Brazilian Journal of Microbiology (2012): 187-200
ISSN 1517-8382

GROWTH, FATTY ACID PROFILE IN MAJOR LIPID CLASSES AND LIPID FLUIDITY OF *AURANTIOCHYTRIUM MANGROVEI* SK-02 AS A FUNCTION OF GROWTH TEMPERATURE

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Submitted: February 01, 2011; Returned to authors for corrections: March 29, 2011; Approved: August 15, 2011.

ABSTRACT

Aurantiochytrium mangrovei Sk-02 was grown in a medium containing glucose (40 g/l), yeast extract (10 g/L) and sea salts (15 g/L) at temperatures ranging from 12 to 35°C. The fastest growth (μ_{max}= 0.15 h^{-1}) and highest fatty acid content of 415 mg/g-dry cell weight were found in the cells grown at 30°C. However, the cells grown at 12°C showed the highest percentage of polyunsaturated fatty acid (PUFA) (48.6% of total fatty acid). The percentage of docosahexaenoic acid (DHA) and pentadecanoic acid (C15:0) decreased with an increase in the growth temperature, whereas, palmitic acid (C16:0), stearic acid (C18:0) and DPA (C22:5n6) increased with an increase in the growth temperature. The composition of the major lipid class (%w/w) was slightly affected by the growth temperature. The fluidity of the organelle membrane or intracellular lipid (by DPH measurement) decreased with an increase in the growth temperatures, while the plasma membrane fluidity (by TMA-DPH measurement) could still maintain its fluidity in a wide range of temperatures (15 - 37°C). Furthermore, the distribution of DHA was found to be higher (36 – 54%) in phospholipid (PL) as compared to neutral lipid (NL) (20 - 41%).

Key words: Anisotropy measurement; *Aurantiochytrium* sp.; Docosahexaenoic acid; lipid class composition; membrane fluidity.

INTRODUCTION

Docosahexaenoic acid (C22:6n3; DHA) is an omega-3 polyunsaturated fatty acid (PUFA) and one of the essential fatty acids necessary for infants during brain and retinal development. In mammals, DHA can be either acquired directly from the diet or there is a limited ability to synthesize it from linoleic acid (C18:3n3) or eicosahexaenoic acid (C20:5n3) obtained from the diet as precursors, with DHA finally obtained via a retro-conversion of C24:6n3 to C22:6n3 by the Sprecher pathway (17, 19). The benefits of DHA (C22:6n3) have been extensively studied in aspects of nutritional and clinical effects in humans, animals and aquaculture. It is found as a major constituent of the phospholipid membrane in the brain and in the rod outer segment in the retina (up to 60% DHA) (9). DHA has been shown to improve cognitive performance in healthy adults and also play an important role in a circulatory system as DHA can

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increase flexibility and strength of arterial walls (3). The main source of DHA is from fish and seafood. In addition, many researches have been found that a number of microalgae in the group of thraustochytrids produce a significant amount of DHA. *Crypthecodinium cohnii* and the thraustochytrid, *Schizochytrium* sp., are now commercially applied to DHA production (21). Although, the production of fish oil does not involve high technologies in the cultivation and oil extraction like the production of DHA oil from microalgae. However, the population of fish is reduced and fish could get contaminated with mercury. Furthermore, the production of fish oil might affect the balance of marine ecosystems. Thus, DHA-containing oil from marine microalgae could be a sustainable source of omega-3 oil. In addition, many studies have shown that DHA-containing oil from marine microalgae is effective and probably safer than fish oil in order to meet the demand for human consumption (3).

Aurantiochytrium (formerly *Schizochytrium*) *mangrovei* Sk-02, a thraustochytrid, was isolated from a mangrove forest in Hong Kong. Previous reports on growth and fatty acid production of this strain showed that high DHA production (up to 4.7 g/L or 22.5% of dry cell weight) could be obtained from a glucose-yeast extract-sea salt medium in shake flask cultivation (24). Other studies with *A. limacinum* OUC88 showed the effect of temperature and salinity on the biomass and total fatty acid production (27). In a more recent study, the effect of temperature on growth and total fatty acid composition in *Aurantiochytrium* sp. strain mh0186 was reported (23). However, there are no reports on the fatty acid profile of the major lipid changes or the membrane fluidity in Thraustochytrids as a function of temperature. Therefore, the objectives of this research were to investigate the effect of cultivation temperature on growth, cellular fatty acid composition, fatty acid composition in major lipid classes and lipid fluidity. The results from this research might be useful for further optimization of DHA production by this strain in both triacylglycerol and phospholipid form. Moreover, the results could add more physiological information in terms of the lipid characterization and lipid fluidity of *A. mangrovei* Sk-02 as a function of growth temperature.

MATERIALS AND METHODS

Microorganism and culture conditions

Aurantiochytrium mangrovei Sk-02 (formerly *Schizochytrium mangrove*i Sk-02) was provided by Prof. Lilian L.P. Vrijmoed and Prof. Gareth Jones (City University of Hong Kong). The pure culture was maintained on a glucose-yeast extract-peptone (GYP) agar slant medium containing 10 g/L glucose, 1 g/L yeast extract, 1 g/L peptone and 15 g/L each of agar and artificial sea salts (50 % artificial seawater) and subcultured every month (24). For obtaining 18S rRNA gene sequence from this strain, a single colony from a GYP agar plate was transferred to 50 ml tube containing 10 ml of liquid medium consisting of 1 g/L peptone, 2 g/L yeast extract, 4 g/L glucose and 15 g/L sea salts. The culture was cultivated at 26°C with 150 rpm for 1 week. Then the cells were harvested and lyophilized. After that the genomic DNA of the cells was extracted by using DNeasy Plant Mini Kit (Qiagen, Germantown, MD, USA). Polymerase chain reaction was done to amplify 18S rRNA gene in the genomic DNA by using two primers: 16S1N (forward, 5′-TCCTGCCAGTAGTCATATGC-3′) and 16S2N (reverse, 5′-TGATCCTCT/CGCAGGTTCAC-3′) (23). The polymerase chain reaction (PCR) mixture composed of 50 µl 1 × Taq PCR buffer (New England Biolabs, UK), 0.2 mM dNTP, 0.2 µM of each primer (16S1N and 16S2N), 2.3 U Taq polymerase (New England Biolabs, UK) and sterile distilled water. The PCR program started from 1 cycle of DNA denaturing at 94°C for 5 min. Then the PCR was run for 40 cycles of 30 s at 94°C, 30 s at 54°C, 2 min at 72°C and followed by 1 cycle of final extension at 72°C. The results of the 18S rRNA gene sequence were aligned and compared to other microorganisms in GenBank database of the National Center for Biotechnology Information (NCBI) by using

BLAST (Basic local alignment search). The neighbor-joining (NJ) tree was constructed by MEGA4 (22) software and CLUSTAL W. The bootstrap values were obtained from 1,000 replications of NJ analyses.

An inoculum was prepared by transferring 1 ml of zoospore suspension in the well from a GYP plate to 250 ml Erlenmeyer flask containing 50 ml of GYP medium. The inoculum was incubated at 25°C in an orbital shaker at 200 rpm for 18 – 20 h. Basal medium consisted of 40 g/L glucose, 10 g/L yeast extract (Difco, USA), 0.001 g/L $MnCl_2$ with 15 g/L artificial sea salts (SS) (Sigma, USA) with an initial pH of 6.5. The cultivation was performed in 500 mL baffled flasks containing 100 ml culture medium. All flasks were inoculated with 5 mL inoculum and incubated in an orbital shaker at 200 rpm at either 12°C, 15°C, 20°C, 25°C, 30°C or 35°C. Duplicate cultivation flasks were sampled and the pH was measured and adjusted to 6.5 by 1 M KOH or 1 M HCl during the cultivation. Virtually no growth was observed at 10°C, whereas growth at 38°C resulted in a large reduction (ca. 50%) in the biomass yield, hence these conditions were not included. During cultivation, one ml samples were taken for assay of the optical density (at 660 nm, indicated as OD_{660}) or 4 mL for biomass dry weight after washing with distilled water (24). When glucose in the culture medium had decreased to approximately 2 to 4 g/L, 40 ml of culture was taken for assay of cell count and cell size by haemacytometer, maximal biomass, overall fatty acid profile as well as composition of major lipid classes.

Optical density measurement, biomass determination and residual glucose assay

The culture broth (1 mL) was collected in 2 mL Eppendorf tubes and centrifuged at 10,000×g for 10 min. The pellet was washed twice with distilled water. The optical density was measured by spectrophotometer (Novaspec II, Pharmacia Biotech, Sweden) at 660 nm. For biomass determination, cell pellets were collected by centrifugation and washed with distilled water. The weight of dried biomass was determined after freeze-drying and expressed as mg/L. The supernatants, after collecting cell pellets by centrifugation, were used for glucose measurement by a glucose oxidase kit (Glucose liquicolor, Human, Germany).

Fluorescence anisotropy measurement

Diphenyl-heaxatriene (DPH, Sigma) and trimethylammonium-diphenyl-hexatriene (TMA-DPH, Sigma) were used as probes to measure whole-cell fluorescence anisotropy of *A. mangrovei* Sk-02. Briefly, fresh cells were collected from cells cultivated at 15°C and 30°C with 15 g/L SS at the early fatty acid formation phase. Cells were washed twice with distilled water. After centrifugation at 10,000×g for 10 min, cells were resuspended in 10 mM phosphate buffered saline (PBS) to have a final OD_{660} of approximately 0.1 (ca.1 mg wet weight / mL). The stock solutions of DPH and TMA-DPH were dissolved in tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO), respectively (6). A final concentration of 2 µM and 5 µM of DPH and TMA-DPH, respectively were added to 1 mL cell suspension (final concentrations of THF and DMSO were less than 0.1%). The same volumes of THF and DMSO without probe were added to a cell suspension as a blank control. After a fluorescence probe was added, the cells were incubated at 15°C, 30°C and 37°C for 20 min. Steady state fluorescence anisotropy was measured with a Perkin-Elmer LS 55 luminescence spectrofluorometer connected to a MP-50 Open Bath Circulator (JULABO). For DPH and TMA-DPA, the excitation and emission wavelengths were 360 nm and 450 nm, respectively. The anisotropy (r) was calculated according to the equation:

$$r = (I_{vv} - GF \times I_{vh}) / (I_{vv} + 2GF \times I_{vh})$$

where: $GF = I_{hv} / I_{hh}$ is the grating factor that is an instrumental correction factor; I is the intensity of fluorescence emitted; while v or h refer to the vertical and horizontal direction, respectively. A higher value of anisotropy (r) indicates a

lower fluidity.

Lipid classes separation and fatty acid analysis

Lipids from dried cells were extracted by chloroform-methanol-distilled water (1:2:0.8 v/v/v) described by (2). The solvents were added to the dried cells in a sequence starting from chloroform, methanol and distilled water as described by (15). A mixture of dried cells and solvents was sonicated in a sonication bath for 15 min, and then the mixtures were incubated at room temperature for 18 h. Finally, lipids were separated by adding 1.25 mL chloroform and 1.25 mL of 1 M NaCl in a mixture. After centrifugation at 1000×g, lipids in the chloroform phase were taken out and dried with nitrogen gas in a new vial. Twenty milligram of lipids were separated into neutral lipid (NL), free fatty acid (FFA), non-acidic phospholipid (non-aPL) and acidic phospholipid (aPL) by aminopropyl-bonded phase via a column chromatography method described by (12). Neutral lipid was first eluted from the column by adding 16 ml of chloroform-isopropanol (2:1 v/v) and 8 mL of 1% v/v acetic acid in diethyl ether was then used to separate free fatty acid. Then 16 mL of methanol was added to the column to elute non-acidic phospholipids (non-aPL) (phosphatidyl choline; PC and phosphatidyl ethanolamine; PE). The last fraction was acidic phospholipids (aPL) (phosphatidyl inositol; PI and phosphatidyl serine; PS), which was eluted by 8 ml of chloroform-methanol-3N HCl (100:200:1 v/v/v). The solvent was evaporated in a vacuum incubator (Gallenkamp, Germany). Dried lipid samples from every fraction were weighed and resuspended in chloroform-methanol (2:1 v/v). The purity of each lipid class was checked by thin layer chromatography (TLC) using silica gel 60 plates. The solvent used for TLC was hexane-diethyl ether-acetic acid (70:30:1 v/v/v). The plate was stained with 3 % w/v copper sulfate in 30% v/v sulfuric acid and heated at 120°C for 15 min.

Lipids extracted from dried cells as well as lipid from individual lipid classes were methyl esterified in 4% v/v conc. sulfuric acid in methanol with 0.1% of 2,6-Di-tert-butyl-4-methylphenol (BHT) (24). The internal standard was C19:0. Fatty acid methyl esters (FAMEs) were injected on a Shimadzu GC-17A gas chromatography equipped with an Omegawax 250™ (30m **x** 0.25 mm) column (Supelco, USA), an auto-injector and flame ionization detector. Injector and detector temperatures were 250°C and 260°C respectively, with helium as carrier gas at a linear velocity of 30 cm/s. Column temperature was held at 200°C for 10 min, then increased to 230°C at 10°C /min and kept at this temperature for 14 min. For routine analysis, peak quantification was performed by comparison with four dilutions of DHA–FAME (Sigma, USA) with C19:0-FAME (Sigma, USA) as internal standard. Retention time of each peak from the sample was compared with a mixed PUFA-standard (Supelco no. 189-19). The results of total fatty acid composition in each lipid class were compared to the results of total fatty acid composition found in cellular lipids extracted by Bligh and Dyer method.

Statistical analysis

The data was statistically analyzed by using one-way analysis of variance, and significant differences were identified by the Duncan and Tukey's test ($P < 0.05$). The analysis was done by SPSS Version 17.0 software (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Effect of culture temperature on growth

The neighbor-joining (NJ) tree showed the phylogenic position of *Aurantiochytrium mangrovei* Sk-02 (JF260953) and its related species based on 18S rRNA gene sequences (Fig. 1). The morphology and fatty acid profile of this strain suggested that the genus and specie of this microalga was closely related to *Aurantiochytrium mangrovei* according to the taxonomy rearrangement of *Schizochytrium* sp. (26). In this study, *A. mangrovei* Sk-02 was grown on 40 g/L glucose at various

temperatures. Exponential growth was observed up till an OD_{660} of ca. 12 and the specific growth rate (μ) was calculated for all conditions (Fig. 2a). At this point, the cell count reached a plateau (data not shown). The cells accumulated lipid until glucose was exhausted in the culture medium with a linear increase in biomass. At this point, the cells were in the late lipid accumulation phase and the maximum OD_{660}, the maximum biomass (mg/L) and the maximum total fatty acid content (mg/g-dry cell weight (DCW)) were obtained at 108 h, 93 h, 50 h, 28 h, 23 h and 32 h in the cells cultivated at 12°C, 15°C, 20°C, 25°C, 30°C and 35°C, respectively (Fig. 2a and 2b). The maximum specific growth rate and the consumption rate of glucose in an early growth phase were increased with the growth temperature except for cells grown at 35°C. The highest μ_{max} of 0.15 h^{-1} and the highest cell number of 2.1×10^8 cells/ml were found in the cells grown at 30°C (Fig 2c). In contrast to the present study, the growth rates of *Aurantiochytrium* sp. strain mh0186 were similar even though the temperature was varied from 15 - 30°C and the cultivation was finished at 96 h for all conditions (23). Thus, the latter results suggested that conditions used for growing mh0186 were oxygen-limited at all temperatures tested.

The biomass significantly increased from 13.2±0.4 to 14.7±0.5 g/L (p<0.05). Total fatty acid was analyzed by GC and the GC chromatogram of total fatty acid from cells grown on various growth temperatures was shown in Figure 3. Total fatty acid (TFA) was significantly increased from 300 to 415 mg/g-DCW with an increase in growth temperature from 12 to 30°C but TFA decreased to 375 mg/g-DCW as the growth temperature was increased to 35°C (Table 1). The highest total fatty acid yield of 160 mg TFA/g glucose was found in the cells grown at 30°C (Table 1). Previously, *A. mangrovei* Sk-02 was shown to have an optimum temperature for maximal biomass and DHA-formation at ca. 25 -30°C (24). Another report of the biomass and total fatty acid content of *A. limacinum* OUC88 cultivated in the range of 16 - 37°C were reduced significantly (2.6 and 1.6 times, respectively), as the temperature increased from 30 to 37°C (27). The results from previous studies agree with the results found in this study suggesting that *A. mangrovei* could grow well at a wide range of growth temperatures (12 to 35°C).

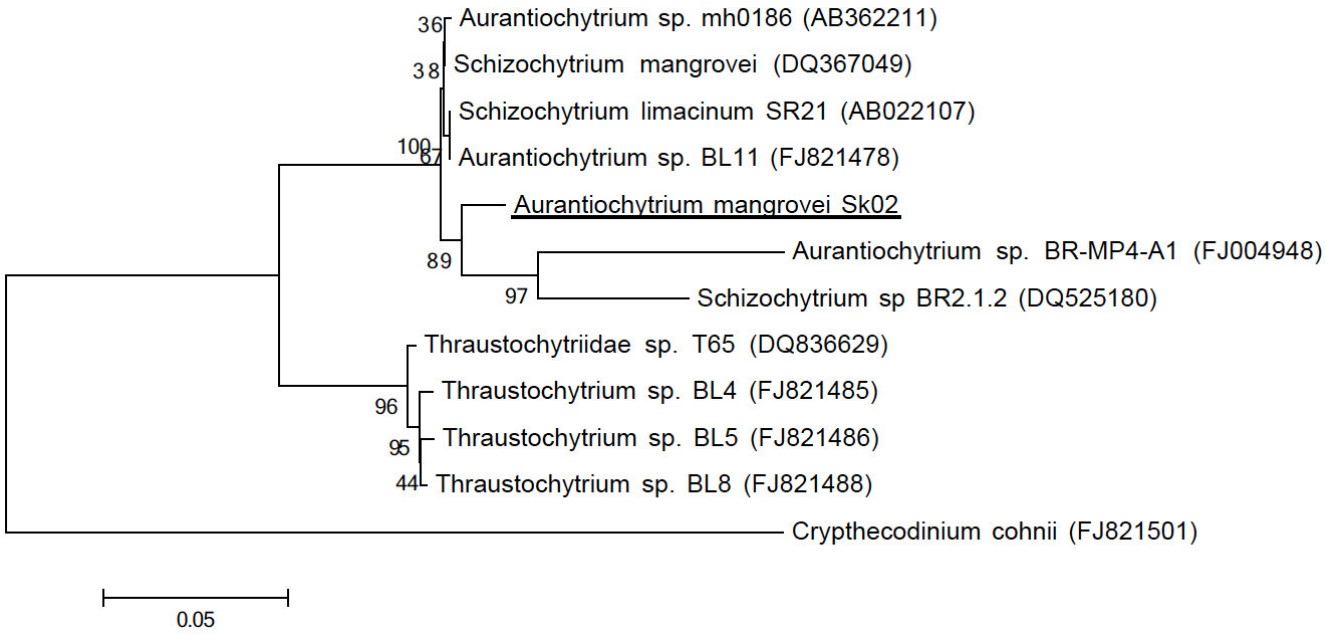

Figure 1. Phylogenic tree of Labyrinthulomyteces using 18S rRNA genes with *Crypthecodinium cohnii* as out group.

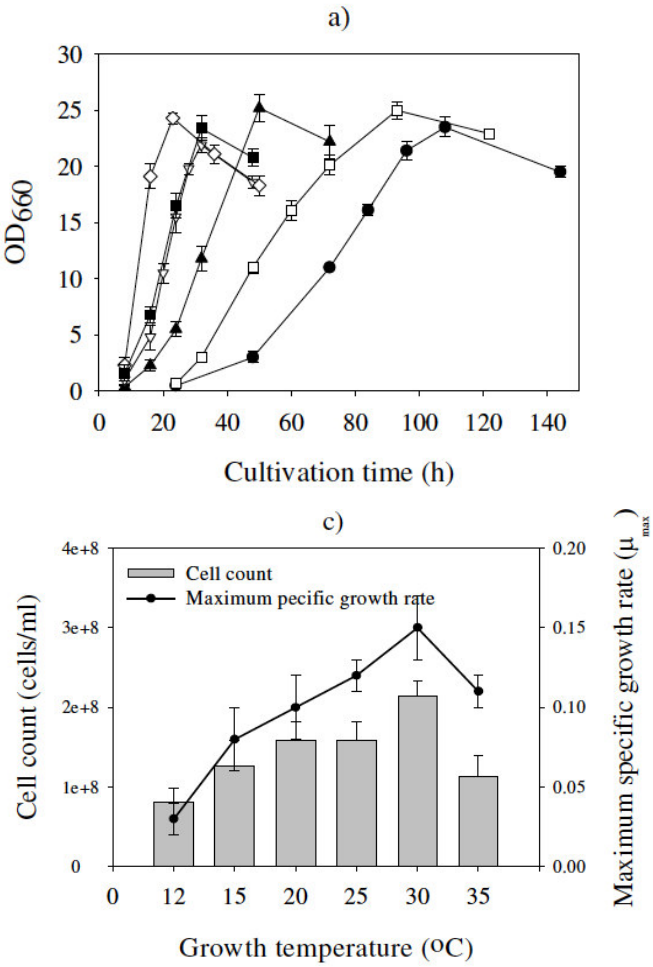

Figure 2. Effect of cultivation temperature on cell growth (a), the residual glucose concentration (g/L) (b) and the maximum specific growth rate (μ_{max}) (line) and cell count (cells/mL) at the maximum OD_{660} (bars) (c) of *Aurantiochytrium mangrovei* Sk-02 as a function of growth temperatures (12°C - 35°C).

Table 1. Fatty acid composition (mg/g-dry cell weight *DCW*) and the total fatty acid yield (mg/g glucose) of *A. mangrovei* Sk-02 as a function of growth temperature. (The data was obtained from direct acid methylation and the percentages of C16:1 and C18:1 were less 1%).

Fatty acid	Temperature (°C)					
(mg/g DCW)	12	15	20	25	30	35
C14:0	8.8±1.6^{b}	10.1±0.9^{b}	8.4±0.8^{b}	11.1±2.3^{b}	14.3±1.8^{a}	11.3±1.5^{b}
C15:0	34.5±0.3^{b}	37.9±1.2^{a}	33.9±0.9^{b}	30.3±0.4^{c}	20.4±0.4^{d}	22.2±0.3^{d}
C16:0	108.9±9.8^{e}	145.6±10.3^{d}	137.4±7.5^{d}	175.6±9.7^{c}	228.3±5.4^{a}	206.9±8.5^{b}
C18:0	2.0±0.4^{c}	2.2±0.5^{c}	2.2±0.2^{c}	4.0±0.3^{b}	4.9±0.6^{a}	4.2±0.2^{ab}
C20:3n6	3.7±2.1^{a}	4.3±0.3^{a}	2.6±0.1^{a}	2.7±0.8^{a}	3.2±2.1^{a}	3.9±0.2^{a}
C20:5n3	1.5±0.3^{a}	1.1±0.1^{a}	0.5±0.4^{a}	1.1±0.7^{a}	1.1±0.3^{a}	0.8±0.3^{a}
C22:5n6	15.1±1.5^{d}	14.0±0.8^{d}	13.1±0.7^{d}	19.9±0.1^{c}	22.9±0.3^{b}	27.8±0.6^{a}
C22:6n3	125.6±5.2^{a}	107.8±8.8^{b}	105.0±5.6^{b}	107.4±2.5^{b}	119.9±4.6^{a}	97.7±7.7^{b}
Saturated	154.2±11.5^{d}	195.8±10.7^{c}	181.9±8.4^{c}	221.0±9.5^{b}	268.0±7.5^{a}	244.6±10.6^{b}
Unsaturated	146.0±1.9^{a}	127.3±4.3^{b}	120.7±2.4^{c}	131.1±0.7^{b}	147.0±2.7^{a}	130.2±5.5^{b}
TFA (mg/g DCW)	300.2±10.3^{c}	323.1±11.3^{d}	302.6±9.5^{de}	352.1±11.3^{c}	415.0±7.7^{a}	374.9±9.7^{b}
TFA (mg/g glucose)	99.0±3.4^{e}	117.9±4.1^{cd}	108.2±3.4^{de}	126.7±4.1^{c}	159.8±2.9^{a}	137.8±3.8^{b}
Biomass (g/L)	13.2±0.3^{b}	14.6±0.5^{ab}	14.3±1.1^{ab}	14.4±0.9^{ab}	14.7±0.3^{a}	14.7±0.9^{a}
C22:6n3/C22:5n6	8.3	7.7	8.0	5.4	5.2	3.5
C16:0/C22:6n3	0.9	1.4	1.3	1.6	1.9	2.1

* Alphabetic superscripts within the same row but among the different groups indicate statistical difference ($p< 0.05$) analyzed by One-way ANOVA using the Tukey and Duncan's test.

Effect of culture temperature on total fatty acid profile

As shown in Table 1 and Figure 3, the major saturated fatty acid found at all cultivation temperatures was palmitic acid (C16:0) and the major polyunsaturated fatty acid was DHA (C22:6n3). However, the ratio of C16:0 (mg/g-DCW) to C22:6n3 (mg/g-DCW) increased from 0.9 to 2.1 as a growth temperature increased from 15 to 35°C. In addition, some minor UFAs like C16:1 and C18:1 were also found but they were not included in the data, since the sum of them was less than 1% of total fatty acid (TFA) or 4 mg/g-DCW (data not shown). An increase in growth temperature was accompanied by an increase in the percentages of C16:0, stearic acid (C18:0) and DPA (C22:5n6), except for the condition at 35°C (Table 2). The percentage of total saturated fatty acid (SFA) increased from 51.4 to 61.3% of TFA with an increase in growth temperature from 12 to 35°C. In contrast, the percentage of total unsaturated fatty acid (UFA) was negatively correlated with an increase in the culture temperature (Table 2). The percentage of DHA in total fatty acid (TFA) decreased significantly from 41.8 to 26.1% whereas, C16:0 increased significantly from 36.3% to 55.2% of TFA with an increase in the growth temperature from 12 to 35°C, respectively (Table 2). These results were in accordance with previous reports. For example, in *Aurantiochytrium* sp. strain mh0186, the amount of UFA and DHA decreased 1.2-times and at 1.3-times, when the temperature increased from 10 to 15°C, respectively (23). Similarly, in *A. limacinum*, the amount of DHA decreased from 37.6 to 24.8% of TFA but C16:0 increased from 38.0 to 41.6% of TFA when the cultivation temperature increased from 16 to 30°C (27).

In order to study, whether growth was oxygen limited, experiments were conducted in which the initial liquid volume was varied in 500 mL baffled flasks containing 50 to 250 mL in 50 mL increments at 30°C. Only at a volume of larger than 150 mL, a decrease in μ_{max} or linear growth was observed (data not shown). Hence, oxygen was not limited in the conditions used in this study. Alternatively or additionally, the FA profile might be a function of the specific growth rate (μ), which was strongly dependent on the cultivation temperature (Fig 2c). Hence it can be argued that experiments should be performed in a chemostat (continuous culture, chemostat) at a fixed dilution = growth rate. However, experiments with various oleaginous microbes suggest that in chemostat the biomass and/or lipid content decreases rapidly with increasing growth rates in N-limited media as shown for instance with an oleaginous mould *Entomophthora exitalis* (13) as well as *Schizochytrium* sp. GS13/2S (7). In the context of Fig 2c, this would imply that dilution rate would have to be set as low as 0.01 h^{-1} to avoid wash-out at low cultivation temperatures. It would be difficult and very time-consuming to get a steady state under such conditions, hence this was not pursued. Furthermore, trends in TFA profiles versus cultivation temperature reported for *Aurantiochytrium* sp. mh0186 where similar to those reported in the present study (23). However, for mh0186, growth curves were linear and similar for all temperatures tested, suggesting that specific growth rate might not be the major factor determining FA-profiles, although this cannot be ruled out.

Unfortunately, the actual intermediates of PUFA production by PKS pathway have not been identified so far. However, the enzyme reactions in PKS pathway showed that DPA was not an intermediate for DHA synthesis in the PKS pathway (16), hence a separate pathway can be postulated. In our study a clear effect of temperature on the ratio of DHA (C22:6n3) to DPA (C22:5n6) was observed, for example, the ratio was 8.3 at 12ºC and 3.5 at 35°C (Table 1). These results were in agreement with the trend reported for *A. limacinum* SR21 and *Aurantiochytrium* sp. strain mh0186, respectively (27, 23). Hence, the regulation of the DPA versus DHA formation appeared to be quite different and it can be concluded that DPA was not a precursor for DHA as already suggested (20). The odd-number fatty acid (pentadecanoic acid, C15:0) was synthesized from propionyl CoA derived from specific amino acids present in yeast extract. Propionyl

CoA was then condensed with malonyl CoA by KS, the first enzyme in the FAS pathway. In this study, C15:0 decreased from 37.9 to 20.4% with an increase in the cultivation temperature from 15 to 30°C (Table 2). So, the production of C15:0 might be dependent on the nitrogen metabolism which was controlled by the growth rate during nitrogen assimilation as well as the growth temperature.

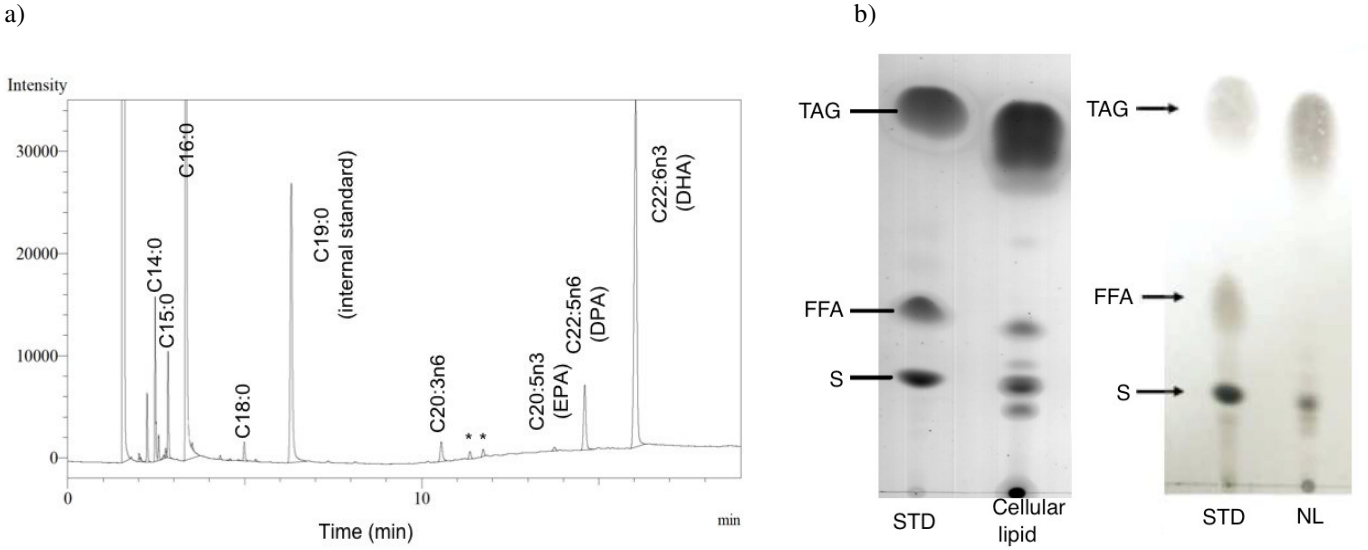

Figure 3. GC chromatogram of methyl-esterified fatty acids from glucose grown cells and from lipid class separation (NL, FFA and PL) at various growth temperatures (Star indicates unidentified peak) (a) and TLC of neutral lipid (NL) class (major lipid class) separated from cellular lipids compared to standard triacylglycerol (TAG), free fatty acid (FFA) and sterol (S) (b).

Table 2. Fatty acid composition (% total fatty acid) of *A. mangrovei* Sk-02 as a function of growth temperature. (The data was obtained from direct acid methylation and the percentages of C16:1 and C18:1 were less 1%).

Fatty acid	**Temperature (°C)**					
(%)	**12**	**15**	**20**	**25**	**30**	**35**
C14:0	2.9±0.3^{a}	3.1±0.2^{a}	2.8±0.3^{a}	3.1±0.6^{a}	3.5±0.2^{a}	3.0±0.2^{a}
C15:0	11.5±0.2^{a}	11.7±0.1^{a}	11.2±0.1^{b}	8.6±0.2^{c}	4.9±0.1^{e}	5.9±0.1^{d}
C16:0	36.3±0.9^{d}	45.1±0.4^{c}	45.4±0.3^{c}	49.9±0.3^{b}	55.0±0.8^{a}	55.2±0.2^{a}
C18:0	0.7±0.1^{b}	0.7±0.0^{b}	0.7±0.1^{b}	1.1±0.2^{a}	1.2±0.1^{a}	1.1±0.1^{a}
C20:3n6	1.2±0.5^{a}	1.3±0.5^{a}	0.9±0.1^{a}	0.8±0.4^{a}	0.8±0.4^{a}	1.0±0.1^{a}
C20:5n3	0.5±0.1^{a}	0.3±0.0^{a}	0±0.0^{a}	0.3±0.3^{a}	0.2±0.1^{a}	0.2±0.2^{a}
C22:5n6	5.0±0.2^{c}	4.3±0.1^{d}	4.3±0.4^{d}	5.7±0.1^{b}	5.5±0.2^{b}	7.4±0.3^{a}
C22:6n3	41.8±1.8^{a}	33.4±0.2^{b}	34.7±0.1^{b}	30.5±0.1^{c}	28.9±1.8^{c}	26.1±0.1^{d}
Saturated	51.4±1.1^{d}	60.6±0.3^{c}	60.1±0.2^{c}	62.8±0.3^{b}	64.6±1.2^{a}	65.3±0.1^{a}
Unsaturated	48.6±0.9^{a}	39.4±0.2^{b}	39.9±0.2^{b}	37.2±0.3^{c}	35.4±0.8^{d}	34.7±0.2^{d}
UI*	2.8	2.3	2.3	2.2	2.0	2.0

* Unsaturation index (UI) = (1.0(%monoene)) + (2.0(%diene)) + (3.0(%triene)) + (4.0(%tetraene)) + (5.0(%pentaene) + (6.0(%hexaene))/100

Alphabetic superscripts within the same row but among the different groups indicate statistical significant ($p < 0.05$) analyzed by One-way ANOVA using the Tukey and Duncan's test.

Fatty acid composition of major lipid classes

The major lipid classes of *A. mangrovei* Sk-02 grown at various temperatures in the late lipid accumulation phase were separated. The results of fatty acid composition in lipid extracted from the cells by Bligh and Dyer method before lipid class separation were compared to the result of fatty acid composition in each lipid class after lipid classes were separated by column chromatography. The results of the sum of fatty acid composition in all classes after lipid class separation were similar to the result found in total lipid extracted before lipid class separation. Moreover, the results of GC analysis of cells by direct methylation method were similar to the results of fatty acid composition of lipid extracted from the Bligh and Dyer method followed by acid methylation for GC analysis (Fig. 3a). The data from this study showed that cells stored lipid predominantly in the form of neutral lipid (NL) (> 75% of total cellular lipid). NL was composed of triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), sterol ester (SE) and sterol (S) as shown by TLC of NL from cells grown at various temperatures (Fig .3b). In addition, FFA and PL were also visible on TLC (Fig. 3b). In order to exclude the possibility that FFA arose from lipase activity during handling, fresh cells were heated at 70°C for 15 min which abolished intracellular lipase as assayed in cell-free extracts by pNPP measurement (data not shown) but gave identical TLC patterns as non-heated cells. FFA accounted for 3 – 5% of the total cellular lipid weight and total PL was approximately 18% of total cellular lipid. The data was independent of the cultivation temperature (Fig. 4).

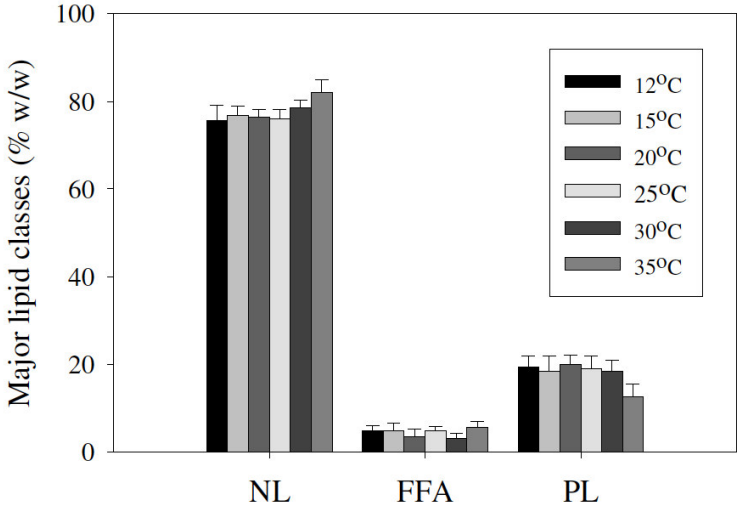

Figure 4. The weight percentage (+/- S.D.) of individual major lipid classes from *A. mangrovei* Sk-02 as a function of growth temperature (12 - 35°C). NL = neutral lipid, FFA = free fatty acid and PL = phospholipid.

Figure 5 shows the influence of growth temperature on the fatty acid composition in NL. Similar to the results of fatty acid composition from overall cellular fatty acid profile, C16:0 slightly increased with an increase in the growth temperature from 12 to 25°C and remained constant from 30 to 35°C. In contrast, C15:0 and DHA decreased from 13.6 to 6.7% of TFA and 40.7 to 24.9% of TFA in NL, respectively when the growth temperature increased from 12 to 35°C (Fig. 5). In phospholipids, the percentage of DHA decreased significantly from 53.5 to 35.5% of TFA in total PL whereas C16:0 increased markedly from 39.9% to 52.0% of TFA in PL as the growth temperature increased from 12 to 35°C (Fig. 6). The major fatty acid composition in NL and PL as a function of temperature were basically similar but the percentage of DHA in total fatty acid of total PL was higher as compared to that in NL (Fig. 5 and Fig. 6). For C20 PUFAs (C20:3n6 and

C20:5n3), a similar decrease with increasing growth temperature would be expected but due to the low absolute levels of these two PUFAs a significant trend could not be established (Fig. 5 and Fig. 6). Phospholipids from cells grown at 15°C and 30°C were chosen as examples of cells grown at low and high growth temperature for the separation of non-acidic phospholipid (non-aPL) and acidic phospholipid (aPL). Previous research has shown that the predominant PL in *A. mangrovei* consisted of PC and PE (5). In Sk-02, 60 - 70% of total PL consisted of non-acidic PL under all growth conditions (data not shown). In phospholipids, DHA in non-aPL was much higher than in aPL in which C16:0 was the main fatty acid. DHA (%) in non-aPL also decreased from 68.5 to 48.5% of TFA with an increase in the growth temperature from 15 to 30°C (Fig. 7). These results were in agreement with the results found in *A. mangrovei* FB3 grown at 25°C as non-aPL in this microalgae made up of ca. 60% of PL (5). Moreover, the percentage of DHA in total PC (a non-aPL) of the thraustochytrid-like strain 12B was approximately 54%, while only 20% of total PL was found in PI (an acidic phospholipid) in cells cultivated at 30°C (18).

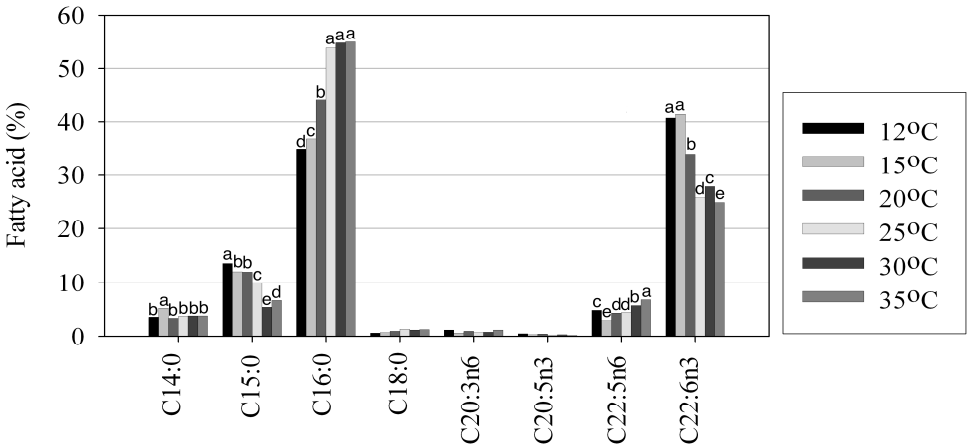

Figure 5. Fatty acid composition in neutral lipid of cells grown at different growth temperature (S.D.< 1.0). Alphabets above each bar graph indicate a significant difference (p<0.05).

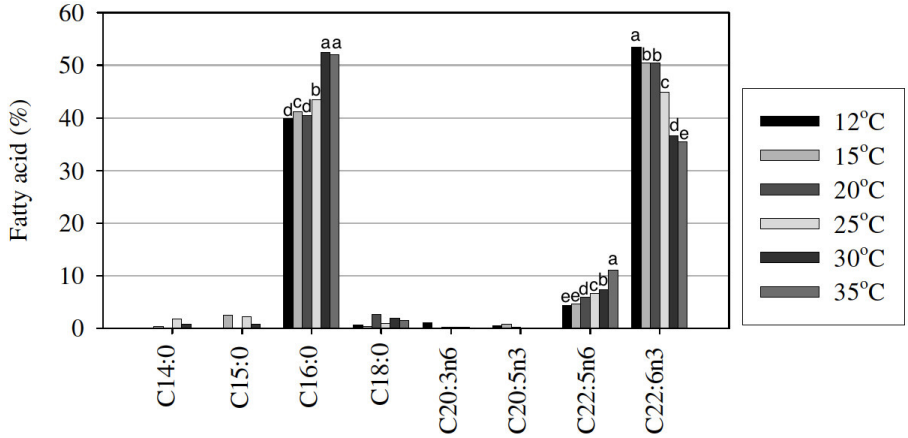

Figure 6. Fatty acid composition in total phospholipids of cells grown at different temperatures (S.D. <1.0). Alphabets above each bar graph in a group of C16:0, C22:5n6 and C22:6n3 indicate a significant difference (p<0.05).

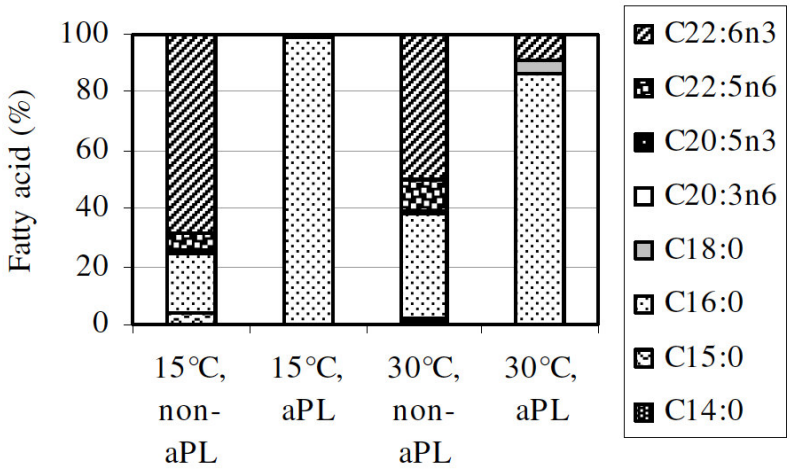

Figure 7. Fatty acid composition of non-acidic phospholipid (non-aPL) and acidic phospholipid (aPL) of cells grown at 15°C and 30°C.

Lipid fluidity measurement

Fresh cells cultivated at 15°C and 30°C until the late lipid accumulation stage were chosen as examples of cells grown at low and high growth temperature, respectively. In order to study the lipid fluidity of the cells grown at two different temperatures, the temperatures for fluidity measurement were set at 15°C, 30°C and 37°C. The common used probes -DPH and TMA-DPH- had different in the polarities. DPH had no head group whereas TMA-DPH was a cationic derivative of DPH molecule, which can anchor at the surface region of the plasma membrane (4). As the temperature for measurements increased, the lipid fluidity measured with DPH from both conditions increased significantly (p<0.05) (Fig. 8). For TMA-DPH, the anisotropy value was only slightly affected by the temperature of measurement. Cells cultivated at 30°C had less fluidity as compared to cells cultivated at 15°C when measured at 15°C by TMA-DPH probe. Thus, this study showed that *A. mangrovei* Sk-02 can maintain a constant plasma membrane fluidity at a wide range of the environmental temperatures. In contrast, lipid fluidity in the intracellular lipid as well as the intracellular membrane was highly affected by the growth temperature as indicated by significant changes in anisotropy value of DPH as a function of growth temperature. These results were in agreement with a study on the effect of temperature on crab plasma membrane fluidity measured by DPH and TMA-DPH as the temperature of measurement increased from 5 to 35°C (4).

Highly unsaturated fatty acids such as DHA and DPA can induce a change in the lipid bilayer structure as their kinked molecules might loosen the packed structure of the membrane lipid bilayer However, the properties and functions of DHA in terms of increasing membrane fluidity in Thraustochytrids still remain unclear (11) as a very high fluidity of membrane itself might not be necessary for microorganisms living in such shallow waters and moderate water pressure as present in mangrove forests where *Aurantiochytrium* sp. are commonly isolated. In addition, not only DHA and DPA as found in *Aurantiochytrium sp*. but also monounsaturated fatty acids like C18:1 could also increase the fluidity of the plasma membrane. This was clearly shown in the deep sea bacterium *Photobacterium profundum* SS9 from which the PKS gene cluster for EPA-production was deleted and oleic acid (C18:1n9) added to the medium. This allowed good growth at either low temperature or high pressure, suggesting that highly unsaturated fatty acids were not essential for the cells to survive in extreme conditions (1). Hence, the function of DHA/DPA in thraustochytrids remains unclear.

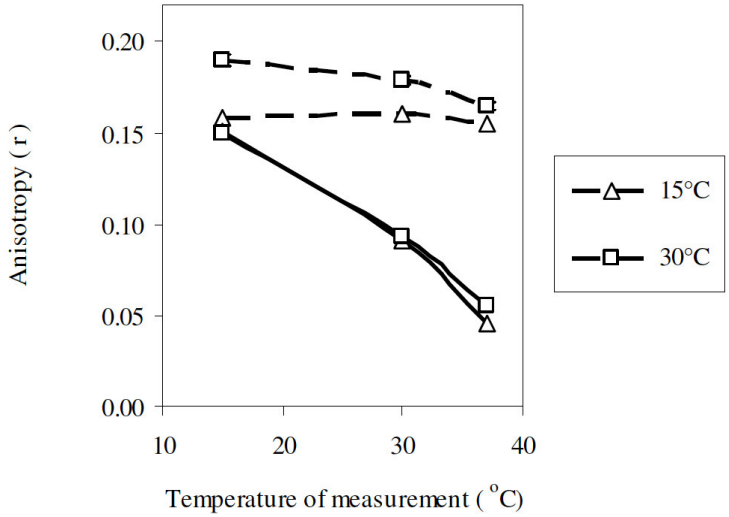

Figure 8. The temperature dependence of fluorescence anisotropy measured by DPH (solid lines) and TMA-DPH (broken lines) of cells cultivated at 15°C and 30°C (S.D. < 0.01).

Implications for commercial production of DHA from *Aurantiochytrium mangrovei*

From this study, the growth temperature for the industrial scale production of DHA by *A. mangrovei* was in the range of 25 to 30°C. However, the temperature for producing DHA-rich cells (ca. 34.2% to 43.8% of TFA) could be in the range of 12 to 20°C. But the cost for cooling the reactor down to a very low temperature was high and the time to finish the cultivation was longer as compared to that at high temperature (25 to 35°C). Therefore, it might be not worth for the large scale production to cultivate the microorganism at low temperature and effort should rather be focused on screening for a strain with a high DHA/DPA content. For example, DHA of strain G13/2S was 43% of TFA in batch fermentation at 27°C (7). The forms of lipid containing in *A. mangrovei* were mainly NL, FFA and PL. The percentage of DHA (68.5% or 65 mol% of TFA) in non-aPL at 15°C (Fig. 7) showed that it might be possible to have PC with DHA at both *sn*-1 and *sn*-2 position as one molecule of phospholipid is composed of 2 moles of free fatty acids. Therefore, PL in particularly DHA-containing non-aPL, especially PC in both the residual oil after TAG purification and the cell debris could be an interesting by-product to add more value to large scale production of DHA from *Aurantiochytrium* sp. It has been suggested that DHA-containing phospholipids are a better form of DHA as they are two times more bioavailable in the brain than in triacylglycerol or ethyl esters forms (14). Moreover, a report on the development of fish larvae by feeding DHA in the form of TAG and PL showed that DHA-containing PL was more effective than TAG-containing DHA (8).

CONCLUSIONS

This study was carried out to show the growth, total fatty acid profile, the distribution of DHA in major lipid classes and lipid fluidity of the plasma membrane of *A. mangrovei* Sk-02 as a function of growth temperature. Cells could grow well over wide range of temperature (12 to 35°C). Moreover, the production of PUFA/TFA was inversely related to the growth temperature with similar trends were also found in NL and PL classes. In addition, cells could maintain their plasma membrane fluidity in a wide range of growth temperature, and only the intracellular lipid and organelle membrane were affected by the growth temperature. Furthermore, the results in terms of growth and lipid characterization as a function of temperature could imply for the commercial production of

DHA from *A. mangrovei*. Thus, *A. mangrovei* could be one of the potential candidates for DHA production in the form of dried cells and extracted oil for animal feed and human consumption in both triacylglycerol form and especially, phospholipid form as PC (non-aPL).

ACKNOWLEDGEMENTS

This research work was partially supported by a RA scholarship from the Faculty of Graduate Studies, Mahidol University. The authors are grateful to Asst. Prof. Noppawan Phumala Morales for her kind support with anisotropy measurements and Dr. Thipa Asvarak for her kind support with 18S rRNA technique.

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