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Original Article

Effect of nitrogen concentration on growth, lipid production and fatty acid profiles of the marine diatom *Phaeodactylum tricornutum*

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ABSTRACT

The marine diatom, *Phaeodactylum tricornutum* had a high lipid content accumulation under photoautotrophically nitrogen-deficient cultivation. The lipid content ($Y_{P/X}$; 53.04 ± 3.26%) was highest with a specific rate of lipid production (q_P; 1.50 ± 0.12 × 10^{-3} mg/mg h), attained at the minimized specific growth rate (μ; 0.87 ± 0.13 × 10^{-2}/h) after 504 h of cultivation. When the specific growth rate (μ; 2.47 ± 0.02 × 10^{-2}/h) was maximized in nitrogen-sufficient culture (32.09 mg/L $NaNO_3$), the specific rate of lipid production (q_P; 0.42 ± 0.19 × 10^{-3} mg/mg h) was lowered. In this work, the nitrogen concentration with fixed phosphorus concentration was used to monitor the lipid accumulation, as the lower nitrogen concentration favored a higher lipid content percentage, compared with a higher nitrogen concentration. Under nitrogen-deficient conditions, *P. tricornutum* produced a large amount of saturated fatty acids, mainly as palmitic acid (C16:0), while palmitoleic acid (C16:1c) was found to be the sole unsaturated fatty acid. On the other hand, eicosapentaenoic acid (C20:5ω3c) was produced in large amounts when there was sufficient nitrogen. Since the biodiesel was qualified based on the fatty acid methyl ester composition, the oil from algae cultured under nitrogen-deficient conditions were considered to meet the biodiesel standard. Thus, *P. tricornutum* optimally cultivated under nitrogen-deficient conditions can accumulate a high oil content, which demonstrates its potential as a biodiesel feedstock.

Introduction

Renewable energy has become an alternative source of energy, especially because it is environmentally friendly (Collet et al., 2011). Algal oil is one of the candidates in this strategy as algal oil has high potential and superior advantages to other fuels derived from energy crops (Chisti, 2007, 2013). However, the qualification of algal oil should be standardized. Generally, microalgae store lipids solely in the form of neutral lipids such as triacylglycerol (TAG) according to Valenzuela et al. (2012). This neutral lipid, used as the substrate, reacts with alcohol via a transesterification reaction under appropriate conditions for biodiesel production (Chisti, 2007). Thus, the cultivation of algae to produce higher amounts of TAG is needed.

Phaeodactylum tricornutum is a marine model diatom, which has been used in various studies in the fields of ecology, physiology, biochemistry and molecular biology (Valenzuela et al., 2012; Ge et al., 2014). The diatom can grow photoautotrophically by using CO_2 and light as carbon and energy sources, respectively. It is unicellular and can accumulate lipids in the range 20–60% under suitable conditions such as nutrient depletion (Valenzuela et al., 2013). In addition, it can store carbon and energy in the form of neutral lipids, especially TAGs which can make up approximately 20–30% of the dry cell weight (Valenzuela et al., 2012). Thus, this diatom has potential as a source for biodiesel production. Moreover, it may also be a source of omega-3, long chain, polyunsaturated fatty acids, especially eicosapentaenoic acid (EPA; 20:5 $\Delta^{5,8,11,14,17}$) according to Hamilton et al. (2014).

Generally, nitrogen plays the key role in the macromolecular synthesis pathway in microorganisms, which involves proteins, nucleic acids and chlorophyll biosynthesis. In the diatom, nitrogen assimilation and carbon metabolism are linked together with

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two intermediates—2-oxoglutarate and oxaloacetate—via the TCA cycle. The amino acids are catabolized yielding various TCA intermediates that could enter the TCA cycle. In this case, acetyl-CoA is produced and used in fatty acid elongation (Hockin et al., 2012). The lack of a nitrogen source affects not only cell growth, but also other pathways, and several researchers have reported that under different nutrient stresses, such as nitrogen or phosphate depletion, oleaginous cells such as algae (*P. tricornutum*), can accumulate up to 70% lipids in their cells (Burrows et al., 2012; Valenzuela et al., 2012). Moreover, nitrate depletion had a greater effect on lipid accumulation than phosphate depletion, but the accumulation increased when both nutrients were depleted concomitantly (Valenzuela et al., 2013). However, that particular research focused on the effect of the nitrogen concentration on the accumulation of lipid in the marine diatom, where the process was optimized by considering high biomass and a high lipid content.

Materials and methods

Algal strain and preculture

Phaeodactylum tricornutum (obtained from the National Research Institute of Aquaculture, Minami-Ise, Japan) was cultivated in 250 mL flasks containing 100 mL sterilized culture medium f/2 (solely prepared from sea water) containing 75 mg/L $NaNO_3$, 5 mg/L $NaH_2PO_4 \cdot 2H_2O$, 30 mg/L $Na_2SiO_3 \cdot 9H_2O$, 1 mL of stock trace metal solution (per 1 L; 3.15 g $FeCl_3 \cdot 6H_2O$, 4.36 g $Na_2EDTA.2H_2O$, 1 mL 9.8 g/L $CuSO_4 \cdot 5H_2O$, 1 mL 6.3 g/L $Na_2MoO_4 \cdot 2H_2O$, 1 mL 22.0 g/L $ZnSO_4 \cdot 7H_2O$, 1 mL 10.0 g/L $CoCl_2 \cdot 6H_2O$ and 1 mL 180.0 g/L $MnCl_2 \cdot 4H_2O$) and 0.5 mL of stock vitamin solution (per 1 L; 200 mg thiamine HCl (vitamin B_1), 1 mL 1.0 g/L biotin (vitamin H) and 1 mL 1.0 g/L cyanocobalamin (vitamin B_{12})) in sea water. The algal cells were incubated statically at 20 °C and pH 7.8 under a light intensity of 80–100 μmol/m^2 s. A 14:10 h light and dark cycle was applied. The preculture was incubated for 1 wk and then transferred as 10% of the starter into a 2-L flask containing 1 L sterilized culture medium f/2 under the same conditions to obtain a high biomass. The incubation was performed for 1 wk before being used as the starter for the next step.

Algal growth

The starter was harvested using centrifugation (2300×*g*, 15 min) and then washed twice with sterilized culture medium f/2 without $NaNO_3$. The biomass was dissolved in 150 mL sterilized culture medium f/2 without $NaNO_3$. Then 10% of the biomass was transferred to a 2 L flask containing 1.5 L sterilized culture medium f/2 with different $NaNO_3$ concentrations (0 mg/L, 16.45 mg/L, 32.09 mg/L and 64.29 mg/L) and fixed using 5 mg/L $NaH_2PO_4 \cdot 2H_2O$. The cultures were cultivated under the conditions as described above in triplicate for 3 wk. The samples were collected daily for subsequent analyses.

Algal sampling and harvesting

The cultures were withdrawn daily for analysis of the cell density, chlorophyll content and lipid concentration. Some other cultures were also collected and dewatered using centrifugation (2300×*g*, 15 min). The fraction of pastes was quickly frozen at −80 °C and then freeze-dried for fatty acid profile analysis. The supernatants were also frozen at −80 °C for measurements of $NaNO_3$ and NaH_2PO_4.

Analytical methods

Algal growth

Algal growth was measured with the optical density at 665 nm (OD_{665}). The dry cell weight was measured using a modified method (Sirisansaneeyakul et al., 2011) to establish the calibration curve. The cell concentration was calculated by comparison with the calibration curves of cell concentration (mg/L) with cell density (coefficient of determination, $R^2 = 0.99$) as shown in Eq. (1):

$$\text{Cell concentration (mg/L)} = 687.91 \times \text{OD}_{665} \quad (1)$$

Lipid accumulation

The lipid concentration was determined using Nile red measurement (Hitachi f-2700 fluorescence spectrophotometer at EX 530 and EM 575) according to Chen et al. (2009). The lipid concentration was calculated by comparison with standard triolein concentrations as shown in Eq. (2):

$$\text{Lipid concentration (mg/L)} = \frac{A - B}{slope} \quad (2)$$

where A and B are the measured value and the y-intercept, respectively.

$NaNO_3$ and NaH_2PO_4 measurement

The $NaNO_3$ concentration was measured solely in NO_3^- form using the modified method from American Public Health Association (2005). The ultraviolet spectrophotometric method enables the rapid determination of NO_3^-, however the trace dissolved organic matter and various inorganic ions may interfere, especially if the culture medium is prepared from sea water as in this work; therefore, the experiment carried out at 0 mg/L $NaNO_3$ was considered as the contaminant NO_3^- (N_0). The concentration of $NaNO_3$ was calculated by comparison with the standard curve as shown in Eq. (3):

$$\text{NaNO}_3 \text{ concentration (mg/L)} = \frac{(A_{220} - A_{275}) \times D}{slope} - N_0 \quad (3)$$

where A_{220} and A_{275} are the absorbance at 220 nm and 275 nm, respectively, D is the dilution factor and N_0 is the concentration of contaminant NO_3^- carried out at 0 mg/L $NaNO_3$ (measured in milligrams/liter).

The NaH_2PO_4 concentration was measured in PO_4^{2-} form using the modified method from American Public Health Association (2005). The concentration of NaH_2PO_4 was calculated by comparison with the standard curve as shown in Eq. (4):

$$\text{NaH}_2\text{PO}_4 \text{ concentration (mg/L)} = \frac{A_{450} \times D}{slope} \quad (4)$$

where A_{450} is the absorbance at 450 nm and D is the dilution factor.

Kinetic parameter calculation

The corrected cell, lipid and substrate concentrations from observation were calculated using the measured concentrations that had been corrected for the volume changes using Eq. (5) and the kinetic parameters were calculated based on the calculation method described previously (Sirisansaneeyakul et al., 2013):

$$\text{Corrected concentration} = \text{Measured concentration} \times \frac{V_t}{V_0} \quad (5)$$

where V_0 and V_t are the initial volume and volume at any time, respectively, both measured in milliliters.

Fatty acid profile determination

The preparation of fatty acid methyl ester (FAME) was performed with the harvested and extracted dry biomass using the modified method described by Ríos et al. (2013). The organic phase containing FAME was mixed with an internal standard (10 g/L methyl nonadecanoate (C19:0) in toluene) in order to calculate the FAME concentration. The obtained FAMEs were analyzed using the modified method from Sigma-Aldrich Co (2003) using gas chromatography-flame ionization detection (GC 2014; Shimadzu Corp.; Kyoto, Japan) equipped with Agilent J&W gas chromatography columns (HP-FFAP, length: 25 m; diameter: 0.320 mm; film: 0.50 μm; temperature limits: 60 °C–240 °C; Agilent Technologies; Santa Clara, CA, USA). The chromatographic conditions were as follows: injection volume, 1 μL; split ratio, 10:1; inlet temperature, 250 °C; oven temperature, isocratic at 200 °C for 40 min; detector temperature, 260 °C; carrier gas, He. The FAMEs were qualified by comparison with the standard PUFA No. 1 marine source (Supelco LC 01565; Sigma–Aldrich Co.; St. Louis, MO, USA).

Biodiesel quality

The biodiesel qualification was determined based on the FAME compositions. The saponification number (SN), iodine value (IV) and cetane number (CN) of FAMEs were calculated empirically using Eqs. (6)–(8), respectively, which were established to predict their suitability for use as biodiesel (Zhou et al., 2013):

$$SN = \sum(560 \times A_i)/MW_i \quad (6)$$

$$IV = \sum(254 \times Db \times A_i)/MW_i \quad (7)$$

$$CN = 46.3 + (5,458/SN) - 0.225 \times IV \quad (8)$$

where A_i, Db and MW_i are the fatty acid percentage, number of double bonds and molecular weight of each fatty acid, respectively.

Statistical analysis

The effects and the regression analysis of the experimental data obtained from separately triplicated experiments were calculated using the Excel (version 2007; Microsoft Corp.; Redlands, WA, USA), SigmaPlot (version 10.0, 2008; Systat Software, Inc.; Erkrath, Germany) and SPSS (version16.0; SPSS Inc.; Chicago. IL, USA) software packages.

Results and discussion

Effect of nitrogen concentration on algal growth and lipid accumulation

A microalga, *P. tricornutum*, was grown in the culture medium f/2 with different $NaNO_3$ concentrations (0–64.29 mg/L $NaNO_3$ with a fixed 5 mg/L $NaH_2PO_4 \cdot 2H_2O$; Table 1). The initial pH was 7.8 and was not controlled throughout all cultivation. The growth of *P. tricornutum* differed in all treatments (Fig. 1A). An exponential phase showed clearly in the first 96 h of cultivation in all $NaNO_3$ concentrations, followed by the stationary phase until the end of cultivation (Fig. 1A). The maximal cell concentration and algal volumetric production rate (Q_X) were 227.05 ± 20.23 mg/L and 0.39 ± 0.03 mg/L h, respectively, found at 480 h of cultivation using an initial 64.29 mg/L $NaNO_3$ (Fig. 1A; Table 1). As reported previously, *P. tricornutum* (FACHB-863) grown in a 2 L flask containing complete f/2 medium with CO_2 addition, produced a lower biomass of 530 mg/L (Yu et al., 2016). However, the strain has potential to scale up biomass production, that is to 0.88 g/L and 1.29 g/L of dry *P. tricornutum* from the autotrophic and mixotrophic cultivations, respectively (Morais et al., 2009) and to 0.98 g/L in an outdoor photobioreactor (Ak et al., 2015). There was no significant difference ($p \geq 0.01$) among the biomass productivity (Q_X) results obtained at 32.09 mg/L $NaNO_3$ and 64.29 mg/L $NaNO_3$ (Q_X; 0.35 ± 0.04 mg/L h and 0.39 ± 0.03 mg/L h, respectively; Table 1). It was clear that higher nitrogen concentrations resulted in higher biomass productivity which was suitable for the production of algal biomass (C_X, approximately 203–226 mg/L; Table 1). The specific algal growth rate (μ) calculated from the exponential growth phase during 72–96 h of cultivation was considered. The maximal μ found at 32.09 mg/L $NaNO_3$ was 2.47 ± 0.02 × 10^{-2}/h which was higher than at 64.29 mg/L $NaNO_3$ (μ; 2.24 ± 0.02 × 10^{-2}/h; $p < 0.05$). The higher μ, the higher possible concentration and productivity of algal biomass that were obtained. As a result, 32.09 mg/L $NaNO_3$ was best for the production of algal biomass.

It was clear that the first 168 h of cultivation were sufficient to attain the greatest biomass (Fig. 1A). However, without providing $NaNO_3$ initially, the cell concentration and Q_X were at their lowest levels of 79.31 ± 7.84 mg/L and 0.08 ± 0.01 mg/L h, respectively, at 216 h of cultivation (Fig. 1A, Table 1). An insufficient amount of nitrogen limited the algal growth; thus *P. tricornutum* only grew slightly without additional nitrogen being supplied ($NaNO_3$; Fig. 1A). From this result, the contaminant nitrogen source (N_0) from the vitamin solution found in the f/2 medium prepared from sea water, the dissolved organic matter and some inorganic ions from sea water could support the further growth of *P. tricornutum* until these were completely consumed. As expected, the cell concentration increased with an increase in the $NaNO_3$ concentration (Fig. 1A). These results indicated that nitrogen is the major

Table 1
Summary of the kinetic parameters affected by the $NaNO_3$ concentration during the cultivation of *P. tricornutum* in the culture medium f/2 for 504 h.

Parameter	$NaNO_3$ concentration (mg/L)[c]			
	0	16.45	32.09	64.29
C_X (mg/L)	68.57 ± 7.57	165.29 ± 3.78	203.48 ± 21.08	225.81 ± 16.58
Q_X (mg/L h)	0.08 ± 0.01	0.27 ± 0.01	0.35 ± 0.04	0.39 ± 0.03
Q_P (mg/L h)	0.07 ± 0.00	0.08 ± 0.01	0.04 ± 0.02	0.01 ± 0.00
μ[a] (× 10^{-2} /h)	0.87 ± 0.13	2.33 ± 0.03	2.47 ± 0.02	2.24 ± 0.28
q_P (× 10^{-3} mg/mg h)	1.50 ± 0.12	0.95 ± 0.05	0.42 ± 0.19	0.11 ± 0.02
% Maximum lipid content[b]	53.04 ± 3.26	23.02 ± 1.25	9.61 ± 3.89	2.79 ± 0.35

[a] Calculated from an exponential growth phase.
[b] Calculated from the highest lipid concentration.
[c] The concentration subtracted from the contaminant NO_3^- at 0 mg/L $NaNO_3$; ND = not determined; values are shown as means ± SD.

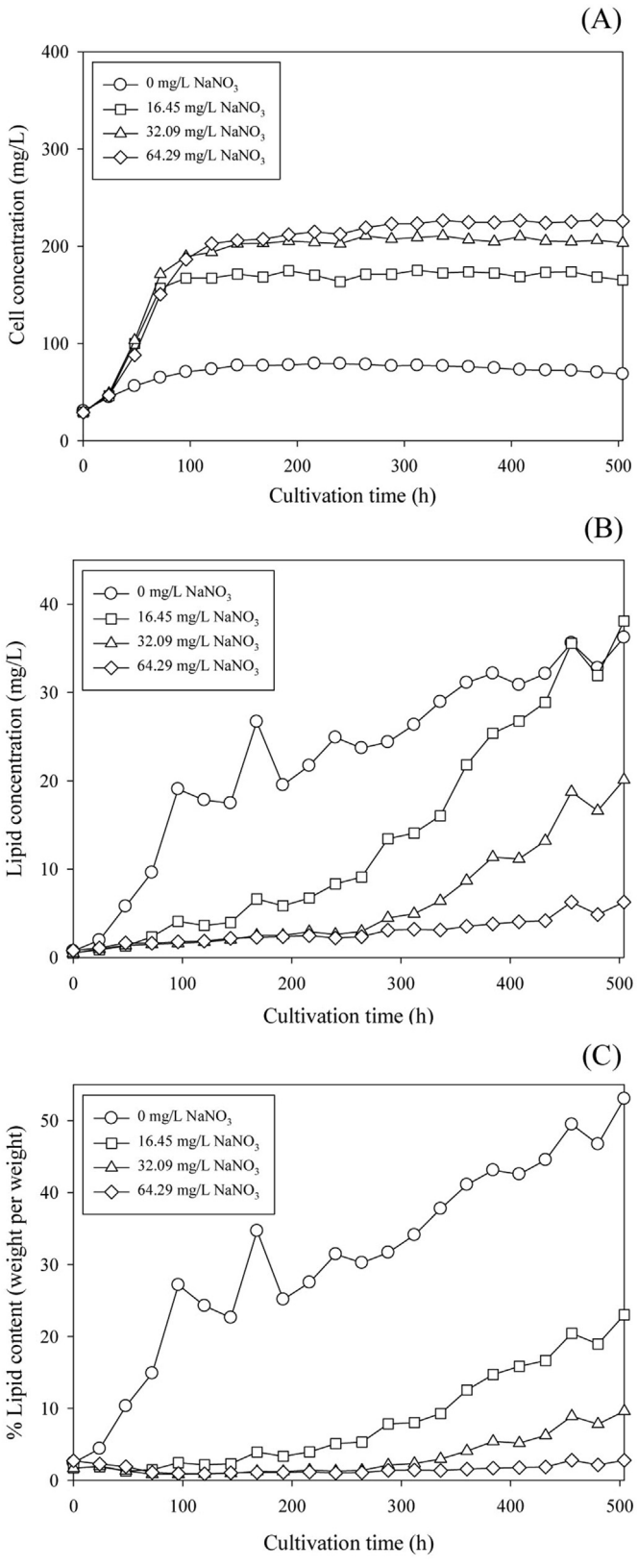

Fig. 1. Changes in (A) cell; (B) lipid concentration; and (C) % lipid content from *P. tricornutum* cultured for 504 h in f/2 medium with varying $NaNO_3$ concentrations (0 mg/L, 16.45 mg/L, 32.09 mg/L and 64.29 mg/L) and a fixed 5 mg/L $NaH_2PO_4 \cdot 2H_2O$.

component for algal growth due to its function in the structural composition of cells and functional proteins such as enzymes in algal cells (Barsanti and Gualtieri, 2006).

The concentration of algal lipid was also observed as shown in Fig. 1B. Though the profiles of the lipid concentration increased with the decreased $NaNO_3$ concentration (Fig. 1B), it differed from the cell concentration profiles (Fig. 1A). The maximal lipid concentration and algal lipid productivity (Q_P) were 38.08 ± 2.91 mg/L and 0.08 ± 0.01 mg/L h, respectively, with 16.45 mg/L $NaNO_3$ at 504 h of cultivation, similar to the lipid concentration (36.23 ± 2.68 mg/L) and Q_P (0.07 ± 0.00 mg/L h) obtained without supplying $NaNO_3$. Although their final concentrations of lipid and Q_P were not significantly ($p > 0.05$) different, their specific rates of lipid production (q_P) were. The maximal q_P was $1.50 \pm 0.12 \times 10^{-3}$ mg/mg h at 0 mg/L $NaNO_3$ attained with the highest lipid content (53.04 ± 3.26%; Fig. 1C) while q_P at $0.95 \pm 0.05 \times 10^{-3}$ mg/mg h was obtained from 16.45 mg/L $NaNO_3$ (Table 1). Clearly, the lower nitrogen concentrations (0 mg/L $NaNO_3$ and 16.45 mg/L $NaNO_3$) provided the higher Q_P, q_P and % lipid content (Table 1). Previously, the nitrogen concentration at 475.5 mg/L KNO_3 produced the maximum lipid content (31.14%) when *Chlorella* sp. TISTR 8990 was grown photoautotrophically in nitrogen-minimal medium, compared with the control condition (Yodsuwan et al., 2015). Therefore, to monitor lipid production, the lower nitrogen concentration was particularly beneficial.

The high lipid accumulation in this study showed similar trends as *P. tricornutum* Bohlin (CCMP2561) cultivated in f/2 medium containing 0.5 mM $NaNO_3$. TAGs were reported to increase after the nitrate depletion at 60 h of cultivation (Ge et al., 2014). The degradation of storage starch might occur, being partially converted to lipid a few days after the stress condition (Zhu et al., 2014). The nitrogen starvation affected algal growth by limiting the protein synthesis rate, resulting from the feedback inhibition of the citric acid cycle. Additionally, it causes insufficient proteins in the photosynthesis system, which decreases the carbon fixation reaction through photosynthesis (Valenzuela et al., 2013). An accumulation of storage lipid (TAGs) is found under such a stress condition, as acetate is assimilated to produce an intracellular carbon via the glyoxylate cycle; therefore, carbohydrate intermediate metabolites are utilized to produce TAGs through the Kennedy pathway (Deng et al., 2011). Another presumed pathway suggested was that under nitrogen depletion, the cellular content of the thylakoid membrane tends to decrease, the acyl hydrolase enzyme is activated and the hydrolysis of phospholipid occurs which may cause an increased content of fatty acid acyl-CoA intracellularly, the precursor in β-oxidation (Goldberg and Cohen, 2006). In addition, the diacylglycerolacyltransferase which is the key enzyme converting acyl-CoA to TAG is also activated (Goldberg and Cohen, 2006). Besides nitrogen depletion, phosphorus depletion also had an impact on lipid accumulation in *P. tricornutum*, however, the effect was less substantial than the nitrogen depletion (Valenzuela et al., 2013). Thus far, *P. tricornutum* could store phosphate as polyphosphate available for cell activity and hence, internal phosphate was not completely limited (Valenzuela et al., 2013). However, the combined depletion of nitrogen and phosphorus had a greater impact on lipid accumulation than that of the individual nitrogen and phosphorus.

It was concluded that the higher nitrogen concentrations (32.09 mg/L $NaNO_3$ and 64.29 mg/L $NaNO_3$ with a fixed 5 mg/L $NaH_2PO_4 \cdot 2H_2O$) and the lower nitrogen concentrations (0 mg/L $NaNO_3$ and 16.45 mg/L $NaNO_3$ with a fixed 5 mg/L $NaH_2PO_4 \cdot 2H_2O$)

provided higher μ and q_P, respectively, which can possibly be exploited as a monitoring parameter in a two-stage culture system consecutively controlled for the separate production of algal biomass—at higher nitrogen concentrations (32.09 mg/L $NaNO_3$ and 64.29 mg/L $NaNO_3$) for the higher μ—and of lipid—at lower nitrogen concentrations (0 mg/L $NaNO_3$ and 16.45 mg/L $NaNO_3$) for the higher q_P. Interestingly, it was also concluded that q_P was a function of μ, which resulted in (i) the higher μ (also higher Q_X) providing the lower q_P (also lower Q_P) as indicated in the growth phase, and vice versa, (ii) the lower μ (also lower Q_X) gave the higher q_P (also higher Q_P) as indicated in the lipid phase. Thus, the higher q_P and Q_P expected in the lipid phase with deficient $NaNO_3$ (0 mg/L $NaNO_3$) is desired in the second-stage culture, while the highest Q_X obtained from the growth phase with the highest μ is desired at 32.09 mg/L $NaNO_3$ rather than 64.29 mg/L $NaNO_3$ in the first-stage culture.

Fatty acid profile of algal oil and the biodiesel quality based on fatty acid methyl ester composition

The fatty acid profiles in microalgal oil obtained from the algal cultures with varying $NaNO_3$ concentrations were investigated (Table 2). The main saturated fatty acid was palmitic acid (C16:0), while palmitoleic acid (C16:1c) was the main unsaturated fatty acid, as reported previously in the wild type of *P. tricornutum* (Pt_WT) grown in the stationary phase with varying temperatures (Hamilton et al., 2014), and from some similar works (Řenzanka et al., 2012; Valenzuela et al., 2013). A large increase in C16:0 and C16:1 with aged culture was also found in the diatom *Thalassiosire pseudonana*. As the cell division altered, both C16:0 and C16:1 could be formed and provided as a form of energy storage (Fisher and Schwarzenbach, 1978). In the current study, the lower nitrogen concentration cultures (0 mg/L $NaNO_3$ and 16.45 mg/L $NaNO_3$) resulted in higher levels of saturated fatty acids than in the higher nitrogen concentration cultures (32.09 mg/L $NaNO_3$ and 64.29 mg/L $NaNO_3$) in the exponential growth phase (first 96 h of cultivation). The saturated fatty acids in the cultures added with various amounts of $NaNO_3$ were clearly higher in the first 144 h of cultivation, and the unsaturated fatty acids were higher in the latter phase (data not shown). Not only C16:0, but also oleic acid (C18:0) increased in older cultures, especially those with a higher nitrogen concentration (32.09 mg/L $NaNO_3$ and 64.29 mg/L $NaNO_3$) as shown in Table 2. Oleic acid was found to play a key role in the lipid metabolism of *P. tricornutum* as a precursor of long chain polyunsaturated fatty acids (PUFAs) during the exponential growth phase (Hamilton et al., 2014). Therefore, greater amounts of PUFAs were found in the exponential growth phase in all cultures (Table 2). However, among the unsaturated fatty acids, monounsaturated fatty acid (MUFA) was higher than PUFA in all algal cultures, especially, at 0 mg/L $NaNO_3$ where it was 5–6 fold higher, whereas 1–2 fold more MUFA was produced from the cultures with a higher nitrogen concentration. At the 0 mg/L $NaNO_3$ level, there was profound lipid production in the second stage algal cultivation, producing qualified algal oil (with higher levels of saturated fatty acids and MUFA) for the biodiesel production.

The biodiesel quality specified using the saponification number (SN), iodine value (IV) and cetane number (CN) was calculated from FAME compositions (Eqs. (6)–(8), respectively) according to Hoekman et al. (2012) and the results are shown in Fig. 2A–C, respectively. The calculated SN was maximally obtained from 16.45 mg/L $NaNO_3$ at 480 h culture time, when a large amount of fatty acid content was produced (Fig. 2A). The calculated SN increased in all algal cultures with the addition of $NaNO_3$, except in the culture where no $NaNO_3$ was added. The fatty acid contents from the algal culture without $NaNO_3$ decreased visibly in later

Table 2
Fatty acid profiles (means ± SD) of algal oil from different culture times of *P. tricornutum* in f/2 medium containing different $NaNO_3$ concentrations.

% Fatty acid composition	$NaNO_3$ concentration (mg/L)											
	0			16.45			32.09			64.29		
	Cultivation time (h)			Cultivation time (h)			Cultivation time (h)			Cultivation time (h)		
	0	96	504	0	96	504	0	96	504	0	96	504
C14:0	2.11 ± 0.95	4.82 ± 0.46	3.43 ± 1.67	2.40 ± 0.82	6.91 ± 0.97	7.49 ± 0.77	1.62 ± 0.73	5.18 ± 2.40	8.30 ± 1.68	2.10 ± 0.14	8.59 ± 1.70	7.56 ± 0.75
C16:0	5.63 ± 0.71	24.55 ± 0.89	17.73 ± 8.40	3.11 ± 1.54	19.31 ± 2.36	21.64 ± 4.74	5.01 ± 3.33	13.23 ± 6.81	17.08 ± 2.84	8.86 ± 0.71	19.35 ± 0.99	17.81 ± 3.19
C16:1	1.97 ± 0.37	17.47 ± 1.92	13.07 ± 6.03	0.92 ± 0.25	13.41 ± 2.46	21.45 ± 4.85	0.80 ± 0.22	8.71 ± 4.25	18.87 ± 3.82	0.83 ± 0.13	11.99 ± 3.04	17.83 ± 3.92
C18:0	1.26 ± 1.74	0.77 ± 0.29	0.53 ± 0.33	0.40 ± 0.22	1.84 ± 0.41	1.39 ± 0.11	0.35 ± 0.28	2.22 ± 0.75	2.67 ± 0.65	0.11 ± 0.02	2.51 ± 1.28	3.52 ± 0.92
C18:1n9c	0.05 ± 0.05	1.39 ± 1.26	0.87 ± 0.31	0.17 ± 0.07	0.42 ± 0.03	0.57 ± 0.03	0.09 ± 0.06	0.59 ± 0.19	0.59 ± 0.05	0.13 ± 0.07	0.50 ± 0.14	0.77 ± 0.04
C18:1ω7c	0.08 ± 0.09	0.76 ± 0.36	1.04 ± 0.43	0.31 ± 0.16	0.74 ± 0.16	0.48 ± 0.17	0.07 ± 0.04	0.82 ± 0.28	0.45 ± 0.13	0.15 ± 0.09	0.80 ± 0.23	0.49 ± 0.15
C18:2ω6c	0.03 ± 0.04	0.61 ± 0.13	1.65 ± 1.45	0.12 ± 0.13	0.97 ± 0.16	0.86 ± 0.13	0.09 ± 0.09	1.27 ± 0.32	0.96 ± 0.07	0.08 ± 0.06	0.91 ± 0.18	1.25 ± 0.16
C18:4ω3	0.05 ± 0.08	0.31 ± 0.11	1.22 ± 1.54	0.20 ± 0.32	0.22 ± 0.14	0.26 ± 0.07	0.04 ± 0.02	0.26 ± 0.06	0.18 ± 0.10	0.11 ± 0.03	0.26 ± 0.17	0.15 ± 0.05
C20:1ω9c	0.02 ± 0.03	0.09 ± 0.01	0.68 ± 0.60	0.17 ± 0.13	0.28 ± 0.21	0.40 ± 0.56	0.03 ± 0.04	0.39 ± 0.10	0.19 ± 0.13	0.08 ± 0.02	0.46 ± 0.15	0.17 ± 0.09
C20:5ω3c	0.17 ± 0.27	3.26 ± 0.60	1.81 ± 1.25	0.13 ± 0.16	4.15 ± 0.61	5.11 ± 1.65	0.07 ± 0.03	4.84 ± 2.61	8.22 ± 2.82	0.15 ± 0.06	3.31 ± 1.24	9.24 ± 2.31
C22:1n11	0.20 ± 0.26	0.05 ± 0.08	0.07 ± 0.08	0.13 ± 0.16	0.79 ± 0.08	1.16 ± 0.46	0.07 ± 0.05	0.56 ± 0.11	0.94 ± 0.03	0.12 ± 0.15	0.90 ± 0.67	1.18 ± 0.38
C22:1ω9c	0.14 ± 0.20	0.07 ± 0.12	0.05 ± 0.05	0.16 ± 0.06	1.32 ± 0.23	1.56 ± 0.57	0.03 ± 0.02	0.77 ± 0.42	1.30 ± 0.80	0.13 ± 0.14	1.32 ± 0.93	1.59 ± 0.59
Sat.[a]	9.00 ± 3.40	30.14 ± 1.61	21.69 ± 10.40	5.91 ± 2.58	28.06 ± 3.74	30.52 ± 5.62	6.97 ± 4.34	20.64 ± 9.96	28.05 ± 5.17	11.07 ± 0.87	30.45 ± 3.97	28.89 ± 4.86
Unsat.[b]	2.71 ± 1.39	24.01 ± 4.59	20.46 ± 11.74	2.31 ± 1.44	22.30 ± 4.08	31.85 ± 8.49	1.29 ± 0.57	18.21 ± 8.34	31.70 ± 7.65	1.78 ± 0.74	20.45 ± 6.75	32.67 ± 7.69
MUFA[c]	2.46 ± 1.00	19.83 ± 3.75	15.78 ± 7.50	1.86 ± 0.83	16.96 ± 3.17	25.62 ± 6.64	1.09 ± 0.43	11.84 ± 5.35	22.34 ± 4.96	1.44 ± 0.59	15.97 ± 5.16	22.03 ± 5.17
PUFA[d]	0.25 ± 0.39	4.18 ± 0.84	4.68 ± 4.24	0.45 ± 0.61	5.34 ± 0.91	6.23 ± 1.85	0.20 ± 0.14	6.37 ± 2.99	9.36 ± 2.99	0.34 ± 0.15	4.48 ± 1.59	10.64 ± 2.52

[a] Sat. = saturated fatty acid.
[b] Unsat. = unsaturated fatty acid.
[c] MUFA = monounsaturated fatty acid.
[d] PUFA = polyunsaturated fatty acid.

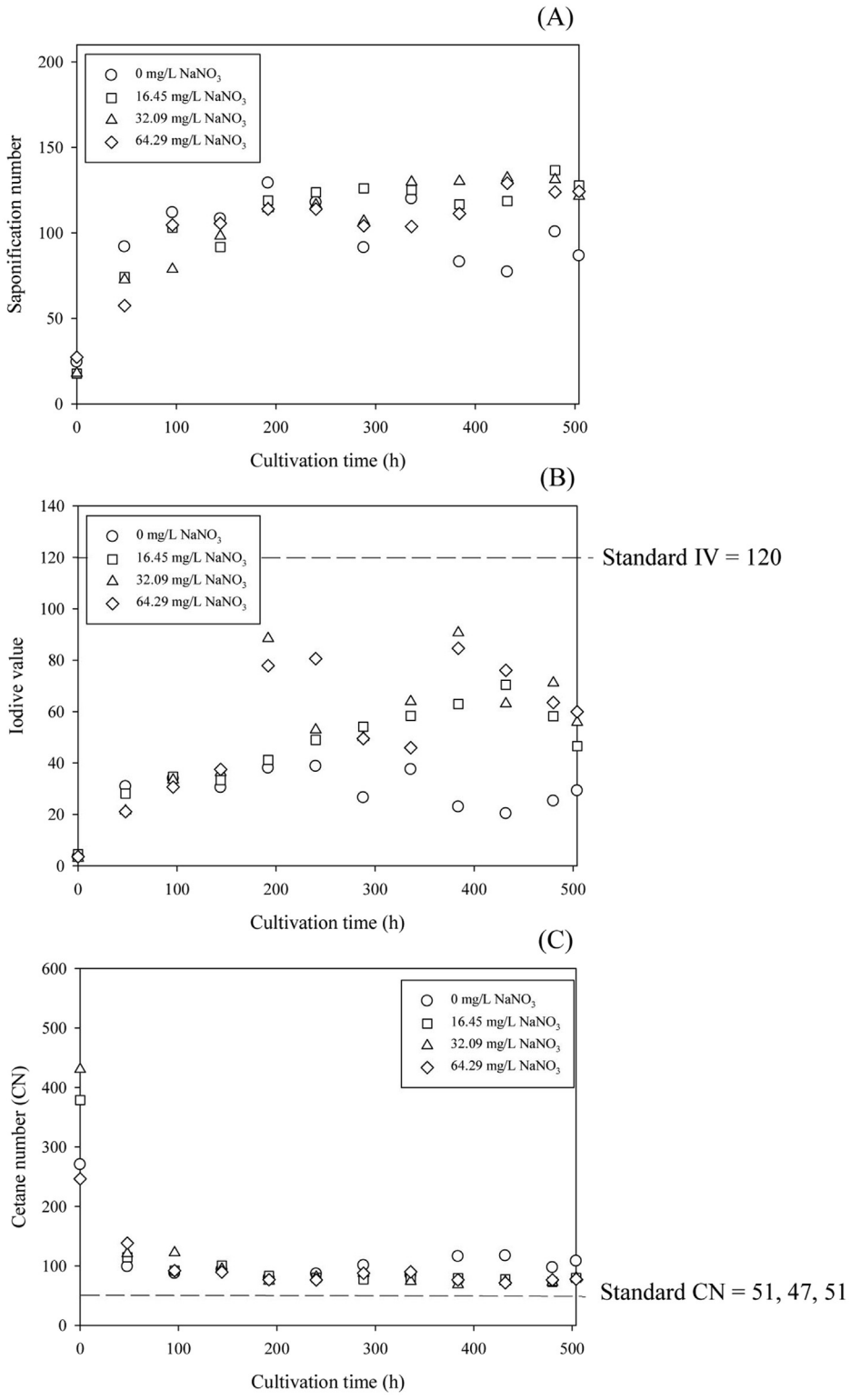

Fig. 2. Characterization of biodiesel verified using fatty acid methyl ester composition from *P. tricornutum* cultivated for 504 h in f/2 medium with varying $NaNO_3$ concentrations (0 mg/L, 16.45 mg/L, 32.09 mg/L and 64.29 mg/L) and a fixed 5 mg/L $NaH_2PO_4 \cdot 2H_2O$. (A) Saponification number; (B) iodine value; (C) cetane number.

cultivation. The calculated IV values varied in all algal cultures (Fig. 2B). The algal oils obtained from the higher nitrogen concentration cultures with the higher unsaturated fatty acids resulted in higher IV values. These calculated IV values were lower than the 120 IV level specified by the European (EN 14214) standard (Hoekman et al., 2012). The CN values were high, when calculated from early cultivation in all algal cultures (Fig. 2C). However, standard biodiesel is also qualified by SN and IV values. The CN values were calculated to be highest in the first 48–96 h of cultivation from all algal cultures except for the culture without any $NaNO_3$ added, where it was highest after 432 h of cultivation. The CN values calculated from all algal cultures were acceptable and

exceeded the minimum specification value of the biodiesel standards for Thailand (2007), the USA (ASTM D6751-08) and Europe (EN 14214) being 51, 47 and 51, respectively (Hoekman et al., 2012). Additionally, the maximal CN values were attainable whenever the amount of saturated fatty acids was greater than the unsaturated fatty acids.

Considering biodiesel quality, the algal cultures obtained from the lower nitrogen concentration cultures (0 mg/L $NaNO_3$ and 16.45 mg/L $NaNO_3$) attained with high saturated/low unsaturated fatty acids were candidates for biodiesel production. The saturated fatty acids had a less negative effect on transesterification; however, their biodiesel quality can be considerably improved (Hu et al., 2008). Biodiesel produced from oil containing a high level of saturated fatty acid shows higher oxidative stability, as well as a higher CN value, which provide high quality biodiesel (Hu et al., 2008; Hoekman et al., 2012). On the other hand, oils containing high levels of unsaturated fatty acids are not resistant to oxidation during prolonged storage (Hu et al., 2008). Generally, for biodiesel production, oils are preferred with a higher degree of saturation as substrates because there is less oxidation (Knothe, 2005; Chisti, 2007). Here, the algal culture without adding $NaNO_3$ gave a higher level of saturated fatty acids and lower unsaturated fatty acids, which resulted in the lower IV value compared with that from algal culture with 16.45 mg/L $NaNO_3$. However, both algal oils had lower SN values. Unfortunately, their CN values were higher than the biodiesel standards. The CN value calculated from the algal culture without adding $NaNO_3$ with 192–240 h of cultivation was lower than that in the algal culture with 16.45 mg/L $NaNO_3$, which did meet the standards (no significant difference at $p < 0.01$). This suggested that the algal oil obtained from the algal culture without adding $NaNO_3$ should be harvested at 240 h culture time as the best potential candidate for biodiesel production. This corresponded concomitantly to the algal lipid content described above.

Interestingly, PUFA, *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA; C20:5ω3c, omega-3), was noteworthy in the algal oil obtained from the higher nitrogen concentration cultures; for example, with levels of 17.17% and 16.26% from the algal cultures with 32.09 mg/L $NaNO_3$ and 64.29 mg/L $NaNO_3$, respectively. Thus, the algal culture with the higher added nitrogen concentration was recommended for accumulating PUFA, that is, EPA (C20:5ω3c), which was the major PUFA in *P. tricornutum* (approximately 35%) according to Hamilton et al. (2014). It is known that PUFA plays a key role in human health, especially in infant growth and development, maintaining cardiovascular health in adults and the prevention and treatment of other medical disorders such as inflammation and cancer (Voigt et al., 2000; Das, 2002; Calder, 2003). The highest % EPA in the current work is the first reported based on the authors' knowledge along with the potential to be controlled using an optimal level of $NaNO_3$, for example, 32.09 mg/L $NaNO_3$ as reported here.

This work illustrated that the algal biomass produced and lipid accumulated from the culture of *P. tricornutum*, were enhanced clearly by using sufficient (up to 32.09 mg/L $NaNO_3$) and by a deficiency or no $NaNO_3$, consecutively. As a state-of-the-art development, the specific growth rate was used successfully to monitor algal growth. The high oil accumulation in algal cells (in the biomass cultured using sufficient-$NaNO_3$ during growth) and later under deficient-$NaNO_3$ stress conditions, showed promise for the use of qualified feedstock/oil for biodiesel production. The current study indicated that two-stage cultivation (consecutively under the sufficient-/deficient-$NaNO_3$ conditions) has potential to produce high algal biomass containing high quality oil.

Conflict of interest

The authors declare no conflict of interest.

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