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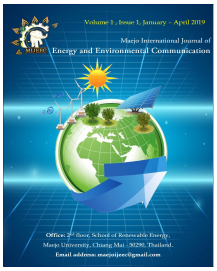

ARTICLE

A selective microalgae strain for biodiesel production in relation to higher lipid profile

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ABSTRACT

Biodiesel have become the important asset by the country especially to build up their economy. Currently, microalgae have been choosing as the source for production of biodiesel based on their advantages. Microalgae are a photosynthetic organism that use light as an energy source and able to produce their own food. These microalgae also produce a lipid that can be used to produce a biodiesel. Using microalgae that contain high lipid profile are very important to make sure the biodiesel can be produce in large quantity in short time and more cost saving. Although many microalgae species have been identified and isolated for lipid production, there is currently no consensus as to which species provide the highest productivity. Different species are expected to function best at different aquatic, geographical and climatic conditions. So, this experiment is conducted to identify which strain of microalgae contains high lipid profile that can be used to convert into the biodiesel. There are three main objectives that involve in this experiment which is to isolate and identify different strain of microalgae from Kuantan Coast, East Coast Peninsular Malaysia, to convert the lipid from microalgae into biodiesel through transesterification, and to estimate higher lipid profile of microalgae species for biodiesel production. Two species of green microalgae were isolated, which is *Nannochloropsis sp* and *Coelastrum sp*. Based on lipid extraction and lipid analysis, it shows that the *Nannochloropsis sp.* have more concentrated of lipid and higher lipid profile compared to *Coelastrum sp*. Hence, *Nannochloropsis sp.* are most suitable species that can be used as a biodiesel feedstock due to higher lipid profile of MUFA.

1. Introduction

As worldwide petroleum reserves diminish due to consumption exceeding discoveries, many countries are becoming increasingly dependent upon imported sources of oil. The demand for energy is growing worldwide especially in many of the rapidly developing countries such as in China and India. Furthermore, the continued combustion of fossil fuels has created serious environmental concerns over global warming due to the increased release of greenhouse gases (GHG) (Chuanchai and Ramaraj, 2018). Biofuels are one of the potential options to reduce the world's dependence on fossil fuels but biofuels have their limitations. One of the recent concerns with respect to increased biofuels production is the availability of land (Hill et al., 2006). It is recognized that the GHG benefits of biofuels can be offset if land with existing high carbon intensity is cleared for the production of

biofuel feedstock. Biofuels that could be produced without large increases in arable land or reductions in tropical rainforests could be very attractive in the future (Chen et al., 2018).

The basic concept of using algae as a renewable feedstock for biofuels production has been known for many years. Microalgae appear to be a promising source of biodiesel as they have high photosynthetic rates and can accumulate substantial amount of lipids in their biomass (up to 77% of dry cell mass in some species). However, historical efforts in this field have been inadequate to facilitate the development of a robust algal biofuels industry. Realizing the strategic potential of algal feedstock will require breakthroughs, not only in algal mass culture and downstream processing technologies, but also in the fundamental biology related to algal physiology and the regulation of algal biochemical pathways (Yu et al., 2011).

2. Materials and methods

2.1 Isolation of microalgae from kuantan coast.

Microalgae sample are isolate from three different places along Kuantan coast which is Pantai Batu Sepat, Tanjung Api and Pantai Batu Hitam. 50 ml of water sample were collected and store in centrifuge tube. Water sample only take from the sea shore by using spatula and plankton net. Then the water samples are bringing to the Faculty Industrial of Science and Technology laboratory for further experiment. At laboratory, the water sample was observed under light microscope to make sure the sample contain the microalgae.

2.2 Mass cultivation of microalgae

After the strains of the microalgae were identified, then the microalgae were mass cultivated. Mass cultivation of microalgae also using BG 11 medium. At first the single colony that was diluted was cultured in 10 ml of BG 11 media. Then after a week the culture was added with 90 ml of BG11 media. After a week another 500 ml of media was added into the 100 ml of microalgae culture. After that, the microalgae culture was mass cultivated by maintaining the culture. Cultures was placed in a room culture with temperature 25°C.

2.3 Lipid extraction of microalgae

The algal lipid extraction procedures are described by Bligh and Dryer in 1959. The microalgae tissue was homogenized with chloroform/methanol (2/1) to a final volume 20 times the volume of the tissue sample (1 g in 20 ml of solvent mixture). After dispersion, the whole mixture is agitated during 15-20 min in an orbital shaker at room temperature. The homogenate was filtrated (funnel with a folded filter paper) and centrifuged to recover the liquid phase.

Nomenclature and Abbreviation

MUFA	Monounsaturated fatty acids
PFA	Polyunsaturated fatty acids
SFA	Saturated fatty acid
FAME	Fatty acid methyl esters
GC	Gas chromatography
GHG	Greenhouse gas emission
GC-FID	Chromatography flame ionized detection
SEM	Scanning electron microscope

Then, the solvent was washed with 0.2 volumes (4 ml for 20 ml) of distilled water. After vortexing some seconds, the mixture was centrifuged at low speed (2000 rpm) in order to separate the two phases. Then the upper phase was removed by siphoning. Then the interface was rinse one or two times with methanol/water (1/1) without mixing the whole preparation. After centrifugation and siphoning of the upper phase, the lower chloroform phases that containing lipid and chloroform was collected. After that, the lipids were stored in GC vial for further analysis.

2.4 Gas chromatography flame ionized detection (GC-FID)

GC analysis was performed for identifying the fatty acid composition. Fatty acid analysis was done by Gas Chromatograph 2010 Plus (Shimadzu, Japan) using Flame Ionization Detector (FID). Injector and detector temperature was set at 5°C and 5°C respectively. One microlitre of the sample was injected in a split less mode at a flow rate of 1 ml/min with Nitrogen as the carrier gas onto a J&W 122-7062, DB-WAX with column (250 m x 250 μm x 0.25 μm, total run time 65 min). The fatty acid methyl esters were identified using fatty acid standards (Sigma, Supleco, 37 FAMEs).

(a) The reaction vessel was allowed to stand for 1 h to enable its contents to settle. The reaction mixture was filtered and the residues was washed twice by using re-suspension in

methanol (30 ml) for 10 min to recover any traces of FAME product left in the residues. (b) Then water (50 ml) was added to the filtrate, to facilitate the separation of the hydrophilic components of the extract, and then transferred into a separating funnel. (c) Then further extraction of the FAME product was achieved by extracting three times for 15 min using 30 ml of hexane and the pooled hexane extracts are washed with water (to remove left-over traces of the acidic catalyst and methanol), separated, and then dried using anhydrous sodium sulphate. (d) The FAME product was then filtered and evaporated to obtain the FAME yields. (e) Extracted FAME products were determined using GC-MS and compared with that of the microalgae oil to monitor the extent of the conversion process.

2.5 Fatty acid analysis by GC-MS

GC analysis was performed for identifying the fatty acid composition. Fatty acid analysis was done by Gas Chromatography using column DB WEX. Injector and Detector temperature was set at 2250 °C and 2500 °C, respectively. 0.5 ml of the sample was injected in a split mode (35:1) at a flow rate of 184.9 ml/min with Nitrogen as the carrier gas onto a FAMEs-RTX- column. Peak areas were integrated using the GC solution software. The fatty acid methyl esters were identified using fatty acid standards (Sigma, Supleco, 37 FAMEs).

2.5.1 Statistical analysis

The data collected are calculated and expressed as mean SD. Regression analysis was done using Microsoft Excel 2007. An analysis of variance was performed by means of the one-way ANOVA.

3. Results and discussions

3.1 Isolation and identification of microalgae

The morphological identifications of microalgae involve Fluorescence microscope and Scanning Electron Microscope (SEM) has been used to identify the species of microalgae. The species is known as *Nannochloropsis sp.* and *Coelastrum sp.* Under fluorescence microscope, *Nannochloropsis sp.* have spherical shape in green color and small compared to *Coelastrum sp.* (Bong and Loh, 2013). *Nannochloropsis sp.* is a small green alga, characterized by spherical or slightly ovoid cells, about 2-5 μm in diameter (Figure.1 (b)). Generally, *Nannochloropsis sp.* is a genus of alga within the heterokont line of eukaryotes that is being investigated for biofuel production. One marine *Nannochloropsis sp.* has been shown to be suitable for algal biofuel production due to its ease of growth and high oil content (28.7% of dry weight), mainly unsaturated fatty acids and a significant percentage of palmitic acid. It also contains enough unsaturated fatty acid linolenic acid and polyunsaturated acid (>4 double bonds) for a quality biodiesel.

Nannochloropsis sp. is a genus of alga comprising 6 known species. The genus in the current taxonomic classification was first termed by Hibberd (1981). The species have mostly been known from the marine environment but also occur in fresh and brackish water. All of the species are small, non-motile spheres which do not express any distinct morphological feature, and cannot be distinguished by either light or electron microscopy.

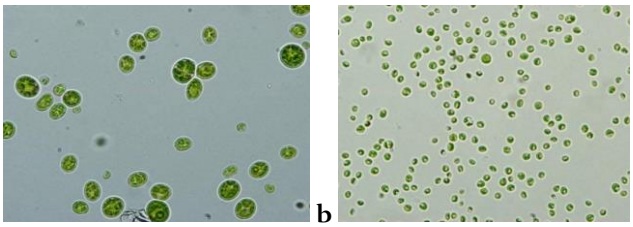


Figure. 1 (a). *Coelastrum* sp. Figure. (b) *Nannochloropsis* sp.

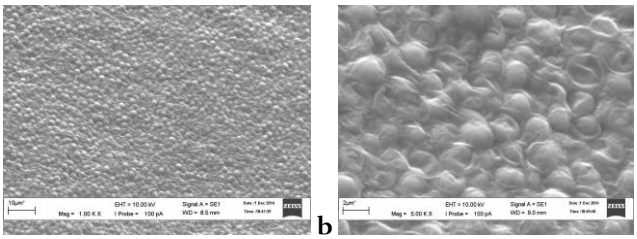


Figure.2 (a) Scanning Electron Microscope view of *Coelastrum* sp. at magnification 1000X. (b) at magnification 5000X

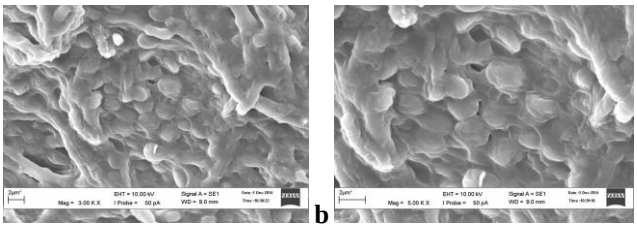


Figure.3 (a) Scanning Electron Microscope view of *Nannochloropsis* sp. at magnification 1000X. (b) at magnification 5000X

The characterization is mostly done by rbcL gene and 18S rDNA sequence analysis. While *Coelastrum sp.* size larger than *Nannochloropsis sp*. This is because *Coelastrum sp.* are builds up from colonies of spherical shape microalgae. *Coelastrum sp.* usually have geometric shape and sphere with cell size 5-7 µm diameter reported by (Sathya and Srisudha. 2013). Under SEM, the structure of the *Coelastrum sp.* are unified (Figure.1.a). The genus *Coelastrum sp*. was characterized by a group of cells unified in a mostly spherical structure, the so-called coenobium. The usually pelagic *Coenobia* contain autospores formed by isolated cells that develop into (significantly smaller) daughter-*Coenobia* in the original mother coenobium. Thus, *Coenobia* and isolated cells simultaneously occur in very different sizes.

3.2 Mass cultivation of microalgae

Microalgae growth was determined by measuring the concentration of microalgae culture at A 665 nm at interval of 2 days for 20 days using a GENESYS 10S UV-Vis spectrophotometer (Figure. 4) show the concentration of the microalgae. The lipid accumulation in microalgae cells was associated with the growth phase of the microalgae. The relationship between the age of culture and biomass concentration was established by growth curve. The cells will undergo stressful conditions and more amount of lipid will accumulate in the microalgae (Hu et al., 2008). Generally, almost all cultures presented an exponential growth phase with the same duration. However, the duration of exponential growth phase can become longer if the microalgae under stressful condition. The various phases represent the reaction of the algae population to the changes of the environmental conditions and depend on the inoculums, the actual cultivation method, nutrient concentration, light intensity and temperature.

Based on the result, *Nannochloropsis sp.* and *Coelastrum sp.* have same duration time of exponential growth phase. *Nannochloropsis sp.* and *Coelastrum sp.* have maximum growth at day 12 with 0.243 A and 0.282 A respectively. The similar *nannochloropsis sp* growth rate was observation reported (Fret et al., 2017). After day 12, the concentration of microalgae starts to decrease slowly. The microalgae growth starts to decrease due to nutrient depleted and some microalgae was die due to the toxin that was released from other microalgae. On the other hand, statistical analysis was provided using single ANOVA which is to show the differences and variation in growth between two species of microalgae. The negative effect of growth was not observer in this species. The similar observation was reported (Rodolfi et al., 2003).

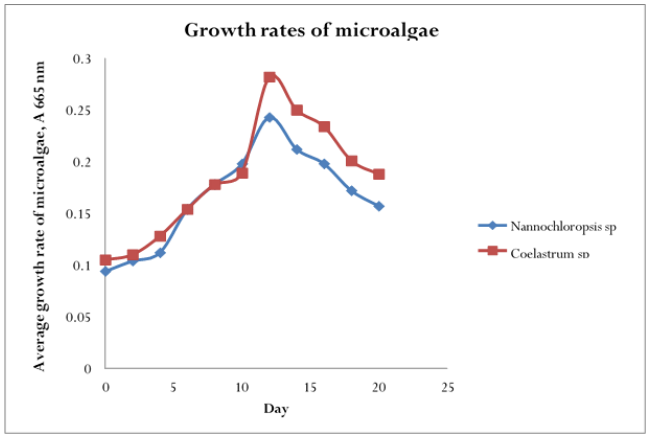


Figure. 4 The growth rates of microalgae *Coelastrum sp* and *Nannochloropsis sp.*

Table 1. Statistical analysis of microalgae growth

ANOVA: Single Factor

SUMMARY

Groups	Count	Sum	Average	Variance
Nannochloropsis sp	**11**	**1.822**	**0.165636**	**0.002244**
Coelastrum sp	**11**	**2.019**	**0.183545**	**0.003258**

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.001764	1	0.001764	0.64127	0.432662	4.351243
Within Groups	0.055017	20	0.002751			
Total	0.056781	21				

3.3 Harvesting of microalgae

In this experiment, centrifuge method was used since this method is one of the cost effective method to obtain concentrated microalgae. Even centrifugation methods are effective method to obtain concentrated biomass, but it the energy requirement is higher than filtration method (Ramanathan et al., 2015). However, it clearly shows that

the choice of harvesting is depend on the biomass type, if the cell size is large enough, then filtration is likely to be the most effective method. Otherwise it is likely that a process stream involving flocculation, sedimentation, flotation or centrifugation is necessary. Both microalgae culture was harvested by centrifuge the sample at 5000 rpm for 5 minutes. The pellets of microalgae were stored in freezer for 5 days and then freeze dry. Then the dried sample was weight using analytical balance. Based on the result, *Coelastrum sp* have higher dry weight which is 0.8837 g compared to *Nannochloropsis sp* only 0.347 g. Usually, *Coelastrum sp.* have higher weight compared to *Nannochloropsis sp*. due to their large structure that consist of a few colonies microalgae in spherical shape (Chan and Wong, 1975).

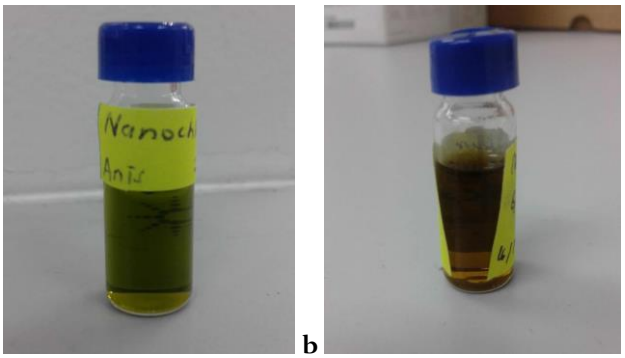
Table 2. Dry cell weight of microalgae *Coelastrum sp* and *Nannochloropsis sp*

Species	Dry weight (g)
Coelastrum	0.883
Nanochloropis	0.347

3.4 Lipid extraction of microalgae

After obtained the dry weight of microalgae, lipid in microalgae was extracted for lipid screening. There are a few methods that can be used to extract the lipid which is solvent extraction, mechanical pressing, and supercritical fluid extraction. However, for this experiment solvent extraction method was used by using chloroform and methanol as a solvent. The advantage of using these solvents for lipid extraction is that they are inexpensive and very efficient and are commonly used for oil extraction. Dried microalgae were mixed with chloroform: methanol with ratio 2:1 respectively (Folch et al., 1957).

After that, the mixtures are place on orbital shaker at room temperature and after 20 minutes the mixture was centrifuge at 2000 rpm for 5 minutes. Liquid phase that contain lipid was collected and the biomass pellet was removed. Then the liquid phases are washing with distilled water: methanol using ratio 1:1 to separate it into two layers. Then the lower layer that contains lipid and chloroform are collected and evaporated in fume hood. Based on the result, lipid from *Nannochloropsis sp*. are more concentrated than *Coelastrum sp*. The structure of the *Coelastrum sp.* is bigger than *Nannochloropsis sp.* due to chlorophyll and other structure. Even *Nannochloropsis sp.* are more small but it contains more fatty acid compared to *Coelastrum sp*. Various *Nannochloropsis sp*. are currently used primarily as energy rich food for fish larvae. Their ability to generate high quantities of lipids (naturally occurring fats) and other molecule used for energy storage, coupled with rapid growth rate, make them ideal candidate for biofuel production (Ma et al., 2016).



a **b**

Figure. 5 (a). Lipid from *Nannochloropsis sp*. (b). Lipid from *Coelastrum sp*.

3.5 Screening lipid profile of microalgae

There are a few approaches that can be used to identify fatty acids or derivatives (fatty acid methyl esters, FAME), including gas chromatography- flame ionized detector (GC-FID), high performance liquid chromatography (HLC), gas chromatography- mass spectrometry (GC-MS), nuclear magnetic resonance (NMR) and thin layer chromatography (TLC), (Sathya, 2017). These methods have been used widely in detecting fatty acid of animal, plant and other organism that have carbon chain from 12 to 24 carbon atoms. After the lipid was extracted from microalgae, the lipid was send to laboratory for further analysis. To determine lipid profile of microalgae, gas chromatography flame ionized detector (GC-FID) was used. 0.5ml lipid from *Nannochloropsis sp.* and *Coelastrum sp.* was transfer into the GC vial. After a week, the result of lipid analysis was obtained. The retention time of lipid are compared to standard manual (Knothe, 2009).

Based on the result, *Nannochloropsis sp.* have various type of fatty acid including MUFA, PUFA and SFA. The highest type of fatty acid that present in this sample was SFA and MUFA. Two SFA that was presented in these microalgae which is pentadecanoic acid, and palmitic acid. Two MUFA that present in these microalgae were 9-hexadecanoic acid and heptadecanoic acid (cis-10).

According to (Ma et al., 2016) the predominant fatty acids of *Nannochloropsis sp.* were palmitic acid (16:0), palmitoleic acid (C16:1) and EPA (C20:5). Only one PUFA was presented in his sample which is hexadecatrienoic acid. Microalgal fatty acids or triacylglycerols used as a feedstock's for biofuel production (Hu et al., 2008). The lipid content *in Coelastrum sp.* Most of fatty acid in *Coelastrum sp* are PUFA and SFA. PUFA includes linoleic acid and docosapentaenoic acid, while for SFA was docosanoic acid and myristic acid (Table 3). According to (El Maghraby et al., 2015) majority fatty acid *Coelastrum sp* known as oleic acid, palmitic acid and followed by myristic acid.

Another lipid that was presented in *Coelastrum sp.* was docosanoic acid. Docosanoic acid known as Behenic acid was detected. This docosanoic acid was saturated fatty acid (SFAs) that have 21 carbon chains with formula $C_{21}H_{43}COOH$. (Sivakumar et al., 2012) reported that high quality of biodiesel also can be obtained from 8 carbon fatty acid, including "oleic acid" methyl ester and "octadecenoic acid" methyl ester (Aida et al., 2016) that both the quantity and quality of lipids produced will vary with identity of the algal species. From comparison of lipid profile between both microalgae species, it shows that *Nannochloropsis sp.* have more MUFA and PUFA (Hibberd, 1981) compared to *Coelastrum sp.* that have more SFA and PUFA. It suggested that both of microalgae can be used as al biodiesel, but required a few genetically modification so that it can produce more MUFA.

Table 3. Fatty acid analysis of *Nannochloropsis sp*. by GC-FID

Retention time (Minutes)	Number of carbon	Name of fatty acid	Type of fatty acid
ND	ND	ND	ND
11.768	C15:0	Pentadecanoic acid	SFA
12.938	C16:0	Palmitic acid	SFA
13.663	C16:1	Palmitoleic acid	MUFA
14.267	C16:3	Hexadecatrienoic acid	PUFA
15.002	C17:1	Heptadecanoic acid (cis-10)	MUFA

ND= non detected

3.6 Analysis of fame by GC-MS

The typical analysis of fatty acids is done by analyzing the methyl esters of the fatty acids by gas chromatography (GC). The fatty acids in the oil must be converted (derivative) to fatty acid methyl esters (FAME) in order for them to be volatile enough to be analyzed by GC. The result show that *Nannochloropsis sp.* have higher profile of n-hexadecanoic acid and pentadecanoic acid. Hexadecanoic acid have higher match with library which is 98 % that can have proved this species have this fatty acid. Fatty acids (FAs) are one of the primary metabolites of microalgae, which enrich their utility both in the form of food and fuels. Additionally, the vast structural diversity coupled with taxonomic specificity makes these FAs as potential biomarkers (Sahu et al., 2013).

Furthermore, from lipid screening using GC-FID, it also shows that *Nannochloropsis sp.* have palmitic acid that was detected at retention time 12.938 minute. Other name of n-hexadecanoic acid (IUPAC name) is palmitic acid with 16 carbon chain. Another fatty acid that was detected in *Nannochloropsis sp.* was pentadecanoic acid. Pentadecanoic acid actually a saturated fatty acid with molecular formula $CH_3(CH^2)_{13}COOH$. According to (Islam et al., 2013) saturated fats can make excellent biodiesel. The *Coelastrum sp.* are suitable for biodiesel production since both of microalgae have palmitic acid or hexadecanoic acid which is can be converted into biodiesel. It also reported by (Sahu et al., 2013) that oleic acid (C18: 1), linoleic acid (C18:1), and palmitic acid (C16:0) were the three main compounds in the high acid biodiesel.

4. Conclusion

The two microalgae species of *Nannochloropsis sp.* and *Coelastrum sp*. was isolated and identified from Pantai Batu Hitam, the east coast region of Kuantan, Malaysia. More biodiesel was produced from *Nannochloropsis sp.* compared to *Coelastrum sp. Nannochloropsis sp.* produce higher amount of hexadecanoic acid methyl ester (methyl hexadecanoate, IUPAC) and pentadecanoic methyl ester (methyl pentadecanoate, IUPAC). However, from GC-FID analysis, *Coelastrum sp.* have higher content of C18:2 making it a less desirable candidate for biodiesel production in terms of oxidative stability. *Nannochloropsis sp.* are more preferred to be used as biodiesel feedstock in the future.

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