivina et al., 2024]. Therefore, conducting parallel molecular genetic studies is a standard practice when investigating representatives of this genus, as well as other green microalgae with similar phenotypes [Kawasaki et al., 2020; Mikhailyuk et al., 2019; Wang et al., 2019]. The issue of the most suitable genomic regions for studying this genus remains unresolved. However, an increasing number of researchers use the ITS1–5.8S–ITS2 region of 18S rRNA gene cluster when describing new strains [Kawasaki et al., 2020; Krivina et al., 2024; Maltsev et al., 2021]. For the IBSS-156 strain isolated from a Crimean epilithic lichen, a nucleotide sequence of 411 base pairs was obtained from this DNA region (GenBank NCBI accession number is PV022508).

Phylogenetic analysis of this region allowed constructing a phylogenetic tree (Fig. 4). It unequivocally supports the classification of the studied strain within the genus *Coelastrella*.

With robust support, the strain is placed within the *Coelastrella rubescens* (Vinatzer) Kaufnerová & Eliás, 2013 clade, which constitutes a well-supported lineage in the genus phylogeny. The obtained sequence shows full identity with the corresponding fragment of an authentic strain *C. rubescens* CCALA 475. These two sequences form a subgroup sister to *C. rubescens* var. *oocystiformis* Qinghua Wang, Huiyin Song, Xudong Liu, Guoxiang Liu & Zhengyu Hu, 2019 FACHB-2297a, with a K2P genetic distance of 0.017. This value is close to the minimum interspecific threshold for the ITS1–5.8S–ITS2 region based on pairwise comparisons of authentic strains *Coelastrella corcontica* (Kalina & Puncochárová) Hegewald & N. Hanagata, 2002 CCALA 308 and *Coelastrella multistriata* (Trenkwalder) Kalina & Puncochárová, 1987 CCALA 309.

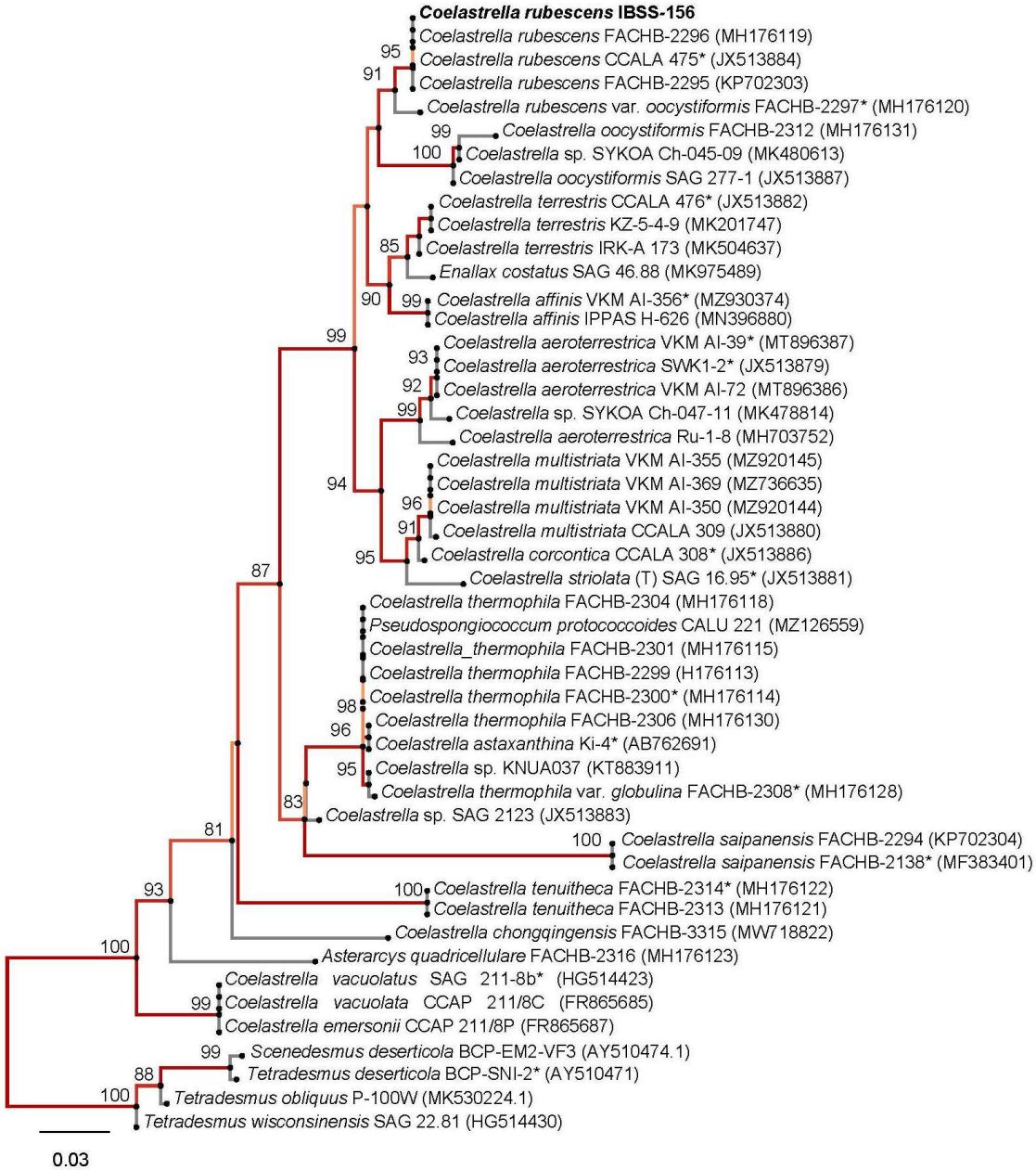

Fig. 4. The phylogenetic position of the IBSS-156 *Coelastrella* strain (Scenedesmaceae, Sphaeropleales) based on the maximum likelihood (ML) inference from partial ITS1–5.8S–ITS2 region of the 18S rRNA gene cluster. Bootstrap support values (> 80) are shown at the nodes. Authentic strains are marked with *; the type species is marked with T

The obtained topology of the tree constructed from a relatively small ribosomal DNA fragment agrees well with phylogenies based on complete 18S rRNA gene sequences [Krivina et al., 2024; Maltsev et al., 2021; Wang et al., 2019]. Specifically, the *C. rubescens* lineage belongs to a subgroup with species usually assigned to a so-called core subclade. The latter one is strongly supported as a sister one to a clade comprising *Coelastrella thermophila* Qinghua Wang, Huiyin Song, Xudong Liu, Guoxiang Liu & Zhengyu Hu, 2019 and *Coelastrella saipanensis* N. Hanagata, 2001; this is fully consistent with phylogenies presented in [Krivina et al., 2024; Maltsev et al., 2021]. Thus, the strain isolated from a Crimean lichen can be assigned with high confidence to *C. rubescens*. This conclusion is corroborated by morphological data: in terms of maximum linear sizes, number and position of cell wall ribs, and number of autospores *per* sporangium, our strain matches *C. rubescens* var. *rubescens*, with its key taxonomic traits summarized in [Andreyeva, 1998; Kaufnerová, Eliáš, 2013; Krivina et al., 2024]. Accordingly, this isolate was registered in the general catalogue of the collection of living cultures of carotenogenic microalgae at IBSS (https://algae.ibss-ras.ru) as the IBSS-156 strain *Coelastrella rubescens* (Vinatzer) Kaufnerová & Eliás, 2013 (Scenedesmaceae, Sphaeropleales).

Study of the growth characteristics of the IBSS-156 strain in batch culture and under stress conditions. A two-stage cultivation scheme is the main approach for growing microalgae to obtain valuable products of secondary carotenogenesis: ketocarotenoids of the astaxanthin group and neutral lipids [Solovchenko, Minyuk, 2021]. At the I, vegetative ("green") stage, the alga is maintained under optimal growth conditions to maximize accumulation of vegetative cell biomass. Upon reaching the stationary growth phase and the onset of nutrient depletion, cultures are transferred to the II ("red") stage by inducing multifactorial stress that triggers the biosynthesis of astaxanthin and neutral lipids. The most commonly applied stress factor is acute nitrogen and phosphorus deprivation, along with a significant increase in light intensity and temperature. To study the growth performance of the IBSS-156 strain, an optimized two-stage cultivation scheme was chosen [Minyuk et al., 2016]. This approach enables investigation of the morphophysiological traits of the alga during the "green" stage and assessment of its capacity to accumulate valuable target products under stress conditions.

The growth dynamics of the IBSS-156 strain in batch culture at the vegetative stage is shown in Fig. 5A. The exponential growth phase lasted from day 1 to day 5, with an average specific growth rate of (0.52 ± 0.11) day^{-1} and a maximum of (0.62 ± 0.11) day^{-1} on day 3. From day 7, the culture remained at the stationary phase. Cell abundance increased 8-fold over 11 days of vegetative growth. High growth rates and the ability to rapidly accumulate biomass are characteristic of *Coelastrella* species [Corato et al., 2022; Goecke et al., 2020; Maltsev et al., 2021; Nayana et al., 2022]. For a reference strain (a free-living one) Vinatzer/Innsbruck V 195 (IPPAS H-350 = CCALA 475) *Coelastrella rubescens* (Vinatzer) Kaufnerová & Eliás, 2013, the maximum specific growth rate at the "green" stage was 0.95 day^{-1} under cultivation conditions comparable to those in our study [Minyuk et al., 2017].

After transfer to abiotic stress conditions, by the day 3 of the "red" stage (Fig. 5B), cell abundance rose 1.6-fold relative to the initial concentration. No further growth occurred. A similar response of *C. rubescens* culture at the onset of the "red" stage under the same stress protocol was reported previously [Minyuk et al., 2017]. This increase may be attributed to the algae utilizing nutrients introduced when the concentrated cell suspension was diluted with a medium, despite the medium being depleted in nitrogen and phosphorus. The first signs of secondary carotenogenesis were observed on day 3 after stress. Visually, the culture in flasks changed its color from green to brown-orange; light microscopy revealed a rise in cell size and altered coloration (see Fig. 3B).

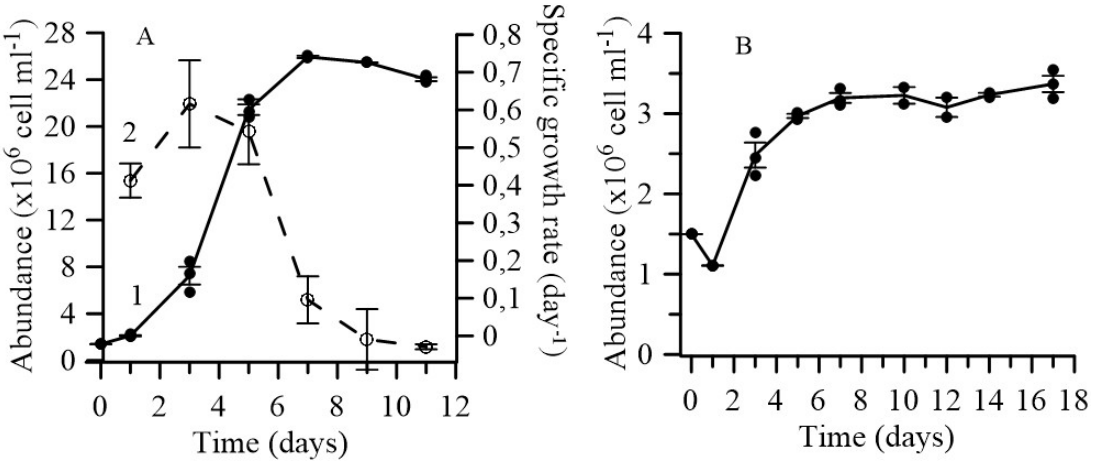

Fig. 5. Dynamics of the cell abundance (1) and the average daily specific growth rate (2) for the IBSS-156 *Coelastrella rubescens* strain during the vegetative stage (A) and secondary carotenogenesis stage (B)

Changes in pigment composition definitely indicate the development of secondary carotenogenesis in cells in response to abiotic stress. This phenomenon is manifested in a decrease in the specific content of chlorophyll and associated primary carotenoids in cells relative to secondary carotenoids [Lemoine, Schoefs, 2010; Shah et al., 2016]. To quantitatively evaluate the dynamics of these processes, researchers use the ratio of absorption peaks of pigment extracts at wavelengths corresponding to the absorption maximums of chlorophyll and secondary carotenoids [Solovchenko, Minyuk, 2021]. To carry out a rapid assessment, we analyzed the absorption spectra of living microalgal cultures at the "green" stage (Fig. 6A) and the "red" one (Fig. 6B). It is evident that spectral profiles of a cell suspension of a living *C. rubescens* culture differ significantly under different cultivation conditions.

At the "green" stage, distinct absorption maximums attributable to chlorophyll *a* were observed in its blue (425 nm) and red (680 nm) regions. An additional shoulder noted at 650 nm in the red region (at 650 nm) is related to chlorophyll *b* absorption: an accessory photosynthetic pigment of green algae. In the blue region, this pigment contributes to a peak at 470–480 nm together with primary carotenoids. Absorption intensities at blue-region maximums exhibited a continuous increase throughout the experiment reaching their highest levels on day 11. Absorption intensities at red-region maximums remained unchanged after day 5 (Fig. 6A).

During the "red" stage, a progressive smoothing of the absorption spectrum was noted in chlorophyll absorption bands, culminating in a unimodal profile with a broad peak shifted towards the blue-green region by day 12 of the experiment. At 480–490 nm, a progressive increase in peak intensity was recorded reflecting accumulation of secondary carotenoids. By day 17, the values dropped sharply without spectral change. Concurrently, initial indicators of the alga's transition to a resting state were observed: complete cessation of cell division and thickening of the outer cell membrane. The observed spectral dynamics aligns with the established concept of gradual degradation of the photosynthetic apparatus in microalgae during their transition to a resting state, and also with the accumulation of ketocarotenoids in cells. The absorption maximums of these pigments are shifted to longer wavelengths compared to those of primary carotenoids [Lemoine, Schoefs, 2010]. Thus, changes in the absorption spectra of living cultures of carotenogenic microalgae during cultivation can be used for rapid assessment of the physiological state of cells and for monitoring the accumulation of ketocarotenoids in a culture.

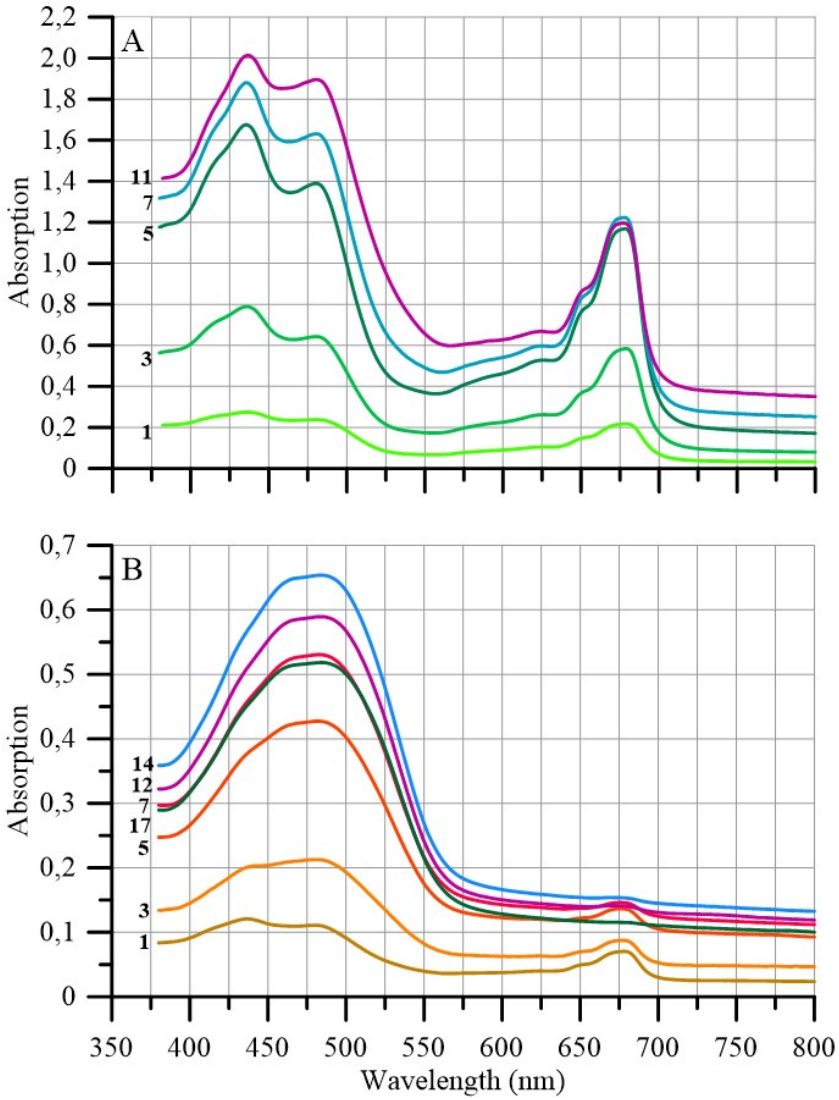

Fig. 6. Changes in the shape of optical absorption spectra of a cell suspension of a living culture of the IBSS-156 *Coelastrella rubescens* strain during the "green" (A) and "red" (B) stages of cultivation (digits on curves indicate the experiment duration in days)

Conclusions:

1. This study reports the first successful isolation of *Coelastrella* cells (Scenedesmaceae, Sphaeropleales) from a thallus of a Crimean epilithic lichen into a unialgal culture. The demonstrated simplicity and reproducibility of the isolation protocol substantiate the utility of the modified method of density gradient centrifugation described in the paper for routine isolation of lichen photobiont cells into a culture.
2. The taxonomic position of the isolated strain within Chlorophyta was confirmed by molecular genetic identification and scanning electron microscopy. Based on a comprehensive combination of morphological and molecular traits, the strain is assigned to the genus *Coelastrella* of the family Scenedesmaceae within the order Sphaeropleales. Phylogenetic analysis of the ITS1–5.8S–ITS2 fragment of the ribosomal DNA cluster with high statistical support assigns the strain to the species *C. rubescens*. The isolate has been deposited in the collection of living cultures of carotenogenic microalgae at IBSS (https://algae.ibss-ras.ru) as IBSS-156 *Coelastrella rubescens*.

3. The strain demonstrated high specific growth rate, up to (0.62 ± 0.11) day^{-1}, and the capacity to accumulate both green biomass and secondary carotenoids under two-stage cultivation conditions. Absorption spectra of cell suspensions at the "red" stage clearly reflect the degradation of green photosynthetic pigments and biosynthesis of secondary carotenoids. Spectral monitoring of living microalgal cultures is a method for rapid assessment of their physiological state and for analysis of ketocarotenoid accumulation during cultivation.

These findings indicate the possibility of isolating promising astaxanthin producers adapted to local conditions from Crimean lichens. Further research is required on the biological properties and commercial potential of the new strain IBSS-156 *Coelastrella rubescens* as a candidate biotechnological object for mass cultivation under the climatic conditions of Crimea and southern Russia, with the aim of producing ketocarotenoids of the astaxanthin group, which are of high medical and nutritional value.

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НОВЫЙ ШТАММ ЗЕЛЁНОЙ КАРОТИНОГЕННОЙ МИКРОВОДОРОСЛИ *COELASTRELLA RUBESCENS*, ПЕРСПЕКТИВНЫЙ ДЛЯ КУЛЬТИВИРОВАНИЯ В КЛИМАТИЧЕСКИХ УСЛОВИЯХ ЮГА РОССИИ

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Впервые из таллома эпилитного крымского лишайника выделена альгологически чистая культура зелёной каротиногенной микроводоросли рода *Coelastrella* Chodat (Scenedesmaceae, Sphaeropleales), которая характеризуется выраженной способностью к накоплению вторичных каротиноидов в условиях острого абиотического стресса. Детально описаны методы выделения фикобионта из малых навесок образца лишайника, получения альгологически чистой и аксеничной культур, адаптации клеток к условиям лабораторного культивирования и длительного хранения в коллекционном фонде. Выполнена таксономическая идентификация фикобионта с использованием морфологических подходов (световой и сканирующей электронной микроскопии) и молекулярно-генетических методов (анализа фрагмента последовательности региона ITS1 — 5.8S — ITS2 гена 18S rRNA). Полученный штамм внесён в каталог коллекции живых культур каротиногенных микроводорослей ФИЦ ИнБЮМ (https://algae.ibss-ras.ru) как IBSS-156 *Coelastrella rubescens* (Vinatzer) Kaufnerová & Eliás, 2013. Проведена оценка ростовых характеристик штамма IBSS-156 в условиях двухстадийной накопительной культуры. Получены данные, свидетельствующие о высоких, до (0,62 ± 0,11) сут$^{-1}$, удельных скоростях роста культуры

на вегетативной стадии. Динамика пигментного состава водорослей при действии комплексного стресса свидетельствует о накоплении вторичных каротиноидов. Показано, что регистрация спектров поглощения живых культур микроводорослей в видимой области может быть эффективным способом экспресс-оценки их состояния и скорости накопления кетокаротиноидов в биомассе в процессе двухстадийного культивирования. Результаты работы указывают на возможность выделения из крымских лишайников перспективных продуцентов астаксантина, которые могут быть предложены для массового культивирования в зонах с засушливым и жарким климатом, в том числе в Крыму и прилегающих районах юга России.

Ключевые слова: каротиногенные микроводоросли, фикобионты, *Coelastrella*, двухстадийное культивирование