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The Effect of Stress Due to Nitrogen Limitation on Lipid Content of *Phaeodactylum Tricornutum* (Bohlin) Cultured Outdoor in Photobioreactor

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Abstract

The effect of nitrogen limitation on the cell density, biomass, chlorophyll *a*, total carotene, protein and lipid content of *Phaeodactylum tricornutum* (Bohlin), Bacillariophyceae, cultured in photobioreactors outdoor was investigated. *Phaeodactylum tricornutum* was cultivated in an appropriate medium as the control group, at the same time it was cultured in a medium of which nitrogen was reduced to 50%. At the end of the study, it was determined that 35.04% lipid with 0.980±0.02 gL^{-1} biomass and 8.87% protein content, meanwhile, in the control group, 16.93% lipid, 1.036±0.025 gL^{-1} biomass and 31.05% protein were determined. As a result, it was observed that while nitrogen limitation increased to the lipid amount, decreased the biomass and chlorophyll *a*, total carotene and protein of the cell.

Keywords: *Phaeodactylum tricornutum,* lipid, N limitation, tubular photobioreactor, outdoor culture.

Azot Eksikliğine Bağlı Stresin, Fotobiyoreaktörde Kültüre Alınan *Phaeodactylum Tricornutum* (Bohlin)'un Lipid Miktarina Etkisi

Özet

Azot sınırlamasının, dışarı ortamda, tubular fotobiyoreaktörlerde kültüre alınan Bacillariophyceae sınıfına ait *Phaeodactylum tricornutum* (Bohlin)'da, hücre yoğunluğu, biyomas, klorofil *a*, total karoten, protein ve lipit miktarına etkisi araştırılmıştır. *Phaeodactylum tricornutum*, kontrol grubu olarak uygun ortamda ve aynı zamanda azotu %50 eksiltilmiş ortamda kültüre alınmıştır. Çalışmanın sonunda, %35,04 lipid, 0,980±0,02 gL^{-1} biyomas ve %8,87 protein içeriği saptanırken, kontrol grubunda %16,93 lipid, 1,036±0,025 gL^{-1} biyomas ve %31,05 protein belirlenmiştir. Sonuç olarak, azot sınırlamasının lipit miktarını artırırken, hücrenin biyomas, klorofil *a*, toplam karoten ve protein miktarında azalmaya neden olduğu gözlenmiştir.

Anahtar Kelimeler: *Phaeodactylum tricornutum,* lipid, azot sınırlaması, tubular fotobiyoreaktör, dışarı kültürü.

Introduction

Microalgae have the ability to use solar energy in combination with water and carbon dioxide to create biomass. In recent years, an increasing interest in microalgal biotechnology was noticed due to the accumulation of cells metabolites in large amounts. Microalgae are used for various purposes mainly as food supplement, fertilizer for improving soil structure, in animal feed due to protein, vitamins, fatty acids, carbohydrates, minerals and pigments, hydrocarbons, polysaccharides, antibiotics, and for other metabolites (Becker, 2007). Microalgae are able to double their weights daily, can easily be used in biotechnological processes, have low cost, contain many useful substances which have an economic value and their ability to resist against environmental factors are the reasons of their significance. Microalgae use sunlight and carbondioxide to produce oil as terrestrial plants, however they possess more productivity than terrestrial plants due to their fast reproduction which take only few hours and the fact that they can be harvested throughout the year.

Recently in many countries, different studies have been carried out on microalgae lipid as renewable fuel sources (Chisti, 2007). Many microalgae strains capable of producing high amount of lipids have been identified and their lipid production metabolisms have been determined and reported (Sheehan *et al.,* 1998). As a source of

renewable energy, opportunities to benefit from non-toxic microalgae are underway for a biodiesel fuel source. For this purpose, high lipid content and better growth rate of microalgae species, besides stress conditions that stimulate content of lipid are being researched in many countries (Bulut Mutlu *et al.*, 2011).

Many studies have shown that, the quantity and quality of lipids within the cell can vary as a result of changes in growth conditions such as temperature and light intensity, or nutrients, especially concentrations of nitrogen, phosphates and iron (Illman *et al.*, 2000; Liu *et al.*, 2008; Xin *et al.*, 2010). Different nitrogen (N) sources and concentrations can affect the growth and biochemical composition of algae and change the amount of carotene and fatty acid (Fidalgo *et al.*, 1998). While N limitation decreases, the cell density of the cultures, amount of biomass and amount of chlorophyll *a*, increase the organic carbon compounds such as the lipid in the biochemical structure of microalgae (Kilham *et al.*, 1997; Pruvost *et al.*, 2009). It was known that decreasing of N or completely removing it from the culture media, caused an increase in lipid content of the cell, thus, N limitation is suggested as the stress factor (Sukenik, 1991).

The aim was to investigate the effect of N limitation on the cell density, biomass, chlorophyll *a*, total carotene, protein and lipid content of *Phaeodactylum tricornutum* (Bohlin) that belongs to the Bacillariophyceae cultured outdoor in tubular photobioreactors.

Materials and Methods

Algal Material and Culture Conditions

The diatom *Phaeodactylum tricornutum* UTEX 640 (Bohlin) was used in this study. The initial culture was obtained from the Algal Biotechnology Laboratory, at the Faculty of Fisheries, Cukurova University. *Phaeodactylum tricornutum* (Bohlin) cells were of two characteristic types, oval and fusiform, each of which remained constant for many cell divisions in clonal culture (Lewin *et al.*, 1958). *Phaeodactylum tricornutum* (Bohlin) is classified as a pennate raphid diatom, but only the oval form has a raphe (Lewin, 1958). The most notable characteristics of this strain are its ability to produce a high proportion of its fatty acids as EPA and its capacity to grow to high cell densities (Yongmanitchai *et al.*,1991).

The cultures were grown in Si-F/2 medium (Guillard and Ryther 1962, Guillard 1975). The medium consist of following composition:75gL^{-1} $NaNO_3$, 5gL^{-1} $NaH_2PO_4H_2O$, 30gL^{-1} $Na_2SiO_39H_2O$ and trace metal solution (9.8gL^{-1} $CuSO_45H_2O$, 6.3gL^{-1} $Na_2MoO_42H_2O$, 22gL^{-1} $ZnSO_47H_2O$, 10gL^{-1} $CoCl_26H_2O$, 180gL^{-1} $MnCl_24H_2O$) were added to 1 ml/L, and the vitamin solution (1gL^{-1} biotin, 1gL^{-1} cyanocobalamin) was added 0.5 mL to 1L. The sources of nitrogen of the medium is $NaNO_3$. In the experiment the amount of $NaNO_3$ was reduced by 50%, 37.5 gL^{-1} $NaNO_3$ was added.

The inoculums for the tubular photobioreactors was grown under laboratory conditions in F/2 medium with silicium, that is, 1 mL/l culture from stock solution of Na_2SiO_3 (30gL^{-1}) (Guillard, 1975), and acclimated cells were used at 20°C under continuous vessel-surface light intensity of 80 $\mu molm^{-2}s^{-1}$. The irradiance was measured by a Radiation Sensor LI-COR (LI-250, Inc. USA). In the laboratory, *Phaeodactylum tricornutum* was cultured in 500 mL glass erlenmeyer flasks firstly, and then increased to the 2L glass flasks, later on the cultures were increased to the 6L volume in balloons, in a continuous culture system. Cultures were placed on the shelves and aerated by a compressor, continuously.

The study was conducted in tubular photobioreactors outdoor of the Algal Biotechnology Pilot Plant at the Fisheries Faculty, Cukurova University. While the control group was cultured in Si-F/2 medium, the other group was cultured in modified medium of which N was reduced by 50% and with 20% inoculation ratio. The volume of each photobioreactor was 300 liters. The diameter of the transparent acrylic tubes was 2.6 cm and tubes were set up horizontally. The volume of collection tanks were 150 L. The CO_2 gas inlet was provided with flow meter. The pH and the flow rate of the cultures were adjusted to 8 and 0.3 m sec^{-1}, respectively.

Analytical Methods

Samples were taken daily for analyses of the optical density, biomass, chlorophyll *a* and total carotene. *Phaeodactylum tricornutum* cell concentration was recorded daily by optical density measurement at 625nm by a UV-visible spectrophotometer (Shimadzu, UV mini, 1240 model, Japan). Biomass was determined according to the method developed by Boussiba *et al.* (1992) with 10 mL of microalgae culture through glass fiber filter (Whatman GF/C, 1.2 µm, UK). Algal biomass on the filter was dried at 105 °C for two hours and weighed (Boussiba *et al*., 1992). For pigment analyses, 10 mL samples were centrifuged at 3500 rpm for 10 min, and the pellet extracted with 5 mL acetone (Parsons and Strickland, 1963). The extracts were centrifuged again and chlorophyll *a* and total carotene were measured spectrophotometrically, recording the absorption at 665, 645, 630 and 480 nm and using the equations of Parsons and Strickland (1963). All measurements were repeated in five replicates.

For lipid and protein analyses, samples of microalgae were collected at the stationary phase of the growth. *Phaeodactylum tricornutum* cells were separated from the medium by centrifugation at 7500 rpm for 10 min, using the centrifuge model of Hereaus Supragufe 22. However, biomass was dried

at 55°C for 2 h, pulverized in a mortar and stored at -20°C for later analysis. Dry extraction procedure according to Zhu *et al.* (2002) with a modification of the wet extraction method developed by Bligh and Dyer (1959) was used to extract the lipid from microalgae cells. Cells were harvested by centrifugation at 7500 rpm for 10 min. After drying, the samples were pulverized in a mortar and extracted using a mixture of chloroform: methanol (2:1, v/v), overnight. About 120 mL of solvents were used for every gram of dried sample in each extraction step. The solid phase was separated carefully using filter paper (Advantec filter paper, no. 1, Japan) in which two pieces of filter papers were applied twice to provide complete separation. The solvent phase was evaporated in a rotary evaporator by vacuum at 60°C. The amount of total protein (Nx6.25) was determined by Kjeldahl method (AOAC, 1995).

The one-way analysis of variance (ANOVA) was used to t-test the effects of N deficiencies of culture on optical density, biomass, chlorophyll *a*, total carotene, lipid and protein amounts of the mean differences by the Statistical Package for the Social Sciences (SPSS) (Version 12.0, SPSS, Chicago, IL) (Zar,1999). As such, the differences were considered to be significant at $P \leq 0.05$.

Results

In this study, the effects of N deficiency on the optical density, biomass, chlorophyll *a*, total carotene, lipid, and protein contents of *Phaeodactylum tricornutum* were investigated. The growth was continued for 15 days for the control group and 12 days for the 50% N deficiency group. In the control group, the highest, lowest and average temperatures were determined as 24±1°C, 19±0°C, and 21±1°C, respectively. The highest, lowest and average light intensities were determined as 558±61, 153±4 and 313±32 $\mu molm^{-2}s^{-1}$, respectively. For the 50% N deficiency group, the highest, lowest and average and average light intensities were determined as 615±9, 163±4 and 386±7 $\mu molm^{-2}s^{-1}$, respectively.

Comparison of main parameters of optical density, biomass, chlorophyll *a* and total carotene contents between the two groups of N deficiency and control in tubular photobioreactors are summarized in Table 1.The optical density was not significantly affected in the two groups ($P>0.05$).While the 0.747±0.007 gL^{-1} of biomass was determined on the first day, 1.036±0.025 gL^{-1} of biomass was obtained on the last day for the control group. Initially, 0.761±0.02 gL^{-1} of biomass was collected, while 0.980±0.02 gL^{-1} of biomass was recorded on the last day for the 50% N deficiency group. The initial chlorophyll *a* and total carotene were determined as 288±1 μgL^{-1} and 0.225±0.001 μgL^{-1} and at the end of the experiment chlorophyll *a* and total carotene were determined as 415±2 μgL^{-1} and 0.620±0.002 μgL^{-1} for the control group, respectively. The initial chlorophyll *a* and total carotene were determined as 293±2 μgL^{-1} and 0.265±0.001 μgL^{-1} and at the end of the experiment chlorophyll *a* and total carotene were determined as 178±3 μgL^{-1} and 0.463±0.001 $\mu gL^{-1}$for the 50% N deficiency group, respectively (Table 1).

The biomass, lipid and protein contents of *Phaeodactylum tricornutum* for the control and 50% N deficiency groups in tubular photobioreactor were summarized in Table 2. For the culture to which N limitation was applied, 35.04% lipid, 0.980±0.02 gL^{-1}

Table 1. Main parameters of optical density, biomass, chlorophyll *a* and total carotene contents of *Phaeodactylum tricornutum* for control groups and in growth medium with N deficiency, in tubular photobioreactors

Parameters	Tubular Photobioreactor Control (15 Day)	Tubular Photobioreactor 50% N (-) (12 Day)
OD_{625nm}*(initial)*	0.251±0.002[a]	0.270±0.007[a]
OD_{625nm}(*end*)	0.537±0.009[a]	0.526±0.004[a]
Biomass (gL^{-1})*(initial)*	0.747±0.007[a]	0.761±0.02[a]
Biomass (gL^{-1})*(end)*	1.036±0.025[a]	0.980±0.02[b]
Chlorophyll *a* (μgL^{-1}) *(initial)*	288±1[a]	293±2[a]
Chlorophyll *a* (μgL^{-1})*(end)*	415±2[a]	178±3[b]
Total carotene(μgL^{-1})(*initial)*	0.225±0.001[b]	0.265±0.001[a]
Total carotene(μgL^{-1})*(end)*	0.620±0.002[a]	0.463±0.001[b]

Means values, n=5; *Different letters between the cloumns indicate significant difference ($P<0.05$).

Table 2. Main parameters of biomass, lipid and protein content of *Phaeodactylum tricornutum* for control group and 50% N deficiency in the growth medium in tubular photobioreactors

Parameters	Tubular Photobioreactor (Control group)	Tubular Photobioreactor (50% N)
Biomass (gL^{-1})	1.036±0.025[a]	0.980±0.02[b]
Lipid (%)	16.93±0.1[b]	35.04±0.01[a]
Protein (%)	31.05±0.1[a]	8.87±0.5[b]

Means values, n=5; *Different letters between the cloumns indicate significant difference ($P<0.05$).

biomass and 8.87% protein were determined, for the control group, 16.93% lipid, 1.036±0.025 gL^{-1} biomass and 31.05% protein were determined (Table 2). According to the results, the stress factor of N deficiency, increased the lipid contents twice in the cells compared to the control group.

Discussion

Despite the advances in algal biotechnology, there are some difficulties about in the microalgae culture. Basically, the main objective of the production of phototrophic organisms is to provide the continuous culture. It is known that the microalgae species are affected from changing environmental factors. The microalgae cells react to these changing factors, continuously. The biochemical composition of biomass depends on growth conditions such as nutrient medium, temperature, salinity, pH, and light (Sukenik, 1991; Cohen *et al*., 1988; Brown *et al*., 1989; Roessler, 1990, Lourenço *et al*., 1998; Hu, 2004).

Algae cultures require the nutrients, mainly nitrogen, phosphorus and potassium for growth (Slade and Bauen, 2013). The stress caused by nitrogen deficiency slows the cell division and growth in the cultures. Therefore, while the growth was continued for 15 days for the control group, the growth entered the stationary phase on the 12th days of the culture of which 50% N deficient.

Nitrogen limiting conditions were in fact reported to significantly increase the lipid content of many microalgae (Illman *et al*., 2000). It is known that different N sources and concentrations can affect the growth and biochemical composition of microalgae (Fidalgo *et al*., 1995). When the N deficiency was applied to the culture, the microalgae biomass decreased and lipid content increased (Xin *et al*., 2010). When *Phaeodactylum tricornutum* was cultured in N deficient medium at different light paths, the biomass decreased (Uslu *et al*., 2014). In similar studies, it was reported that the N deficiency decreased optical density and biomass (Kilham *et al*., 1997; Pruvost *et al*., 2009). In this study, N concentration was reduced to fifty percent rate in F/2 media described in method. It was observed that the optical density was not affected significantly ($P>0.05$), while the biomass decreased in the N-deficient medium statistically. Even so, the biomass was recorded as 1.036±0.025 gL^{-1} for the control group and 0.980±0.02 gL^{-1} was determined for the N-deficient culture.

The limitation of N in the medium caused a significant change in cell composition, increased the accumulation of lipid and decreased protein content in *Phaeodactylum tricornutum* cells during the batch growth. Nitrogen deficiency increased the amount of lipid, in different microalgae species such as *Isochrysis affinis galbana*, *Nannochloropsis* sp., *Phaeodactylum tricornutum*, *Neochloris oleoabundas*, *Chlorella vulgaris*, *Spirulina platensis* (Sukenik and Wahnon, 1991; Reitan *et al*., 1994; Fidalgo *et al*., 1995;Pruvost *et al*., 2009; Bulut Mutlu *et al*., 2011; Uslu *et al*., 2011). Similarly, Griffiths and Harrison (2009) reported that when N deficiency was applied to the culture at laboratory conditions, lipid content might be increased. Similar studies indicated that while N is deficient, protein decreases in general (Shifrin and Chisholm, 1981). Thomas *et al.* (1984) cultured the microalgae *Phaeodactylum tricornutum* in N sufficient and deficient mediums. Nitrogen-sufficient medium contained 55% protein, 10% carbohydrate, 20% lipid, 12% ash and 4.8 calories mg^{-1} dry weight. Nitrogen deficiency changed these values to 25%, 15%, 30%, 16% and 5.0, respectively. In another study, *Isochrysis affinis galbana* was cultured and it was reported that when the N was raised the lipid ratio decreased from 22% to 16.9% (Utting, 1985). Weldy and Huesemann (2007) cultured *Dunaliella salina* in photobioreactors under high light and low light as well as N deficiency and N sufficient mediums. They reported that the lipid amount of *Dunaliella salina* ranged from 16% to 44% at high light and N deficiency conditions and they found that 38% lipid under only high light culture conditions. Bulut Mutlu *et al.*, (2011) studied the effect of 50% N, 100% N, 50% N-P, 50% P deficiencies and nitrite addition on the lipid, protein and biomass of *Chlorella vulgaris*, in laboratory conditions. The highest lipid and the lowest protein amounts of 35.6% and 13.0%, respectively were reported for the culture in which 100% N deficient. The lowest biomass of 0.12 gL^{-1} was found in the group which contained nitrite. Uslu *et al*. (2011) investigated the effect of N deficiencies (50 and 100%) on the lipid, protein and biomass of *Spirulina platensis* in at laboratory conditions. They found that, 67.4%, 53.5%, 5.6% protein and 5.78%, 13.66%, 17.05% lipid were found for the control group, 50% N(-) and 100% N(-), respectively. The highest lipid, 17.05% and 1.00gL^{-1} biomass were recorded from the culture with 100% N deficiency. Uslu *et al.* (2012) cultured three microalgae species *Isochrysis affinis galbana*, *Phaeodactylum tricornutum* and *Porphyridium cruentum.* The effect of 50% N and 100% N deficiencies in laboratory conditions was investigated.They found that the highest lipid content was given by *Isochrysis affinis galbana* and *Phaeodactylum tricornutum* in 50% N deficiency. While 30.91% lipid and 0.755±0.03 gL^{-1} biomass were determined for *Isochrysis affinis galbana*, 30.18% lipid and 0.978±0.02 gL^{-1} biomass were reported for *Phaeodactylum tricornutum*. It was observed that *Porphyridium cruentum* treated with the 100% N deficiency contained 11.23% lipid that was lower than the other two species. Uslu *et al.* (2013), cultured *Chlorella vulgaris* in 50% reduced N medium in tubular photobioreactors. They found 12.34% lipid for the control group while the highest lipid content of 38.16% was reported for the 50% N

reduced culture. Uslu *et al.* (2014) investigated that lipid, protein and biomass of *Phaeodactylum tricornutum* using different light path length of 1, 3, 5, 7 and 10 cm in panel photobioreactor systems (PBR) and 50% N deficiency in the medium. They reported that the highest lipid, protein and biomass were of 34.6%, 8.50% and 1.064gL^{-1}, respectively for *Phaeodactylum tricornutum* treated to deficiency of 50% N in the panel PBR system with 7 cm light path. In this study, 35.04% lipid, 0.980±0.02 gL^{-1} biomass and 8.87% protein were determined for *Phaeodactylum tricornutum* with 50% N limitation, while 16.93% lipid, 1.036±0.025 gL^{-1} biomass and 31.05% protein were determined for the control group. According to the results obtained, lipid amount in the cells with N deficiency compared to the control group were doubled. In this study, N deficiency caused the diminution of protein content and biomass amount while the lipid content increased (Table 2). Nitrogen limitation caused the increasing of lipid in *Phaeodactylum tricornutum* cells.

Marin *et al*. (1998), investigated the effects of different rates of N on the amounts of carotene and chlorophyll *a* of *Dunaliella salina*. They reported decreasing total carotene and chlorophyll *a* when the N deficiency was increased. Durmaz (2006), reported that a reduction in the chlorophyll *a* and total carotene of *Nannochloropsis oculata* cells in 50% N deficient condition. In this study, N deficiency of 50% caused a decrease in the amount of chlorophyll *a* and the amounts of total carotene, while the lipid ratio of the cells increased.

In conclusion, microalgae biomass can be source of biodiesel as a renewable energy. The studies about microalgae lipid production are mostly conducted at the full-controlled conditions in the laboratories. However, it is important that to achieve the mass production of microalgae for industrial production. And also it is important that decreasing of the cost of culture to produce lipid. To determine the microalgae species contained high lipid and to obtained high biomass are studied in many countries. In our country, also we have to study about isolation of the microalgae contained high lipid in our seas. Our country has planty of sunlight and subtropic climate, and the photosynthetic microalgae biomass can produce easily. Finally, the results obtained from this study showed that lipid content of *Phaeodactylum tricornutum* can be considered as a contribution to biodiesel industry and the supply facilities.

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