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CHANGES IN THE CELL GROWTH, LIPID CONTENT AND LIPID PROFILE OF *CHLORELLA PROTOTHECOIDES* UNDER DIFFERENT MEDIUMS

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ABSTRACT

Chlorella protothecoides is a precious lipid source that can be used as a biodiesel feedstock. Calcium, carbon, iron, hydrogen, magnesium, nitrogen, oxygen, phosphorus, potassium and sulfur are among the various elements have to be supplied for the growth of microalgae. Especially, nitrogen and phosphorus have significance in handling the growth ratio and lipid production of microalgae. In this study, the effect of different mediums (with/without nitrogen) on cell growth and lipid content of *Chlorella protothecoides* was investigated. Highest lipid extraction efficiency was obtained by using hexane at ambient temperature. The maximum lipid content of *Chlorella protothecoides* fed with the nitrogen free Bold Basal medium was 48% (dry weight percent). The composition of fatty acid methyl esters was influenced by alteration of medium.
Keywords: *Chlorella* protothecoides, lipid, microalgae.

FARKLI ORTAMLAR ALTINDA *CHLORELLA* PROTOTHECOIDES'İN HÜCRE BÜYÜMESİ VE LİPİD İÇERİĞİNDEKİ DEĞİŞİMLER

ÖZ

Chlorella protothecoides biyodizel ham maddesi olarak kullanılabilecek değerli bir lipid kaynağıdır. Kalsiyum, karbon, demir, hidrojen, magnezyum, nitrojen, oksijen, fosfor, potasyum ve kükürt mikroalglerin büyümesi için gereken çeşitli elementlerin arasında yer almaktadır. Özellikle, azot ve fosfor microalglerin büyüme oranı ve lipit üretiminde öneme sahiptir. Bu çalışmada farklı ortamların (azotlu ve azotsuz) *Chlorella* protothecoides'in hücre büyümesi ve yağ içeriğine etkisi araştırıldı. En yüksek lipit ekstraksiyon verimi, oda sıcaklığında hekzan kullanılarak elde edildi. Azotsuz Bold Bazal ortamında beslenen *Chlorella protothecoides*'in maksimum lipid içeriği % 48 (kuru ağırlık yüzdesi)'dir. Ortamdaki değişim yağ asidi metil esterlerinin bileşimini değiştirdi.
Anahtar Sözcükler: Chlorella protothecoides, lipid, mikroalg.

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1. INTRODUCTION

Microalgae are unicellular organisms that are attracting more interests in both academic and industrial fields as promising feedstock candidates for generation of biofuels including biodiesel, bioelectricity, bio-hydrogen, bio-oil and bio-syngas and high value products especially food supplements and pharmaceuticals [1-3]. Moreover, microalgae are used for purification of wastewater effluents [4].

Microalgae are renewable and environmental-friendly feedstocks. Since microalgae is rich in biochemically stable isotopes, vitamins (biotin, vitamin C, vitamin E, etc.), fatty acids and pigments (carotenoids and ficobiliproteins), the suitable microalgal biomass production is a very important issue. Nowadays, some metabolites of microalgae are used in medicine due to their anticholesterolemic, antitumoral, immunomodulatory, antibacterial, antimycotic and pharmacological activities [5].

Biodiesel production is another aspect of microalgae feedstocks due to their high biomass production, high photosynthetic efficiency and rapid growth [6]. By the heterotrophic growth of some microalgae, accumulation of lipids in higher levels can be achieved. This will provide an alternative way to reduce microalga oil production and thus commercialization of large scale biodiesel production.

The algal biomass cultivation, harvesting and processing are the key parameters for production of microalgae with suitable cell composition and price. The carbon source, light, growth medium with nutrients are essential for microalgal cell growth. Nitrogen and phosphor sources are the most important nutritional elements of microalgae culture. Nitrate, ammonium and urea are the most preferred nitrogen sources for microalgae cultivation.

This research is aimed to study the effect of different growth mediums, with/without nitrogen compound, on the autotrophic growth of microalgae Chlorella protothecoides, its lipid content and lipid profile. The dry matter content, optical density, cell count analyses were carried out daily and also growth kinetics were investigated.

2. EXPERIMENTAL SECTION

2.1. Materials

The culture of *Chlorella protothecoides* was purchased from Ege Biotechnology Inc. (İzmir, TURKEY).

2.2. Algal Growth Medium

Bold Basal medium (BBM) was used for *Chlorella protothecoides* growth. The composition of Bold Basal medium was given in Table 1. The stock solutions were stored in the refrigerator at 4°C after prepared by using the chemicals (Table 1) and distilled water.

To prepare Bold Basal medium, 10 mL of each stock solution and 1 mL of each trace element solution were completed to 1 L with distilled water. The pH was adjusted to 7.5 by using 1 M hydrochloric acid or sodium hydroxide solution. The media (200 mL) was transferred to 250 mL Erlenmeyer flasks and then autoclaved at 121°C (1 atm) for 20 min.

2.3. Inoculation and Cultivation of *Chlorella* Protothecoides

The *Chlorella protothecoides* colonies were inoculated into 10 mL stock solution containing tubes. Then, they were added to Erlenmeyer flasks (250 mL) containing 150 mL sterilized Bold Basal media at 28°C under 40 W fluorescence light. Bold Basal medium with or without nitrogen was tested.

Table 1. Components of the Bold Basal medium

Stock solutions	**Concentration per liter of distilled water (g/ L)**
$NaNO_3$	25
$MgSO_4.7H_2O$	7.5
NaCl	2.5
K_2HPO_4	7.5
KH_2PO_4	17.5
$CaCl_2.2H_2O$	2.5
Trace element solutions	
$ZnS0_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49
H_3BO_3	11.42
EDTA	50.0
KOH	31.0
$FeSO_4.7H_2O$	4.98
H_2SO_4	1 mL

2.4. Microalgae Harvesting

Chlorella protothecoides cells were harvested using a Hitachi centrifuge at 4800 rpm for 20 minutes and then washed with distilled water for three times to remove excess medium.

2.5. Characterization

The dry weight was performed in a way that was similar to the procedure reported by Vonshak et al. [7]. Briefly, 50 mL of homogenous suspension of *Chlorella* samples were dried at 105°C for 1 hour in an oven. The semi-dried samples were filtered under vacuum through a Whatman GF 6 glass fibre filter and then washed with a solution (pH 4). The samples were oven dried again at 105°C for 2 hours. The dried filter paper containing *Chlorella* samples were cooled in desiccator and weighted. The dry weight was calculated from the difference between the initial

and final weight. The dry weights were expressed in terms of percentage.

Optical density was determined spectrophotometrically at 500 nm using a UV/Visible spectrophotometer (Scınco S-3100). The determinations were made daily in triplicate for each sample.

A microscopic count was performed on the microalgal suspensions using a Olympos CX40 microscope.

For the lipid extraction, three different methods were used. Firstly, the harvested cells were dried at 55°C for 2 hours in an oven and then lyophilized. Lyophilized cells were stored at -20°C until the analysis. Extraction methods were given below:

i) The method of Bligh and Dyer [8] was used. The samples (0.1 g) were extracted with the mixture of chloroform: methanol (2:1, v/v, 60 mL). The mixture was mixed with $CaCl_2$ solution (0.4%,10 mL) and then was separated carefully by filtering through a Whatman GF 6 glass fibre filter.

ii) The samples (0.1 g) were extracted with hexane (6 mL). The mixture was stirred in a homogenizer for a minute and then left in a waterbath (25°C). After 24 hours, the mixture was centrifuged and filtered.

iii) The samples (0.1 g) were extracted with hexane using a Soxhlet extraction apparatus for 8 hours.

At the end of all extractions, the solvent phase was evaporated in a rotary evaporator at 60°C to remove organic solvent. The remained part from the evaporation was weighed to calculate the lipid content and the results were expressed in terms of percentage.

Gas chromatography was used to evaluate the fatty acid composition of hexane extracts (at 25°C) of *Chlorella protothecoides* samples. The BF_3/methanol method was performed to convert fatty acids into fatty acid methyl esters [9]. The analyses were executed on a Shimadzu 2025 GC system with a 100m x 25mm x 2 µm capillary column and flame ionization detector. The flow rate of the carrier gas (helium) was 1.2 ml/min. The temperatures of the detector and injector were 225°C and 250°C, respectively. The analysis was repeated for three times and the avarege of three measurements were given.

3. RESULTS AND DISCUSSION

3.1. Cell Count

The type of growth medium is known to be critical factor influencing the growth rate of algae in terms of biomass productivity. In this study, the effect of two growth mediums was examined on the growth of *Chlorella protothecoides*. The results show that the maximum biomass production under Bold Basal and nitrogen free Bold Basal culture conditions were same (71.3 10^5 cells/ mL) at the end of sixth day (Fig. 1). Rosenberg et al.[10] also observed that the growth of *Chlorella protothecoides* under autotrophic conditions reached to cell density of 10 million cells ml^{-1} within one week. As can be seen from Figure 1, higher biomass productivities are achieved within 0–6 days which is a required trend for successful harvesting of algal biomass [11]. These data suggested that sodium nitrate as a nitrogen source was not efficient to enhance cell growth.

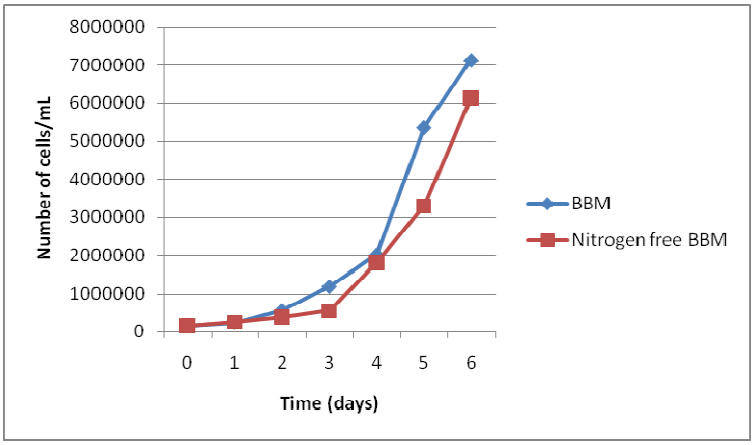

Figure 1. Effect of medium on biomass production of *Chlorella protothecoides* microalgae a) Bold Basal b) Nitrogen free Bold Basal

3.2. Optical Density

Optical density measurement is one of the most used method to determine the cell growth of algae rapidly. Optical densities were detected within 0-9 days. It was observed that the use of Bold Basal media resulted in an increase in biomass concentration. Optical densities in the Bold Basal medium reached a maximum of 1.52 at 500 nm (Figure 2). For nitrogen free Bold Basal medium, it was determined as 1.47.

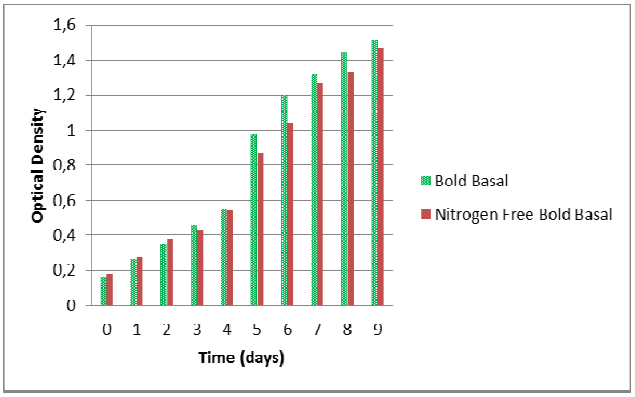

Figure 2. Effect of medium on optical density of *Chlorella protothecoides* microalgae

3.3. Dry Weight

The effects of nitrogen on biomass dry weight of *Chlorella protothecoides* are shown in Figure 3. The biomass concentration raised significantly after 3 days. The highest biomass dry weight values were obtained in nine days as 0.67 g/ L and 0.66 g/L in Bold Basal and nitrogen free Bold Basal medium, respectively. It was determined that there is not a significant difference between the biomass dry weights.

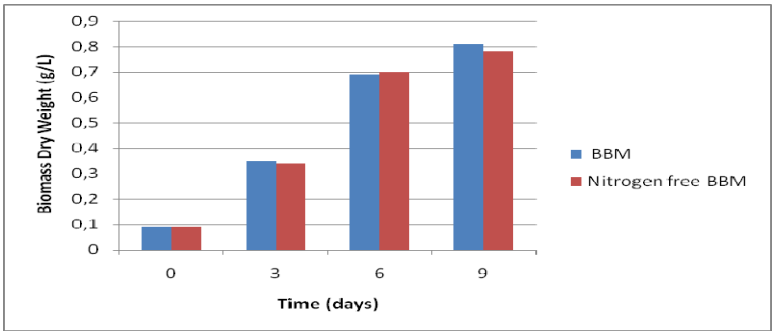

Figure 3. Effect of medium on biomass dry weight of *Chlorella protothecoides* microalgae a) Bold Basal b) Nitrogen free Bold Basal

3.4. Lipid

The lipid accumulation in *Chlorella protothecoides* changes due to various factors such as light intensity, pH, speed of agitation, sources of nutrients, salinity, and temperature [1]. As shown in Table 2, the medium is one of the key factors influencing the lipid content of microalgae. When hexane extraction methods were used the biomass obtained from the Bold Basal medium with sodium nitrate as nitrogen source has higher lipid content than the nitrogen free one. The maximum lipid amount on a dry weight basis (29%) was obtained for Bold Basal medium sample after extraction with hexane at ambient temperature. Similar results were reported by Heredia-Arroyo et al. [12] for the lipid production of autotrophic *Chlorella protothecoides* using potassium nitrate as nitrogen source that was determined using Soxhlet extraction with hexane. Also, the values of lipid content was even similar (21.5-34 %) obtained by choloroform:methanol extractraction of the lyophilized algal powder of hetotropic *Chlorella protothecoides* cells that was cultured using urea as nitrogen source and sugarcane bagasse hydrolysate as carbon source [13].

Table 2. Effect of medium on the lipid productivity of *Chlorella protothecoides* microalgae

Extraction Solvent/Method	**Lipid content (dry weight %)**	
	Bold Basal medium	Nitrogen free Bold Basal medium
Choloroform:methanol	23	21
Hexane / Ambient temperature	29	26
Hexane / Soxhlet extraction	25	22

The lipid metabolic profiles of *Chlorella* protothecoides are summarized in Table 3. The composition of fatty acid methyl esters showed that the extracts were rich in palmitoleic (16:1), heptadecenoic (17:1), oleic (18:1 acid), linoleic (18:2) gadoleic (20:1) and eicosadienoic (20:2) acids. In the present study, the fatty acid composition varied with the change in Bold Basal medium.

Table 3. Fatty acid composition of hexane/ambient temperature extracts of Chlorella protothecoides

Distribution of fatty acids (%, of total fatty acids)	**Hexane extract at ambient temperature**	
	Bold Basal medium	Nitrogen free Bold Basal medium
13:0 (Tridecylic acid)	-	0.30
14:0 (Myristic acid)	0.51	0.78
14:1 (Myristoleic acid)	1.69	2.02
15:1 (Pentadecenoic acid)	4.25	3.29
16:0 (Palmitic acid)	1.77	3.70
16:1 (Palmitoleic acid)	8.43	9.56
17:1 (Heptadecenoic acid)	11.53	12.76
18:0 (Stearic acid)	0.57	-
18:1 trans-9 (Elaidic acid)	0.36	0.41
18:1 cis-9 (Oleic acid)	17.80	14.45
18:2 trans-9,12 (Linoelaidic acid)	0.55	0.68
18:2 cis-9,12 (Linoleic acid)	13.84	14.10
18:3 cis-6,9,12 (γ-Linolenic acid)	-	0.36
20:1 (Gadoleic)	11.44	11.15
18:3 cis-9,12,15 (α-Linolenic acid)	1.14	1.32
21:0 (Heneicosylic acid)	0.83	0.89
20:2 (Eicosadienoic acid)	8.94	8.05
20:3 cis-8,11,14 (Dihomo-γ-linolenic acid)	-	0.36
22:1 (Erucic acid)	6.83	6.06
23:0 (Tricosylic acid)	-	0.55
22:2 (Brassic acid)	4.68	3.98
24:0 (Lignoceric acid)	-	0.39
24:1 (Nervonic acid)	2.73	1.98
20:5 (Eicosapentaenoic acid)	1.41	1.23
22:6 (Docosahexaenoic acid)	0.68	0.59

4. CONCLUSION

Nitrogen is one of the essential elements for the growth of microalgae. The Bold basal medium had increased the biomass productivity and lipid content of the *Chlorella protothecoides*

biomass. The maximum biomass dry weight was achieved at the end of the 9 days. Hexane extraction at ambient temperature was found to be the best extraction method for lipid recovery. It was determined that lipid accumulation is intensified under Bold Basal medium. Moreover, the distribution of the fatty acids was affected by the alteration of medium. The results indicated that the lipid recovered from *Chlorella protothecoides* is a promising candidate for further biodiesel applications.

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