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Growth and lipid accumulation in response to different cultivation temperatures in *Nannochloropsis oculata* for biodiesel production

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Abstract

Background: Microalgal lipid is a promising feedstock for biodiesel production. The aim of the present study was to investigate the effects of cultivation temperature on the growth and lipid accumulation properties of *Nannochloropsis oculata* microalgae.

Methods: *Nannochloropsis oculata* can grow in a wide range of temperatures (5 ~ 35°C). Late in the stationary growth phase of microalgae, biomass production and lipid accumulation were measured. The methanol-chloroform extraction method was used to extract total lipids from dried cells. The direct esterification method was used to measure fatty acids. Constituents were identified by gas chromatography.

Results: The results show that the maximum specific growth rate at 20°C was 0.1569 day^{-1}, and the maximum biomass production of microalgae at 25°C was 2.2667 g/L. The highest percentage of biomass conversion into lipid (35.71%) occurred at 30°C. Maximum lipid productivity was seen at temperatures of 15°C, 20°C, and 25°C, but the analysis of fatty acids in the three temperatures shownare maximum accumulations of triglycerides in the microalgae cells at 20°C and 25°C.

Conclusion: In the cultivation of *Nannochloropsis oculata*, the optimal temperature range for maximum efficiency in biodiesel production from lipids is 20°C to 25°C.

Keywords: Freshwater microalga, *Nannochloropsis oculata*, Cultivation temperature, Lipid accumulation

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Introduction

Finding sufficient supplies of clean energy for the future is one of society's most daunting challenges and is intimately linked with global stability, economic prosperity, and quality of life (1). Fuels represent around 70% of total global energy requirements, particularly in transportation, manufacturing, and domestic heating. At present, electricity accounts for only 30% of global energy consumption (2). In the European Union (EU), the transport sector is responsible for almost one quarter of greenhouse gas emissions (3). Concerns about decreasing fossil fuel sources, especially oil and natural gas, along with climate changes and global warming necessitate giving more attention to renewable energy (2, 4). Recently, biodiesel (green fuel) as a renewable and non-toxic energy which produced low amounts of pollution has been proposed as a replacement for fossil fuels. Its use leads to a decrease in the harmful emissions of carbon monoxide, hydro-

carbons, and particulate matter and to the elimination of SO_x emissions, with a consequent decrease in the greenhouse effect, in line with the Kyoto Protocol agreement (5-8). Microalgae are photosynthetic organisms that have an extraordinary potential for cultivation as energy crops. They are also able to produce a wide range of commercially interesting by-products such as fats, oils, sugars, pigments, and many other functional bioactive compounds (9-12). The microalgal lipid productivity/biomass is about 15–300 times that of conventional crops (6). Production and accumulation of lipids by microalgae is one of the important sources of biodiesel (13). Microalgal oil is mostly accumulated as triglycerides, and its reaction with simple alcohols (a chemical reaction known as "transesterification") leads to the formation of a chemical compound known as an alkyl ester, more generically known as biodiesel (10,14,15). Several types of microalgae are able to accumulate a large amount of lipids in their cells. *Nanno-*

*chloropsis oculata*is a marine unicellular alga of the class Eustigmatophyceae and is most often considered (16-19). The growth rate, cell density, and lipid content of the microalgae are essential parameters contributing to lipid productivity and reducing the total cost of biodiesel production (6,20). Therefore, determining the optimum microalgae culture conditions helps increase lipid productivity, promoting biodiesel investigation levels and its usage on an industrial scale (20,21). The quantity and proportion of different types of lipids within *Nannochloropsis* cells under a variety of growth conditions (salinity, light intensity) and medium specifications (nitrogen density, phosphate, and iron) have been determined (14,22,23). One of the important factors of microalgae cultivation is temperature; it affects the structural components of the cells (particularly lipids and proteins), controls the basic rates of all chemical reactions, and causes significant changes in metabolic regulatory mechanisms, specificity of enzyme reactions, cell permeability, and cell composition (18). This typicality will ultimately be effective on biomass and lipid production rates. There is no consistent pattern in relation to the effect of temperature on lipid production. Increasing culture temperature in some microalgae causes an increase in lipid rates; however, low temperatures cause greater lipid production in *Nibshiapleacea* microalgae (19).

Studies done on *Nannochloropsis oculata* have only investigated the effects of various factors on biomass production or cellular lipid rate; less attention has been given to lipid productivity. The current study investigated the effect of temperature on lipid productivity as one of the effective factors in economical biodiesel productivity. Moreover, this study analyzed the lipid extracted from microalgae for the quality and quantity of its compositions, and its applicability for biodiesel production was investigated.

Methods

The current study was conducted during the second half of 2012 at the Environmental Health Engineering Research Center of Kerman University of Medical Science using laboratory-scale pilot plants.

Nannochloropsis oculata

Stock culture of *Nannochloropsis oculata* was obtained from the Research Institute for Aquaculture in the south of Iran. This microalgae is a eukaryotic photosynthetic microorganism and, given its simple structure, has a fast growth rate (22). An image of *Nannochloropsis oculata* is shown in Figure 1. In their study conducted in China, Chen et al used high density microalgae (25×10^6 cell/ml) to determine cell density on microalgae growth and lipid composition (9).

Culture conditions

Stock culture was cultivated in a liquid Walne medium ($MnCl_2$, $CoCl_2$, $CuSo_4$, $FeCl_3$, $(NH_4)_6Mo_7O_{24}$, $ZnCl_2$, $NaNO_3$, H_3BO_3, Na_2EDTA, NaH_2PO_4), and simulated sea water was attained by dissolving sea salt in sterile water.

Figure 1. Image of *Nannochloropsis oculata* used in this study.

All tests were repeated three times in a ficolab equipped with temperature and light intensity controls under sterile conditions and in a 2-liter Meyer flask. In this study, the effect of temperature on microalgae was investigated at seven temperatures (5°C, 10°C, 15°C, 20°C, 25°C, 30°C, and 35°C) that were controlled with a thermometer. According to the studies of Sen et al and Banerjee et al, continuous illumination at 70 µE m^{-2} S^{-1} with a 12-hour light cycle and medium salinity of 25 ppt were considered in this study (24,25). Fluorescent lamps (FL20D, OSRAM, Korea) were used as the light source for microalga growth. Sterile-air containing 2% (v/v) $CO2$ and filtered using glass fiber was aerated into the column through an air sparger at the bottom of the column at a flow rate of 0.5 v/v.

Microalgae cell counting and dry weight

Cell density (cells mL^{-1}) was measured using a UV/Visible spectrophotometer (Shimadzu Corporation). Absorbance of cell density in wavelengths 100 to 800 nm was measured. Maximum absorbance was observed at wavelength 680 nm. Each sample was diluted to give an absorbance in the range of 0.1–1.0, if optical density was greater than 1.0. Microalgae dry weight per liter (g L^{-1}) was measured according to the method previously reported. Microalgae cells were collected by centrifugation of wet biomass for 30 minutes at 15°C with 3000 RPM. The dry weight of marine microalgae samples was affected by the salt absorbed on the cell surface, and its presence in the intercellular water ensured error in estimating the amount of biomass. This explains the differentiations in the amount of dry cell weight in various papers. Hence, before gravimetric analysis was performed, the salts were removed by again solving the centrifuged cells in 200 ml ammonium formate (0.5M, pH 8.0, adjusted with 1M NaOH) and centrifuging under the mentioned conditions. Microalga pellets were dried at 100°C for 4 hours for dry weight measurements. The specific growth rate of microalgae in the logarithmic phase was calculated as follows:

$$\mu = \frac{lnN_f - lnN_i}{t_2 - t_1} \tag{Eq. 1}$$

where µ (day^{-1}) is the specific growth rate, lnN_i and lnN_f

are cell densities (cell/ml) at the beginning and end of the logarithmic growth phase, respectively, and t is the time (day) (26).

Extraction and measurement of lipid content and TAG

The methanol-chloroform (1/1, V/V) extraction method was used to extract total lipids from the dried cells. To remove residual microalgae, the extraction lipids were filtered on membranes with 0.45 μm mean pore size. After washing twice with methanol and its complete evaporation, gravimetric analysis was done, and part of the lipid fraction was expressed as the percentage of dry cell weight. Lipid productivity was calculated by:

$$P_{Lipid} \left(gl^{-1}day^{-1}\right) = \frac{(C_f \times DCW_f) - (C_i \times DCW_i)}{T} \qquad (Eq. 2)$$

Where P_{Lipid} is lipid productivity; C_f and DCW_f are lipid content (g/g) and biomass (g/l) of the microalgae in the final stationary growth phase, respectively; Ci and DCW_i are lipid content (g/g) and biomass (g/l) of the microalgae in the initial stationary growth phase, respectively; and T is cultivation time (day). After total lipid amount was measured, the dried lipid was solved in 0.4 ml of isopropyl alcohol, and TAG was estimated by an enzymatic colorimetric method using a commercial kit from BHKT Clinical Reagent Co., Ltd., No. 2400076 (27).

Determination of fatty acid profiles

The direct esterification method was used to measure fatty acid properties. A mixture of 100 mg lyophilized microalgae and 8 ml KOH was sonicated for 3 minutes. For saponification, the mixture was heated to 100°C for 15 minutes and then cooled to room temperature. For esterification, 8 ml of 0.7 NHCl in methanol and BF_3/CH_3OH was added to the mixture (14% V/V), and it was again heated to 100°C for 15 minutes. After cooling to room temperature, emulsification was avoided by adding 2 ml of a saturated solution of sodium chloride to the mixture. FAMEs were extracted by adding aliquots of n-hexane. The FAMEs in the hexane layer were analyzed using standard gas chromatography (Agilent technologies 7890A-5975c) with a capillary column and a flame ionization detector. Nitrogen was used as the carrier gas and delivered at a rate of 1.5 mL min^{-1}. The temperature was programmed to increase from 130°C to 180°C at a rate of 10°C min^{-1} and thereafter ramped to 210°C at a rate of 2°C min^{-1}. The injector and detector were kept at 220°C and 250°C, respectively (27).

Statistical analysis

Data was analyzed using one-way analysis of variance (ANOVA).To determine the statistical difference between temperatures, the Tukey test was used. A value of $P < 0.05$ was considered statistically significant (16,28).

Results

Effects of temperature on cell growth rate of *Nannochloropsis oculata*

Figure 2 shows the cell growth rate of *N. oculata* in dif-

ferent cultivation temperatures. No increase was observed in the number of microalgae cells at temperatures of 5°C and 35°C. At 20°C and 25°C, the microalgae cells entered into the logarithmic growth phase after one day, and the maximum cell growth rate was seen at the end of the stationary growth phase. At temperatures of 10°C and 15°C, the microalgae cells entered into the logarithmic growth phase after three days and at the beginning of the fourth day. The results also showed that microalgae cells had very low growth at the temperature of 30°C. As seen in Figure 2, maximum cell growth rates were seen at the temperatures of 20°C and 25°C. The results of one-way ANOVA showed that the differences between cell growth rates at different temperatures were significant ($P < 0.05$). Tukey test results confirmed that the cells were not put in homogeneous groups and growth rates at temperatures of 20°C and 25°C had significant differences with temperatures of 10°C ($P = 0.001$, $P = 0.000$) and 15°C ($P = 0.043$, $P = 0.01$). There was no significant statistical difference in growth rates between temperatures of 20°C and 25°C ($P = 0.599$), nor between 10°C and 15°C ($P = 0.725$).

Effects of temperature on biomass and lipid production

As seen in Figure 3, the results of tests of temperature effects on biomass production in *Nannochloropsis oculata* at the end of the stationary growth phase showed that the maximum amount of biomass was produced at the temperature of 25°C. Because of unsufficient microalgae growth, there were no differences between the initial and final biomass amounts at 5°C, 30°C, and 35°C. The results of one-way ANOVA showed that the differences between
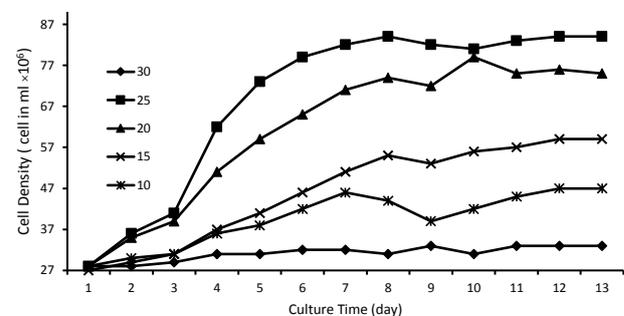

Figure 2. Cell growth rate of *Nannochloropsis oculata* in different cultivation temperatures.
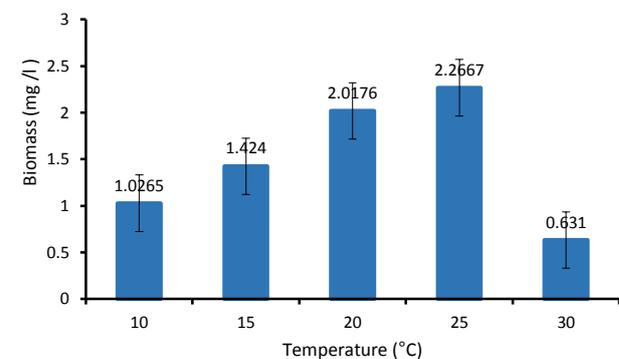

Figure 3. Biomass production by Nannochloropsis oculata in different cultivation temperatures.

biomass production rates at different temperatures were significant ($P = 0.000$), and Tukey test results confirmed that the cells were not put in homogeneous groups. Figure 4 shows that, at the 20°C and the end of the stationary growth phase, despite the large amount of biomass production, the percentage of converting biomass to lipid was minimum. Moreover, at 30°C, despite the small amount of biomass production, the percentage of converting biomass to lipid was maximum. In this study, the results of one-way ANOVA showed that lipid production in the stationary growth phase and at 20°C was significantly different from other temperatures ($P = 0.000$); the minimum lipid content was seen at this temperature (16.93%), and the maximum amount of biomass to lipid conversion was seen at 30°C.

Effect of temperature on fatty acids composition

The results of the fatty acids composition of *N. oculata* grown in different cultivation temperatures are shown in Table 1.

Discussion
Effects of temperature on cell growth rate

Temperature is a very sensitive factor for microalgal growth and metabolic activities in microalgal cells. It is also an easy-control factor in the practical operation of microalgae cultivation (6,29). In this study, a different temperature regime was used and temperature was considered as an independent variable. Most microalgae respond to increases in temperature with increased exponential growth rates until reaching their optimum temperature, after which point loss of structural integrity tends to lead to sharp declines in growth rates (30). In this study, cell density increased along with temperature increases from 10°C to 25 °C and reached its maximum growth at 25°C
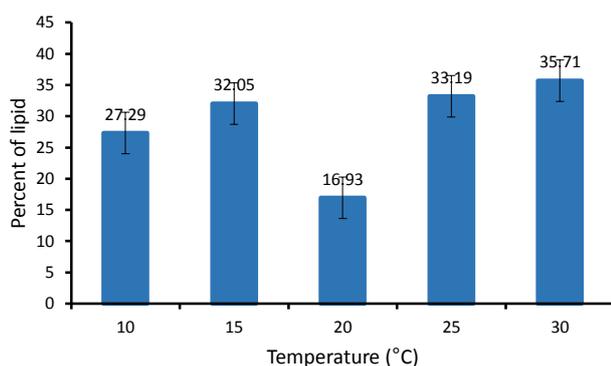

Figure 4. Lipid production by *Nannochloropsis oculata* in different cultivation temperatures

(Figure 2). Abu-reza et al referred to 24°C to 26°C as the optimal temperature for *Nannochloropsis* growth (31). Sukenik et al in their study on *Nannochloropsis* sp. referred to the temperature of 25°C as the optimal temperature for growth (32). Wagenen et al also studied *Nannochloropsis salina* and referred to the temperature of 23°C as the optimal temperature for growth; this result matches the results of the current study (30).

Investigations of the special growth rate in the studied temperature regime showed that the maximum special growth rate in the logarithmic growth phase was at 25°C and was 0.1569 day^{-1}; for 20°C it equaled 0.1388 day^{-1}, for 15°C it equaled 0.1016 day^{-1}, and for 10°C, it equaled 0.0645 day^{-1}. In their study on *Nannochloropsis oculata*, Converti et al found that by decreasing the temperature from 20°C to 15°C, the special growth rate decreased from 0.13 to 0.06 day^{-1}. By increasing temperature to 25°C, the special growth rate decreased to 0.07 day^{-1}; this result matched the findings of the current study (22). In a study by Chiu et al, after 6 to 8 cultivation days of *Nannochloropsis oculata* in F/2 medium, light intensity of 300 µE m^{-2} S^{-1}, and a temperature of 26°C, the special growth rate reached 0.571 day^{-1} (15). Banerjee et al cultivated *Nannochloropsis oculata* in Walne medium with a light intensity of 150 µE m^{-2} S^{-1} and a temperature of 20°C. They attained a special growth rate of 0.004 day^{-1} (25). The differences in special growth rates between studies could have been caused by differences in cultivation type, light intensity, previous microalgae medium, photo-bio reactor type or medium salinity (14,17).

Effects of temperature on biomass production

Microalgae are unicellular photosynthetic organisms that use light energy and carbon dioxide with a higher photosynthetic efficiency than plants for the production of biomass (20). In this study, as shown in Figure 3, the maximum amount of biomass was attained in the stationary growth phase at a temperature of 25°C (2.2667 g/L). Chiu et al, after 6 to 8 cultivation days of *Nannochloropsis oculata* in F/2 medium and using 2% carbon dioxide at 26°C, could produce 1.277 g of biomass per liter (15). In the study of Su et al on a single-stage batch culture of *Nannochloropsis oculata* in Walne medium and at a temperature of 25°C through the logarithmic growth phase, the biomass density was about 0.58 g/L which increased in the stationary growth phase to 0.654 g/Ll (33).

Effects of temperature on lipid productivity

Environmental factors such as temperature, light, pH, salinity, and nutrient status of the culture medium not only

Table 1. Fatty acid composition in dry weight, percentage of *Nannochloropsis oculata* grown in 15, 20, 25°C and end of stationary growth phases

Fatty acid	Scientific name	15°C	20°C	25°C
C14:0	Myristic acid	2.16± 0.04	6.02 ± 0.03	6.17 ± 0.19
C16:0	Palmitic acid	28.66 ± 0.84	33.57 ± 0.85	39.93 ± 0.09
C16:1 n-7	Palmitoleic acid	35.17 ± 0.29	34.92 ± 0.1	29.56 ± 0.41
C18:0	Stearic acid	1.87 ± 0.01	1.87 ± 0.00	1.86 ± 0.00
C18:1 n-9	Oleic acid	5.53 ± 0.13	4.07 ± 0.05	4.13 ± 0.01

affect the photosynthesis and productivity of algal cells, but also influence the pattern, pathway, and activity of cellular metabolism and, thus, dynamic cell composition (20). Temperature is one of the effective factors on lipid accumulation in cell and fatty acid percentages in lipids. The results of this study showed that the highest biomass to lipid conversion was achieved at 30°C. These results match those of Sukenik et al (32). In a study by Converti et al on *Nannochloropsis oculata*, lipid content was increased from 7.9% to 14.9% by increasing cultivation temperature from 20°C to 25°C (22). These results also match those of the current study in which increasing the temperature from 20°C to 25°C increased lipid content from 16.93% to 33.19%. In the study by Enebo and Iwamoto on *Rhodotorula gracilis*, however, increasing cultivation temperature from 27°C to 35°C decreased the lipid content (34). That result showed that the effects of temperature on different types were not similar. Increasing lipid accumulation with increasing cultivation age (reaching the stationary growth phase) was caused by cell division and the diversion of photosynthetic energy into TAG production (14).

The results showed that more cell biomass production by microalgae did not cause higher lipid production. Thus, lipid content or biomass amount was not singly a proper scale for microalgae lipid productivity. The roles of both factors should have been considered beside each other using Eq. 2 (19). The results of the current study showed that lipid productivity at 30°C, 25°C, 20°C, 15°C, and 10°C was, sequentially, 0.01647, 0.0395, 0.0394, 0.03841, and 0.0206 gl^{-1} day^{-1}. These results show that the maximum lipid productivity was at 25°C, 20°C, and 15°C and was significantly more than lipid productivity at 10°C and 30°C. Furthermore, there was no difference in lipid productivity at 15°C, 20°C, and 25°C. These results match those of Converti et al (22), Griffiths and Harrison (7), and Huerlimann (14).

Effects of temperature on fatty acid composition
An important characteristic of microbial fat production is the possibility of changing the fatty acid composition by altering culture conditions. Onefactor affecting fatty acid composition is temperature. Under unfavorable conditions, microalgal growth is arrested, photosynthetic activity decreases, and excess energy might be stored in the form of lipids and/or carbohydrates (28). The microalgae oil in the form of triglycerides is convertible to biodiesel, and vegetable oils currently used for biodiesel are mainly C16 and C18 (7). In a study by Olofsson et al (5), the fatty acids appropriate for producing biodiesel were identified as myristic acid, palmitic acid, palmitoleic acid, stearic acid, and oleic acid, which comprise more than 45%-78% of total fatty acids. As seen in Table 1, in this study, these compositions comprised more than 70%-80% of fatty acid compositions at the temperatures of 15°C, 20°C, and 25°C. Furthermore, at all studied temperatures, the palmitic acid and palmitoleic acid comprised more than 60%-70% of fatty acids; these results match those of Kleinschmidt

et al (29).

The TAG of *Nannochloropsis* consist mainly of saturated and monounsaturated fatty acids and are stored in vacuoles within the cell (5, 28). Most microalgae accumulate very little TAGs during exponential growth, but substantial amounts of TAGs can accumulate during the stationary phase (5). As the result, in this study, the fatty acid composition analysis was done in the microalgae stationary growth phase. According to Table 1 of this study, TAG content at the end of the stationary growth phase equaled 73.39% at 15°C and was significantly less than the TAG amount at 20°C (80.45%) and 25°C (81.65% fatty acid). In a study on *Nannochloropsis* sp., Wang and Wang achieved a TAG amount of 41.1% of fatty acids (35). The EN14214 standard for biodiesel quality attached the limitation of less than 12% for the composition of linolenic acid. This composition in the current study at all temperatures was less than one percent of fatty acids.

Conclusion
The amount of biomass and lipid produced in a cell was not alone a proper criterion to choose microalgae for economical biodiesel production. However, the maximum *Nannochloropsis oculata* lipid productivity was achieved at temperatures of 15°C, 20°C, and 25°C. The results of fatty acid analyses at the aforementioned temperatures showed that the triglyceride amount was significantly more at the temperatures of 20°C and 25°C than at 15°C. Therefore, temperatures of 20°C to 25°C were the best temperatures for lipid productivity and biodiesel production from *Nannochloropsis oculata* microalgae.

Ethical issues
We certify that all data collected during the study is presented in this manuscript, and no data from the study has been or will be published separately.

Competing interests
Authors declare that they have no competing interests.

Authors' contributions
SD and BH conceived and designed the study. MM and AR performed the literature search and wrote the manuscript. All authors participated in data acquisition, analysis, and interpretation. All authors critically reviewed, refined, and approved the manuscript.

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