Genetics and Plant Physiology – 2015, Volume 5(1), pp. 29–38
Special Issue – Project BG051PO001-3.3.06-0025 "Support for training and development of young competitive experts in the field of physiology, phytochemistry, genomics, proteomics and biodiversity of eukaryotic organisms"

Available online at http://www.ifrg-bg.com

# NEW STRAIN *HAEMATOCOCCUS* CF. *PLUVIALIS* ROZHEN-12 – GROWTH, BIOCHEMICAL CHARACTERISTICS AND FUTURE PERSPECTIVES

***Gacheva G.**, *P. Dimitrova, P. Pilarski***

*Institute of Plant Physiology and Genetics, BAS, Acad. G. Bonchev Str., bldg. 21, 1113, Sofia, Bulgaria*

Received: *17 October 2014* Accepted: *13 January 2015*

**Summary:** Growth and biochemical composition of a newly isolated Bulgarian strain *Haematococcus* cf. *pluvialis* Rozhen-12 were studied in relation with culture media composition and temperature regime. The best growth and the highest carotenoid content (5.6 mg/g biomass) during the vegetative phase were obtained when the alga was cultivated at the highest salt concentration in the medium (with urea and $NH_4NO_3$ as nitrogen sources). The strain showed a significant adaptive capacity, retaining high biomass productivity in a wide temperature range 25 – 38°C and a photon flux density of of 2 x 132 μmol $m^{-2}$ $s^{-1}$, which makes this alga suitable for outdoor mass cultivation.

**Citation:** 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.

**Key words**: carotenoids, culture media, growth, *Haematococcus*, temperature.

## INTRODUCTION

The world's needs of carotenoids (used as natural dyes and nutraceuticals) are continuously growing whereas the demands for biotechnologically produced pigments (astaxanthin, canthaxanthin, lutein, etc.) are also increasing. The main producers of carotenoids among microalgae belong to Chlorophyceae - *Chlorella, Chlamydomonas, Dunaliella, Haematococcus* and *Muriellopsis* (Pulz and Gross, 2004). These algae can accumulate carotenoids inside their cells and therefore are an alternative to the chemically synthesized pigments (Bhosale and Bernstein, 2005).

Microalgal carotenoids are successfully applied in the food industry and human health care, with their antioxidant properties being the focus. For example, natural β-carotene is a preferred health product because it is a mixture of *cis* and *trans* isomers and has an antitumour effect (Demming-Adams and Adams, 2002).

*Corresponding author: gergana_gacheva@abv.bg

Astaxanthin is a xanthophyll carotenoid, which is found in various microorganisms and marine animals. It exhibits strong antioxidant, anti-lipid peroxidation, anti-diabetic and anticancer activities, as well as cardiovascular disease prevention and immune-modulation properties (Ranga Rao et al., 2014). The green alga *Haematococcus pluvialis* accumulates a higher quantity of astaxanthin under stress conditions such as high salinity, nitrogen deficiency, high temperature and light (Sarada et al., 2002; 2012; Ranga Rao, 2011). Astaxanthin produced from *H. pluvialis* is a main source for human consumption (Kidd, 2011). Astaxanthin products in forms of tablets, capsules, syrups, oils, soft gels, creams are available on the market.

Bulgaria is one of the countries in the world where mass outdoor cultivation of microalgae (*Chlorella, Scenedesmus* and *Arthrospira*) has been achieved. In the recent years, despite of the great interest in *Haematococcus* biomass production, including a number of Bulgarian companies, no extensive research has been done.

The aim of this work was to isolate and characterize a new local strain from *Haematococcus* genera that could be used for commercial production.

## MATERIALS AND METHODS

The studies were conducted with the strain *Haematococcus cf. pluvialis* Rozhen-12. Samples were collected from an old granite bed of a dried fountain near Rozhen village (Blagoevgrad region). The isolation technique included streaking cells across agar plates (Andersen and Kawachi, 2005) resulting in the formation of single colonies. The strain was kept in the collection of the Department of Experimental Algology at the Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences.

Monoalgal, non-axenic cultures of *Haematococcus* cf. *pluvialis* strain Rozhen-12 were grown autotrophically for 8 days (192 h) in different culture media: $BG_{11}$ (Rippka et al., 1979); modified $BG_{11}$ (Boussiba and Vonshak, 1991) and medium of Šetlik (1967), modified by Georgiev at al. (1978) at ½ concentrations of nutrients, noted as ½ ChR (Table 1). A carbon source was provided by bubbling 2% $CO_2$ (v/v) in air through the suspensions. A block for intensive cultivation of algae at set temperatures (21.5 - 40.5°C) and a photon flux density of 2 x 132 µmol $m^{-2}$ $s^{-1}$ was used for 4 days (96 h)-cultivation of *H.*cf. *pluvialis* strain Rozhen-12.

Biomass for biochemical analysis was harvested at the end of the experiments.

The concentration of the dried algal biomass was analyzed gravimetrically. Protein content was measured following the method of Lowry (1951). Carbohydrate content was determined following the phenol-sulfuric acid method of Dubois et al. (1956). 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). Pigments were extracted with boiling methanol and their content was determined spectrophotometrically. Pigment quantity and growth rate were calculated based on Mackinney's formula (1941) and Levasseur et al. (1993), respectively.

**Table 1.** Chemical composition of different culture media used for cultivation of *Haematococcus* cf. *pluvialis* Rozhen-12.

| № | Elements | $BG_{11}$ [mg $l^{-1}$] | mod.$BG_{11}$ [mg $l^{-1}$] | ½ ChR [mg $l^{-1}$] |
|---|---|---|---|---|
| 1 | $NaNO_3$ | 1500 | 1500 | - |
| 2 | $K_2HPO_4$ | 40 | 320 | - |
| 3 | $KH_2PO_4$ | - | - | 340 |
| 4 | $MgSO_4.7H_2O$ | 75 | 200 | 984.5 |
| 5 | $CaCl_2.2H_2O$ | 36 | 36 | - |
| 6 | EDTA-$Na_2$ | 1 | 1 | - |
| 7 | $Na_2CO_3$ | 20 | 100 | - |
| 8 | NaCl | - | - | - |
| 9 | $H_3BO_3$ | 2.86 | 2.86 | 31 |
| 10 | $MnCl_2.4H_2O$ | 1.81 | 1.81 | - |
| 11 | $ZnSO_4.7H_2O$ | 0.22 | 0.22 | - |
| 12 | $Na_2MoO_4.2H_2O$ | 0.39 | 0.39 | - |
| 13 | $CuSO_4.5H_2O$ | 0.08 | 0.08 | 2.25 |
| 14 | $Co(NO_3)_2.6H_2O$ | 0.05 | 0.05 | - |
| 15 | $NH_4NO_3$ | - | - | 800 |
| 16 | $CO(NH_2)_2$ | - | - | 600 |
| 17 | $Fe_2(SO_4)_3.9H_2O$ | - | - | 1.40 |
| 18 | $MnSO_4.4H_2O$ | - | - | 1.10 |
| 19 | $ZnSO_4.5H_2O$ | - | - | 1.45 |
| 20 | $CoSO_4.7H_2O$ | - | - | 1.40 |
| 21 | $(NH_4)_2MoO_4.7H_2O$ | - | - | 1.20 |
| 22 | $NaHCO_3$ | - | - | 2000 |
| 23 | Citric acid | 6 | 6 | - |
| 24 | Ammonium-ferric citrate | 6 | 6 | - |
| | Sum [mg $l^{-1}$] | 1689.41 | 2174.41 | 4764.30 |

## RESULTS AND DISCUSSION

The first step of our work was to determine the most suitable culture medium for optimal growth of *Haematococcus cf. pluvialis* Rozhen-12. The intensive cultivation was carried out at 36°C and a photon flux density of 2 x 132 μmol $m^{-2}$ $s^{-1}$. The results are shown in Fig. 1.

The biggest linear phase and the maximal culture density were reached when the medium with the highest salt concentration was used (½ ChR medium). These results are of interest for outdoor mass cultivation, especially when using technology for thin layer cultivation (5-10 mm) and increased density (8-12 g $l^{-1}$) of the algal suspension (Livansky and Pilarski, 1993; Livansky et al., 1993, 1995).

Qualitative composition of microalgal biomass is an important feature in mass cultivation. Differences in the content of some metabolites in algal biomass depending on the culture media are an expected result of the high plasticity of cellular metabolism. The chemical

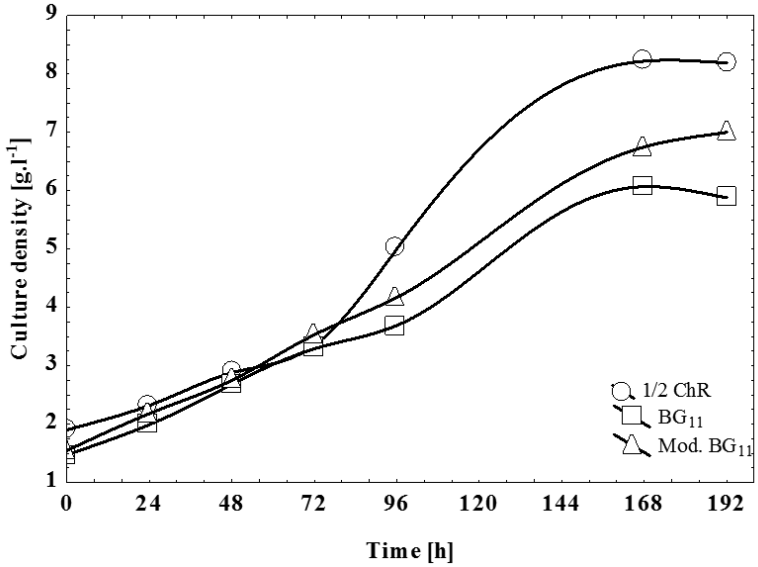


**Figure 1.** Growth curves of *Haematococcus* cf. *pluvialis* Rozhen-12 cultivated in three different media (the average data are shown).

composition of *Haematococcus cf. pluvialis* Rozhen-12 is presented in Table 2. The main component that we were interested in was carotenoids. It is important to underline the two times higher carotenoid content in ½ ChR medium (in which urea and $NH_4NO_3$ were used as nitrogen sources). Our data are in contrast to the results reported by Göksan et al. (2011). The authors studied the effect of various nitrogen sources in vegetative *Haematococcus* cultures and observed the lowest dry weight and pigment (chorophyll and carotenoids) content in the urea-containing medium in comparison with $NaNO_3$, $KNO_3$ and $NH_4NO_3$-containing growth media.

The better biomass productivity and the higher carotenoid content in *H. cf. pluvialis* Rozhen-12 cultivated in ½ ChR medium determined the use of this medium in our further experiments.

*Haematococcus cf. pluvialis Rozhen-12* was cultivated on a block with a temperature gradient (21.5 – 40.5°C) and a photon flux density of 2 x 132 μmol $m^{-2}$ $s^{-1}$. The results on algal growth are shown in Fig. 2.

The growth rate (μ) during the 3rd day was highest at 35°C. In the temperature range 25-38°C the decline in cell growth rate was 19%, while in the range 31-38°C it was only 5%, indicating that the strain retained high biomass productivity in a wide temperature range. These results demonstrate the adaptive capacity of the strain as an advantage in mass outdoor

**Table 2.** Chemical composition of *Haematococcus* cf. *pluvialis* Rozhen-12 depending on the culture media (data presented as % of dry weight).

| | Proteins [% DW] | Carbohydrates [% DW] | Lipids [% DW] | Chlorophyll „a“ [% DW] | Carotenoids [% DW] |
|---|---|---|---|---|---|
| ½ ChR | 21.23±2.98 | 47.20±3.82 | 16.17±2.13 | 1.57±0.17 | 0.66±0.09 |
| Mod. $BG_{11}$ | 13.78±0.14 | 64.22±3.94 | 8.29±1.20 | 0.43±0.03 | 0.37±0.02 |

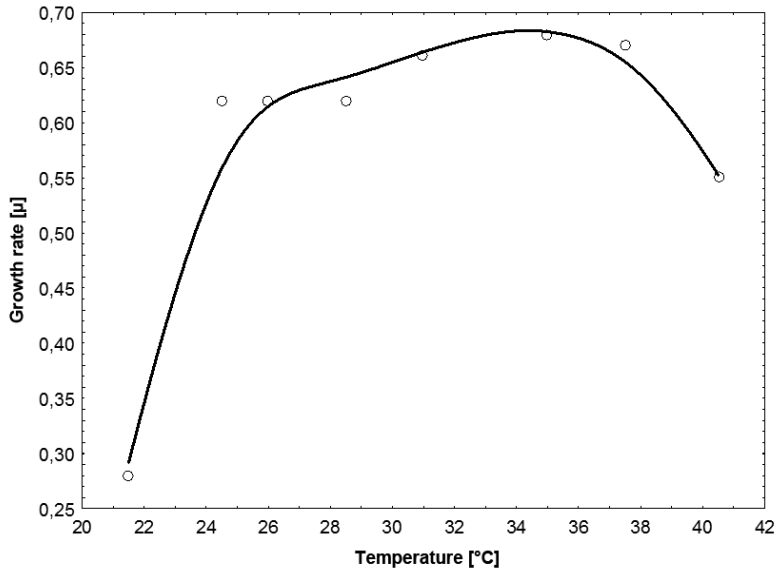


**Figure 2.** Growth rate (72h) of *Haematococcus* cf. *pluvialis* Rozhen-12, cultivated at different temperatures (the average data are shown).

cultivation where diurnal and seasonal fluctuations are significant. The extreme temperatures (21.5°C and 40.5°C) led to growth retardation that was stronger at the low temperature. Neither microscopic changes nor changes in the suspension color were observed.

Unlike *Chlorella* spp. and *Scenedesmus* spp. in which cell proteins represent 50% of dry weight (Livansky et al., 1995; Gacheva and Pilarski, 2008), protein content in *Haematococcus cf. pluvialis* Rozhen-12 was much lower (by average 23.6%) (Fig. 3). Our results

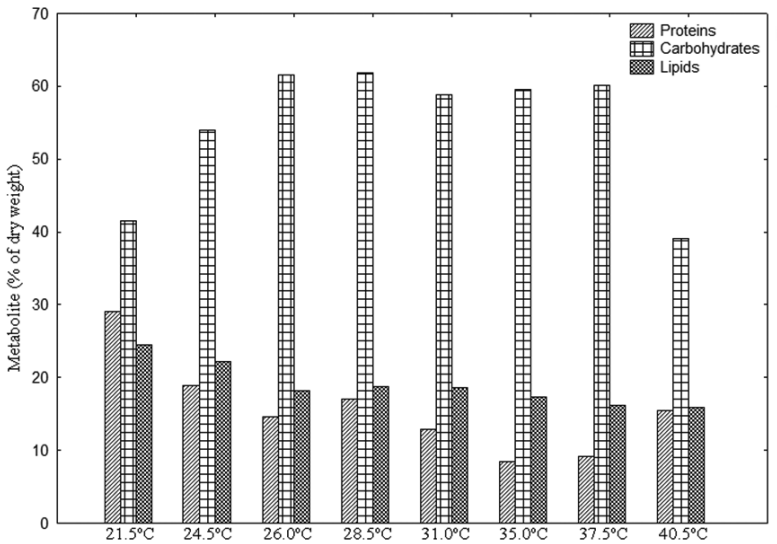


**Figure 3.** Main cellular components (% of dry weight) of *Haematococcus* cf. *pluvialis* Rozhen-12 cultivated at different temperatures (the average data are shown).

confirmed the data presented in a technical review on *Haematococcus* algae where an average protein content of 23.62% on a dry weight basis was pointed (Lorenz, 1999).

Carbohydrate content in *H. cf. pluvialis* Rozhen-12 cells varied from 39% to 62%, but it was relatively constant in the range 24-38°C. Lorenz (1999) indicated 40% carbohydrates in *Haematococcus* cells, but a serious increase in these metabolites (almost 60% of dry weight) could be achieved in nitrogen deficiency conditions during the first day of impact (Recht et al., 2012). The accumulation of carbohydrates in *H. pluvialis* Rozhen-12 (up to 62% of dry weight) could be explained by intensive photosynthesis.

Lipid content in *H. cf. pluvialis* Rozhen-12 was highest (24.5% of dry weight) at 22°C and decreased at 37.5 and 40.5°C to 16.2% and 15.9% of dry weight, respectively (Fig. 3). Low-temperature stress induced the accumulation of lipids in the green microalga *Dunaliella salina* (Norman and Thompson, 1985). This result was confirmed also in our study - the highest lipid level was measured at the lowest temperatures of *Haematococcus pluvialis* Rozhen-12 cultivation. Usually, the conditions which induce lipid synthesis reduce the algal growth as reported for other microalgae (Rodolfi et al., 2009).

The most significant variations (from 1.10 to 1.98% of dry weight) depending on the growth temperature were observed in chlorophyll *a* content (Fig. 4). Cifuentes et al. (2003) reported lower levels of chlorophyll *a* in *H. pluvialis* cultivated in $NaNO_3$ and $NH_4Cl$-containing media under 85 µmol photons $m^{-2}$ $s^{-1}$ (0.38% and 0.63% of dry weight, respectively). Carotenoid levels in *H. pluvialis* Rozhen-12 cells also increased with temperature enhancement and reached a maximum at 37.5°C (0.53% of dry weight). Total carotenoid content estimated in this work may be considered low when compared to data reported in the literature, but the carotenoid accumulation

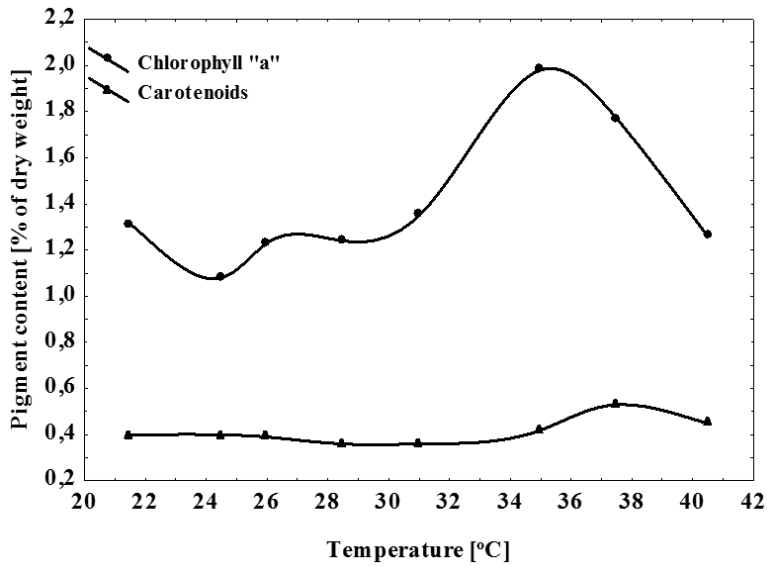


**Figure 4.** Pigment content (% of dry weight) in the cells of *Haematococcus* cf. *pluvialis* Rozhen-12 cultivated at different temperatures (the average data are shown).

depends on the strain and the stress conditions applied: salt stress, nitrate-deprived medium, high light intensity (Cifuentes et al., 2003). Our experiments were conducted under relatively favorable conditions allowing the achievement of optimal microalgal growth, but without a significant stimulation of carotenoid synthesis.

Fatty acid composition of *Haematococcus pluvialis* was also analyzed (Fig. 5). Palmitic and α-linolenic acids were among the most pronounced fatty acids. Zhekisheva et al. (2002) reported the presence of fatty acids with more carbon atoms (20 and 22) in *Haematococcus* cells, however, our results with strain *H. cf. pluvialis* Rozhen-12 did not confirm these data.

The industrial production of

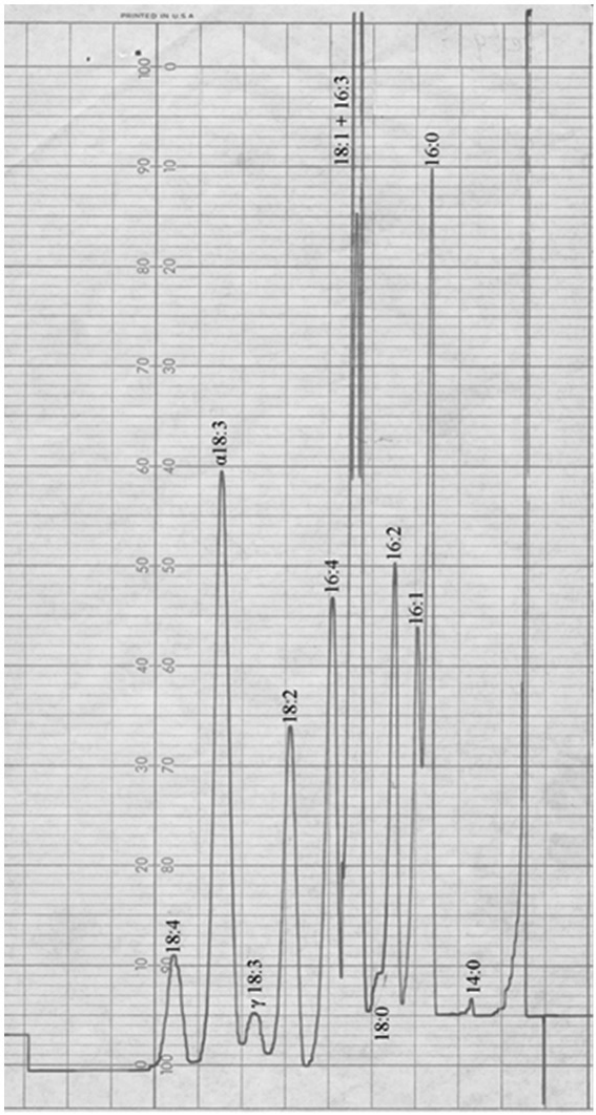


**Figure 5.** Gas-chromatographic record of fatty acid composition of *Haematococcus* cf. *pluvialis* Rozhen-12 cultivated at 35°C and a photon flux density of 132 µmol $m^{-2}$ $s^{-1}$.

astaxanthin from the green alga *Haematococcus pluvialis* is a two-step process: the first step (green phase) is the production of a sufficient volume of green vegetative cell suspension under optimal growth conditions and the second step (red phase) is astaxanthin accumulation in microalgal cells at stress conditions (deprivation of nitrate and phosphate, increased temperature and light intensity, salt stress, etc.). Mass cultivation of *H. pluvialis* is conducted in open ponds or closed photobioreactors mainly at autotrophic conditions.

The main problem with *Haematococcus pluvialis* mass outdoor cultivation is the low growth rate exhibited by the alga under autotrophic conditions and its sensitivity to the climate changes during the cultivation period. Our study was entirely focused on the vegetative growth stage of the local strain *H. cf. pluvialis* Rozhen-12 as an important part of biotechnological astaxanthin production. The obtained results showed that this strain provided biomass with a stable biochemical composition even when cultivation was carried out either at a very low (24.5°C) or high temperature (40.5°C). This feature makes this strain competitive with other *Haematococcus* strains. Further research on the red phase of *H. cf. pluvialis* Rozhen-12 life cycle is needed.

## ACKNOWLEDGEMENTS

The Operational Programme Human Resources Development (2007–2013), the European Social Fund and the Ministry of Education and Science of Bulgaria (Grant № BG051PO001-3.3.06-0025) are thanked for financial support.

## REFERENCES

Andersen R. A, M. Kawachi, 2005. Traditional microalgae isolation techniques. In: Algal culturing techniques. Ed. R. A. Andersen, Elsevier Academic Press, 83–100.

Bhosale P., P. S. Bernstein, 2005. Microbial xanthophylls. Appl. Microbiol. Biotechnol., 68: 445–455.

Boussiba S., A. Vonshak, 1991. Astaxanthin accumulation in the green alga *Haematococcus pluvialis*. Plant Cell Physiol., 32 (7): 1077–1082.

Cifuentes A. S., M. A. González, S. Vargas, M. Hoeneisen, N. González, 2003. Optimization of biomass, total carotenoids and astaxanthin production in *Haematococcus pluvialis* Flotow strain Steptoe (Nevada, USA) under laboratory conditions. Biol. Res., 36: 343–357.

Demming-Adams B., W. W. Adams, 2002. Antioxidants in photosynthesis and human nutrition., 298: 2149–2153.

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. 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).

Göksan T., İ. Ak, C. Kılıç, 2011. Growth characteristics of the alga

*Haematococcus pluvialis* Flotow as affected by nitrogen source, vitamin, light and aeration. Turkish J. Fish. Aquatic Sci., 11: 377–383.

Kidd P., 2011. Astaxanthin, cell membrane nutrient with diverse clinical benefits and anti-aging potential. Altern. Med. Rev., 16: 355–364.

Levasseur M., P. A. Thompson, P. J. Harrison, 1993. Physiological acclimation of marine phytoplankton to different nitrogen sources. J. Phycol., 29 (5): 587–595.

Livansky K, P.S. Pilarski, 1993. Carbon dioxide supply to algal cultures. II. Efficiency of $CO_2$ absorption from a natural gas supplied to the recirculation pipe of a cultivation unit. Archiv fur Hydrobiologie, 97 (Algological studies 69): 113–123.

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.

Lorenz T., 1999. A Technical Review of *Haematococcus* Algae. http://www.cyanotech.com/pdfs/bioastin/axbul60.pdf.

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.

Norman H.A., G. A. Thompson Jr., 1985. Effects of low-temperature stress on the metabolism of phosphatidylglycerol molecular species in *Dunaliella salina*. Plant Physiol. Biochem., 242: 168–175.

Petkov G., 1990. Lipids of photoautotrophically cultivated microalgae. Ph. D. Thesis, IPP, BAS, 119 pp. (In Bulgarian).

Pulz O., W. Gross, 2004. Valuable products from biotechnology of microalgae. Appl. Microb. Biotechnol., 65: 635–648.

Ranga Rao A., 2011. Production of astaxanthin from cultured green alga *Haematococcus pluvialis* and its biological activities. Ph.D. Thesis, University of Mysore, Mysore, India.

Ranga Rao A., M. P. Siew, R. Sarada, G. A. Ravishankar, 2014. Astaxanthin: sources, extraction, stability, biological activities and its commercial applications - a review. Mar. Drugs, 12: 128–152.

Recht L., A. Zarka, S. Boussiba, 2012. Patterns of carbohydrate and fatty acid changes under nitrogen starvation in the microalgae *Haematococcus pluvialis* and *Nannochloropsis* sp. Appl. Microbiol. Biotechnol., 94: 1495–1503.

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.

Rodolfi L., G. Zittelli, N. Bassi, G. Padovani, N. Biondi, G. Bonini, M.

R. Tredici, 2009. Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. Biotechnol. Bioeng., 102: 100–112.

Sarada R., A. Ranga Rao, B. K. Sandesh, C. Dayananda, N. Anila, V. S. Chauhan, G. A. Ravishankar, 2012. Influence of different culture conditions on yield of biomass and value added products in microalgae. Dyn. Biochem. Proc. Biotechnol. Mol. Biol., 6: 77–85.

Sarada R., U. Tripathi & G. A. Ravishankar, 2002. Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions. Process Biochem., 37 (6): 623–627.

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.

Zhekisheva M., S. Boussiba, I. Khozin Goldberg, A. Zarka, Z. Cohen, 2002. Accumulation of oleic acid in *Haematococcus pluvialis* (Chlorophyceae) under nitrogen starvation or high light is correlated with that of astaxanthin esters. J. Phycol., 38: 25–331.