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Outdoor Photoautotrophic Cultivation of *Chlorella* sp. TISTR 8990 in Nitrogen- and Phosphorus-Minimal Media for Lipid Accumulation

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ABSTRACT

The nitrogen/phosphorus pool available during phases of microalgal growth plays an important role in obtaining higher biomass and lipid accumulation. The current study was based upon the optimization of the microalga, *Chlorella* sp. TISTR 8990 using a photoautotrophic culture in an open 6 L drinking water bottle with a total working volume of 4 L and continuous sparging with 1% (v/v) CO_2 mixed air at a flow rate of 0.67 volume per liquid volume per minute. The effects were investigated of nitrogen (0, 258.5, 317.0 and 475.5 mg.L^{-1} KNO_3 at fixed 1 g.L^{-1} KH_2PO_4) and phosphorus (0, 19.4, 38.8 and 58.2 mg.L^{-1} KH_2PO_4 at fixed 2 g.L^{-1} KNO_3) concentrations on the biomass and lipid production. The results showed that 258.5 mg.L^{-1} KNO_3 and 38.8 mg.L^{-1} KH_2PO_4 maximized the cell concentrations after 144 hr of cultivation at 473.7 and 455.0 mg.L^{-1}, respectively. On the other hand, 31% and 22% lipid contents were obtained in the basal medium with 475.5 mg.L^{-1} KNO_3 and 38.8.mg.L^{-1} KH_2PO_4, respectively. The biomass concentration and lipid content were highest in the nitrogen-minimal medium, but at different initial concentrations of KNO_3 (258.5 and 475.5 mg.L^{-1} KNO_3, respectively). In addition, palmitic acid (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3) were mostly found in the microalgal oil obtained from all treatments. It was concluded that KNO_3 and KH_2PO_4 in the basal media assisted in producing the optimal microalgal growth and lipid accumulation in cells. However, the nitrogen-minimal medium showed potential for lipid production on the basis of the lipid content. In addition, the biodiesel quality specified using the saponification number, iodine value and cetane number were calculated from the fatty acid methyl ester composition. It was also found that fatty acids derived from the nitrogen-minimal medium are a higher potential source for biodiesel production than those from the phosphorus-minimal medium.

Keywords: lipid accumulation, microalgal growth, nitrogen, KNO_3, phosphorus, KH_2PO_4

INTRODUCTION

Currently, the energy crisis has become a major problem, especially the predicted shortfall in fossil fuels (Leung *et al*., 2010). Thus, environmentally friendly energy sources are gaining more attention and recently, microalgae have been suggested as being able to play a key role in the energy crisis as lipids can accumulate in their cells as triacylglycerols (TAGs), and so microalgae are

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interesting as feedstock for biodiesel production (Powell and Hill, 2010; Collet *et al*., 2011; Yang *et al*., 2011). Moreover, microalgae can be grown with minimal requirements for cultivation area in comparison with other energy crops (Rubens, 2008).

Generally, microalgae can grow phototrophically and store neutral lipids as TAGs, which are a main substrate for biodiesel production (Chen *et al*., 2011). This lipid form can be reacted with alcohol under appropriate conditions via a transesterification reaction and produce biodiesel (Chisti, 2007). Thus, a high amount of TAG is needed. There are several methods that can be used for lipid induction during microalgal cultivation. One of the stress conditions on microalgal cultivation that produces high TAG is nitrogen and/or phosphorus starvation (Roessler, 1990; Weldy and Huesemann, 2007; Wu and Hsieh, 2008; Widjaja *et al*., 2009; Chen *et al*., 2011). Many researchers have reported that microalgae cultivated under nitrogen-starved conditions could accumulate lipids in their cells with the oil yield depending on the species (Weldy and Huesemann, 2007; Wu and Hsieh, 2008; Meng *et al*., 2009; Chen *et al*., 2011; Wu *et al*., 2012; Liang *et al*., 2013).

Microalgae can be cultivated under various conditions including photoautotrophic, heterotrophic and mixotrophic conditions. However, photoautotrophic cultivation has been found to be technically and economically feasible for large scale microalgal biomass production (Brennan and Owende, 2010). In addition, open pond systems such as a raceway pond are well known for microalgal biomass production (Chisti, 2007; Brennan and Owende, 2010). In the current study, the effects were investigated of nitrogen and phosphorus concentrations on the lipid accumulation of *Chlorella* sp. TISTR 8990 cultivated under outdoor conditions. In addition, the fatty acid profile from algal biomass was studied and the feasibility of derived microalgal oil for biodiesel production was also determined. The obtained information from the outdoor cultivation in drinking water bottles should indicate the potential for scaling up in raceway ponds for larger amounts of biomass and lipid production.

MATERIALS AND METHODS

Microalgal strain and preculture

Chlorella sp. TISTR 8990 (Sirisansaneeyakul *et al*., 2011) was cultivated in 50 mL Horikoshi basal medium containing 2 $g.L^{-1}$ KNO_3, 1 $g.L^{-1}$ KH_2PO_4, 1 $g.L^{-1}$ $MgSO_4 \cdot 7H_2O$, 2 $mg.L^{-1}$ $FeSO_4 \cdot 7H_2O$, 2.86 $mg.L^{-1}$ H_3BO_3, 1.81 $mg.L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.22 $mg.L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.08 $mg.L^{-1}$ $CuSO_4.5H_2O$ and 0.021 $mg.L^{-1}$ Na_2MoO_4 (Horikoshi *et al*., 1981) in a 200 mL microalgal tube. The microalga was incubated at 30 ºC and pH 6.0 under a light intensity of 15 klux (260 $\mu mol.m^{-2}.s^{-1}$). A diurnal cycle of 16:8 hr light:dark was applied. The medium was continuously sparged with air mixed with 1–2% (volume per volume, v/v) CO_2 at a flow rate of 0.67 gas volume per liquid volume per minute (vvm). The inoculum was incubated for 7 d and then transferred into 150 mL Horikoshi basal medium and cultivated under the same conditions as described previously for 7 d.

Microalgal growth

Microalgal inoculum was photoautotrophically cultured in an open 6 L drinking water bottle with a working volume of 4 L. The culture was grown outdoors and continuously sparged with air mixed with 1% (v/v) CO_2 at a flow rate of 0.67 vvm for 7 d. The effects of nitrogen- and phosphorus-minimal media varying with four levels of an initial KNO_3 concentration (0, 258.5, 317.0 and 475.5 $mg.L^{-1}$) at a fixed 1 $g.L^{-1}$ KH_2PO_4, and with four levels of initial KH_2PO_4 concentration (0, 19.4, 38.8 and 58.2 $mg.L^{-1}$) at a fixed 2 $g.L^{-1}$ KNO_3, respectively, in accordance with all treatments were considered. Samples were collected daily for subsequent analysis.

Analytical methods

The microalgal growth was measured at an optical density of 680 nm and its dried weight was measured using the method described by Sirisansaneeyakul *et al*. (2011) and Puchcha *et al*. (2012). The lipid content was measured using the modified method from Işik *et al*. (1999). The nitrogen (KNO_3) and phosphorus (KH_2PO_4) concentrations were determined using the modified methods from American Public Health Association (2005). The kinetic parameters were calculated based on the method described by Sirisansaneeyakul and Wongkongkatep (1995) and Sirisansaneeyakul (2011).

The free fatty acids were prepared by harvesting and extracting wet microalgal biomass followed using the modified method described by Tran *et al*. (2012). Then, the preparation of fatty acid methyl ester (FAME) was carried out using the modified method of Klinkeson *et al*. (2004). The obtained FAMEs were analyzed using the modified method from Sigma-Aldrich Co. (2003) using a gas chromatography-flame ionization detector, GC-FID (Network GC system, Agilent Technologies 6890N; Agilent Technologies; Santa Clara, CA, USA) equipped with Agilent J&M GC columns, (HP-FFAP, length, 25 m; diameter, 0.320 mm; film, 0.50 µm; temperature limits: 60–240 °C). The chromatographic conditions were: injection volume 1 µL; split ratio 50:1; inlet temperature 250 °C; initial oven temperature was maintained at 140 °C for 5 min, then increased with flow rate at 4 °C.min^{-1} until 230 °C and held at 250 °C for 5 min; with a detector temperature of 250 °C using helium as the carrier gas at a flow rate at 1 mL.min^{-1}. The FAMEs were fitted for comparison with the external standard FAME mixes of C8:0–C22:0 (Supelco LB80284) and C8:0–C24:0 (Supelco LB94466).

The saponification number (SN), iodine value (IV) and cetane number (CN) of the FAMEs were calculated empirically using Equations 1–3, respectively, which were established to predict their suitability for use as biodiesel (Mohibbe Azam *et al*., 2005; Zhou *et al*., 2013).

$$SN = \Sigma(560 \times A_i) / MW_i \quad (1)$$

$$IV = \Sigma(254 \times D \times A_i) / MW_i \quad (2)$$

$$CN = 46.3 + 5458 / SN - 0.225 \times IV \quad (3)$$

where A_i is the fatty acid percentage, D is the number of double bonds and MW_i is the molecular weight of each fatty acid.

Statistical analysis

The effects and the regression analysis of the experimental data obtained from the duplicated experiments were calculated using Microsoft® Excel 2007 (Microsoft Corporation; Redlands, WA, USA) and SigmaPlot® 10.0 (2008 Systat Software, Inc.; Erkrath, Germany).

RESULTS AND DISCUSSION

Microalgal growth and lipid accumulation of *Chlorella* sp. TISTR 8990

Cell concentrations showed an increasing trend for almost all treatments (Figures 1a and 1c), except with that of 0 mg.L^{-1} KNO_3 concentration in nitrogen-minimal medium, which decreased after 96 hr cultivation (Figure 1a). The highest cell concentrations were obtained from the treatment which had the KNO_3 concentration at 258.5 mg.L^{-1} (473.7 mg.L^{-1} biomass, Figure 1a) and the KH_2PO_4 concentration at 38.8 mg.L^{-1} (455.0 mg.L^{-1} biomass, Figure 1c) after 144 hr cultivation. The microalgae cultivated in the phosphorus-minimal medium showed a higher growth trend than in the nitrogen-minimal medium because of its higher nitrogen content (2 g.L^{-1} KNO_3) and sufficient phosphate sources for growth requirements during the cultivation which agreed with Chen *et al*. (2011). These results indicated that nitrogen is essential for cell growth due to its function in the components of all structural and functional proteins in algal cells (Barsanti and Gualtieri, 2006).

In addition, the lipid concentrations fluctuated in both medium types. The highest lipid concentrations were found after 96 hr and 120 hr

cultivation in nitrogen- (80.0 mg.L^{-1} lipid, Figure 1b) and phosphorus- (90.8 mg.L^{-1} lipid, Figure 1d) minimal media, respectively. It was thought that it would be economical if the microalgae could be cultivated in either nitrogen- or phosphorus-minimal medium for a short period (approximately 120 hr or 5 d) to attain high lipid productivity.

Generally, nitrogen depletion resulted in a decreasing rate of protein synthesis resulting in feedback inhibition in the citric acid cycle and also photosynthesis impairment which led to insufficient proteins for the photosystem reaction center and for photosynthetic electron transport. These conditions caused a substantial decrease in carbon fixation through photosynthesis. The acetate assimilation produces the mainly intercellular carbon via the glyoxylate cycle in which carbohydrate intermediate metabolites are utilized to produce TAGs through the Kennedy pathway with the accumulation of TAGs in the microalgal cell (Deng *et al*., 2011).

Besides the reduction of growth and photosynthetic rates as described above, nitrogen depletion leads to a reduction in the respiratory rate. Moreover, such conditions may cause a decrease in the cellular content of the thylakoid membrane, activation of acyl hydrolase and stimulation of the phospholipid hydrolysis. These phenomena may increase the intracellular content of fatty acid acyl-CoA (Goldberg and Cohen, 2006; Rodolfi *et*

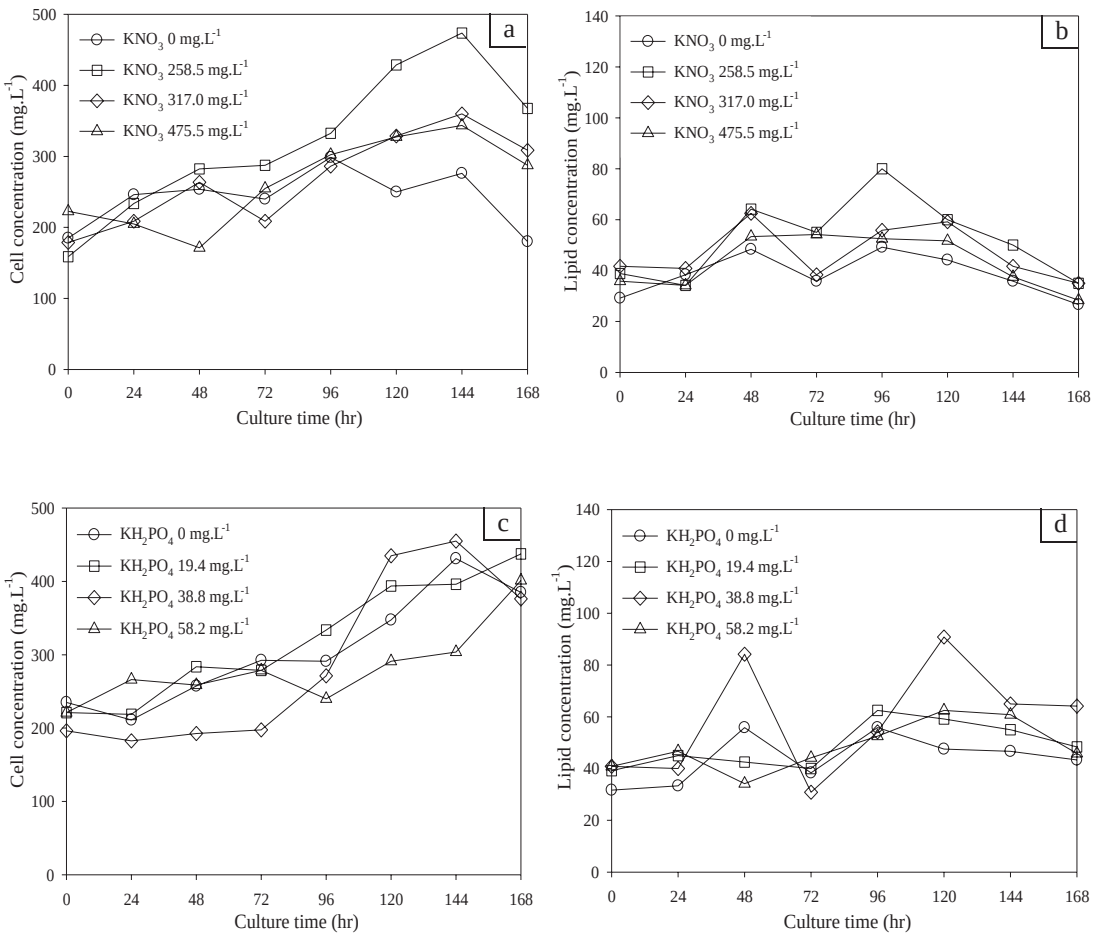

Figure 1 Changes in cell (a and c) and lipid (b and d) concentrations of *Chlorella* sp. TISTR 8990 cultivated in Horikoshi basal medium with different KNO_3 (a and b) and KH_2PO_4 (c and d) concentrations for 168 hr.

al., 2009). Furthermore, nitrogen limitation could also activate diacylglycerolacyltransferase which converts acyl-CoA to TAG (Barsanti and Gualtierj, 2006).

Phosphorous is also a vital constituent of DNA and RNA. The lack of this element causes incomplete cell division, leading to the interruption of cell growth and also the reduction of phospholipids synthesis which will promote the synthesis of TAGs (Deng *et al*., 2011).

Kinetic parameters for microalgal growth and lipid accumulation

The maximum specific growth rates occurred at 0.0076 and 0.0058 hr^{-1} in nitrogen- (258.5 $mg.L^{-1}$ KNO_3) and phosphorus- (38.8 $mg.L^{-1}$ KH_2PO_4) minimal media, respectively (Table 1). In addition, the nitrogen-minimal medium without KNO_3 produced the lowest specific growth rate, consistent with the necessity of a nitrogen source for growth as described above. The greatest volumetric rates of biomass production were 2.19 and 1.80 $mg.L^{-1}.hr^{-1}$ in nitrogen- (258.5 $mg.L^{-1}$ KNO_3) and phosphorus- (38.8 $mg.L^{-1}$ KH_2PO_4) minimal media, respectively. They were higher than the biomass productivity (1.5 $mg.L^{-1}.hr^{-1}$) maximized from *C. zofingiensis* cultured in a 10 L photobioreactor under outdoor conditions (Feng *et al*., 2012). However, these highest volumetric rates of biomass production were 23 and 19% of that obtained from *Chlorella* sp. (9.27 $mg.L^{-1}.hr^{-1}$) which was cultivated in a 70 L photobioreactor under outdoor conditions using 0.1 $g.L^{-1}$ urea as a nitrogen source (Zhou *et al*., 2013).

The highest volumetric rate of lipid production ($Q_{P,N}$) was obtained with the nitrogen-minimal medium (Table 1). The KNO_3 concentration at 317.0 $mg.L^{-1}$ produced the highest $Q_{P,N}$ of 0.434 $mg.L^{-1}.hr^{-1}$, while the lowest occurred in the absence of KNO_3 (0.208 $mg.L^{-1}.hr^{-1}$). In the phosphorus-minimal medium, the highest volumetric rate of lipid production ($Q_{P,P}$) was 0.417 $mg.L^{-1}.hr^{-1}$ at 38.8 $mg.L^{-1}$ KH_2PO_4, while the other KH_2PO_4 concentrations gave lower $Q_{P,P}$ values than those $Q_{P,N}$ values found in the nitrogen-minimal medium. In comparison, *Chlorella* sp. grown in 27.5 and 41.3 $mg.L^{-1}$ KH_2PO_4 showed higher lipid productivity at

Table 1 Summary of kinetic parameters: cell concentrations ($C_{X,N}$ and $C_{X,P}$), specific growth rates (μ_N and μ_P), volumetric rate of biomass production ($Q_{X,N}$ and $Q_{X,P}$), volumetric rate of lipid production ($Q_{P,N}$ and $Q_{P,P}$) and maximum lipid content percentage affected by nitrogen (KNO_3) and phosphorus (KH_2PO_4) sources.

Initial KNO_3 concentration[1] ($mg.L^{-1}$)	N/P ratio[3]	$C_{X,N}$ ($mg.L^{-1}$)[4]	$Q_{X,N}$ ($mg.L^{-1}.hr^{-1}$)[4]	μ_N (hr^{-1})[4]	$Q_{P,N}$ ($mg.L^{-1}.hr^{-1}$)[5]	Maximum lipid content (%)
0	0.005	276.2±160.9	0.63±0.48	0.0028±0.0007	0.208	19.05
258.5	0.460	473.7±61.9	2.19±0.83	0.0076±0.0035	0.429	24.46
317.0	0.523	360.0±70.7	1.26±0.83	0.0049±0.0002	0.434	23.70
475.5	0.835	343.7±93.7	0.84±0.21	0.0030±0.021	0.255	31.14
Initial KH_2PO_4 concentration[2] ($mg.L^{-1}$)	N/P ratio[3]	$C_{X,P}$ ($mg.L^{-1}$)[4]	$Q_{X,P}$ ($mg.L^{-1}.hr^{-1}$)[4]	μ_P (hr^{-1})[4]	$Q_{P,P}$ ($mg.L^{-1}.hr^{-1}$)[5]	Maximum lipid content (%)
0	340.90	431.3±26.5	1.36±0.45	0.0042±0.0024	0.252	21.68
19.4	137.61	396.2±19.4	1.22±0.25	0.0040±0.0014	0.243	20.57
38.8	101.41	455.0±21.2	1.80±0.77	0.0058±0.0036	0.417	21.92
58.2	65.36	303.7±79.6	0.57±0.61	0.0022±0.0040	0.181	21.87

[1,2] = Initial KH_2PO_4 and KNO_3 were 1 and 2 $g.L^{-1}$, respectively.

[3] = Calculated from experimental data.

[4] = Calculated from 0-144 hr cultivation.

[5] = Based on maximum lipid concentration.

0.371 and 0.267 $mg.L^{-1}.hr^{-1}$, respectively (Liang *et al*., 2013). In addition, the biomass and lipid productivities from both minimal media showed higher values than those obtained from the marine unicellular microalgae, *Chaetoceros muelleri* and *Dunaliella salina*, cultured under nutrient-deprived conditions, but their lipid contents were lower (Gao *et al*., 2013). Lipid productivity is suggested as the key parameter to use in the selection of algal species for biodiesel production (Griffiths and Harrison, 2009).

The lipid yield based on algal biomass (namely, the maximum lipid content percentage) was somewhat superior in the nitrogen-minimal medium compared to the phosphorus-minimal medium (Table 1). This result indicates that the nitrogen limitation affected lipid accumulation more than the phosphorus limitation did. In the nitrogen-minimal medium, the maximum lipid content was 31.14% (at 475.5 $mg.L^{-1}$ KNO_3); however, the volumetric rate of lipid production ($Q_{P,N}$, 0.255 $mg.L^{-1}.hr^{-1}$) was not attractive in all treatments (Table 1). The results were similar to those for *Scenedesmus* sp. LX1 cultured in a nitrogen-minimal medium, which resulted in a highest lipid content of 30% (Xin *et al*., 2010), while *C. vulgaris* was reported to produce a highest lipid content of 40% in low nitrogen-containing medium (Illman *et al*., 2000). *C. vulgaris* ESP-31 was found to increase the lipid content from 20.9 to 55.9% when the nitrogen concentration decreased from 1.25 to 0.31 $g.L^{-1}$ (Yeh and Chang, 2011), whereas, *C. vulgaris* grown in a minimal medium supplemented with 1 mM KNO_3 enhanced the lipid production by 2.5-fold (Lv *et al*., 2010). In the current study, the maximum lipid content percentage was better than the highest lipid content of 26% in *C. pyrenoidosa* cultured with 50 $mg.L^{-1}$ KNO_3 (Nigam *et al*., 2011). On the other hand, the maximum lipid content of approximately 22% in the phosphorus-minimal medium was at 38.8 and 58.2 $mg.L^{-1}$ KH_2PO_4, but at 38.8 $mg.L^{-1}$ KH_2PO_4 gave the highest lipid productivity ($Q_{P,P}$, 0.417 $mg.L^{-1}.hr^{-1}$) as shown in Table 1. Interestingly, these maximum lipid contents were higher than those from *Chlorella* sp. grown in lower phosphorus concentrations that varied from 16 to 240 μM K_2HPO_4 (Liang *et al*., 2013). However, Liang *et al*. (2013) reported that *Chlorella* sp. increased both the lipid content and lipid productivity under low phosphorus conditions. Furthermore, they was also found that lipid accumulation in cells decreased by supplementing the growth media with K_2HPO_4 in the late growth phase. This implies that lipid accumulation shows a contradictory trend to the lipid production in microalgal cells (Sheehan *et al*., 1998; Rodolfi *et al*., 2009). This was in agreement with the lipid content percentage and lipid productivity of *C. muelleri* and *D. salina* grown under insufficient nutrient conditions (Gao *et al*., 2013).

Lower N/P ratios (N atoms per atom of P) of approximately 0.5 were optimal for the cell concentration ($C_{X,N}$, approximately 360–470 $mg.L^{-1}$) and volumetric rate of lipid production ($Q_{P,N}$, approximately 0.43 $mg.L^{-1}.hr^{-1}$, with 23–24% maximum lipid content percentage) at 260–320 $mg.L^{-1}$ KNO_3 (Table 1). However, in the current investigation, the maximum lipid content of 31% with $Q_{P,N}$ at approximately 0.25 $mg.L^{-1}.hr^{-1}$ was apparently at a 1.6-fold higher N/P ratio (approximately 0.8, with approximately 480 $mg.L^{-1}$ KNO_3) as shown in Table 1, resulting possibly from the starvation conditions (with the lower μ_N). On the other hand, a higher N/P ratio (for example approximately 100) was found to be best for the cell concentration ($C_{X,P}$, approximately 460 $mg.L^{-1}$), volumetric rate of lipid production ($Q_{P,P}$, approximately 0.42 $mg.L^{-1}.hr^{-1}$) and 22% maximum lipid content at approximately 40 $mg.L^{-1}$ KH_2PO_4 (Table 1). This might be useful to monitor the algal growth and lipid accumulation with the specific growth rate (μ_N).

In the current research, the optimal μ_N was in the range 0.0049–0.0076 hr^{-1} using either a lower (approximately 0.5) or higher (approximately 100) N/P ratio by statistical analysis at $P < 0.1$

(Table 1). However, the precise mechanism for starving nitrogen or phosphorus could not be well explained here. Neither starving nitrogen nor phosphorus produced any clear advantage for algal biomass and lipid production (Table 1). Nevertheless, to reduce the production cost of algal biomass and lipids careful consideration should be given to determining which alternative is superior between a limiting nitrogen or phosphorus source or both.

Fatty acid profiles of microalgal oil

The fatty acid composition was analyzed of the microalgal oil presenting in microalgae which were cultivated with nitrogen- and phosphorus-minimal media. The fatty acid profiles were compared with standard FAME mixtures, as shown in Table 2. The fatty acid profile obtained from nitrogen-minimal medium contained 43–51% saturated fatty acids and 49–57% unsaturated fatty acids (Table2, which was similar to palm oil according to Knothe, 2005), while the phosphorus-minimal medium had a lower level of saturated (31–37%) and a higher level of unsaturated (63–69%) fatty acids, respectively. More than 90% of the fatty acid composition was composed of C16–C18 which is the predominant component of biodiesel (Li *et al*., 2010). The most abundant saturated fatty acid was palmitic acid (C16:0), but this varied in the two minimal media (37–42% and 27–33% in nitrogen- and phosphorus-minimal media, respectively). These palmitic acid contents were similar to the levels of 33–42% found in the microalgal oil from *C. vulgaris* cultured in media supplemented with 0.03–0.05 mM nitrate (Cha *et al*., 2011),

Table 2 Fatty acid composition of microalgal oil resulting from gas chromatographic analysis and various biodiesel qualities calculated from fatty acid methyl ester compositions.

Fatty acid (%)	KNO_3 concentration[1] ($mg.L^{-1}$)				KH_2PO_4 concentration[2] ($mg.L^{-1}$)				Palm oil[3]
	0	258.5	317.0	475.5	0	19.4	38.8	58.2	
Saturated fatty acids									
C16:0	37.0	38.1	42.3	37.8	31.3	32.3	27.0	30.1	40–47
C18:0	4.9	2.4	4.6	2.4	2.8	1.7	2.3	0.8	3–6
Other	1.6	2.6	4.6	2.8	1.8	2.2	1.8	1.2	-
Total	43.5	43.2	51.5	43.0	35.9	36.2	31.1	32.1	43–53
Unsaturated fatty acids									
C18:1n9c	24.4	7.8	4.6	6.8	15.2	5.4	3.8	4.1	36–44
C18:2n6c	21.9	31.8	26.3	31.2	30.3	30.4	34.6	34.0	6–12
C18:3n3	9.5	15.4	14.2	17.0	17.5	26.6	29.2	28.7	-
Other	0.7	1.9	3.4	2.0	1.1	1.4	1.3	1.1	-
Total	56.5	56.9	48.5	57.0	64.1	63.8	68.9	67.9	42–56
MUFAs	25.1	9.7	8.1	8.8	9.00	16.3	6.9	5.2	-
PUFAs	31.5	47.1	40.5	48.2	42.2	47.9	56.9	63.7	-
Biodiesel quality by calculation									
SN	196.1	197.2	198.0	197.3	195.8	196.3	195.4	196.0	-
IV	84.1	103.2	89.4	105.8	112.1	127.6	140.2	138.0	-
CN	55.2	50.8	53.8	50.2	49.0	45.4	42.7	43.1	56–61

SN = Saponification number; IV = Iodine value; CN = Cetane number; MUFAs = Monounsaturated fatty acids; PUFAs = Polyunsaturated fatty acids

[1] = initial KH_2PO_4 was 1 g L^{-1}

[2] = initial KNO_3 was 2 $g.L^{-1}$

[3] = Sourced from Knothe (2008).

and from *D. tertiolecta* grown under nitrogen-starvation (Chen *et al*., 2011). Moreover, C16:0 was also found abundantly in *Chlamydomonas reinhardtii* grown in nitrogen-deprived medium, which differed from *Coccomyxa* sp. C-169 where C18:0 was mostly found under similar conditions (Msanne *et al*., 2012). In contrast, *Chlorella* sp. cultured in medium with sodium chloride and acetate produced the unsaturated fatty acid, oleic acid (C18:1) at 29–44% which was higher than for the saturated fatty acids (Zhou *et al*., 2013).

Moreover, the most abundant unsaturated fatty acid was linoleic acid (C18:2) which was found in both minimal media (22–32% and 30–35% in nitrogen- and phosphorus-minimal media, respectively). Also, C18:2 was found mainly as a polyunsaturated fatty acid (PUFA) in *C. vulgaris* and *C. sorokiniana* cultured in nitrate-minimal medium (Cha *et al*., 2011). These results were different from the oil profile of *D. tertiolecta* which produced the most (39.6%) linolenic acid (C18:3) under nitrogen-starved conditions (Chen *et al*., 2011). However, C18:3 was found to be an inferior fatty acid in this study (10–17% and 18–29% in nitrogen- and phosphorus-minimal media, respectively). Nutrient availability has a significant impact on the proliferation of microalgae and has broad effects on their lipid and fatty acid composition (Sharma *et al*., 2012).

Microalgal biodiesel quality based on fatty acid methyl ester composition

The fatty acid composition should be considered for microalgal biodiesel production. The biodiesel quality values specified with SN, IV and CN were calculated from FAME compositions and are summarized in Table 2. SN depends on the percentage concentration of fatty acid components present in FAME and IV is related to the unsaturated fatty acid component which is measured by the reaction of I_2 and carbon-carbon double bonds in oil (Mohibbe Azam *et al*., 2005; Hoekman *et al*., 2012). The calculated SN and IV values had ranges of 196–198 and 84–106, respectively, with fatty acid profiles that resulted from the nitrogen-minimal medium, whereas different values of SN (195–196) and IV (112–140) were obtained from the phosphorus-minimal medium. These IV values are comparable to those from microalgal biodiesel obtained from *Chlorella* sp. grown under different concentrations of sodium chloride and acetate (IV = 95.33–112.03) as reported by Zhou *et al*. (2013). As expected, the higher CN gives the fuel a better ability to ignite quickly after injection (Mohibbe Azam *et al*., 2005; Hoekman *et al*., 2012). It was found that the calculated FAMEs obtained from the nitrogen-minimal medium (CN = 50–55) were higher than those from the phosphorus-minimal medium (CN = 43–49) but these values showed similar CN (49.11–52.70) values to those reported recently (Zhou *et al*., 2013). Interestingly, the estimated CN level produced using the nitrogen-minimal medium was found to be close to the level of CN from palm biodiesel (CN = 55–61) according to Knothe (2008) as shown in Table 2.

The saturated fatty acid does not have an effect on the transesterification process to produce biodiesel, but it does affect the biodiesel properties (Hu *et al*., 2008). The biodiesel produced by saturated fatty acids showed higher oxidative stability and a larger cetane number, but had rather poor low-temperature properties which meant it was more likely to be a gel at ambient temperature. In contrast, biodiesel produced by polyunsaturated fatty acids is particularly susceptible to oxidation, so it has instability problems during prolonged storage (Hu *et al*., 2008). In general, the lower unsaturated fatty acid content in algal lipids causes less of an oxidation reaction which is an advantage for the production of biodiesel (Knothe, 2005; Chisti, 2007). The comparison between these lipids obtained from the two minimal media showed that the lipids obtained from the nitrogen-minimal medium would be a better potential substrate for biodiesel production than those from the phosphorus-minimal medium due to the higher level of saturated fatty acids (Chisti, 2007; Isleten-Hosoglu *et al*., 2012).

The CN values obtained from the nitrogen-minimal medium are acceptable with regard to the biodiesel standards of Thailand (2007), USA (ASTM D6751-07a) and European Standards Organization (EN 14214:2003) (51, 47 and 51, respectively) according to Winayanuwattikun *et al*. (2008); however, the CN expected from the phosphorus-minimal medium was considered to meet the standards. On the other hand, the IV values obtained from the nitrogen-minimal medium were remarkably at the maximum limit of the biodiesel standard of European Standards Organization (EN14214: 2003) (IV < 115) but nevertheless, the contents of linolenic acid were higher than set by the standard (C18:3 < 12%) according to Mohibbe Azam *et al*. (2005).

CONCLUSION

The microalga *Chlorella* sp. TISTR 8990 was photoautotrophically cultivated in open 6 L drinking water bottles with a culture working volume of 4 L. Both the concentrations of nitrogen and phosphorus as co-nutrients had a clear effect on algal growth and lipid accumulation. Moreover, the profiles of the lipids from microalgal cells cultured in nitrogen- and phosphorus-minimal media contained various saturated and unsaturated fatty acids. The greatest amounts of fatty acids were palmitic acid (C16:0), linoleic acid (C18:2) and linolenic acid (C18:3), respectively. Interestingly, an appropriate lower N/P ratio (such as 0.5) which affected the specific growth rate (μ_N of approximately 0.005–0.008 hr^{-1}) was associated with a fatty acid profile (C16:0 of approximately 38–42%) and satisfied the qualifications for the biodiesel standard making it quite useful as a means of organizing algal cultivation efficiently. Nevertheless, further work is forthcoming to confirm adequate scaling up for larger biomass and lipid production and to verify the specific growth rate as a useful parameter for monitoring algal culture.

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