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Investigation of *Haematococcus Pluvialis* for Microalgae Cultivation Using the Flashing Light Method

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Abstract: In view of factors such as their suitability for biofuel production, high oil content, easy accessibility and resistance to contamination, *Haematococcus pluvialis* species of Chlorophyta class was the choice of microalgae selected for this study. Time-based voltage, voltage-based light intensity calibration study and the amount of increase in cell concentration in time have been specified using the flashing method for the examined species. As a result of voltage calibration studies conducted under the trial method, light intensity for flashing practice was detected as 850 $\mu E.m^{-2}s^{-1}$. In order to apply light intensity continuously and at an equivalent level, a FMT-150 photobioreactor was employed and on the first day when the microalgae species was introduced into the photobioreactor, a cell count of 350 $mg.ml^{-1}$ was obtained. The growth process was examined through the cell counts carried out every 48 hours, including the initial phase, under a constant temperature of 25°C. Having applied all these parameters, the *Haematococcus pluvialis* species, which had a cell concentration of 350 $mg.ml^{-1}$, displayed a tendency to grow until the end of the 32th day. While the highest number of cells was reached on the 32nd day with 565 $mg.ml^{-1}$, the population started to decline after the 32nd day, and the trial was terminated on the 38th day.

Keywords: Biodiesel, *Haematococcus pluvialis*, microalgae, renewable energy sources, flashing method

Mikroalg Üretimi İçin *Haematococcus Pluvialis* Türünün Flashing Yöntemi ile İncelenmesi

Öz: Bu çalışmada, biyoyakıt üretimine uygunluk, yağ oranı miktarının fazla olması, kolay bulunabilirlik ve kontaminasyona dayanıklılık gibi faktörler göz önüne alınarak, Chlorophyta sınıfına ait *Haematococcus pluvialis* mikroalg türü seçilmiştir. İncelenmeye alınan bu türün; flashing (yanıp sönen) yöntemi ile zamana dayalı voltaj, voltaja bağlı ışık yoğunluğu kalibrasyon çalışması ve zamana bağlı hücre yoğunluğundaki artış miktarları belirlenmiştir. Deneme yönteminde yapılan voltaj kalibrasyon çalışmaları sonucunda flashing uygulaması ışık şiddeti 850 $\mu E.m^{-2}s^{-1}$ olarak belirlenmiştir. Sürekli ve eş yoğunlukta ışık şiddeti uygulayabilmek için FMT-150 tipi fotobiyoreaktör kullanılmış ve mikroalg türünün fotobiyoreaktöre verildiği ilk gün 350 $mg.ml^{-1}$ hücre sayımı yapılmıştır. Başlangıç süreci ile birlikte her 48 saatte bir 25°C sabit sıcaklıkta hücre sayımları yapılarak büyüme süreci incelenmiştir. Tüm parametreler uygulandıktan sonra 350 $mg.ml^{-1}$ hücre yoğunluğuna sahip *Haematococcus pluvialis* türü 32. günün sonuna kadar büyüme eğilimi göstermiştir. En yüksek hücre sayısına 32. günde 565 $mg.ml^{-1}$ ile ulaşılırken, 32. günden sonra populasyonda düşme görülmeye başlanmış, 38. günde deneme sonlandırılmıştır.

Anahtar Kelimeler: Biyodizel, *Haematococcus pluvialis*, mikroalg, yenilenebilir enerji kaynakları, yanıp-sönme yöntemi

1. Introduction

It is a known fact that the energy resources in the world will be depleted in the near future. Besides significant climate issues, many developing countries are faced with problems such as shortage of energy, raw materials and agricultural production. Moreover, the orientation towards the use of new and renewable energy

sources has become even more important due to causes such as global climate change. This is as a result of higher levels of CO_2 emissions being released into the atmosphere, caused by the ever-growing use of fossil fuels, in order to meet the energy requirements of the steadily increasing global population. For these countries, simple, inexpensive and sustainable environmental protection systems in accordance with local conditions should be developed. While researching new and renewable energy sources, particular attention must be paid to avoiding the use of resources that aggravate food shortages, or further contaminate and deplete other key natural resources, such as soil, water and oxygen. In this respect, biofuel production from microalgae-based biomass, which is one of the renewable energy sources, appears as a method that offers a high potential (Scragg et al. 2002).

As research on biomass has become even more important, due to increasing oil prices in recent years, microalgae have come to be recognized as a promising source of energy (Tapan, 2006). In this research, drawing on data available in the literature, cultivating parameters based on light intensity for the *Haematococcus pluvialis* species, which is among the microalgae species that are suitable for biofuel production, are revealed (Demirbaş, 2010).

Algae are aquatic eukaryotes that have a relatively simple structure, absorb light through photosynthesis and transform inorganic substances to organic substances. They vary from tiny, unicellular species to complex multi-cellular structures. While these multi-cellular (ligneous) structures are called algae, the unicellular structures are called microalgae. *Haematococcus pluvialis*, which was evaluated in terms of increase in cell count depending on light intensity, is a unicellular microalgae species (Sforza et al. 2010).

Microalgae have a rich protein, carbohydrate and fatty acid content. These organisms, which have a nutritional value, are the most essential source of macronutrients, vitamin and trace elements for aquatic animals (Eliçin et al. 2009; Richmond, 2004). The most important main component in the composition of microalgae is protein. Moreover, it should also be noted that microalgae are rich in vitamins, especially vitamin B_{12}. Having a rich protein content, algae also contain an abundance of minerals, such as K, Na, Mg, Ca, P, S and Fe; however, the ratio of these minerals may vary according to the season (İlgi and Şebnem, 2007).

In large-scale cultures, it is necessary to compare the main issues, such as the effective use of light, temperature, maintenance of hydrodynamic equilibrium in microalgae culture, and ensuring the sustainability of the culture. Ideal development of each microalgae species is idiosyncratic. In Table 1, average conditions of microalgae cultivation are presented (Eliçin et al. 2009).

Table 1. Average conditions for microalgae cultivation
Çizelge 1. *Mikroalglerin ortalama üretim şartları*

Parameters	Limit values	Optimal conditions
Temperature (^oC)	17 – 42	18 – 24
Salinity (g/l)	12 – 40	20 – 24
Light intensity (lux)	1000 – 100000	2500 – 5000
Duration of light exposure (day:night h)	-	16:8 min. / 24:0 max.
pH	6 – 9	8.2 – 8.7

Amongst the examples of microalgae species and culture media, we may refer to Spirulina, which exhibits optimal growth under high pH and bicarbonate concentration; Chlorella, which exhibits optimal growth in nutrient-rich media, and *Dunaliella salina*, which exhibits optimal growth better under high salinity. Today, in microalgae cultivation for commercial purposes,

microalgae such as Spirulina and Chlorella are usually preferred as these species are both edible and used in pharmaceuticals. Table 2 presents oil and protein contents of various microalgae species (Xu, et al. 2006). Microalgae can be cultivated in either open or closed systems. While natural ponds, pools and tanks, which can be made from any type of material, are employed in open systems, small-size bags, tubular and horizontal-plate photobioreactors can be given as examples of closed systems. Owing to fatty acids, such as oleic acid (C18:1) and palmitoleic acid (C16:1), which comprise more than 50 % of their structure, microalgae contain a high amount of energy.

Table 2. Oil and protein contents of various microalgae species (Xu, et al. 2006)
Çizelge 2. *Bazı mikroalg türlerinin yağ ve protein içerikleri*

Microalgae species	Oil	Protein
Anabaena cylindrica	4–7	43–56
Arthrospira maxima	6–7	60–71
Botryococcus braunii	86	4
Nannochloropsis salina	21	48
Chlorella ellipsoidea	84	5
Chlorella vulgaris	14–22	51–58
Dunaliella salina	6	57
Haematococcus pluvialis	22-38	30-45
Scenedesmus obliquus	12–14	50–56
Spirulina platensis	4-6	46-63
Spirulina maxima	6-7	60-71

Table 3 presents fatty acid compositions of microorganisms, which are of fundamental importance for biodiesel production (Tawfiq et al. 2004). Microalgae are quite suitable for transformation into biofuel. Table 4 compares biofuel generation from various biodiesel sources (Chisti, 2007).

Table 3. Fatty acid compositions of microorganisms (Tawfiq et al. 2004)
Çizelge 3. *Mikroorganizmaların yağ asit kompozisyonları*

	Microalgae	Yeast	Fungi	Bacteria
16:0 Palmitic acid	18-21	11-37	7-23	8-10
16:1 Palmiteloic acid	55-57	1-6	1-6	10-11
18:0 Stearic acid	1-2	1-10	2-6	11-12
18:1 Oleic acid	58-60	28-66	19-81	25-28
18:2 Linoleic acid	4-20	3-24	8-40	14-17
18:3 Linolenic acid	14-30	1-3	4-42	-

Table 4. Comparison of various biodiesel sources (Chisti, 2007)
Çizelge 4. *Bazı biyodizel kaynaklarının karşılaştırılması*

Product	Oil production (l/ha)
Corn	172
Soybeans	446
Canola	1190
Jatropha	1892
Coconut	2689
Palm	5950
Microalgae (70% oil content)	136900
Microalgae (30% oil content)	58700

2. Materials and Methods

In the research, considering factors such as their high oil content, easy accessibility, resistance to contamination and temperature, the *Haematococcus pluvialis* species of Chlorophytaclass was selected as material (Johnson and Wen, 2009). Having procured the specified species from the algae culture collection in University of Texas (UTEX, 2011). Austin, the species was modified and cultivated in Bold & Basal nutrient media (Z-Hun et al. 2005; Metting and Pyne, 1986).

The microalgae species used in the trial was subjected to several phases, the first of which was the acclimatization phase, before being introduced to the photobioreactor. During this phase the *Haematococcus pluvialis* species was cultured in petri dishes and cultivated for a specific time in in vitro culture. During the acclimatization phase, the growth of microalgae is relatively slow. The adaptation phase was another phase through which *Haematococcus pluvialis* has undergone. Microalgae collected from petri dishes were initially transferred to 100 ml volumetric flasks. After putting the microalgae solution into volumetric flasks, which were sterilized in an autoclave, the flasks were placed in a plant growth regulator. Having left the solutions in 100 ml volumetric flasks for 72 hours, the solutions were diluted and transferred into 250 ml volumetric flasks. After keeping the microalgae for a further 72 hours in plant growth regulator, the solutions were diluted once more and transferred into 1000 ml volumetric flasks. After another 72-hour period, the number of cells was counted and the microalgae were introduced into the FMT-150 photobioreactor.

The flashing method was implemented using the FMT-150 photobioreactor. The study was performed in Pyrex glass and designed as column-type in FMT-150, which has a vessel volume of 1000 ml (Figure 1).

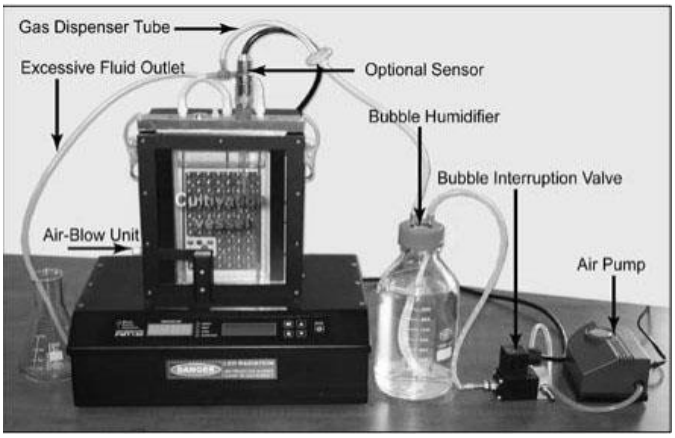

Figure 1. FMT-150 photobioreactor
Şekil 1. *FMT-150 tip fotobiyorekatör*

The FMT-150 includes programmable light, temperature and gas (CO_2) features. The systems offer two types of temperature options, i.e. static (18-50 ^oC) and variable temperature (0-70 ^oC). The static temperature option was selected and during the research the temperature was kept at a constant 25 ^oC. In order to avoid precipitation issues, a magnetic stirrer was used in the cultivation vessel of the photobioreactor (Naz and Gökçek, 2006). During the trials, the cell counts performed periodically were recorded through a user-defined software.

The most important component of the photobioreactor is the lighting compartment. The compartment contains three types of lighting panels: White, White-Red and Red-Blue, respectively. Both static and fluctuating lighting regimes can be employed. The research was

carried out according to white (daylight) flashing methodology. The LED light source on the FMT-150 has a precision that allows to apply 1800 – 2500 µmol (photon)/m^2s under white light (Z-Hun et al. 2005). The research was designed in two parts: i.e. an application of time-based voltage and voltage-dependent light intensity at the first stage, and identification of variations in cell counts, under the light intensity specified, as a result of the initial trial, at the second stage.

3. Results and Dicussion

Measuring the light intensity from a flashing light source is a rather difficult task, as the intensity of the flashing light changes constantly as a function of time. Flashing creates a cycle in which the calibration of the intensity of light and the voltage must first be determined in order to accurately measure the intensity of the flashing lights during this cycle. The changes in voltage with respect to time are given in Figure 2.

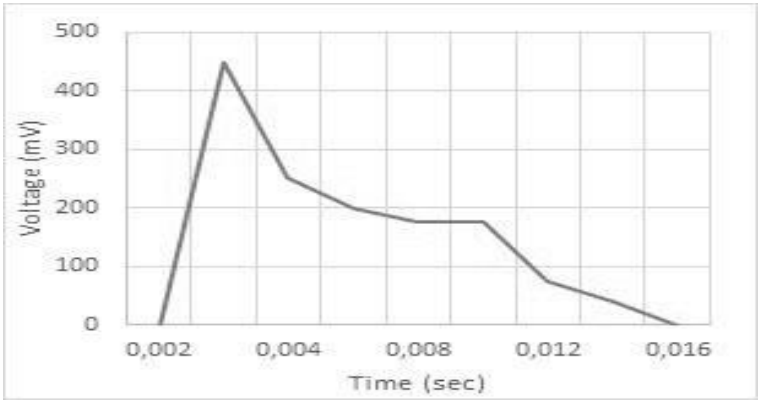

Figure 2. The time-voltage curve
Şekil 2. *Zaman-Voltaj Eğrisi*

For different voltage values, flashing light intensity profiles were recorded (Figure 3). A digital oscilloscope (TDS3032, Tektronix) was used to carry out this procedure (Z-Hun et al. 2005). In order to verify the accuracy of the flashing light intensity, the calibration curve was obtained separately. The calibration curve was obtained using continuous light and the voltage output of the quantum sensor (TDS3032 Oscilloscope) (Lababpour et al. 2004).

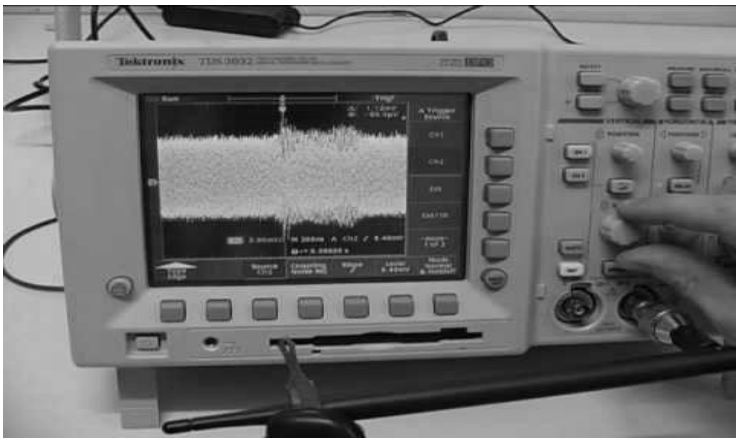

Figure 3. TDS3032 Oscilloscope
Şekil 3. *TDS3032 Osiloskop*

In the calibration curve obtained, light intensity is given in µE $m^{-2}s^{-1}$ and the voltages given in mV. An investigation of the curve reveals that the lowest light intensity of 50 µE.m^{-2}

s^{-1} is achieved at 60 mV, whereas the highest light intensity of 850 µE.$m^{-2}s^{-1}$ was achieved at 325 mV (Figure 4). During the second phase of the research, based on the calibration curve obtained, cell concentrations were measured for 30 days using the light intensity achieved at the highest voltage (Z-Hun et al. 2005) (Figure 5).

At the beginning of the trial 850 ml of microalgae was placed in a FMT-150 photobioreactor. The initial cell count, conducted before the microalgae were placed in the photobioreactor, was recorded as 350 mg.ml^{-1}. At the next stage, the highest light intensity of 850 µE.$m^{-2}s^{-1}$ was chosen, which was obtained by conducting voltage-light intensity calibration tests.

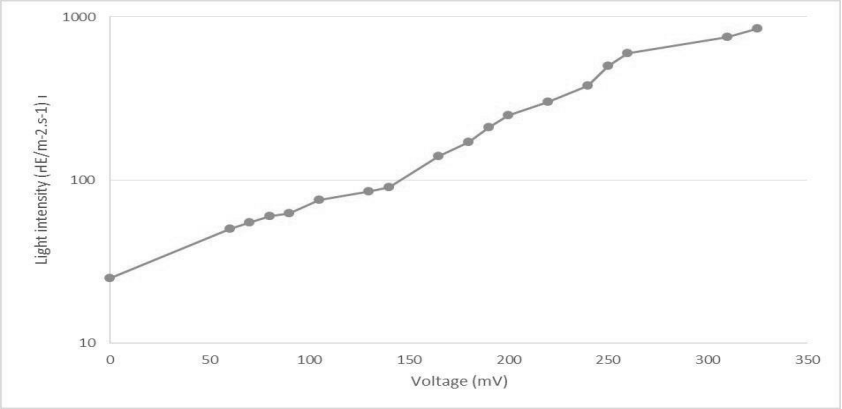

Figure 4. Calibration curve
Şekil 4. *Kalibrasyon eğrisi*

Using a white (daylight) LED source through the flashing method, 850 µE.$m^{-2}s^{-1}$ light intensity was continuously applied for 24 hours. Counting the cells under the microscope, in samples taken from the photobioreactor by means of special software, cell concentrations were measured every 48 hours and saved on a computer.

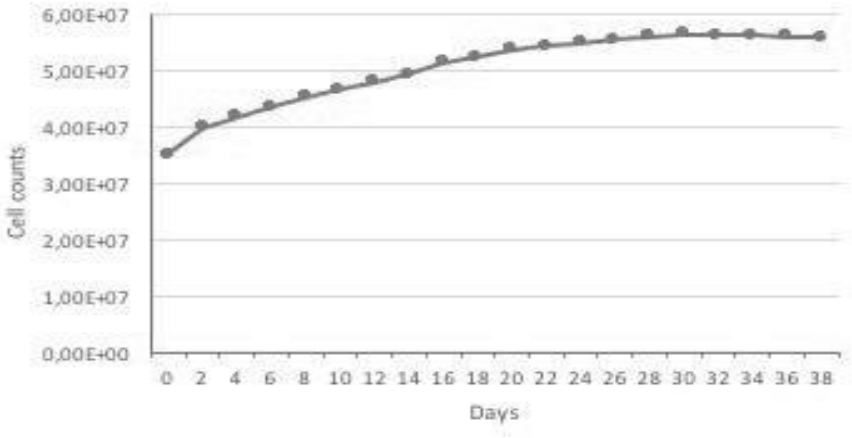

Figure 5. Cell concentration curve under time-dependent constant light intensity
Şekil 5. *Zamana bağlı sabit ışık yoğunluğunda hücre yoğunluğu eğrisi*

From all these data, it was revealed that the *Haematococcus pluvialis* species, which had a cell concentration of 350 mg.ml^{-1} on the first day, exhibited a tendency to grow until the end of the

32nd day under a constant temperature of 25 ^{o}C and flashing white light of 850 µE $m^{-2}s^{-1}$ intensity, obtained at 325 mV (Z-Hun et al. 2005). At the mid-point of the trials, i.e. the 16th day, the concentration increased from 350 $mg.ml^{-1}$ to 510 $mg.ml^{-1}$. While the highest number of cells was reached on the 32nd day with 565 $mg.ml^{-1}$, the population started to decline after the 32nd day, and the trial was terminated on the 38th day. In a previous study we observed that the cell count was 148 $mg.ml^{-1}$ at the end of 15 days under continuous white light (daylight) applied uninterruptedly for 24 hours (Eliçin et al. 2013).

4. Conclusions

The study indicated that high-intensity light is the most important factor leading to the increase of the cell count, and furthermore, high-intensity light and flashing method is much more effective than continuous and low-intensity light. In addition to this, it was revealed that the *Haematoccocus pluvialis* species exhibited an effective growth until the 32nd day, which implies that the microalgae should be harvested on the 32nd day. Since the cell count dropped dramatically after the 32nd day, the amount of oil that can be extracted would also decrease considerably after this duration.

5. Acknowledgements

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