Increasing content of lipid in tropical microalgae *Chlorella sorokiniana* and *Closterium* sp. with variation of nitrogen content and extraction temperature

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Abstract. This research was aimed to obtain algae biofuels as an alternative energy which comes from tropical microalgae biomass *Chlorella sorokiniana* and *Closterium* sp. The cultivation was performed at a controlled room in batch culture *photobioreactor*, temperature 27°C, pH 6, aeration with air flow rate of 150 mL/sec, and light intensity at 2400 lux. These nutrient sources used artificial PHM with KNO₃ variation as a nitrogen source of 0 grams; 0.25 grams; 0.5 grams; 1 gram. The research involved microalgae lipid extraction with *Blight & Dyer* method and also carried out support of alcohol, chloroform and distilled water (1:1:1) with optimum temperature 30°C. Oil yields that obtained were analyzed with *Gas Chromatography Mass Spectrometry* (GC-MS) method, dry weight with a gravimetric method and cell density with spectrophotometry. The result showed that the reduction of KNO₃ materials (0,25 gr) can produce biomass and highest total lipid content at 0,41 g/L and 20,31 %/(w/w). The results showed that KNO₃ decreased the amount of biomass which was not significant but the total lipid content of microalgae was increased. Lipid content and fatty acids extracted from *Blight & Dyer* showed oil content that could potentially be the raw material of biodiesel.

1 Introduction

The use of fossil fuels in many industries like transportation is reported to become one of the main factors that caused an increase in carbon dioxide (CO₂) gas emissions in the atmosphere that cause global warming. Fossil energy is a limited energy and less environmentally friendly. The burning process results in the greenhouse effect because it contains carbon dioxide (CO₂), sulfur dioxide (SO₂), and nitrogen oxide (NO_x) [1]. The price of fuels, which will be getting an increase, and pollution problem causing all parties to look for alternative fuels. In the recent decades, there is much research about the using of biomass and waste as the alternative fuels because of their eco-friendly and non-toxic nature [2].

Biodiesel is one of the alternative fuels which can be produced from plant biomass, for instance, corn, oil palm, canola, jatropha, and animal fat [3]. However, the use of vegetable oil for biodiesel production needs a big land, high maintenance cost, and cause a competition towards food needs [4].

Biodiesel is a mono-alkyl ester of long chain fatty acids, which originated from transesterification of biology materials [5]. Microalgae have the potential to produce higher amounts of biomass and oil than other plants. Biodiesel from microalgae has the greatest potential to replace crude oil because of its role as

producer, microalgae require only sunlight and other simple food sources for their growth [6]. Microalgae have a chemical composition of cells composed of proteins, carbohydrates, and fatty acids. This fatty acid component will be extracted into lipid.

The method that can be used to convert lipid contained in the microalgae into biodiesel is by extraction or separation and isolation of triglycerol fats from the harvested microalgae. The transesterification method happened when one molecule of each triglyceride in the algae oil reacts with three molecules of methanol, resulting in three molecules of methyl ester (product of biodiesel) and one glycerol molecule, thus biodiesel is obtained [7]. Therefore, microalgae are the appropriate alternative raw materials for biodiesel production [8].

The lipid content in microalgae biomass can reach above 50% with very rapid growth [9]. High amounts of lipids can be obtained by testing the growth of microalgae in nutrient-lacking condition. Generally, nitrogen plays a key role in the synthetic pathway of macromolecules in microorganisms, because the growing cell will be disrupted if there is a lack of nitrogen sources [10].

At the time when the source of nitrogen is lacking, microalgae will accumulate lipids. Several studies have reported that due to lack of nutrition, such as nitrogen depletion in *Phaeodactylum tricornutum*, microalgae can

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increase lipid production by 50%. The increased lipid content in nutrient deficiency is due to low cell component production rates, yet oil production remains high [11].

Based on the description above, this research was conducted to determine the need for KNO₃ as the N source which affects the production of lipid as an important compound that can be converted into biodiesel.

2 Research methodology

2.1 Materials

The samples used were seedlings of *Chlorella sorokiniana* and *Closterium sp.* Cultivation media of microalgae using artificial media (PHM) with the composition of KNO₃ as nitrogen source 1 gr, 0,2 gr KH₂PO₄, and 0,2 gr MgSO₄, which were dissolved in one L distilled water. After that, the composition was homogenized using a stirrer while two drops of Fe stock solution and two drops of *Tracelement* were being dropped to the stirrer.

2.2 Microalgae cultivation analysis

Microalgae cultivation process used a limited system (batch culture) in photobioreactor one L within 14 days. The composition contained in the photobioreactor is a total culture volume with amount 80% of the volume of the container. PHM media used 90% of culture volume, pure *Chlorella sorokiniana* and *Closterium sp* microalgae seedlings were added by 10% of culture volume, and the addition of nutrient molasses by 5% of culture volume.

KNO₃ as a source of nitrogen in the cultivation medium is varied as much (gram) 0; 0,25; 0,5; 1 gr. The research condition is arranged as follows: pH 6, temperature 27°C, aeration 150 mL/sec, and light intensity 2400 lux. The cultivated microalgae were measured for growth using a UV-Vis spectrophotometer at a wavelength of 680nm daily.

2.3 Lipid extraction analysis

Microalgae lipid extraction process was carried out at 30°C, using *Blight & Dyer* method with the alcohol solvent, chloroform, and distilled water (1: 1: 1). The harvested microalgae were centrifuged with a rotation speed of 6000 rpm. Then 10 ml of methanol was added, after that, all of them were shaken. The next step, 10 ml of chloroform was added, following that all of them were shaken, later 10 ml of distilled water were added. Like in the previous step, the recent mix was shaken and finally, the mix was centrifuged one more time. This process generated three phases, which were a mixture of methanol and distilled water in the upper layer, cell or biomass in the middle layer, and a mixture of chloroform with oil in the lower layer. The middle and bottom layers

were used for lipid analysis and dry weight analysis using the following formula.

$$\% \text{ Lipid} = \left(\frac{A \times B}{C} \right) \times 100\% \quad (1)$$

A : The weight of lipid (gram)

B : The weight of biomass (gram)

$$\% \text{ Dry Weight} = \left(\frac{A - B}{A} \right) \times 100\% \quad (2)$$

A : The empty container weight + sample

B : The empty container weight

2.4 Transesterification analysis

The rest of the harvesting was being used to obtain biodiesel oil, by adding 2% NaOH 4 ml and methanol, then was refluxed with Soxhlet, which then was resulted to two layers, the upper layer was oil and the bottom layer was glycerol.

3 Result and discussion

Fig 1. shows growth curve on the mixed culture of *Chlorella sorokiniana* and *Closterium sp* with a variation of N source addition which is KNO₃ on growth media using UV-Vis spectrophotometer.

Based on the growth curve it can be seen that the highest growth in the mixed culture of *Chlorella sorokiniana* and *Closterium sp* with KNO₃ source variation of 100% and 50% amount, each occurred on the 6th day. While on the KNO₃ source variation of 25% amount occurred on the 5th day.

Microalgae *Chlorella pyrenoidosa* cannot grow without any nutrient content of nitrogen source and its growth is directly proportional to the addition of N concentration in the medium [12]. On the growth curve, it is seen that in a mixed culture of tropical microalgae with KNO₃ variations of 100%, 50%, and 25% amount have longer lag phase and longer death phase. In contrast to a mixed culture of tropical microalgae with KNO₃ variation of 0% amount, has a shorter lag phase, as well as experiencing a phase of death faster than other variations. The result of the study is shown from the growth curve that the growth of the mixed culture of tropical microalgae is directly proportional to the addition of N concentration in the medium.

This may be caused as described [12] that microalgae cannot grow without the appropriate nutritional content, proper and complete ratio, for instance, the microalgae must have elements C, N, H, P, K, Mg, Ca and S. For that, microalgae still needed addition of nitrogen source in its growth.

Fig 1. Growth Curve of Mixed Culture at Different KNO₃ Concentration.

The results of lipid and dry weight analysis can be seen in **Table 1** and **Table 2**.

Table 1. The Effect of KNO₃ as N-Inorganic Source on Dry Weight.

Total Sources of N (gr)	Dry Weight (g/L)			
	R1	R2	R3	RX
0,00	0,05	0,12	0,17	0,11
0,25	0,40	0,40	0,42	0,41
0,5	0,39	0,44	0,46	0,43
1,00	0,45	0,47	0,48	0,47

Based on the results shown in Table 1, a decrease in KNO₃ levels by 50% and 25% of normal requirements gives a non-significant increase in the amount of biomass. The highest number of dry biomass occurs in a medium of normal KNO₃ addition, which is 100%, with amount 0,47 g/L. If KNO₃ is added only by 50% and 25% there is a non-significant increase in dry biomass, with amount 0,43 g/L and 0,41 g/L, respectively. The lowest dry biomass occurs when the growth medium is not given the addition of N source.

The amount of N concentration reduced to 50% in growth media does not provide a significant reduction compared to normal requirements [13]. The result of this research shows that there is no significant decrease in a normal requirement that is 100% with KNO₃ which is added only by 50%.

Inadequate amounts of nitrogen will limit the growth of algae, thereby biomass that is produced by *Phaeodactylum tricornutum* resulted in only small amount without the addition of nitrogen [13]. The results of the study showed that there was a decrease in the amount of biomass at 0% KNO₃ levels.

Table 2. The Effect of KNO₃ as N-Inorganic Source on Lipid Content.

Total Sources of N (gr)	Lipid Weight (%w/w)			
	R1	R2	R3	RX
0,00	16,24	16,00	16,10	16,11
0,25	20,96	19,75	20,23	20,31
0,5	17,48	18,40	17,30	17,73
1,00	15,28	15,85	15,37	15,5

Table 2 shows that the highest lipid content in the condition of the source of nitrogen decline by 25% is 20.31 %(w/w). The decrease of 50% and 0% give the amount of lipid which are not much different, which are 17,73 %(w/w) and 16,11 %(w/w).

When the source of N is limited to the growth of microalgae *Scenedesmus sp.*, dry biomass decreases while lipid production increases [14]. The result shows that the mixed culture of *Chlorella sorokiniana* and *Closterium sp.* if KNO₃ was added only by 50% and 25%, the resulting dry biomass decreased, whereas lipid production increased.

The highest lipid content was obtained in *Phaeodactylum tricornutum* under optimal source nitrogen reduction conditions of 50% [11] while in *Scenedesmus sp.*, it was found that the highest lipid content in nitrogen administration was only 10% [14]. In this study, it can be seen that the mixed culture of *Chlorella sorokiniana* and *Closterium sp.* produced the highest lipid when KNO₃ as the source of N is reduced to 25% of the normal requirement.

The results of this study are consistent with the previous study [10], that in conditions of lack of nitrogen source in the medium, the microalgae will accumulate lipids and high fatty acids. Increased lipid content in nutrient deficiency is due to low cell component production rates, though oil production remains high [11].

4 Conclusion

The results of this research showed that the mixed culture of *Chlorella sorokiniana* and *Closterium sp.* with the addition of KNO₃ amount 25% of normal requirement resulted in higher dry biomass and total lipid microalgae content of 0.47 g/L and 20.31 %(w/w), respectively.

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