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# Isolation Oil Producing Micro algae *chlamydomonas snowii* from Tropical Fresh Water, Indonesia

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## ABSTRACT

Microalgae are organisms that have the potential as a producer of raw materials that are environmentally friendly biofuels. Microalgae contain lipids and fatty acids that can be converted to biodiesel via transesterification reaction. In this research, samples are collected at Batusangkar district freshwater, Sumatera Barat, Indonesia. Nile red staining was used to select microalgae containing lipid. The isolated microalgae species were identified by microscopic and molecular identification. Based on microscopic identification, the isolated microalgae is *Chlamidomonas snowii*. The detected fatty acids of *Chlamidomonas snowii* are C3:0, C15:0, C27:0, C16:0 ,C18:0 and C14:0. The effect of nitrogen stress on the lipid content of *Chlamidomonas snowii* was determined. The lipid content on *C. snowii* at nitrogen concentration of 22, 5 mg/L, 25 mg/L and 27, 5 mg/L were 21, 46%, 21, 46%, and 19, 84%, respectively.

**Keywords**: Microalgae, *Chlamydominas snowii*, Nile red, Lipids, Fatty acids.

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## INTRODUCTION

Concerns about shortage of fossil fuels, increasing crude oil price, energy security and accelerated global warming have led to growing worldwide interests in renewable energy sources such as biofuels. An increasing number of developed and rapidly developing nations see biofuels as a key to reducing reliance on fossil fuel, lowering emissions of greenhouse gases (GHG), mainly carbon dioxide ($CO_2$) and methane ($CH_4$), and meeting rural development goals [1].

Biofuels are referred to solid, liquid or gaseous fuels derived from organic matter. They are generally divided into primary and secondary biofuels. While primary biofuels such as fuel wood are used in an unprocessed form primarily for heating, cooking or electricity production, secondary biofuels such as bioethanol and biodiesel are produced by processing biomass and could be used in vehicles and various industrial processes. The secondary biofuels are categorized into three generations: first, second and third generation biofuels on the basis of different parameters, such as the type of processing technology, type of feedstock or their level of development [2].

Microalgae are potential as a producer of biodiesel feedstock. Microalgae grows quickly and have a very large capacity to produce natural oils (lipids) approximately 60% of the dry weight. Natural oil produced by the microalgae is generally the same as the natural oils of higher plants [3].

The idea of using microalgae as a source of biofuel is not new, but it is now being taken seriously because of the rising price of petroleum and, more significantly, the emerging concern about global warming that is associated with burning of fossil fuels. The utilization of microalgae for biofuels production offers the following advantages over higher plants: (1) microalgae synthesize and accumulate large quantities of neutral lipids (20–50 % dry weight of biomass) and grow at high rates; (2) microalgae are capable of all year round production, therefore, oil yield per area of microalgae cultures could greatly exceed the yield of best oilseed crops; (3) microalgae need less water than terrestrial crops therefore reducing the load on freshwater sources; (4) microalgae cultivation does not require herbicides or pesticides application; (5) microalgae sequester $CO_2$ from flue gases emitted from fossil fuel-fired power plants and other sources, thereby reducing emissions of a major greenhouse gas (1 kg of dry algal biomass utilise about 1.83 kg of $CO_2$); (6) microalgae could be applied as bioremediation agent of wastewater by removal of $NH_4^+$, $NO_3^-$, $PO_4^{3-}$ from a variety of wastewater sources (e.g. agricultural run-off, concentrated animal feed operations, and industrial and municipal wastewaters); (7) combined with their ability to grow under harsher conditions and their reduced needs for nutrients, microalgae can be cultivated in saline/brackish water/coastal seawater on non-arable land, and do not compete for resources with conventional agriculture; (8) depending on the microalgae species, other compounds may also be extracted, with valuable applications in different industrial sectors, including a large range of fine chemicals and bulk products, such as polyunsaturated fatty acids, natural dyes, polysaccharides, pigments, antioxidants, high-value bioactive compounds, and

proteins [4]. One of the characters that form the basis in selecting microalgae as biodiesel feedstock because microalgae can growth in extreme environments [5]

There are several types of microalgae that have been known to have a high lipid content, such as *Botryococcus braunii*, *Chlorella* sp, *Schizochitrium* sp, *Nannochloropsis* sp. However, Indonesian microalgae has not been explored and studied intensively as feedstock biodiesel makers

## MATERIAL AND METHODS

### Sampling

Samples of microalgae were collected from freshwater at Batusangkar district, West Sumatera, Indonesia. Freshwater sample containing microalgae was collected from open pond for 3 L at the depth of ±2.5 m. Microalgae in the water were collected and then stored in 250 mL Bold Basal Medium [6].

### Isolation and Identification of Microalgae

Isolation of microalgae were done by series dilution method of freshwater sample. The freshwater sample containing microalgae was diluted serially in the range of $10^{-1}$-$10^{-9}$, and then was incubated at 27-30$^{o}$C. Then allowed to grow approximately for one week to obtain pure species [7]. The isolated microalgae was identified morphologically and then molecularly [8].

### Nile Red staining

Oil containing microalgae was screened by using solution of Nile red (9-diethylamino-5H-benzo[α]phenoxazin-5-one) in acetone (1 mg in 1 ml acetone). Microalgae (0.5 ml) were centrifuged and washed with physiological saline solution several times. Nile red was added to resuspended microalgae in physiological saline (1:100 v/v) and incubated for 20 minutes [9]. Lipid staining was observed under fluorescence microscope (ZEISS).

### Microalgae Cultivation and Growth

Microalgae were cultivated in shake flask culture in BBM medium at 27-30$^{o}$C, 250 rpm. Microalgae growth was monitored by spectrophotometer at $OD_{680}$ every day for 21 days. The isolated microalgae was grown in BBM medium containing various concentration of $NaNO_3$, namely 22.5 mg/L, 25 mg/L, and 27.5 mg/L) [10].

**Molecular identification**

DNA of microalgae was extracted using Dneasy® mini plant kit (Qiagen). The extract of DNA was amplified in PCR (Takara, Japan): initialing in 96$^{o}$C for 5 minutes, denaturation in 96$^{o}$C for 30 s, annealing at 57$^{o}$C for 30 s, extension in 72$^{o}$C for 30 s, and termination in 72$^{o}$C for 4 minutes. All reactions were carried out for 35 cycles using universal primer for the amplification of the D1-D2 rRNA region, fw1 (5'-AGCGGAGGAAAAGAAACTA-3') as forward primer and rev1 (5'-TACTAGAAGGTTCG-ATTAGTC-3') as reverse primer were used. Electrophoresis was carried out in agarose gel 1%. Family tree created by maximum likelihood Test[11].

**Productivity and lipid content**

The lipid content of microalgae was determined gravimetrically. The culture of microalgae (200 mL) was centrifuged at 6000 rpm for 10 minutes, and the pellet was dried in oven at 80°C for 24 hours and weighted. Next, the dried microalgae biomass was suspended in the mixture of 4 mL of deionized distilled water, 10 mL of methanol and 5 mL chloroform, and then was shake for 24 hours. Next, into the suspension was added 5 mL of deionized distilled water and 5 ml of chloroform, and then was centrifuged at 6000 rpm for 10 minutes. The lipid buildup was placed in a test tube and heated to replace mixed chemical solutions were added previously, and was weighted [12].

**Fatty acid analysis by GC-MS**

Isolated microalgae (10 ml) were centrifuged at 3000 rpm for 10 min (TOMY LC-200) and separated from the supernatant. Pellets were dried with nitrogen gas and 5 µL of internal standard (C19:0) 0.5 mg/ml were added. Methyl esterification was performed by adding 0.1 ml of 0.5% HCl-methanol, 0.4 ml of dehydrated methanol. Fatty acid methyl esters (FAMEs) were extracted using n-hexane and separated by centrifugation. n-Hexane layer was dried with nitrogen gas. For GC-MC analysis, hexane (40 µL) was added and input 1µL sample to GC column. GC-MS analysis was conducted by using DB WAXTR column (30 m, ϕ = 0.250 µm) and the temperature about 130$^{o}$C [13].

## RESULTS AND DISCUSSIONS

**Isolation and identification of microalgae**

One species of microalgae had been isolated and observed under light microscope as shown in Figure 1. Based on the microalgae morphology, the microalgae was identified as *C. snowii* with a typical cell elliptical to lanceolate (long and slender), usually green, and has eye spots [8].

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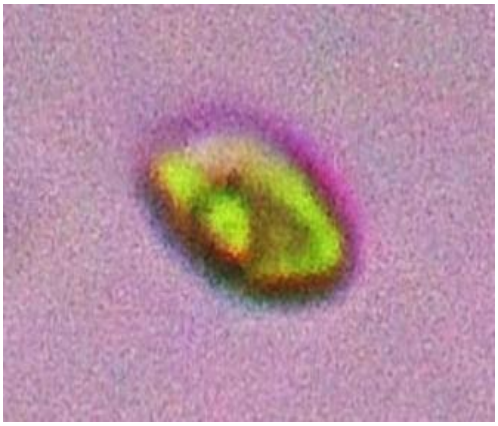
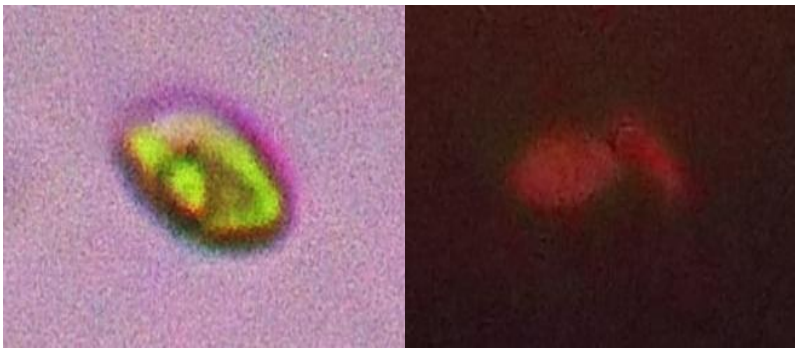


**Figure 1. Microalgae species contained in the sample**

Nile Red staining was performed on isolated species grown on BBM medium to determine the intensity of lipid content. The yellow or orange fluorescence on the Nile red stained microalgae indicated the content of lipid in the microalgae. This color change occurs because Nile red reacts with lipid contained in microalgal cells by changing the color of red to yellow or orange [14]. Nile Red staining results on isolates of microalgae can be seen in Figure 2A dan 2B.



**A** **B**

**Figure 2. (A) The isolated microalgae (B) Microalgae with Nile red staining**

## Molecular Identification

Identification of the species with molecular done with spherical morphology to confirm the name of the species and see kinship species. Determination of species is done by comparing the sequences obtained with the data in the NCBI gene (Blast). Alignment is done by using the program ClustalX2, distance between species analysis were performed using the MEGA5. Made family tree based on Maximum Likelihood Tests is shown in Figure 3.

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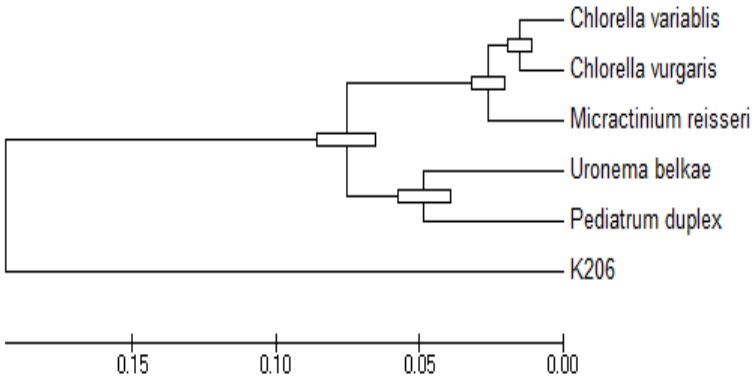


**Figure 3. Family tree Maximum Likelihood Test**

Based on the analysis of the P-distance MEGA5 program, it is known that the *C. snowii* has a range of isolates with *Micractinium reisseri* 34.2%, 37.1% with *Chlorella variablis, Chlorella vurgaris* with 37.9%, 38.2% with *Uronema belkae*, and 40,2% with *Pediatrum duplex.* This shows away kinship between isolates with those species, thus naming the isolates could not be ascertained molecularly. However, based on morphology structure similar to *Chlamydominas snowii*.

In this study, molecular identification is done by using universal primers for regions D1-D2 LSU rDNA. Primers used were universal primers FW1 (5'- AGCGGAGGAAAAGAAACTA-3') as forward primer and rev1 (5'- TACTAGAAGGTTCGATTAGTC-3') as the reverse primer. Regions D1-D2 LSU rDNA potential for use in taxonomy. This region can be amplified with universal primers, showing the diversity and suitable to distinguish closely related species [11].

Name the molecular species of microalgae using local D1-D2 LSU rDNA could not be determined with certainty as molecularly. This is due to the remoteness of kinship. According *Dzikowski* (2004), sequence differences >2 % is a different species, but if the 0.1% sequence divergence within a species is still classified. The distance between the bases is still less than 2% can be classified into a single species, but if the distance is more than 2% is including different species. In a study of the genus *Prototheca* using LSU rDNA, sequence differences obtained for the species *P.zopfii* and *P.moriformis* more than 2% [15]. So it can be stated that the isolates is not one species based on the results of the blast. The vagueness of kinship on microalgae may be caused by the unavailability of data for these species gene.

**The Content and productivity lipid**

Lipid microalgae generally in the form of esters of glycerol and fatty acids with chain lengths $C_{14}$-$C_{22}$ [16]. Fatty acids in microalgae include intracellular molecules as present in the cell in the chloroplast. Here is the data amount of lipids extracted from samples *C. snowii* grown in various nitrogen concentration as shown in Figure 4.

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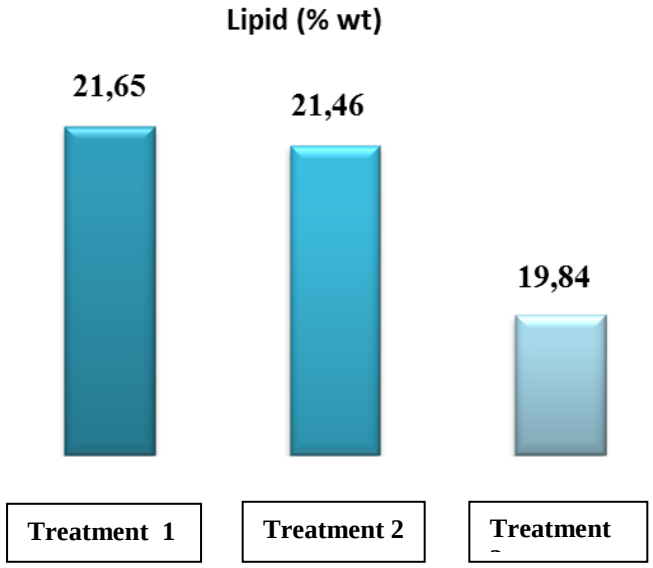


**Figure 4. Effect of N source ($NaNO_3$) variation on the lipid content of microalgae, Variation 1: 22.5 mg/L, 2: 25 mg/l, and 3: 27.5 mg/L $NaNO_3$**

As shown in Figure 4, the microalgae that was cultivated on BBM medium with N concentration (22.5 mg/L) had the highest lipid content (21.65%). While microalgae in BBM medium with N concentration (25 mg/L) reached 21.46% and the concentration of N (27.5 mg/L) 19.84%. The nitrogen stress induces the biosynthesis of lipid in microalgae. Nitrogen concentrations also affect lipid productivity of microalgae, for variation 1 ($NaNO_3$= 22.5 mg/L) produced 15.468 g/L/day, variation 2 ($NaNO_3$=25 mg/L) produced 15.325 g/l/day and variations 3 ($NaNO_3$ = 27.5 mg/L) produced 14.168 g/L/day. However, the lipid productivity of microalgae at that condition was not significantly different [10].

**Microalgae growth with variation total nitrogen**

Microalgae growth is influenced by several things: nutrients, light intensity, temperature, salinity, and pH. Microalgae growth patterns at different nitrogen concentrations can be seen in the Figure 5. Figure 5 shown that *C. snowii* microalgae grown on higher nitrogen 27.5 mg/L produced higher amount of biomass. The amount of nitrogen that much, producing microalgae growth rate is high. Nutrient needs are met and environmental factors that support will result in a good growth rate

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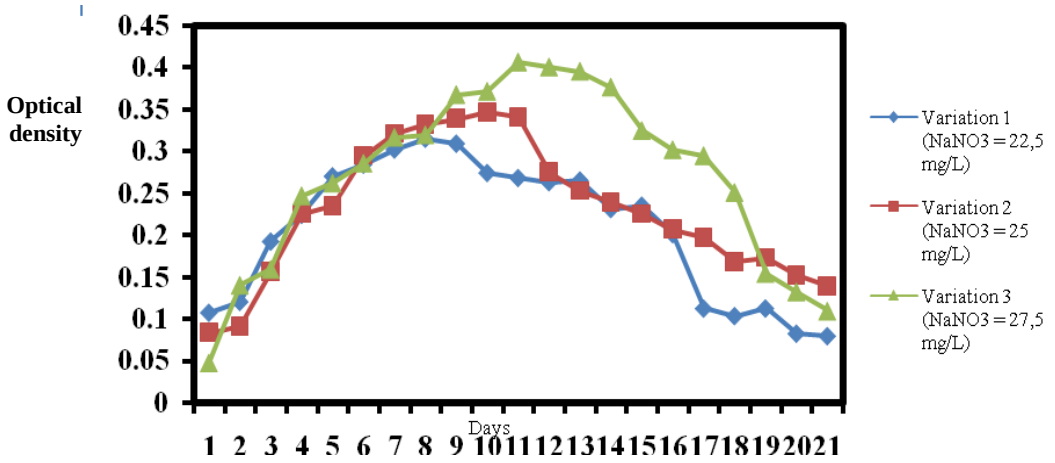


**Fig 5: Microalgae growth charts with variations of the number of sources N**

Comparison of fatty acid composition of the three variations $NaNO_3$ amount of fuel contained in the medium in the form of % can be seen in the Figure 6.

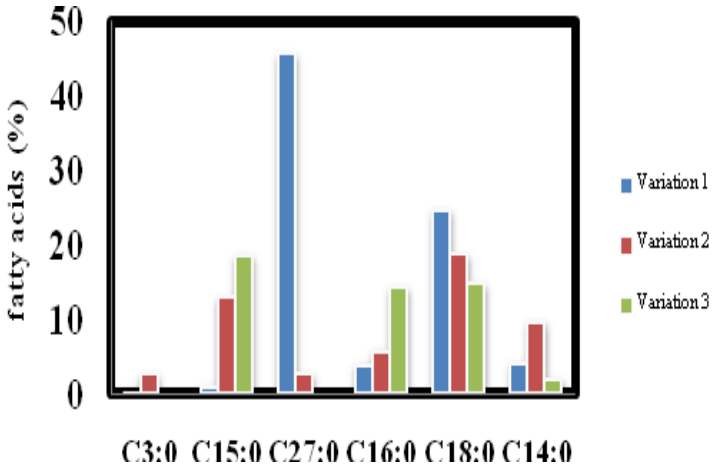


**Fig 6: The amount of fatty acids in%**

From the Figure 6 it can be seen that the fatty acids found in species *C. snowii* are fatty acids C3:0, C15:0, C27:0, C16:0, C18:0 and C14:0. The concentration of nitrogen in the growth medium affect significantly the lipid content and the fatty acid type on the microalgae. Low nitrogen concentration in the medium induced the production of high molecular weight of fatty acid.

**Fatty acid analysis**

The results in Table 1 shows that the fatty acid type was also affected by the nitrogen concentration in the growth medium of *C. snowii*.

| Types of fatty acids | Amount of fatty acids (nmol) | | |
|---|---|---|---|
| | variation 1 ($NaNO_3$ 22,5 mg) | variation 2 ($NaNO_3$ 25 mg) | variation 3 ($NaNO_3$ 27,5 mg) |
| C3:0 | 0,00046 | 0,0112 | 0,00000 |
| C15:0 | 0,00063 | 0,0529 | 0,05880 |
| C27:0 | 0,03080 | 0,0112 | 0,00000 |
| C16:0 | 0,00266 | 0,0238 | 0,04500 |
| C18:0 | 0,01600 | 0,0763 | 0,04725 |
| Types of fatty acids | Amount of fatty acids (nmol) | | |
| | variation 1 ($NaNO_3$ 22,5 mg) | variation 2 ($NaNO_3$ 25 mg) | variation 3 ($NaNO_3$ 27,5 mg) |

**Table 1: Fatty acid composition on *C. snowii* grown at various nitogen concentration medium**

The C18:0 production was high in the medium containing 25 mg/mL $NaNO_3$, in the other hand the C2:0 was highest.

## CONCLUSSION

Nile red staining can be used to select a lipid containing microalgae as a candidate for the production of biodiesel. The detected fatty acids in *C. snowii* were the fatty acids of C3:0, C15:0, C27:0, C16:0, C18:0 and C14:0. The availability or concentration of nitrogen in the growth medium affected the growth of *C. snowii* . It also affect amount of each fatty acid type that was produced by microalgae significantly.

## REFERENCES


[1] Koh LP, Ghazoul J. Biofuels. Biolog Conservation 2008; 141:2450-2460.
[2] Nigam PS, Singh A. Production of liquid biofuels from renewable resources. Progress in Energy and Combustion Science. 2010;In press.DOI: 10.1016/j. pecs.2010.01.003
[3] [NREL] National Renewable Enerfy Laboratory. 2003. A look back at the U.S. department of energy's aquatic species program-biodiesel from algae. Colorado: NREL.
[4] Hu Qiang Hu, M.S., Eric Jarvis, Maria Ghirardi, Matthew Posewitz, Michael Seibert, Al Darzins. The Plant J 2008; 54: 621-639.
[5] Chisti Y. Biotechnol Adv 2007; 25: 294-306.
[6] Welch, P.S. 1948. Limnology methods. Mc graw-Hill Book Company. Inc. New York.
[7] Waluyo, L. 2008. Teknik dan Metode Dasar dalam Mikrobiologi. Malang : UNM Press.
[8] Presscott, G.W., 1978. How to know the freshwater algae. 3rd edition. WMC. Brown Company. Lowa.
[9] Cooksey KE, Guckert JB, Williams SA, Collis PR. J Microbiol Methods 1987; 6: 333-345.
[10] Li X, Hu Y, Ghan Ket al. Bioresour Technol 2010; 101: 5494-5500.
[11] Sonnenberg R, Nolte AW, Tautz D. Front Zool 2007; 4: 6-12.
[12] Bligh EG, Dyer WJ. Can J Biochem Physiol 1959; 37: 911-917.
[13] Matsunaga Tadashi MM, Yoshiaki Maeda, Hiroshi Sugiyama, Reiko Sato, Tsuyoshi Tanaka. Biotechnol Lett 2009; 31: 1367–1372.
[14] Carman KR, Thistle D, Ertman SC, Foy M. Mar Ecol Prog Ser 1991; 74: 307-311.
[15] Dzikowski R, Levy MG, Poore MF, Flowers JR. J Parasitol 2004; 90: 413-414.
[16] Borowitzka MA. Fats, oils and hydrocarbons. In: Microalagal Biotechnology, (Eds. M.A. Borowitzka, L.J. Borowitzka). Cambridge University Press. 1998: p.257-287.