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Effects of Temperature and Nitrogen Concentration on Growth and Lipid Accumulation of the Green Algae *Chlorella vulgaris* for Biodiesel

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ABSTRACT

This study investigated the effect of different temperatures and different nitrogen concentrations on the lipid content and biomass of *Chlorella* microalgae. In this study, algae were cultured in five media with different amounts $NaNO_3$ as 3, 1.5, 0.80, 0.40 g/L, and three temperatures (10, 20, 30 °C). The results of the experiments showed that the optimal temperature and nitrogen concentration for the biomass increase in *Chlorella vulgaris* are 30°C and 3 g/L, respectively. It was observed that biomass decreased and lipid amount increased due to the decrease in nitrogen concentration. The high lipid amount of 20.80% dry weight (DW) was obtained from the algae produced at 30°C in the free-nitrate medium. The contribution of temperature change to lipid production was not as effective as nitrogen deficiency in the study. According to the fatty acid analysis results made by GC-FID, *C. vulgaris* seems suitable for biodiesel production because it contains medium-length (C16-C18) fatty acid chains.

INTRODUCTION

The world population is increasing day by day, and it is predicted that the world population will increase by 1.5 times in 2050 (Sajjadi et al., 2018). Fossil fuel reserves, which use for a significant part of the energy need in the world, are rapidly being depleted. The efficient use of energy is even more vital today due to the rapid decrease in fossil fuels and the increase in fuel demand (Widjaja et al., 2009). In addition to the depletion of oil reserves, another essential issue that should not neglect is the rapidly emerging environmental pollution (Liew et al., 2014). Therefore, the production and use of environmentally friendly, renewable, and sustainable energy resources are supported worldwide. Most of the renewable energy sources such as biodiesel, bioethanol, and biohydrogen are produced from plant sources. However, since more than 95% of its production produces in vital soil resources or arable lands required for living creatures and depletes freshwater used in irrigation, it is more harmful to both environment and economy (Sajjadi et al., 2018). In this case, the importance of alternative biofuel sources has increased. The most striking of these alternative sources recently are algae. Microalgae can produce lipids without the need for arable land. Microalgae are much more advantageous than terrestrial plants for potentially producing biodiesel in all regions of the World (Metting, 1996). Therefore, microalgae are considered an important

source of raw material for biodiesel (Li et al., 2008). However, not every microalgae species can be produced easily and quickly. The microalgae species selected for biodiesel production should both be able to increase its biomass rapidly and have high lipid content. However, algae containing a high number of lipids grow more slowly than those with low content (Vasudevan & Briggs, 2008; Deng et al., 2009). Chlorella is the first to come to mind because it can both produce rapidly, and its lipid content reaches approximately 14 to 22% of its dry weight when produced under normal conditions (Illman et al., 2000; Spolaore et al., 2006). Nevertheless, the quality and quantity of algal lipids exhibit changes depending on various environmental conditions such as temperature, nutrient, light intensity (Illman et al., 2000; Liu et al., 2008; Seyhaneyildiz Can et al., 2015).

Many studies have been conducted on the role of nitrogen and temperature in algal lipid accumulation (Dong et al., 2013; Olofsson et al., 2014). In the study conducted to determine the effect of nitrogen on lipid accumulation, it was observed that microalgal growth was negatively affected by nitrogen deficiency, whereas lipid accumulation increased (Lombardi & Wangersky 1991). The increases in lipid content vary by species (El-Baky et al., 2004; Pal et al., 2011; Olofsson et al., 2014). Also, nitrogen is the limiting factor for developing many species (Li et al., 2010; Park et al., 2012). Another important factor is temperature. Temperature is one of the environmental factors affecting algal development and lipid accumulation. Again, the effect of temperature on biomass and lipid accumulation varies from species to species (Xin et al., 2010; Park et al., 2012; Roleda et al., 2013).

The main objective of this study is to examine the synergistic or antagonistic effects of nitrogen and temperature on growth rate and lipid productivity of *Chlorella vulgaris*. For this purpose, *C. vulgaris* was cultivated at three temperatures using nutrient media containing five nitrogen concentrations. Additionally, optimum temperature and nitrogen concentration have been determined for both biomass production and lipid accumulation to determine the suitability for biodiesel production.

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MATERIAL AND METHODS

Microalgae

C. vulgaris Beijerinck (Chlorophyceae) used in the study was isolated from a fish pond in Ege University, Faculty of Fisheries, Izmir, Turkey. The isolated algae were transferred from agar plate prepared with f/2 nutrient medium (Guillard, 1975) to liquid culture medium.

Culture System

Trials were conducted in triplicate in 1000 mL Erlenmeyer flasks with 500 mL f/2 medium at different temperatures (10, 20, 30 °C) and different nitrogen concentrations (3, 1.5, 0.8, 0.4 g/L, and nitrate-free) to investigate the effects of nitrogen deficiency and temperature on biomass and lipid yield. All glassware and nutrient media used in the trials were sterilized by autoclaving for 15 min, at 121°C, 1-atmosphere steam pressure, to prevent contamination during production, and they were kept at room temperature for 24 h before inoculation. Algae in 20 mL test tubes were inoculated into 1mL Erlenmeyer flasks. The continuously aerated culture medium was kept constant at pH 7.5 with sodium bicarbonate and HCl buffer solution. In the 18-day experiment, the light intensity of 33.6 µmol photon was applied continuously to all experimental groups, and the cultures were shaken twice a day to prevent algae precipitation.

Microalgal Biomass Concentration

Algal dry weight was measured daily by a precision balance (Precisa XB 220A) for 18 days. Before measuring the dry weight, the algae were filtered with filtration papers, then washed with distilled water and dried at 100°C for 12 h (Lee, 1998). All measurements were conducted in triplicate. At the end of the culture period, all algal biomass was centrifuged at 4000 rpm for 5 min using a TD3 (800B) centrifuge. The algal biomass was washed with distilled water three times to cleaned biomass from nutrient media, and they were dried stored at −20°C for later analysis (Lee, 1998).

Cell Disruption and Lipid Extraction

Before starting to break up dried algae cells, 5 mL phosphate buffer solution (pH 7.4) was added to prevent side reactions. An aliquot (1 g) of the dry cell biomass was broken up in Bead-beater at 4800 rpm for 3 min. The algae broken up in Bead-beater were removed into centrifuge tubes, and 6 ml hexane per 1 g dry algae was added and centrifuged at a high-speed of 4000 rpm for 5 min. After 24 h, residual microalgae were separated from the lipid-hexane mixture using a filter paper with 0.50 µm mean pore diameter. The

hexane in the hexane-lipid mixture was evaporated at 60°C, and the remaining lipid was measured gravimetrically. Lipid amount was determined in terms of the percentage of dry weight (Lee, 1998).

Fatty Acid Composition

To determine the fatty acid compositions of lipids were used Perkin Elmer Clarus 500 gas chromatography (GC-FID) and fused silica RTX-2330 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Restek Corp., Bellefonte, PA, USA). Instrument check-out and determination of fatty acids of the samples was accomplished with a 37-component mixture (Supelco number 18919). As a carrier, helium was used at an injection-split ratio of 1/50 and a gas flow rate of 1.0 mL/min. The gas chromatography column was started to be heated. When the column temperature reached 100°C, 1 μL of the extracted lipids were injected. The column temperature was increased at 5°C/minutes from 100 to 180°C (for 10 min), and then to 250°C (for 20.7 min) at 3°C/minutes.

Statistical Analysis

The dry weight and the extracted lipid contents from the five groups were compared using a one-way ANOVA and Duncan's multiple comparison method. The level of significant difference was at $P<0.05$.

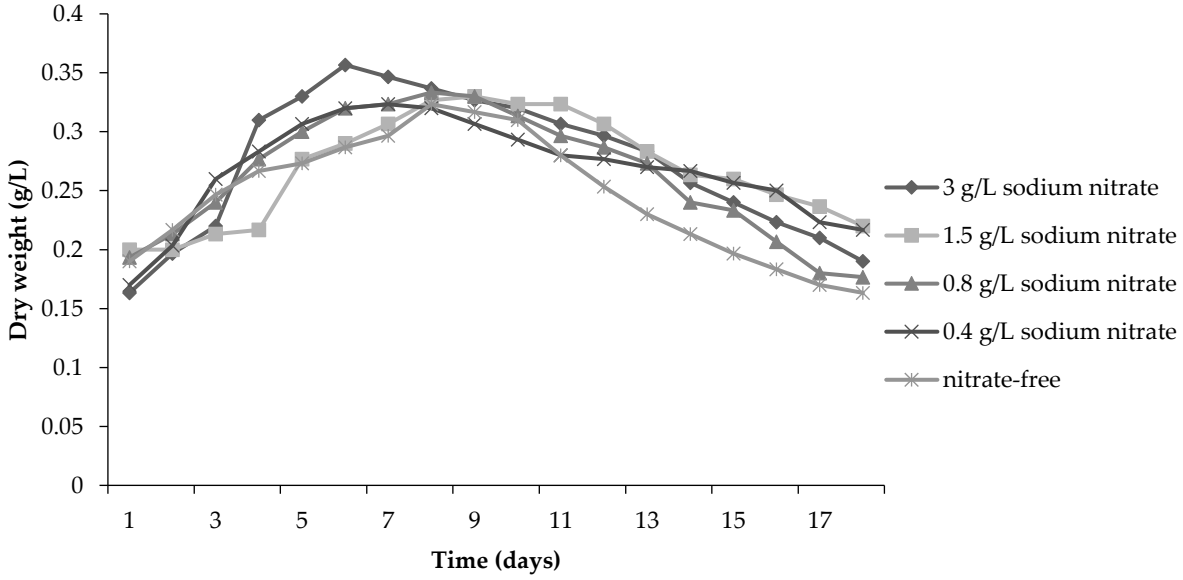

Figure 1. The dry weight of *C. vulgaris* cultured at 10°C

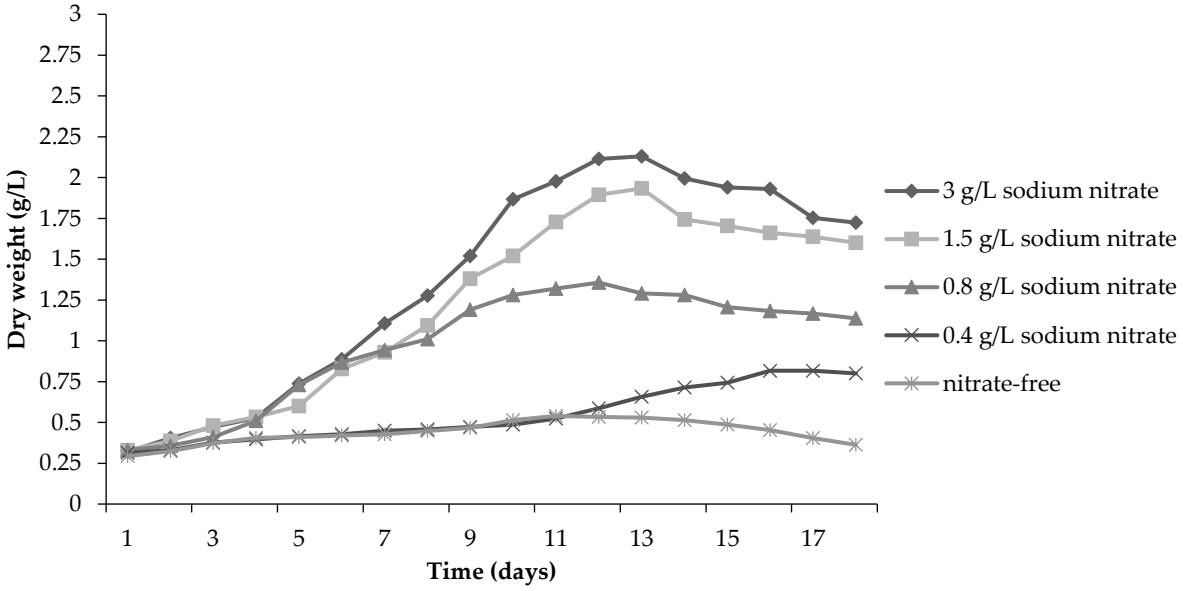

Figure 2. The dry weight of *C. vulgaris* cultured at 20°C

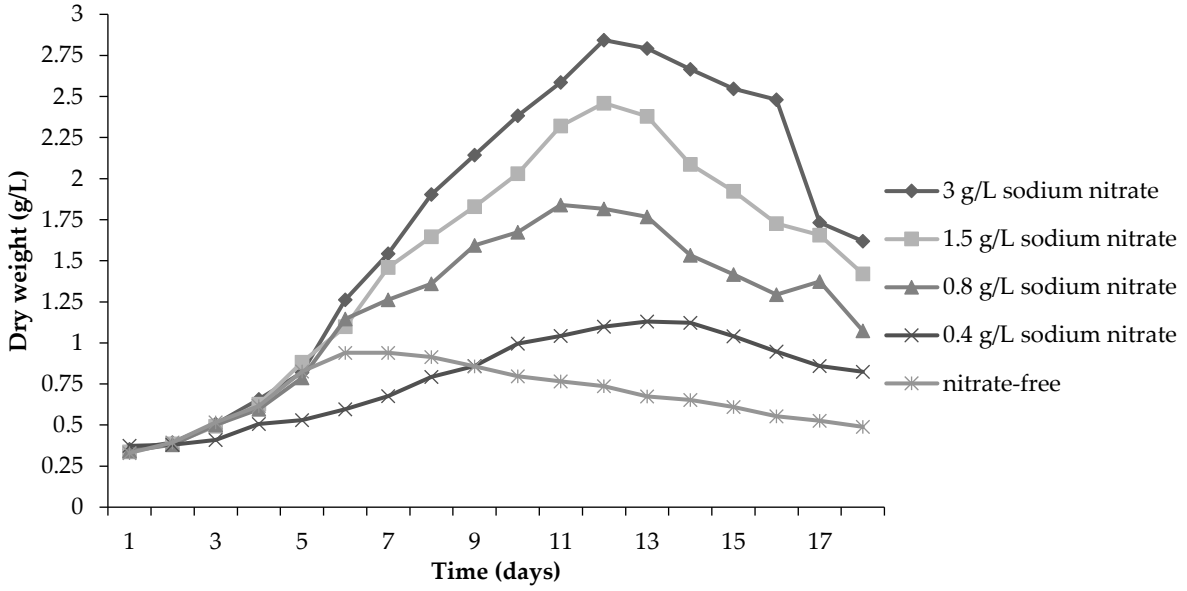

Figure 3. The dry weight of *C. vulgaris* cultured at 30°C

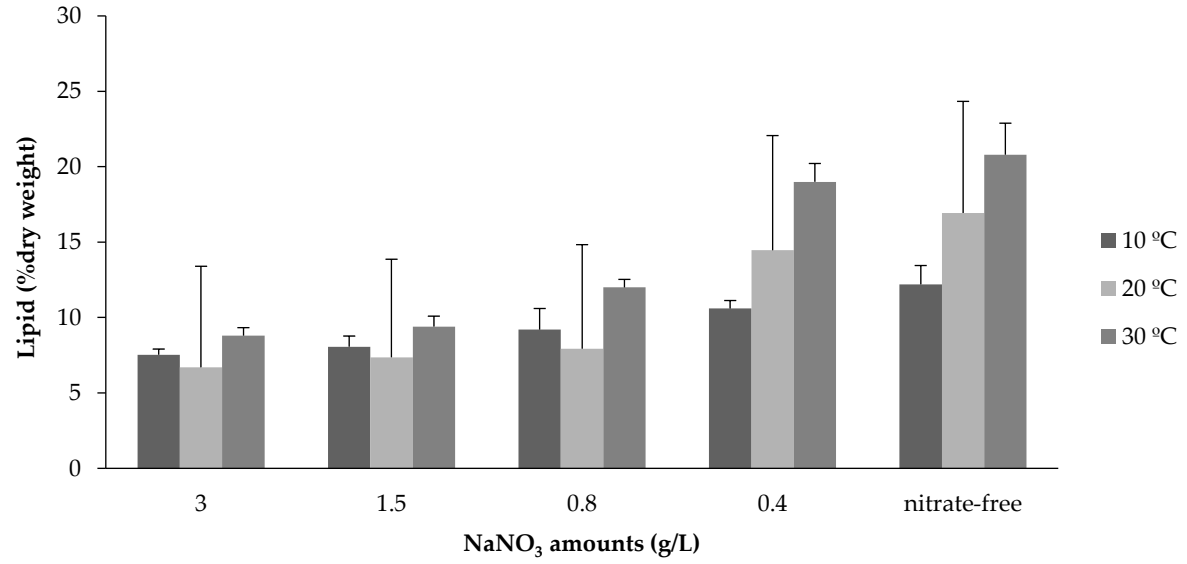

Figure 4. The total lipid amount of *C. vulgaris* cultured at different temperatures

RESULTS

Effect of Temperature and Nitrogen Concentration on Algal Biomass

Biomass slowly increased in algae culture at 10°C until day 9th and decreased in the following days (Figure 1). The maximum biomass obtained during the 18-day-old trial was determined as 0.357 g/L on the 6th day, in the nutrient medium containing 3 g/L $NaNO_3$ concentration. When the results of the experiment conducted at 20°C were examined, it was noted that the logarithmic phase continued until the 13th day in medium containing 3 g/L $NaNO_3$ concentration, and the maximum biomass was 2.13 g/L on the 13th day (Figure 2). The logarithmic phase continued until the 12th day for the trial conducted at 30°C, and again, maximum development was achieved as 2.84 g/L on the 13th day, with 3 g/L $NaNO_3$ concentrations (Figure 3).

The Effect of Temperature and Nitrogen Concentration on Algal Lipid Production

The changes in temperature and nitrate concentration led to significant changes in the cell composition, facilitating the accumulation of lipid components in microalgae during batch culture. In this study, reducing nitrate was more effective than the temperature for lipid accumulation. When the results of the study are examined, it was determined that

Table 1. Growth and lipid values for *C. vulgaris* microalgae cultured at different temperatures (mean ± standard deviation)

$NaNO_3$ (g/L)	10°C		20°C		30°C	
	DW (g/L)	*Lipid (% DW)*	*DW (g/L)*	*Lipid (% DW)*	*DW (g/L)*	*Lipid (% DW)*
3	0.273^{a}±0.06	7.53^{c}±0.38	1.371^{a}±0.75	6.700^{c}±0.20	1.735^{a}±0.87	8.80^{c}±0.53
1.5	0.268^{a}±0.05	8.07^{c}±0.71	1.221^{a}±0.59	7.367^{c}±0.25	1.487^{b}±0.69	9.40^{c}±0.69
0.8	0.263^{ab}±0.06	9.20^{bc}±1.40	0.976^{b}±0.44	7.933^{c}±0.50	1.209^{c}±0.49	12.00^{b}±0.53
0.4	0.2681^{a}±0.05	10.6^{ab}±0.53	0.544^{c}±0.17	14.467^{b}±1.10	0.788^{d}±0.34	19.00^{a}±1.22
Nitrate-free	0.245^{b}±0.05	12.2^{a}± 1.25	0.439^{c}±0.07	16.933^{a}±1.10	0.674^{d}±0.22	20.80^{a}±2.09

Note: *Different superscript letters (^{a}, ^{b}, ^{c}, ^{d}) indicate that the values of the means in the table are significantly different P<0.05*

the most efficient group in terms of lipid is the algae cultured in the nitrate-free nutritional medium at 30°C (Figure 4). In terms of biomass, the most productive group is algae cultured in a nutrient medium containing 3 g/L nitrate at 30°C (Table 1).

In this study, both the optimum and adverse conditions were created on algae to observe the change in lipid amounts. The following equation (1) was used to find the most efficient group in terms of lipid production in studies.

$$Lipid\ amount\ (g) = \frac{DW(g) \times lipid(DW\%)}{100} \quad (1)$$

According to the equation, it was determined that the most efficient group for both biomass and lipid is the culture in a nutrient medium of 3 g/L nitrates at 30°C (Table 1).

Table 2. The fatty acid content and concentration of *C. vulgaris*

Fatty acids	Fatty acid concentration (%)
Linoleic acid	23.89±0.04
Margaric acid	0.86±0.06
Miristoleic acid	1.66±0.03
Oleic acid	4.24±0.08
Palmitic acid	32.72±0.06
Palmitoleic acid	3.88±0.007
Stearic acid	10.15±0.006
Trans linolenic acid	20.76±0.004
Undecanoic acid	1.84±0.004

Fatty acid compositions of the lipids obtained from the most efficient group were determined by GC-MS (Table 2). Biodiesel is defined as fatty acid alkyl esters (FAAEs) derived from vegetable and animal oils. Biodiesel is mainly esters of six fatty acids: palmitic acid (C16: 0), stearic acid (C18: 0), oleic acid (C18: 1), linoleic acid (C18: 2), and linolenic acid (C18: 3) (Chuck et al., 2009). Table 2 shows that most fatty acids of *C. vulgaris* are essential fatty acids for biodiesel production. These results demonstrate that *C. vulgaris* is an appropriate species for biodiesel production.

DISCUSSION

Differences in the culture environment affect the biomass and cell contents of algae. Changes in algal growth and lipid production of cells have been more pronounced, particularly in nitrate deficiency, the essential nutrient (Pinto et al., 2003; Ip and Chen, 2005). All studies conducted with microalgae, it has been observed that algae accumulate lipids, especially triglycerides, despite nitrogen deficiency (Hsieh and Wu, 2009; Yeh and Chang, 2011; Sun et al., 2014). In this study, the response of *C. vulgaris* to varying nitrate concentrations was investigated. Dry biomass weight was used for *C. vulgaris* growth assessment. Results indicated that biomass decreased in response to declining nitrate concentrations, but conversely, lipid production increased. Similarly, other studies were reported that the growth of *Chlorella pyrenoidosa* and *Scenedesmus obliquus* decreased growth under nitrogen-deficient conditions (Mandal and Mallick, 2009; Nigam et al., 2011). In this study, there is an inverse relationship between the lipid produced by algae and nitrate concentration. The reason can be expressed as algae modifying their lipid metabolism to adapt to adverse conditions occurring in culture conditions (Su et al., 2011).

However, one of the main factors affecting the growth of fatty acids, lipids, and species produced by microalgae is the temperature (Renaud et al., 2002; Converti et al., 2009; Taoka et al., 2009). In this study, besides the nitrate concentration, the effect of different temperatures on the algae biomass and lipid content was also investigated. Algal biomass increased with temperature. However, the effect of temperature on lipid production was not significant as on algal growth. However, lipid production at 30°C was higher in all groups compared to other temperatures. The differences in both temperature and nitrogen concentration in the experiments made stress on *C. vulgaris*, and this stress caused a response as increased lipid accumulation. As seen in the literature, Illman et al. (2000) and Liu et al. (2008) reported that the amount and content of lipids inside the cell vary depending on factors temperature or light intensity. Temperature affects the physiological process by changing the speed and stability

of the chemical reactions of cellular components. However, this effect also depends on the strain species (Sandnes et al., 2005; Griffiths and Harrison, 2009; Van et al., 2012).

In this study, the suitability of *C. vulgaris* for biodiesel production was also tried to be determined. Therefore, the answer to the question of how to increase biomass and the amount of lipid was sought. Because biomass production is as significant as lipid accumulation for the efficiency of the study. We observed that the most productive group in terms of both biomass and lipid was the algae grown in medium with 3 g/L nitrogen at 30°C. Fatty acid analysis of this most productive group was made. The fatty acid profile of an alga determines the usage areas of that alga. For example, it is harmful to use an oil with a high erucic acid content as a food raw material. On the other hand, polyunsaturated fatty acids are highly nutritionally valuable (Sissener et al., 2018). Oils containing excessive amounts of free fatty acids reduce biodiesel yield by producing soap in reactions with alkali catalysts. Therefore, triglycerides containing long-chain fatty acids and oils containing less free fatty acids are preferred for biodiesel production (Ekin, 2019). According to the fatty acid analysis results of *C. vulgaris* in our study, it was seen that it contains high amounts of long-chain fatty acids (Table 2).

CONCLUSION

It was interesting data that lipid production increases by reducing nitrogen in the nutrient media. It was also noteworthy that the temperature was not as effective as nitrogen. However, if we want to produce fuel that can compete with petroleum both ecologically and economically, we must make a product that is more cost-effective and more environmentally friendly. For example, algae can be produced in wastewater. Thus, while the nutrient medium required for algal biomass is taken from wastewater, the polluting effects of wastewater are also reduced. Or, algae production facilities can be established in areas where the industry is concentrated, and thus, algae can reduce industry-source carbon dioxide. As the number of such applications to be conducted on a commercial scale increases, the biodiesel obtained from algal lipids will be superior to petroleum-based fuel.

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Compliance With Ethical Standards

Authors' Contribution

ŞSC designed the study and performed algae culture and lipid extraction from algae. EK prepared the literature. SC wrote the first draft of the manuscript. GT performed statistical analyses. HT assisted in laboratory work. TS helped in all phases of the studies. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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