Turk. J. Fish.&Aquat. Sci. 19(9), 785-791
http://doi.org/10.4194/1303-2712-v19_9_07

RESEARCH PAPER

Turkish Journal of FISHERIES and AQUATIC SCIENCES

Isolation and Cultivation of a Newly-Discovered Astaxanthin-Rich Green Microalga - *Haematococcus sp*. Flotow Strain from Homeros Valley (Bornova Creek, Izmir, Turkey)

Gamze Turan [1,*]

[1] Ege University, Fisheries Faculty, Aquaculture Department, 35100 Bornova, Izmir, Turkey.

Article History
Received 24 September 2017
Accepted 25 September 2018
First Online 28 September 2018

Corresponding Author
Tel.: +902323115217
E-mail: gamze.turan@ege.edu.tr

Keywords
Haematococcus
Green alga
Isolation
Pigments
Astaxanthin

Abstract

Samples of the green unicellular microalgae *Haematococcus* Flotow 1844 were collected from freshwater ponds in Homeros Valley, Bornova Creek, Izmir, Turkey. Specimens were isolated using the micropipetting method and were microscopically examined for morphological features. One isolate was identified as a local strain of *Haematococcus* sp. In addition, *Scenedesmus acuminatus* (Lagerheim) Chodat 1902, *Scenedesmus dimorphus* (Turpin) Kützing 1834 and *Chlorella elipsodae* Gernerck 1907 were found. DMSO (dimethyl sulfoxide) extracts of the local *Haematococcus* strain contained ~ 3.6 % total astaxanthin in dry weight.
The isolate's culture was scaled up in greenhouse raceway ponds in batch mode and several parameters of biomass yield (cell density, optical density, dry weight content and chlorophyll *a* content) were measured during a two weeks growth trial. The Turkish isolate of *Haematococcus* sp. seems to be a promising candidate for the commercial production of astaxanthin for food and pharmaceuticals.

Introduction

Exploration and exploitation of potential microalgae for various industrial applications from food to biofuels has been increasing (Spolaore, Joannis-Cassan, Duran, & Isambert, 2006). One of the commercially most successful genera has been the green astaxanthin-rich *Haematococcus* spp., predominant in freshwater, brackish lakes, reservoirs and ponds (Boussiba, 1999).

Haematococcus synthesizes and accumulates high levels of astaxanthin under stress conditions such as high light irradiance and nitrogen starvation (Ceron *et al*., 2006; Göksan & Gökpinar, 2005 and 2010; Göksan, Ak, & Kılıç, 2011; Hagen, Grunevald, Xylander, & Rothe, 2001; Hata, Ogbonna, Hasegawa, Taroda, & Tana, 2001; Kang, Lee, Park, & Sim, 2005 and 2007). An astaxanthin content of up to 5% in dry weight has been reported under commercial culture conditions (Aflola, Meshulam, Zarka, & Boussiba, 2007; Cifuentes, Gonzalez, Vargas, Hoeneisen, & Gonzalez, 2003; Orasa, Frangueira, Cid, & Abalde, 2000; Orasa, Valero, Herrero, & Abalde, 2001; Torzillo, Göksan, Faraloni, Kopecky, & Masojidek, 2003; Torzillo, Göksan, Işık, & Gökpınar, 2005).

Astaxanthin is used in food, feed, pharmaceutical, nutraceutical and cosmetic applications (Cysewski & Lorenz, 2004; Ergun & Erdem, 2000; Kobayashi *et al*., 1997, Lorenz & Chsewski, 2000; Mayne, 1996; Montsant, Zarka, & Boussiba, 2001; Naguib, 2000; Querin, 2003; Renstorm, Borch, Skulberg, & Liaaen-Jensen,1981; Spolaore *et al*., 2006).

Haematococcus is a cosmopolitan genus, having been found in Europe, North America and Africa, and in different climatic zones like continental, temperate, tropical and alpine. To date four *Haematococcus*

species - *H. pluvialis*, *H. lacustris*, *H. droebakensis* and *H. capensis* with 11 strains have been cultivated in laboratory and commercial cultures. There have been several studies on *H. pluvialis* cultivation in Turkey (Göksan & Gökpınar, 2005; Göksan *et al*., 2010; Göksan *et al*., 2011). In view of the economic importance of the genus, the present study describes the isolation and first examination in culture of a promising indigenous strain of *Haematococcus* for its astaxanthin content with a culture scale up in greenhouse raceway ponds.

Materials and Methods

Isolation and Acclimatization to Greenhouse Conditions

Water samples were collected from algal blooms in freshwater ponds in Homeros Valley (Latitude 38°29' 44.32'' North by Longitude 27°13' 45.03'' East) Bornova Creek, Izmir, Turkey; Figure 1). The red color cells or cysts, *Scenedesmus acuminatus*, *Scenedesmus dimorphus* and *Chlorella ellipsoidae* were identified in the water samples.

Isolation of the cells was performed with a Pasteur pipette under a microscope (Andersen and Kawachi, 2005). The pipette was heated in a flame, extended, and broken. The target organisms (red cells) were picked up individualy from the samples and collected in clean 20 mL test tubes using the micropipettes.

A total of 20 mL of red algal stock culture was transferred to 200 mL of enriched Bold Basal Medium (BBM) in a 250 mL Erlenmeyer flask. The red cell cultures were incubated at 25°C under a 16/8 hours light/dark cycle (120 µmol/m^2 /s fluorescent white lights 4000K) with constant shaking. When green cells started to grow, they were microscopically examined for morphological features. After it was found that the cells were belonging to the genus *Heamatococcus*, the samples were cultured in Optimal *Heamatococcus* Medium (OHM) developed by Fabregas, Dominquez, Requerio, Maseda, and Otero (2000) and subjected to serial dilutions in order to get *Heamatococcus* unialgal culture stocks. Pure cultures were obtained from single cells and the unialgal cultures were established in both liquid and on agar slants of OHM, incubated at 20±1°C under 90 µmol photons/ m^2/s irradiance and continuous light. The purity of the culture was ensured by repeated plating and by regular microscope observations. Some samples of isolated red cells of *Haematococcus* sp. were labeled as EGE-SUF-ALGAE-27 were deposited at the Algae Culture Collection of Ege University Fisheries Faculty Aquaculture Department.

Haematococcus sp. established in agar plates were inoculated into 150-mL Erlenmeyer flasks and incubated for two weeks at 25±1°C under 180 µmol photons/ m^2/s irradiance and continuous light. The cultures were sub-cultured at two-week intervals at least ten times prior to its scale up in 500, 1000 and 2000-mL Erlenmeyer flasks in OHM.

The stock cultures were moved to a greenhouse in carboys of 20 L capacity for 2 -3 weeks, before serving as the inocula for 100 L tanks. The algae in the tanks were subjected to at least 4 cycles of sub-culturing at

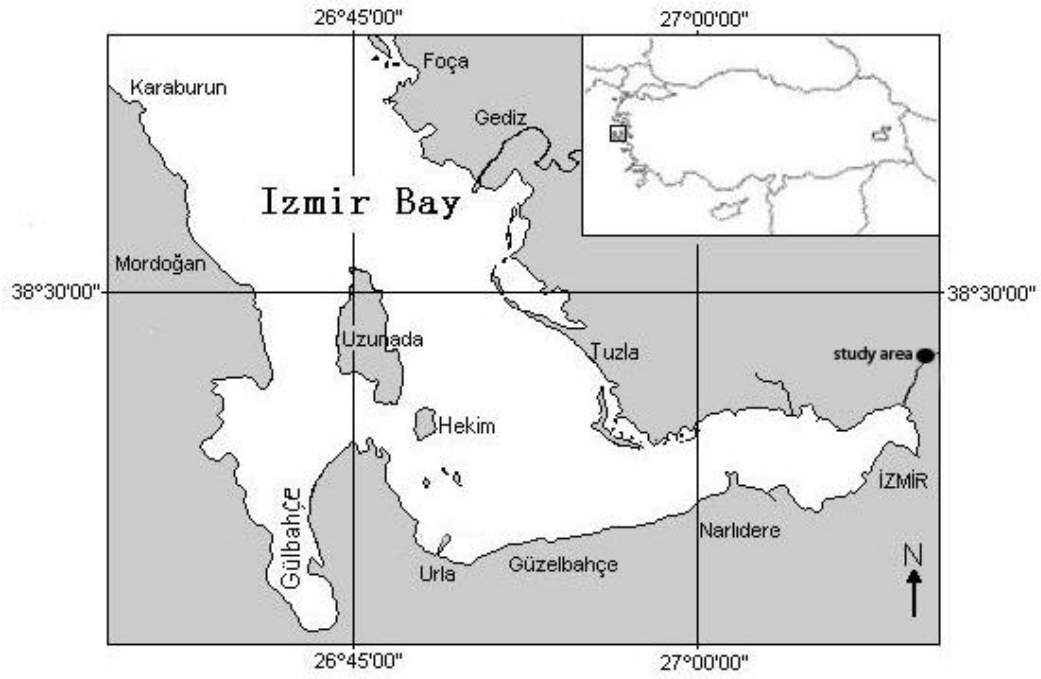


Figure 1. The map of study area.

intervals of two weeks at ambient temperature. The culture tanks were covered with transparent covers having four to five holes to facilitate aeration and to avoid condensation. Cultures were periodically examined under a microscope for detecting contaminants. Algal cultures in carboys and tanks were used to start cultures in 6,000 L capacity raceway ponds.

Cultivation of *Haematococcus sp*. in Greenhouse Raceway Ponds

OHM was prepared and the pH was adjusted to 7-7.5. The culture was inoculated at 10 -15% (v/v) in to a raceway pond of 6,000 L capacity and the volume was made up to 3,000 L. The paddle wheel was set to 15 rpm, which provided good mixing. Cultures were monitored daily for two weeks for the physical parameters and their growth - cell density, optical density (OD), chlorophyll *a* and astaxanthin contents. The tanks were periodically examined under a microscope for detection of contaminants.

Biomass Yield Estimation

Cell density: Well-mixed samples of algal cultures were taken daily by a pipette and algal cell numbers were estimated using a haemocytometer under a microscope.

Optical density (OD**):** Well-mixed samples of algal cultures were taken daily and optical density was measured at 680 nm (Kang *et al*., 2005).

Chlorophyll *a*: Algae were filtered through a 0.45-µm membrane filter, which was then ground in a glass grinding vessel in 34 mL of spectrophotometric grade acetone (90%) with a motor-driven Teflon pestle for 3 min at room temperature. After grinding and rinsing with 90% acetone, the volume was doubled, mixed, and centrifuged for 10 min at about 5,000 g. The absorption spectrum of the supernatant was similar to that of pure chlorophyll *a*. Therefore, absorption at 630, 645, and 665 nm in 90% acetone was used as in Parsons and Strickland (1963) to calculate chlorophyll *a* concentrations.

Dry weight (DW): A volume of culture was centrifuged at 5000 rpm for 5 min at 40°C, the pellet was washed at least twice with distilled water, dried in a ventilated stove (Ecocell, MMM Group) for 4 h at 105°C, and weighed (Vonshak, 1997).

Astaxanthin Extraction and Estimation

A known volume of culture was centrifuged at 5000 rmp for 5 min at 4°C

and the resultant pellet was treated with a known volume of dimethyl sulfoxide (DMSO) and kept in a water bath for 30 min at 21°C. Absorbance of the pooled extracts was measured by a Spectrophotometer (Model Boeco S-20, Germany) at 489 nm. Total astaxanthin was estimated using the formula of Boussiba, Fan, & Vonshak (1992).

Experimental Design and Data Analysis

Each value was delivered from three representative samples. All the observations and calculations were made separately for each set of experiments and were expressed as a mean±standard deviation .

Results and Discussion

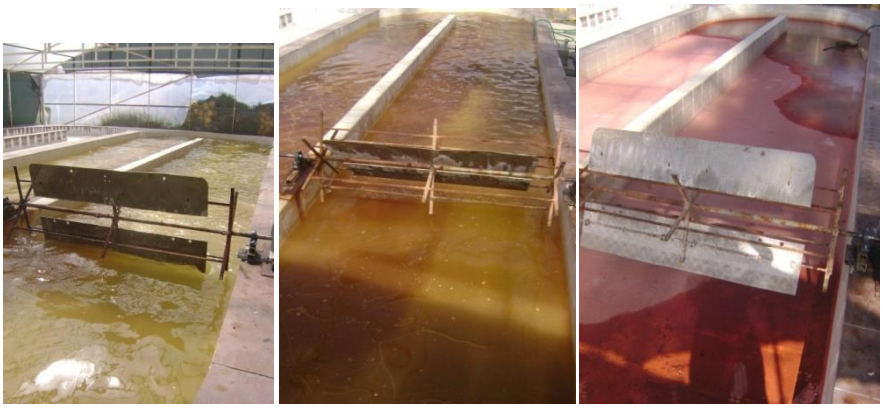
Isolation and Acclimation of *Haematococcus sp*. to Greenhouse Conditions

The cell characteristics and the morphological features of the Turkish isolate demonstrated its close similarity with the genus *Haematococcus*. The individual cell size ranged between 10- 18 µm, depending upon the growth phase, with bigger aggregates of cells observed in the natural habitat and during culture stationary growth phase. Cells are spherical in shape, generally green to yellowish green, turning red under stressed conditions. The Turkish isolate of *Haematococcus* was determined by size and shape to be *Haematococcus*. An astaxanthin analysis confirmed the *Haematococcus* designation.

Biomass and astaxanthin yields in the greenhouse raceway pond cultures Haematococcus sp. grew exponentially (Figure 2-a, b, and c) up to day 12, with a cell concentration of 1.6±0.15 X 10^6 cells/ mL (Figure 3), OD 1.92±0.01 (Figure 4) and 0.504±0.002 g DW /L (Figure 5). The results showed similarities to those found by Ceron *et al*. (2006), Cysewski and Lorenz (2004), Dominquez-Bocanegra, Legareta, Jeronimo, & Campocosio, 2004, Göksan and Gökpınar (2005 and 2010), Göksan *et al*. (2011), Hagen *et al*. (2001), Hata *et al*. (2001), Kang *et al*. (2005 and 2007), Kobayashi *et al*. (1993 and 1997), Wang *et al*. (2003). The small differences between the study results can be explained by the different culture methods employed. For example, in the study of Hata *et al*. (2001) a sequential heterotrophic-photoautotrophic cultivation method of *Haematococcus pluvialis* was applied and a cell concentration of 7 g DW/ L was obtained.

The chlorophyll *a* concentration dropped from 22.33±0.23 mg/ g DW to 3.43±0.25 mg/g DW during the cultivation period (Figure 6). Similar results were also found in the study of Boussiba *et al*. (1999) where it was concluded that astaxanthin accumulation was accompanied by a drop in Chlorophyll *a* concentration per cell down to 50% of its original value (30 pg/cell) in nitrogen-starved culture whereas in the phosphate-starved culture this parameter did not change.

The astaxanthin content of the cultures was 3.6±0.1 % in DW (Figure 7). This result was similar to the finding that the astaxanthin content of *H. pluvialis* under commercial culture conditions can reach up to 5% in dry weight (Aflola *et al*., 2007, Cifuentes *et al*., 2003, Boussiba *et al*., 1992, Boussiba, 1999, Boussiba *et*



A B C

Figure 2. Cultivation of green stage cells (a), green to redish color early stage palmelloid cells (b) and harvested red color aplanospores (Haematocyts) and astaxanthin yield (c) of *Haematococcus* sp. in greenhouse raceway ponds.

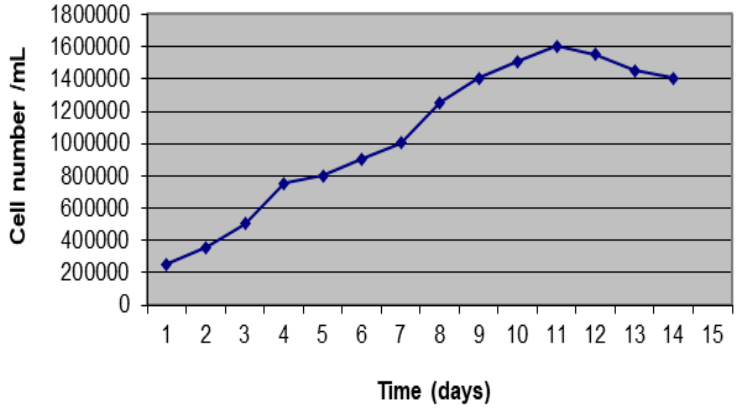


Figure 3. Mean cell numbers of representative samples in *Haematococcus* sp. cultures during cultivation in greenhouse raceway ponds.

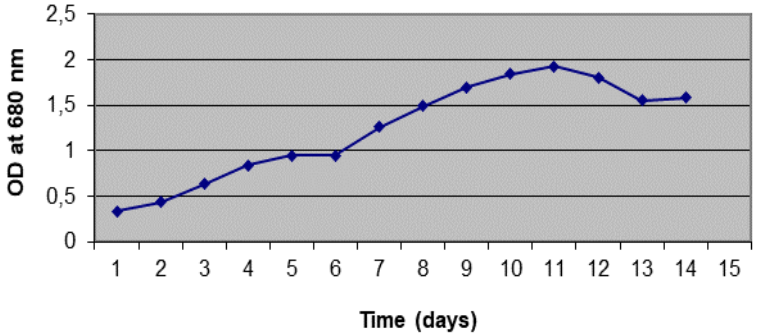


Figure 4. Mean optical densities of representative samples in *Haematococcus* sp. cultures during cultivation in greenhouse raceway ponds.

al.,1999, Orasa *et al*., 2000 and 2001, Torzillo *et al*., 2003 and 2005).

Conclusions

The astaxanthin content of *Haematococcus* cultures is relatively high and makes this isolate promising for exploitation. Indigenous species are most likely desirable for successful industrial exploitation of organisms, and make the present investigation important for commercial development. Our further studies will identifiy this genus at species level with

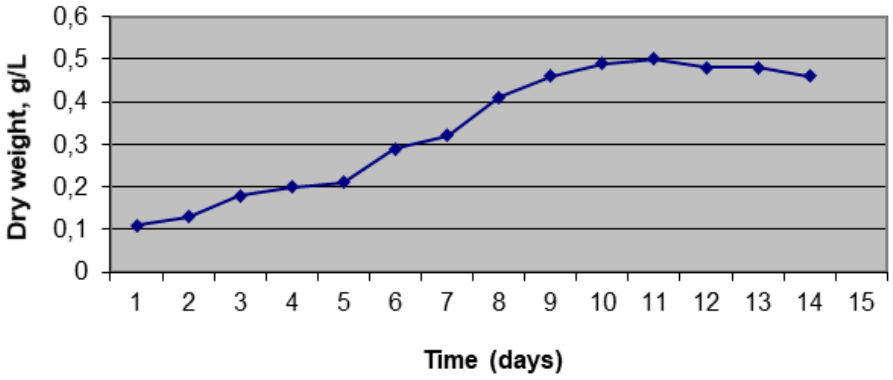


Figure 5. Mean dry weights of representative samples in *Haematococcus* sp. cultures during cultivation in greenhouse raceway ponds.

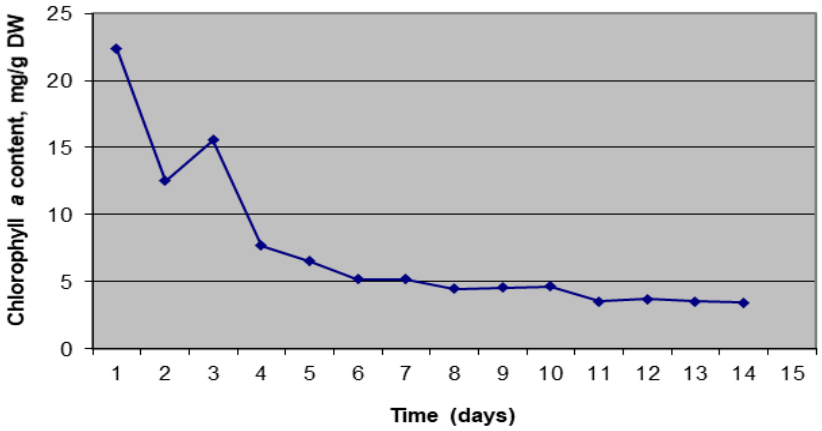


Figure 6. Mean total Chlorophyll *a* contents in dry weight of representative samples in *Haematococcus* sp. cultures during cultivation in greenhouse raceway ponds.

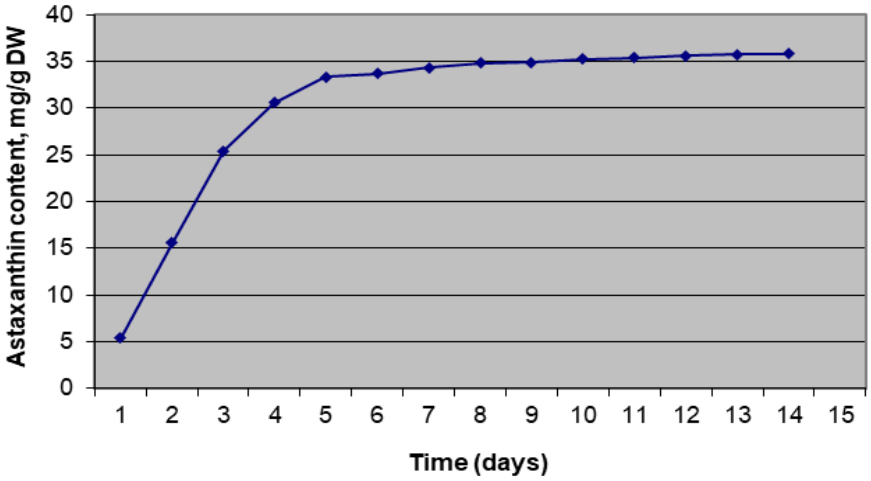


Figure 7. Mean total astaxanthin content in dry weight of representative samples in *Haematococcus* sp. cultures during cultivation in greenhouse raceway ponds.

molecular studies, optimization of culture parameters and identification of protocols for high yield biomass and astaxanthin production. Utilizing this technology provides a variety of economic incentives to a farm operator, such as product diversity, and the production of a valuable by-product.

Acknowledgements

The author is grateful to Prof. Dr. Semra CİRİK for identifications of the algal species, Dr. Amir NEORI for valuable information and suggestions in the preparation of this manuscript and Prof. Dr. Stephen Thomas ASTLEY for editing this manuscript.

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