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Enhancement of some culture conditions for optimizing growth and lipid production in the diatom *Nitzschia palea*

Lawrence Victor D. Vitug*[3] *& Susana F. Baldia*[1,2,3]

[1]Department of Biological Sciences, College of Science;
[2]Research Center for the Natural and Applied Sciences; [3]Graduate School
University of Santo Tomas
España Boulevard, 1015 Manila, PHILIPPINES

Diatoms are unicellular and colonial microalgae which are currently explored as one of the potential algal species for biofuel production since they are known to accumulate high lipid content under different environmental stresses. This paper focuses on the diatom *Nitzschia palea* subjected under various culture conditions of light intensity, temperature, and pH, using batch culture experiments for optimizing their growth, lipid content and lipid productivity. *Nitzschia palea* attained a maximum growth of 3.08×10^6 cells/mL under the optimum conditions of 18 mmol photons $m^{-2}\,s^{-1}$ light intensity, temperature of 30°C, and pH 9.

The highest lipid content (1.23 mg mL^{-1}) was attained at 30°C. Lipid productivity increases with increased light intensity, temperature and pH. This was positively correlated with both lipid content and culture conditions (p <0.05), while growth of *N. palea* was negatively correlated with lipid content (p <0.05). *Nitzschia palea* had shown a relatively high growth rate and lipid content which are important characteristics for consideration as a potential for biofuel resource.

Keywords: *Nitzschia palea*, culture conditions, biofuel resource, lipid content, lipid productivity

INTRODUCTION

Microalgae are one of the potential alternative energy resources. These organisms are currently being explored for biofuel production because of the advantages of higher photosynthetic efficiency, higher biomass production and faster growth [1]. Some algal species are exceedingly rich in oil which can reach 15–70% of the dry weight of their algal biomass [2].

Among these microalgae, diatoms have been identified as strong candidates for significant biodiesel production because they produce up to 60% of their cellular mass as triacylglycerols (TAG) which can easily be converted into biodiesel through transesterification reaction [3].

**To whom correspondence should be addressed*
sfbaldia@yahoo.com

They contain polymerized silica in their cell walls. Moreover, diatoms store carbon in the form of natural oils or as a polymer of carbohydrates known as chrysolaminarin. *Nitzschia palea* is a freshwater diatom that was found to exhibit high lipid production according to the results of red nile fluorescence in Aquatic Species Program [4].

Environmental conditions affect growth and lipid production of algae which may vary in different algal species. Light intensity influences the cell growth and lipid accumulation of microalgae [5]. With increasing light intensity, photoinhibition occurred during the cultivation of some algal strains [6]. Hu *et al*. [7] observed that by further increasing light to 800 μmol photons m^{-2} s^{-1}, a decline in cell density of the *Pseudochlorococcum* culture occurred. In contrast to the study made by Lv [8], the specific growth rate of 0.04 h^{-1} and lipid content of 44–47% were achieved for *Skeletonema costatum* under 50 and 100 μmol photons m^{-2} s^{-1}, whereas 0.01 h^{-1} and 35–40% were obtained under 20 or 400 μmol photons m^{-2} s^{-1} due to light limitation or inhibition. Furthermore, during dark incubations, total fatty acid yields decreased steadily in both *Isochrysis* (with constant cell density) and *Gymnodinium* (with declining cell density) cultures but total neutral lipid yields decrease only in *Gymnodinium* culture [9].

Most commonly cultured microalgae can tolerate temperatures between 16°C and 27°C. Temperature lower than 16°C will slow down growth, whereas those higher than 35°C are lethal to some species. Optimal growth temperatures of 15–26.8°C have been reported for some species, with maximum cell densities obtained at 23.8°C [10]. In the work of Cho *et al.* [11], temperatures ranging from 15–30°C, has been found to be optimal for growth of the green alga *Chlorella ellipsoidea* and a 25°C temperature range promoted the highest growth rate of the algal species. Further increasing the culture temperature caused a serious inhibition of both the cell growth and fatty acid biosynthesis [12]. Under all temperatures tested, TAGS was the predominant lipid constituent (64.5–69.1% of total lipid) and was highly saturated. Lower temperature favored the formation of polar lipids. The highest content of phosphatidylcholine (PC), the major phospholipids component, was reached at 15°C (10.9% of total lipid). Similarly, the lipid production of *Nannochloropsis oculata* and *Chlorella vulgaris* was strongly influenced by variation in temperature [13]. An increase in temperature from 20°C to 25°C practically doubled the lipid content of *N. oculata* (7.9–14.92%), while an increase from 25°C to 30°C brought about a decrease of the lipid content of *C. vulgaris* from 14.71% to 5.9%.

Among the environmental factors, pH plays a significant role in the biomass production of microalgae. The change in environmental factors such as phosphate and pH strongly affects biomass production due to their physiological requirement. Although pH regimes not only influence dissociation and chemistry of media, they also affect physiology of cell and biomass production [14]. For cultures of microalgal species, the pH regime ranges between 7 and 8, while its optimal is 8.2–8.7 [15]. The pH regimes can be modified to a certain extent during algal cultivation in order to increase its biomass production. Most microalgal species are favored by neutral pH, whereas some species are tolerant to higher pH (e.g. *Spirulina platensis* at pH 9) or lower pH (e.g. *Chlorococcum littorale* at pH 4) [10]. This increase in pH can be beneficial for inactivation of pathogens in microalgal wastewater treatment, but can also inhibit microalgal growth.

For *Chlorella* spp., alkaline pH stress resulted in TAGS accumulation and a decrease in membrane lipid classes (and presumably, membranes). The fatty acids in polar lipids in the unidentified *Chlamydomonas* sp. were more saturated than those of *C. reinhardtii*. The

relative percentage of TAG in the total lipid content in *Chlamydomonas* spp. grown in medium at pH 1 was higher than that in cells grown at higher pH. It was suggested, that the increase in saturation of fatty acids in membrane lipids of *Chlamydomonas* may represent a possible adaptation mechanism for low pH in order to decrease membrane lipid fluidity [16].

The present study focuses on the effects of the different culture conditions on growth, lipid content and lipid production, of the diatom *N. palea*. The research offers to diversify the biofuel resources as an alternative source of energy. In addition, the study is envisioned to contribute further research and development to the sustainability of algae based biofuel.

Experimental

Acquisition of stock cultures. *Nitzschia palea* which was originally isolated from Laguna de Bay, was acquired from the Culture Collection of the Research Center for the Natural and Applied Sciences of the Thomas Aquinas Research Complex. This culture was grown and maintained in BRSP medium [17] and continuously renewed every 12–14 days throughout the experiment. The algal cultures were incubated in culture shelves under cool white fluorescent lamps with a light intensity of 9 μmol photons m^{-2} s^{-1} at a temperature of 23±2°C.

***Media preparation and maintenance of* Nitzschia.** Inorganic medium (BRSP medium) with the following composition in g L^{-1} was used as culture medium for *N. palea* [17]: 0.1258 g $CaNO_3$, 0.0654 g $MgCl_2$, 0.0450 g, $MgSO_4.7H_2O$, 0.0191 g KCl, 0.0812 g NaCl, 0.0229 g Na_2SiO_3, 0.2573 g $NaNO_3$, 0.1861 g Na_2HPO_4, 0.0003 g $FeCl_2$ and with trace elements (mg 200 mL^{-1}) of 400 mg H_3B0_3, 300 mg $MnCl_2$. H_2O, 40 mg $ZnSO_4.7H_2O$, 20 mg $CaCl.5H_2O$, and 2 mg $NaMoO_4$. The pH of the medium was adjusted to 7.4 and sterilized at 15 lbs psi at 121°C for 15 min. The volumes of the said medium depended on the amount of cultures necessary for the up-scaling procedure. All cultures were maintained in lighted culture shelves 9 μmol photons m^{-2} s^{-1} at 23±2C.

Up-scaling of cultures. About 10 pieces of 16×125 mm test tubes which contained the stock cultures were transferred regularly every 12 days and up-scaled to three 125 mL flasks which were later on transferred to 250 mL flasks after 1 to 2 weeks. Cultures from 500 mL flasks were used as inocula for the experiment.

Experiments on different culture conditions

Variations in light intensity. A batch culture experiment using *N. palea* was conducted in 250 mL Erlenmeyer flasks containing the BRSP medium. *Nitzschia* was exposed to three different light intensities: 9, 18, and 27 μmol photons m^{-2} s^{-1}. The experiment was conducted in continuous LD cycle at 23±2°C.

Temperature variations. Using the best light intensity, *Nitzschia* was grown in batch culture at three different temperatures:18°C, 23°C, and 30°C. The experiment was done in 250 mL Erlenmeyer flasks containing the BRSP medium. For the 15°C and 23°C system, an air conditioner was used to maintain the temperature. For the 30°C, the cultures were subjected to an enclosed non-airconditioned room. All experimental set-ups were provided with 18 mmol photons m^{-2} s^{-1} light intensity and the culture conditions were maintained.

Variations in pH. *Nitzschia palea* were grown at different pH levels such as 6, 7, 8, and 9 using the best light intensity and temperature. The experiment was done in 250 mL Erlenmeyer flasks containing the BRSP medium. For the 30°C experimental set-up, the cultures were positioned to an enclosed non-airconditioned room. The culture medium was adjusted by using a 12 M NaOH and 6 M HCl and checked with a water

quality instrument (PASCO GLX Explorer) for the correct pH. The experimental set-ups were maintained at 18 μmol photons m^{-2} s^{-1} in continuous LD cycle at 30°C

Data measurements. Growth in terms of cell density was monitored every 2 days taking about 1 mL algal sample from each replicate of 250 mL Erlenmeyer flasks. Few drops of Lugol's solution was mixed to the 1 mL sample and shaken so that the cells were allowed to sink and settle. Samples were counted using a haemacytometer [18] and a compound microscope.

For measurement of dry weight, about 10 mL aliquot from *Nitzschia* cultures were taken from three replicates of each batch experiment. Samples were obtained during the exponential growth phase, and were filtered in glass fiber filters (Whattman, GF/C 47 mm) by vacuum filtration, washing twice with distilled water to remove nonbiological materials [19]. These were dried at 80°C until constant weight has been achieved. Likewise, the biomass was harvested during this time in which all replicates of each various culture conditions were pooled together. Pooled cultures were subjected to natural sedimentation and stored in one liter beaker in a refrigerator overnight. Algal cultures were harvested using a micro fiber glass filter and the filtrate was further collected through vacuum filtration. Algae were placed in a 47 mm micro fiber filter papers (Whatman GF/C) and kept in the freezer for lipid analysis. This method involved the extraction of lipid from the frozen biomass of *N. palea* using hexane. Ten (10) mL of hexane was added to each sample in centrifuge bottles. After standing the algae overnight, samples were subjected to rotary evaporation for the determination of lipid content at 49°C. Samples were placed in a pre-weighed scintillation vials.

Data analysis. Cell density was computed following the paper of Martinez *et al*. [18]. To evaluate the dried algal biomass at various culture conditions, the formula used by Shen [20] was followed. Lipid content and lipid productivity were assessed based on the method described by Wang *et al.* [21].

All statistical analyses were calculated using the Paleontological Statistics Software [22, 23] and Statistical Package for Social Science 17.0. Past Software was used to analyse the normality test (Shapiro Wilks Test), one-way analysis of variance, Kruskal-Wallis and Levenes. Pearson *r* Correlation Coefficient were calculated using SPSS 17.0 Software.

One-way ANOVA was used to compare the means of growth, dry weight, lipid content, lipid productivity, and culture conditions of *N. palea*. Shapiro Wilks was used for normality test while Levenes test was for the analysis of homogeneity. Pearson correlation coefficient (*r*) was used to find out the relation between culture conditions, growth, dry weight, lipid content and lipid productivity of *N. palea*.

Results and Discussion

Culture growth. The diatom *N. palea* achieved its optimum growth at 18 μmol photons m^{-2} s^{-1} (Fig. 1) showing its exponential phase on the 7th day with a maximum growth of 1.93×10^6 cells mL^{-1}. While at 9 and 27 μmol photons m^{-2} s^{-1}

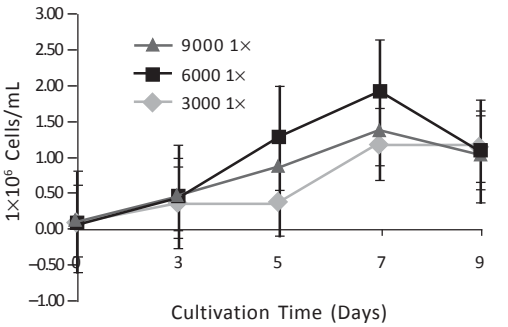

Figure 1. Growth of *N. palea* in various light intensities: 9 μmol photons m^{-2} s^{-1} (3000 l×), 18 μmol photons m^{-2} s^{-1} (6000 l×), and 27 μmol photons m^{-2} s^{-1} (9000 l×)

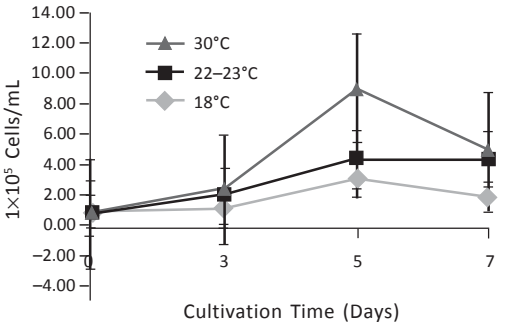

Figure 2. Growth of *N. palea* in various temperatures

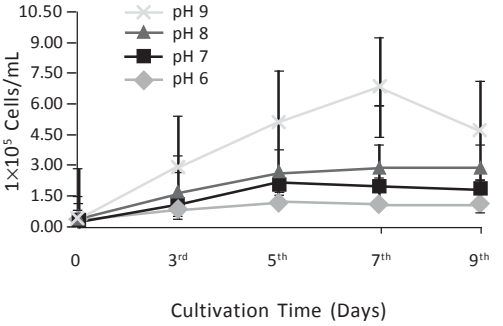

Figure 3. Growth of *N. palea* in various pH concentrations

light intensities, a maximum growth of 1.18×10^6 and 1.39×10^6 cells mL^{-1} was achieved, respectively. It was interesting to note that at both 18 and 27 μmol photons $m^{-2}\,s^{-1}$, there had been an increasing trend on the 3rd day until it reached its exponential phase on the 7th day. All cultures incubated at three light intensities had reached their exponential phase on the 7th day at the same time. At higher light intensities, the cultures of *N. palea* rapidly declined on the 9th day which may be due to photo inhibition. While at low light intensity, the growth of *N. palea* was constant after the exponential phase on the 9th day. The growth of *P. brevistriata* was significantly affected by light intensity ($p<0.05$).

Among the environmental factors, light has a marked effect on the lipid production and fatty acid composition in algae [24–30]. Generally, low light intensity favors the formation of polar lipids such as the membrane lipids associated with the chloroplast, whereas high light intensity benefits the accumulation of neutral storage lipids in particular TAGS.

Studies regarding the effects of light on lipid synthesis in microalgae have been restricted to different light conditions, showing that various light intensities are associated with alterations in cellular structures relevant for lipid synthesis. Several researches demonstrated that high light conditions could alter the lipid storage strategies of several algal species [26, 31], but also low light conditions had an effect on algal lipids [27]. Nevertheless, in these studies, the availability of light was considered to be homogeneous and was only manipulated regarding intensity.

Nitzschia palea favors a temperature of 30°C with a maximum growth of 9.03×10^5 cells mL^{-1} rather than 18°C and 22–23°C with maximum growth of 3.15×10^5 and 5.49×10^5 cells mL^{-1}, respectively (Fig. 2). The growth at the three temperatures used in this experiment showed similar increasing trend during the exponential phase on the 5th day. At 18°C and 30°C, maximum growth occurred on the 5th day, however the growth rapidly decline on the 7th day. While with the temperature of 22–23°C, the growth of the *N. palea* was constant after achieving its exponential phase on the 5th day. Moreover, the growth of *N. palea* was significantly affected by temperature ($p<0.05$).

The results had demonstrated that diatoms especially *N. palea* are capable of adapting at higher temperatures, and did not cause inhibition unlike the results obtained by Chen *et al.* [12] in which increasing the temperature to 27°C causes an inhibition of cell growth of *Nitzschia laevis,* as revealed by its low maximum biomass concentration. The result was congruent to the study conducted by Chaffin *et al.* [32] which suggested that *Fragilaria capucina,* a diatom

species similar to *N. palea* is a eurythermal genus which is capable of surviving in a wide range of temperatures.

The growth of *N. palea* was significantly affected by pH (p <0.05). The diatom *N. palea* had an optimum growth at pH 9 with a maximum cell density of 6.9×10^5 cells mL^{-1} during its exponential phase which occurred on the 7th day of the culture period (Fig. 3). At both pH 9 and pH 8, the growth of the diatom reached its exponential phase on the 7th day as well. The growth of *N. palea* at pH 6 and pH 7 reached an earlier exponential phase on the 5th day with a maximum growth of 1.24×10^5 cells mL^{-1} and 2.21×10^5 cells mL^{-1}, respectively.

Variation in pH can affect algal growth in a number of ways. It can change the distribution of carbon dioxide and carbon availability, alter the availability of trace metals, and essential nutrients, and at extreme pH levels; potentially cause direct physiological effects.

Our results showed that *N. palea* can tolerate a wide range of pH, which coincides with the observation of Barinova [33] in her own work for *Fragilaria vaucheriae*, a similar diatom species which is considered to be an alkaliphile and can tolerate high levels of pH. In the study done by Luszczek [34], it was found out that a species also of the same taxon, *Fragilaria capucina*, can tolerate low level of pH which can be considered as an acidophile. This means that distinct species of diatoms varies and depended among various pH ranges. Although *N. palea* did not grow well at a lower pH in the present study, it has been observed that diatom silicification occurs at a low pH or acidic condition to form their frustules and uptake of silicic acid [35].

The taxon of *Nitzschia* can tolerate both acidic and alkaline conditions based on the specific taxon. In this case, *N. palea* can be considered an alkaliphile since it can tolerate high level of pH.

Lipid content and dry weight. Both *N. palea* grown in 18 μmol photons m^{-2} s^{-1} and 27 mmol photons m^{-2} s^{-1} had relatively close dry weight values, while those grown in 9 mmol photons m^{-2} s^{-1} had the lowest dry weight (Fig.4). When it comes to lipid content, similar results were observed, wherein those cultured in both 18 mmol photons m^{-2} s^{-1} and 27 mmol photons m^{-2} s^{-1} had relatively close values for their lipid content with 1 and 0.91 mg/mg dry weight, respectively. While at 9 mmol photons m^{-2} s^{-1}, the lipid content was at its lowest value, i.e., 0.17 mg mg^{-1} dry weight, which suggested that at higher light intensity, the dry weight increases.

Increased light intensity raises the cellular content of dry weight and lipids due to enhanced formation of TAGS [36]. Light intensity influences the cell growth and lipid accumulation of microalgae [5]. Both lipid content and dry weight of *N. palea* significantly varies by light intensity (p <0.05). Hu [37] suggested that lipid storage (mainly TAGS) could be increased by increasing light intensity. In photoautotrophic cultures, there are many culture conditions for increasing lipid content which includes high light intensity [38], high iron concentration [39], and high carbon dioxide concentration [40].

The dry weight of *N. palea* was significantly affected by temperature (p <0.05). In terms of

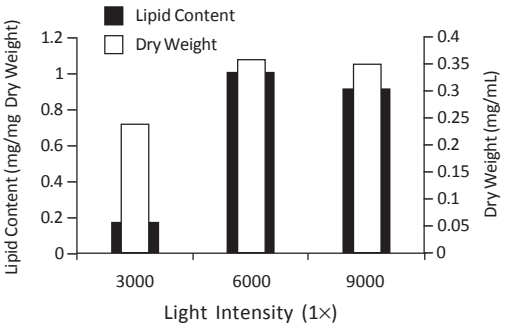

Figure 4. Dry weight and lipid content of *N. palea* at varying light intensities: 9 μmol photons m^{-2} s^{-1} (3000 l×), 18 μmol photons m^{-2} s^{-1} (6000 l×), and 27 μmol photons m^{-2} s^{-1} (9000 l×)

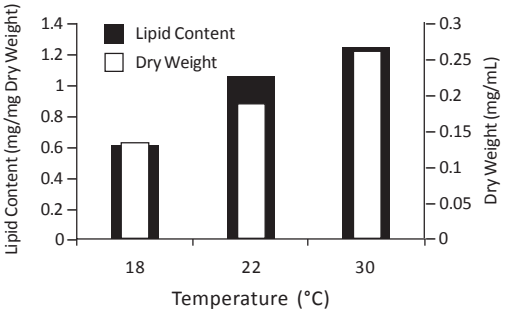

Figure 5. Dry weight and lipid content of *N. palea* at different temperatures

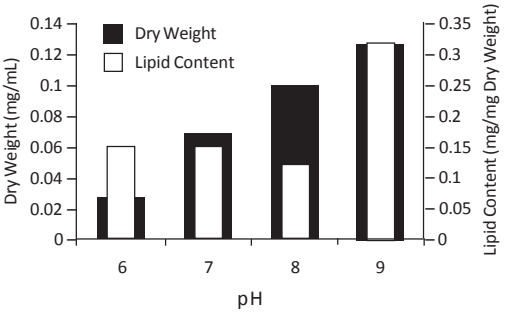

Figure 6. Dry weight and lipid content of *N. palea* at various pH concentrations

dry weight, there was an increasing trend from 18°C to 30°C (Fig. 5). The lipid content of *N. palea* had displayed an increasing trend as temperature increases, having the highest lipid content of 1.23 mg mg^{-1} dry weight at 30°C.

Temperature is another important environmental factor that affects profiles of algal lipids and fatty acids. In general, an increase in temperature causes an increase in fatty acid saturation and a decrease in the fatty acid unsaturation. This effect t has a high impact on the lipid content of microalgae.

The role of total lipids may be important at high temperatures or that there is greater use of lipids as a storage product at higher temperatures, especially in some cases where there had been an increase of lipids in algal cells. Temperature shifts induce intraspecific variation in microalgal production and biochemical composition in response to temperature shift. In relation to this, algae commonly alter the physical properties and thermal responses of membrane lipids to maintain fluidity and function of membranes [41]. In this study there was an interaction between dry weight and lipid content (p <0.05).

The dry weight of *N. palea* displayed an increasing trend as the pH of culture medium increased from pH 6 to pH 9 (Fig. 6). The lipid content at pH 6 and pH 7 were relatively similar, that is, 0.15 mg mg^{-1} dry weight. The lipid content of 0.12 mg mg^{-1} dry weight slowly declined at pH 8, while this was highest at pH 9 (0.32 mg mg^{-1} dry weight). Thus, dry weight is significantly affected by pH (p <0.05).

Lipid content and lipid productivity. The lipid content of *N. palea* was significantly affected by light intensity (p <0.05). At a lower level of light intensity (9 µmol photons m^{-2} s^{-1}), the atom exhibited a low lipid content of 0.17 mg mg^{-1} dry weight with a lipid productivity of 0.0044 mg mL^{-1} day^{-1}, respectively (Fig. 7). As lipid productivity began increasing at higher light intensities, *N. palea* started to accumulate lipids at 18 µmol photons m^{-2} s^{-1} with a lipid content of 1 mg mg^{-1} dry weight and lipid productivity of 0.04 mg mL^{-1} day^{-1}. Both lipid productivity and lipid content

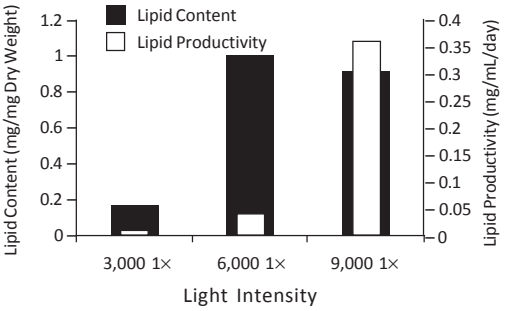

Figure 7. Lipid content and lipid productivity of *N. palea* at variious light intensities: 9 µmol photons m^{-2} s^{-1} (3000 l×), 18 µmol photons m^{-2} s^{-1} (6000 l×), and 27 µmol photons m^{-2} s^{-1} (9000 l×)

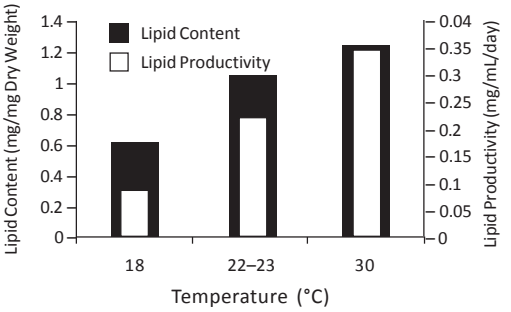

Figure 8. Lipid content and lipid productivity of *N. palea* under varying temperatures

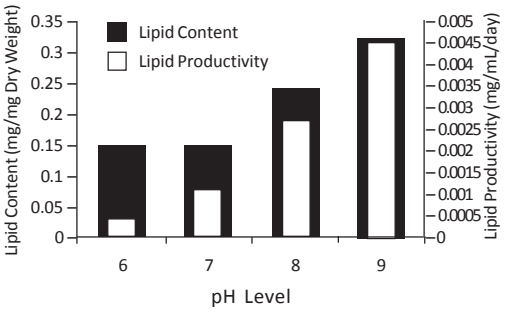

Figure 9. Lipid content and lipid productivity of *N. palea* at different pH levels

slowly declined when reaching 27 μmol photons m^{-2} s^{-1}, with values of 0.91 mg mL^{-1} dry weight and 0.035 mg mL^{-1} day^{-1}, respectively.

Cells started to accumulate lipid when light had good penetration (at low cell density). When individual cells were exposed to a large quantity of light energy, this resulted in more metabolic flux generated from photosynthesis to be channelled to lipid accumulation on an unit biomass basis. Fatty acid synthesis is normally stimulated by light. Thompson [42] explained that TAGS is mostly synthesized in the light and then re-utilized for polar lipid synthesis in the dark. Therefore, the overall lipid content of microalgae reflects this change under different light conditions. Hence, lipid productivity was significantly affected by light, growth, dry weight, and lipid content (p <0.05).

The lipid content and lipid productivity of *N. palea* were significantly affected by temperature (p <0.05). The microalgae began to accumulate lipids as temperature increases. The highest lipid content obtained was 1.23 mg mg^{-1} dry weight with lipid productivity of 0.035 mg mL^{-1} day^{-1} (Fig. 8). While at lower temperature, the lipid production of *N. palea* tended to accumulate lipids slowly with lipid productivity of 0.0088 and 0.022 mg mL^{-1} day^{-1} at 18°C and 22–23°C, respectively. On the other hand, lipid content at 18°C and 22–23°C is 0.61 and 1.05 mg mg^{-1} dry weight, respectively.

Several studies had shown that lower environmental temperatures generally cause variations in lipid content [42–44]. The lipid composition is also influenced by shifts of temperature in the environment [16]. In this study, lipid productivity was significantly affected by growth, dry weight and lipid content (p <0.05).

The lipid content and lipid productivity of *N. palea* were significantly affected by pH (p <0.05). The lipid production seemed to be lower at pH 6 and pH 7 with lipid productivity of 0.00045 and 0.0011 mg mL^{-1} day^{-1}, respectively (Fig. 9). Both lipid productivity and lipid content slowly increased as it reached higher pH levels. The lipid productivity at pH 8 was 0.013 mg mL^{-1} day^{-1} with a lipid content of 0.12 mg mg^{-1} dry weight. The highest lipid productivity of 0.0045 mg mL^{-1} day^{-1} was attained at pH 9 with a lipid content 0.32 mg mg^{-1} dry weight. In general, growth of *N. palea* at varying pH in terms of dry weight, lipid content and lipid productivity was highly significant (p <0.05).

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