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Cell Biomass and Lipid Productivities of *Meyerella planktonica* under Autotrophic and Heterotrophic Growth Conditions

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***Abstract*—**Microalgae *Meyerella planktonica* is a potential biofuel source because it can grow in bulk in either autotrophic or heterotrophic condition. However, the quantitative growth of this algal type is still low as it tends to precipitates on the bottom. Besides, the lipid concentration is still low when grown in autotrophic condition. In contrast, heterotrophic condition can enhance the lipid concentration. The combination of autotrophic condition and agitation treatment was conducted to increase the density of the culture. On the other hand, a heterotrophic condition was set up to raise the lipid production. A two-stage experiment was applied to increase the density at the first step and to increase the lipid concentration in the next step. The autotrophic condition resulted higher density but lower lipid concentration compared to heterotrophic one. The agitation treatment produced higher density in both autotrophic and heterotrophic conditions. The two-stage experiment managed to enhance the density during the autotrophic stage and the lipid concentration during the heterotrophic stage. The highest yield was performed by using 0.4% v/v glycerol as a carbon source (2.9±0.016 x 10^6 cells w/w) attained 7 days after the heterotrophic stage began. The lipid concentration was stable starting from day 7.

***Keywords*—**Agitation, Glycerol, Heterotrophic, Lipid Productivity, *Meyerella planktonica*.

I. Introduction

ENERGY crisis is one of the most concerning problems for the survival of human civilization these days. Energy problems have arisen because almost all new technological products rely on energy derived from fossil fuels. The need for energy increases along with the increase of human population. High human population growth implies higher rate of various needs, including vehicles, air conditioners, heaters, etc. The fact that oil price surpassed 100 USD per barrel indicates an increase in demand for the commodity. Another serious problem concerning the use of fossil fuels is its impact on the environment. Fossil fuels are blamed to be the main agents of global warming and climate changes that have been a global concern over the last two decades. Thus, the need for alternative sources of energy is inevitable. The ideal alternative sources of energy are those which have little or negative impact on the environment and those which are renewable.

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One example of renewable energy proposed to substitute for fossil fuel energy is biofuel [1], [2]. Biofuel, composed of acyl esters (Fatty Acid Methyl Ester, FAME and Fatty Acid Ethyl Ester, FAEE), is derived via transesterification of short chain fatty acids into longer ones.

Biofuel can be produced from plants, such as *jarak 'Ricinus communis',* sunflowers, corns, sugarcanes, and other agricultural products. The use of agricultural commodities for biofuel production, however, is considered unsustainable [1], and time consuming due to its long waiting period [3]. In addition, using agricultural products for this purpose is detrimental to the environment. One significant example is the use of palm oil as an energy source. Mass plantation of the species will undoubtedly reduce the forest size, cause the extinction of various plants and animal species, and reduce the availability of water [4]. Moreover, using crops as sources of biofuel will also threaten the availability of land for planting crops for food.

One alternative source of biofuel is microalgae. Microalgae are considered to be a prospective choice because of their abundance in the ocean and their ability to grow rapidly when treated with proper care and nutrition. A microalgae species which are newly found yet has much potential for lipid production is *Meyerella planktonica*. Belonging to phylum *Chlorophyceae*, the species is known to have high exceptional tolerance towards its environment for growing as the cells are able to grow both in freshwater and brackish water. Sharing the same genera with other *Chlorella* species, this unicellular microalgae is able to grown both autotrophically and heterophically, thus increasing its cellular density and lipid accumulation significantly compared to other microalgae species [5].

As for the cell production quantity, the cultivation of *Meyerella planktonica* encounters two main problems, i.e. it precipitates easily and it sticks to the bottom of the culture vessel. Recent studies have shown that the production of *Meyerella planktonica* was hampered because this species can only utilize bottom of the culture vessel as it settles or even sticks to the bottom of the vessel [6]. The *Meyerella planktonica* cells, which settle on the bottom of the culture vessel, will later stick on the vessel. This means that *Meyerella planktonica* can only be grown at the bottom surface of the culture vessel and its growth is relatively slow. Therefore, it is necessary to develop a culture system which prevents the cells from precipitating at the bottom of the vessel. When the cells do not precipitate at the bottom of the

vessel, they can disperse evenly across the vessel. A semi bioreactor method, which relies on agitation and aeration systems, can help the spread of algal cells to all parts of the tank and prevent the buildup on the bottom of the container. The agitation and aeration systems have been applied for rotifer production and have managed to increase its production by 32% [7].

In terms of the quality, the lipid content of algae can be increased through heterotrophic growth. In heterotrophic condition, algae use the carbon available in the environment as their source of energy, substituting for the energy from light [5]. In such an environment, carbon assimilation increases and the excess of carbon is converted into lipid [8]. The source of carbon in this environment can be enhanced by adding glucose ($C_6H_{12}O_6$) or glycerol ($C_3H_8O_3$). The high content of C element in these two compounds can stimulate lipid accumulation in algal cells. The application of the heterotrophic growth methodon *Chlorella protothecoides* using glucose as a carbon source has been claimed to successfully increase the lipid content, four times as much as that of the autrotroph method [5]. Although glucose contains more C element, the assimilation process of C element of glucose requires the presence of transport protein hexose/H^+ symport system which requires energy (1 ATP) [9]. In contrast, that is not the case with glycerol. Glycerol does not require the transport protein to enter the cells. The assimilation of glycerol into the cells occurs through diffusion process of osmotic pressure difference and this process does not require energy. In addition, glycerol is an osmotic solution and therefore, it is not toxic to the cells, even in high concentration [10].

The objectives of this study were (i) to increase the density of *M. planktonica* via an autotrophic semibioreactor culture approach and (ii) to increase the lipid content of *M. planktonica* through heterotrophic culture system using glycerol as its source of carbon.

II. Materials and Method

The *M. planktonica* algae used for the experiment were isolated and purified from gut of soft coral *Dendronephtya* sp. [7]. The cells were maintained in *f/2* Guilard (1962) and grown in 250-ml Erlenmeyer flasks in room temperature.

The semi bioreactor vessel was made from a cylindrical glass aquarium with no cover, measuring 30 centimeters in diameter and 60 centimeters in height. A small plastic tube was placed on the bottom of the vessel to supply air. Two baffles were attached on both inner sides of the vessel to prevent vortex forming when the agitator is spinning. A specially designed motor was attached to the agitator positioned on the bottom of the vessel to prevent precipitation. Speed controlling mechanism was applied to adjust the agitation speed. As for heterotrophic culture, the vessel was tightly wrapped with a dark piece of plastic to prevent light from entering.

To determine optimal growth conditions for quantitative growth, the factorial design of 2 x 2 was applied. The first treatment was with autotrophic and heterotrophic conditions. The second one was with agitation and non-agitation. The experiment was repeated four times. Therefore, 16 units of experiment (2 x 2 x 4) were needed. During the autotrophic growth, the cells were grown in a 10 L semi-bioreactor vessel under constant fluorescent light with the intensity of 100 Lux. Culture under the autotrophic growth condition served as the initial inoculum. Agitation was attained using an agitator with rotation set to 60 rpm. The cell growth and density were monitored every other day using a hemocytometer.

To increase the lipid content qualitatively, another set of experiments was conducted heterotrophically using 1% (w/v) glucose, and 1% (v/v) glycerol as carbon sources and the autotrophic condition served as a negative control. The cell growth and density were monitored every other day using a hemocytometer. After the cells reached the stationary phase, samples were extracted to measure their lipid concentration. The experiment was also repeated four times. Therefore, 12 units of experiment were conducted.

A two-stage growth experiment was conducted to determine the total lipid resulted from culture under various glycerol concentrations (0.1%, 0.4%, 0.7% v/v). During the first stage, the algae was cultured in an autotrophic condition and agitated with the rotation set to 60 rpm until the growth reached the stationary phase. During the second stage, the culture process (from the first stage) was followed by heterotrophic condition using glycerol as an energy source. This heterotrophic condition was kept by blocking the light source from entering the vessel using a black plastic cover. Cell counts were made every other day, and the lipid samples were then extracted after the cells reached the stationary phase.

The lipid was quantified using soxhlet extraction method. The dry weight of samples was taken by centrifugation of the culture samples in a 50-ml tube at 8000 rpm for 10 minutes. The supernatant was removed and cell pellets were dried overnight in a drying oven at the temperature of 80oC. Lipid extraction was performed using the soxhlet method. About 5 grams of dried cells were coated using a thin cotton layer and a piece Whattman paper, and then they were inserted into the huls. The soxhlet was assembled and lipid extraction was run for 6 hours using n-hexane as solvent. The solvent, containing lipid extracted from the sample, was then separated from the lipid using a rotavapor. The weight of the lipid was measured by subtracting the weight of empty flask before the extraction from the weight after the extraction. The cellular lipid concentration was measured by comparing the extracted lipid weight and the sample dry weight.

III. Results

The growth kinetics of *M. planktonica* closely resembles the sigmoid growth curve and the growth peak was reached on day 14. The autotrophic treatment showed higher density of *M. planktonica* and this was significantly different (p=0.00) from the one resulted in the heterotrophic one. As for the semi-bioreactor, the agitation treatment resulted higher density of *M. planktonica* and was significantly different (p=0.005) compared to the non-agitation treatment, both in autotrophic and heterotrophic conditions. Hence, the highest density was

performed by the combination of autotrophic and agitation treatments. The highest density was 4.393 x 10^6 cells/ml. It was obtained on the 14th day (Fig. 1).

M. planktonica cultured in the heterotrophic condition practically did not show any growth. The density remained constant, or even declined; and the lowest growth rate was observed when treated with glycerol 3% (w/w). However, cellular lipid content of cells grown in the heterotrophic condition was three times (50% dry weight) higher compared to that in the autotrophic condition (17% dry weight). In terms of the heterotrophic energy source, the use of glycerol as carbon yielded higher lipid content than the use of glucose in the heterotrophic condition (Fig. 2).

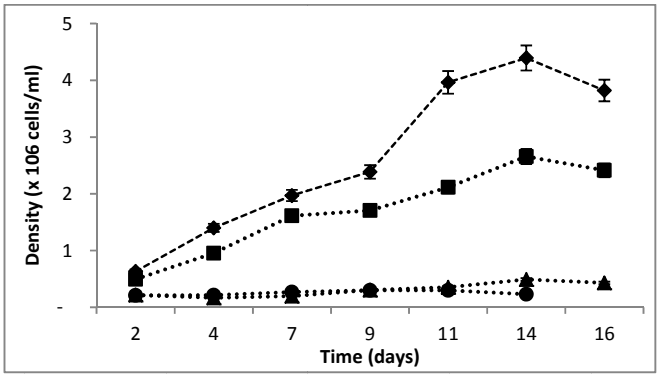

Fig. 1 *M. planktonica* cell growth with different treatments against the growth period (♦: autrotroph agitation; ■: autrotroph non-agitation; ▲: heterotroph agitation; ●: heterotroph non-agitation)

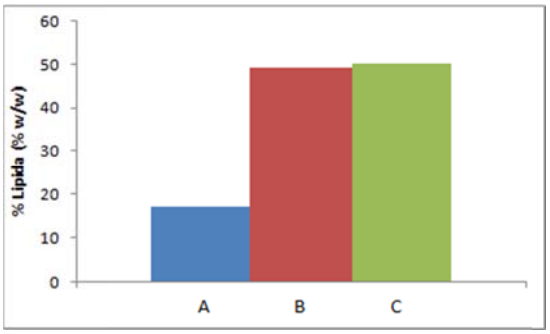

Fig. 2 Lipid concentration in autotrophic treatment (A), heterotrophic glucose treatment (B), and heterotrophic glycerol treatment (C)

Considering the previous results, the two-stage experiment was set up by combining an autotrophic condition to increase density (quantitative optimization) followed by a heterotrophic condition to increase the cellular lipid content of *M. planktonica* (qualitative optimization). During the autotrophic growth stage, the growth peak was achieved on day 14 and it entered the decline phase on day 16 (Fig. 3). Heterotrophic growth stage began on day 16 and *M. planktonica* then re-entered the exponential growth phase until day 21, then it was followed by the decline phase on day 23. In the heterotrophic growth stage using glycerol 0.4% as a carbon source yielded higher cellular growth (6.51±0.007 x 10^6 cells/ml) compared to cells grown with 0.1% glycerol (5.59±0.070 x 10^6 cells/ml) or 0.7% glycerol (5.48±0.070 x 10^6 cells/ml).

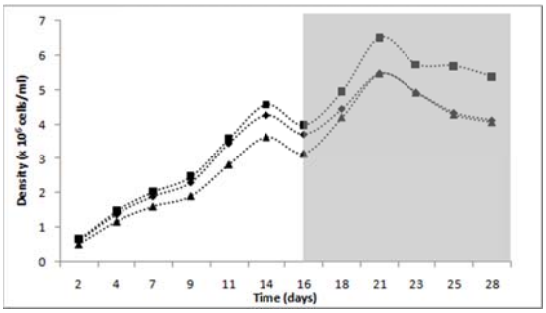

Fig. 3 *Two-stage M. planktonica* growth at autotrophy stage (light) and heterotrophy stage (dark) (♦: gliserol 0.1%; ■: gliserol 0.4%; ▲: gliserol 0.7%)

The cellular lipid concentration was stable at 49% 7 days after the heterotrophic treatment. No significant difference was observed in terms of the cellular lipid concentration measured on day 7, day 9, and day 12 after the heterotrophic treatment. In fact, a slight decline was observed on the day 12th (Fig. 4).

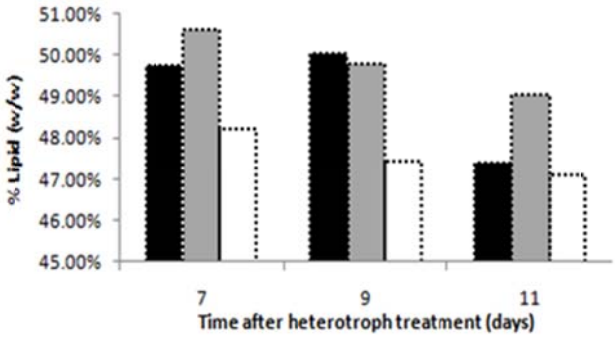

Fig. 4 Lipid concentration after the hetero- trophic phase with different treatments: 0.1% glycerol (dark), 0.4% glycerol (grey) and 0.7% glycerol (white)

The total lipid yield (cell density x cellular lipid concentration) showed that *M. planktonica* culture using 0.4% glycerol as a source of carbon resulted in higher yield (2.9±0.016 x 10^6 cells w/w) and that was significantly different when compared to the one using 0.1% glycerol treatment (2.432±0.05x 10^6 cells w/w) or 0.7% glycerol treatment (2.373x 10^6 cells w/w).

IV. Discussion

The density of *M. planktonica* triggered by the autotrophic treatment is higher than the one by the heterotrophic treatment. The growth rate of the heterotrophic culture could be inhibited due to the inherent nature of *M. planktonica*. This species is a phototrophic organism, which obtains energy for its growth mostly from light through photosynthesis [11]. The absence of light in a heterotrophic condition could inhibit the growth of *M. planktonica*. As for semi-bioreactor growth treatment, the agitated culture resulted higher density

compared to the non-agitated one. *M. planktonica* is a non-motile organism [11], resulting in precipitation of cells at the bottom of the vessel and its growth was then limited only on the bottom surface of the vessel. The role of agitation was to help the cell to disperse evenly throughout the water column; and this could increase the efficiency of the cellular chemical diffusion and light penetration [12]. In the non-agitated treatment, *M. planktonica* cell tended to be suspended at the bottom of the vessel. This means that the growth of the cell mostly depends on only the space around the bottom of the vessel.

The combination treatment, i.e. agitated autotrophic culture, performed the highest density. The maximum density was 4.393 x 10^6 cells/ml, and it was obtained on day 14. This result, however, was still lower compared to the result yielded by similar species [13] which could reached the density of 16 x 10^6 cells/ml. This significant difference might due to the different intensity of light and species used. While this experiment employed *M. planktonica* and the intensity of light was 100 Lux, [13] study used *Chlorella* sp. with the light intensity of 2500 Lux. Although the cellular density of *M. planktonica* was lower compared to that of *Chlorella* sp., the lipid concentration of the latter was double (49%). Reference [14] showed lower lipid concentration (22%) than this percentage.

Although autotrophic culture yielded higher cellular density, the cellular lipid concentration derived from cells grown in the autotrophic condition was lower than the cells grown heterotrophically (17% compared to 49%). In heterotrophy, cellular photosynthesis does not completely come to a halt. The environment where light is not available creates stress for the cells; and thus, lipid synthesis of cells will be induced [15]. Although the environment lacks of light and no energy is produced, the photosynthesis which has naturally occurred drives the *M. planktonica* cells to maintain $NADP^+$ as an essential electron acceptor for photosynthesis. No-growth condition resulted in the accumulation of NADPH since no NADPH breakdown was observed. The process to synthesize lipids helps *M. planktonica* to maintain $NADP^+$ concentration in the heterotrophic growth [16].

Under heterotrophic conditions, glycerol, used as a carbon source, resulted in higher lipid concentration than glucose did. This confirms the existing literature, which stated that glycerol, as a carbon source for cells, is easily absorbed actively and passively [17]. This characteristic allows glycerol to be readily used as an energy source. The presence of glycerol inside cells allows better lipid synthesis, for glycerol can also be used by cells as precursor for creating triacylglycerols [18]. Based on these findings, the next step of research only used glycerol as a carbon source.

As for glycerol concentration used, the addition of 1% glycerol into the culture yielded better result compared to addition of 3% (higher) glycerol concentration. This finding contradicted [14] in which they suggest that adding 2% glycerol into the culture generates the best result. However, high concentration of glycerol could be detrimental to the growth of *M. planktonica* [6]. This may be due to sub-optimal culture conditions leading to cellular stress, slowing growth to a halt and immediately induce lipid metabolism. In this research the optimal glycerol concentration for the lipid production by *M. planctonica* was found to be 0.4%.

By taking these data into account, another option for maximizing growth as well as lipid concentration was set up by combining the autotrophic and heterotrophic conditions, where the cells first grown autotrophically with agitation until the culture reached maximum cellular density (day 16), then continued with heterotrophic growth stage using glycerol as the energy source. This experiment was also conducted to optimize the glycerol concentration for cellular growth and lipid concentration. On day 7 after heterotrophic growth stage began, supplementation with 0.4% glycerol offered the best result compared to 0.1% glycerol and 0.7% glycerol (Fig. 3). The use of 0.4% glycerol as supplement for heterotrophic growth yielded optimal result, whereas at concentration higher than 0.4% the addition of glycerol posed an inhibiting effect on growth.

V. Conclusion

For density, the autotrophic treatment yielded 10-20 times higher than the heterotrophic one did; and the agitation treatment resulted higher density than the non-agitation treatment did. However, in terms of the quality, heterotrophic treatment produced lipid concentration three times higher than autotrophic treatment, either using glucose or glycerol as a carbon source (50% compared to 17%). In a two-stage experiment, adding glycerol as a carbon source in the heterotrophic stage managed to increase both the density of cells and the lipid content. The density increased until day 5 after the heterotrophic condition and the addition of glycerol, and the lipid content ascended the lipid content three times higher compared to autotrophic treatment. 0.4% glycerol as a carbon source in the heterotrophic condition was found to be the optimum concentration for the lipid production. The lipid concentration remained stable starting from day 7 after the heterotrophic condition and tended to decrease after day 9.

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