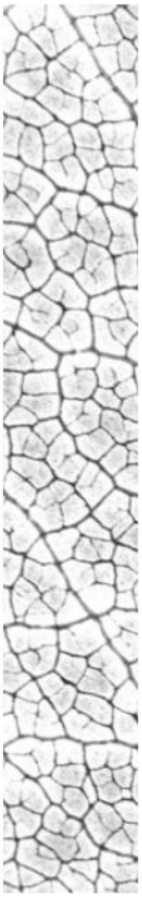

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Exploration of freshwater microalga as bioenergy candidate

Didi Jaya Santri [1], Hilda Zulkifli [2*], Aldes Lesbani [3], Hermansyah [3]

[1] Biology Education Department, Universitas Sriwijaya, Palembang, INDONESIA
[2] Biology Department, Universitas Sriwijaya, Palembang, INDONESIA
[3] Chemistry Department, Universitas Sriwijaya, Palembang, INDONESIA
*Corresponding author: hilda.zulkifli@yahoo.com

Abstract

Oleaginous microalgae with high biomass productivity, lipid content, and lipid productivity are desirable for sustainable biofuel production. Rapid and accurate quantification of lipid content facilitates the identification of promising microalgae candidates. In the present study, 8 freshwater microalgae species from four region in South Sumatera were isolated and identified based on their morphological. Their carbohydrate and lipid content were characterized and screened using Shoxlet method. Based the morphological features, the cultures were initially identified as *Spirulina* sp., *Microcystis* sp., *Scenedesmus* sp., *Dictyosphaerium* sp., *Straurastrum* sp., *Pandorina* sp., *Chlorella* sp., and *Ankistrodesmus* sp. Total carbohydrate content from isolates found in the range 0,1 to 0,7% with the highest content of carbogydrate is *Chlorella* sp. Total lipid contentshowed prominent variation between all of isolates and found in the range of 3 to 15 % of DW with the highest level is *Chlorella* sp. Thus, our findings serve as a baseline data on the bioprospecting potential of isolates from freshwater region, an unexplored area of South Sumatera.

Keywords: microalgae, freshwater, biomolecul, South Sumatera

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INTRODUCTION

The use of fossil fuels has caused various problems, i.e. non-renewable, environmental impacts caused such as global climate change due to accumulation of carbon dioxide in the atmosphere, high prices of crude oil and also the availability of fossil fuel reserves that are rapidly decreasing (Chisti, 2008; Wu, et al., 2012). Therefore, efforts to search for renewable and environmentally friendly alternative fuels are intensively implemented. One of them is the development of Biofuel derived from plants, animals or microorganisms. One potential source of biofuel is microalgae.

Research on renewable and carbon neutral fuels has developed in recent years using various sources that come from living things, especially cultivated plants that contain oil. Biofuels from plant cultivation cannot be optimally expected because they also threaten food security, and also production results that do not meet needs. Attention now shifts to the use of microalgae biomass as a source of biofuel. This is because it has greater photosynthetic efficiency than other biomass sources (Hannon, et al., 2010; Minowa, et al., 1995; Sayre, 2010; Shen, et al., 2010). The productivity of biodiesel produced from microalgae is much greater than other cultivated plants. For this reason, research is needed to find quality microalgae with high lipid productivity characteristics and good adaptability. The superior microalgae strain was obtained as an exploration of natural isolates, and then the efficiency was improved through selectivity and transformation (Wu et al., 2012).

The potential use of microalgae as a raw material for biofuel has been widely reviewed (Alam, et al., 2015; Amin, 2009; Chisti, 2008; Wu et al., 2012). Microalgae is very potential as a BBH-producing ingredient due to its high lipid content, easy cultivation and fast growth rate. Microalgae can double its biomass in 24 hours and microalgae oil content can reach 80% of the biomass dry weight (Chisti, 2008). Hilda et al (2016) have conducted research on the chemical composition of culture media with chemical compositions of Spirulina as metal adsorbents.

As a province with 30% of its total area is fresh water, South Sumatra has a large potential of microalgae. Several previous studies on algae in the waters of South Sumatra have been carried out, but generally only assess the diversity of algae (Isnaini, 2012, Munthe, et al., 2012, Nita & Syaiful, 2015, Elvandari, et al., 2015).

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Proprietary growth of algae biomass from South Sumatra has been carried out by Apriyani (2017), but this multiplication is still multispecies (mixcultured).

So far there have been no attempts to isolate potential microalgae as a source of biomass and biofuels originating from the local waters of South Sumatera, given that South Sumatera has many habitats suitable for microalgae. Therefore, research is needed to find microalgae that have the potential as biomass raw materials and materials. biofuel, and have good adaptability that comes from the swamp waters of South Sumatra. Microalgae superior strains were obtained as an exploration of natural isolates, and then the efficiency was improved through selectivity and transformation.

This study aims to produce pure local algae isolates which are potential as biomass raw materials and biofuels sourced from the freshwaters of South Sumatra. Specifically, this study aims to: i) Produce pure local microalgae isolates from the swamp waters of South Sumatra; ii) obtain pure microalgae culture from the swamp waters of South Sumatra with the characteristics according to the requirements of renewable energy sources; iii) obtained Environmental conditions suitable for algal propagation culture (light, pH, temperature); and v) algae isolates were obtained with the characteristics of potential lipids and / or carbohydrates.

MATERIAL AND METHODS

Sample Collection

Sampling was carried out at several locations of freshwater waters in South Sumatra, including in the cities of Palembang, Banyuasin, Ogan Ilir, and Ogan Komering Ilir. The collection was taken in the morning between 08.00 to 11.00 a.m. from the top and bottom of the waters using plankton net, with the aim of getting the dominant microalgae in each place. The collection of water samples is carried out using a 10 liter bucket and the collected water is then filtered with a plankton net. Each sample was collected in a 100 ml, and carried in low temperature conditions to the laboratory. Phenotypic identification was carried out based on morphological characters to determine the diversity of microalgae species. Calculation of abundance is done to get the dominant type of microalgae using Sedgwick Rafter Counting Cell (SRCC).

Microalgae Isolation

In order to get a single type of microalgae from the water samples, a standard plating methods were used to seperate algal population (Cho, et al., 2011; Lee et al., 2014; Muhammad, et al., 2014).

Samples from the field were first dilluted to isolated the coloni. Nutrient medium BG11, BBM, Fraquil, and several standard mediums are mixed in agar medium. Forty mililiters of agar medium is poured into a sterile petri dish as a growing medium for the samples. One mililiter of the sample is flattened on the surface of the agar medium. The inoculated microlalga colonies were placed at room temperature (20-25 °C) and grown for 14 days. The grown algae culture is then separated in vaous growing media under sterile conditions and placed at room temperature in the laboratory. This step is repeated until getting a single culture from algae. Stock culture is maintained once a month by re-growing in agar media. The results of this stage were local microalgae isolates from freshwater of South Sumatera.

Cultivation and harvesting

Microalgae are cultured in various types of medium. Culture medium uses pond water and swamp from microalgae origin habitat as the main medium. This water is filtered using plankton net with the aim of removing sedimentation particles in the water. The filtered water in the erlenmayer is then stored for later use. The growing media used are BBM Medium (Bold Basal Medium), BG-11 (Blue green-11), AF6, Fraquil and simple medium such as NPK, Pellets and EM4. Especially for cyanobacteria, BG 11 medium is used (Lee et al., 2014; Muthulakshmi & Meenatchisundaram, 2015). Each culture sample is monitored every two days by measuring the abundance of microalgae using SRCC. Cultured aerated using aerator, and culture media added every week according to treatment. Culture temperature is maintained at room temperature (25-30 °C). Lighting is provided through a 40 watt fluorescent lamp with a lighting duration of 10 hours a day overnight (10:14) for 14 days. Microalgae growth is seen as the abundance of microalgae cells which are counted every 2 days for 16 days of observation.

Microalgae harvesting was carried out by centrifugation method at 4000 rpm for 2 minutes. The samples obtained were weighed to get the wet weight. Then the sample was dried in an oven at 40 °C for 24 hours after first being desiccated in a vacuum pump machine. Dry biomass is then weighed as dry weight.

Lipid Content Analysis

Lipid content was extracted by *Soxhlet* method (Pargiyanti, 2019) with few modification. Briefly, algal dry biomass 2 g was mixed with 8 g of sand that has been heated and chloroform, distillation for 4 h. The supernatant extract was mixed with chloroform for 2 h. Dried at 100 °C and weight noted as W3. The lipid content was measured.

Carbohydrates Content Analysis

Carbohydrates content was measured based on total sugar content by Cleg-Anthrone method (Apriyantono et al. 1989). 1 g dry sample or 2.5 g wet sample added 10 ml water and dissolved with 13 ml perchloric acid 52%. And the solution diluted to 100 ml and filtered. The extract was diluted until 25 ml. The extract was taken 10 ml and diluted until 100 ml of solution. A total of 1 ml of this solution was put into each test tube and added 5 ml of anthrone reagent., and then heated in a water bath

Table 1. Microalgal Cultures were Isolated from South Sumatera

Region	Genus			
Palembang	*Actinastrum*	*Eudorina*	*Monoraphidium*	*Scenedesmus*
	Ankistrodesmus	*Euglena*	*Mougeotia*	*Selenastrum*
	Aphanothece	*Eugtimatos*	*Navicula*	*Spirogyra*
	Chlamydomonas	*Golenkenia*	*Nitzschia*	*Spirulina*
	Chlorella	*Gomphosphaeria*	*Oocystis*	*Staurastrum*
	Coelastrum	*Marssoniella*	*Oscillatoria*	*Tabellaria*
	Cosmarium	*Merismopedia*	*Pandorina*	*Tetraedon*
	Desmidium	*Micractinium*	*Pediastrum*	*Tetrastrum*
	Dictyosphaerium	*Microcystis*	*Pyrobotrys*	*Zygnema*
Banyuasin	*Actinastrum*	*Chlorella*	*Microcystis*	*Oscillatoria*
	Anabaenopsis	*Euglena*	*Monoraphidium*	*Phacus*
	Aphanothece	*Merismopedia*	*Nitzschia*	*Scenedesmus*
Ogan Ilir	*Ankistrodesmus*	*Monoraphidium*	*Scenedesmus*	*Straurastrum*
	Dictyospharium	*Pediastrum*	*Selenastrum*	
OKI	*Scenedesmus*	*Nitchzia*		

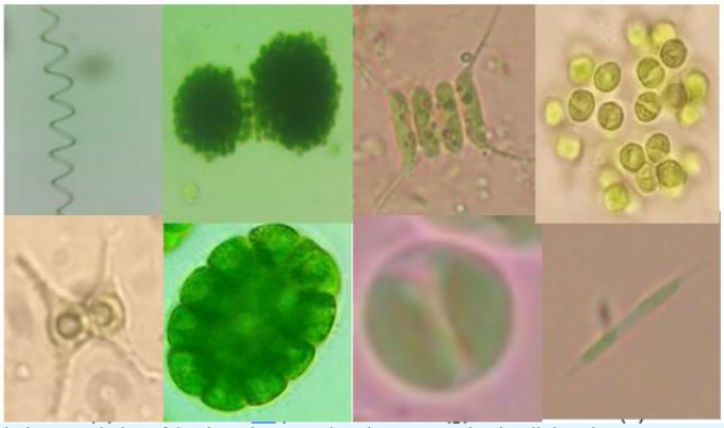

Fig. 1. Morphological characteristics of 8 microalgae strains documented using light microscope at × 40x magnification

of 100 °C for 12 min. Measurement of the absorbance at a wavelength of 630 nm. The results were compared with glucose standard solutions at concentrations of 10, 20, 40, 60, 80, and 100 ppm. The starch content can be calculated by the formula from Goni et al. (1997) as follows

Starch content = sugar content x 0.9

RESULT AND DISCUSSION

In this study, a total of 40 microalgal cultures (**Table 1**) were isolated from the four region of freshwater in South Sumatera, and 8 microalgal cultures (**Table 2**) were selected based on their culture purity and distinct morphological characteristics. Microscopic observations of the algal isolates revealed its colonial existence and purity (**Fig. 1**). Based on the morphological features, the cultures were initially identified as *Spirulina* sp., *Microcystis* sp., *Scenedesmus* sp., *Dictyosphaerium* sp., *Straurastrum* sp., *Pandorina* sp., *Chlorella* sp., and *Ankistrodesmus* sp.

Microalgae include various groups of organisms that can be classified into several types such as Cyanobacteria (Cyanophyceae), green algae (Chlorophyceae), diatoms (Bacillariophyceae), yellow-green algae (Xantophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaephyceae), dinoflagellates (Dinophyceae), and "picoplankton" (Prasinophyceae and Eustigmatophyceae) (Venkatesan, et al., 2015; Zhu, et al., 2014; Suganya et al., 2016). Microalgae growth depends on the presence of light intensity, temperature, nutrient concentration, salinity, and pH (Sayre, 2010). Based on isolation in variation medium such as BG 11, BBM, Fraquil, AF6, fish pellets, NPK and EM4 there are some species is found (**Table 2**).

Based on growth rate in various medium, *Ankistrodesmus* sp. was the fastest grow up in NPK+ medium. Thus showed that *Ankistrodesmus* sp. most adaptable in artificial habitat. Meanwhile, *Dictyosphaerium* sp. was the slowest grow up in BG 11 medium. BG11 media and BBM media showed a higher mortality rate from microalgae isolates than other media. This indicates that the content of BG and BBM media is needed by microalgae, especially the genus Ankistrodesmus and Dictyosphaerium.

Samples were taken from four districts waters in South Sumatra that have high abundance of microalgae, The samples were first biologically isolated to separate microalgae from other microorganisms. Then the

Table 2. Growth Rate of Freshwater microalgae in batch culture

No	Genus	Medium	Growth rate (ind/cc)					
			T_0	T_1	T_2	T_3	T_4	T_5
1	*Spirulina*	BG 11	4.841	4.366	7.209	7.495	8.452	
2	*Spirulina*	AF6	4.761	5.071	5.763	5.615	5.089	3.090
3	*Microcystis*	AF6	1.050	1.793	2.195	1.749	1.326	956
4	*Scenedesmus*	BG11	774	871	1.286	1.411	1.596	
5	*Dictyosphaerium*	BG11	338	453	671	887		
6	*Straurastrum*	AF6	6.663	6.730	9.336	6.461	6.530	6.380
7	*Pandorina*	Fraquil	10.138	13.608	15.661	16.871	16.587	16.053
8	*Chlorella*	NPK +	12.364	76.400	283.055			
9	*Scenedesmus*	BBM	467	1.890	3.795			
10	*Ankistrodesmus*	NPK +	15.237	20.149	27.891	33.802	45.674	38.356

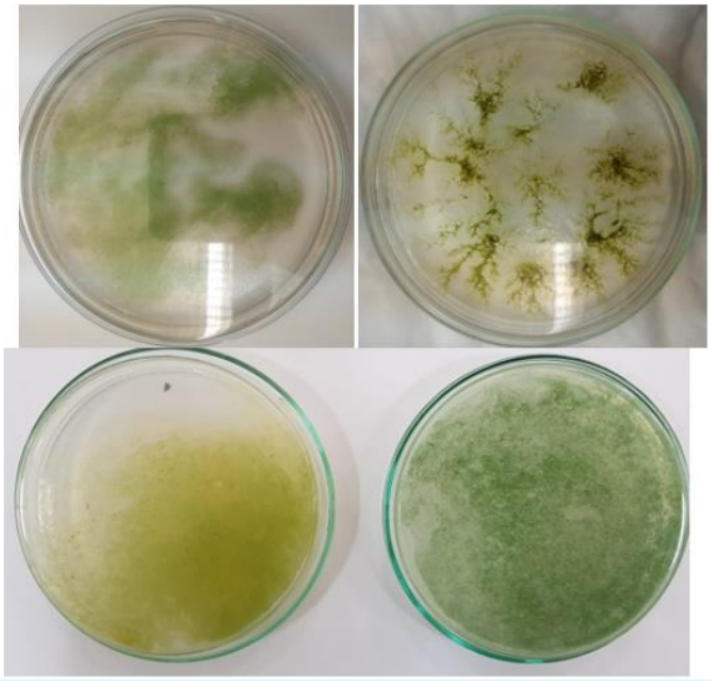

Fig. 2. Freshwater microalga isolate *a. Spirulina b. mycrocystis c. Anabaena d. Pandorina e. Pandorina*

sample was taken 1 mL and then added to 40 mL of solid agar medium in the petridish using pourplate method. Incubation of samples at room temperature control (20-25 °C) and grown for 14 days. As a result, we have several isolate microalgae i.e *Spirulina, Microsystis, Pandorina, Anabaena, and Ankistrodesmus*. The abundance of each species before isolation were 8337/cc, 542/cc, 13,610/cc, 5275/cc and 23.253/cc, respectively. There were differences in the characteristics of growth for each species. The isolate colony in the plates showed different morphological characteristics. *Spirulina* and *Microsystis* isolates showed the bluish green colors with microalgae colonies in spots shaped in the medium. The color of Pandorina isolates were dark green with unified colonies on the surface of the medium, while for Anabaena isolates the color was green yellow green which forms thin plates on the surface of the medium (**Fig. 2**).

Algae Culture in Simple Photobioreactor

The increasing number of the human population will have a real impact on the increasing needs of human life, one of which is the need for fuel (Zou, et al., 2016). It is undeniable that for almost a century humans have been very dependent on fossil fuels (Deliismail et al., 2018) and have caused the world economy to increase. However, this increase is very inversely proportional to the condition of the environment which is increasingly degraded and has decreased environmental quality. This is evidenced by the increasing number of greenhouse gas (GHG) emissions (Sankaran, et al., 2018) and continues to accumulate in the atmosphere which has an impact on climate change. The gas most commonly produced from human activity emissions is CO_2 with levels at a level of 405.0 ppm in the atmosphere in 2017 based on research results from the National Oceanic and Atmospheric Administrator. Based

Fig. 3. Simple Photobioreactor model for microalgae culture

on the results of the analysis of CO_2 levels in the present era, they have been in a very high condition since 800,000 years (Lindsey, 2018) this will have a very negative impact on earth conditions. So that the use of renewable and environmentally friendly resources is very necessary and cannot be postponed, because the real impact of CO_2 accumulation is increasing which will have an impact on human life, one of which is the use of microalgae to become biofuel.

In this follow-up study, pure microalgae biomass culture from isolation was carried out using a simple photobioreactor and a lipid and carbohydrate potential test and characterization of DNA isolates were carried out. The results of this study found five types of microalgae that can be cultured, namely *Nannochloris*, *Dictyosphaerium*, *Chlorella Scenedesmus*, and *Stigeoclonium*. The liquid culture of pure microalgae *Nannochloris*, *Dictyosphaerium* and *Chlorella* using BG-11 medium and microalgae *Scenedesmus* and *Stigeoclonium* were cultured using BBM medium. The use of simple photobioreactors and the addition of CO_2 (**Fig. 2**) has been shown to accelerate the growth rate of microalgae so that harvesting can be done earlier. Growth rates of culture microalgae are shown in **Fig. 3**.

In addition, the characteristics of algal biofuel which are environmentally friendly, pollution-free and cost-friendly make it a very suitable alternative fuel (Chisti, 2007). The results of other studies show that in the process of cultivating microalgae, carbon sources for growth can use carbon dioxide from emissions produced by power plants compared to using carbon dioxide in the atmosphere (Schenk, et al., 2008), this is of course very beneficial for the power plant industry. electric power because it can indirectly reduce the emission produced. The benefits of increasing the number of microalgae for life are also very large, with the use of microalgae as biofuels, microalgae cultivation will increase, this will also have an impact on the environment because microalgae have better CO_2 fixation capabilities than land plants (Klinthong et al., 2015; Fulke, et al., 2014) so that the impact can reduce the amount of CO_2 in the atmosphere and increase the amount of O_2. So that the potential of the microalgae that has been described can be a very big opportunity to make microalgae into a renewable energy resource that can meet human energy needs which will continue to increase every time.

Microalgae are microscopic creatures that are very abundant and have the ability to reproduce and replicate very quickly and have the ability to adapt very well to unfavorable environmental conditions (Adeniyi et al., 2018; Rittman, 2008). Furthermore, there have been many studies showing the potential of microalgae which can be used as renewable biofuels (Deliismail et al., 2018; Kanna et al., 2019; Sakthi et al., 2020), studies show that the content of trigislerides and polysaccharide in algae is very suitable for used as a biofuel (Sakthi et al., 2020). In addition, the content in microalgae can be modified according to human needs by using various modification techniques during the growth process (Meher et al., 2006; Leong et al, 2018). Each type of microalgae has a different length of the growth process. In some types of microalgae there are experienced a long growth phase. While there are also several types of microalgae that experience a growth phase in a short time.

Based on **Fig. 4**, it can be observed that the culture of *Nanochloropsis* sp. microalgae is very adaptable with new environments. It's caused the nutrition, pH, and light intensity of the medium supported of *Nanocloropsis* sp. Cleavage of microalgal rapidly and continuously in exponential phase until the 6th day, and the tationary phase on the 8th until 10th day, and deacreased on the 12th to the 14th day. *Stigeoclonium* sp. experienced relatively little addition from 2th to 10th day. This means that *Stigeoclonium* sp. growth phase, which is the adaptation phase, occurs in quite a long time. Then on

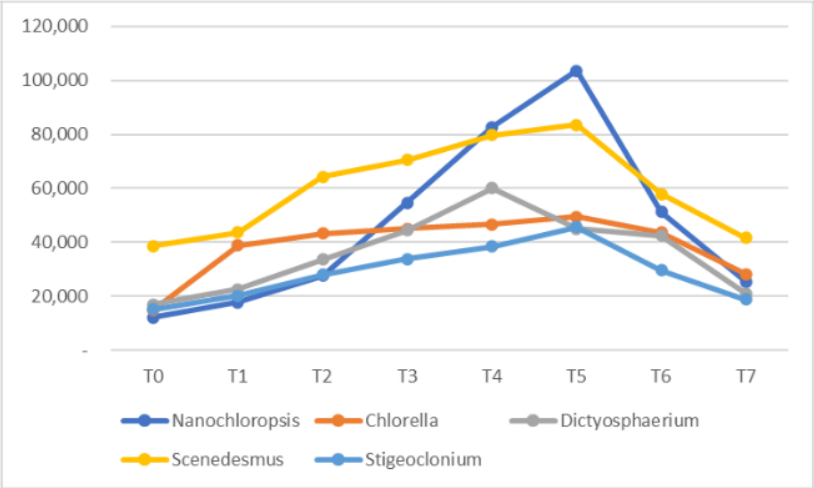

Fig. 4. Growth rate of five Species Microalgae from freshwater culture

Table 3. Total Lipid and Carbohydrate Content of microalgae

No	Species	Medium	Lipid Content	Total Carbohidrat
1	*Nanochloropsis* sp.	BG 11	6,94%	0,20%
2	*Chlorella* sp.	BG 11	15,3%%	0,70%
3	*Dictyosphaerium* sp.	BG11	8,95%	0,10%
4	*Scenedesmus* sp.	BBM	6,34%	0,10%
5	*Stigeoclonium* sp.	BBM	3,21%	0,30%

the 12th day a high number of cells was added. Furthermore, on the 14th day the growth of microalgae, 3872 cells/ml, occurred in the stationary phase because the number of microalgae was still considered stable. In *Chlorella* growth, the number of cells does not dominate the microalgae culture. It can be seen in **Fig. 4** that shows the lag phase or the adaptation phase when cells adjust to the medium used. This is characterized by the characteristics of the least increase in cell abundance on the 2nd day. Furthermore, on the 10th day the abundance of cells experienced a decrease that occurred continuously until the last day, namely the 14th day.

Besides that, in the culture of *Dictyoshaerium* sp, the exponential phase occurs until the 8th day because division occurs very quickly and continuously. The stationary phase occurs on the 8th day through the 12th day of culture experiencing a relatively constant density. This happens because of nutritional conditions that begin to limit growth. Furthermore, observations on the 14th day began to enter the phase of death. *Scenedesmus* growth is also more significant and relatively longer, that is on the 2nd day, the number of cells continues to increase until it reaches the highest exponential phase on the 12th day of 82600 cells /ml. This is because the culture medium supports the growth of *Scenedesmus*. On the 14th day there was a phase of death which caused the abundance of cells to decrease. Death is caused by very high competition in the live media.

Lipid and Carbohydrates Content

Microlagal biomass is carried out at the to[17]hase of each type. Harvesting is done to test the content of carbohydrates, lipids and DNA characterization in microalgae. The carbohydrate test is based on the total sugar content method using a refractometer. Testing of lipid levels in microalgae is carried out by the method of determining the levels of fats and oils with Soxhlet (Woodman, 1941). **Table 3** shows the results of tests conducted for five types of microalgae from culture results.

The test results for fat and carbohydrate content are still not satisfactory because of the low results obtained. Of the five species tested, only Chlorella received the highest fat content, 15.3%. This is because the influence of culture is not optimal. In the next research, nutrition which can increase microalgae fat levels will be improved. If we compared with the results of freshwater alga fat test results by Verawaty et al (2017), the two species tested also produced a fat content below 10%. In the type of Oediogenium, fat content ranges from 3.75 to 6.90%. As for the type of Cladophora, it ranges from 3.75 to.d. 6.75%.

CONCLUSION

Based on the morphological features, the cultures [8]ere initially identified as *Spirulina* sp., *Microcystis* sp., *Scenedesmus* sp., *Dictyosphaerium* sp., *Straurastrum* sp., *Pandorina* sp., *Chlorella* sp., and *Ankistrodesmus*

sp. Total carbohydrate content from isolates found in the range 0,1 to 0,7% with the highest content of carbogydrate is *Chlorella* sp. Total lipid content showed prominent variation between all of isolates and found in the range of 3 to 15 % of DW with the highest level is *Chlorella* sp. Thus, our findings serve as a baseline data on the bioprospecting potential of isolates from freshwater region, an unexplored area of South Sumatera.

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