Effect of Different Culture Media and Light Intensities on Growth of *Haematococcus pluvialis*

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Abstract

The growth rate of *H. pluvialis* is controlled or regulated by the physical and chemical parameters. The aim of this study was to investigate and compare the effect of various culture media and light intensities on the growth of *H. pluvialis* in batch culture. The experiments were achieved by five different culture media and three different light intensities. The maximum cell concentration of 9.50 x 10^5 cells ml^{-1}, which corresponds to the growth rate of 0.195 d^{-1}, was obtained in RM culture medium at the light intensity of 40 µmol photons m^{-2} s^{-1}. The cell concentration decreased by only 7% in Basal culture medium compared to RM culture medium under the same light intensity.

Key words: Batch cultivation, Growth rate, *Haematococcus pluvialis*, Irradiance, Nutrient, Microalgae.

INTRODUCTION

The unicellular fresh water microalga, *Haematococcus pluvialis* Flotow (Volvocales, Chlorophyceae) is green-colored, biflagellate, and motile in its vegetative stage [1]. In its growth stages, it has both motile and non-motile forms [2]. In the algal life cycle of *H. pluvialis*, green vegetative cells with two flagella can grow autotrophically in the light [3] and heterotrophically on acetate in the dark [4,5]. This microalga shows low growth rates and low final cell densities under optimal growth conditions [6].

In recent years the green microalga *H. pluvialis* has been considered as a possible natural source for the production of astaxanthin and it has been widely studied. However, one of the main problems, according to Bubrick [7], in the production of astaxanthin from *Haematococcus* is contamination with fast-growing unicellular green and/or blue-green algae due to the relative slow growth of *Haematococcus* [8]. *Haematococcus* cells are sensitive to high hydrodynamic stress and changes in cell morphology under various environmental conditions [9].

No toxicity associated with *Haematococcus* has ever been reported in the literature. The general composition of *Haematococcus* algae consists of common carotenoids, fatty acids, proteins, carbohydrates, and minerals [10].

Both of the main physical and chemical parameters, especially nutrient medium and light, directly control the growth rate of *H. pluvialis*. Different studies have been performed on the growth conditions of *H. pluvialis* [1,6,9]. This comprehensive study on the determination of the culture medium and the light intensity was carried out to maximize the growth of *H. pluvialis* for batch cultivations.

MATERIALS and METHODS

Algal strain and inoculum preparation

Haematococcus pluvialis Flotow EGE MACC-35 was obtained from the Culture Collection of Microalgae at the University of Ege, Izmir, Turkey. Stock culture of *H. pluvialis* was grown photoautotrophically in BG11 medium [11,12] at 25 °C under continuous illumination (100 µmol photons m^{-2} s^{-1}) in 10-L flask. For the preparation of the inoculum, the cells from the stock culture were collected and concentrated by centrifugation (1,160 x g, 2 min) and the supernatant was removed. The collected cells were transferred, incubated aseptically in a 1000 ml Erlenmeyer flask containing 800 ml of fresh BG11 medium under continuous illumination (75 µmol photons m^{-2} s^{-1}), at 25 °C for 4 days. Air was supplied to the culture at a flow rate of 1 L min^{-1} (1.25 vvm). 4-day old culture (at vegetative cell growth phase) was used as inoculum at 10% volume for all experiments.

Growth conditions

The temperature was measured in the centre of the flask with a thermocouple (Dixell-XT115), controlled by air conditioner. Illumination was provided by standard cool white fluorescent lamps (18 W) from one side of the flask. Irradiance was measured in the centre of the flask with a quantum meter (Lambda L1-185). Continuous aeration was provided by bubbling air, using a blower (Nitto Kohki Co, LTD). Rotometers (Özgül-air Co. Izmir -Turkey) were used to provide the desired air flow rate. Pure CO_2 was added intermittently, using timer (Akboru BND-50/G1), for 10 s in every 10 min to the air stream (1.5% v/v) in order to provide inorganic carbon to the culture and keep the pH value below 8. Styrofoam was used to avoid the light penetration between the experiments when studies dealing with the different light intensities.

The experiments were performed in 250 ml Erlenmeyer flasks. 4-day old culture (20 ml, approximately 1 x 10^6 cells ml^{-1}) was inoculated into 200 ml sterilized fresh media in 250 ml Erlenmeyer flasks. The flasks were incubated for 12 days at 25 °C under various light intensities. Air was supplied to the culture at a flow rate of 0.8 L min^{-1}(4 vvm).

Samples were taken at indicated times, and following growth parameters were measured immediately; the cell concentration was determined by counting triplicate samples in a Neubauer haemocytometer. The specific growth rate (μ) of the cells was calculated from the initial logarithmic phase of growth for at least 48 h, as $\mu = \ln X_2 - \ln X_1/dt$, where X_2 is the final cell concentration, X_1 is the initial cell concentration and dt is the time required for the increase in concentration from X_1 to X_2. Doubling time was also calculated as $DT = \ln 2/\mu$.

Five culture media: BG11 [11,12], Modified BG11 (Mod. BG11) [13], OHM [14], Basal [9], and Rudic's medium (RM) [15] were tested at three light intensities: 40, 50 and 60 μmol photons m^{-2} s^{-1}, resulting in a total of 15 different conditions. All media were prepared and autoclaved separately and added to sterilized water. If necessary, the vitamins were added aseptically to the final medium after autoclaving. All Chemicals (Merck Co.) were used analytical grade. The compositions of culture media were shown in Table 1.

RESULTS

The effects of 15 different conditions on the growth of *H. pluvialis* were simultaneously investigated for 12 days of cultivation period. The experiments were performed under the same growth conditions.

As shown in Fig. 1, there were significant differences on the growth of cells beginning from 7 days of cultivation period in different culture media at the light intensity of 40 μmol photons m^{-2} s^{-1}. The cell concentration reached the maximum value (9.50 x 10^5 cells ml^{-1}) in RM culture medium on the 10th day of cultivation and then started to decrease slightly. On the other hand, in Basal culture medium the cell

Table 1. Nutrient composition of different culture media: BG11, Modified BG1, Basal, OHM and RM

Constituents	BG11 (mg/L)	Mod. BG11 (mg/L)	OHM (mg/L)	Basal (mg/L)	RM[a] (mg/L)
$NaNO_3$	1500	1500	-	-	300
K_2HPO_4	40	320	-	-	80
KH_2PO_4	-	-	-	-	20
$Ca(NO_3)_2.4H_2O$	-	-	-	150	-
KNO_3	-	-	410	100	-
Na_2HPO_4	-	-	30	-	-
β-Na_2glycerophosphate	-	-	-	50	-
$MgSO_4.7H_2O$	75	200	246	40	10
$CaCl_2.2H_2O$	36	36	110	-	58.5
Citric Acid	6	6	-	-	-
Ammonium Ferric Citrate	6	6	-	-	-
EDTA-Na_2	1	1	-	2.71	-
EDTA	-	-	-	-	7.5
Na_2CO_3	20	100	-	-	-
NaCl	-	-	-	-	20
Vitamin B_{12}	-	-	0.0150	0.0001	-
Biotin	-	-	0.025	0.0001	-
Thiamine-HCl	-	-	-	0.01	-
Thiamine	-	-	0.0175	-	-
H_3BO_3	2.86	2.86	-	-	0.3
$MnCl_2.4H_2O$	1.81	1.81	0.98	0.108	-
$MnSO_4H_2O$	-	-	-	-	1.5
$ZnSO_4.7H_2O$	0.22	0.22	-	0.066	0.1
$Na_2MoO_4.2H_2O$	0.39	0.39	0.12	0.0075	-
$(NH_4)6Mo7O_{24}.4H_2O$	-	-	-	-	0.3
$CuSO_4.5H_2O$	0.08	0.08	0.012	-	0.08
$Co(NO_3)_2.6H_2O$	0.05	0.05	-	-	0.26
$FeCl_3.6H_2O$	-	-	-	5.888	17
$CoCl_2.6H_2O$	-	-	0.011	0.012	-
Trisaminomethane	-	-	-	500	-
Fe(III)citrateH_2O	-	-	2.62	-	-
Cr_2O_3	-	-	0.075	-	-
SeO_2	-	-	0.005	-	-

[a] The composition of RM culture medium was obtained from the MD Patent, 2000, Nr. a 2000 0154, belonging to Rudic V[22].

concentration increased to about 8.85 x 10^5 cells ml^{-1} at the end of the period. The similar cell concentrations of 7.75 x 10^5 cells ml^{-1} and 7.65 x 10^5 cells ml^{-1} were obtained on the10th day in BG11 medium and OHM medium, respectively. The cell concentration was significantly lower in Mod.BG11 medium compared to RM culture medium under the same light intensity.

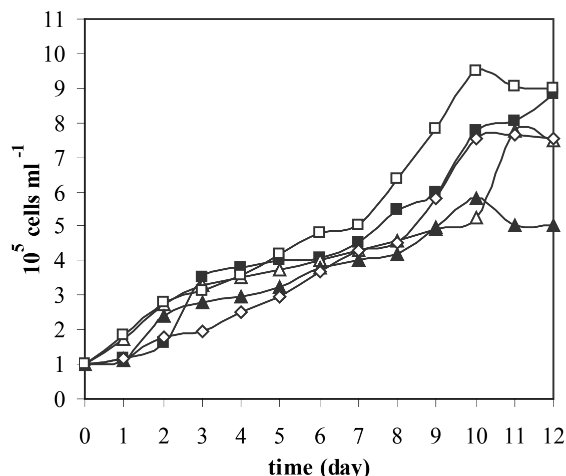

Figure 1. Effect of different media on the growth of *H. pluvialis* at a light intensity of 40 µmol photons m^{-2} s^{-1}. (Δ) BG11, (▲) Mod. BG11, (◊) OHM, (■) Basal, (□) RM

The maximum cell count, 8.10 x 10^5 cells ml^{-1}, was obtained in RM culture medium and the lowest in Mod.BG11 medium (5.20 x 10^5 cells ml^{-1}) under the light intensity of 50 µmol photons m^{-2} s^{-1}. The cell concentration decreased by only 11% in Basal medium in comparison with RM medium. During the cultivation, the growth increased 7.90 times in terms of the initial cell concentration in BG11 medium at 50 µmol photons m^{-2} s^{-1} (Fig. 2).

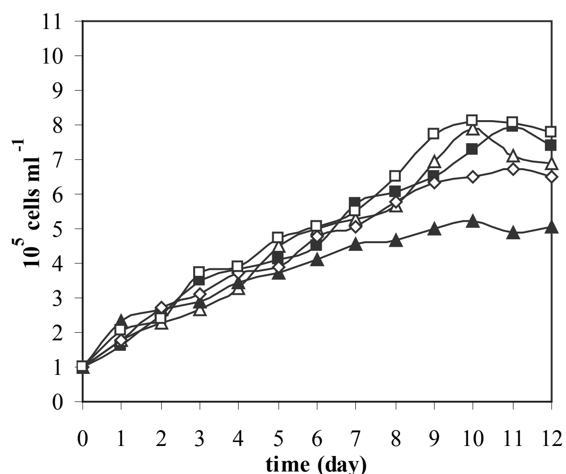

Figure 2. Effect of different media on the growth of *H. pluvialis* at a light intensity of 50 µmol photons m^{-2} s^{-1}. (Δ) BG11, (▲) Mod. BG11, (◊) OHM, (■) Basal, (□) RM

As shown in Fig. 3, after 10 days of cultivation period, the curves lost regular increase and nearly remained at constant levels due to the light intensity of 60 µmol photons m^{-2} s^{-1} for each medium. When the cell concentration reached value of 6.8 x 10^5 cells ml^{-1} in BG11 medium, growth increased by 6.6% in RM medium with respect to BG11 medium on the10th day under the same light intensity.

The cells grown in RM medium were effective under different light intensities when compared to those grown in other media commonly used for the growth of *H. pluvialis*. In the cultivation period correct growth parameter selection is necessary to obtain maximum growth rates.

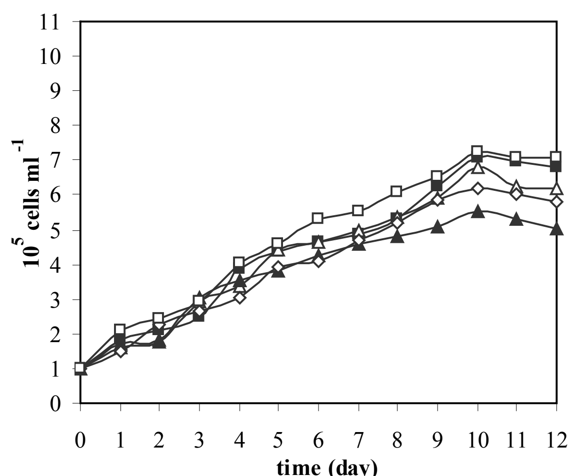

Figure 3. Effect of different media on the growth of *H. pluvialis* at a light intensity of 60 µmol photons m^{-2} s^{-1}. (Δ) BG11, (▲) Mod. BG11, (◊) OHM, (■) Basal, (□) RM

When the effects of different light intensities on the growth of *H. pluvialis* were taken into consideration, little decrease in cell concentration was observed with increasing light intensity from 40 µmol photons m^{-2} s^{-1} to 50 µmol photons m^{-2} s^{-1} for each culture medium, as shown in figures, generally. The cell concentration in RM medium increased to about 9.50 x 10^5 cells ml^{-1} at the light intensity of 40 µmol photons m^{-2} s^{-1} while this concentration was somewhat lower, 8.10 x 10^5 cells ml^{-1}, at 50 µmol photons m^{-2} s^{-1} on the10th day. As expected, increasing light intensity to 60 µmol photons m^{-2} s^{-1} reduced to the growth rate of *H. pluvialis*.

After 12 days of growth period, the maximum cell concentrations of 9.50 x 10^5 cells ml^{-1} and 8.85 x 10^5 cells ml^{-1}, which correspond to the growth rates of 0.195 d^{-1} and 0.177 d^{-1}, were obtained in RM medium and Basal medium, respectively, at the light intensity of 40 µmol photons m^{-2} s^{-1} (Table 2).

Table 2. Cell concentrations and growth rates of *H. pluvialis* cultured in different media under different light intensities

Culture medium	Light intensity (µmol photons m^{-2} s^{-1})	Cell con. (x 10^5 cells ml^{-1})	µ (d^{-1}- h^{-1})	Doubling time (h)
BG 11	40	7.75	0.115-0.0048	144
Mod. BG 11	40	5.85	0.111-0.0046	150
OHM	40	7.65	0.138-0.0058	119
Basal	40	8.85	0.177-0.0074	93
RM	40	9.50	0.195-0.0081	85
BG 11	50	7.90	0.159-0.0066	105
Mod. BG 11	50	5.20	0.086-0.0036	192
OHM	50	6.75	0.102-0.0043	161

Basal	50	7.92	0.131-0.0055	126
RM	50	8.10	0.154-0.0064	108
BG 11	60	6.80	0.130-0.0054	128
Mod. BG 11	60	5.55	0.104-0.0043	161
OHM	60	6.20	0.118-0.0049	141
Basal	60	7.05	0.134-0.0056	124
RM	60	7.25	0.137-0.0057	121

DISCUSSION

Several experiments have been carried out by authors on the growth of *Haematococcus* sp. in order to obtain maximum growth rate [1,6,9,14]. By using repeated fed-batch processes, the cells could be maintained in the vegetative form, leading to more than two times increase in cell number output rate[9]. In batch cultures the optimal *Haematococcus* media (OHM) produced a cell density of 6.25 x 10^5 cells ml^{-1} after 14 days of culture with no astaxanthin being accumulated [16,14]. One of the other results was reported by Domìnguez-Bocanegra et al. [17], maximal growth of *H. pluvialis* (green cells) was obtained 3.5 x 10^5 cells ml^{-1} in the BBM medium under continuous illumination (177 µmol photons m^{-2} s^{-1}) with continuous aeration (1.5 vvm).

In developing an optimal process for microalgal products, two major aspects are usually considered for improvement. One is the effect of environmental factors such as temperature, light intensity, pH, aeration and agitation, while another is the selection of a suitable nutrient medium [18,19]. It is well known that the culture medium not only affects the cell productivity, but also affects cell composition and yield of specific products [20,19]. However, medium formulation studies are usually time consuming and expensive. To overcome these problems, better experimental design techniques should be implemented [19].

Light is essential for the life cycle of *H. pluvialis*. Higher light intensities can lead to photoinhibition. Light penetration (which is inversely proportional to cell concentration) is another problem in the phototrophic cultivation of microalgae [21]. Under optimal growth conditions, light absorbed by antenna pigments is converted to chemical energy forming ATP and NADPH through a photosynthetic electron transport chain. This chemical energy is finally stored in starch by fixing CO_2 through the Calvin cycle [2].

The effect of light intensity is dependent on the nutritional state of the cultures. The interference between nutrient status and light intensity has been already reported in carotenogenesis studies [4,16], indicating the importance of using an optimized culture medium for correct interpretation of experimental results [6].

As cell concentration changes, the light requirements change. Light intensity might be adjusted in the range of about 30-150 µmol photons m^{-2} s^{-1}, depending on the volume of the culture for vegetative growth of *H. pluvialis*.

The choice of medium used depends on several factors: the growth requirements of the algae, how the constituents of the medium may affect final product quality, and cost [22]. In this experimental study, the best results were obtained in RM culture medium and Basal culture medium, respectively, at the light intensity of 40 µmol photons m^{-2} s^{-1} for the growth of *H. pluvialis*. Basal and OHM media contain three vitamins- vitamin B1 (thiamine HCl), vitamin B_{12} (cyanocobalamin), and vitamin H (biotin) - are usually expensive, whereas RM culture medium contain none. Among vitamins, thiamine was established as a growth factor for this microalga, while B_{12} stimulated growth but was not essential [23]. Other authors have found that biotin, thiamine and B_{12} have no significant effect on growth rate and final dry weight [19,6]. In conclusion, RM culture medium is more economical than Basal culture medium in terms of containing the constituents. No experimental studies had been encountered concerning the RM culture medium in the scientific literature.

These results have demonstrated that maximal growth of *H. pluvialis*, 9.50 x 10^5 cells ml^{-1}, was obtained in RM medium under continuous illumination (40 µmol photons m^{-2} s^{-1}) at 25 °C during 12 days of growth period.

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