






Production of α -linolenic Acid by an Oleaginous Green Algae *Acutodesmus obliquus* Isolated from Malaysia

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Abstract

The main purpose of this study is to provide information on the biochemical composition of the total lipid extract of three different native freshwater microalgae namely *Acutodesmus obliquus* CN01, *Chlorella sp.* Carolina-15-2069, and *Chlorella vulgaris* NIES-1269 at different growth phases, cultured under standardized conditions that will be useful for commercialization purposes due to the content of α -linolenic acid (ALA), a type of omega-3 fatty acids. We monitored growth, biomass accumulation, chlorophyll content, lipid content and fatty acids composition in all three freshwater microalgae. In addition, this study seeks to throw some light on the lipid content of a newly isolated freshwater green alga *Acutodesmus obliquus* CN01 which have shown to give significant quantities of ALA, an added nutritional value to this microalga oil. *Acutodesmus obliquus* CN01 exhibited the fastest growth rate among the three microalgae used. Biomass was shown to be enhanced in the nitrogen enrichment medium. The outcomes from this study indicated that newly isolated *Acutodesmus obliquus* CN01 from Malaysia cultured under normal condition showed fast growth rate with high lipid content and also high ALA content. Therefore, from this study, *Acutodesmus obliquus* CN01 was identified as species of choice to be further explored for its potential to produce omega-3 fatty acids.

Keywords: *Acutodesmus obliquus*, *Chlorella sp.*, *Chlorella vulgaris*, Lipid, Malaysia.

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INTRODUCTION

Polyunsaturated fatty acids (PUFA) are the essential nutrients that humans need to consume in order to obtain PUFA in their diet since humans cannot synthesise PUFA. Prominent derivative products of PUFA are omega-3 fatty acids (or PUFA n-3) and omega-6 fatty acids (or PUFA n-6)¹. Omega-3 fatty acids have first double bond located between the third and fourth carbon atom counting from the methyl end of the fatty acid². The shortest chain of omega-3 fatty acids is α -linolenic acid (ALA, C18:3, n-3)³. The main long chain of omega-3 fatty acids (ω -3 LC-PUFA) are eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3) and docosahexaenoic acid (DHA; 22:6n-3)⁴.

Omega-3 fatty acids have a pivotal role in brain development, cardiovascular function and immune system regulation⁴. There is evidence that omega-3 fatty acids also play a crucial role in treating arteriosclerosis, cancer and rheumatoid arthritis⁵.

In the new global economy, increase of the human population has become a central issue for the needs of new sources for omega-3 fatty acids. Currently, the main commercial source for omega-3 fatty acids is marine fish⁵. However, evidence suggests that marine fish is not sufficient for the world's requirement of omega-3 fatty acids. Fish may not be the best source of omega-3 fatty acids due to its scarcity, odour, geographical and seasonal variations in quality⁶. Besides, there is an increasing interest in recent years among vegetarians for omega-3 fatty acids from non-animal sources⁷. Growing demand for new sources of omega-3 fatty acids is also a continuing safety concern within the accumulation of heavy metals and lipo-soluble compounds such as organic mercury compounds and polychlorinated biphenyls (PCBs) from the contaminants in the marine fish oil⁷. A much-debated question is whether marine fish will be used as a source of food to humans or as a source for production of omega-3 fatty acids supplement to human for health care. Hence, several microalgae such as *Chlorella* sp. and *Chlorella vulgaris* have been studied due to their known ability to produce omega-3 fatty acids^{3,8}.

Recently, microalga biomass has been attracting a lot of interest because of its rich

source of nutrients such as omega-3 fatty acids, omega 6 fatty acids, proteins, minerals and other essential nutrients⁹. There is a growing body of literature that recognises microalgae as a suitable alternative source of omega-3 fatty acids due to their continuity of raw materials, easily developed in the tropics, not susceptible to chemical contamination¹, being produced in a carefully controlled environment, and suitable for those following a vegetarian diet¹⁰.

Existing research recognizes *Acutodesmus* sp. as one of the ideal oleaginous freshwater microalgal species for research due to luxurious growth⁸ with easy cultivation¹¹⁻²¹, highly immune to bacterial infection¹², dominate other algal species and can flexibly adapt to wide range of environmental condition¹³. This present study, therefore, set out to assess biochemical composition of three different native freshwater microalgae with respect to different growth phases cultured under standardized conditions that might be useful for further commercial exploitation. Additionally, this present study seeks to throw some light on the lipid content of newly isolated freshwater green alga *Acutodesmus obliquus* that contain significant quantities of ALA after lipid accumulation, which could give nutritional added value to this microalgae oil.

MATERIALS AND METHODS

Microalgae strains

The freshwater microalgae species are *Chlorella* sp. (Carolina-15-2069, Carolina Biological Supply Company, USA), *Chlorella vulgaris* (NIES-1269, National Institute for Environmental Studies) and *Acutodesmus obliquus* (CN01, MJIIT-UTM, Malaysia). *Chlorella* sp. (Carolina-15-2069, Carolina Biological Supply Company, USA) was obtained from Carolina Biological Supply Company. Meanwhile, *Chlorella vulgaris* (NIES-1269, National Institute for Environmental Studies, Japan) was obtained from National Institute for Environmental Studies, Japan. *Acutodesmus obliquus* (CN01, MJIIT-UTM, Malaysia) was obtained from a freshwater sampling site at Hulu Langat River near to Langat River Dam in Selangor, Malaysia by researchers from Universiti Teknologi Malaysia.

Growth of microalgae

Chlorella sp. (Carolina-15-2069, Carolina Biological Supply Company, USA) and *Chlorella*

vulgaris (NIES-1269, National Institute for Environmental Studies, Japan) were cultured in BG-11 medium²². *Acutodesmus obliquus* (CN01, MJIT-UTM, Malaysia) was cultivated in AF6 medium²². The pH of the BG-11 medium and AF6 medium were adjusted to pH 7.5 and pH 6.6, respectively. Cultures were grown at 25°C for 12 h photoperiod with white fluorescent lamp at 80 μmol photons/m²/s with continuous aeration. The cultures were grown for 30 days otherwise stated.

The cultures with 10% (v/v) inoculum size were grown separately under two conditions, which are under normal medium (BG-11, AF6) and with the addition of 4 mM of sodium nitrate (nitrogen rich conditions)²³ in normal medium to establish nitrogen enrichment medium in identifying the effects on biomass production.

The growth of the freshwater microalgae was monitored every other day at 750 nm optical density (OD) using UV-spectrophotometer (Hach DR 6000, Hach, U.S.A.). Specific growth rate (μ, day⁻¹) for each strain was determined at the exponential growth phase²⁴.

Dry cell weight

Dry cell weight was determined at different cultivation time points (10, 20, 30 days) adopting the method of Fan *et al.*⁵ Briefly, cultures were centrifuged at 3500 x *g* for 10 min. The pellet cells were then dried in the drying oven at 65°C for 24 hours. The dry cell weight was measured after drying the cell pellet at 65°C and was expressed as gram of dried weight per 1 ml of medium⁵.

Chlorophyll concentration measurement

Chlorophyll concentration was measured every ten days. 1 ml of algae culture was centrifuged at 4°C for 10 minutes at 16000 x *g*. Supernatant was removed. 1.0 mL of absolute (100 %) methanol was added. The samples were vortexed and centrifuged again at 4°C for 10 minutes at 16000 x *g*. The OD of the supernatant was measured by spectrophotometer at 645 nm and 663 nm to identify the chlorophyll content, which was calculated and expressed as μg/mL⁷.

Lipid staining

Pellet from 1 ml of culture was washed twice with 1 mL of phosphate buffer saline (PBS). PBS was prepared by dissolving 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄·12H₂O and 0.24 g KH₂PO₄ in 1000 ml distilled water and was autoclaved. After that,

the cell suspension was diluted with distilled water to 1:100 serial dilutions for staining by Nile red (9-diethylamino-5-benzo[α]zphenoxazinone). The staining stock solution was prepared by dissolving 0.5 mg of Nile red powder into 1 ml of acetone. After adding 1.8 μL Nile red stock solution to the diluted cell suspension, it was kept in the dark for 20 minutes to ensure that the pigment reacted towards the lipid in the algae. Then, the samples were assayed using fluorescence spectrometer (LS55, Perkin Elmer, USA) by recording the fluorescence intensity (a.u.). The excitation and emission wavelength used are 488 nm and 520 nm, respectively²⁵.

Total Lipid Extraction

The lipid was extracted according to the modified Folch's lipid extraction method²⁶. 50 mL of algae suspension were harvested by centrifugation at 12,000 x *g* for 5 minutes at 25°C. Before the pellet was freeze-dried using freeze dryer (FDU-1200, EYELA, Japan) for 1 day, it was stored at -80°C. The freeze-dried cells were weighed and re-suspended in 6 mL methanol-chloroform mixture (1:2 v/v). 100 μL of C17 fatty acid (internal standard) with the concentration of 500 μg/mL was added to the crude lipid, which was then mixed by vortexing prior to centrifugation at 25°C for 10 minutes at 1000 x *g*. The supernatant was removed and 1.25 mL of 0.1M KCl was added to the new tube containing the supernatant, which was then mixed by vortexing for 30 seconds and centrifuged again at 25°C for 10 minutes at 1000 x *g*. Lipid content in the bottom chloroform phase was transferred to the rotary evaporator (VC-15s, Taitec, Japan) for evaporation under vacuum for 2 hours to the constant weight. Lipid content in the microalgae cells was calculated by weighing the dried lipid extract obtained from the evaporation using the electronic scale and expressed as the percentage of dry cell weight.

Analysis of Fatty Acid Content

Fatty acid methyl esters (FAMES) were extracted from the total intracellular lipid according to Kotajima *et al.*²⁷ 4 mL of 0.1 N methanolic hydrochloric acid (MeOH: HCl) was added to the dried lipid and kept in water at 100°C for 1 hour to obtain FAMES from the saponification and methyl-esterification of the free fatty acids and acyl-groups in the lipids. Then, 4 mL of hexane was added to the cooled FAMES sample to recover the resultant

FAMES with vigorous shaking for separating the two layers. The upper FAMES containing layer was kept in a new micro centrifuge tube. The lower MeOH:HCl phase, which is the remaining layer, was re-extracted with 2 mL distilled water and 2 mL hexane. The re-extracted upper FAMES containing layer was combined with the first upper layer extracted that was kept earlier in the micro centrifuge tube and then transferred to rotary evaporator for evaporation under vacuum for 30 minutes. 300 μ L of hexane was added to the dried FAMES. The solution was transferred into a glass vial for determining the fatty acid composition using gas chromatography.

Gas Chromatography Analysis

FAMES were quantified and identified by gas chromatograph equipped with mass spectrometer detector (GC/MS: G 3171A, Agilent, USA) and a HP-5M5 column (Agilent). The carrier gas used was helium in a splitless mode with constant flow rate of 7.10 mL/min. The initial temperature was 100°C for 1 min then raised to 200°C at a ramping rate of 25°C/min and held for 1 minute, which was then further increased to 250°C at a rate of 4°C/min and held again for 7 minutes²⁸. The FAMES content was calculated according to the method of Corro *et al.*²⁹ by comparing the peak area of the total FAME chromatogram with the peak area of internal standard (C17:0).

RESULTS AND DISCUSSION

Growth of microalgae

Table 1 displays the specific growth rate of microalgae in normal medium and those in nitrogen enriched medium. The specific growth rate quantified the growth potential as a number indicating the number of times an organism

Table 1. Specific growth rate of microalgae in normal medium and nitrogen enriched medium

Microalgae	Specific growth rate (μ , day ⁻¹)	
	Normal medium	Nitrogen enriched medium
<i>Acutodesmus obliquus</i> CN01	0.0781	0.0931
<i>Chlorella</i> sp. Carolina-15-2069	0.0581	0.0690
<i>Chlorella vulgaris</i> NIES-1269	0.0533	0.0721

reproduces in the time unit³⁰. *Acutodesmus obliquus* CN01 was shown to exhibit the fastest growth rates, which are 0.0781/day (normal media) and 0.0931/day (nitrogen enriched media) for both media compared to *Chlorella* sp. Carolina-15-2069 (0.0581/day and 0.0690/day) and *Chlorella vulgaris* NIES-1269 (0.0533/day and 0.0721/day). From previous study, it was shown that high doubling time corresponds to a low specific growth rate³¹. Mujtaba *et al.*²³ reported that *Acutodesmus* sp. has a tendency for easy cultivation and has a fast growth rate. Fast and high growth rate encourages high biomass productivity, competitive advantage over competing species and reduces required culture area³¹.

Dry cell weight

Dry cell weight of the microalgae was measured to identify the differences in biomass production by the normal medium and nitrogen enrichment medium. Fig. 1a and Fig. 1b illustrate the dry cell weight of microalgae in normal medium and in nitrogen enriched medium, respectively.

Fig. 1a and 1b indicate enhanced biomass production in the nitrogen enrichment medium. This was due to high nitrogen concentrations in the medium (4 mM of sodium nitrate) that can significantly enhance the biomass production³². *Acutodesmus obliquus* CN01 has the highest biomass production at day 20 in normal AF6 medium (0.0051 g/mL) and nitrogen enrichment medium (0.0066 g/mL) compared to *Chlorella* sp. Carolina-15-2069 and *Chlorella vulgaris* NIES-1269. These results are consistent with those of other studies and suggest that *Acutodesmus obliquus* CN01 has a fast growth rate which promotes high biomass productivity that can reduce cost for large-scale lipid production³³. Biomass for *Acutodesmus obliquus* CN01 was shown to sharply increased during 10-30 days of cultivation in nitrogen enrichment medium if compared to the normal AF6 medium.

For normal AF6 medium, the maximum production of biomass for *Chlorella* sp. Carolina-15-2069 (0.0022 g/mL) and *Chlorella vulgaris* NIES-1269 (0.0033 g/mL) was obtained at day 30 (Fig. 1a and 1b). For nitrogen enrichment medium, the maximum production of biomass for *Chlorella* sp. Carolina-15-2069 (0.0034 g/mL) was at day 30 while *Chlorella vulgaris* NIES-1269 (0.0041 g/mL) at day 30. As shown in Fig. 1a and

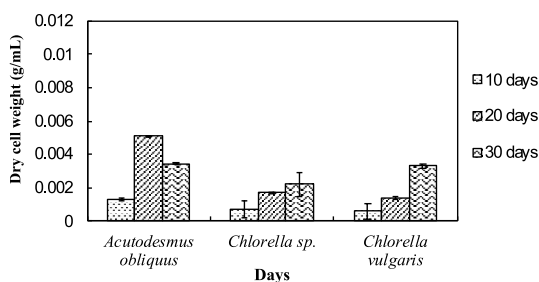


Fig. 1a. Dry cell weights of microalgae in normal medium.

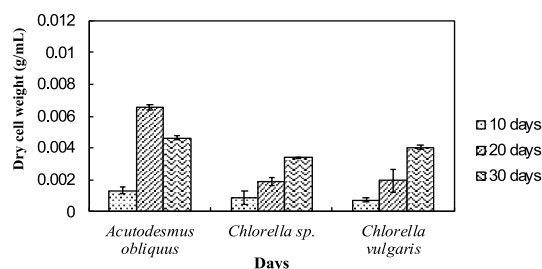


Fig. 1b. Dry cell weights of microalgae in nitrogen enrich medium.

Fig. 1b, the maximum algal biomass for these three microalgae was obtained after 10-30 days of cultivation. The results, as shown in Fig. 1a and 1b, indicated that the medium enrichment and the age of the microalgae culture can affect the biomass production.

Chlorophyll concentration measurement

The health of photosynthetic cells in microalgae are assessed by measuring the chlorophyll content of microalgae⁷. In summary, from Fig. 2, these results show that the highest total chlorophyll content was observed in *Chlorella sp.* Carolina-15-2069 (21 mg/L) followed by *Chlorella vulgaris* NIES-1269 (13 mg/L) and *Acutodesmus obliquus* CN01 (3 mg/L). Interestingly, the chlorophyll content was observed to increase with cultivation time. These results are in line with those of previous studies. Chlorophyll is one of the cellular compounds that is used for estimating biomass of microalgae in culture and can be used to measure growth where the chlorophyll content per cell is increased due to the reduction of the irradiance into the culture by the shading effect produced by the high cellular density of the culture³⁴. On day 30, the total chlorophyll content of *Acutodesmus obliquus* CN01 suddenly decreased to 1.2 mg/L. This could be due to nutrient depletion.

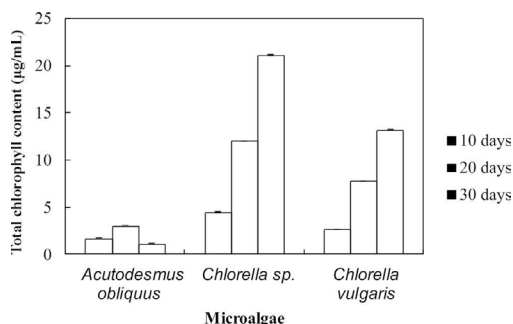


Fig. 2. Total chlorophyll content in microalgae.

Nile red staining

Nile red is a red fluorescent lipophilic dye³⁵ where it fluoresces indicates the present amount of neutral lipid³⁶. The colour of the Nile red able to penetrate cell walls, cytoplasmic membrane and dissolve in the intracellular neutral and polar lipids to give the desired yellow fluorescence colour and will give a red fluorescence colour without staining cells³⁷. From the lipid analysis using Nile red staining, *Chlorella sp.* Carolina-15-2069 showed strongest fluorescence signals at day 20, whereas both *Chlorella vulgaris* NIES-1269 and *Acutodesmus obliquus* CN01 yielded strongest fluorescence emissions at day 30 (Fig. 3).

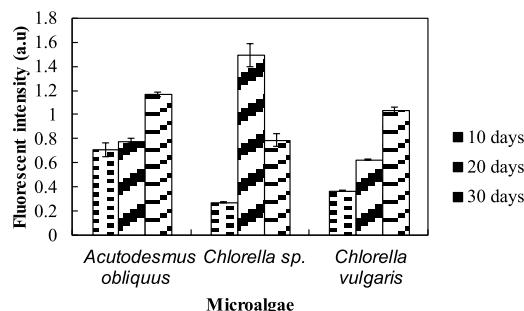


Fig. 3. Nile red staining results of microalgae.

The current study found that after 20 days, the microalgae was still able to accumulate neutral lipid. This is an interesting outcome. High fluorescence intensities indicated high neutral lipid content in these organisms. Another interesting finding is the lipid content changed along with growth. Measuring lipid content by Nile red staining is the rapid³⁶, simplest and cost-effective method³⁵.

Lipid contents

The amount of lipid in microalga was quantified to identify the lipid content in the microalgae. Lipids are synthesized as energy

and carbon reserve to stress conditions and accumulated as triacylglycerols (TAG) in the cytoplasm³⁸. Besides, lipid production was enhanced by a number of factors including aeration to the culture, increases in carbon dioxide supply and depleted with a depletion of carbon source in the medium²³. The most striking result to emerge from the data is that the lipid content for *Acutodesmus obliquus* CN01 was the highest (47%) if compared to the other two algae (Fig. 4). The lipid content value in this study was the highest lipid content reported for *Acutodesmus obliquus* CN01 growth under normal condition^{12,19,39}. This result is somewhat counterintuitive. *Acutodesmus* sp. is of interest as *Acutodesmus* sp. was the ideal species for lipid accumulation^{7,40,41,42}. It has been reported that low nutrient concentration and good penetration of light to the individual cells would cause photosynthesis to generate more metabolic flux that will be channelled to lipid accumulation in *Acutodesmus* sp.⁷

There was a significant difference in lipid class and fatty acid composition of microalga cells at different growth phase due to variations in culture conditions including nutrient status (nitrogen or phosphorus starvation)³⁷, temperature, salinity, pH, photoperiod, light intensity and light quality⁶. Prior studies have noted that reduction in lipid content of *Chlorella* sp. Carolina-15-2069 and *Chlorella vulgaris* NIES-1269 are due to triglyceride utilization during cell division cycle, mortality of

algal cells at later stage, and also depletion of nutrients in the culture medium²¹.

FAMES profiles

The percentage of fatty acid composition in the three species of microalgae used in this study was summarised in Table 2. Fatty acids were extracted after 15 days of cultivation and analysed by GC/MS for the identification of the fatty acids profile in the freshwater microalgae. A possible explanation for this is that longer cultivation time was beneficial for the accumulation of unsaturated fatty acids²¹. As illustrated in Table 2, fatty acids that were synthesized have chain lengths that range from C16 to C18.

These result suggest that saturated fatty acid (SFA) content was higher in *Chlorella* sp. Carolina-15-2069 if compared to the other two microalgae. Surprisingly, monounsaturated fatty acid (MUFA) was not found in *Chlorella* sp. Carolina-15-2069 and *Chlorella vulgaris* NIES-1269. According to data obtained from Table 2, we can conclude that *Chlorella vulgaris* NIES-1269 has the highest amount of polyunsaturated fatty acid (PUFA) followed by *Chlorella* sp. Carolina-15-2069 and *Acutodesmus obliquus* CN01. Variations that were observed in the fatty acid amount in each microalga are possibly due to extraction technique, lipolysis due to long term storage of microalgal paste and culture condition of the microalgae such as temperature, nutrients, dilution rate, carbon dioxide concentration and growth phase of microalgae at the time of harvest².

In the present study, *Acutodesmus obliquus* CN01 showed high amount of α -linolenic acid (ALA), palmitic acid, and oleic acid. The results of this study also indicated that the main fatty acids found in *Chlorella* sp. Carolina-15-2069 were palmitic acid, ALA, and hexadecatrienoic acid. *Chlorella vulgaris* NIES-1269 consisted of mostly ALA, palmitic acid, and linoleic acid. It is apparent from this table that ALA and palmitic acid were the main components of the fatty acid profile in these three microalgae.

The highest amount of ALA was found in *Acutodesmus obliquus* CN01 (38 %) followed by *Chlorella vulgaris* NIES-1269 (35 %), and *Chlorella* sp. Carolina-15-2069 (17.9 %). Another important finding was that ALA content in *Acutodesmus obliquus* CN01 reported in this study was higher than other study^{6,9}. It has been suggested that

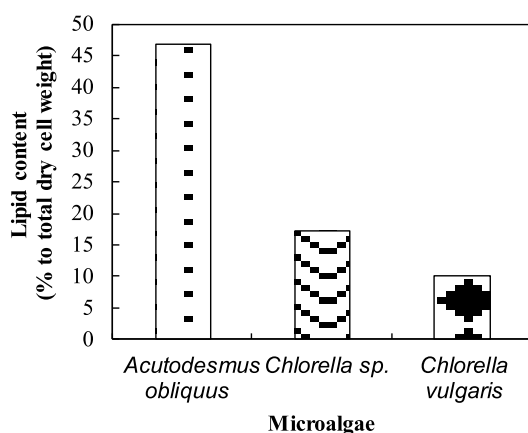


Fig. 4. Lipid content of *Acutodesmus obliquus* CN01 (percentage to total dry cell weight). Dotted bar: *Acutodesmus obliquus* CN01, wavy line bar: *Chlorella* sp. Carolina-15-2069, solid diamond: *Chlorella vulgaris* NIES-1269.

Table 2. Fatty acid profile (FA) (percentage to total fatty acids) of freshwater microalgae

Fatty acids	Common name	<i>Acutodesmus obliquus</i> CN01	<i>Chlorella</i> sp. Carolina-15-2069	<i>Chlorella vulgaris</i> NIES-1269
Saturated				
C16:0	Palmitic acid	32.4±2	33.9±3.7	25.7±0.8
C18:0	Stearic acid	ND	ND	8.7±0.3
Sum		32.4±2	33.9±3.7	34.4±1.1
Monounsaturated				
C18:1	Oleic acid	32.0±2	ND	ND
Sum		32.0±2	ND	ND
Polyunsaturated				
C16:2 (n-6)	Hexadecadienoic acid	ND	13.1±1.45	ND
C16:3 (n-3)	Hexadecatrienoic acid	7.4±0.45	17.3±1.9	6.1±0.2
C16:4		ND	ND	5.9±0.45
C18:2 (n-6)	Linoleic acid	13.6±0.85	11.6±1.25	12.8±0.4
C18:3(-3)	α-linolenic acid	38.0±13.5	17.9±6.55	35.4±1.1
Sum		32.0±14.8	59.9±11.15	60.2±2.15

ND= not detected

ALA is a precursor or starting molecule for the synthesis of EPA and DHA⁴³ and vital for normal development of infant's brains and nervous systems³. It is interesting to note that in this study, the most compelling finding is *Acutodesmus obliquus* CN01 has a fast growth rate with a high lipid content and also high ALA content compared to the other two species of microalgae tested. This is an encouraging and positive result which provide important insights into *Acutodesmus obliquus* CN01 as another significant source of nutraceutical supplements especially ALA and has an extra advantage than the other two microalgae (*Chlorella* sp. Carolina-15-2069 and *Chlorella vulgaris* NIES-1269) for its easy upscale. This result is somewhat is counterintuitive. *Acutodesmus obliquus* CN01 that was isolated from Malaysia was shown to well adapt to the temperature in Malaysia, which can reduce the upscale cost if to be used for production purposes. Apart from ALA, palmitic acid which is another main component of the fatty acid profile in these three microalgae has an outstanding antioxidant activity⁴⁴ and has been identified as antitumor agent⁴⁵.

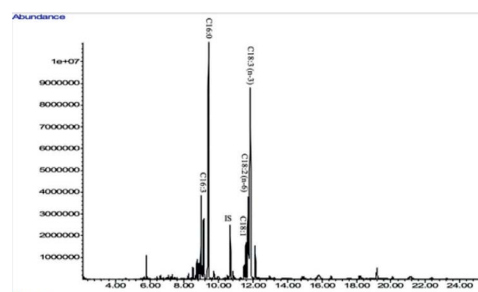


Fig. 5a. GC-MS chromatograms of FAME derived from freeze-dried biomass of freshwater green algae *Acutodesmus obliquus* CN01 (see Table 2 for peak identification). *Heptadecanoic acid (C17:0) was used as an internal standard.

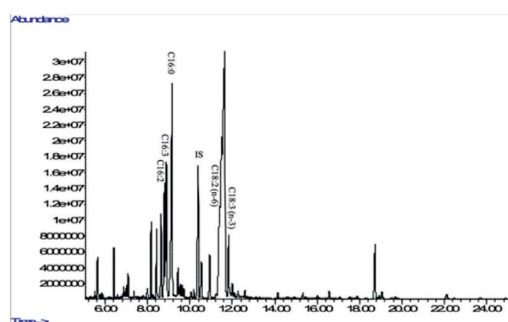


Fig. 5b. GC-MS chromatograms of FAME derived from freeze-dried biomass of freshwater green algae *Chlorella* sp. Carolina-15-2069 (see Table 2 for peak identification). *Heptadecanoic acid (C17:0) was used as an internal standard.

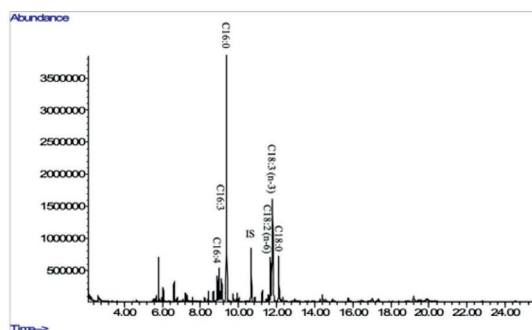


Fig. 5c. GC-MS chromatograms of FAME derived from freeze-dried biomass of freshwater green algae *Chlorella vulgaris* NIES-1269 (see Table 2 for peak identification). *Heptadecanoic acid (C17:0) was used as an internal standard.

CONCLUSION

The purpose of this study is to provide information on the biochemical composition of the total lipid extracted from freshwater microalgae. From this study, newly isolated *Acutodesmus obliquus* CN01 from Malaysia exhibited fast growth rate with a high lipid content and also high ALA content compared to *Chlorella* sp. Carolina-15-2069 and *Chlorella vulgaris* NIES-1269. Further research on *Acutodesmus obliquus* CN01 is well underway in defining its ability to yield omega-3 fatty acids.

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AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

FUNDING

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Fig. 6a. Morphological observation of *Acutodesmus obliquus* CN01 with 40X magnification.



Fig. 6b. Morphological observation of *Chlorella* sp. Carolina-15-2069 with 40X magnification.

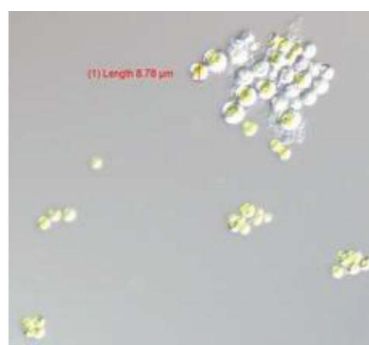


Fig. 6c. Morphological observation of *Chlorella vulgaris* NIES-1269 with 40X magnification.

Takasago Thermal Engineering Co. Ltd from 2015 to 2017.

DATA AVAILABILITY

All datasets obtained or studied during this study are incorporated in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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