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Effect of Temperature and Nutrient Limitation on the Growth and Lipid Content of Three Selected Microalgae (*Dunaliella tertiolecta, Nannochloropsis* sp. and *Scenedesmus* sp.) for Biodiesel Production

Nita Rukminasari

Department of Fisheries, Marine Science and Fisheries Faculty, Hasanuddin University, Jl. Perintis Kemerdekaan Km. 10, Makassar-90245, South Sulawesi–Indonesia

Corresponding author email: nitasari_02@hotmail.com

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Abstract Microalgae is one of potential source for biodiesel due to high efficiency of solar energy conversion to chemical energy. Several microalgae also have high lipid content per dry weight of biomass. The aims of the present work to study the effects of temperature and nutrient depletion on the growth and lipid content of three selected microalgae (*Dunaliella tertiolecta*, *Nannochloropsis sp* and *Scenedesmus sp*) in view of their possible utilization as raw materials for biodiesel production. In addition, various lipid analysis methods were applied, such as gravimetric, Nile Red staining and FTIR spectroscopy. Algal growth and lipid content was strongly influenced by the variation of tested parameters; indeed, an increase or decrease temperature from ambient temperature and nutrient depletion practically increase in lipid content. Nile Red staining and FTIR spectroscopy are effective tool to analyze rapidly of lipid content from selected microalgae.

Keywords Biodiesel; *Dunaliella tertiolecta*; *Scenedesmus* sp.; *Nannochloropsis* sp.; Lipid; Nile Red staining; FTIR spectroscopy

1 Introduction

Nowadays, the global energy system is predominantly based on utilization of fossil fuels, coal oil and natural gas. This system has several problems, such as: 1) it creates pollution on local., regional and global scales, 2) the reserves of fossil fuel are limited while on the other hand the demand for fossil fuel increases dramatically with the increasing population as a consequence creating a global energy crisis and 3) fossil fuel produces greenhouse gas emissions (NO_x, CO_2 and SO_x) that cause global warming and climate change problems (Barbir, 2009).

For the past ten years, fuel production from biomass (biofuel) has received considerable attention from researchers and scientists as it is a biodegradable, renewable and non-toxic fuel (Mutanda et al., 2010). Biofuel based on vegetable oil, bioethanol and biodiesel represent promising energy sources to displace fossil fuel (Lardon et al., 2009). Biodiesel from microalgae seems to be a promising renewable biofuel that has the potential to completely displace petroleum-derived transport fuel without adversely affecting the supply of food and other crop products (Chisti, 2008). Shay (1993) reported that algae were one of the best sources of biodiesel. In fact algae are the highest yielding feed stock for biodiesel. It can produce up to 250 times the amount of oil per acre as soybean and could produce 500 to 1 500 gallons of biodiesel per acre per year in open ponds (Shehan et al., 1998). Algae produce 7 to 31 times greater yields of oil than palm oil due to their ability to accumulate lipid and their very high actual photosynthetic yield: about 3%~8% of solar energy can be converted to biomass whereas observed yields for terrestrial plants are about 0.5% (Hossain et al., 2008; Lardon et al., 2009; Huntley et al., 2007; Li et al., 2008). Microalgae are estimated to produce biomass greater than the fastest growing terrestrial plant with a rate account for 50 times (Li et al., 2008). In addition microalgae also have a varies lipid content of 1%~85% by dry weight (Chisti, 2007; Sheehan et al., 1998; Rodolfi et al., 2009). Biodiesel yield depends strongly upon the lipid content of the algal strain. Some algal strains can contain lipid up to 60% of dry mass (Shehan et al., 1997; Chisti, 2007). Algal oils are usually accumulated

as membrane components, storage products, metabolites and sources of energy under some special production conditions (Deng et al., 2009).

For biodiesel product, the economic feasibility of microalgal mass culture have to be taken into consideration, the searching of microalgal species with high lipid content and high cell growth is a great importance (Lv et al., 2010). However, there are two categories of microalgae that used for lipid production such as: 1) high lipid content but low growth rate, for example *Botryococcus braunii* with lipid content of 50% but had low biomass productivity of 28 mg/L/day (Dayananda et al., 2007); 2) high growth rate but low lipid content, such as, *Chlorella vulgaris* (Griffiths and Harrison, 2009).

Biodiesel consists of fatty acid methyl esters, which commonly are derived from triacyglycerols (TAGs) by transesterification with a short chain alcohol such as methanol, with glycerol as a by product (Chen et al., 2010). Most microalgal species produce largae amounts of TAG under stress condition, e.g, under nutrient depletion and temperature stressed (Chen et al., 2010).

Enhancement lipid production in cell at various cultivation condition such as nitrogen deprivation and phosphate limitation (Rodolfi et al., 2009; Phadwal and Singh, 2003; Cheng et al., 2010), light intensity and temperature stressed (Tsovenis et al., 2003; Norman et al., 1985), iron supplementation and silicon deficiency (Liu et al., 2008; Griffiths and Harrison, 2009) and different CO_2 concentration (Chiu et al., 2009; Ho et al., 2010) had been tested. Among them, nutrient deprivation has been the most studied aspect. Cheng et al (2010) reported that nitrogen starved cell of *Dunaliella tertiolecta* had accumulated significant amounts of neutral lipids by day 3 and reached maximum lipid content per OD unit by day 4 of culture. Rodolfi et al (2009) found that N-deprived could stimulate the lipid accumulation with low biomass productivity. Nitrogen starvation created an environmental stresses for microalgae to increase a lipid production as a consequence of inhibiting cell division (Sukenik and Livne, 1991). It showed that lipid accumulation was commonly correlated by nitrogen limited growth rates and due to overall the low lipid production (Lv et al., 2010).

Most previous study have investigated separately the effect of those factors to cell growth or lipid production of various microalgal species, they had hardly been investigated simultaneously and comprehensively between cultivation condition, especially for three different species (*Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp.) which are promising species as a lipid production from microalgae. Noteworthy that chlorophyll play an essential role for capturing CO_2 and solar energy to generate the metabolic flux for not only cell growth but also lipid accumulation of microalgae photosynthesis (Cohen et al., 1988; Li et al., 2008). Therefore, to get better understanding of the relationship between cell growth and lipid accumulation, the measurement chlorophyll a (Chl *a*) of cell during the cultivation process is important to be done.

In this study, the batch culture of three species microalgae (*Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp.) were carried out. The variation methods of lipid measurement from cultured species were applied, including gravimetric method of Bligh and Dyer (1959), Nile Red staining method (Cooksey et al., 1987; Elsey et al., 2007; Chen et al., 2009; Chen et al., 2011) and FTIR method (Dean et al., 2010; Giordano et al., 2001). The effects of cultivation conditions including nutrient and temperature condition on cell growth, Chl *a* content and lipid content of *Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp. were thoroughly investigated. The influence of culture conditions on the cell growth, chl *a* content and lipid production were further discussed. Finally, the different method of lipid analysis were compared and discussed thoroughly.

2 Materials and Methods

2.1 Microalgae and culture medium

Three microalgal species were used in this study, specifically *Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp. (all species from culture collection of Algae, Algal Physiology Laboratory, Biological Science, Monash University). All microalgae are eukaryotic photosynthesis microorganisms that grow rapidly as a consequence of their simple structure (Li et al., 2008). *Dunaliella tertiolecta* and *Nannochloropsis* sp. are marine microalgal were cultured in PhK medium, consisting of 2L of pasteurized artificial seawater which has the following composition (per liter): 22 g NaCl, 5.0 g $MgSO_4.7H_2O$, 0.6 g KCl,

0.5 g $NaNO_3$, 0.25 g $CaCl_2.2H_2O$, 0.2 g tris-base, 0.165 g $NaHCO_3$, 1.0 mL of 3% solution KH_2PO_4 and 6.0 mL of trace elemental solution. The trace elemental solution (per liter) includes 0.02 g $CuSO_4.5H_2O$, 0.0125 g $NaMO_4.2H_2O$, 9.0 g Fe citrate, 9.0 g Citric acid, 0.046 g $ZnSO_4.7H_2O$, 0.289 g $MnCl_2.4H_2O$, 0.0081 g $CoCl_2.6H_2O$, 0.1001 g $FeSO_4.7H_2O$, 2.0027 g EDTA, 2.2877 g H_3BO_3, 0.010 g vitamin B_{12}, 0.005 g Biotin and 0.02 g Thiamine HCl. *Scenedesmus* sp. is freshwater microalgal was cultured in MLA medium, consisting of 2 L pasteurized distillated water which has the following composition (per liter): 49.4 g $MgSO_4.7H_2O$, 85 g $NaNO_3$, 6.96 g K_2HPO_4, 2.47 g H_3BO_3, 0.00129 g H_2SeO_3, 16.9 g $NaHCO_3$, 29.4 g $CaCl_2.2H_2O$, 10 mL micronutrients. The micronutrient solutions (per liter) includes 4.36 g Na_2EDTA, 1.58 g $FeCl_3.6H_2O$, 0.6 g $NaHCO_3$, 0.36 g $MnCl_2.4H_2O$, 1.0 g $CuSO_4.5H_2O$, 2.2 g $ZnSO_4.7H_2O$, 1.0 g $CoCl_2.6H_2O$, 0.6 g $Na_2MoO_4.2H_2O$, 0.010 g Biotin, 0.010 vitamin B_{12} and 0.010 g Thiamine HCl. For the treatments of N and P depletion, the culture media were not added of N and P elements, such as $NaNO_3$ and K_2HPO_4.

2.2 Culture system

Growth experiments were done at different temperatures and nutrient conditions in 500 mL-Erlenmeyer flasks. The medium and flasks were sterilized in an autoclave for 20 mins at 121℃ in order to prevent any contamination during the early stages of growth.

The cell cultured was kept at incubator room at 18℃ and 25℃ equipped with artificial lightening. Each autotrophic batch cultivation was carried out in triplicate for 9 and 10 days at a continuous photon flux density of 180 μmol m^{-2} s^{-1}, which was measured by a light intensity meter (LICOR Model LI-1400 data logger) for 24 hours.

Temperature and nutrient condition in the medium were selected as a treatments (independent variables). Two different temperatures condition were selected, such as 18℃ and 25℃, while three different nutrient conditions were control (deplete nutrient medium), nitrogen and phosphate depletion medium.

2.3 Microalgal cell counting and dry weight

A direct microscopic cell count was performed daily with Brightline Hemocytometer (Neubauer, Weber England) and a Olympus CHS model microscope (Olymphus Optical Co. Ltd, Japan). Algae were cultivated in flasks for biomass determination. 10 mL samples were filtered on to pre-dried and weighed GF/C fibre filters every second day of culture. Filters were oven dried overnight at 60℃, and reweighed using an analytical scale.

2.4 Measurement of growth rate

Specific growth rate (μ d^{-1}) was calculated as follows: $\mu=\ln(Wt/Wo)/\Delta t$, where Wt and Wo were the final and initial biomass concentration, respectively. Δt was the cultivation time in day (Ono and Cuello, 2007).

2.5 Measurement of chlorophyll *a*

The measurement of chl *a* was taken every second day of cell cultivation. Sample (10 mL) of the culture was filtered using a 25 mm GF/C filter. Filtered cell was placed into 10 ml centrifuge tube and 9 mL of 100% cold acetone was added. The tubes were wrapped in foil and placed at a fridge overnight for extracting chlorophyll. The next day, the samples were sonicated with probe sonicator (Bronson Sonifier 450, Unisonic, Australia) for 3 min and adding 1 mL dH_20. Samples were centrifuged (Heraeus Multifuge 3SR, Thermo Scientific, Australia) at 3000 rpm for 10 min. Chlorophyll a concentration (μg/mL) was determined using Spectrophotometer (Cary 50 Bio UV-Visible Spectrophotometer) with the wavelength of 630, 647, and 750. The relationship of the Chl *a* amount in supernatant with those wavelength was correlated according to Jeffrey and Humphrey (1975): Chlorophyll $a=11.85E_{664}–1.54E_{647}–0.08E_{630}$.

2.6 Lipid analysis

2.6.1 Nile Red Fluorescence assay

A fluorescence spectrometric method was applied for fast determination of lipid content using a Hitachi F-2000 Fluorometer. Measurements were recorded daily. In the method, the microalgal cells were stained with Nile Red (Sigma, St.Louis, MO, USA) followed the protocol reported by Elsey et al (2007). In brief, 3 mL of culture was pipetted into a glass cuvette. A fluorescence measurement as a standard culture was noted. Excitation wavelength was set at 486 nm and the emission wavelength was 570 nm. Fluorescence intensity was recorded over a period of 30 seconds and the result of fluorescence intensity reading vs time was displayed as a graph using a Toshiba Satellite 4080XCDT laptop computer with a software program of Logger Pro. The average of fluorescence intensity

was also noted. A 15μL aliquot of 7.8 μM Nile Red dissolved in acetone was added. A second fluorescence of the stained samples were measured for 600 seconds or until a peak fluorescence reading was indicated.

2.6.2 FTIR spectroscopy

For FTIR spectroscopy a 50 mL sample was taken from each replicate flask for each treatment in the end of cell cultivation, mixed and centrifuged at 3000 rpm for 10 mins. The supernatant removed and the cells re-suspended in approximately 100 μL of distilled water, 30 μL of which was then deposited on CFR Low-e microscope slide. The samples were then desiccated under vacuum for overnight. The dried cellular deposit on the CFR Low-e microscope slide was placed on the FTIR microscope stage for spectral acquisition (Chen et al., 2010; Giordano et al., 2001; Wagner et al., 2010). FTIR spectra collected using FTIR spectrometer (Varian 7000, FTIR Stingray Imaging Series) coupled to an infrared microscope (Varian 6000 UMA FTIR microscope) and equipped with a mercury-cadmium-telluride detector cooled with liquid N_2. Spectra were collected over the wavenumber range 4000 – 700 cm^{-1}. The Varian FTIR system was controlled by a Window-compatible PC running Spectra Pro software.

2.6.3 Gravimetric determination of neutral lipids

To determine the total content of lipids in cells, 50 mL of cells were harvested by centrifugation (4000 g for 20 min, Heraues Multifuge 3SR) in the end of cell cultivation. Lipid were extracted by adding 28 mL of 50 mM phosphate buffer (pH 7.4) into the cell pellets, resuspended and sonicated for 1 min. The samples were transferred to a separatory funnel, added 35 mL chloroform and 70 mL methanol and shaked rigorously and leaved for 18 hours. After 18 hours has passed, the samples were added by 35 mL chloroform and 35 mL distilled water, respectively. Allowed the sample to stand for a further 18 hours and collect the lower chloroform layer in a round bottom flask which having previously recorded the weight of the empty flask. The extract was evaporated in a water bath (50℃) using a rotary evaporator (Buchi, Switzerland) to remove solvents and weight the flask for determining lipid content. The lipid extraction followed the protocol reported by Guckert et al (1988); Wagner et al (2010) and Lee et al (1998).

2.7 Statistical analysis

Mean comparison were conducted by one-way analysis of variance (ANOVA), followed by LSD test to determine significance. In all cases, comparisons that showed a p value <0.05 were considered significant.

3 Results and Discussion

3.1 Impact of nutrient depletion and temperature stress on algae biomass

The impact of nutrient depletion and temperature stress was examined on three biomass indicators, cell density, relative growth rate, chlorophyll *a* concentration and dry weight. *Dunaliella tertiolecta, Nannochloropsis* sp. and *Scenedesmus* sp. were grown in batch culture under three nutrient conditions: nutrient replete, no nitrate and no phosphate and two temperature conditions: 18℃ and 25℃.

Figure 1, 2 and 3 illustrates the effect of nutrient and temperature cultivation conditions on the (a) cell growth and (b) Chl *a* content of three microalgae. The growth behavior of *Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp. under different nutrient and temperature conditions showed a varied trend (Figure 1). Cell growth in terms of cell density was higher at control treatment and normal temperature than under nutrient depletion and temperature stress. The peak of cell density all microalgals cultured were occurred at day 6 or day 7. The effect of temperature on cell density of microalgae varied within species. Cell density was higher at ambient temperature for marine (*Dunaliella tertiolecta* and *Nannochloropsis* sp.) and freshwater species (*Scenedesmus* sp.), such as 18℃ and 25℃, respectively. *Dunaliella tertiolecta* showed a higher cell density at 18℃ than 25℃. Eighteen degree of temperature for *Dunaliella tertiolecta* showed an optimum temperature for cell growth. *Dunaliella tertiolecta* has a lower temperature for optimum growth than others species (*Dunaliella salina* and *D. viridis*), such as 22℃ and 26℃, respectively (Garcia et al., 2007). Like others species of Dunaliella, *D. tertiolecta* showed a positive response on cell growth in terms of cell density to increase of temperature. Figure 1(a) showed that there was a significant decrease in cell density of *D. tertiolecta* to increase temperature. This result was supported by Garcia et al (2007) who found that growth of *D. salina* and *D. viridis* decreased significantly with increasing temperature.

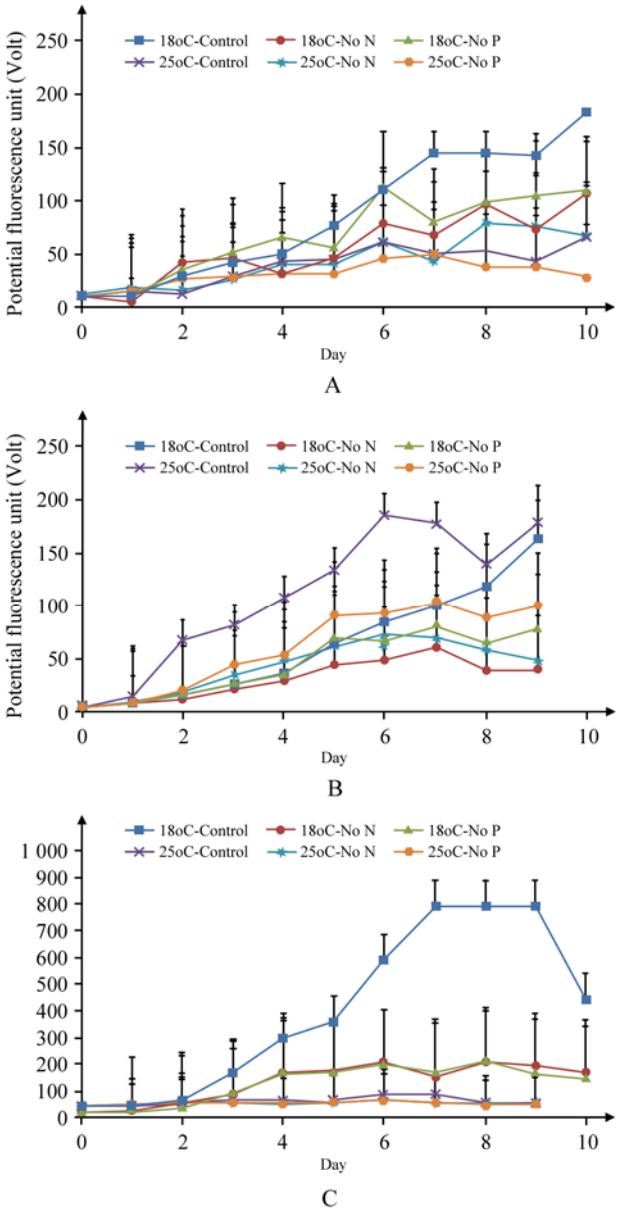

Figure 1 Cell growth in response to varying nutrient and temperature condition. Cell count of *Dunaliella tertiolecta* (A), *Scenedesmus* sp. (B) and *Nannochloropsis* sp. (C)

The response of *Dunaliella tertiolecta* to nutrient limitation on cell growth was also investigated. The growth curve in terms of cell density of three microalgal cultured for different nutrient condition are shown at Figure 1. Reasonably, the maximum algal density increased with deplete nitrogen and phosphorus condition. Range of cell density of *Dunaliella tertiolecta* were 7.3×10^4 to 182×10^4, 5.7×10^4 to 106.7×10^4 and 10.3×10^4 to 109.7×10^4 cell/mL for replete, −N and −P culture medium, respectively (Figure 1A). Similar with temperature stress condition, the highest growth of cell was occurred at replete nutrient condition for both temperature treatment. The highest number of cell account for 182×10^4 cell/mL at replete nutrient treatment with the temperature cultured was 18℃ at day 10 of cultivation. Statistically, there was a significant different of cell density over cultivation period between nutrient treatments ($p<0.05$).

The growth curves of *Scenedesmus* sp. in the growth medium for different cultivation temperature and nutrient condition are shown in Figure 1 (B). After 9 days of cultivation *Scenedesmus* sp. grew into the decreasing phase and reached a maximum cell density at day 6 of cultivation for all temperature and nutrient treatments. The study found that the optimal temperature for *Scenedesmus* sp. growth was 25℃. Through Paired-samples t-test, microalgal densities in the cultivation process at the temperature of 25℃ was significantly higher than at 18℃ ($p < 0.001$). At 18℃ *Scenedesmus* sp. grew very slowly at the beginning till the end of cultivation. The peak of cell density at low temperature was one day later than higher temperature. The result was supported by previous study by Xin et al (2010) who found that at 25℃ *Scenedesmus* sp. LX1 got the maximum specific growth rate and at 30℃ it had the maximum carrying capacity K and population growth rate Rmax. Cell density of *Scenedesmus* sp. was lower at deplete nutrient condition than replete nutrient condition not only at 18℃ but also at 25℃.

Nannochloropsis sp. growth appeared to be affected at temperatures above 18℃ (Figure 1C). At 25℃, this microalgae exhibited a very low and almost no grow in terms of cell density, this temperature led to an abrupt interruption of microalgal growth and later the cell dead on further period of cultivation. Previous studies by Sayegh and Montagnes (2010); James et al (1989) and Brown and Jeffrey (1992) found that *Nannochloropsis* sp. grown optimally at temperature around 20℃.

In nutrient deplete, exponential phase of *Nannochloropsis* sp. are similar to those in the literature, under comparable conditions (Figure 1C). *Nannochloropsis* sp have maximum cell growth in terms of cell density of 792×10^4 cell/mL at day 7 of cultivation.

The effect of temperature on microalgal growth was species-dependent. Previous studies by Converti et al

(2009); Chen et al (2008) and Westerhoff et al (2010) found that the optimal cultivation temperature for the specific growth rate of *Nannochloropsis oculata* and *Nitzschia laevis*, was 20℃ and 23℃, respectively. In Westerhoff et al.'s study showed that the exponential growth rate constant (l = 0.03 h1) did not vary between 27 ℃ and 39 ℃ of *Scenedesmus* and *Chlorella*, but at 42℃ the microalgae could not grow.

Chlorophyll *a* concentration of three microalgal cultured showed a similar pattern in respond to temperature stress and nutrient limitation (Figure 2). Three microalgal cultured exhibited two times higher chl *a* concentration. Chl *a* concentration in *Dunaliella tertiolecta* ranged from 8.56×10^{-7} to 7.67×10^{-6} pg cell^{-1} and decreased with time at both temperature and nutrient conditions (Figure 2A). No significant different of chl *a* concentration in relation to culture time (0, 2, 4, 6, 8 and 10 day) for the temperature and nutrient condition ($p<0.05$). Chl *a* concentration expressed per cell at the end the cultivation period ranged from 8.15×10^{-7} to 1.68×10^{-6} pg cell^{-1}. Chl *a* concentration at *Dunaliella tertiolecta* in this experiment was lower than other *Dunaliella* species, such as *Dunaliella salina* and *D. viridis* that reported by Garcia et al (2007). Photosynthetic limit to temperature was affected by different in size and morphological feature between these species (Coles and John, 2000). Temperature cultivation condition showed a slightly affect to chl a concentration at *Dunaliella tertiolecta*. This result supported by previous study of Garcia et al (2007) at different Dunaliella species. However, nutrient depletion showed a significant effect to chl a concentration of *Dunaliella tertiolecta.*

Chl *a* concentration trend over cultivation period of *Scenedesmus* sp. was similar with *Dunaliella tertiolecta* (Figure 2B). There was two peak of chl *a* per cell, which were initial and day 4 of cultivation. Chl a concentration in *Scenedesmus* sp. ranged from 4.55×10^{-9} to 2.76×10^{-6} pg cell^{-1}. Chl *a* concentration at 25℃ temperature cultivation and replete nutrient condition was higher than at 18℃. Statistically, there was a significant different of chl *a* concentration between 25℃ and 18℃ and between nutrient treatment ($p<0.05$).

Chl *a* concentration of *Nannochloropsis* sp. over cultivation period was showed at Figure 2C. Peak of

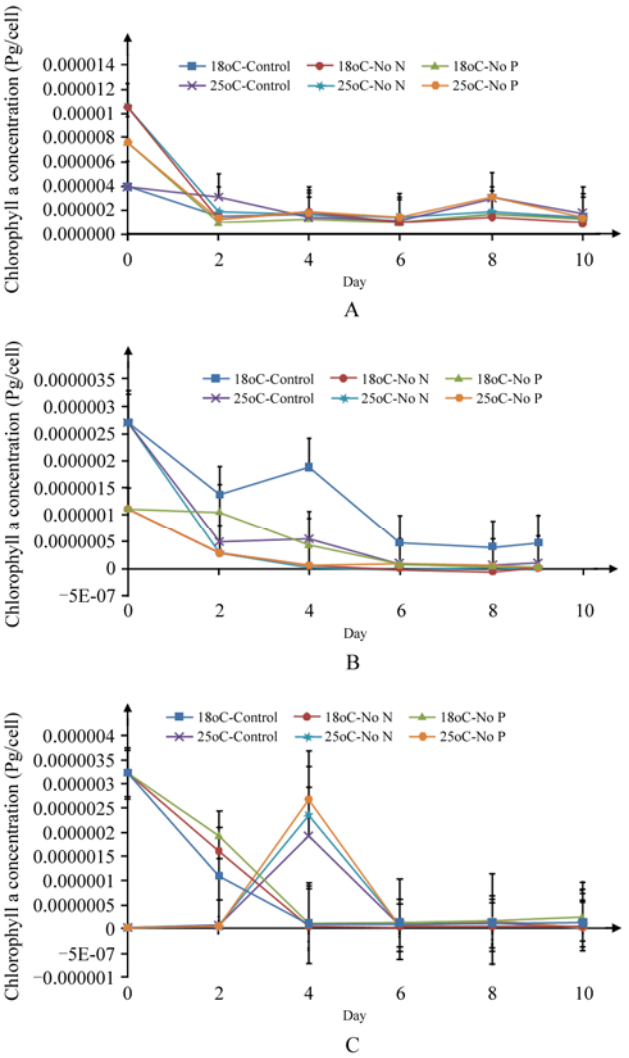

Figure 2 Chlorophyll *a* concentration per cell in response to varying nutrient and temperature condition. Chl *a* per cell of (A) *Dunaliella tertiolecta*, (B) *Scenedesmus* sp. and (C) *Nannochloropsis* sp.

chl a concentration per cell was occurred at initial and day 4 of cultivation for 18℃ and 25℃, respectively. The range of chl *a* concentration was 4.94×10^{-9} to 3.24×10^{-6} pg cell^{-1}. There was a significant different of chl *a* concentration at day 4 of cultivation between 18℃ and 25℃ ($p<0.05$). The result of chl *a* concentration in *Nannochloropsis* sp. was consistent with previous study by Sukenik et al (1993) that showed the highest chlorophyll *a* content was recorded in cultures grown at 25℃, whereas the lowest value was measured in cultures grown at 18℃.

3.2 Effect of temperature and nutrient depletion on the lipid content

The cell growth and lipid accumulation were closely

related with cultivation condition including temperature, nutrient limitation, CO_2 concentration and light intensity (Lv et al., 2010). In this study we also investigated the effect of different temperature and nutrient conditions of media cultured on lipid content using three different methods of lipid analysis, such as Nile Red Staining, Gravimetric and FTIR spectroscopy.

3.2.1 Lipid analysis by Nile Red Fluorescence

Lipid content of three selected microalgae was measured daily using Nile Red fluorescence method (Figure 3). Figure 3 showed the total neutral lipid content, as measured by Nile Red fluorescence, of cultured subjected to these conditions. As shown, cell

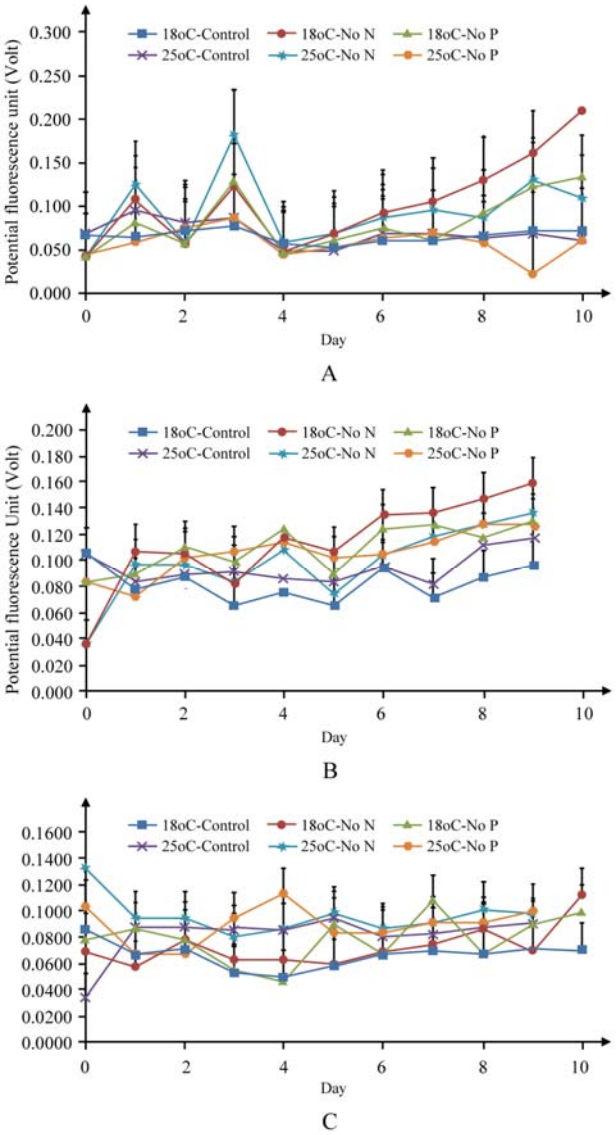

Figure 3 Lipid concentration based on Nile Red Fluorescence reading in response to varying nutrient and temperature condition. Nile Red Fluorescence reading of (A) *Dunaliella tertiolecta*, (B) *Scenedesmus* sp. and (C) *Nannochloropsis* sp.

cultured in -N medium cultured at 18℃ and 25℃ temperature cultivation condition contained a higher amount of neutral lipid than in control and –P medium for all algal cultured. The largest amount of neutral lipid was recorded at day 9 and day 10 of cultivation for *Scenedemus* sp., *Dunaliella tertiolecta* and *Nannochloropsis* sp., respectively. The pattern of neutral lipid content based on Nile Red fluorescence reading of three selected microalgae were similar in response to different temperature and nutrient conditions. However, *Dunaliella tertiolecta* showed the highest amount of neutral lipid for −N medium treatment than *Scenedesmus* sp. and *Nannochloropsis* sp. account for 0.210 potential fluorescence unit. Using our Nile Red method we found that the cells grown under nitrogen depletion condition exhibited 3 times, 2 times and 1.5 times more fluorescence than cells grown under nitrogen-sufficient conditions for *Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp., respectively. The fluorescence reading in this study was lower than previous study by Elsey et al (2007) which recorded 6.7 times more fluorescence of cell grown under nitrogen deficient condition than cells grown under nitrogen-sufficient conditions. Low Nile Red fluorescence reading for neutral lipid content in this study probably due to no modification conducted to Nile Red assay. Elsey et al (2007) reported that to improve ability of screening for neutral lipids, either across multiple microalgal strains grown under identical conditions, or across varying growth conditions through conducting modification to the Nile Red assay.

3.2.2 Lipid analysis by Gravimetric method

Figure 4 showed the relative neutral lipid content, as measured by gravimetric method for different temperature and nutrient conditions of three selected microalgae. Lipid content was higher at −N medium cultured than −P and sufficient nutrient treatments. Culture with −P medium behaved similarly to culture in −N medium at *Scenedesmus* sp. and *Nannochloropsis* sp. The result also indicated that temperature stress increased lipid content for *Dunaliella tertiolecta* and *Scenedesmus* sp. (Figure 4A and 4B). Statistically, there was a significant different of lipid content at –N medium cultured between 18℃ and 25℃ ($p<0.05$). *Dunaliella tertiolecta* showed a higher lipid content at –N medium cultured than *Scenedesmus* sp. and *Nannochloropsis* sp. account for 0.352 g/L. Lipid accumulation under this condition appear as a response

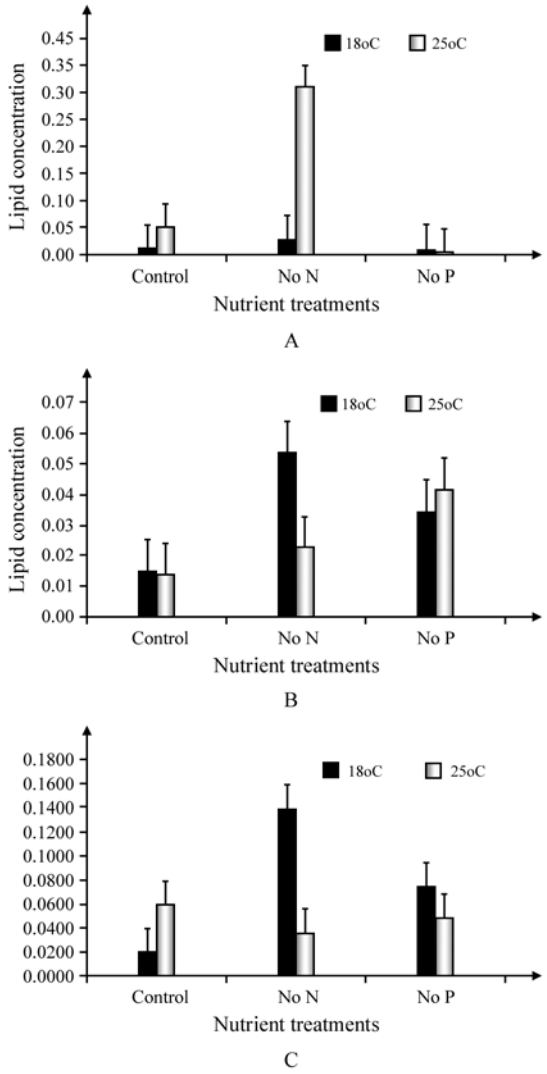

Figure 4 Lipid concentration based on Gravimetric method in response to varying nutrient and temperature condition. Gravimetric lipid reading of (A) *Dunaliella tertiolecta*, (B) *Scenedesmus* sp. and (C) *Nannochloropsis* sp.

to the depletion of nutrients from the medium. The result is confirmed by previous report by Chen et al (2011) who recorded that cells incubated in nitrogen-deficient medium accumulated substantial amounts of lipid by day 3, but this had declined by day 7. Rossler (1988) reported that the nutrient deficiency induced an increase in the rate of lipid synthesis in a diatom, *Cyclotella cryptica*, and resulted in lipid accumulation in the cells.

3.2.3 Lipid detection by FTIR spectroscopy

FTIR spectroscopy can be used to monitor the biochemical content of phytoplankton cells in very short time intervals of less than one hour (Wagner et al., 2010). In this study, FTIR spectroscopy was used to determine relative neutral lipid content in three selected microalgae (*Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp.) (Figure 5).

Neutral lipid content was measured using this method for the end of algal cultivation period, such as day 9 and day 10 for *Scenedesmus* sp. and *Dunaliella tertiolecta* and *Nannochloropsis* sp., respectively. At FTIR spectroscopy, the bands were assigned to specific molecular groups on the basis of biochemical standards and published studies, as described previously (Stehfest et al., 2005). Bands were attributed to υ(C=O) stretching of amides from proteins (amide I,~1655 cm^{-1}); δ(N–H) bending for amides from proteins (amide II, ~1545 cm^{-1}); $\delta_{as}(CH_2)$ and $\delta_{as}(CH_3)$ bending of methyl from protein (~1455 cm^{-1}); and $\delta_s(CH_2)$ and $\delta_s(CH_3)$ bending of methyl and υ_s (C–O) stretching of COO-groups (~1380 cm^{-1}) and υ_{as} (>P=O) stretching, associated with phosphorus compounds (~1260 cm^{-1}). Two bands were of particular interst, the band at 1740 cm^{-1} which was associated with υ(C=O) of ester groups, primarily from lipid and fatty acids and the region from 950~1200 cm^{-1} associated with υ(C–O–C) stretching of poly-saccharides.

Relative lipid content was determined by calculating the ratio of the lipid (1740 cm^{-1}) band to the protein band (amide I) (Figure 5). The range of lipid:protein ration for all microalgal culture from 0.67 to 3.77. The highest lipid:protein ration was recorded at *Scenedesmus* sp. for −N nutrient treatment at 25℃ cultivation temperature account for 3.78. Interestingly, *Dunaliella tertiolecta* showed the highest lipid:protein ratio at replete nutrient treatment for 18℃ cultivation temperature. However, there was no significant different of lipid:protein ratio between temperature condition for all nutrient treatments except for *Scenedesmus* sp. This result indicated that *Scenedesmus* sp. had higher lipid production at N limitation condition than others two species (*Dunaliella tertiolecta* and *Nannochloropsis* sp.) based on FTIR spectroscopy reading. Recent study of *Scenedesmus subspicatus* found lipid production reduced markedly in the low-N culture by over 50% compare to high −N culture (Dean et al., 2010). These experiments indicated that optimization of nutrient limitation conditions rather than complete starvation offers the potential to increase lipid productivity.

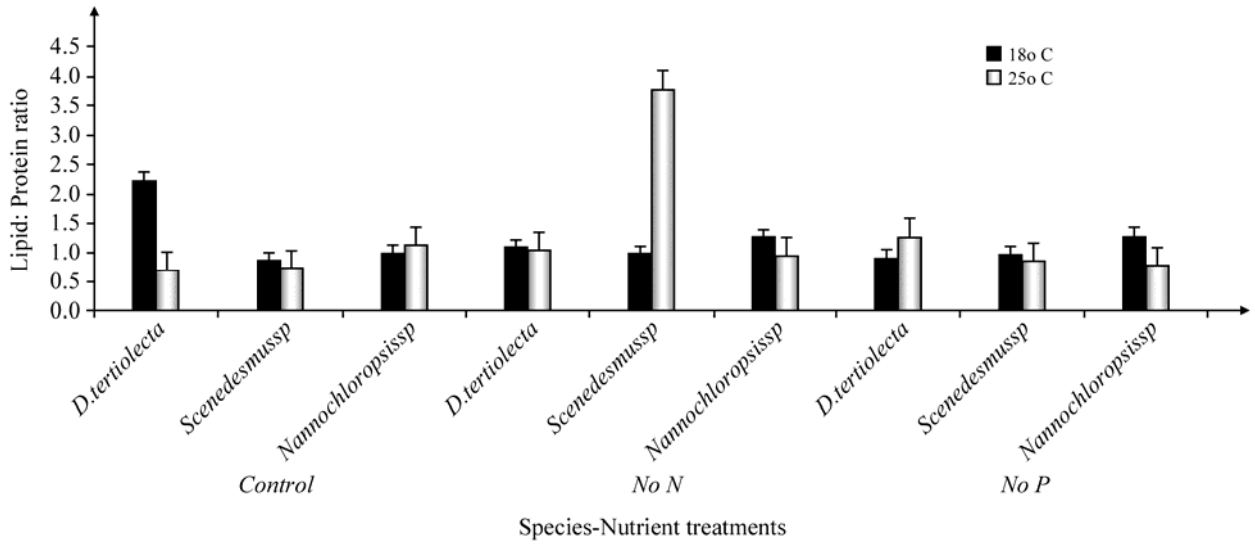

Figure 5 Changes in lipid:protein ratio within cell of *Dunaliella tertiolecta, Scenedesmus sp* and *Nannochloropsis sp* in response to varying temperature and nutrient conditions

Note: For data sets, each point is the mean (±SE) of three FTIR spectra from pooled samples (derived from three replicate flasks). Each of the spectra was separately derived from randomly selected small groups of cells within the vacuum-dried preparation

4 Conclusions

The variation of parameters tested (temperature and nutrient limitation) strongly influenced the lipid content of microalgae. All the stress condition investigated led not only to the accumulation of lipids, but also to a reduction in microalgae growth, thereby affecting the lipids productivity.

This study also has shown that *Dunaliella tertiolecta, Scenedesmus* sp. and *Nannochloropsis* sp. can respond and distinctive changes in growth and lipid content with temperature and nutrient stress conditions. These study also demonstrated that Nile Red staining and FTIR analysis can effectively identify change in lipid content in algal cells in response to temperature and nutrient treatments. This demonstrated that FTIR and Nile Red will be efficient tool for rapidly monitoring lipid accumulation of microalgae and will have applications for algal biofuel production processes.

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