

Enhanced Lipid Production in *Dunaliella salina* Grown under High Light Intensity by Shifting the Culture from High to Low Nitrogen Concentration

¹Rokeya Akter, ²Shah Md Monir Hossain, ¹Wu Zhe, ³Prasart Kermanee, ^{1,3,4}Niran Juntawong

¹Interdisciplinary Graduate Program in Bioscience, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

²Tropical Agriculture International Program, Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.

³Department of Botany, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand

⁴Center for Advanced Studies in Tropical Natural Resources, National Research University- Kasetsart University (CASTNAR, NRU-KU), Kasetsart University, Bangkok 10900, Thailand

Address For Correspondence:

Shah Md Monir Hossain, Tropical Agriculture International Program, Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
Tel: +66 889576818; Fax: + 663 4281086.; E-mail: monirsmh@yahoo.com

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ABSTRACT

Dunaliella salina (*D. salina*) is a potential source of lipids and subsequent biodiesel production. However lipid production from *D. salina* is dependent on biomass production rate and lipid content. To get more lipid, production efficiency need to be improved. The aim of this study was to application of two-stage culture strategy for improved microalgal growth and lipid Production. In this study the culture was grown under four different nitrogen concentrations (2 mM, 5 mM, 10 mM and 20mM) and also compared the culture by shifting from high (20 mM) to low nitrogen (2 mM) concentration under high (800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) and low light (200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) intensity in order to get the high lipid yield. The result showed that maximum lipid contents (46%) and lipid per cell (37.40 pg cell^{-1}) was obtained in the culture grown under low nitrogen concentration whereas high nitrogen culture showed maximum lipid concentration 0.73 g L^{-1} and lipid productivity 56.32 $\text{mg L}^{-1}\text{day}^{-1}$ when grown under high light intensity. The culture grown under high nitrogen with high light intensity increased the overall lipid productivity due to increase the biomass dry weight (2.13 g L^{-1}) and biomass productivity (66.01 $\text{mg L}^{-1}\text{day}^{-1}$). However the culture shifted from high nitrogen to low nitrogen concentration grown under high light intensity showed the highest contents of lipids (56.47%), concentration of lipid (1.169 g L^{-1}) and lipid productivity (89.92 $\text{mg L}^{-1}\text{day}^{-1}$). From the above results conclude that shifting of *D. salina* culture from high to low nitrogen concentration grown under high light intensity is the suitable condition to increase the overall lipid production in *D. salina*. This strategy can be used for outdoor cultivation for efficient algal biofuels production.

KEYWORDS: Biomass, lipid, *Dunaliella salina*, light stress, nitrogen limitation

INTRODUCTION

The current environmental concerns for generating renewable energy sources had grown dramatically due to global CO₂ increased, fossil fuel depletion and the crude oil prices increased. Crude oil reserves will be depleted within 50 years as the world oil consumption rate is 80–90 million barrels per day and the estimated value of world oil reserves is 1.3 trillion barrels [1]. Due to this problem it is essential to find ways to supply reliable, economically feasible, sustainable and renewable fuels and enhance economic prosperity and sustainability.

Microalgae is a third generation biofuel source provides a promising alternative to petroleum fuels that is potentially renewable, sustainable, carbon neutral, and can have a significantly smaller footprint in land area demand, compared to plant-based first and second generation biofuels. Microalgae cultivation is very simple, can grow autotrophically using inorganic carbon and solar energy to produce lipid. Their photosynthetic efficiency is higher and the final algal oil production per unit of surface may reach 200 times that of common plant oils [2-4]. Microalgae using solar energy by photosynthesis increase in the amount of neutral storage lipids, mainly triacylglycerols (TAGs) that can be converted to biodiesel as fatty acid methyl esters (FAME) through transesterification [5].

The lipid content in microalgae varies from about 1–85% of the dry weight [6-7], typically over than 40% lipid contents achieved under nutrient limitation. The lipid-rich species *Botryococcus braunii*, which is known to accumulate lipid up to more than 70% of biomass, but its doubling time is usually in the range of 5–7 days [8]. The marine microalgae *Dunaliella* sp. is used in biodiesel production due to their faster growth rate and easier cultivation and non-sterile open pond condition [9]. However, *D. salina* accumulates only 30% lipid content under nitrogen sufficient growth conditions with high light intensity. The lipid content of *D. salina* can increase 14% under nitrogen limitation [10]. Large scale production of microalgae has remained economically infeasible due to the large costs of nutrient and sterile growing conditions. Moreover, low lipid productivity is not sufficient for outdoor biodiesel production.

To make microalgal-based biofuel production more economic, it is necessary to enhance both microalgal biomass and overall lipid productivity. Marine microalgae accumulates lipid, especially neutral lipids/triglycerides (TAGs) under nitrogen starvation [11], phosphate limitation [12], high salinity [13], carbon source concentration [12] and so on. *D. salina*, is candidate feed stocks for biofuel production [14] because mass production systems for this alga have been established already worldwide. *D. salina* can be manipulated easily for either fatty acid content or composition by high irradiance, salinity stress and N-deprivation [10]. Their fatty acid profiles reported are mainly C16:0 (23.7%), and C18:0 (7%) total lipids. Highly saturated fatty acids give an excellent cetane number and oxidative stability to biodiesel [15]. Stearic acid content of *D. salina* is higher than *Botryococcus braunii*, *Scenedesmus* sp. [16], *Chlamydomonas*, *Chlorella* sp. and *Navicula* sp. [17].

In addition the light intensity is an important factor affecting the growth and lipid accumulation, and it could also affect fatty acid composition in the microalgae *Parietochloris incise* and *Dunaliella viridis* [18]. Therefore light intensity not only greatly affect algae photosynthesis as well as cell composition and metabolic pathway, but also the economic efficiency of the algae cultivation process [19], and consequently, the supply and efficient utilization of light energy have been the greatest scientific and technological challenge in research and development on commercial cultivation of microalgae. Rabbani *et al.*, [20] observed that under nitrogen deprivation in high-light treated *D. salina* cells increases beta-carotene and total fatty acid [21]. *Dunaliella* cells produced beta-carotene as a co-product which protects the cells against photoinhibition [22]. The algal cells cultivated in nitrogen deficient medium with high light intensity induced biomass and pigment accumulation in the reactor [14].

However, this is generally known that any stress concomitant with lower biomass productivity and thus low overall lipid productivity [3-4]. Meanwhile a replacing nitrogen source is necessary for maintaining a high cell growth rate and achieving high cell density [23].

Therefore, economical production of algal biodiesel will require optimization of two-stage of microalgal growth for enhancing lipid productivity. In the first stage, the algae are grown under nitrogen-sufficient condition to obtain a maximum cell density as quickly as possible and, in the second stage, application of stress conditions to induce lipid synthesis. The objective of this work was to maximize the biomass content in order to increase the lipid productivity by shifting the culture condition from high to low nitrogen concentration under high light intensity. This experiment could make large scale biodiesel production economically feasible by decreasing the nutrient cost by increasing the lipid productivity under high light intensity. The results of this research could be used for future commercial algal production in outdoor cultivation.

MATERIALS AND METHODS

Microalgae source, medium and cultivation:

Marine microalgae *D. salina* was obtained from gene bank, department of botany, faculty of science, Kasetsart University, Thailand. The *D. salina* was grown in the modified medium from the original recipe of Weldy and Huesemann (2007) [10] and the composition as follows; 3 mM KCl, 5 mM KNO₃, 0.5 mM MgSO₄·7H₂O, 0.3 mM CaCl₂, 0.1 mM K₂HPO₄, 0.4 mM FeCl₃, 4 mM EDTA, and 1 mL (per L) of micronutrient solution. The micronutrient solution is composed of 50 mM H₃BO₃, 10 mM MnCl₂·4H₂O, 0.8 mM ZnSO₄·7H₂O, 2 mM NaMoO₄·2H₂O, 0.2 mM CoCl₂·6H₂O, 2 mM NaVO₃ and 0.2 mM CoCl₂·6H₂O with 1 M NaCl and 1 M NaHCO₃.

For preparation of starter, single colony was picked and inoculated into modified *Dunaliella* growth medium containing 1 M NaCl for further experiment. The strain was incubated for 7 days at 25±2°C ambient temperature with continuous photoperiod at 56 μmol photons m⁻² s⁻¹ under white fluorescent lamps.

Experimental setup:

D. salina microalgae were grown in bubble column photobioreactor at 25±2 °C temperature with a working volume of 250 mL. Twenty milliliters of starter cells at the density of 0.8×10⁶ cells mL⁻¹ was transferred into 230 mL medium containing 20 mM, 10 mM, 5 mM and 2 mM KNO₃ concentrations in combination with high (800 μmol photons m⁻² s⁻¹) and low light intensity (200 μmol photons m⁻² s⁻¹). The light intensity was measured by the Lux meter (Lab facility, LX101, 50000LX). Multiply the Lux by the conversion factor to get photosynthetic photon flux (PPF). The culture was aerated with 95% air containing 5% CO₂ (v/v) at approx. 0.01 vvm (volume of air per volume of medium per minute) for 15 days.

The experiment was conducted in 4 different nitrogen concentrations under high and low light intensity in order to compare and evaluate the influence of nitrogen contents on growth and lipid production. For two-stage cultivation: 1st stage *Dunaliella* cells were cultivated in 20 mM nitrogen concentration under high and low light intensity for 7 days. The culture was grown up to the stationary phase and 2nd stage shifted to low nitrogen condition (2 mM). Cells were recovered from the high nitrogen medium through centrifugation at 10,000×g for 5 min and then removed the spent medium. Collected cells were washed thoroughly 3 times with distilled water. Cells were transferred to modified medium containing 2 mM KNO₃ and incubated under selected conditions.

Growth and biomass dry weight (DW) determination:

The cell growth was determined from the cell numbers during the cultivation period. Samples were collected every 2 days for 15 days and the cell number was counted by using a neubauer hemocytometer (Marienfeld, Germany) and a light microscope (CX21, Olympus Imaging Corp., Japan). The specific growth rate was calculated from the slope of the linear regression of cell density (cells mL⁻¹) against time (days) and as specified by Wood *et al.*, (2005) [24] i.e.:

$$\mu = \ln(N_t/N_0)/(t-t_0)$$

where N_t is cell density at time (t), and N₀ is cell density at the start of the exponential phase (t₀).

Ten mL of algal suspension in a pre-weighed centrifuge tube was centrifuged at 2,500×g for 10 min to produce biomass pellets. The pellets were washed with distilled water (twice) to remove salt. After removing the supernatant and the pellets were dried in an electric oven at 65°C for 24 hr. and then weighed. Biomass productivity (mg L⁻¹day⁻¹) was calculated as the dry biomass produced at the cultivation period using the formula:

$$\text{Biomass productivity} = \text{amount of biomass (mg L}^{-1}\text{)}/\text{Number of days}$$

Chlorophyll content measurement:

Every 2 day the chlorophyll content was measured. Ten ml of algal culture was centrifuged at 2,500×g for 10 min. Discard the medium and then add 10 ml of 100% ice cold acetone to the pellet. Then the tube was vortex thoroughly until the pellet completely dissolved. The tubes were placed at a refrigerator overnight to extract chlorophyll. The next day the sample was centrifuged at 5,000×g for 10 min. Chlorophyll a concentration (μg mL⁻¹) was measured using Spectrophotometer (UV-Visible 8453 Spectrophotometer) with the wavelength of 664 and 647 [25]. The chlorophyll a content was calculated by using the equation [26].

$$\text{Chlorophyll a (}\mu\text{g mL}^{-1}\text{)} = \frac{(11.93 \times \text{OD}_{664} - 1.93 \times \text{OD}_{647}) \times \text{Volume of acetone}}{\text{Volume of culture}}$$

Total lipid extraction:

Lipid extraction from *D. salina* was carried out by slightly modification of the protocol by Bligh and Dyer (1959) [27]. Lipid was extracted from biomass pellets by using methanol, chloroform, and water. Lipid analysis was done at three points during the experiments. Fifty milliliters of culture were harvested by centrifugation at 5,000×g for 10 min. The supernatant was removed by pipette; the pellets were then suspended with 4 mL of dH₂O. After that, 10 mL of methanol and 5 mL of chloroform were added in sequence, resulting in a 10:5:4 ratio of methanol: chloroform: water. After an overnight incubation on a shaker, 5 mL of water and 5 mL of chloroform were added which results in a 10:10:9 ratio of methanol: chloroform: water. The samples were centrifuged for 10 min at 5,000×g to obtain two layers. At this solvent ratio, two layers were formed, a water methanol upper layer and a lipid containing chloroform lower layer was removed by pipetting and placed into

another pre-weighed vial. The chloroform was evaporated by heating on a hot plate. After evaporation, the vial was weighed again. The difference between the weight before and after evaporation was represented as the weight of the microalgal lipids from the cultured samples. Lipid content and lipid productivity were then calculated by the following formula:

$$\text{Lipid content (\% dry weight)} = \frac{\text{Weight of lipid (mg L}^{-1}\text{)}}{\text{Weight of biomass (mg L}^{-1}\text{)}} \times 100$$

$$\text{Lipid productivity (mg L}^{-1}\text{day}^{-1}\text{)} = \text{Biomass productivity (mg L}^{-1}\text{day}^{-1}\text{)} \times \text{Lipid content}/100$$

Statistical analysis:

All the data were analyzed using Microsoft Excel 2010. Statistical analyses were performed by using the SAS version 9, followed by a Duncan's multiple range tests. Differences were considered significant at $p < 0.001$. Every experiment was conducted in triplicate.

Results:

Effect of KNO₃ concentration and light intensity on growth and dry weight:

To investigate the effect of KNO₃ concentration on the growth of *D. salina*, the culture was grown at $25 \pm 2^\circ\text{C}$ under high and low light intensity. The maximal cell number (25.6×10^7 cells mL⁻¹) was observed in the culture grown in 20 mM KNO₃ under high light intensity on 9th day of inoculation. In contrast, the microalgal growth (1.04×10^7 cells mL⁻¹ and 1.80×10^7 cells mL⁻¹) was decreased under low concentrations of KNO₃ (2 mM and 5 mM) when compared to the high concentrations (20 mM) of KNO₃. The result indicated that *D. salina* grow better in high concentration of nitrogen with high light intensity when compared to the culture grown in low nitrogen concentration with low intensity of light (Fig.1). The highest specific growth rate (0.71 μ/day) was achieved in the culture grown in high concentration of KNO₃ under high light intensity.

Biomass of *D. salina* was determined by the dry weight. During the cultivation period on the 9th day the highest biomass content was obtained in the culture grown in high concentration of nitrate (20 mM KNO₃) under high light intensity (2.13 g L⁻¹) when compared to the low KNO₃ (2 mM KNO₃) concentration under low light intensity (0.71 g L⁻¹). The biomass increased dramatically with increase in the cell number (Fig. 1 and 2). The maximum biomass productivity was found in the culture grown in high KNO₃ concentration with high light intensity (163.76 mg L⁻¹day⁻¹) (Table 1). After 9th day of inoculation the growth tends to slow down and attained the stationary due to nutrient depletion and the algae started to accumulate lipid in the culture medium.

Effect of different KNO₃ concentrations and light intensity on chlorophyll content:

The chlorophyll content was determined to show the effects of nitrogen on algal growth. The chlorophyll content was increased under high nitrogen concentration. Among the different nitrogen concentrations highest chlorophyll content was obtained (75.25 μg mL⁻¹) in high nitrogen concentration with high light intensity whereas the lowest chlorophyll content was obtained (47.40 μg mL⁻¹) in low nitrogen concentration under low light intensity (Table1). The result showed that the nitrogen limitation had effects on the algal growth rate.

Effect of different KNO₃ concentrations under high and low light intensity on lipid production:

Fig. 3 and 4 showed the lipid content of *D. salina* grown in different concentrations of KNO₃ under high and low light intensity. As the concentration of KNO₃ was decreased, an increasing trend was observed in percentage (%) of lipid and lipid content (pg cell⁻¹) under high light intensity (Fig. 3 and 4). But the overall lipid productivity was higher in high KNO₃ concentration when compared to low KNO₃ concentration when the culture grown under high light intensity. The highest lipid concentration 0.73 g L⁻¹ was obtained in 20 mM KNO₃ under high light intensity. Moreover, when the nitrogen source was decreased in the medium from 20 mM to 2 mM KNO₃, the % of lipid content was increased from 24 to 46% and lipid content also increased from 28.55 to 37.40 pg cell⁻¹ (Fig. 1 and 2).

Effect of shifted culture from 20 mM to 2 mM KNO₃ conditions grown under high and low light intensity on biomass and lipid content:

On 9th day of cultivation the biomass and biomass productivity of the shifted *D. salina* culture was not statistically different in comparison to non-shifted culture under high or low light intensity. Moreover a sharp rise in lipid accumulation (56%) was achieved when the cultures were grown in shifted condition (Fig. 2). The maximum lipid concentration (1.16 g L⁻¹), which was 1.5 times higher was obtained in the shifted culture than the non-shifted condition (0.73 g L⁻¹) grown under high light intensity. The result indicated that the shifted culture condition significantly improved the lipid content.

Lipid accumulation rates:

To determine the lipid productivity by *D. salina* grown under various nitrogen concentration cells were cultured up to 15 days. The $\text{mg L}^{-1}\text{day}^{-1}$ was used to lipid production rates on 0-6, 6-9 and 9-13 days. The second day of shifting from 20 mM KNO_3 to 2 mM KNO_3 the culture grown under high light intensity showed the maximum lipid production $89.92 \text{ mg L}^{-1}\text{day}^{-1}$ whereas the lowest lipid production rate was obtained ($77 \text{ mg L}^{-1}\text{day}^{-1}$) under low light intensity (Fig. 5). After second day of shifting, the lipid content was decreased dramatically. The limitation of nutrients from the medium had increased the accumulation of lipid by the two-stage strategy system.

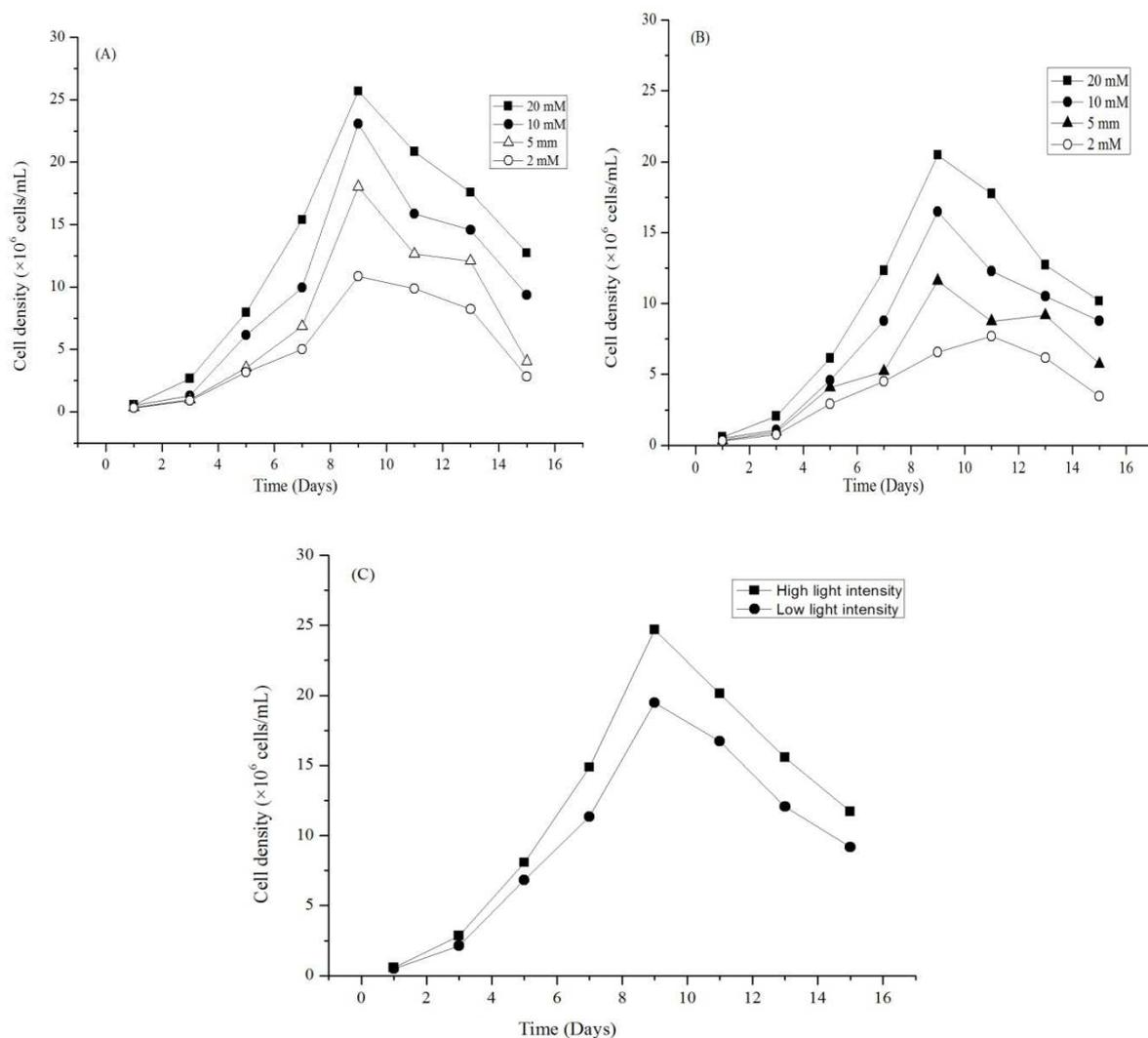


Fig. 1: Cell density in *D. salina* cultures grown under different KNO_3 concentrations with (A) high and (B) low light intensity and (C) shifting culture condition

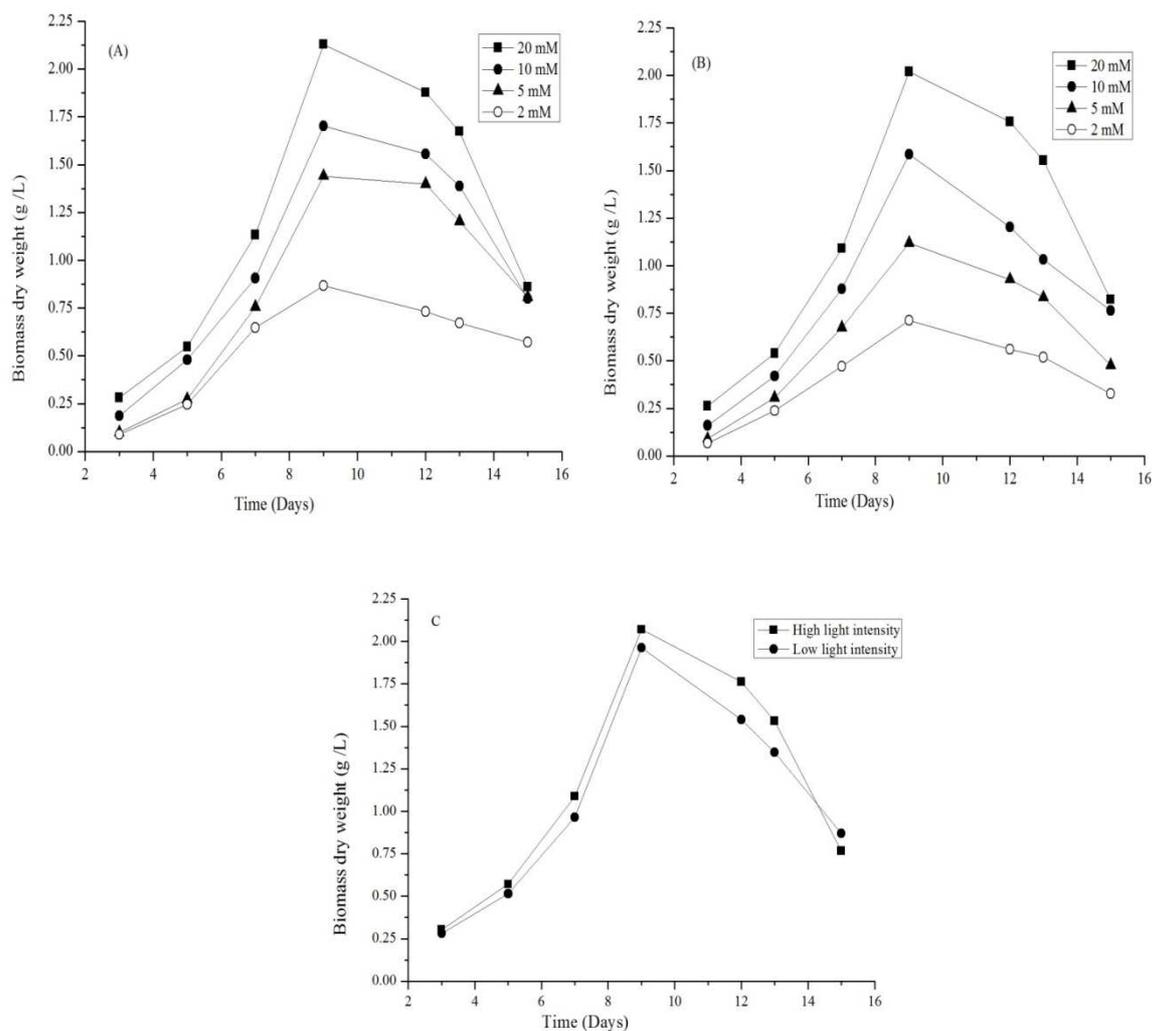


Fig. 2: Biomass in *D. salina* cultures grown under different KNO_3 concentrations with (A) high and (B) low light intensity and (C) shifting culture condition

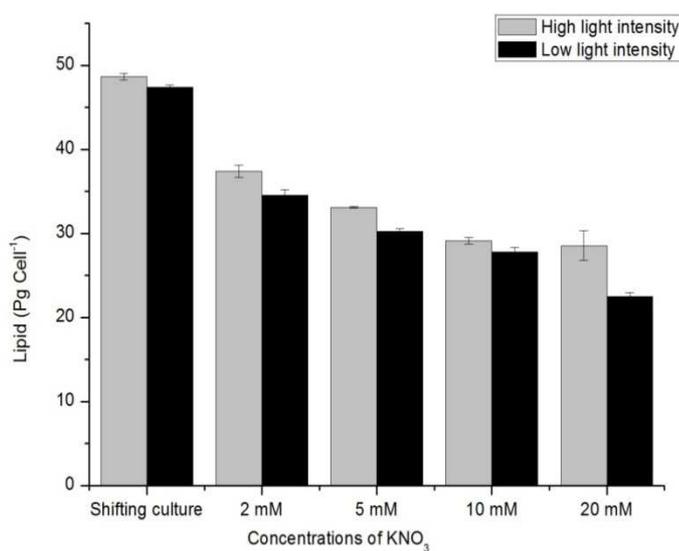
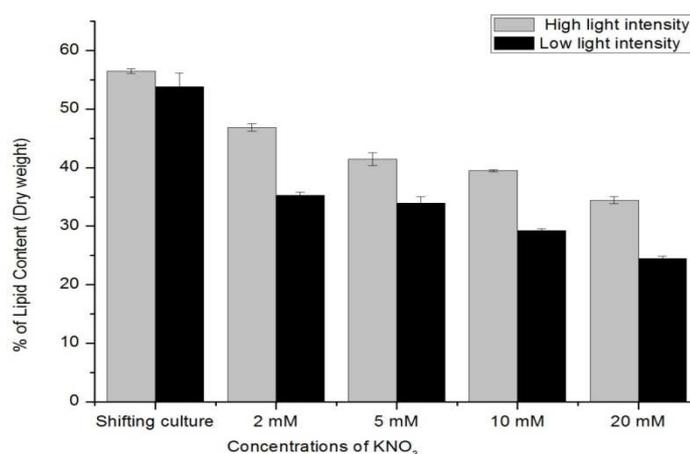
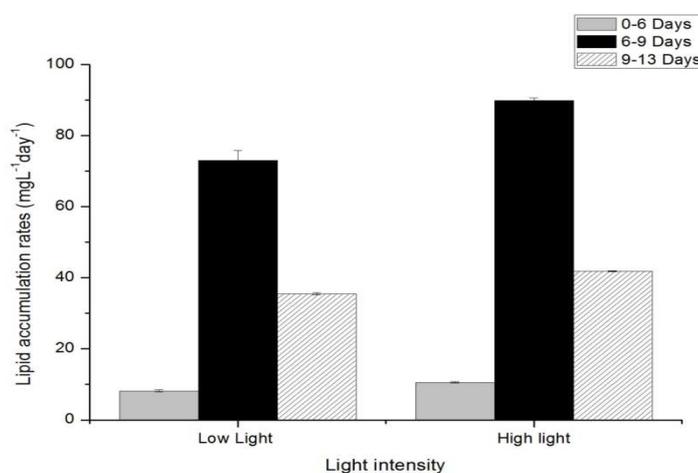


Fig. 3: The lipid (pg cell^{-1}) in *D. salina* cultures grown under different KNO_3 concentrations and shifting culture with high and low light intensity

Table 1: Comparison between culture grown different concentrations of KNO₃ and shifted culture under high and low light intensity on growth and lipid content

	Light Source	Concentration of KNO ₃				
		2 mM	5 mM	10 mM	20 mM	Shifting culture
Specific growth rate (μ Day ⁻¹)	L-Light	0.51±0.03 ^d	0.57±0.02 ^c	0.68±0.02 ^{ab}	0.70±0.02 ^a	0.65±0.03 ^b
	H-Light	0.53±0.03 ^{cd}	0.56±0.02 ^c	0.69±0.01 ^{ab}	0.71±0.03 ^a	0.68±0.01 ^{ab}
Cell density (×10 ⁶ cells mL ⁻¹)	L-Light	6.56±0.01 ^j	11.60±0.02 ^g	16.49±0.15 ^h	20.47±0.10 ⁱ	19.47±0.10 ^e
	H-Light	10.75±0.10 ⁱ	18.03±0.01 ^f	23.10±0.03 ^c	25.64±0.03 ^a	24.64±0.10 ^b
Chlorophyll content (μg mL ⁻¹)	L-Light	51.03±0.19 ^g	56.47±0.71 ^f	59.72±0.09 ^e	69.95±0.95 ^c	67.23±1.78 ^d
	H-Light	52.57±0.21 ^g	60.60±0.34 ^e	66.75±0.22 ^d	75.25±0.62 ^a	72.08±1.54 ^b
Biomass (g L ⁻¹)	L-Light	0.71±0.03 ^h	1.12±0.06 ^f	1.58±0.02 ^d	2.02±0.04 ^{ab}	1.93±0.08 ^b
	H-Light	0.87±0.06 ^g	1.44±0.01 ^e	1.70±0.05 ^c	2.13±0.12 ^a	2.07±0.01 ^b
Biomass productivity (mg L ⁻¹ day ⁻¹)	L-Light	59.33±0.77 ^h	100.28±1.6 ^f	120.33±1.25 ^d	154.62±0.77 ^b	153.27±1.83 ^b
	H-Light	66.01±2.31 ^g	110.79±0.8 ^e	131.03±3.87 ^c	163.41±1.81 ^a	161.74±1.77 ^a
Lipid content (pg cell ⁻¹)	L-Light	34.45±3.25 ^c	30.23±0.65 ^d	27.78±0.32 ^e	22.47±0.52 ^f	48.74±1.73 ^a
	H-Light	37.40±0.41 ^b	33.12±0.73 ^c	29.09±0.09 ^{de}	28.55±0.40 ^{de}	47.37±0.45 ^a
Lipid (g L ⁻¹)	L-Light	0.39±0.36 ^h	0.47±0.50 ^g	0.56±0.56 ^{ef}	0.61±0.62 ^{de}	0.91±0.95 ^b
	H-Light	0.45±0.47 ^g	0.59±0.60 ^e	0.68±0.67 ^{cd}	0.73±0.73 ^c	1.17±1.17 ^a
Lipid productivity (mg L ⁻¹ day ⁻¹)	L-Light	32.10±2.08 ⁱ	40.14±1.91 ^g	45.23±0.6 ^f	48.38±1.13 ^e	77.07±1.47 ^b
	H-Light	37.23±0.92 ^h	45.91±0.99 ^f	51.65±0.7 ^d	56.33±0.84 ^c	89.92±0.75 ^a

L- Light = Low light intensity; H-light = High light intensity. Different letters are significant according to Duncan's multiple range test

**Fig. 4:** The percentage of lipid content in *D. salina* cultures grown under different KNO₃ concentrations and shifting culture with high and low light intensity**Fig. 5:** Lipid accumulation rates during the cultures grown in shifted condition under high and low light intensity

Discussion:

From this study we found that shifting of culture from high to low nitrogen concentration grown under high light intensity has the positive effect on both growth rate and lipid productivity.

The pattern of growth indicates that high nitrogen concentrations with high light intensity increased the high biomass. Cell growth was decreased by low nitrate concentration under low light intensity. Weldy and Husesemann, [10] reported that *D. salina* showed better growth in the culture grown high concentration of KNO_3 (1.5 g L^{-1}) under high light intensity than the low concentration of KNO_3 (0.4 g L^{-1}) under low light intensity. The result showed that the % of lipid and lipid content (pg cell^{-1}) were increased under low nitrogen concentration than the high concentration of nitrogen. Moreover the overall high lipid productivity was found under high nitrogen concentration due to higher biomass.

The result showed that low nitrogen concentrations develop higher % of lipid and lipid content (pg cell^{-1}) than the high concentration of nitrogen. But the overall high lipid productivity was found under high nitrogen concentration due to higher biomass. From the results the lipid accumulation by *D. salina* ranges from 24-56% depending on growing conditions and growth phase. This result was similar to the previously result of *Dunaliella* sp. which had the ability to accumulate 45-55% of lipid [28]. From the result it is clearly showed that the nitrogen limitation increased lipid content. This result confirmed previous reports that nitrogen limitation increase lipid production in *Dunaliella* species [29-30]. Nitrogen is one of the most important nutrients affecting lipid metabolism in algae. Nitrogen limitation leads to decrease the growth rate, protein synthesis, photosynthesis and increase in lipid contents [8]. *Dunaliella* species are composed largely of triacylglycerols [20]. In addition, under nitrogen deficient conditions algal cell size increased, accumulate carbon metabolites as lipids and nitrogen containing macromolecules and carbon reserve compounds like carbohydrates and fats [31].

Large scale production of biodiesel from microalgae is economically infeasible due to the cost of nutrients and sterile growing conditions. Therefore improve the overall lipid content for economic production of biodiesel two-stage culture strategy has to be applied. Biomass production was largely unaffected in that conditions ranging from 1.93 and 2.07 g L^{-1} under low and high light intensity. Lipid content gradually increased from 34% to 56% at nitrate limitation condition and lipid per cell (pg cell^{-1}) also increased from 34.45 - $47.74 \text{ pg cell}^{-1}$. The overall lipid productivity increased from 56.33 - $89.92 \text{ mg L}^{-1}\text{day}^{-1}$ due to the high biomass and cell number. Mujtaba *et al.*, [12] also applied two stage strategies in *Chlorella vulgaris* to get the fast cell growth under nutrient rich conditions and incubation under nitrogen starvation for enhancing overall lipid productivity (lipid content 24.6-53% and lipid productivity 31.5 - $77.1 \text{ mg L}^{-1}\text{day}^{-1}$). Their results support with the Thai *D. salina* isolates lipid productivity. The present study revealed that shift from high to low nitrogen concentration would be an effective technique to maintain sufficient biomass for subsequent biodiesel production. So large scale cultivation of *D. salina* for production of lipid as well as biodiesel, it may be more economical to subject the cultures to a short period of nitrogen limitation to induce high level neutral lipids. This will reduce the use of more nutrients and finally reduce the cost.

Factors inducing lipid production under high light intensity is the basic energy source for photoautotrophic microalgae. The present study showed that high light intensity develops high biomass and lipid content than low light intensity. An increased growth rate corresponding to the light intensity was also noted in *Dunaliella viridis* when it was cultivated at 250 - $700 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ but decreased when the light intensity changed from 700 to $1,500 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ [32]. The photosynthesis of aquatic plants is saturated in the range of 20–50% of full sunlight, or about 400 – $1000 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Thus, light harvesting efficiency is important for bioreactor engineering encompassing those microorganisms [33]. There are wide variations in the nature of algal strains and their dependence on light intensity for growth.

Light intensity influences not only the microalgal growth but also alter the biochemical composition [34]. Harwood *et al.*, [35] reported that high light intensity leads to oxidative damage of polyunsaturated fatty acids (PUFA), and is also required for the synthesis of C16:1 (3 *trans*) and alters the level of this fatty acid (FA) in microalgae. Low light intensity induces the formation of polar lipids, particularly the membrane polar lipids associated with the chloroplast, whereas high light intensity decreases total polar lipid content with a simultaneous increase in the amount of neutral storage lipids, mainly TAGs. TAG production under high light conditions might serve as a protective mechanism for the cell. Increased FA synthesis which in turn is stored as TAG, potentially helps the cell to re-generate its electron acceptor pool. High light intensity is important representative of the large scale outdoor cultivation of microalgae for lipid production. This result showed that high light intensity could improve the nitrate limitation to start to accumulate lipid early. It was related with the proposed function of beta-carotene content of *D. salina* as a protection of the cell against injury by high light intensity. However the ability of *D. salina* produces a high value co-product beta-carotene with lipid to be potential economic feasibility of mass culturing.

The present study revealed that the use of two-stage cultivation under high light intensity, composed of fast cell growth under high nitrogen condition and then transfer low nitrogen condition is beneficial for enhancing overall lipid productivity. The results from this study suggest that *D. salina* can be cultured to produce high lipids. So the two-stage strategy under high light intensity would be suited for large scale production of lipids

for biodiesel with *D. salina*. It is expected that the results could serve as a reference for further larger-scale production. Moreover, further research on what the effects of phosphate, carbon resource and light quality on lipid accumulation during nitrogen starvation is recommended. It is possible that the two-stage strategy also to be applicable to other lipid producing algae. However, feasibility to commercialize microalgae biofuels is still questionable as high energy input to harvest microalgae biomass is required especially in two-stage culture process. Therefore, especially for big-scale microalgae culture, more effective ways to harvest microalgae such as continuous centrifugation, microfiltration and immobilization technology will have to be implemented. Besides, in larger commercial production system longer N-starvation time and higher illumination, though they could enhance the lipid production, may lead to more manpower and energy (such as electronic power) consumption and thus increase cost for biofuels production, for a conventional method. Therefore, although most studies have suggested that the application of a two-stage process is most likely the most promising culture strategy, more efforts should be taken to reduce the capital costs.

Conclusion:

D. salina has the ability to accumulate significant amount of lipid under low nitrogen concentration. To enhance biomass and overall lipid production in *D. salina* we had applied two-stage strategy. In the first stage of the cultivation to increase the biomass the culture was grown in higher nitrogen concentration in the second stage cultivation the cell was shifted to the nitrogen limitation condition to obtain the maximum lipid and lipid productivity. The highest lipid accumulation was attained after 2 days of the culture shifted to the low nitrogen condition. The results showed that culture grown in low nitrogen concentration accumulate higher % of lipid content and lipid per cell than the culture grown in high nitrogen concentration. The culture grown under the high light intensity increased biomass content than the low light intensity grown culture. Due to higher biomass the culture grown under high nitrogen concentration accumulates highest lipid concentration and lipid productivity. From this result we conclude that two stage processes is one of the best ways to enhance both the algal biomass and overall lipid productivity. This strategy can be used for large scale outdoor cultivation to develop microalgal biomass and as possible to strengthen the stress on each cell to enhanced lipid production to become economically valuable for renewable oil and biodiesel production. This process can be applicable to any species of algae to enhance both biomass and the targeted products.

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