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Effect of Gibberellin on Some Fatty Acid Profiles Under Nitrogen Starvation in Green Algae *Chlorella vulgaris*

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Abstract: Plant growth substances could be stimulating algal growth rate and alter lipid compositions. In the present study, we tested hypothesis that exogenous gibberellin (GA) has any effect on growth rate and some fatty acid profiles in green algae *Chlorella vulgaris*. In Bold Basal Medium with 100 µM GA_3, cell density increased to 68.57% on third day as compared to the control cells. These results indicated that GA_3 enhanced microalgal growth and cell size. The lipid profile was also altered compared to control using Gas Chromatography-Mass Spectrometry (GC-MS). GA_3 promotes the production of C16:0, C18:0, C18:1 and C18:3 on day-3 and-5. Under nitrogen starvation condition, application of GA_3 provide enhanced algae growth and stimulated C16:0 and C18:1 production. In conclusion, this study demonstrated that gibberellin could be a good candidate as a hormone for increasing lipid production in microalgae culture system.

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1. INTRODUCTION

Microalgae are photosynthetic microorganisms with a high growth rate and the ability to convert carbon dioxide into biomass. Microalgae can synthesize high levels of metabolites that play an important role in biodiesel production, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), carotenoids (β-carotene, lutein and astaxanthin), and phycocyanin (Mata et al., 2016). In this respect, it is widely accepted today as a potential sustainable biomass raw material source for biofuel production (Borowitzka & Moheimani, 2013). Although microalgae are a rich source of potential molecules (such as lipids, carbohydrates and proteins) that can be converted into fuel substitutes that are renewable, non-toxic, biodegradable and carbon-neutral; therefore, they are regarded as an environmentally friendly fuel source (Dillschneider et al., 2013), microalgal biofuels are still not seen as an alternative to fossil fuels. Because the main obstacle to successfully implementing microalgal biofuels as a replacement for fossil fuels is their high cost to produce. Today, many researchers focused on obtaining cheaper and high efficiency microalgal biofuels.

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The manipulation of culture conditions or genetic engineering approaches are widely used to increase targeted compounds such as lipids, pigments, proteins and PUFAs from microalgae (Sreekumar et al., 2018). One of the most widely used approaches is enhanced the cultivation of microalgae or biomass production. This approach is the most expensive and technically difficult step in the implementation of algae biofuel production (Leite et al., 2013). Increasing the biomass productivity and/or lipid and carbohydrate production of microalgae can increase algae cultivation's economic feasibility (Abdelaziz et al., 2013). The cellular accumulation of lipids in microalgae can also be induced by different environmental factors such as high light and salinity (Solovchenko et al., 2008; Rodolfi et al., 2009; Ren et al., 2014; Benvenuti et al., 2015). Previous studies demonstrated that application of metal stress such as copper, magnesium, iron and cadmium enhanced total lipid content (Liu et al., 2008; Li et al., 2013; Ren et al., 2014). It has also known that N-starvation causes alternation on carbon flux through the pathway of protein synthesis to lipid and/or carbohydrate metabolisms (James et al., 2013; Li et al., 2013; Jerez et al., 2016). Thus, high lipid accumulation in microalgae can be occurring under N starvation (Rodolfi et al., 2009; Benvenuti et al., 2015). Previous studies demonstrated that lipid content enhanced a 2 to 4-fold under N-starvation in microalgae such as *Chlorella, Chlamydomonas*, *Dunaliella* and *Nannochloropsis* species (Rodolfi et al., 2009; Cakmak et al., 2012; Illman et al., 2000). However, algae growth, development and metabolism effected negatively under nitrogen starvation.

Exogenously applying of plant growth regulators could be one of the alternative strategies to stimulate the synthesis fatty acids (Park et al., 2013; Lu & Xu, 2015). Some research groups reported that auxin and jasmonic acid altered to fatty acid composition in *Chlorella* species (Jusoh et al., 2015[a]; 2015[b]). Gibberellins (GAs) are diterpenoid acids that affect many areas of plant growth, such as leaf growth and flower and seed development. They promote stem elongation, fruit generation and seed germination in higher plant (Nakajima et al., 2006). It has also known that gibberellins found in macro-and microalgae (Lu & Xu, 2015). Previous studies demonstrated that active GAs, GA_1 and GA_3 in brown algae *Fucus vesiculosus* and *F. spiralis* (Radley, 1961; Jennings, 1968). Additionally, many studies have focused on gibberellin's effect on the growth of microalgae and their bioproducts. In addition, previous studies have reported that GA increases biomass accumulation and triacylglycerol content in *microalgae* (Mekhalfi et al., 2014; Du et al., 2015). Although increased growth in response to GAs has been documented in some algae (Jennings, 1968; Joseph & Chennubhotla, 1999), little evidence for GAs activity on growth and developmental processes has been observed in green algae (Lu & Xu, 2015). Moreover, the effects of GA_3 under normal conditions and N-starvation on fatty acid production have not demonstrated yet. In the present work, we tested two hypotheses; (i) exogenously GA_3 altered fatty acid composition under normal conditions, (ii) exogenous GA_3 changes the fatty acids composition) under N-starvation.

2. MATERIAL and METHODS

2.1. Culture Conditions

Chlorella vulgaris was obtained from the EGEMAC culture collection, Ege University, Izmir, Turkey. Five of the 250 mL Erlenmeyer flasks of *C. vulgaris* were used for the experiment. All experiments were carried out using cells in the exponential phase. It set up four different experimental group. The first group was the culture that was grown in a Bold Basal media (BBM, as a control). The second group was 250 mL Erlenmeyer flask of *C. vulgaris* grown in a BBM containing 100 µM GA_3. Third group was that *C. vulgaris* culture was collected with centrifuge and grown in BBM without any nitrogen sources. Finally, fourth group was that *C. vulgaris* culture was grown in BBM without nitrogen and with GA_3 in a growth chamber under continuous illumination at 80 µmol $m^{-2}s^{-1}$ light intensity and 24 °C for 1, 3, 5 and 7 days.

2.2. Growth Rate and Cell Size

The absorbance of cell growth was measured on UV-spectrophotometer at 663 nm, and cell number was counted with Neubauer hemocytometer. Each experiment repeated three times.

2.3. Methyl Esters of Fatty Acids (FAMEs)

FAMEs were carried out according to the modified procedure of Bligh and Dyer (1959) and Kattner and Fricke (Kattner & Fricke, 1986). Briefly, the extraction mixture with the dissolved lipids was evaporated to dryness and trans-esterified with 2 mL of 3% H_2SO_4 in methanol (Kattner & Fricke, 1986) four hours at 70°C. After cooling to room temperature, 2 mL of hexane was added for extraction of FAMEs. The solvent was evaporated, and 50 µL of hexane was added. Each experiment repeated three times.

2.4. GC-MS Analysis

The methyl esters of fatty acids were quantified by a gas chromatograph (Shimadzu QP2010 ultra model) equipped with a flame ionization detector (FID). The GC-MS column (TRB-5MS model) was fused 30 mm x 0.25 mm x 0.25 µm. Injector and FID inlet temperature were 270°C and 250°C, respectively. Column temperature was programmed to hold at 40°C for 4 min, then rise at 8°C min^{-1} increase to 280°C and was held at this temperature for 20 min. The column head pressure of carrier gas (helium) was flow rate 0.8 mL min^{-1}. Each experiment repeated three times.

2.5. Statistical Analysis

Statistical analysis was performed with one-way analysis of variance (ANOVA) or Student's t-test followed by *post-hoc* Tukey test as appropriate (SPSS for Windows, version 11.0).

3. RESULTS and DISCUSSION

3.1. Effect of Gibberellin on Cell Growth and Size

Exogenously plant growth regulators induced cell growth and algal biomass in microalgae (Joseph & Chennubhotla, 1999). GAs is a phytohormone and essential for plant growth and development processes (Sasaki et al., 2003; Tyler et al., 2004). In the present study, the stimulation effect of GA_3 on the growth was tested depend on time (Table 1). As shown in Table 1, the cell density of *C. vulgaris* at early stationary growth phase was 4.92 $x10^6\pm$ cells/mL. In BBM with 100 µM GA_3, cell density increased to 9.74 $x10^6\pm$ cells/mL on third day. The cell density also increased 127. 99% on the seventh day as compared to the control cells (Table 1). Previous studies demonstrated that GA_3 stimulated biomass production in *Chlamydomonas reinhardtii* (Park et al., 2013). Falkowska et al. (2011) also showed GA_3 had a stimulating influence on the cell number in *C. vulgaris.* Similarly, it was observed that GA treatment increased biomass productivity by 8.7-fold and 5.3-fold, respectively, in *C. ellipsoidea* and *Scenedesmus abundans* (González-Garcinuño et al., 2016). In this study, microscopic analysis in the present study showed that application of GA_3 significantly affected cell size in *C. vulgaris* culture (Table 1). These results indicated that GA_3 could be a very useful phytohormone for improving algal cell density. Similarly, Yu et al. (2016) reported that an increase in growth/biomass due to GA treatment might increase glucose uptake rate. Still, this consumption may occur with inhibition of glycolysis and the tricarboxylic acid cycle. However, more studies are needed to determine which gibberellins promote metabolic pathways.

3.2. Effect of Gibberellin on Fatty Acid Composition

Previously studies reported that plant growth regulators and growth stage altered oil compositions in microalgae and higher plants (Joseph & Chennubhotla, 1999; Lu & Xu, 2015). In the normal conditions, our results demonstrated that production of C18:0 increased on the

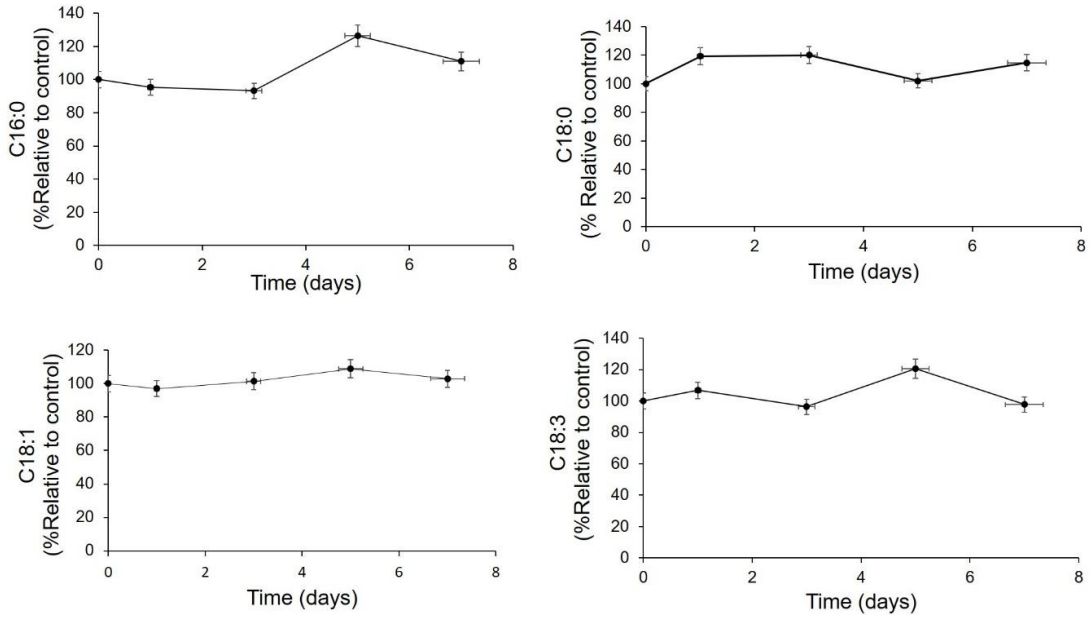
first and third days; however, interestingly, the increased amount of C16:0, C18:1, and C18:3 occurs especially on fifth day (Figure 1) under normal conditions. Grindstaff et al. (1996) demonstrated that GA_3 stimulates the degree of unsaturation of fatty acid in barley aleuronic layers in the higher plant. González-Garcinuño et al. (2016) also demonstrated the application of Gibberellins enhanced lipid productivity in *C. ellipsoidea*. In addition, treatment with GA_3 induced the amount of polyunsaturated fatty acid (especially, C18:2, and C18:3) and decreased the amount of saturated (16:0 and 18:0) fatty acid in isolated ER microsomal membranes (Grindstaff et al., 1996). In contrast, in the present study, GA_3 enhanced the production of C18:0, C18:1 and C16:0 on the first day and up to maximum production on the fifth day ($p<0.05$, Figure 2). These results indicated that application of gibberellin significantly enhanced fatty acid production in algae.

Table 1. The growth parameter of *Chlorella vulgaris* culture applied with GA_3 or control

Days after treatment	Cell density		Cell density % relative to control	Cell Size (µm)		Cell size % relative to control
	Control X±SD	GA_3 X±SD		Control X±SD	GA_3 X±SD	
1.day	4.92 x10^6± 0.14	5.33 x10^6± 0.18 ^{c}	108.33%	2.92 ± 0.43	3.17 ± 0.58	8.56%
3.day	7.61 x10^6± 0.12^a	9.74 x10^6±0.30^{bc}	127.99%	2.64 ± 0.63	3.05 ± 0.54	15.53%
5.day	14.20 x10^6± 0.21^a	18.54 x10^6±0.21^{bc}	130.56%	2.93 ± 0.62	2.95 ± 0.60	0.68%
7.day	16.07 x10^6 ± 0.28^a	22.45 x10^6±0.90^{bc}	139.7%	2.65 ± 0.43	3.01 ± 0.56	13.58%

"a" is a significant value when compared to control 1-day, "b" is a significant value when compared to GA_3 1-day, and "c" is a significant value when compared to control.

Figure 1. Fatty acid profile of *Chlorella vulgaris* grown under normal culture condition.



3.3. Effect of Gibberellin on Growth and Fatty Acid Composition Under N-Starvation

Effect of N-starvation on microalgae growth rate was demonstrated as seen in Table 2. Zhu et al. (2014) reported that N-starved cells increased twofold in number within the first two days. Similarly, our results showed that cell density of *C. vulgaris* increased 2-fold within three days under N-depletion (Table 2). It could be used for nitrogen storage during growth processes. However, cell density decreased up to approximately 1.66-fold in day-7 when compared to day-3. Application of GA_3 provides to increasing to cell growth under N-starvation (Table 2). After

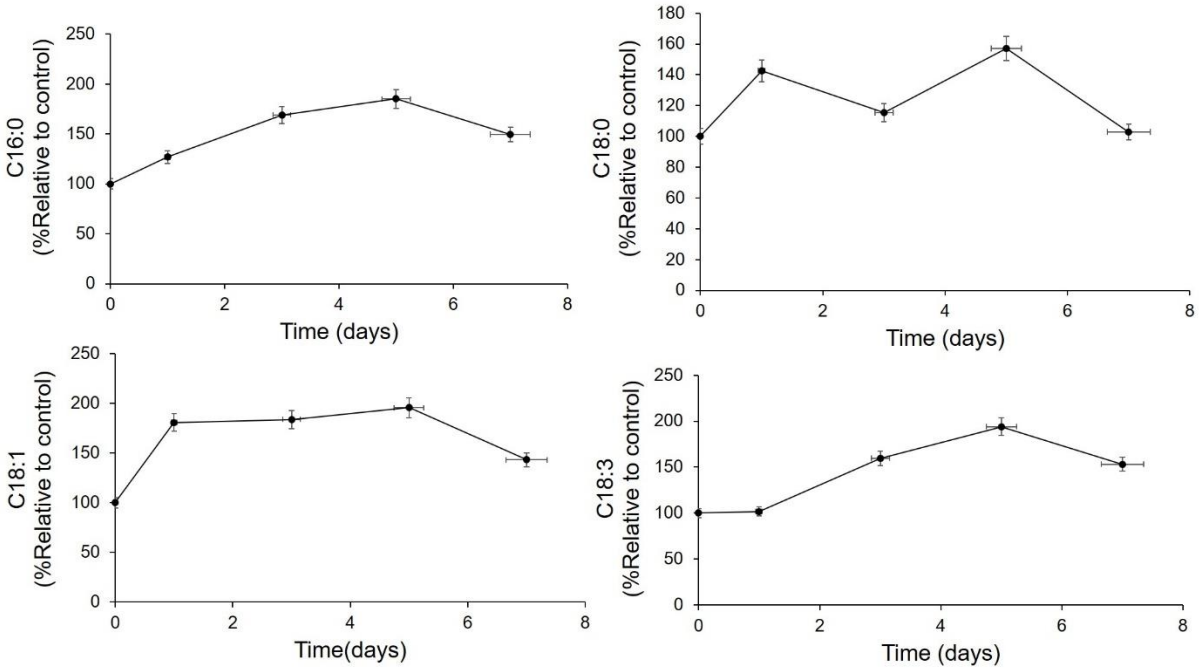
5 days, the cell growth started to decrease because of the lack of nitrogen sources. This effect could be explained that gibberellin as a phytohormone could trigger some metabolic pathways which are involved in response to nitrogen starvation. On the other hand, this hypothesis is speculation and needs to be testing in future studies.

Table 2. The growth parameter of *Chlorella vulgaris* culture applied with GA_3 or control

Days after treatment	Cell density		Cell density % relative to N-starvation
	N-starvation X±SD	N-starvation GA_3 X±SD	
1.day	4.67 $x10^6$± 0.12	4.83 $x10^6$± 0.05	103.43%
3.day	9.33 $x10^6$± 0.16^a	11.29 $x10^6$±0.12^{bc}	121.01%
5.day	7.51 $x10^6$± 0.25^a	14.02 $x10^6$±0.18^{bc}	186.68%
7.day	6.63 $x10^6$ ± 0.09^a	12.06 $x10^6$± 0.09^{bc}	181.9%

"a" is significant value when compared to control 1-day, "b" is significant value when compared to GA_3 1-day, and "c" is significant value when compared to control.

Figure 2. Fatty acid profile of *Chlorella vulgaris* grown under normal culture condition with 100 µM GA_3



N-starvation is very a general approach for changing fatty acid composition in algae. Previous studies demonstrated that N-starvation induced fatty acid production especially C16:0 and C18:1 in microalgae (Benvenuti et al., 2015; Babu, Wu, Kabra & Kim, 2017). In the present study, the fatty acid profile changed under N-starvation. The C16:0 and C18:1 increased within the first day under N-depletion (Figure 3). However, C18:3 and C18:0 profile in *C. vulgaris* significantly decreased after 3 days (Figure 3). Babu et al. (2017) demonstrated that phytohormone's application under N-limitation is a useful cultivation strategy to improve the lipid production rate of microalgae. Similarly, application of GA_3 with N-starvation significantly increased C16:0, C18:0, C18:1, and C18:3 at first and three days ($p<0.01$, Figure 4). Our results also showed that the application of GA_3 under normal conditions provided approximately 1.02, 1.18, 1.24 and 1.16 fold higher results C18:0, C18:1, C18:3 and C16:0

compared to N-starvation with GA_3 within 3 days, respectively (Figure 4). This study showed that other combinations of growth medium supplement with GA_3 100 µM and one or more published strategies such as nitrogen starvation could further increase the unsaturated fatty acid synthesis productivity of *C. vulgaris*, making its use industrially viable.

Figure 3. Fatty acid profile of *Chlorella vulgaris* grown under N-starvation condition.

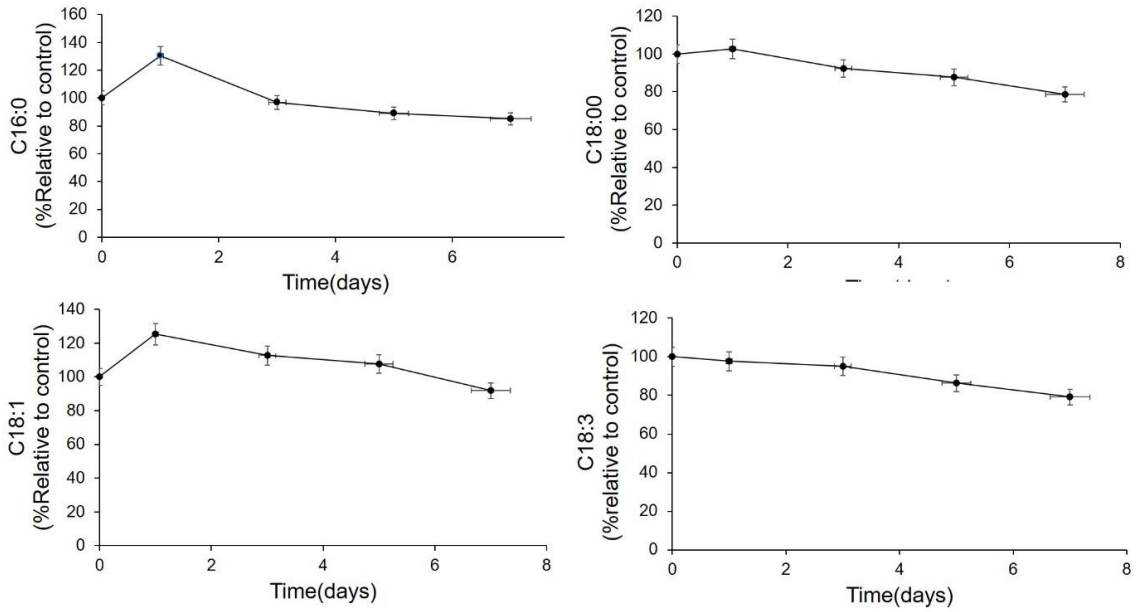
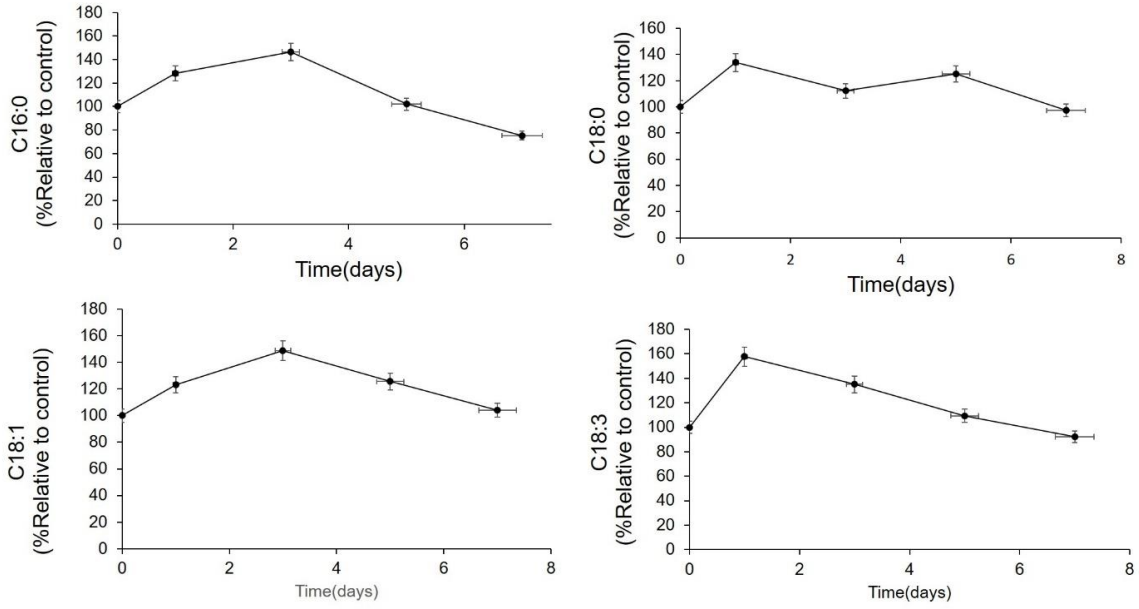


Figure 4. Fatty acid profile of *Chlorella vulgaris* grown under N-starvation condition with 100 µM GA_3



4. CONCLUSION

The combination of plant growth regulators and abiotic stress is a general approach for enhanced the accumulation of fatty acids and maintaining the microalgal biomass. In the present study, our results indicate that GA_3 supplementation increased microalgal growth rate, algal cell size and lipid production especially involved in biodiesel production under nitrogen starvation. These results showed the potential of application GA_3 in algal culture as a utilizer

for biodiesel application. This data also demonstrated that gibberellin could play a role in response to stress in algae physiology. In addition, the use of Gibberellic acid can bring us one step closer to making *C. vulgaris* suitable for biodiesel production. This study proposes using plant regulators to increase unsaturated fatty acids in combination with different stress conditions and help develop growing strategies for higher microalgal biodiesel production. Therefore, it seems necessary to study this subject in future research to create ideal culture conditions for biodiesel production.

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Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the author(s).

Authorship contribution statement

Uygar Kabaoglu: Investigation, Microscopic studies, Analysis of fatty acid. **Ufuk M. Aslan:** Cell growth, Fatty acid analysis. **Dilek Unal:** Experimental design, Writing-orginal draft, Statistical analysis, supervision.

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