Effects of increasing temperature and nitrate concentration on cell abundance, growth rate, biomass and free fatty acid of *Tetraselmis* sp

Nita Rukminasari, Sharifuddin Bin Andy Omar and Muhammad Lukman

Faculty of Marine Science and Fisheries, Hasanuddin University, Indonesia

Email: nita.r@unhas.ac.id

Abstract. It is well known that marine microalgae have a high diversity and potency as natural food for aquaculture, a high bioactive compound, and source of biofuel. *Tetraselmis* sp. is one of the marine-green algae, which has a high lipid content. A completely randomized design was used in this experiment with four levels of temperature treatments (28°C, 30°C, 32°C, and 34°C) and three levels of nitrate treatments (0 g/L, 0.2 g/L and 0.4 g/L) with length of culture was 21 days. The result of our study showed that the highest growth rate was found at 30°C with double nitrate concentration account for 0.20 cell/day. The highest biomass was found at 0.20 g/L nitrate treatment for 34°C temperature treatment account for 0.17 g/L, while the lowest dry weight was 0.08 g/L that was found at 0.0 g/L nitrate treatment for 28°C temperature treatment. The highest free fatty acid percentage was found at 30°C of temperature treatment at the 0.40 g/L of nitrate concentration treatment, account for 8.81 %. Statistically, results showed that increasing temperature affected significantly to biomass and free fatty acid while increasing nitrate was different significantly on growth rate, and biomass.

1. Introduction

Microalgae have been explored as an alternative for biofuel sources due to has a high growth rate and high lipid content. Microalgae also have several characteristics that are valuable as a photosynthetic organism, such as high photosynthetic efficiency, high product accumulation rate[1]. High biomass production rate, contain Carbon, Nitrogen and Phosphorus that fulfill the Redfield Ratio [2,3] To harvest microalgae only required several days and it was less time for harvesting compared to terrestrial plans, such as jatropha, that takes 2–3 years. Some species of microalgae such as *Botryococcus* sp. have high lipid content per dry weight account for 70%, which is very potential for oil production, with simultaneous production of biorefinery, several microalgae have others side products include glycerin and fertilizers, that is very useful for industrial applications. Biofuel based microalgae would not compete with food, so it helps in resolving food versus fuel associated with food crops. Mass production of microalgae only require small area and it will be effective in using land. Furthermore, microalgae also can grow at wastewater with high nutrient, it could be used as wastewater treatment and also produce biofuel and can be grown on unproductive land [4].

Mass production of microalgae has been produced several chemical products and health food i.e.: pigments, vitamins, long long-chain unsaturated fatty acids, antioxidants for cosmetic, human and animal food industry [5]. Nitrogen concentration is very important in microalgae culture due to this element is a component of amino acid and also part of chlorophyll as a consequent, available of nitrogen in media
culture will affect microalgae growth rate. Previous studies have shown that when culture media of microalgae is nitrogen depleted, nitrogen metabolism in algal cell will decrease, this condition could contribute to increasing synthetic activity of carotenoid and other non-nitrogen compounds. This condition also will increase lipid content due to lack of nitrate, which plays important role in biosynthesis, reduce biomass of algae and in contrary it will promote lipid/protein ratio [6].

*Tetraselmis* is well-known as natural food for fish fry oysters and mollusks at aquaculture industries. It is a green flagellate single-cell alga with the size ranging between 10 and 20 μm, and it has good characteristics for mass production. This microalgae has a high tolerant of broad salinity changing, has a less nutrient requirement for their growth (it does not require vitamins for growth; it could use several sources of nitrogen, such as ammonia, urea, nitrates or amino-acids) and finally, it can live at a wide range of temperatures (2-34°C) [7]. Several previous studies have been conducted related to *Tetraselmis* sp. in particular manipulating media culture and environmental stress, such as depleted nutrient (nitrate, phosphor, and iron) for increasing lipid production [7–16], however there was a few studies of *Tetraselmis* sp. to examine the effect of increasing nitrate concentration and temperature medium culture on growth rate, biomass, and free fatty acid. This study aimed to determine impact of increasing temperature and nitrate concentration at culture media on cell abundance, growth rate, biomass and free fatty acid of *Tetraselmis* sp.

2. Materials and methods

2.1. Microalgae culture and medium

Microalgae stocks were provided from the Research Institution for Coastal Aquaculture and Fisheries Extension, Maros, South Sulawesi, Indonesia. The culture medium used was the Conway medium mixing 1 L of macro- and micronutrient stock solution with 100 mL of vitamin solution. The composition of the nutrients stock solution is 1.3 g FeCl₃·6H₂O, 0.36 g MnCl₂·4H₂O, 33.6 g H₃BO₃, 45.0 g EDTA, 20.0 g NaH₂PO₄·2H₂O, 100.0 g NaNO₃, 21 mg ZnCl₂, 20 mg CoCl₂·6H₂O, 9 mg (NH₄)₂MoO₄·4H₂O and 20 mg CuSO₄·5H₂O in 1 L of distilled water. The stock solution of vitamins contains 1.0 g thiamine and 0.05 g cyanocobalamin in 1 L of distilled water. *Tetraselmis* sp. was stored in a 400 mL Erlenmeyer flask as a continuous culture and was exposed to continuous artificial light illumination.

2.2. Cultivation of marine microalgae under different temperature and nitrate concentration

*Tetraselmis* sp. was cultivated in Conway medium for 21 days under three different nitrate concentrations: 0.0 g/L, 0.20 g/L and 0.40 g/L. There were four temperature treatments: 28°C, 30°C, 32°C and 34°C. Each treatment had three replicates. The microalgae growth was monitored in terms of abundance by counting using a Hemocytometer (Improved Neubuer, China) under a light microscope (Olympus CX.23). Samples were taken every three days for counting abundance.

2.3. Growth rate calculation

The growth rate of microalgae was calculated using in the following formula:

\[
\mu = \frac{(\ln c_f - \ln c_i)}{\Delta t} \tag{1}
\]

where:

- \(c_i\) is the initial number of the cell of the tested microalgae (mL⁻¹ cells),
- \(c_f\) is the final number of the cell of the tested microalgae (mL⁻¹ cells), and
- \(\Delta t\) is the time between two calculation (days).

2.4. Biomass/dry weight

The dry weight of microalgae tested was measured at the end of the experiment (day 21) using a gravimetric method [17]. 10 mL sample was centrifuged for 10 minutes (12000 rpm), then biomass was washed with 10 mL distillate water and centrifuged again. Biomass then placed into dish that been weighted. Biomass then was dried using oven at 70°C for 16 hours till weight was constant. The dry weight then calculated using the formula below:
CDW = \((W_1 - W_0) \times 1000/V\)  

Where:  
CDW = Cell dry weight (g/L)  
W1 = Dry weight of dish + cell biomass  
Wo = Dry weight of the dish  
V = Volume of sample (10 mL)  

2.5. Free fatty acid  
The free fatty acid content was calculated using the following formula [11]:  
ALB level = \((25.6 \times N \times V_t)/BS\)  

when:  
N = Normality of standardized KOH  
Vt = KOH volume used for titration (mL)  
BS = Weight of sample (g)  

Meanwhile, the percentage of free fatty acids is calculated using the following formula:  
\[
\frac{(N \text{ KOH} \times \text{BM palmitate acid} \times \text{Titration volume})}{m \text{ sample}} \times 100\% 
\]

where:  
N = Normality  
V = Volume of titer substance  
M = Weight of sample  
The molecular weight of Acid Palmitate = 256  

2.6. Data analysis  
The experimental data were analyzed descriptively and statistically. Statistical analysis was performed to determine the correlation between the above data with other parameters which were analyzed statistically through analysis of variance (ANOVA).  

3. Results and discussion  
3.1. Effect of increasing temperature and nitrate concentration on the cell abundance and growth rate.  
We examined the effect of increasing temperature and nitrate concentration on the cell abundance and growth rate through calculating and monitoring cell abundance every third day of microalgae culture for 21 days of culture and growth rate was calculated at the end of the experiment. We found that there was a different pattern of cell abundance between temperature treatments and nitrate treatments (figure 1). Generally, there were increasing cell abundance with increasing nitrate concentration for all temperature treatments over period of culture, however the significant increase of cell abundance between nitrate concentration treatments for day 9, 12, 15, 18 and 21 was shown at 30°C (figure 1b). The peak of cell abundance was found mostly at day 21 for all nitrate and temperature treatments. This finding inlined with previous study by Teo et al. (2014) [13], which found that *Tetraselmis* sp reached the highest peak of growth at day 20 when the culture under different of nitrate concentration. Temperature treatment of 30°C and 32°C tend to have a higher cell abundance for over period of culture than other two temperature treatments for all nutrient treatments. It found that increasing cell abundance was almost 12% with increasing 0.2 g/L of Nitrate.
Figure 1. The cell abundance of *Tetraselmis* sp. for each temperature and nutrient treatment (Mean ± SE, N = 3).

The growth rate of *Tetraselmis* sp. was measured at the end of the experiment (21 days). The result showed that there was a similar pattern of growth rate between nitrate concentration treatments for all temperature treatments, which was increasing nitrate concentration also increasing growth rate. The growth rate was a higher at 0.2 g/L and 0.4 g/L of nitrate for 30°C of temperature treatment than three other temperature treatments, which the highest of growth rate was account for 0.2036 cell/day. At 34°C of temperature treatment, the growth rate of *Tetraselmis* sp. was increasing by 50% when nitrate concentration in the medium increased from 0.0 g/L to 0.40 g/L, account for 0.068 cell/day to 0.143 cell/day, respectively. Statistically, result showed that there was a significant difference of growth rate between 0.0 g/L nitrate vs 0.20 g/L nitrate and 0.0 g/L nitrate vs 0.4 g/L nitrate for 30°C, 32°C, and 34°C, however for 28°C growth rate was only significant difference between 0.0 g/L nitrate vs 0.4 g/L nitrate (Figure 2). There was a no significant difference in growth rate between 0.20 g/L and 0.40 g/L nitrate for all temperature treatments. Increasing growth with increasing nitrate concentration due to nitrogen is an important factor and important component of amino acids as well as being a key role for microalgae growth [13]. Our finding also showed that the growth rate was significantly lower at the absence of nitrate at culture medium. It assumed that lack of nitrate caused termination of cell division as a consequent, there was an inhibition of microalgae growth. This finding supported by previous study by [14] which found that microalgae in the environmental stress condition such as the absence of nitrate will lead to termination of cell division and when this condition continues for long period of time will cause the dead phase of microalgae. Result found that the highest growth rate occurred at 30°C and growth rate was tended to decrease with increasing temperature for all nitrate concentration treatment. It assumed that the optimum temperature for maximum growth of *Tetraselmis* ssp was 30°C. Lee and
Kim (2009) [15] mentioned that microalgae growth is affected by temperature directly through affected cell growth and death and indirectly by affecting the solubility of CO₂ and O₂ within medium. They also found that Tetraselmis sp. was some eurythermal microalgae that could live at wide range of temperature from 2°C to 34°C.

![Figure 2](image_url)

**Figure 2.** Growth rate of Tetraselmis sp. for each temperature and nitrate concentration treatment (Mean ± SE, N=3). * = significant difference at P<0.05, ** = significant difference at P<0.01, *** = significant difference at P<0.01, ns = no significant difference.

3.2. Effect of increasing temperature and nitrate concentration on biomass, and free fatty acid (FFA)

Biomass and free fatty acid were measured at the end of the experiment. Figure 3a showed that increasing temperature and nitrate concentration of Tetraselmis sp. media tend to have higher biomass, however the biomass was a significant difference only between 0.0 g/L vs 0.20 g/L and 0.0 g/L vs 0.40 g/L for 32°C and 34°C. While there was no significant difference in Tetraselmis sp biomass between nutrient treatments for 28°C and 30°C. This finding supported by previous study by [13] who found that the microalgae cultivated in 0.18 g/L nitrate concentration displayed the highest cell dry weight on day 18 of cultivation. The cell dry weights were low in the absence of nitrate but increased when nitrate concentration was increased from 0.1 g/L and 0.18 g/L. The highest biomass was found at 0.20 g/L nitrate treatment for 34°C temperature treatment account for 0.17 g/L, while the lowest dry weight was 0.08 g/L that was found at 0.0 g/L nitrate treatment for 28°C temperature treatment. At 30°C of temperature treatment, biomass of Tetraselmis sp. increased from 0.12 g/L to 0.13 g/L when nitrate concentration was increased from 0.20 g/L to 0.40 g/L. Our biomass was lower than previous study by [11] who found that biomass of Tetraselmis glacialis increased from 0.32 g/L to 0.62 g/L when nitrate concentration increased by 0.05 g/L. The difference of biomass with previous study due to the difference of species.

Figure 3b showed that there is a slightly different of the free fatty acid percentage of Tetraselmis sp. between nitrate and temperature treatments. There was a tendency that 0.20 g/L nitrate had a higher FFA than other nitrate concentration for all temperature treatments. FFA increased with increasing nitrate...
concentration from 5.86% to 8.81% for nitrate concentration of 0.0 g/L and 0.40 g/L, respectively for 30°C temperature treatment. Free fatty acid in our finding was much lower than previous study by [10] who found at the highest nitrate concentration of 2.65 mM, the lipid content of *Tetraselmis* sp increased from 19.0% to 26.5% within 2 days, and reached the highest content of 27.6% on the 5th day. Lower FFA of our study than previous study due to the different method that been used for measuring lipid content in the microalgae cell. However, our result found that the depleted of nitrate concentration caused decreasing of FFA, which was supported by previous study by [10] that conducted a study about nitrate repletion strategy for enhancing lipid production from marine microalga *Tetraselmis* sp. In general, there was no significant difference of FFA between nitrate and temperature treatment except for 30°C temperature treatment, there was a significant difference of FFA between 0.0 g/L nitrate vs 0.20 g/L and 0.0 g/L nitrate vs 0.40 g/L nitrate concentration treatment. However, our result showed a contradiction with previous research relate to nitrate depletion affected in decreasing lipid production by [13]. Our result found FFA was higher at a high nitrate concentration treatment than 0.0 g/L of nitrate (the absence of nitrate). This contradiction result was assumed due to the different method of microalgae culture, which we used a continuous culture with 24 hours light exposure.

**Figure 3.** Dry weight and Free fatty acid (FFA) of *Tetraselmis* sp. for each temperature and nitrate concentration treatment (Mean ± SE, N=3). * = significant difference at P<0.05.
4. Conclusions
This study demonstrated that by manipulating culture medium of Tetraselmis sp. through increasing temperature and nitrate concentration could be increasing cell abundance by 12% and growth rate by 50%. However, statistically showed that increasing temperature affected significantly to biomass and free fatty acid while increasing nitrate was different significantly on the growth rate, and biomass. This result strongly suggested that to increase biomass and free fatty acid for biofuel sources from marine microalgae, Tetraselmis sp. it could be conducted through manipulating temperature medium of culture.

Acknowledgment
We would like to thanks to the Center for Research and Development for Marine, Coastal and Small Islands, Hasanuddin University who provided us with a room for running our experiments. This research was funded by Directorate General for Higher Education, Ministry of Research, Technology and Higher Education, Republic of Indonesia under the research scheme of “National Strategy Research” 2017. We also thanks to Nutrition Laboratory, Animal Husbandry Faculty, Hasanuddin University who assist us with samples analysis and to Research Institute for Coastal Aquaculture and Fisheries Extension who provided us with microalgae stocks.

References
[1] Minowa T, Yokoyama S and Kishimoto M 1995 Oil production from algal cells of Dunaliella tertiolecta by direct thermochemical liquefaction 74 1735–8
[2] Shurin J B, Mandal S and Abbott R L 2014 Trait diversity enhances yield in algal biofuel assemblages 603–11
[3] Haag A L 2010 Algae bloom again IN BRIEF 447 2007–8
[4] Katiyar R, Gurjar B R, Biswas S, Pruthi V, Kumar N and Kumar P 2017 Microalgae: An emerging source of energy based bio-products and a solution for environmental issues Renew. Sustain. Energy Rev. 72 1083–93
[5] Pal D, Khozin-goldberg I, Cohen Z and Boussiba S 2011 The effect of light, salinity, and nitrogen availability on lipid production by Nannochloropsis sp. 1429–41
[6] Illman A M, Scragg A H and Shales S W 2000 Increase in Chlorella strains calorific values when grown in low nitrogen medium 27 631–5
[7] Molina E, Martinez E, Sanchez S, Garcia F and Contreras A 1991 The influence of temperature and the initial N:P ratio on the growth of microalgae Tetraselmis sp. Process Biochem. 26 183–7
[8] Dahmen-Ben Moussa I, Chtourou H, Karray F, Sayadi S and Dhouib A 2017 Nitrogen or phosphorus repletion strategies for enhancing lipid or carotenoid production from Tetraselmis marina Biorean. Technol. 238 325–32
[9] Kawaroe M, Prartono T, Sunuddin A and Saputra D 2016 Marine Microalgae Tetraselmis suecica as Flocculent Agent of Bio-flocculation Method HAYATI J. Biosci. 23 62–6
[10] Kim G, Bae J and Lee K 2016 Nitrate repletion strategy for enhancing lipid production from marine microalga Tetraselmis sp. Biorean. Technol. 205 274–9
[11] Selvakumar P and Umadevi K 2014 Enhanced lipid and fatty acid content under photoheterotrophic condition in the mass cultures of Tetraselmis gracilis and Platymonas convolutae Algal Res. 6 180–5
[12] Michels M H A, Camacho-Rodriguez J, Vermudden M H and Wijffels R H 2014 Effect of cooling in the night on the productivity and biochemical composition of Tetraselmis suecica Algal Res. 6 145–51
[13] Teo C L, Jamaluddin H, Zain NA M and Idris A 2014 Biodiesel production via lipase catalysed transesterification of microalgal lipids from Tetraselmis sp. Renew. Energy 68 1–5
[14] Ji CF, Yu X J, Chen Z A, Xue S, Legrand J and Zhang W 2011 Effects of nutrient deprivation on biochemical compositions and photo-hydrogen production of Tetraselmis subcordiformis Int. J. Hydrogen Energy 36 5817–21
[15] Lee C-G, Kwon J-S and Kim E 2009 Biodiesel production from marine microalga, *Dunaliella tertiolecta*, *Tetraselmis chui* and *Nannochloris oculata* J. Biosci. Bioeng. 108 S130–1

[16] Weiss V, Gromet-Elhanan Z and Halmann M 1985 Batch and continuous culture experiments on nutrient limitations and temperature effects in the marine alga *Tetraselmis suecica* Water Res. 19 185–90

[17] Su C, Fu C, Chang Y, Nair G R, Ye J, Chu I and Wu W 2008 Simultaneous Estimation of Chlorophyll a and Lipid Contents in Microalgae by Three-Color Analysis 99 1034–9