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Freshwater pond microalgae for biofuel: Strain isolation, identification, cultivation and fatty acid content

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Abstract. *Perdana BA, Dharma A, Zakaria IJ, Syafrizayanti. 2021. Freshwater pond microalgae for biofuel: Strain isolation, identification, cultivation, and fatty acid content. Biodiversitas 22: 505-511.* Microalgae have capability to produce fatty acid for biofuel, drugs, and nutraceutical foods development. This study was carried out to obtain a new strain candidate for fatty acid production. The methods were used in this study include isolation of microalgae species from freshwater ponds of Andalas University, Padang, Indonesia. Molecular identification of microalgae was carried out with specific 18S rRNA primer, F-P73, and R-P47. Microalgae growth was measured by cell density and optical density method using various wavelengths (400, 500, and 680 nm). Total lipid was extracted using Bligh & Dyer method. Fatty acid analyses were conducted using gas chromatography-mass spectroscopy. Microalgae were isolated i.e *Chlorella emersonii* MAUA001, *Mychonastes rotundus* MAUA002, *Scenedesmus dimorphus* MAUA003, and *Scenedesmus armatus* MAUA004. The result exhibited *M. rotundus* was the highest lipid content, it was about 28.8% biomass weight. Fatty acid profiles of microalgae were dominated by monounsaturated (MUFA) and saturated fatty acid (SFA). The highest content of fatty acid species found in *C. emersonii* with octadecenoic acid (C18:1) was 47.74% total lipid. This work showed that *C. emersonii* has potential as biodiesel due to high saturated fatty acid.

Keywords: Fatty acids, lipid, microalgae, microalgae isolation, molecular identification

INTRODUCTION

Microalgae is a photosynthetic microorganism that is able to fix CO_2 to produce biomass and has a role as a primary producer in aquatic environment. It has the capability to grow under harsh conditions. Microalgae can be easily cultivated and produced to obtain certain organic material for human necessity, such as biomass, lipid, phycocyanin, astaxanthin, and other useful metabolites. By the year 2004, about 7000 tons of microalgae biomass annually have been produced for market demands (Spolaore et al. 2006). This statistic showed that microalgae have gained popularity among the worldwide industrial productions and will continue to develop utility and trends.

Microalgae have a great diversity that has potential to explore and use as human needs. A conservative approach result estimated the microalgae species about 72500 algal species, about 44000 species have probably been published (Guiry 2012), less than 50 species have been commercially produced by industries (Milledge 2012). Most frequently research topics of microalgae related to biomass, biodiesel, fatty acid, and food due to that topic have high potential and high price for making valuable products (Garrido-Cardenas et al. 2018). Microalgae have capability to rapid growth and accumulating high oil content rather than other plants. Some microalgae species, such as *Botryococcus brauinii* have lipid content 65% of biomass dry weight (Tasić et al. 2016).

Microalgae lipids are appropriate choice to reduce fossil fuel consumption. Fossil fuel consumption will lead the fossil oil reserves to be exhausted in less than 50 years. Besides, fossil fuel is the major greenhouse gasses source responsible for global warming; fossil fuel energy usage should be reduced or limited. Moreover, microalgae oil has high omega-3 fatty acid content, which is good for novel human food development (Topuz 2016). EPA and DHA contents in microalgae oil is able to consume without causing some health risk such as allergy to seafood oil (Martins et al. 2013). Microalgae cultivation and development for biodiesel and food need the appropriate strain to enhance the products. Isolation, identification, and characterization of microalgae growth are important to initiate early microalgae development and production. This study would carry out isolation of some species of freshwater microalgae. The purpose of this research was to obtain the best species for fatty acid production. The new strains would be used for various applications of fatty acid production.

MATERIALS AND METHODS

Isolation and identification of microalgae

Microalgae were isolated from freshwater ponds of Andalas University, Padang, West Sumatra, Indonesia using micropipette washing technique (Parvin et al. 2007). The isolated microalgae were then cultivated using Bold

Basal Medium in 500 mL flask at temperature 27ºC, light intensity of 2500 lux for 24 hours/day, and aerated using 3.5 L/min flow rate. Microalgae were harvested at late exponential phase by centrifugation. The genomics DNA microalgae were isolated using Plant Wizard Genomic DNA Purification Kit (Promega). Extraction procedure was conducted following manufacturer's instruction. The DNA quality was checked using electrophoresis in 0.8% agarose. The 18S rRNA was amplified using a pair primer, forward F-P73 (5'AATCAGTTATAGTTTAATTGRT'3) and reverse R-P47 (5'TCTCA GGCTCCCTCTC CGGA'3) (Gour et al. 2016). PCR was conducted following the steps; initial denaturation of 4 minutes at 94°C followed 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 52.5°C for 55 seconds, primer extension at 72°C for 1 minute, then final extension at 72°C for 5 minutes. PCR products were separated using electrophoresis on 1% agarose. DNA sequencing was conducted using 18S rRNA, then all sequences were compared with GenBank database using BLAST.

Extraction of total lipid and fatty acid analyses by gas chromatography and mass spectroscopy

Total lipid extraction was conducted using Bligh and Dyer method (Cavonius et al. 2014). Amount of 20 mg of microalgae dry biomass were wetted with 80 µL distilled water for 60 min, then added 300 µL of chloroform: methanol (1:2), vortex for 2 min. Then, sample was added 100 µL of chloroform and vortex for 30 s. Sample was centrifuged at 2500 x *g* for 6 min. The water layer was discarded, the chloroform phase recovered, the residue re-extracted with 100 µL of chloroform three times. Then, the extracts were air-dried and total lipid (TL) percentage was determined by following formula:

$$\%TL = \frac{\text{Total lipid weight (g)}}{\text{Total biomass weight (g)}} \text{ x } 100\ \%$$

Lipid extracted from microalgae biomass was methylated by modified methanolic HCl-transesterification method Cavonius et al. (2014). Microalgae lipid was incubated at 90°C for 2 hours with methanol: HCl: chloroform (10: 1: 1). Thereafter, lipid was added 1 mL distilled water and fatty acid methyl ester extracted by adding 2 mL hexane-chloroform (4: 1), vortexed and top layer recovered. The organic layer was injected to GC-MS with specification were column oven temperature 50°C, injection temperature 250°C, pressure 119,3 kPa, total flow 60 mL/min, start time 2.00 min, end time 60.54 min, start m/z 40.00, and end m/z 500.00.

RESULTS AND DISCUSSION

Isolation and identification of microalgae

Four species of microalgae had been successfully isolated from freshwater ponds of Andalas University. Microalgae were observed under microscope using 400x magnification to revealed morphological characteristics and purity (Figure 1). Result from morphological identification showed that the four species were *Chlorella* sp.1, *Chlorella* sp.2, *Scenedesmus* sp.1, and *Scenedesmus* sp.2. Morphological identification was used to assist the next molecular identification pr°C ess to determine the correct primer. Microalgae molecular identification confirmed that *Chlorella* sp.1 as *Chlorella emersonii* MAUA001, *Chlorella* sp.2 as, *Mychonastes rotundus* MAUA002, *Scenedesmus* sp3 as *Scenedesmus dimorphus* MAUA003, and *Scenedesmus* sp4 as *Scenedesmus armatus* MAUA004. All microalgae classified as Chlorophyta, Ordo Chlorococcales.

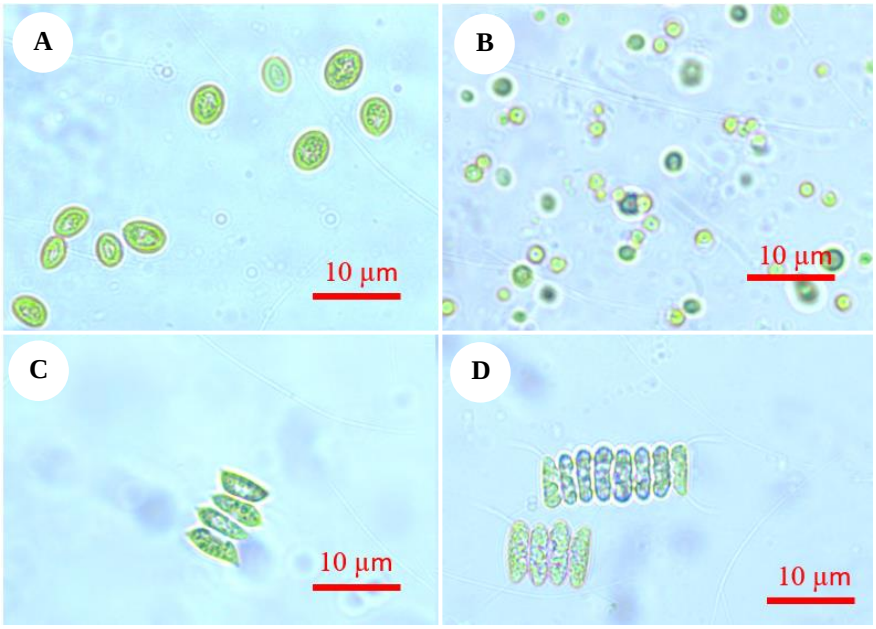

Figure 1. Morphological image using microscope 400x magnification. A. *Chlorella emersonii* MAUA001; B. *Mychonastes rotundus* MAUA002; C. *Scenedesmus armatus* MAUA004; D. *Scenedesmus dimorphus* MAUA003

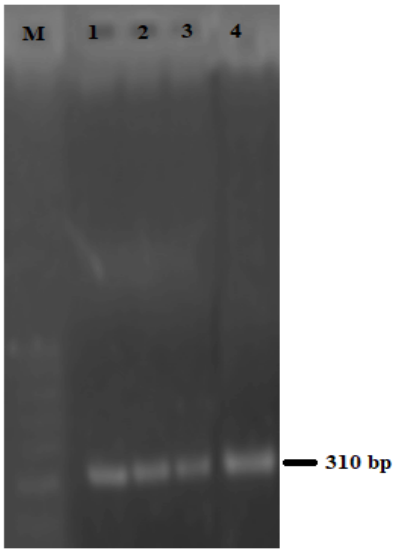

Figure 2. Agarose gel electrophoresis of 18S rRNA PCR products. M: Marker, 1: *S. dimorphus*, 2: *C. emersonii*, 3: *M. rotundus*, 4: *S.armatus* electrophoresis band

Microalgae DNA extracted and amplified by F-P73 and R-P74 primer showed the clear bands (Figure 2). Electrophoresis analyses exhibited the PCR product in amount of 310 bp. The PCR products of the samples were then purified and send to sequencing analyses. Microalgae DNA sequences were compared with other related species in GenBank database with 98-99% sequence similarity and e-value 0.0. Databases were then downloaded, aligned, and constructed using MEGA 6.0 with Neighbor-Joining phylogenetic tree method.

Phylogenetic tree exhibited the bootstrap value 67, 71, 77 and 99 on *S. dimorphus, C. emersonii, M. rotundus*, and *S. armatus* group respectively (Figure 3). The bootstrap value highly supported closest relationship between species in each cluster. Bootstrap value is used for determining appropriate model of phylogenetic tree. The higher of bootstrap value is shown then more valid data used and more acceptable the phylogenetic tree model constructed. Conversely, if bootstrap value is lower, the sequence must be removed from analyses so that the phylogenetic tree can be trusted (Dharmayanti 2011). Figure 3 showed that *S. armatus* MAUA004 is the highest bootstrap value between microalgae isolates. *S armatus* MAUA004 related with *Scenedesmus* sp KL, *Desmodesmus* sp Sp.19.010, and *S. armatus* GonYang4. Likewise the other isolate, the species are grouped in appropriate clusters.

Microalgae growth characteristic

Microalgae were cultivated using 500 mL Bold Basal Medium (BBM) for 10 days (about early stationary phase). Growth rates were measured using cell density and optical density (OD) approaches. Growth rate measurements using cell density are shown in Figure 4.A indicated that microalgae exhibited the late logarithmic phase at day 9, except for *S. dimorphus* on day 8. Late logarithmic phase is signed with drastically high increase of cell density and followed by the lower of cell density accretion. In cell division rate, *M. rotundus* have highest cell density among the other microalgae. *M. rotundus* cell at day 10 was 140 x 10^6 cell/mL. Optical density of microalgae growth was measured using 400, 500, and 680 nm. The choice of those wavelengths aimed to calibrate the appropriate visible wavelength for microalgae growth measurement. At Figure 4.B showed that OD at 400 nm has highest absorbance value. While, the OD 680 nm is lower at all microalgae, except *S. dimorphus* after day 6 cultivation.

Optical density was conducted to simplify the continued measurement of microalgae growth rate. Therefore, in this study, the linear regression correlation was used to analyze relationship between the OD wavelengths with cell density. The cell density was chosen as standard for validation OD growth rate measurement. Linear regression correlation coefficient (R^2) value showed in Table 1. R^2 values range between 0.89-0.99, implicating a high positive relationship between cell density with all OD wavelengths. However, the highest value of R^2 was 680 nm for *M. rotundus*, *S.armatus*, and *S. dimorphus*. Meanwhile, *C. emersonii* has highest R^2 value at 500 nm. The highest R^2 values indicated the most appropriate wavelength for microalgae growth measurement.

Microalgae cell size exhibited fluctuation along cultivation process. Cell size of *M. rotundus* and *C. emersonii* were measured by diameter of cell. However, *S. armatus* and *S. dimorphus* were measured by the width of cell due to more fluctuative than the length of cell. Microalgae cell size diverse along the time of cultivation. There was no clear pattern between microalgae cell size with cultivation time. The higher microalgae cell size exhibited autospore formation for microalgae cells and the lower showed the microalgae finished division process after autospore form. Standard deviation bar in Figure 5 showed the diverse cell size at one time of measurement. The highest diverse cell size for every species indicated that most active division process of cell occurred before measurement.

Lipid content and fatty acids profile of microalgae

Microalgae harvested, extracted and total lipid percentage was determined by lipid per biomass weight. Figure 6 showed that lipid content range between 23-28.8% bw. *M. rotundus* was the highest lipid content among other microalgae, followed by *S. dimorphus, C. emersonii*, and *S. armatus*. Analyses of variance (ANOVA) were conducted to analyze the significance between the microalgae lipid contents, then followed by Bonferroni post hoc test at significance 0.05. *M. rotundus* lipid content has no significant value with *C. emersonii*, but it has a difference with *S. armatus* and *S. dimorphus*. Based on the analysis, *M. rotundus* was the best lipid content for production.

Table 1. Linear regression correlation coefficient (R^2) relationship between OD and cell density

Optical density	*M. rotundus*	*C. emersonii*	*S. armatus*	*S. dimorphus*
400 nm	0.8951	0.8806	0.8393	0.8825
500 nm	0.9732	0.9779	0.8706	0.9155
680 nm	0.9859	0.9623	0.8908	0.9942

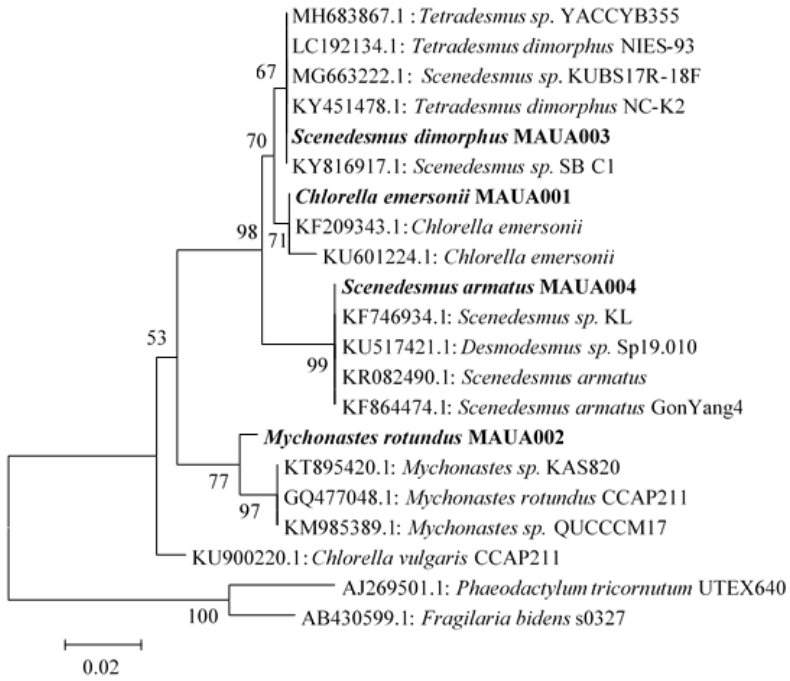

Figure 3. Phylogenetic tree of microalgae constructed using Neighbor-Joining method

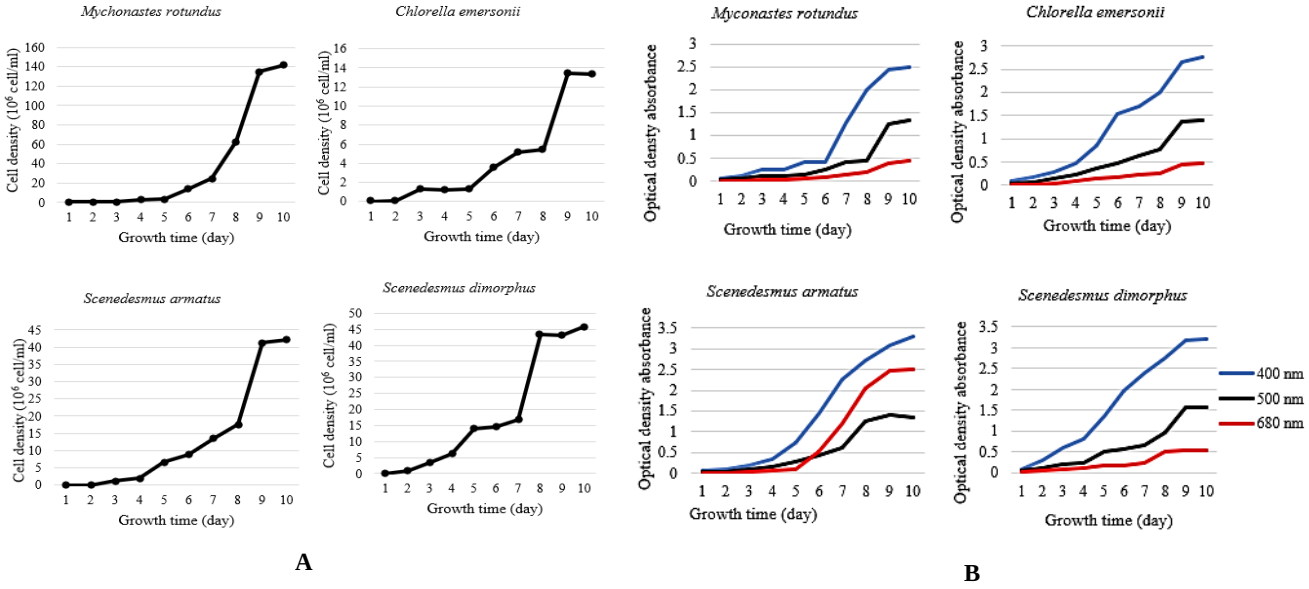

Figure 4. Microalgae growth measured by A. Increasing cell density of microalgae; B. Optical density with 400, 500 and 680 nm wavelength

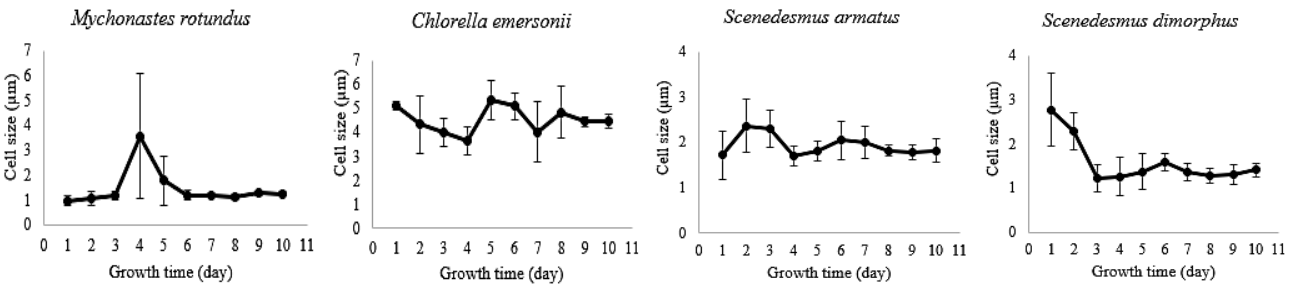

Figure 5. Microalgae cell size and standard deviation bar along cultivation process

GC-MS analyzed microalgae lipid for fatty acids profile. Table 2 exhibited the kind of fatty acid profile. Total fatty acids were recovered range between 42.17-91.23 % total lipid. The highest total fatty acid was found in *C. emersonii* and the lowest ones in *M. rotundus*. Monounsaturated fatty acid (MUFA) was the highest content of *M. rotundus, C. emersonii* dan *S. dimorphus*. Meanwhile, *S. armatus* was dominated by saturated fatty acid (SFA) about 33.47%. Besides MUFA, *M. rotundus* has high polyunsaturated fatty acid (PUFA) and low SFA content. But, *C. emersonii* and *S. dimorphus* had higher SFA contents than PUFA. In terms of fatty acids species, octadecenoic acid (C18:1) was the highest fatty acids content of M. rotundus, C. emersonii, and S. dimorphus. Meanwhile, S. armatus has palmitic acid (C16:0) as the highest content of fatty acids species. Omega fatty acids were found of kind omega-3, omega-6 and omega-12. *M. rotundus* and *C. emersonii* have high omega-6 contents 14.97 % and 13.72 % respectively.

Table 2. Fatty acids profile of microalgae (% total lipid) of *M. rotundus* (MR), *C. emersonii* (CE), *S. dimorphus* (SD) dan *S. armatus* (SA)

Fatty acid species	Formula	Fatty acid content (%TL)			
		MR	***CE***	***SD***	***SA***
Hexadecanoic acid/ Palmitic acid	C16:0	17.60	18.70	17.43	29.26
Hexadecenoic acid	C16:1	-	-	-	2.54
Hexadecadienoic acid	C16:2	1.31	2.58	-	0.70
Hexadecatrienoic acid	C16:3 ω3	3.64	4.57	-	-
Octadecanoic acid/ Stearic acid	C18:0	2.30	4.15	2.02	3.75
Octadecenoic acid	C18:1	23.97	47.74	18.92	24.39
Octadecenoic acid omega 12	C18:1 ω12	-	-	3.80	3.72
Octadecadienoic acid	C18:2	-	-	8.21	9.72
Octadecadienoic acid/ Linoleic Acid	C18:2 ω6	14.97	13.72	-	-
Octadecatrienoic acid/Methyl γ-linolenate	C18:3 ω6	-	-	-	0.43
Eicosatrienoic acid	C20:3	-	0.40	-	-
Docosanoic acid/ Behenic acid	C22:0	-	-	-	0.46
Total FA		42.17	91.86	50.38	74.57
SFA		18.20	22.85	19.45	33.47
MUFA		23.97	47.74	22.72	30.65
PUFA		19.92	21.27	8.21	10.85
Omega-3		3.64	4.75	-	-
Omega 6		14.97	13.72	-	0.43
Omega 12		-	-	3.80	3.72

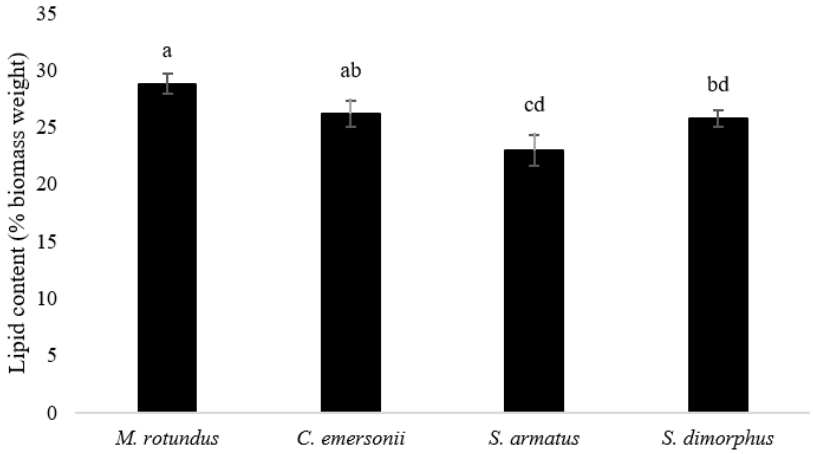

Figure 6. Microalgae lipid contents extracted by Bligh Dyer method. The superscript letter above the bar lipid content showed the significant value by ANOVA analyses with Bonferroni post hoc test at significance 0.05

Discussion

Microalgae were isolated and observed using microscope exhibited clear morphological differences. *M. rotundus* and *C. emersonii* have same coccoid form, but the differences between them both were cell size of *C. emersonii* is larger than that of *M. rotundus*. *C. emersonii* cell size was 5-7 µm while *M. rotundus* 1-3 µm (Figure 5). *Mychonastes* and *Chlorella* were one group of *Chlorella* genus due to the similarities of their morphological characteristic (Krienitz et al. 2011). *S. armatus* and *S. dimorphus* have a clearly different morphological characteristics in present of cetae or spine in tip of *S. armatus* cenobium. *S. armatus* cenobia was ovoid rounded while *S. dimorphus* ovoid pointed. All of morphological characteristics were suited with identification book (Yamaji 1980). Molecular identification was conducted for validation and observation of closest relationship between the previous databases. DNA extraction and amplification using specific 18S rRNA primer, F-P73 and R-P47 exhibited 310 bp in electrophoresis result. The PCR product was a bit larger than previous study was conducted by Gour et al. (2016) with PCR product 270-276 bp for *Chlorella* and *Scenedesmus* microalgae.

Microalgae growth exhibited a high increase in cell density over time. *M. rotundus* has highest cell density (140 x 10^6 cell/ml at day 10) among the microalgae isolates. The *M. rotundus* cell density was higher than previous researches (Šoštariè et al. 2009; Chia et al 2013). Bold Basal medium was used in this study suited for microalgae isolates growth. Wong et al. (2017) has reported about microalgae medium for cultivation. The Bold Basal medium was the best growth medium among the other twelve growth mediums (M-8, BG 11 modified, Spirulina medium modified, N-8, BG 11, RM, Chu modified, Johnson, F/2, Fog, and Fog medium without nitrogen).

Microalgae lipid content ranged between 23-28.8% were classified as adequate if compare to previous research. Commonly, microalgae lipid content ranged between 10-50% biomass weight (Xu and Hu 2013; Ramluckan et al. 2014; Tan et al. 2018; Chi et al. 2019). The difference of microalgae lipid contents depends on microalgae strain, cultivation treatment, and lipid extraction process. Microalgae fatty acid profile showed various types of fatty acid. Based on the common usage of microalgae fatty acid, the microalgae have potential as biodiesel and food production in this study. Some microalgae have high content of SFA and MUFA such as *C. emersonii* were potential for biodiesel production. *C. emersonii* were dominated by C18:1 which has 57.74% total lipid content. Besides, *C. emersonii* and *M. rotundus* have high PUFA contents that have potential as food production.

In conclusion, microalgae isolation and fatty acid profiling were obtained. Microalgae isolate for fatty acid production was successfully isolated. Lipid content of *M. rotundus* was the highest obtain of all other isolates. Fatty acid profiles of microalgae were dominated by monounsaturated (MUFA) and saturated fatty acid (SFA). The highest content of fatty acid species was found in *C. emersonii* with octadecenoic acid (C18:1) content was 57.74% total lipid. Based on application purpose, *C. emersonii* has potential as biodiesel and *M. rotundus* as nutraceutical food.

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