Appl Biochem Biotechnol (2010) 160:764–772
DOI 10.1007/s12010-009-8627-7

Semi-continuous Cultivation of *Haematococcus pluvialis* for Commercial Production

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Received: 20 October 2008 / Accepted: 25 March 2009 /
Published online: 22 April 2009

Abstract The objectives of the present study on the growth of *Haematococcus pluvialis* were to indicate the effects of a long-term semi-continuous cultivation, sterilization, carbon dioxide, and different culture media by using artesian well water. This investigation was an enterprise in order to commercialize the production economically. When the effect of CO_2 was investigated in basal culture medium, the influence of sterilization was also researched in Rudic's culture medium in vertical panel-type photobioreactors for 31 days of semi-continuous cultivation. The maximum cell concentration of 10.55×10^5 cells ml^{-1}, which corresponds to the growth rate of 0.271 day^{-1} with the areal productivity of 3.531 g m^{-2} day^{-1}, was found in non-sterilized RM medium on the 24th day of the third run of semi-continuous cultivation at a renewal rate of 50% in a vertical panel-type photobioreactor.

Keywords Carbon dioxide · *Haematococcus pluvialis* · Nutrient medium · Photobioreactor · Semi-continuous cultivation · Sterilization

Abbreviations
C_b Biomass concentration (g l^{-1})
C_c Cell count (cells ml^{-1})
DT Doubling time (day)
P_{ba} Areal productivity (g m^{-2} day^{-1})
P_{bv} Volumetric productivity (g l^{-1} day^{-1})
μ Specific growth rate (day^{-1})

Introduction

The major market for astaxanthin is as a pigmentation source in aquaculture, primarily salmon, trout, and sea beam. Astaxanthin sells for approximately \$2,500 kg^{-1} with the annual worldwide aquaculture market estimated at \$200 million. Although over 95% of this

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market consumes synthetically derived astaxanthin, consumer demand for natural products makes the synthetic pigments much less desirable and provides opportunity for the production of natural astaxanthin by *Haematococcus* [1].

Additionally, the red ketocarotenoid astaxanthin surpasses the antioxidant activity of other carotenoids like β-carotene, zeaxanthin, and vitamin E. Thus, the pharmaceutical and cosmetic industries are increasingly interested in astaxanthin since it can, for instance, protect against the damaging effects of ultraviolet radiation, and chemically induced cancers, and also enhance the immune system [2].

In bioreactor cultures of *Haematococcus* algae, due to the high cost of capital investment and the requirements for medium sterilization and aseptic operation, only high cell densities are considered to be economically justified [3]. Two types of bioreactor systems are suitable for cultivation of *Haematococcus lacustris* at high cell densities. These include photobioreactors [4] and conventional heterotrophic fermenters [5, 6]. The very cheap method of growing *Haematococcus pluvialis* in open ponds will not materialize because of contamination problems [7].

Batch culture systems are widely applied because of their simplicity and flexibility, allowing to change species and to remedy defects in the system rapidly. Although often considered as the most reliable method, batch culture is not necessarily the most efficient method. Semi-continuous cultures may be indoors or outdoors, but usually their duration is unpredictable. Since the culture is not harvested completely, the semi-continuous method yields more algae than the batch method for a given tank size [8].

The objectives of the present study on growth of *H. pluvialis* were to indicate the effects of (a) a long-term semi-continuous cultivation, (b) sterilization, (c) CO_2, and (d) different culture media. This investigation was an enterprise in order to commercialize the production economically.

Materials and Methods

Algal Strain and Inoculum Preparation

H. 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 [9, 10] at 25 °C under continuous illumination (100 μmol photons m^{-2} s^{-1}) in a 10-l flask. For the preparation of the inoculum, the cells from the stock culture were collected and concentrated by centrifugation (1.160×*g*, 2 min) and the supernatant was removed. The collected cells were transferred and incubated aseptically in a 1,000-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). A 4-day-old culture was used as inoculum at 10% volume for all experiments.

Growth Conditions

The temperature was measured in the center of the reactor with a thermocouple (Dixell-XT115), controlled by an air conditioner. Illumination was provided by standard cool white fluorescent lamps (18 W) from one side of the reactor. Irradiance was measured in the center of the reactor with a quantum meter (Lambda L1-185). Continuous aeration was provided by bubbling air, using a blower (Nitto Kohki Co, Ltd). Rotameters (Özgül-air, Izmir, Turkey) were used to provide

the desired air flow rate. If necessary, pure CO_2 was added intermittently, using a timer (Akboru BND-50/G1, Izmir, Turkey), for 10 s 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 light penetration between experiments.

Vertical Panel-Type Photobioreactor

A schematic diagram of the vertical panel-type photobioreactor is shown in Fig. 1. The photobioreactor was made of Plexiglas (0.5 cm wall thickness). The total volume of the photobioreactor was 1.5 l (25 cm L×2.5 cm W×25 cm H). The gas spargers were placed from both opposite corners of the photobioreactor so as to supply enriched air to the culture. The sparger diameter is 0.4 cm (single nozzle). Chemical sterilization was applied by using 1.5% (*v*/*v*) hypochlorite for the reactors.

A 4-day-old culture (100 ml, approximately 1×10^6 cells ml^{-1}) was inoculated into 1,000 ml fresh media in 1,500 ml panel reactors. The reactors were incubated at 25 °C under the light intensity of 75 μmol photons m^{-2} s^{-1}. Air was supplied to the culture at a flow rate of 1 l min^{-1} (1 vvm). The multifactorial experimental design was achieved by two different culture media for 31 days of semi-continuous cultivation with the following conditions: (a) basal culture medium [11] with the addition of CO_2 under non-aseptic conditions; (b) basal culture medium without the addition of CO_2 under non-aseptic conditions; (c) Rudic's (RM) culture medium [12] with the addition of CO_2 under non-aseptic conditions; and (d) RM culture medium with the addition of CO_2 under aseptic conditions. Each culture medium was prepared and added to

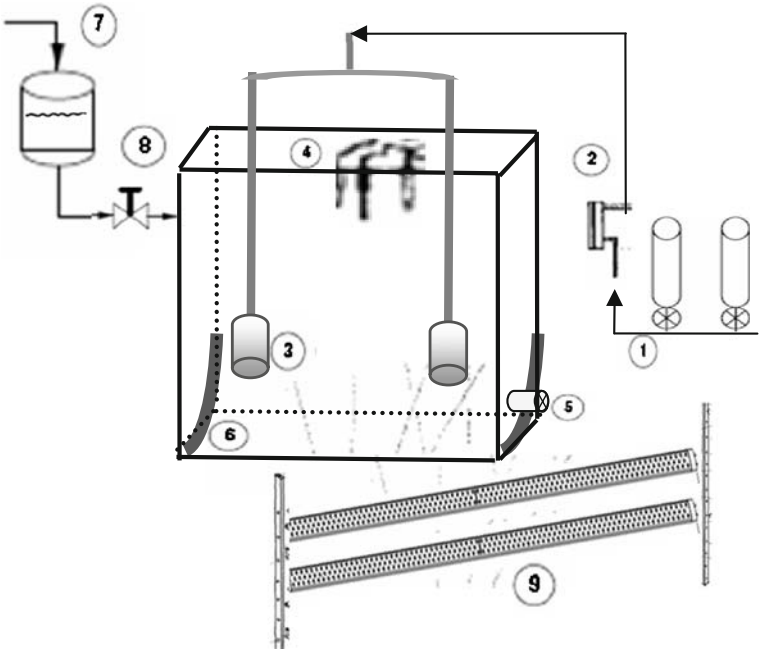

Fig. 1 A schematic diagram of the vertical panel-type photobioreactor: *1* air and CO_2 injection, *2* rotameter, *3* sparger, *4* electrodes and sensors, *5* harvesting outlet, *6* baffle, *7* culture medium vessel, *8* control valve, *9* light source

artesian well water. Furthermore, one of the media was autoclaved. All reagents used were of analytical grade (Merck & Co.). The basal culture medium contained (per 100 ml): 15 mg Ca $(NO_3)_2{\cdot}4H_2O$, 10 mg KNO_3, 5 mg β-Na_2 glycerophosphate, 4 mg $MgSO_4{\cdot}7H_2O$, 1 μg thiamine–HCl, 0.01 μg vitamin B_{12}, 0.01 μg biotin, 0.3 ml PIV metals, and 50 mg trisaminomethane. The PIV metals contained (per 100 ml): 19.6 mg $FeCl_3{\cdot}6H_2O$, 3.6 mg $MnCl_2{\cdot}4H_2O$, 2.2 mg $ZnSO_4{\cdot}7H_2O$, 0.4 mg $CoCl_2{\cdot}6H_2O$, 0.25 mg $Na_2MoO_4{\cdot}2H_2O$, and 100 mg EDTA-Na_2. The Rudic's culture medium contained (per liter): 300 mg $NaNO_3$, 20 mg KH_2PO_4, 80 mg K_2HPO_4, 20 mg NaCl, 47 mg $CaCl_2$, 10 mg $MgSO_4{\cdot}7H_2O$, 0.1 mg $ZnSO_4{\cdot}7H_2O$, 1.5 mg $MnSO_4{\cdot}H_2O$, 0.08 mg $CuSO_4{\cdot}5H_2O$, 0.3 mg H_3BO_3, 0.3 mg (NH_4) $6Mo7O_{24}{\cdot}4H_2O$, 17 mg $FeCl_3{\cdot}6H_2O$, 0.2 mg $Co(NO_3)_2{\cdot}H_2O$, and 7.5 mg EDTA.

For semi-continuous cultivation, a percentage of the volume of the culture (renewal rate) was harvested when a deceleration of the growth velocity was observed. The renewal rates of 40%, 50%, and 60% were applied at the end of the first, second, and third runs of semi-continuous cultivation, respectively, and the same volume of freshly prepared culture medium was added for each experiment. The cell capacity to flocculate made harvesting easier even in the vegetative stage. Before harvesting, the aeration of the photobioreactors was turned off and the cells were allowed to settle. Cultures were harvested by draining the culture media into a plankton collector equipped with 50 to 150 μm mesh. The sediments were stirred up manually following harvest, thoroughly mixing the cultures, to re-suspend cells.

Measurements and Analytical Methods

Samples were taken at indicated times and growth parameters were measured immediately; the cell concentration was determined by counting triplicate samples in a Neubauer hemocytometer. Dry weight was determined in duplicate by filtering a 5-ml culture sample through preweighed Whatman GF/C filters and drying the cell mass at 105 °C for 2 h. The correlation between the cell count (C_c) and the biomass concentration (C_b) was:

$$C_b\left(\mathrm{gl}^{-1}\right) = 0.0668\, C_c\left(10^5 \mathrm{cell/ml}\right) + 0.1304, \tag{1}$$

where C_b is the dry weight biomass concentration. The value of linear regression coefficient is 0.99 for Eq. 1. The specific growth rate (μ) of the cells was calculated from the initial logarithmic phase of growth for at least 48 h, as

$$\mu = (\ln C_{c2} - \ln C_{c1})/\mathrm{delta}\ t, \tag{2}$$

where C_{c2} is the final cell concentration, C_{c1} is the initial cell concentration, and delta t is the time required for the increase in concentration from C_{c1} to C_{c2}. Doubling time (DT) was also calculated as

$$\mathrm{DT} = \ln 2/\mu. \tag{3}$$

The volumetric productivity of the biomass is calculated [13]:

$$P_{bv} = \mu C_b, \tag{4}$$

where P_{bv} is the volumetric productivity and C_b is the concentration of the biomass. The areal productivity P_{ba} is related with volumetric productivity, as follows [13]:

$$P_{ba}A = P_{bv}\,V, \tag{5}$$

where V is the volume of the reactor and A is the land area occupied by it.

Results

The effects of four different conditions on the growth of *H. pluvialis* were simultaneously investigated for 31 days of semi-continuous cultivation period. As shown in Fig. 2, the maximum cell concentration of 8.55×10^5 cells ml^{-1}, which corresponds to the volumetric productivity of 0.135 g l^{-1} day^{-1} (2.109 g m^{-2} day^{-1}) , was obtained in non-sterilized RM medium on the 10th day of the first run of semi-continuous cultivation. It should be noted here that when the volumetric productivity decreased due to the dilute culture broth, this could have caused an increase in the cost of recovering the biomass.

When the second run of semi-continuous cultivation was started with a renewal rate of 40%, all the cells were still in the vegetative form. The maximum cell concentration, 9.25×10^5 cells ml^{-1}, was obtained in non-sterilized RM medium and the lowest in non-sterilized basal medium without the addition of CO_2 (7.25×10^5 cells ml^{-1}) at a renewal rate of 40% on the 17th day. These were equivalent to the growth rates of 0.243 and 0.157 day^{-1}, respectively (Table 1). After that, the cultures in basal and RM media were harvested at about 50% of the volume of the culture on the 17th day of cultivation. This was to control the cell concentration for the next run.

The maximum cell concentration of 10.55×10^5 cells ml^{-1}, corresponding to the growth rate of 0.271 day^{-1} with the areal productivity of 3.531 g m^{-2} day^{-1}, was found in non-sterilized RM medium on the 24th day of the third run of semi-continuous cultivation with a renewal rate of 50%, which indicated that cells could adjust well to the growth conditions. The best growth was obtained in the third run of semi-continuous cultivation. It should be noted here that the optimum carrying capacity of the system was reached in the third run. At the end of the third run, the cultures in basal and RM media were harvested at about 60% of the volume of the culture.

No significant increase in cell concentration was noted at each experimental parameter with a renewal rate of 60% on the 31st day of the fourth run of semi-continuous cultivation. It was recorded that cell division was inhibited in the fourth run of semi-continuous

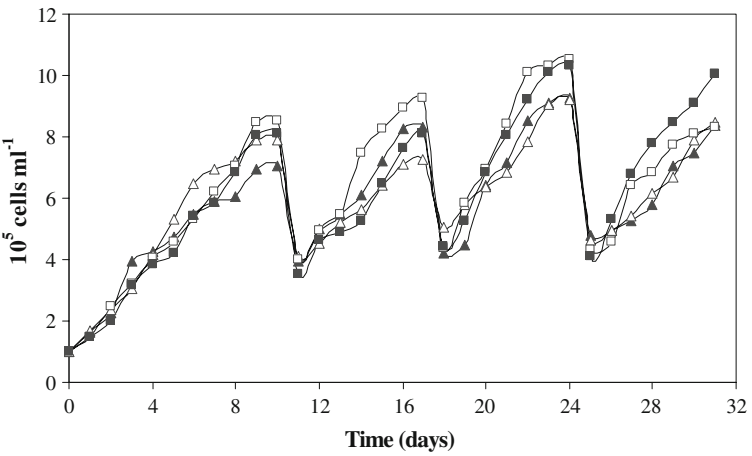

Fig. 2 Effect of different conditions on the growth of *H. pluvialis* at a light intensity of 75 μmol photons m^{-2} s^{-1} for 31 days of semi-continuous cultivation period. *Filled triangle*, basal culture medium with the addition of CO_2 under non-aseptic conditions; *empty triangle*, basal culture medium without the addition of CO_2 under non-aseptic conditions; *empty square*, RM culture medium with the addition of CO_2 under non-aseptic conditions; *filled square*, RM culture medium with the addition of CO_2 under aseptic conditions

Table 1 Results of obtaining kinetic parameters of *H. pluvialis* cultured semi-continuously in different conditions.

	Day	C_b (g l^{-1})	μ (day^{-1})	DT (day)	P_{bv} (g l^{-1} day^{-1})	P_{ba} (g m^{-2} day^{-1})
Run 1						
Basal with CO_2	10	0.601	0.143	4.850	0.086	1.344
Basal without CO_2	10	0.658	0.151	4.590	0.099	1.547
Non-sterilized RM	10	0.702	0.193	3.591	0.135	2.109
Sterilized RM	10	0.672	0.175	3.961	0.118	1.844
Run 2						
Basal with CO_2	17	0.685	0.167	4.151	0.114	1.781
Basal without CO_2	17	0.615	0.157	4.415	0.097	1.516
Non-sterilized RM	17	0.748	0.243	2.852	0.182	2.844
Sterilized RM	17	0.672	0.217	3.194	0.146	2.281
Run 3						
Basal with CO_2	24	0.748	0.180	3.851	0.135	2.109
Basal without CO_2	24	0.745	0.177	3.920	0.132	2.062
Non-sterilized RM	24	0.835	0.271	2.558	0.226	3.531
Sterilized RM	24	0.820	0.221	3.136	0.181	2.828
Run 4						
Basal with CO_2	31	0.688	0.155	4.472	0.107	1.672
Basal without CO_2	31	0.695	0.149	4.652	0.104	1.625
Non-sterilized RM	31	0.685	0.191	3.630	0.131	2.047
Sterilized RM	31	0.802	0.223	3.108	0.179	2.797

cultivation so the experiment was finished on the 31st day of semi-continuous cultivation. It is possible that higher renewal rates could have caused an inhibition of cell division. In other words, the value of the renewal rate had exceeded the saturation point such as the well-known phenomenon of photoinhibition for the fourth run of semi-continuous cultivation.

It is also worth noting that the renewal rate is the main factor affecting the cell concentration in semi-continuous cultivation. To evaluate the semi-continuous cultivation, the runs were compared, as shown in Fig. 2. The second run was 8.2% higher than the first run. The third run was also 24% higher whereas the fourth run was 3% lower than the first run. When a slowdown was observed in the fourth run compared to those grown in other runs, the experiment was over. The result of semi-continuous cultivation means that the next run could be achieved successfully with higher specific growth rates by using correct renewal rates.

Discussion

Batch culture is widely used for commercial cultivation of algae for its ease of operation and simple culture system [1]. Semi-continuous culture is also useful for long-term cultivation. Several experiments have been carried out by different authors on the growth of *Haematococcus* sp. in order to obtain maximum biomass productivity. As reported by Fàbregas et al. [14], green vegetative cells were produced in semi-continuous cultures

maintained with daily renewal rates between 10% and 40%. The highest cell productivity, 64×10^6 cells l^{-1} day^{-1} was obtained with a daily renewal rate of 20%. The semi-continuous culture of *H. pluvialis* was successfully implemented with the optimal airlift bioreactor design and under optimal conditions. The specific growth rate and productivity of the semi-continuous culture were 0.31 day^{-1} and 5.52 cells ml^{-1} day^{-1}, respectively. This result was comparable to that reported by Hata et al. [11], who successfully achieved the semi-continuous culture, but only in the small scale (in 500 ml Erlenmeyer flask) with a productivity of 6.8 cells ml^{-1} day^{-1} [15]. On the other hand, *H. pluvialis* was cultivated under photoautotrophic conditions in a bubble column with fed-batch addition of nutrients, especially nitrate, and a cell number above 5×10^6 cells mL^{-1} with the overall productivity of 0.40 g $l^{-1}day^{-1}$was attained after 300 h [16]. For the outdoor culture of *H. pluvialis* in the tubular photobioreactor, biomass concentrations of 7.0 g l^{-1} (d.wt.) were reached after 16 days, with an overall biomass productivity of 0.41 g l^{-1} day. In the bubble column photobioreactor, the maximum biomass concentration reached was also 1.4 g l^{-1}, with an overall biomass productivity of 0.06 g l^{-1} day. The maximum daily biomass productivity, 0.55 g l^{-1} day, was reached in the tubular photobioreactor for an average irradiance inside the culture of 130 $\mu E/m^2$ s. [17].

The CO_2–H_2CO_3–HCO_3^-–CO_3^{2-} system is the most important buffer generally present in freshwaters, and it is the best means available to control and maintain specific pH levels that are optimal for mass-cultivated species [1]. Carbon dioxide uptake is accompanied by changes in pH. Buffers are used to prevent precipitation in the media with minimal pH change. There were natural buffering agents in basal culture medium. Trisaminomethane plays an important role as a buffer. Sodium glycerophosphate also makes the trace metal salts less prone to precipitation in the basal medium. Therefore, in the present study, the basal medium was selected to indicate the effect of CO_2. Consequently, no significant effect of CO_2 in non-sterilized basal medium was recorded due to its buffering agents. However, the effect of CO_2 on the growth of *H. pluvialis* could change depending on the culture medium used.

Since pure carbon dioxide is expensive, alternative and cheaper sources of this gas, such as waste gas from industrial combustion process, diesel engines, cement plants, or fermentation, have been tried. Ethanol production as the main agro-industrial fermentation process is considered as an ideal source of carbon dioxide for algal cultivation as the gas can be used without costly purification. Therefore, the location of algae ponds near such plants is an important consideration in reducing algae production costs; so far, however, all these possibilities remain unrealized and have not gained any economical importance [18]. As reported in 2007 by Habas Firm, the price of pure CO_2 in Turkey is approximately US \$1 kg^{-1} which is 25% more expensive than in European countries.

The large-scale production of *Haematococcus* cells was achieved by the use of a relatively high initial *Haematococcus* cell concentration which permitted the *Haematococcus* cells to predominate immediately after their inoculation into the photobioreactor, thereby preventing any possible contaminating microorganisms to grow in the photobioreactor. This procedure is thus particularly efficient for growing large-scale cultures of *Haematococcus*, while effectively preventing contamination by other microorganisms without the need for expensive and time-consuming steps such as careful sterilization of all apparatus and media and the use of antibiotics or any other antimicrobial agents [19].

Contaminants have eventually built up in the culture under non-sterilized conditions for the long-term bio-productions. *Haematococcus* sp. has served different industries as astaxanthin producer. Product quality is varied and maintained by adjusting the sterilization and process control for various final products depending on the different industrial

applications. In this study, *H. pluvialis* was considered as a pigment producer for the aquaculture industry. RM culture medium was chosen to also demonstrate the effect of sterilization on the growth of *H. pluvialis*. In the fourth run of semi-continuous cultivation, growth increased by 21% in sterilized RM medium with respect to non-sterilized RM medium with a renewal rate of 60% on the 31st day. Hence, long-term effects of sterilization must be tested especially for large-scale production which the control of the growth conditions is much more difficult during longer-term cultivation.

Conclusion

In this study, by comparing the runs of semi-continuous cultivation, the cells grown in RM medium were more effective than the basal medium. The maximum areal productivity of 3.531 g m^{-2} day^{-1}, obtained in non-sterilized RM medium, was much higher than the 2.828 g m^{-2} day^{-1} obtained in sterilized RM medium on the 24th day of the third run of semi-continuous cultivation, whereas on 31st day of the forth run of semi-continuous cultivation, the areal productivity decreased by only 37% in non-sterilized RM medium in comparison with sterilized RM medium. Hence, *H. pluvialis* must be cultivated under sterilized conditions for the long-term bio-production. Furthermore, the effect of CO_2 was investigated in the basal medium, and similar areal productivities of 2.109 and 2.062 g m^{-2} day^{-1} were obtained on the 24th day of the third run of semi-continuous cultivation in the basal medium with the addition of CO_2 and basal medium without the addition of CO_2, respectively. Considering each run of the semi-continuous cultivation, no significant effect of CO_2 in the basal medium was recorded due to its natural buffering agents.

The results have demonstrated that the maximum cell concentration of 10.55×10^5 cells ml^{-1}, which corresponds to the growth rate of 0.271 day^{-1} with the areal productivity of 3.531 g m^{-2} day^{-1}, was found in non-sterilized RM medium on the 24th day of the third run of semi-continuous cultivation at a renewal rate of 50% under the light intensity of 75 μmol photons m^{-2} s^{-1} in the vertical panel-type photobioreactor.

Acknowledgment We wish to thank Ege University Scientific Research Fund for financial support.

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