PRELIMINARY STUDIES ON THE GROWTH AND BIOCHEMICAL COMPOSITION OF A PROMISING CAROTENOID PRODUCING STRAIN *COELASTRELLA* SP.

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Abstract

The green microalga *Coelastrella* sp. is a promising but poorly studied producer of valuable carotenoids (canthaxanthin, astaxanthin and β-carotene). Our aim was to find the most appropriate mineral medium for optimal algal growth, which is of key importance before the application of stress factors for induction of carotenoid accumulation. Initially the alga was cultivated in: BG_{11}; BG_{11} + 1 g/l $NaHCO_3$; and medium after Šetlik (1967), modified by Georgiev et al. (1978) with ½ concentration of nutrients (noted as ½ ChR). The best growth (5,7 g/l in exponential phase) was achieved in ½ ChR. The next step was studying the effect of dilution of ChR medium on the growth and main cellular metabolites. In stationary growth phase *Coelastrella* sp. accumulated carbohydrates to the highest extent (57,9 ± 2,6% of dry weight) in 4-fold diluted medium. The highest lipid content was detected in 1/8x ChR (36,5 ± 0,9%). The most concentrated media was the most favorable for protein accumulation (35,3 ± 0,3%). Pigments (chlorophyll *a*, *b* and carotenoids) increased with the elevated nutrient concentration in the medium as well as with aging of the cultures. These results could serve as a basis for future implementation of *Coelastrella* sp. strain BGV in mass cultivation process and production of carotenoids.

Key words: *Coelastrella, growth, medium, carotenoids*

Introduction

Carotenoids are among the most common, naturally occurring terpenoid pigments. Their color varies from yellow to orange or red, depending on the number of conjugated double bonds of the polyene chain and corresponds to their ability to absorb photons in the blue and near-UV regions. These pigments are synthesized de novo by all photosynthetic organisms, some bacteria and fungi. Men and animals have no ability to synthesize carotenoids and they should be obtained from the diet.

Microalgae are a major natural source for a vast array of valuable compounds, including a diversity of pigments, for which these photosynthetic microorganisms represent an almost exclusive biological resource (Del Campo et al., 2007; Guedes et al., 2011). Carotenoids have an industrial use in food products and cosmetics as vitamin supplements and health food products and as feed additives for poultry, livestock, fish, and crustaceans (Anunciato et al., 2012; Kovač et al., 2013). Carotenoids are well known as effective agents in prevention and treatment of various human diseases – cardiovascular disorders, cancer, diabetes, cataract, retinal macular degeneration, night blindness, brain ischemia, etc. (Ambati et al., 2014; Zhang et al., 2014).

Carotenoid production has proven one of the most successful branches in microalgal biotechnology (Del Campo et al., 2007). The growing needs of mankind for natural products demand much more efforts in widening the production and improvement of technology for greater carotenoid yields from biological resources. *Dunaliella salina* and *Haematococcus pluvialis* are the best commercial sources of β-carotene and astaxanthin, respectively (Tafreshi & Shariati, 2009; Han et al., 2013). The literature is abundant in articles concerning the conditions for their optimal growth, carotenoid accumulation under stress impact, mass outdoor cultivation techniques, etc. The microalgae of genus *Coelastrella* (Chlorophyta) can also synthesize significant amounts of carotenoids at salt stress and high light intensity (Hu et al., 2013). These algae are less studied than *Dunaliella salina* and *Haematococcus pluvialis* and are promising subject for in-depth

investigation in terms of their potential implementation in industrial production.

The aim of our work was preliminary study on the effect of mineral media composition on the growth and qualitative composition of the biomass of a newly isolated strain *Coelastrella* sp. BGV as initial step for a future production process.

Materials and methods

The alga used in this study was identified as *Coelastrella* sp. and marked as strain BGV. Samples were collected from a metal trough near Varvara village, Bulgaria (N 42° 10′; E 24° 0-7′). The isolation technique was streaking cells across agar plates (Andersen & Kawachi, 2005). Initially monoalgal, non-axenic cultures of *Coelastrella* sp. strain BGV were grown autotrophically in three different culture media (Fig. 1): BG_{11} (Rippka et al., 1979); BG_{11} + 1 g/l $NaHCO_3$ and medium after Šetlik (1967), modified by Georgiev et al., 1978 (noted in the text with the abbreviation ChR) with ½ concentration of nutrients (½ ChR) for 336 hours (14 days). The next step was cultivation of *Coelastrella* sp. in different dilutions of ChR medium (1/8; ¼; ½ and 1x) for 312 hours (13 days) – Figure 2. All cultures were incubated at 30 ± 1°C and continuous lateral illumination with cool-white fluorescent lamps at a photon flux density of 132 μmol m^{-2} s^{-1}. A carbon source was provided by bubbling 2% CO_2 (v/v) in air through the suspensions. Protein content was determined using the method of Lowry et al. (1951); total carbohydrates were analyzed by the phenol-sulphuric acid method (Dubois et al., 1956); pigments (chlorophyll *a* and *b*, carotenoids) were extracted with hot methanol, determined spectrophotometrically and calculated by Mackinney's formulae (1941). Total lipid content was measured as described by Petkov (1990) after 2 times extraction of 20-30 mg of wet biomass with a mixture of chloroform:methanol (2:1, v/v). Received data are shown on Figures 3-7.

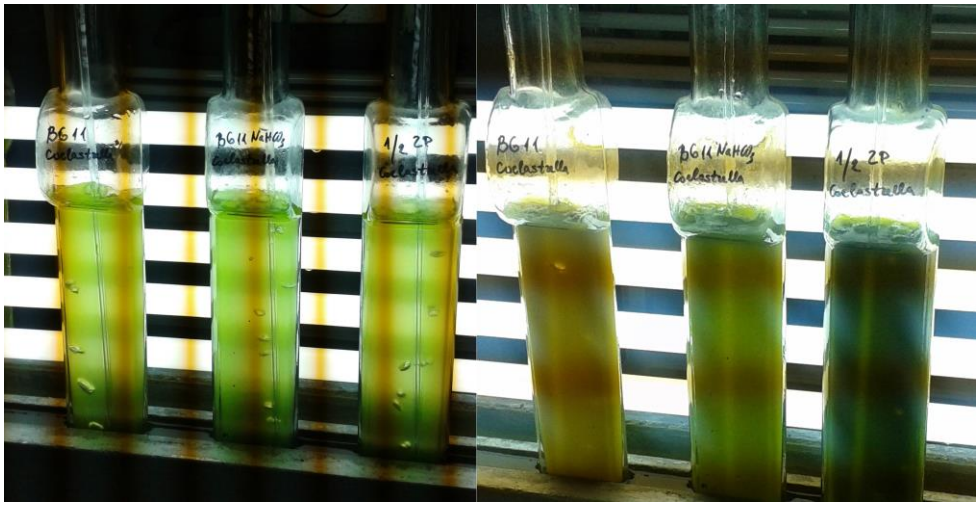

Figure 1. *Coelastrella* sp. strain BGV cultures grown in BG_{11}; BG_{11} + 1 g/l $NaHCO_3$ and ½ ChR media, in the beginning – 0h (left) and at the end of experiment – 336 h (right).

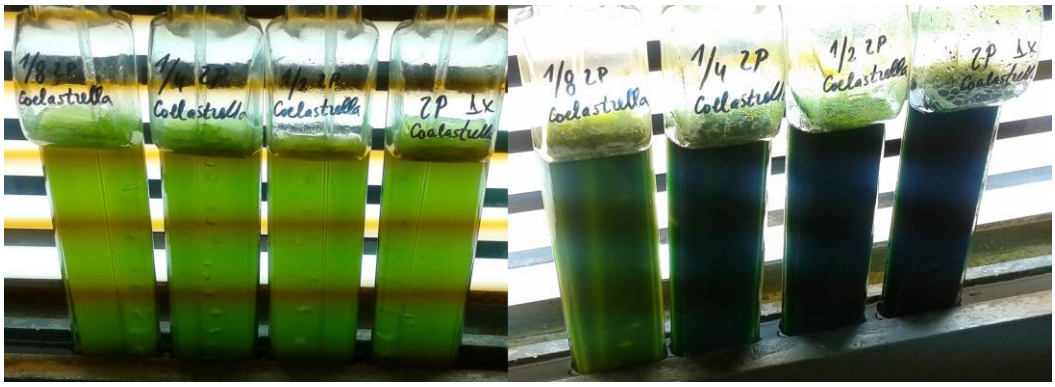

Figure 2. *Coelastrella* sp. strain BGV cultures grown in 1/8; ¼; ½ and 1x ChR media, in the beginning – 0h (left) and in the late exponential growth phase – 168 h (right).

Results and discussion

In their original habitats such as fountains and temporary waterbodies (troughs, roadside ditches), the *Coelastrella* strains are often found co-existing with strains of the green alga *Haematococcus pluvialis* (Neofotis et al., 2016). During the isolation procedure in our study we also have found and tried to isolate *H. pluvialis* but till this moment *Coelastrella* sp. and filamentous blue-green algae dominate and suppress *Haematococcus* growth.

The medium after Šetlik (1967), modified by Georgiev et al. (1978), noted in the text as ChR, is widely used at different dilutions for mass outdoor cultivation of microalgae from Chlorophyceae, like *Chlorella* and *Scenedesmus* (Pilarski, 1994). According to the original recipe ChR medium has the highest nutrient concentration – about 7,5 g/l. For comparison BG_{11} (Rippka et al., 1979); and BG_{11} + 1 g/l $NaHCO_3$ have 4.4-times and 2.8 times less nutrients than 1x ChR. Initially we have chosen BG_{11}, BG_{11} + $NaHCO_3$ and ½ ChR media on the basis of our previous experience with other green microalgae (Gacheva & Pilarski, 2008; Gacheva et al., 2015; Cohen, 2015). Two-times diluted ChR provides the best growth – 5,7 ± 0,4 g/l in late exponential phase (288 h) and the highest pigment content in early stationary stage (1,15 ± 0,1 % of dry weight) – Fig. 3 and Fig. 4.

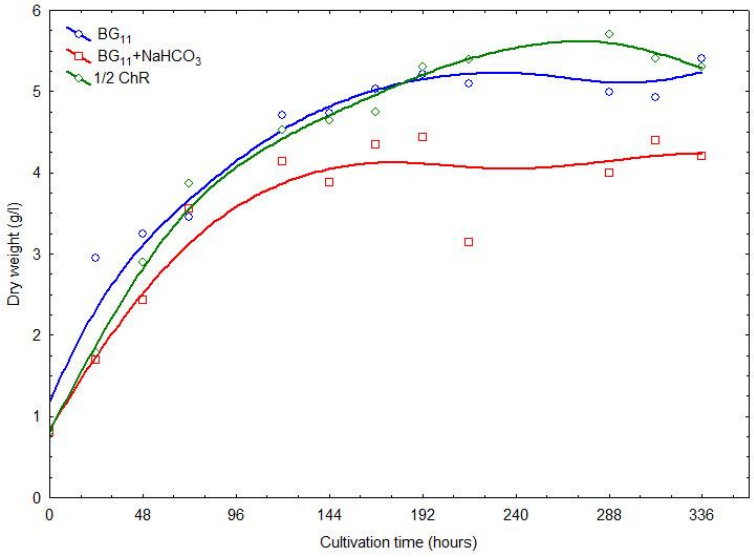

Figure 3. Growth curves of *Coelastrella* sp. BGV cultivated in BG_{11}; BG_{11} + 1 g/l $NaHCO_3$ and ½ ChR media.

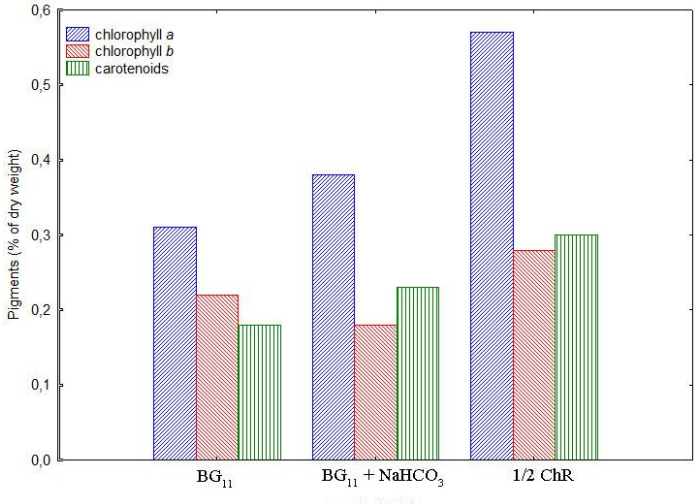

Figure 4. Pigment content (% of dry weight) in *Coelastrella* sp. BGV depending on the mineral medium.

The next step was studying the influence of ChR medium dilution on the growth and main metabolites in *Coelastrella* sp. BGV. It is interesting that no growth delay can be noticed for none of the dilutions during the first days after inoculation (no lag phase), which shows that *Coelastrella* sp. cells adapt very quickly to different nutrient concentrations (Fig. 5). Intensive growth of the cultures continues for more than 288 hours except for the 1/8 ChR variant (192 h). Increased concentrations of mineral elements in the medium (½ ChR and 1x ChR) provide good growth of the strain, that is of interest for the mass outdoor production of microalgal biomass (Livansky et al., 1993, 1995).

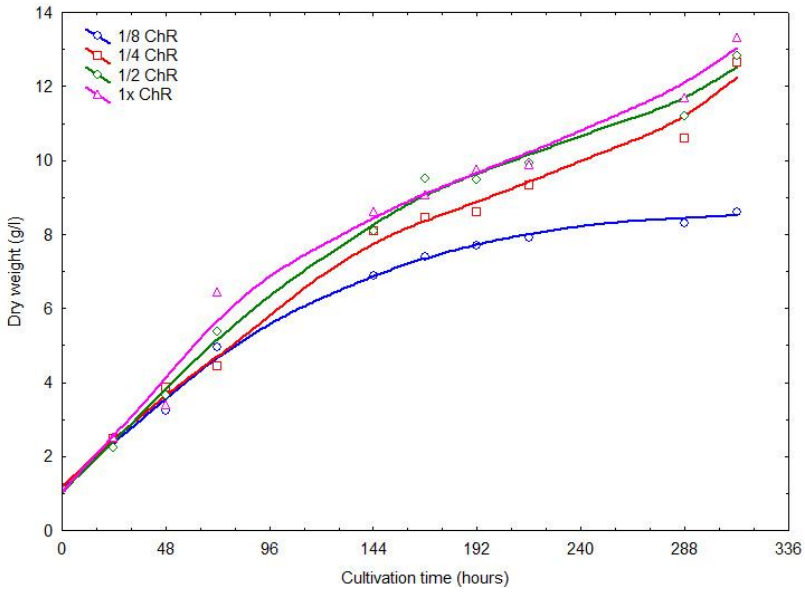

Figure 5. Growth curves of *Coelastrella* sp. BGV grown in different concentrations of mineral medium (1/8; ¼; ½ and 1x ChR) for 312 hours.

Proteins of *Coelastrella* sp. BGV ranged from 18,2 – 38,2% of dry weight in late exponential stage and 19,1 – 35,3% in early stationary stage. The elevated nutrient concentration in the medium stimulates protein synthesis regardless of the growth phase, but with aging of the cultures protein levels decreased, except for 1/8 ChR variant (Fig. 6A). Our results, especially those for ¼ ChR, correspond with protein

content in *Coelastrella oocystiformis* grown in BG_{11} medium with and 3% CO_2 (27% of dry weight = DW) found by Iyer et al. (2015).

Carbohydrates are the most abundant main metabolites in *Coelastrella* sp. BGV cells (35,8 – 68,2 % of DW). There is no correlation between the most intensive growth and carbohydrate content. Gradual decrease of carbohydrate levels can be noted with the increase of nutrient elements and culture aging (Fig. 5B). The lowest nutrient concentration stimulates synthesis of carbohydrates and lipids to the highest extent – Fig. 6B and 6D (68,2% and 36,5%, respectively). The growth stage affects carbohydrate synthesis most considerably in 1/8 ChR variant. Iyer et al. (2015) also determined the highest percent of carbohydrates (44% of DW) in *Coelastrella oocystiformis*.

Total lipids of *Coelastrella* sp. BGV vary from 17,7% to 36,5% of dry weight in early stationary phase. The increased medium concentrations does not have any significant effect on lipid production (Fig. 6D). The lowest nutrient concentration (1/8 ChR) stimulates lipid accumulation. We detect much higher lipid values (36,5% of dry biomass) than those achieved for the thermotolerant strain *Coelastrella* sp. F50 – 16% of DW in early stationary stage and 22% after salt stress and high light intensity (Hu et al., 2013). Neofotis et al. (2016) demonstrate that a few *Coelastrella* strains perform not only well in the laboratory. The strain DOE0202 also has been tested positively in small raceway-type ponds. The authors indicate that these new *Coelastrella* strains might be employed as new platform strains for biofuel and/or bioproduct generation.

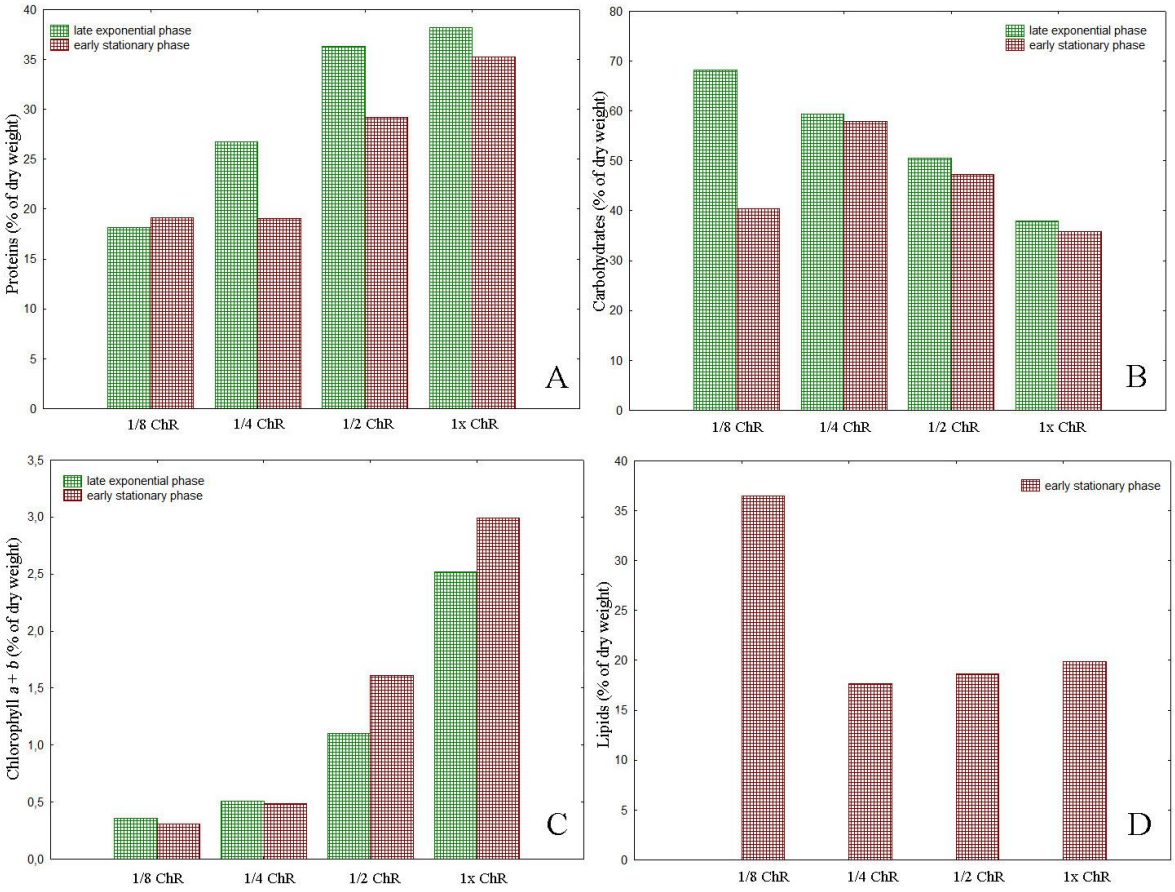

Figure 6. Biochemical composition of *Coelastrella* sp. BGV depending on the dilution of ChR medium. A – proteins; B – carbohydrates; C – chlorophyll *a* + *b*; D – lipids (% of dry weight). Green bars –

late exponential phase; red bars – early stationary phase.

Pigments (chlorophyll *a*, *b* and carotenoids) of *Coelastrella* sp. BGV increase with the elevated nutrient concentration in the medium as well as with aging of the cultures (Fig. 6C and Fig. 7). In the early stationary stage the most concentrated medium (1x ChR) leads to more than 3 times higher carotenoid content. Our results for carotenoids in exponential growth phase (0,1 – 0,13% of DW) correspond with total carotenoids detected in the green cells of *Coelastrella oocystiformis* (Iyer et al., 2015). These authors manage to enhance the carotenoid production to 1,97% in the red cells.

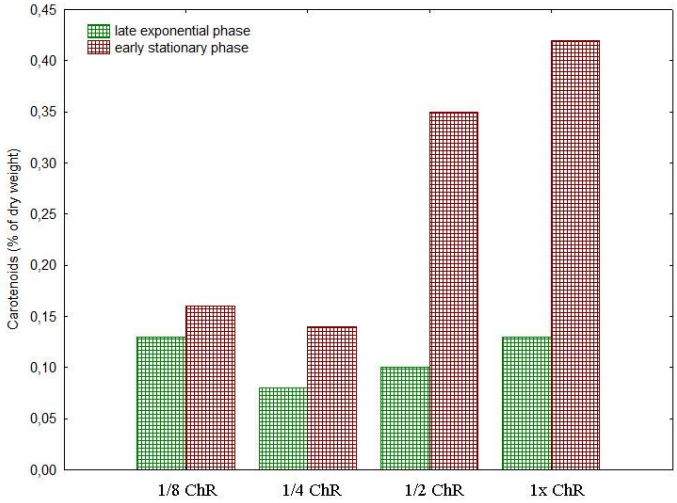

Figure 7. Carotenoid content (% of dry weight) of *Coelastrella* sp. BGV depending on the dilution of the mineral medium. Green bars – late exponential phase; red bars – early stationary phase.

Conclusions

This study deals with the initial step of biotechnology of a promising carotenoid producing strain *Coelastrella* sp. BGV, namely establishment of the appropriate mineral medium composition for optimal growth. The newly isolated *Coelastrella* sp. BGV is a fast growing strain, easily adapted to various concentrations of nutrients in the medium. More concentrated media (½ ChR and 1x ChR) provide the best growth and stimulate the accumulation of pigments together with aging of the cultures. Carbohydrate and lipid values increase significantly when the cultures are grown in 1/8 ChR medium.

The accumulation of sufficient biomass with balanced biochemical quality is a pre-condition for induced carotenoid synthesis. The following step in our experimental work will be applying stress factors like salt stress, high temperatures and light intensities for increase of carotenoid content in *Coelastrella* sp. BGV. We consider the current results concerning the growth and biochemical characterization of this novel strain as a basis for future caretonoid production.

Acknowledgments:

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Literature

Ambati R. R., S.-M. Phang, S. Ravi, R. G. Aswathanarayana. 2014. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications - a review. Mar. Drugs, 12: 128-152.

Andersen R. A, M. Kawachi, 2005. Traditional microalgae isolation techniques. In: Algal culturing techniques. Ed. R.

A. Andersen, Elsevier Academic Press, 83–100.

Anunciato T. P., & P. A. da Rocha Filho. 2012. Carotenoids and polyphenols in nutricosmetics, nutraceuticals, and cosmeceuticals. Journal of Cosmetic Dermatology, 11 (1): 51-54.
Cohen J. 2015. The effect of mineral media composition on the growth and biochemical composition of *Haematococcus pluvialis* BS2. Thesis, 44 pp. (in Bulgarian).

Del Campo J. A., M. García-González,M. G. Guerrero. 2007. Outdoor cultivation of microalgae for carotenoid production: current state and perspectives. Appl. Microbiol. Biotechnol., 74: 1163-1174.

DuBois M., K. A. Gilles, J. K. Hamilton, P. A. Rebers F. Smith, 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem., 28 (3): 350–356.

Gacheva G., P. Dimitrova, P. Pilarski. 2015. New strain *Haematococcus* cf. *pluvialis* Rozhen-12 – growth, biochemical characteristics and future perspectives. Genetics and Plant Physiology, 5 (1): 29-38.

Gacheva G., P. Pilarski. 2008. The resistance of a new strain *Chlorella* sp. R-06/2, isolated from an extreme habitat to environmental stress factors. Gen. Appl. Plant Physiol., special issue, 34(3-4): 347-360.

Georgiev D., H. Dilov, S. Avramova. 1978. Millieu nutritif tamponne et méthode de culture intensive des microalgues vertes. Hydrobiology Bulgaria, 7: 14– 23 (In Bulgarian).

Guedes A. C., H. M. Amaro, F. X. Malcata. 2011. Microalgae as sources of carotenoids. Mar. Drugs, 11 (9): 625-644.

Han D., Y. Li, Q. Hu. 2013. Astaxanthin in microalgae: pathways, functions and biotechnological implications. Algae, 28(2): 131-147.

Hu C. W., L. T. Chuang, P. C. Yu, C. N. N. Chen. 2013. Pigment production by a new thermotolerant microalga *Coelastrella* sp. F50. Food Chem., 138: 2071-2078.

Iyer G., V. Nagle, Y. V. Gupte, S. Desai, M. Iyer, N. Moramkar, V. Sawant. 2015. Characterization of high carotenoid producing *Coelastrella oocystiformis* and its anti-cancer potential. Internat. J. Curr. Microbiol. Appl. Sci., 4 (10): 527-536.

Kovač D. J., J. B. Simeunović , O. B. Babić , A. Č. Mišan , I. Lj. Milovanović. 2013. Algae in food and feed. Food & Feed Res., 40 (1): 21-31.

Livansky K., M. Kajan, P. Pilarski. 1993. pCO2 and pO2 profiles along the flow of algal suspension in open solar culture units: Verification of a matematical model. Archiv für Hydrobiologie, 98 (Algological studies 70): 97-119.

Livansky K., M. Kajan, P. Pilarski. 1995. Productivity, respiration and chemical composition of the green alga *Scenedesmus incrassatulus* grown in outdoor cultivation units with and without baffles. Archiv für Hydrobiologie, 106 (Algological studies 76): 111-128.
Lowry O., N. Rosenbrough, A. Z. Farr, R. J. Randball, 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265–275.

Mackinney G., 1941. Criteria for purity of chlorophyll preparations. J. Biol. Chem., 132: 91–96.

Neofotis P., A. Huang, K. Sury,W. Chang, F. Joseph, A. Gabr, S. Twary, W. Qiu, O. Holguine, J. E.W. Polle. 2016. Characterization and classification of highly productive microalgae strains discovered for biofuel and bioproduct generation. Algal Res., 15: 164-178.
Petkov G. 1990. Lipids of photoautotrophically cultivated microalgae. Ph. D. Thesis, IPP, BAS, 119 pp. (In Bulgarian).

Pilarski P. 1994. Optimization of productivity of green microalgae from genus *Scenedesmus* cultivated outdoor. PhD thesis, IPPG – BAS (in Bulgarian).

Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, R. Y. Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J. Gen. Microbiol., 111:1-61.

Šetlik, I. 1967. Contamination of algal cultures by heterotrophic microorganisms and its prevention. Ann. Rep. Algol. for the Year 1966, Trebon, CSAV, Inst. Microbiol., 89-100.

Tafreshi A. H., M. Shariati. 2009. *Dunaliella* biotechnology: methods and applications. J. Appl. Microbiol., 107: 14-35.

Zhang J., Z. Sun, P. Sun, T. Chen, F. Chen. 2014. Microalgal carotenoids: beneficial effects and potential in human health. Food Funct., 5 (3): 413-425.