THE EFFECTS OF IRON AND LIGHT INTENSITY ON BIOMASS AND PIGMENT SYNTHESIS OF *Heamotococcus pluvialis* UNDER LABORATORY CONDITIONS

MELTEM TUFAN, SELIN SAYIN

Mustafa Kemal University, Fisheries Faculty, 31200, Iskenderun, Turkey, helenselin@yahoo.com

In the present study the effects of iron and light intensity on biomass and pigment synthesis of *Heamotococcus pluvialis* (Chlorophyta) was studied under laboratory conditions. In the culture medium composed different light intensity (50, 200 ve 475 µmol photon $m^{-2}sn^{-1}$) and by suplementing Fe+EDTA, the species *H. pluvialis* which subjected to 11 hours light and 13 hours darkness photoperiod, growing parameters was determined. The experiments were applied in 2 processes and during the experiment period optical density, chlorophyl-a (mg L^{-1}), dry weight(g/mL^{-1}) and astaxsanthin amount was followed. The results are; while astaxanthin was raising, chlorophyl-a was reduced; raising light intensity and Fe+EDTA supplement make raise the amount of astaxsanthin. The highist astaxanthin value (% 0.768) obtained from the study is provided by 200µmol photon $m^{-2}sn^{-1}$ light intensity with Fe+EDTA supplement ($p<0.05$).

Keywords: Haematococcus pluvialis, astaxanthin, light intensity, Fe, growth

INTRODUCTION

Photosynthetic cells are of great importance as primary producers of various organic matters and for their ability to regenerate atmosphere. Many algal biotechnologists have studied the application of the photosynthetic machinery of algal cells to the production of new bioactive compounds and to environmental processes over the last several decades. Microalgae have vast potential as sources for valuable pharmaceuticals, pigments, vitamins, proteins, fatty acids, sterols, polysaccharides and other biologically active compounds, or potential health benefits (Metting and Pyne, 1986; Richmond, 2004). A red ketocarotenoid pigment, astaxanthin (*3,3'-dihydroxy-β,β-carotene-4,4'-dione*), has received an increasing interest from the cosmetics, the food and the feed industries. *Haematococcus pluvialis* is the richest source of natural astaxanthin and is now cultivated at industrial scale. Astaxanthin is a strong coloring agent and a potent antioxidant – its strong antioxidant activity points to its potential to target several health conditions.

A quality of products from *H. pluvialis* has over 70% contents of diester form and over 80% contents of 3S, 3S' form in total astaxanthin. Therefore contents of astaxanthin in total carotenoids are show to over 95% and contents of astaxanthin per cell are show to ~5.0%. But disadvantages of production by microalgae are low cell density by light limitation and low salt medium (Borowitzka, 1988; Orosa *et al.*, 2001). Astaxanthin disperses towards the periphery of *Haematococcus* cells under light induction, and moves back towards the center after illumination is discontinued (Yong and Lee, 1991). No major quantitative or qualitative changes occur during this migration. Red cysts are more resistant to photoinhibition than green cysts, strongly indicating a photoprotective role for astaxanthin. The specific rate of astaxanthin accumulation is a function of the photon flux density *Haematococcus* cultures are exposed (Lee and Soh, 1991). Continuous illumination is most favorable for astaxanthin formation, and carotenoid content is correlated proportionally to light quantity (Kobayashi *et al.*, 1992).

Light intensity showed significant effect on cell growth and level of astaxanthin accumulation in the cells. The optimum photon flux density corresponding to the maximum level of algal biomass production was 60 $\mu E \cdot m^{-2} \cdot s^{-1}$. High light intensity

caused relatively large quantities of astaxanthin to be accumulated in the cells of Haematococcus (Ramirez *et al.*, 2001). Metal ion, oxidative stress and salt stress stimulate the formation of astaxanthin in this alga. The ferrous form of iron is known to give rise to free radical formation via the Fenton reaction, free radicals may play a role in astaxanthin formation and active oxygen species (O_2, H_2O_2, peroxy radical) also enhance the formation of astaxanthin. Factors affecting the bio-accumulation of such pigments were fully understood. Carotenoids accumulation by microalgae depend on both nutritional status (Cero'n *et al.*, 2005; Tittel, 2005) and environmental conditions, such as high light intensity (Bhosale, 2004), type of light (Janhke, 1999). In all cases, the induced algal cells must be used by the log phase growth to avoid the dry weight failure. Also, shifting of photosynthetic metabolism to carotenoids accumulation by lipid biosynthesis should be considered (El-Shafey *et al.*, 1999).

Iron is an essential element for phytoplankton owing to its importance in numerous metabolic processes. Bioavailability of iron depends upon every aspect of Fe chemistry (solubility, complexation, thermodynamics, kinetics of ligand exchange) in addition to phytoplankton uptake mechanisms and kinetics. There is still no conclusive agreement on describing and quantifying "bioavailable iron" (Wells *et al.*, 1995). Some of the operationally defined iron forms may have strong correlations with bioavailability of iron to phytoplankton (Wells and Mayer, 1991). The importance of the different factors inducing the astaxanthin is well known but not completely understood. There are a lot of known factors that affect the astaxanthin accumulation in *H. pluvialis*: nutrient limitation or supplement (Fabregas *et al.*, 2003), oxygen stress (Kobayashi *et al.*, 1992), high light intensity (Park and Lee, 2001), and blue light (Fabregas *et al.*, 2003; Lababpour *et al.*, 2004). Among these, the effect of light is undoubtedly the most important factor in the astaxanthin accumulation (Bubrick, 1991). In order to produce high level of astaxanthin from *H. pluvialis*, a proper design of photobioreactor and illumination by effective light sources are required. The quality of light, such as wavelength and/or emission spectra of light also affects the performance of algae cultivations (Lee, 1999) as well as astaxanthin production.

In order to produce astaxanthin, the process involves two stages which are green stage which involves cultivation of green algae in order to increase its mass. The second stage is referred to carotegenesis stage. In this stage, green alga undergoes transformation to red algae after being exposed to some certain stress conditions *H. pluvialis* with the maximum mass followed by the second stage to produce astaxanthin (Kobayashi *et al.*, 1992) under stress induction. Two stages production process have been proposed due to different culture conditions needed for production of green algae and astaxanthin accumulation. Algal growth related to productivity of astaxanthin. Therefore, this study focused on optimization productivities of *H. pluvialis* biomass so that can enhance productivities of astaxanthin by screening the effect of different light intensity and Fe+EDTA.

MATERIALS AND METHODS

Strain and Culture Conditions

Haematococcus is an ubiquitous green algae classified as: *Chlorophyta* (Phylum), *Chlorophyceae* (Class), *Volvocales* (Order), *Haematococcaceae* (Family), *Haematococcus* (Genus), *pluvialis* (Species).

The unicellular green algae *H. pluvialis* (34/12) was purchased from the Culture Collection of Algae at England and was cultivated photoautotrophically in the modified Bold's Basal Medium (MBBM), whose composition consisted of 246.5 mg·L^{-1} of $NaNO_3$, 24.99 mg·L^{-1} of $CaCl_2 \cdot 2H_2O$, 73.95 mg·L^{-1} of $MgSO_4 \cdot 7H_2O$, 4.98 mg·L^{-1} of $FeSO_4 \cdot 7H_2O$, 74.9 mg·L^{-1} of K_2HPO_4, 175.57 mg·L^{-1} of KH_2PO_4, 25.13 mg·L^{-1} of NaCl, 49.68 mg·L^{-1} of $C_{10}H_{16}N_2O_8$ (EDTA), 1.57 mg·L^{-1} of $CuSO_4 \cdot 5H_2O$, 1.19 mg·L^{-1} of $Na_2MoO_4 \cdot 2H_2O$, 11.13 mg·L^{-1} of H_3BO_3, 1.44 mg·L^{-1} of $MnCl_2 \cdot 4H_2O$, 8.83 mg·L^{-1} of $ZnSO_4 \cdot 7H_2O$, 0.49 mg·L^{-1} of $Co(NO_3)_2 \cdot 6H_2O$, 6.06 mg·L^{-1} of MoO_3, 30.86 mg·L^{-1} of KOH, and 0.98 mg·L^{-1} of H_2SO_4 in distilled water.

Experimental Plan

To examine the effects of Fe+EDTA and different light intensities on the test organism (*H. pluvialis*), $FeSO_4 \cdot 7H_2O$ and EDTA (C10H16N2O8) were added to the growth medium (Modified Bold Basal Medium). Each test was carried out in 2000 ml polyetilene bottles. The initial pH value of the solution was adjusted between 7 by adding nitric acid (0.1M) or sodium hydroxide (0.1 M). The experiments were conducted at 23°C. During the experimental period, optical density, dry weight (g L^{-1}), chlorophyll *a* (mg L^{-1}) and astaxanthin concentration (%) were measured every day as shown in Table 1.

Table 1. Experimental Design

Experiments	Light intensity (μmol photon m^{-2} sn^{-1})	Groups	Parameters
I	30 (Control) 50 (A) 200 (B) 475 (C)	I: Control, A, B, C + added Fe+EDTA II : Control, A, B, C	Optic Density (O.D) Chlorophyll-a (mg L^{-1}) Dry weight(gL^{-1})
II	30 (Control) 200 (B)	I: Control, B + added Fe+EDTA II: Control, B	Dry weight(gL^{-1}) Astaxsanthin (%)

Pigments Extraction Method (Chlorophyll, Astaxanthin)

One milliliter of culture sample was centrifuged at 3000 rpm for 10 min to pellet cell material. Pipetting the supernatant and collect in the tubes. Adding 1 mL of acetone to the centrifuge tube, vortex for 30 sec. Homogenizer for 3 min with 12.000 rpm for broking the cell walls. The sample was stored at 4°C refrigerator for 20 min to extract pigments. These steps were repeated until the color of cell debris became white or colorless. Gently mix and centrifuge at 3,000 rpm for 10 min. to pellet remainder. Measurement of O.D at 475, 663, 645, 750 and 850 nm in spectrophotometer.

Chlorophyll concentration and astaxanthin concentration were analyzed by a spectrophotometer (model HP8453B, Hewlett Packard, Waldbronn, Germany). Chlorophyll concentration was calculated by eq. 1.

chlorophyll a = (12,7 X A663) – (2,69 X A645)
chlorophyll b = (22,9 X A645) – (4,64 X A663) (1)

Astaxanthin concentration was calculated by a calibration curve obtained by synthetic astaxanthin (product number A9335, Sigma Chemical Co., St Louis, MO, USA) as a standard. For astaxanthin concentration less than 10 mg/L, the following calibration was used (eq. 2).

astaxanthin concentration (mg / L) = 0.0045 X OD475 (2)

Optical Density and Dry Weight

Optical density values were obtained according to the procedure reported by Boussiba and Vonshak (1992). The samples were measured at 680 nm absorbents value with spectrophotometer. The sample containing 10 ml algal suspension was filtered through a filters 47 mm (diameter) (Whatman GF/C) that was dried in a microwave oven (105°C in 8 min) and weighed prior to filtration. The filter was put in a glass Petri dish in the oven under the above conditions. After cooling the fitler in a dessicator (20 min.), it was weighed again (Boussiba and Richmond, 1979).

Statistical Analysis

Data were analyzed statistically using one way analysis of variance (ANOVA). When significant treatment effects were detected, Duncan's multiple range test was used to identify specific differences among treatment means at a probability level of 5%.

RESULTS AND DISCUSSION

Experiment I

The results obtained for different light intensities and Fe+EDTA in cultures grown for 15 days are shown in Figure 1. As seen in Figure 1 and Figure 2, the results of this study proved that dry weight of the cells value of *H. pluvialis* was effected by light intensities and iron added and when the results were compared, difference was recorded between groups ($p>0.05$). The highest dry weight values were recorded in Group I (4.43 gL^{-1}).

These results suggest that the amounts of dry weight was strongly effected by the amounts and the chemical speciation of iron (Fe+EDTA). Fe+EDTA and light intensity observed during dry weight increase must have had a strong impact on the iron forms and bioavailability in *H. pluvialis* cells.

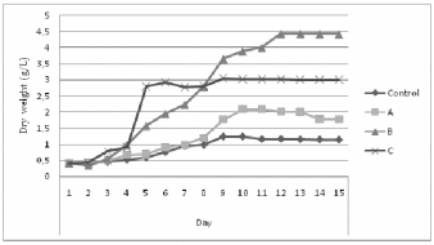

Figure 1. Dry weight values (g/L) of Group I

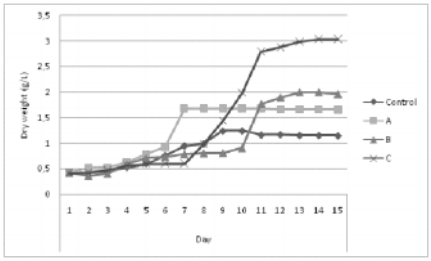

Figure 2. Dry weight values (g/L) of Group II

The maximum optical density value (0.23) was observed for the cultures supplemented with Fe(III) + EDTA and in 200 µmol foton $m^{-2}sn^{-1}$ light intensity . Analysis of variance of OD between groups showed that all groups were significantly different from each other and from the control group ($P<0.05$). (Figure 3, 4).

Dissolved Fe are constantly complexed by strong organic ligands in the aquatic systems. Strong Fe-binding ligands complex >99.9% of total dissolved Fe and can

dramatically increase the solubility of Fe in oxic waters, allowing greatly elevated dissolved Fe concentrations (Buck and Bruland, 2007). Furthermore, these strong Fe ligand complexes in natural waters appear to be largely bioavailable to marine phytoplankton (Maldonado and Price, 1999).

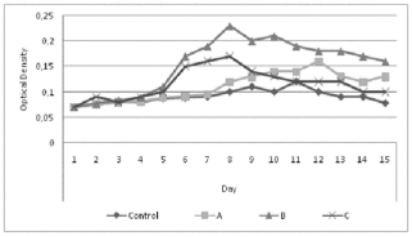

Figure 3. Optical Density Values of Group I

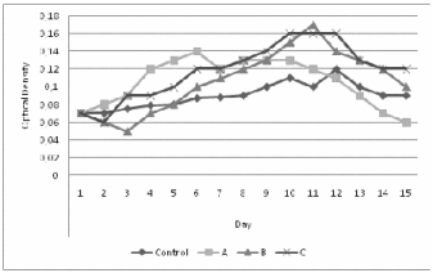

Figure 4. Optical Density Values of Group II

The highest chl *a* values (1.28, 1.57, 5.33 and 2.99 mg L^{-1}) were recorded in Group I. Statistical differences between all studied groups were significant (P<0.05) (Figure 5, 6). Martin and Fitzwater (1988) pointed out that an increase in chl *a* amount can be observed in Fe added culture on the third day of the experiment.

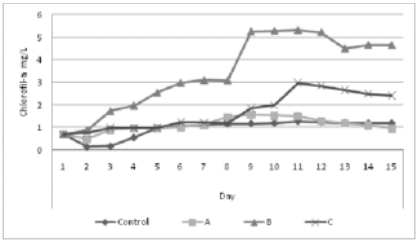

Figure 5. Chlorophyl-a values of Gruop I

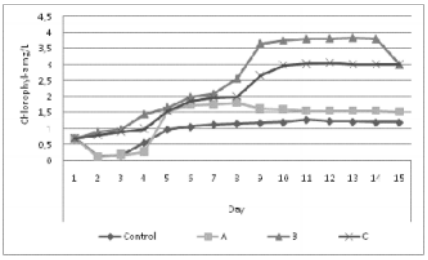

Figure 6. Chlorophyl-a values of Gruop II

Experiment II

The maximum dry weight value respectively 0.19, 0.25 and 0.27g/L was observed for the cultures group. Analysis of variance of dry weight value between groups showed that all groups were significantly different from each other and from the control group (P<0.05).

The maximum astaxanthin amounts respectively % 0.545 (Control), % 0.968 (Group I)) ve % 0.721 (Group II) was observed. Similarly, light induces stimulation of the phytoene synthase and phytoene desaturase in *Chlamydomonas reinhardtii*. An obligate photoautotroph, *Spirulina platensis*, was also reported to disply increased carotenoid levels under strong illumination (Liu, 1984) and in most cases, light causes a quantitative improvement in carotenoid content in microorganisms (Bhosale, 2004). Other hand, a significant enhancement in Chl a concentration was observed in relation to Fe addition.

CONCLUSIONS

Findings of this study indicate that Fe+EDTA and light intensity had relatively higher effect on the growth of *H.pluvialis*. Accordingly, these treatments (added Fe+EDTA and high light intensity) can be proposed for use in optimization of *H.pluvialis* cultures.

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