



The Effect of Temperature and Nitrate Compound on Growth, Biomass and Free Fatty Acid Content on Microalgae Culture of *Spirulina* sp. and *Skeletonema* sp.

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Abstract

Temperature and availability nutrients played an important role on growth and lipid production of microalgae. In this study, we examined the effect of increasing suhu and excessed and depleted NO_3 on growth rate, biomass and free fatty acid concentration in the *Spirulina* sp and *Skeletonema* sp. Two microalgae were culture on Conway and Milne media for 21 days using continuous culture technique. There were four temperature treatments, such as 28°C, 30°C, 32°C and 34°C and three nutrient treatments, which were control nutrient treatment, without NO_3 and two times NO_3 concentrations from control treatments with three replicates for each treatments. Results found that increasing temperature significantly affected on biomass and concentration free fatty acid, meanwhile nutrient treatments affected on growth rate, biomass and concentration of organic Carbon. In general, increasing temperature was more affected on *Spirulina* sp in terms of increasing growth rate, biomass and free fatty acid concentration. However, *Skeletonema* sp was more responsive to availability of NO_3 in the media culture for increasing free fatty acid, and percentage of free fatty acid per dry weight.

INTRODUCTION

Currently many countries in the world are experiencing two major problems, namely the energy crisis and global warming. The energy crisis occurs because of the decrease in fuel supply, on the other hand the need for fuel oil is increasing as the world population increases. The price of fuel oil is skyrocketing and the security conditions of oil-producing countries are very unstable. This situation exacerbates the economic conditions especially of the developing countries.

Indonesia is one of the developing countries that has a relatively high population and requires a large amount of fuel oil. Currently energy crises such as fuel oil (BBM) and electrical energy are becoming hot issues in Indonesia. The fuel crisis has made people aware that Indonesia has been

heavily dependent on petroleum. The limited supply of petroleum causes fuel prices to increase rapidly. Currently oil reserves in Indonesia are only 4.9 billion barrels. With a production rate of 550 million barrels per year, both consumed for community and industry needs, it is predicted within five years the oil reserves will gone. Besides being used as fuel, petroleum is a source of electrical energy. Up to now, the electricity demand for public and industrial consumption is still sufficient. Meanwhile, the petroleum reserves used for the power plant in the next few years will be exhausted (Hayun, 2009).

To overcome those issues, it has become an urgent need to find alternative energy sources to solve the problem of rising fuel prices in the increasingly uncontrolled world market. Since the last two or three years, along with indications of

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rising oil prices in world markets, some countries are beginning to adopt policies to promote biofuels. Initially, biofuel was regarded as the best alternative to oil fuel scarcity, also considered "more environmentally friendly" or "greener" and campaigned as sustainable energy (Subejo, 2009).

Biofuel consists of several types, those are: Bioethanol, Biodiesel and Biogas. Generally these alternative energy sources come from plants (biomass). Potential crops of biofuel are sugar cane, cassava, oil palm, distance and some algae (Hadi et al., 2006). Biofuel research derived from mainland crops has been widely practiced, but biofuel research sourced from algae/microalgae in Indonesia is rarely conducted and published whereas the potential of microalgae as an alternative energy source is enormous. Several studies on the potential of microalgae as biofuel have been widely practiced in developed countries. Christi (2008); Li et al. (2008); Song et al. (2008); Walker et al. (2005) and Wang et al. (2008) found that oil-rich microalgae are a promising alternative source of fat to produce biodiesel. Furthermore Rupprecht (2009) examines the potential of *Clamydomonas reinhardtii* algae as a biohydrogen producer. Sialve et al. (2009) studied the sustainability of microalgae as a source of biodiesel. Wijarnako & Putri (2012) studied the lipid extraction of *Nannochloropsis* sp microalgae with methanol solvents and chloroform and found that the solvent use to produce optimum lipid extracts mixed with solvent ratio methanol and chloroform which produce optimum lipid extract as 2: 3 with 3 hours of extraction time at temperature of 70oC. Sobari et al. (2013) examined the lipid content of several types of marine cyanobacteria and found that *Nostoc* sp showed the highest growth rate while *Oscillatoria* sp had the greatest lipid content compared to other cyanobacteria (*Nostoc* sp, *Synechococcus* sp and *Lynbya* sp) with 4.2 mg lipid content. Hanif & Dewanti (2015) conducted research on designing microalga conversion process into biofuel as an innovation of environmentally friendly technology. Previous research emphasized on lipid content of some microalgae, while research on the content of free fatty acids from microalgae is still rare, whereas free fatty acid content is one indicator to assess the biofuel potential possessed by microalgae. This research was done based on this problem. The purpose of this research is to know the effect of temperature increase and excess and deficiency of NO_3 in culture medium to growth rate,

biomass and free fatty acid content from microalgae *Spirulina* sp and *Skeletonema* sp. While the focus of research is on the content of organic C and fatty acids from the test microalgae.

MATERIAL AND METHODS

To increase fat production from *Skeletonema* sp and *Spirulina* sp microalgae the experiment was conducted by manipulating the culture medium of the microalgae. The manipulation of culture media is manipulation of culture media temperature and manipulation of nutrient content in culture medium.

Experimental Design

Cultural media manipulation aim to increase free fatty acid production from *Skeletonema* sp and *Spirulina* sp. Pure culture of *Skeletonema* sp and *Spirulina* sp was obtained from Brackish Water Aquaculture Research Institute and Counseling, Maros. The experimental design of this culture media manipulation was a randomized block design with three factors: duration of culture (day), temperature and nutrient. For the duration of culture will consist of three treatments, those are: 7 days, 14 days and 21 days for each microalgae *Skeletonema* sp and *Spirulina* sp. As for the temperature factor, the treatments tested were: 28°C, 30°C, 32°C and 34°C. The nutrient treatment factor consists of: without nitrate, normal natrite levels and double nitrate levels. Each factor and treatment will consist of three repetitions. The variables observed were: 1) algae growth rate, 2) total algae production per unit volume, 3) total production of free fatty acids per unit volume, 4) total dry weight of algae per unit volume and 5) ratio of dry weight of fat and total dry weight algae per unit volume.

Calculation of Growth Rate and Biomass

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

$$\mu = (\ln c_1 - \ln c_0) / \Delta t^{-1} \quad (1)$$

whence:

c_0 is the initial number of cell of the tested microalgae (mL-1 cells), c_1 is the final number of cell of the tested microalgae (mL-1 cells) and Δt is the time between two calculation (days).

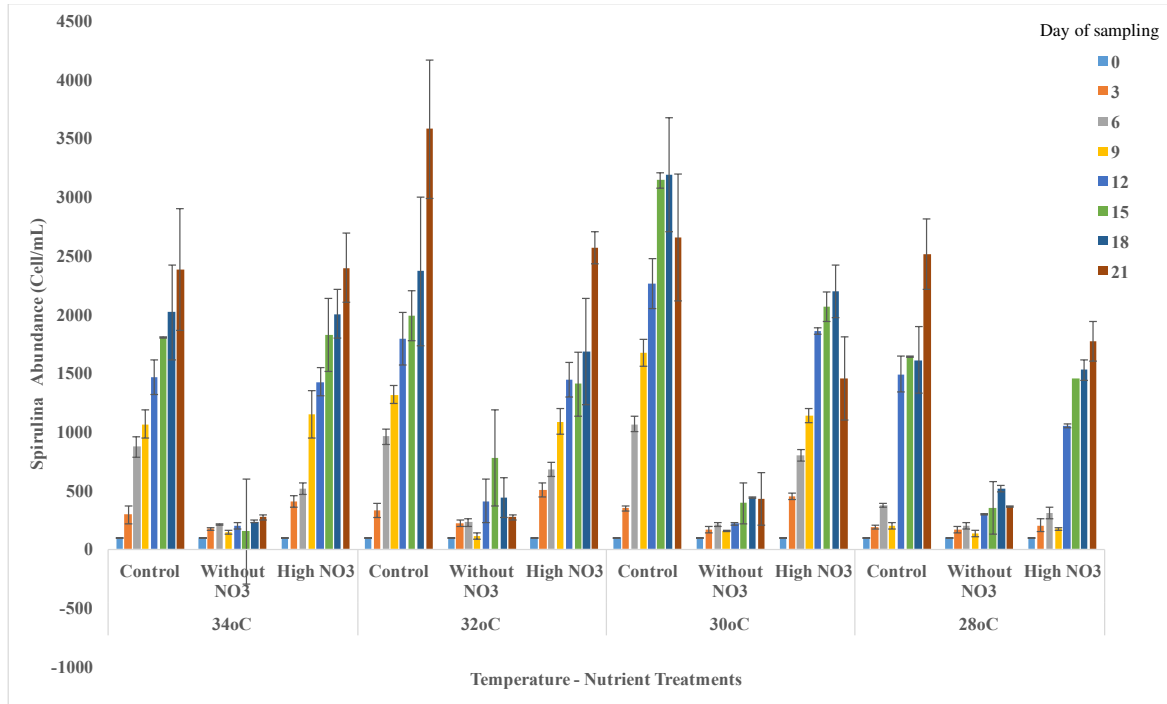


Figure 1. The abundance of *Spirulina* sp for each treatment of temperature and nutrient during culture period ($X \pm SE$, $N = 3$).

The biomass of the tested microalgae was measured using the gravimetric method. Microalgae are harvested by filtering using paper filter that has weighed and rinsed with distillate water. The filter paper and the microalgae were then dried in the oven for 24 hours and weighed. The dry weight of the microalgae harvest was obtained from reducing the dry weight of the filter paper and the microalgae with the dry weight of the filter paper (Vonshak et al., 1985).

Measurement of Free Fatty Acid Content of Tested Microalgae

The free fatty acid content was calculated using the following formula (Kertaren, 1986):

$$ALB \text{ level} = (25.6 \times N \times V_t) / BS \quad (2)$$

where, N is standardized KOH Normality, V_t is KOH volume used for titration (mL) and BS is Weight of sample (g). Meanwhile the percentage of free fatty acids is calculated using the following formula:

$$\%FFA = (N \text{ KOH} \times BM \text{ Palmitic acid} \times V \text{ titration}) / (m \text{ sample} \times 100) \times 100\% \quad (3)$$

where, N is Normality, V is Volume of titration substance, M is Weight of sample and Molecular weight of Palmitic acid is 256.

Analysis Procedure of Sample

Measurement of research variables based on references from Bolling & Fiehn (2005) and Um & Kim (2009), while calculating total algae products per unit volume using SRC (Sandwich Rafter Chamber) tool.

Data Analysis

The experimental data was analyzed descriptively and statistically. Statistical analysis was performed to determine the correlation between the above data with other parameters which was analyzed statistically through regression analysis, correlation and analysis of variance (ANOVA). Data analysis will use SPSS v.12 statistical software.

RESULTS AND DISCUSSION

The Abundance of *Skeletonema* sp. and *Spirulina* sp. During The Culture Period

The test microalgae (*Skeletonema* sp and *Spirulina* sp) were cultured for 21 days and the abundance of the test microalgae was calculated every three days so that the abundance data consisted of the abundance of the 0th, 3rd, 6th, 9th, 12th, 15th, 18th and 21st. There were a different abundance between tested microalgae for each time of calculation, temperature and nutrient treatment.

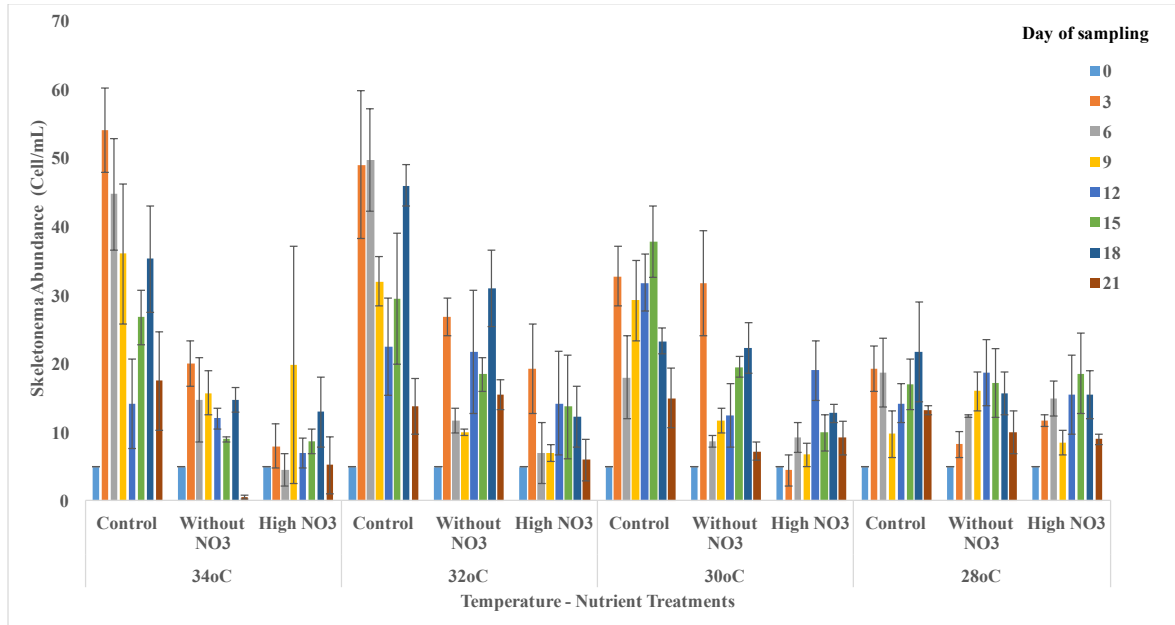


Figure 2. The abundance of *Skeletonema* sp for each treatment of temperature and nutrient during culture period (Mean \pm SE, N = 3).

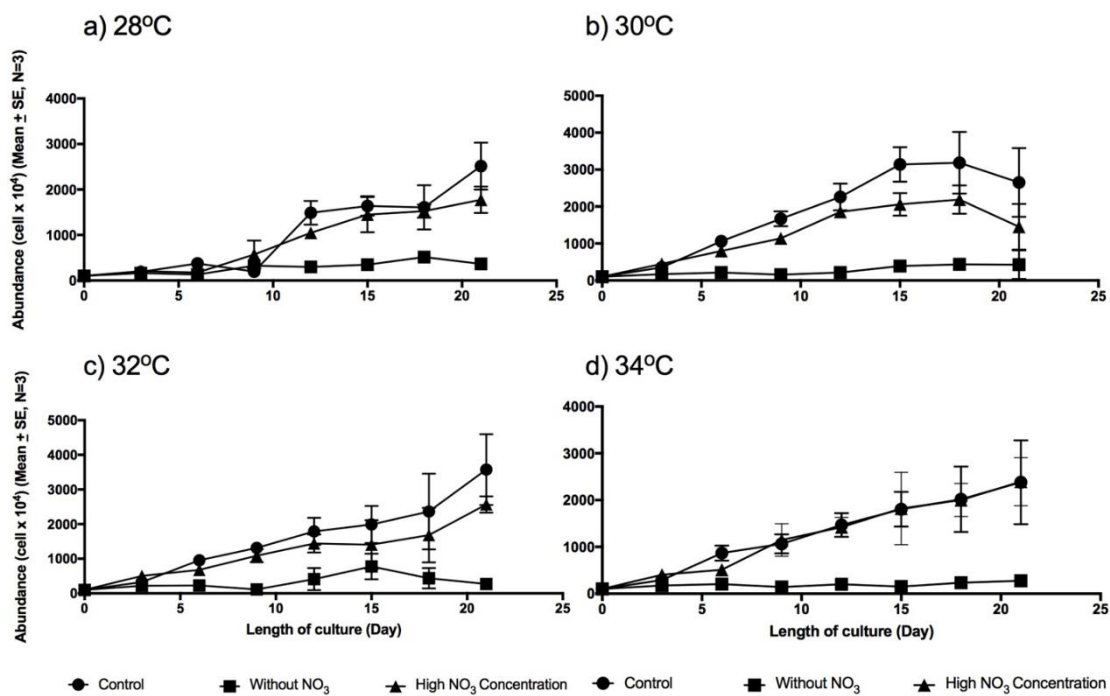


Figure 3. Graph of the abundance of *Spirulina* sp during the culture period for each temperature treatment at three nutrient treatments (X \pm SE, N = 3)

Spirulina sp. abundance tended to be much higher than the abundance of *Skeletonema* sp (Figures 1 and 2).

Spirulina sp. abundance tends to be greater in the nutrient control treatment for all treated temperatures during the culture period. The graph of its abundance tends to be in the form of regression except in the temperature treatment of

30°C the graph of its abundance tends to be parabolic (Figure 3a - d).

The results of statistical analysis showed that the abundance of *Spirulina* sp was significantly different ($P < 0.05$) for each treatment of temperature and nutrient at each time of calculation (Table 1). This suggests that *Spirulina* sp gives a positive response to culture media manipulation

Table 1. ANOVA results of *Spirulina* sp. Abundance at the temperature and nutrient treatment for each time of calculation. *** was significantly different at P <0.001, ** was significantly different at P <0.05 and * significantly different at P <0.1.

Source	df	3 rd Day		6 th day		9 th day		12 th day		15 th day		18 th day		21 st day	
		F	Value of Sig.	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..
Temperature	3	8.62	0.000***	38.11	0.000***	51.91	0.000***	8.27	0.001***	4.91	0.008***	4.25	0.015**	2.29	0.105
Nutrient	2	23.84	0.000***	158.6	0.000***	127.53	0.000***	135.58	0.000***	52.59	0.000***	16.38	0.000***	57.87	0.000***
Temp vs Nutrient	6	1.96	0.111	10.31	0.000***	15.05	0.000***	3.06	0.023**	2.71	0.038**	2.47	0.053	1.49	0.224

Table 2. ANOVA test Results of abundance of *Skeletonema* sp on temperature and nutrient treatment for each time of calculation. *** was significantly different at P <0.001, ** was significantly different at P <0.05 and * was significantly different at P <0.1

source	df	3 rd Day		6 th day		9 th day		12 th day		15 th day		18 th day		21 st day	
		F	Value of Sig.	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..
Temperature	3	7.011	0.002***	3.56	0.029**	1.94	0.15	1.94	0.15	1.42	0.262	4.25	0.015**	0.77	0.523
Nutrient	2	29.18	0.000***	31.13	0.000***	7.29	0.003***	7.29	0.003***	10.52	0.001***	16.38	0.000***	5.89	0.008***
Temp vs Nutrient	6	3.78	0.009***	5.29	0.001***	1.27	0.309	1.75	0.153	2.47	0.053**	2.47	0.053**	1.66	0.174

Table 3. ANOVA test results of growth rate, dry weight, organic C content and free fatty acid content. *** was significantly different at P <0.001, ** was significantly different at P <0.05 and * was significantly different at P <0.1.

Variant source	df	Growth rate		Dry weight		C. Organic content		Free Fatty Acid (FFA) content		Ratio of BK:FFA		% FFA/BK	
		F	Value of Sig.	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..	F	Value of Sig..
Species	1	2.98	0.091	5.04	0.029**	44.27	0.000***	2.78	0.102	2.52	0.119	1.55	0.22
temperature	3	1.22	0.312	25.24	0.000***	3.94	0.014**	37.96	0.000***	1.17	0.33	0.67	0.576
Nutrien	2	36.02	0.000***	4.86	0.012**	18.53	0.000***	0.79	0.458	4.63	0.01*	5.20	0.009***
Sp vs tempt vs Nutrient	12	1.44	0.181	2.45	0.014**	1.69	0.100	4.91	0.000***	1.70	0.142	1.93	0.095*
Species vs Nutrient	2	23.56	0.000***	0.26	0.772	5.96	0.005***	1.72	0.191	0.96	0.392	0.97	0.387
Species vs tempt	3	3.57	0.021**	1.49	0.23	0.17	0.917	1.48	0.232	0.14	0.936	0.403	0.751

(different temperature and concentration of nutrient / NO₃). Based on the results of statistical analysis it showed that *Spirulina* sp is more responsive to manipulation of NO₃ concentration than temperature manipulation. This is evidenced by the obvious difference between the nutrient treatments for each observation time. *Spirulina* sp abundance tends to be low on condition of media without NO₃. Based on the results of this study it shows that in general the availability of nutrients, especially NO₃ directly affect the abundance of microalgae. The results of this invention are in accordance with previous studies by Rukminasari (2013) which show that density of *Dunaliella tertiolecta*, *Nannochloropsis* sp. and *Scenedesmus* sp. were strongly affected by the availability of nutrients and

the density of the three types of microalgae tended to decrease in conditions without N.

Skeletonema sp. has a lower abundance than *Spirulina* sp for each treated temperature and nutrient during the culture period. Treatment of nutrient control abundance was higher than that of two other nutrient treatments (Without NO₃ and 2 times NO₃ concentrations). The graphs of abundance for each treatment of temperature and nutrient tend to be bimodal with several peaks of abundance during the culture period (Figure 4).

The results of ANOVA show that the abundance of *Skeletonema* sp differs significantly between the temperature treatments at the 3rd, 6th and 18th day counts. While the treatment of nutrient abundance was significantly different for all time calculations (Table 2).

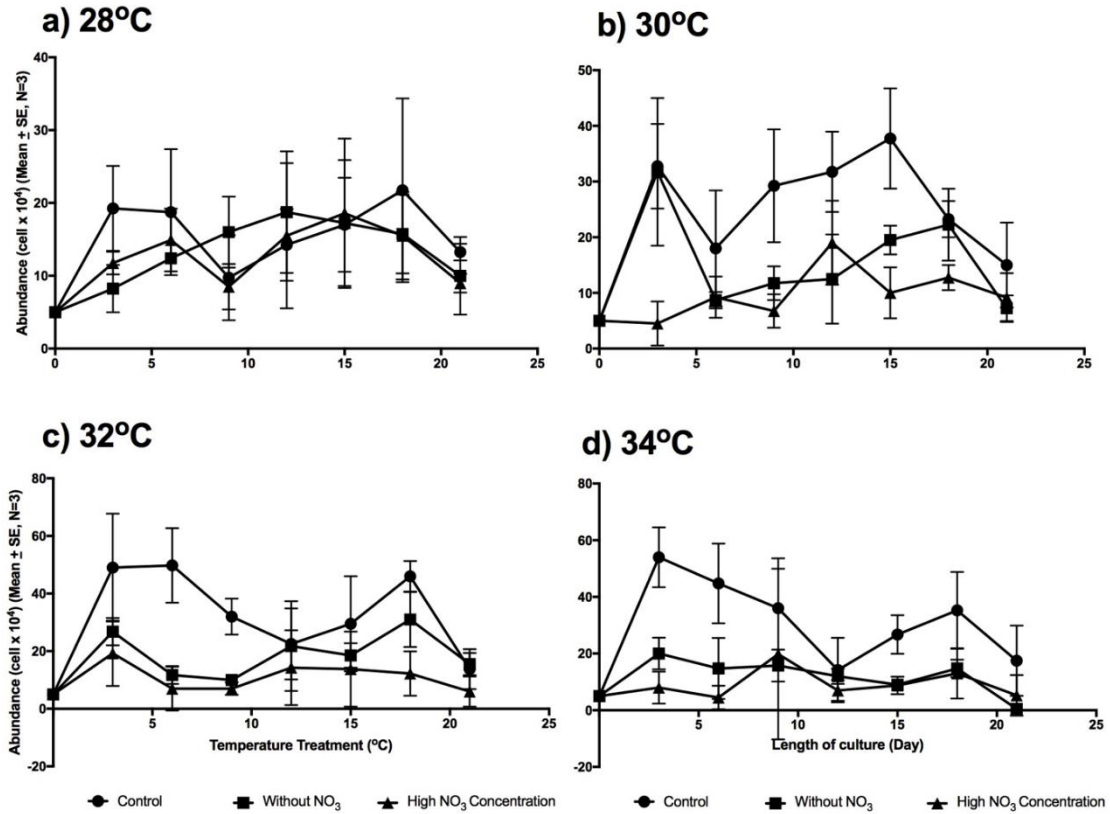


Figure 4. Graph abundance of *Skeletonema* sp. during culture period for each temperature treatment at three nutrient treatments (Mean ± SE, N=3).

Similar to *Spirulina* sp, the abundance of *Skeletonema* sp microalgae is also more responsive to changes in NO₃ concentrations in the medium than with the increase in temperature. This is evidenced by the apparent difference in *Skeletonema* abundance between nutrient treatment at all time calculations (Table 2) and this finding is consistent with previous research by Rukminasari (2013).

Growth Rate, Dry Weight, Organic C Content and Free Fatty Acid (FFA) Content and Dry Weight Ratio and Free Fatty Acid

Growth Rate of *Spirulina* sp. and *Skeletonema* sp.

The growth rate of the tested microalgae was measured after 18 days of culture time (Fig. 5). *Spirulina* sp. growth rates at the temperature and nutrient treatment showed different patterns (Figure 5b). Growth rate at treatment without NO₃ was very low for all temperature treatments, while the highest rate of growth is obtained in the treatment of nutrient control to treat the temperature of 28°C by 0.154 cells/day. The lowest growth rate was obtained on treatment without

NO₃ at 32 °C at 0,011 cells/ day. Based on ANOVA statistic test, *Spirulina* sp spiral growth rate was significantly different at 5% test level (P <0.05) between nutrient treatment (Table 3).

Skeletonema sp. showed different growth rate patterns compared with *Spirulina* sp (Figure 5a). Growth rates tend to be lower in treating high NO₃ concentrations compared with two other nutrient treatments for all treated temperatures. The highest growth rate was obtained in the nutrient control treatment at temperature of 32°C as 0.123 cells/day and the lowest growth rate was obtained at the high NO₃ nutrient treatment at temperature of 32 °C at 0,049 cells/day. Based on the ANOVA results showed that the rate of *Skeletonema* sp acceleration was significantly different at the 5% test level (P <0.05) between the nutrient treatment (Table 3). Meanwhile the growth rate was not significantly different between tested microalgae (*Spirulina* sp. and *Skeletonema* sp.). The interaction between species vs. temperature vs. nutrient was not significantly different. The interaction between species vs. nutrient and species vs. temperature showed significant differences at the test level of (P <0.01 and P <0.05). The results of this study

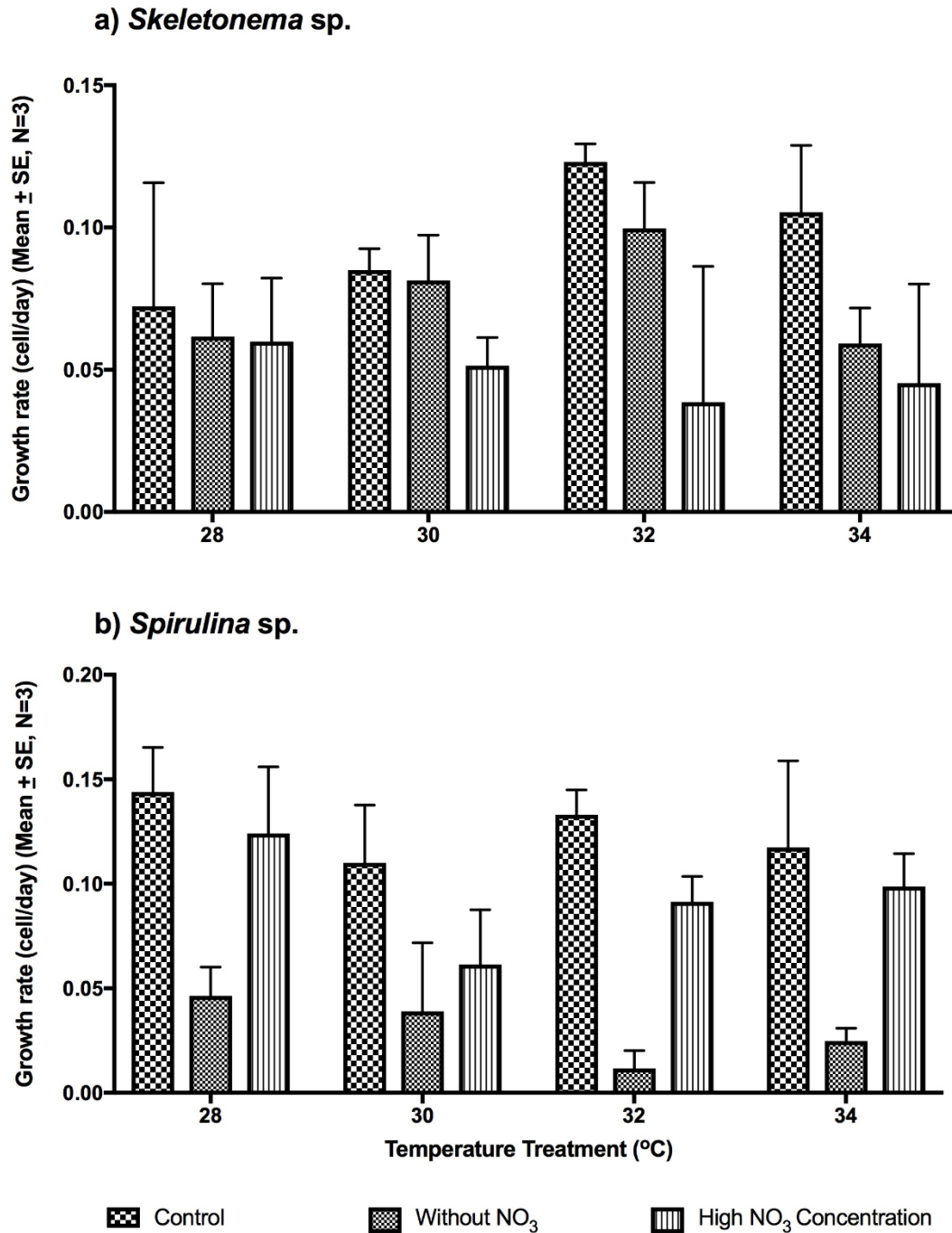


Figure 5. Growth rate of *Skeletonema* sp. and *Spirulina* sp. for each treatment of temperature at three nutrient treatments (Mean±SE, N = 3).

indicate that nutrient manipulation of culture media from the test microalgae has more significant effect on the growth rate of the test microalgae than the temperature manipulation. Interestingly, it was found in this study that there was a difference in response between *Spirulina* sp. and *Skeletonema* sp. on increasing NO₃ concentrations. This is thought to be due to differences in metabolic response of both microalgae to the availability/concentration of NO₃ in culture medium. Based on the results of

Chen et al (2011) research, the availability of primary nutrients (N and P) and micro nutrients (Fe, Mn, and Co) affects the growth rate of *Dunaliella tertiolecta*.

**Comparison of *Spirulina* sp. and *Skeletonema* sp.
Dry Weight**

Dry weight was measured to determine the biomass of the test microalgae per 100 mL. The result showed that there was a marked difference in

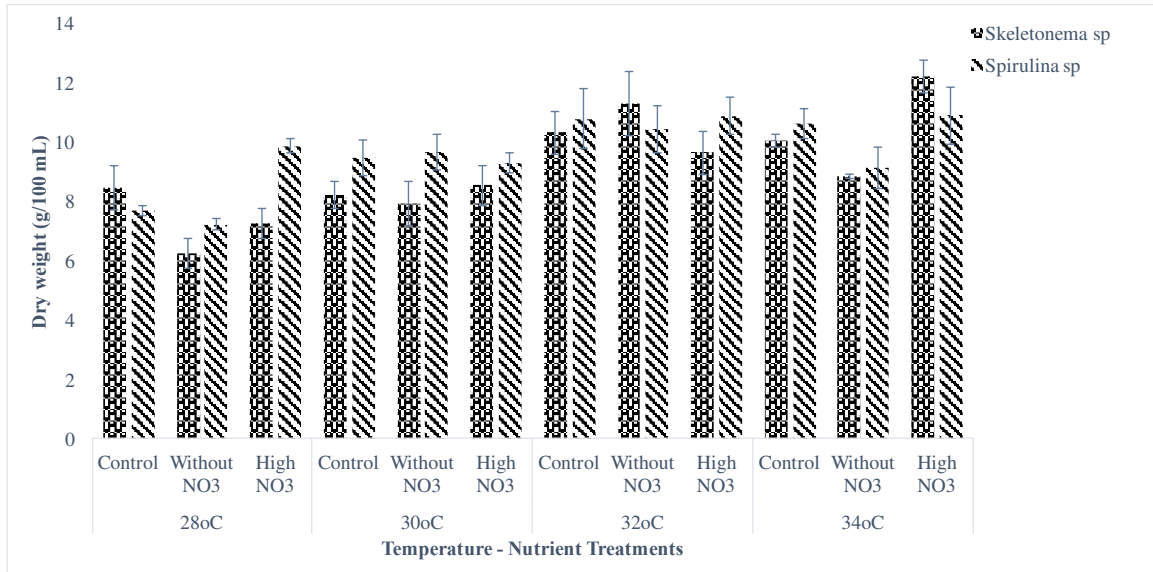


Figure 6. Dry weight of *Spirulina sp.* and *Skeletonema sp.* for each treatment of temperature at three nutrient treatments (Mean \pm SE, N = 3).

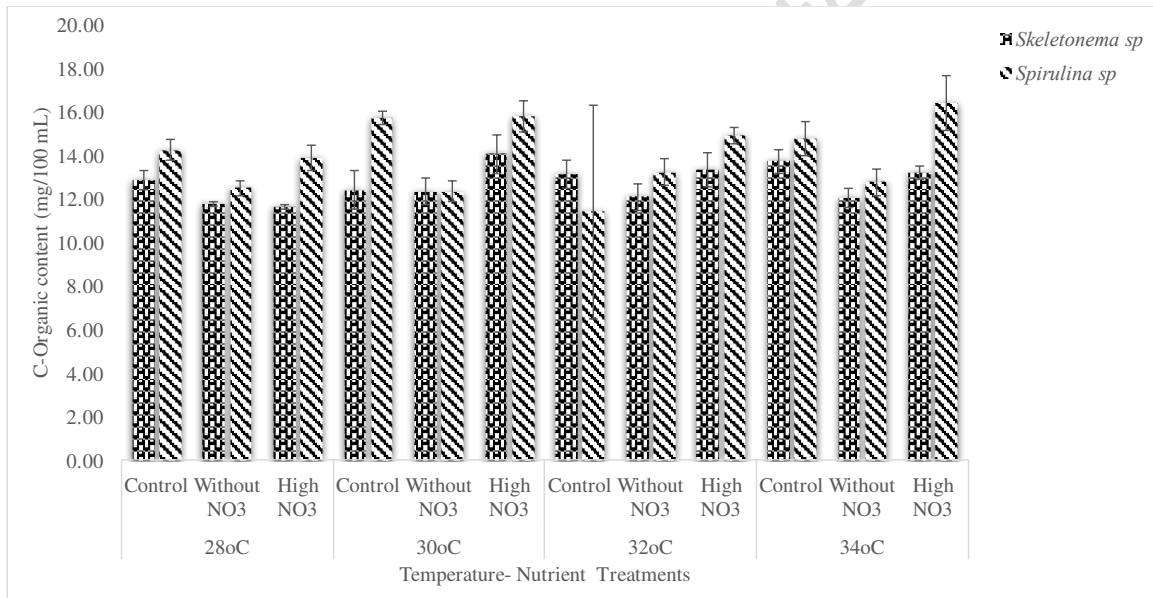


Figure 7. C-organic content of *Spirulina sp.* and *Skeletonema sp.* for each treatment of temperature at three nutrient organic treatments (Mean \pm SE, N = 3).

dry weight between test microalgae tested. There was a tendency *Spirulina sp.* has a greater dry weight than *Skeletonema sp.* on some treated temperatures and nutrients (Figure 8). The highest dry weight was obtained on *Skeletonema* at treated temperature of 34°C and treated with high NO₃, 12.2 g/100 mL. While the lowest dry weight was obtained in *Spirulina sp.* at treatment without NO₃ at 28°C. The ANOVA test results showed significant differences for species variant, temperature, nutrient and interaction between species vs temperature vs nutrient at test level of P <0.01 and P <0.05) (Table 3). While the interaction between species vs

temperature and species vs nutrients did not show any significant difference. The results of this study indicate that there was a marked difference in algae biomass test on temperature and nutrient treatment. *Spirulina sp.* tend to have greater dry weight/biomass compare to *Skeletonema sp.* This is presumably because *Spirulina sp.* has much larger cell abundance than *Skeletonema sp.* per mL. The results of the statistical analysis showed that the two test microalgae responded significantly to the treatment of temperature and nutrient seen from dry weight/biomass. This suggests that treating

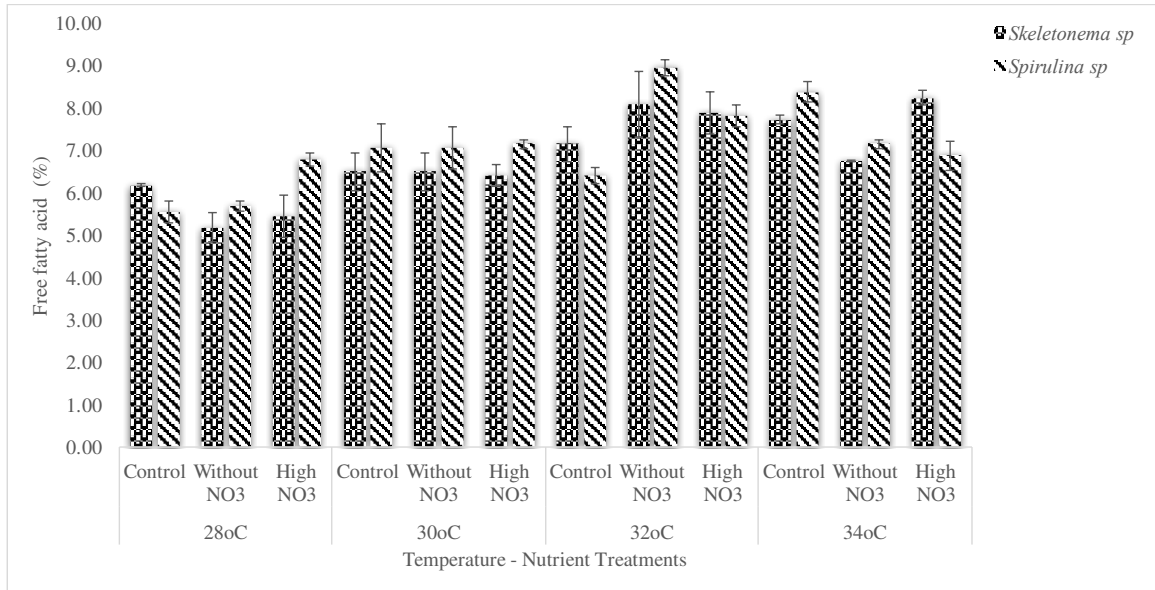


Figure 8. Free fatty acid Percentage of *Spirulina sp.* and *Skeletonema sp.* for each treatment of temperature at three nutrient treatments (Mean±SE, N =3).

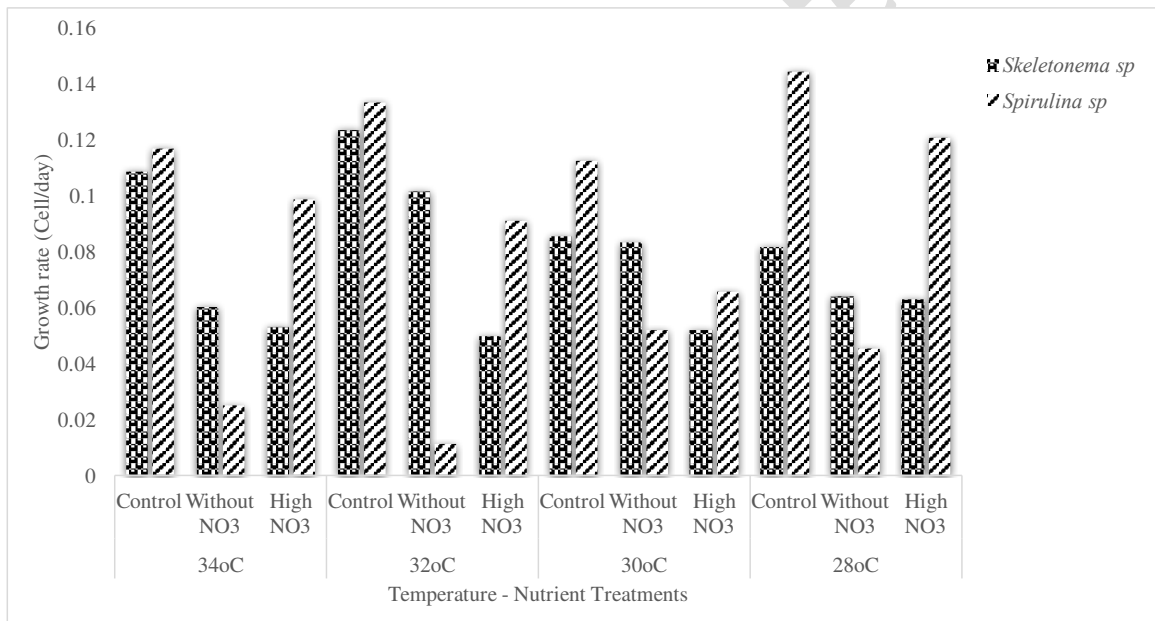


Figure 9. DW: FFA Ratio of *Spirulina sp.* and *Skeletonema sp.* for each treatment of temperature at three nutrient treatments ($X \pm SE$, N =3).

temperature and nutrient concentrations significantly affect the biomass of both test microalgae. This is presumably because the increase in temperature and nutrient availability will affect the growth of the tested microalga so that with increasing rate of growth will increase the biomass of the microalgae.

Comparison of C- Organic Content of *Spirulina sp.* and *Skeletonema sp.*

The C- organic content was measured to determine the potential level of microalgae tested as a biodiesel

source. The organic C content was calculated per mg for every 100 mL of the test microalgae (Figure 7). Figure 7 showed that *Spirulina Sp* has a higher C- organic content than *Skeletonema sp.* for each treatment of temperature and nutrient. The highest C- organic content was obtained on *Spirulina sp.* at treating temperature of 34°C at high NO₃ treatment, account for 16.38 mg/100mL. While the lowest C- organic content obtained at the treatment temperature of 30°C on the nutrient control. The statistical test results showed that there are significant differences for source of variant species, temperature, nutrient and interaction between

species and nutrient at 5% and 1% test level (Table 3). While the interaction between species vs temperature vs nutrient and species vs temperature had no significant difference.

Comparison of Free Fatty Acid (FFA) of *Spirulina* sp. and *Skeletonema* sp.

The percentage of FFA was calculated to see the potential level of tested microalgae as the source of biodiesel. The unit of free fatty acid was % per dry weight. *Spirulina* sp. tend to have higher free fatty acid content than *Skeletonema* sp for each treatment of temperature and nutrient, except for the treatment of 28°C, 32°C and 34°C for high nutrient control and NO₃ treatment (Figure 8). The highest free fatty acid content was obtained at a temperature treatment of 32°C for the treatment of nutrients without NO₃ for *Spirulina* sp where the free fatty acid content was 8.37 %. While the lowest free fatty acid was obtained at a temperature treatment of 28°C for the treatment of nutrients without NO₃ on *Skeletonema* sp, amounting to 5.17 %. The statistical test results showed that the only real difference was found for the source of variant; temperature and the interaction between species vs temperature vs nutrient at the test level of 1% (P <0.01). As for the source of other variants are not significantly different (Table 3).

The results of this study indicated that only temperature treatment which affects the fat content of the two test microalgae. Increased temperatures tend to increase the fat content of the tested microalgae and there is a noticeable difference in the fatty acid content of the two test microalgae between the temperature treatments. Based on the results of further tests of Tukey, it showed that the content of free fatty acids differ significantly between temperature treatment, except between the temperature of 32°C vs 34°C free fatty acid content is not significantly different. This is presumably because the increase in temperature will affect the rate of photosynthesis and metabolism of the test microalgae, so that the production of fat will increase. The results of this study are consistent with the results of Rukminasari (2013) research showing that the increase of culture medium temperature for three types of test microalgae (*Dunaliella tertiolecta*, *Nannochloropsis* sp. And *Scenedesmus* sp.) increased fat production.

Comparison of Biomass/Dry weight (DW): Free Fatty Acid (FFA) Ratio of *Spirulina* sp. and *Skeletonema* sp.

The ratio of DW: FFA was calculated to determine the biodiesel production potential of microalgae tested. *Spirulina* sp. tend to have higher DW: FFA ratio than *Skeletonema* sp. for almost all temperature and nutrient treatment (Figure 9). The highest DW: FFA ratio was obtained at temperature of 32°C temperature treatment for nutrient control treatment account for 1.16, while the lowest ratio of 1.17 was obtained at 32°C for treatment of nutrient without NO₃. The result of statistical analysis showed that the ratio of DW: FFA was significantly different for the variant source of nutrient treatment at the test level of 10% (P <0.1) (Table 3).

CONCLUSION

Treatment of temperature significantly affected dry weight, organic C content and free fatty acid content, whereas nutrient treatment significantly affected growth rate, dry weight and organic C content. Media culture manipulation by increasing the temperature of the media had more significant effect on increasing growth rate, biomass and free fatty acid content for *Spirulina* sp. For increased free fatty acid content and percentage of free fatty acid content per dry weight, the manipulation of culture medium through the manipulation of NO₃ availability was more significant in *Skeletonema* sp and showed potential as biodiesel greater than *Spirulina* sp through NO₃ availability manipulation on culture medium.

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