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Agricultural Fertilizers as Economical Alternative for Cultivation of *Haematococcus pluvialis*

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Abstract A *Haematococcus pluvialis* strain isolated from the ruins of Ephesus in Turkey was investigated as regards its adaptation to laboratory conditions and maximum growth rate. In the first stage of the experiment, the growth of *H. pluvialis* was compared in common culture media. Furthermore, in an effort to minimize the culture costs, the second stage of the experiment compared the growth rate in the culture medium selected in the first stage with that in commercial plant fertilizers. The results demonstrated that the maximum cell concentration of 0.90 g/l, corresponding to a growth rate of 0.150 d^{-1}, was found with an N-P-K 20:20:20 fertilizer under a light intensity of 75 μmol photons m^{-2} s^{-1} on the 12th day of cultivation.

Keywords: Commercial fertilizer, culture medium, growth, *Haematococcus pluvialis*, isolation

Haematococcus is a biflagellate, unicellular green alga with spherical cells. Almgren [1] already described the ecology and distribution of *Haematococcus* in Sweden, where the alga is found in ephemeral rain pools made of rock, generally of small dimensions and based upon firm material, impermeable to water. Meanwhile, Hazen [9] reported that the alga "is very common and widely distributed in Europe, where it is found from Scandinavia to Venice, plus the alga is also distributed from Vermont to Texas and Massachusetts to Nebraska, and probably farther West" [15].

The photosynthetic microalga *Haematococcus pluvialis* is a potential source of astaxanthin, which is used as a feed additive for the pigmentation of cultured salmon and egg yolk, and also as an antioxidative drug [11]. In the algal life cycle of *H. pluvialis*, green vegetative cells with two flagellae grow autotrophically in the light [12] and heterotrophically in the dark [4, 12, 13]. Deficiency of a nutrient, such as nitrogen, induces encystment, with a morphological change from vegetative to cyst cells as the resting stage [10, 12].

Accordingly, the present study isolated a *Haematococcus pluvialis* strain from the ruins of Ephesus in Turkey and investigated its adaptation to laboratory conditions and maximum growth rate. The first stage of the experiment compared the growth of *H. pluvialis* in the most common culture media, whereas the second stage of the experiment compared the growth rate in the culture medium selected in the first stage with that in commercial plant fertilizers as alternative culture media to minimize the costs.

MATERIALS AND METHODS

Isolation of *Haematococcus pluvialis*

The *Haematococcus pluvialis* was isolated from a sample curved from a waterhole in the marble ruins of Ephesus, an ancient city near the modern town of Selcuk, about 50 km south of Izmir in Turkey. The sample (1 ml) was inoculated into 9 ml of a sterilized BG 11 medium in a 70-ml tube and incubated for 7 days at 25°C at a light intensity of 30 μmol photons m^{-2} s^{-1}. The isolation was then accomplished by streaking the natural sample across an agar surface, picking up the isolated colonies from the agar plate using a platinum loop, and restreaking them on a new agar plate, followed by rinsing in a liquid BG 11 culture medium to free the cells. Finally, the isolates were incubated at 25°C at a light intensity of 40 μmol photons m^{-2} s^{-1} in 250-ml Erlenmeyer flasks for 14 days. The isolated *H. pluvialis* from Ephesus was added to the Ege University Microalga Culture Collection (EGE MACC), under the reference EGE MACC-38.

Inoculum Preparation

The stock culture of *H. pluvialis* was grown photoautotrophically in a BG11 medium [17, 19] at 25°C

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under continuous illumination (100 μmol photons m^{-2} s^{-1}) in a 10-l flask. To prepare the inoculum, cells were collected from the stock culture, concentrated by centrifugation (1,160 ×g, 2 min), and the supernatant removed. The collected cells were then transferred and incubated aseptically in a 1,000-ml Erlenmeyer flask containing 800 ml of a 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 (1.25 vvm). For all the experiments, a 4-day-old culture (in the exponential growth phase) was used as the inoculum at a 10% volume.

Growth Conditions

The temperature was measured in the center of the flask with a thermocouple (Dixell-XT115), and controlled using an air conditioner. The illumination was provided by standard cool white fluorescent lamps (18 W) from one side of the flask, and the irradiance measured in the center of the flask with a quantum meter (Lambda L1-185). Continuous aeration was provided with bubbling air supplied by a blower (Nitto Kohki Co., Ltd), and rotometers (Özgül-air) used to provide the desired air flow rate. In addition, a timer (Akboru BND-50/G1) was used to add pure CO_2 for 10 s every 10 min to the air stream (1.5% v/v) to provide inorganic carbon to the culture and keep the pH value below 8.

A 4-day-old culture (80 ml, approximately 1.8 g/l) was inoculated into 800 ml of sterilized fresh media in 1,000-ml Erlenmeyer flasks and incubated for 12 days at 25°C under a light intensity of 75 μmol photons m^{-2} s^{-1}, while air was supplied to the culture at a flow rate of 1 l/min (1.25 vvm).

The 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 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 an increase in concentration from X_1 to X_2. The doubling time was also calculated as $DT = \ln 2/\mu$.

In the first stage of the experiment, five culture media {BG11 [17, 19], Rudic's medium (RM; Rudic and Dudnicenco 2000. MD Patent Nr. a 2000 0154), Basal [8], OHM [5], and Modified BG11 (Mod.BG11; Boussiba *et al.* 2000. US Patent 6,022,701)} were tested at a light intensity of 75 μmol photons m^{-2} s^{-1}. All the components (Merck Co.) used were of analytical grade.

In the second stage of the experiment, three different fertilizers and the selected culture medium were tested under a light intensity of 75 μmol photons m^{-2} s^{-1}. All the components (Horasan Co.) of the selected culture medium were of technical grade. The fertilizers were 0.21 g/l urea with the addition of 0.08 g/l K_2HPO_4 and 0.02 g/l KH_2PO_4, 0.2 g/l N-P-K 20:20:20, and 0.2 g/l GH Gross, all of which are commercial fertilizers used for agriculture. The composition of the N-P-K fertilizer is shown in Table 1.

Table 1. Composition of N-P-K 20:20:20.

Constituents	%
Nitrogen	20
..Ammoniacal Nitrogen	3.89
..Nitrate Nitrogen	6.11
..Urea Nitrogen	10
..H_2O Insoluble	NA
Phosphorus	20
Potassium	20
Magnesium	0.05
Boron	0.0068
Copper	0.0036
Iron	0.05
Manganese	0.025
Molybdenum	0.0009
Zinc	0.0025

RESULTS

Identification of Isolate

During the growth stage, the cells were spherical to ellipsoid and enclosed by a cell wall with a diameter of 30 μm under a light microscope (Olympus B071, 100 times magnification). The cells had 2 flagella of equal length emerging from anterior papilla. According to its age, the cells ceased to be mobile, yet the cellular structure remained the same without the flagella. Under stress conditions, the volume of the cells increased with a diameter of over 40 μm and the cell wall became resistant. The maturation of the cyst cells was accompanied by enhanced carotenoid biosynthesis and a gradual change in cell color to red. When the cystic cells were transferred to optimal growth conditions, daughter cells were released from the cystic cells, and then vegetative cells regenerated from the daughter cells. Based on these morphological features, the isolate was identified as *Haematococcus pluvialis* and added to the Ege University Microalgae Culture Collection (EGE MACC) under the reference EGE MACC-38.

Effect of Different Culture Media

In the first stage of the experiment, the maximum cell concentration of 0.82 g/l, corresponding to a growth rate of 0.141 d^{-1}, was obtained in the Mod.BG 11 medium on the 12th day of cultivation, as shown in Fig. 1. The cell concentration was only 11% lower in the RM medium compared with the Mod.BG 11 medium under a light intensity of 75 μmol photons m^{-2} s^{-1}, yet significantly lower in the basal medium under the same light intensity. Thus, at the end of the first stage, the Mod.BG 11 culture medium was

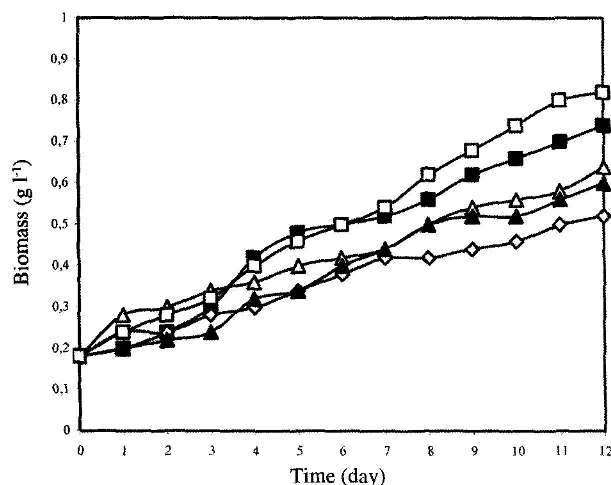

Fig. 1. Effect of different culture media on growth of *H. pluvialis* under a light intensity of 75 μmol photons m^{-2} s^{-1}.
(△) BG11, (■) RM, (◇) Basal, (▲) OHM, (□) Mod. BG11.

selected and prepared with technical grade components for the second stage of the experiment.

Effect of Different Fertilizers and Technical Culture Medium

In the second stage of the experiment, the maximum cell concentration of 0.90 g/l, corresponding to a growth rate of 0.150 d^{-1}, was obtained in an N-P-K 20:20:20 fertilizer on the 12th day of cultivation. In the technical Mod.BG 11 medium, the cell concentration increased to about 0.76 g/l under a light intensity of 75 μmol photons m^{-2} s^{-1}. Cell concentrations of 0.60 g/l and 0.58 g/l were obtained in

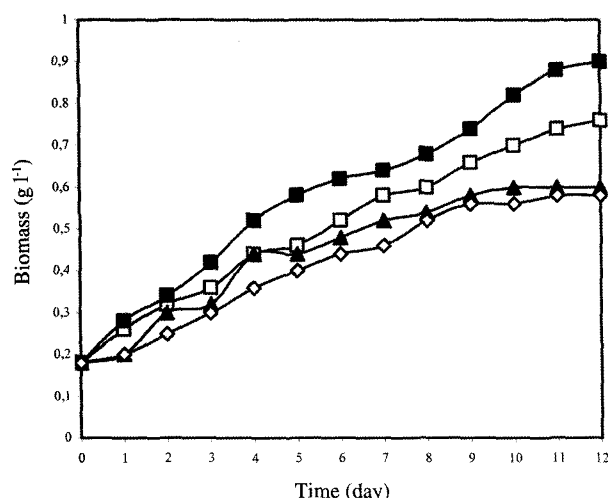

Fig. 2. Effect of different fertilizers and technical culture medium on growth of *H. pluvialis* under a light intensity of 75 μmol photons m^{-2} s^{-1}.
(□) Tech. Mod. BG11, (▲) Urea+K_2HPO_4+KH_2PO_4, (■) N-P-K 20:20:20, (◇) GH Gross.

Table 2. Results of maximum cell concentrations and growth rates for isolated *H. pluvialis* cultured in different media under a light intensity of 75 μmol photons m^{-2} s^{-1}.

Culture medium	Cell con. (g/l)	μ (d^{-1}–h^{-1})	Doubling time (h)
BG 11	0.64	0.116–0.0048	144
RM	0.74	0.131–0.0055	126
Basal	0.52	0.096–0.0040	173
OHM	0.60	0.110–0.0046	151
Mod. BG 11	0.82	0.141–0.0059	117
Tech. Mod. BG 11	0.76	0.133–0.0056	123
Urea+K_2HPO_4+KH_2PO_4	0.60	0.110–0.0046	151
N-P-K 20:20:20	0.90	0.150–0.0063	110
GH Gross	0.58	0.106–0.0044	157

the Urea and GH Gross fertilizers, respectively, with no significant difference between them on the 12th day under the same light intensity (Fig. 2).

When both stages of the experiment were compared based on the maximum cell concentrations, the N-P-K 20:20:20 fertilizer was 9.8% higher than the analytical grade Mod.BG 11 culture medium (Table 2).

DISCUSSION

The results of this study suggest additional benefits from the use of commercial agricultural fertilizers in *H. pluvialis* cultures. For example, the use of N-P-K 20:20:20 for the growth of *H. pluvialis* not only increases the growth rate, but also provides an economic profit.

Several experiments have previously been carried out on the growth of *Haematococcus* sp. For example, Katsuda *et al.* [11] reported that, when growing *H. pluvialis* cells under illumination from LEDs (light emitting diodes), changing from red to blue LEDs after 140 h, and replacing the medium three times, the cell concentration reached above 0.8 mg/ml. Furthermore, in a photobioreactor research program for *H. pluvialis*, the biomass concentration increased from 50 g/m (0.2 g/l) in January to 90 g/m (0.36 g/l) in September with a modified Bolds Basal medium [16], whereas Gong and Chen [7] predicted a dry cell weight of 0.88 g/l with the development of an optimal chemically defined medium for the growth of *H. pluvialis*.

However, when adjusting the light intensity within a range of about 30–150 μmol photons m^{-2} s^{-1}, the light requirements change according to the cell density and shape of the photobioreactor. Therefore, the optimum light requirements need to be determined according to the experimental conditions. In a previous study by the current authors, the optimum light intensity was determined as 75 μmol photons m^{-2} s^{-1} when using 1,000-ml Erlenmeyer flasks (Imamoglu E., Ms Thesis).

It is well known that the culture medium affects not only the cell productivity, but also the cell composition and yield of specific products. However, medium formulation studies are usually time-consuming and expensive. Thus, to overcome these problems, better experimental design techniques need to be used [7]. The choice of medium used depends on several factors: the growth requirements of the algae, how the constituents of the medium may affect the final product quality, and cost [2].

It should also be noted that the appearance of a bacterial population in commercial plant fertilizers is much higher than in other analytical grade culture media. However, the presence of a bacterial population is inevitable and even useful, as it scavenges dead algae and other detritus, thereby preparing the nutrient medium for further generations of algae [6].

As reported by Davies *et al.* [3], the phytoplankton population increased by 56% when using agrolyser and NPK as fertilizers under laboratory conditions. Essentially, the use of agrolyser (micronutrient fertilizer) with NPK (macronutrient fertilizer) increased the phytoplankton population by providing more nutrients.

An outdoor mass culture of *Spirulina* in fertilized seawater was also reported by Tomaselli *et al.* [18], where the control medium was a standard bicarbonate medium, nitrate was the nitrogen source in the control medium, and nitrate and urea were tested in the seawater medium. The mean daily biomass yields (including the winter season) were 8.1 g (dry wt)/m in the control medium, 7.3 g (dry wt)/m in the seawater plus urea, and 5.2 g (dry wt)/m in the seawater plus nitrate. Thus, no great differences were found between the control medium and the seawater plus urea.

Urea is a low-cost nitrogen fertilizer, combining a high nitrogen composition with low transport and storage costs. Thus, urea would seem to be the obvious fertilizer of choice when only nitrogen is needed (James, D.W. 1993. Issued in furtherance of Cooperative Extension work, Utah State University EP/07-95/DF).

As reported by The Fertilizer Institute (TFI) in 2005, the increased global demand for fertilizers has played a large role in the recent increase in fertilizer prices. For example, over the last 4 years, world N demand grew by nearly 10 percent, phosphate demand grew by 13 percent, and potash demand grew by an amazing 25 percent. Thus, when the prices of the culture media used in this study were considered, the price of the N-P-K 20:20:20 fertilizer in Turkey was 98.8% lower than the price of the analytical grade culture medium (Table 3).

Table 3. Analysis of culture media prices in Turkey, 2005.

Culture medium	Cost ($/1000 tonne)
Analytical grade	29,000
Technical grade	2,480
N-P-K 20:20:20	346
Urea+K_2HPO_4+KH_2PO_4	1,716

In conclusion, the present results demonstrated that the maximum cell concentration of 0.90 g/l, corresponding to a growth rate of $0.150\ d^{-1}$, was found with an N-P-K 20:20:20 fertilizer under a light intensity of 75 μmol photons $m^{-2}\ s^{-1}$ on the 12th day of cultivation, thereby confirming the successful use of a fertilizer such as N-P-K 20:20:20 for the growth of *H. pluvialis*.

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