Lipid accumulation on optimized condition through biomass production in green algae

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Abstract. Microalgae, mainly green algae, are well known as sources of lipids for food, feed, and energy. As our institution owned microalgae collections, we performed microalgae screening to find candidate/s, producing high lipid content. In this research, microalgae were screened by various media, and then the medium was optimized. Larger scale cultivation was done in order to find out the yield of the biomass production and lipid content. The condition with phosphate buffer was applied to increase the lipid accumulation. The lipid content was analyzed by Nile red staining. As a result, Chlamydomonas reinhardtii and Chlorella vulgaris showed high biomass production in the Tris-Acetate-Phosphate (TAP) medium. Chlamydomonas reinhardtii at four times concentration and C. vulgaris at the initial concentration of TAP medium demonstrated the most increased biomass production. The maximum biomass production and lipid content was 0.9 g/l and 31% for C. reinhardtii and 1.7 g/l and 27% for C. vulgaris, respectively. Under starvation, the lipid content was increased up to 52% for C. reinhardtii and 34% for C. vulgaris, which were higher than their control. From this study, the TAP medium was found to have the most increased biomass production, and starvation enhanced lipid accumulation.

1. Introduction
Microalgae or microphyte is a unicellular organism that exists individually or in a group, found in marine or freshwater environments. Microalgae have chlorophyll so that this organism can carry out photosynthesis, which plays an essential role in converting carbon dioxide into oxygen. Microalgae are divided into several groups according to their morphology, cell wall, energy storage, pigment, and molecular properties [1]. One of the major groups is Chlorophyta or green algae. Green algae contain chlorophyll a and b and produce starch as food storage in plastids. Because of their ability to make starch, green algae are known to be used for biofuel producers [2]. The starch produced by the microalgae will be converted into lipid and utilized as functional food or feed and energy substitution [3].

Lipid is a primary metabolite that simultaneously produces protein and carbohydrates. Lipid plays a huge role in cell membrane construction. In the late stationary phase, the lipid can be obtained from starch degradation [4,5]. This mechanism was established because of nutrient depletion or severe conditions. Nutrient starvation and stress are also conditions that can lead to the enhancement of lipid production [6]. However, if the stress was applied from the earlier stage, the cells will grow slower.
Even though the lipid content in the cells is increased, the microalgae biomass is decreased due to the severe condition [7]. For that reason, many researchers try to find a formula to get high microalgae biomass with high lipid content, including the application of two stages of cultivation [8,9]. This study is to find a suitable medium for microalgae to grow fast and to produce lipids in higher content by screening several medias and applying different concentration in the cultivation of microalgae.

2. Material and Methods

2.1. Strains, media, and cultivation.

The strains used in this study were *Chlamydomonas reinhardtii* from Chlamydomonas Center No. 124, *Parachlorella kessleri*, *Chlorella sorokiana*, and *Chlorella vulgaris* from NIES collection with number 2125, 2169, and 2170 respectively. The other strains that came from local collection were *Nanochloropsis sp.*, *Tetraselmis sp.*, *C. vulgaris*, and *C. volutis*. *Nanochloropsis* sp. and *Tetraselmis* sp. were collected from Jepara collection. Meanwhile, *C. vulgaris* and *C. volutis* were laboratory collections which were submitted to Indonesian Culture Collection (InaCC). The media which used for cultivation and for screening process were AF6, bold basal medium (BBM), modified Johnson, BG11, tris-acetate-phosphate (TAP), modified Bristol medium (MBM) [10], Zarrouk medium (ZM) [11], and Chu13 [12]. For 100 ml AF6 medium contained, NaNO₃ 14 mg; NH₄NO₃ 2.2 mg; MgSO₄.7H₂O 3 mg; CaCl₂.2H₂O 1 mg; ferric citrate 0.2 mg; citric acid 0.2 mg; KH₂PO₄ 1 mg; K₂HPO₄ 0.5 mg; CaCO₃ 1 mg; biotin 0.2 μg; thiamine HCl 1 μg; vitamin B₆ 0.1 μg; vitamin B₁₂ 0.1 μg; FeCl₃.6H₂O 98 μg; MnCl₂.4H₂O 18 μg; ZnCl₂ 5.2 μg; CoCl₂.6H₂O 2 μg; Na₂MoO₄.2H₂O 1.25 μg; Na₃EDTA.2H₂O 0.5 mg, pH 6.8. For 100 ml of BBM, contained, NaNO₃ 25 mg; KH₂PO₄ 17.5 mg; K₂HPO₄ 10 mg; MgSO₄.7H₂O 7.5 mg; CaCl₂.2H₂O 2.5 mg; NaCl 2.5 mg; KOH 3.1 mg; FeSO₄.7H₂O 0.498 mg; H₂BO₃ 0.142 mg; ZnSO₄.7H₂O 0.882 mg; MnCl₂.4H₂O 0.144 mg; Na₂MoO₄.2H₂O 0.12 mg; CuSO₄.5H₂O 0.157 mg; CoCl₂.6H₂O 0.04 mg; Na₃EDTA.2H₂O 5 mg. For 100 ml of Chu13, contained, KNO₃ 40 mg; K₂HPO₄ 8 mg; MgSO₄.7H₂O 20 mg; CaCl₂.2H₂O 10.7 mg; ferric citrate 2 mg; citric acid 10 mg; CoCl₂.6H₂O 2 μg; H₂BO₃ 0.572 mg; ZnSO₄.7H₂O 0.362 mg; MnCl₂.4H₂O 0.044 mg; CaSO₄.5H₂O 0.016 mg; Na₂MoO₄.2H₂O 8.4 μg; H₂SO₄ 1 drop. For 100 ml of modified Johnson, contained, KNO₃ 100 mg; KH₂PO₄ 3.5 mg; MgCl₂.6H₂O 150 mg; MgSO₄.7H₂O 50 mg; CaCl₂.2H₂O 26.5 mg; KCl 20 mg; NaHCO₃ 4.3 mg; NaCl 3 g; pH 8.5. For 100 ml of BG11, contained, NaNO₃ 150 mg; K₂HPO₄ 4 mg; MgSO₄.7H₂O 7.5 mg; CaCl₂.2H₂O 3.6 mg; ferric citrate 0.6 mg; citric acid 0.6 mg; Na₃EDTA.2H₂O 0.1 mg; Na₂CO₃ 2 mg; H₂BO₃ 0.286 mg; ZnSO₄.7H₂O 22.2 μg; MnCl₂.4H₂O 0.181 mg; Na₂MoO₄.2H₂O 39 μg; CuSO₄.5H₂O 7.9 μg; CoCl₂.6H₂O 4 μg; pH 7.4. For 100 ml of TAP medium, contained, NH₄Cl 40 mg; K₂HPO₄ 11.9 mg; KH₂PO₄ 6.03 mg; MgSO₄.7H₂O 10 mg; CaCl₂.2H₂O 5.1 mg; FeSO₄.7H₂O 0.499 mg; H₂BO₃ 1.14 mg; ZnSO₄.7H₂O 2.2 mg; MnCl₂.4H₂O 0.506 mg; Na₂MoO₄.2H₂O 0.11 mg; CuSO₄.5H₂O 0.157 mg; CoCl₂.6H₂O 0.161 mg; Na₃EDTA.2H₂O 5 mg; pH 6.6-6.8. For 100 ml of ZM, contained, NaNO₃ 250 mg; K₂HPO₄ 50 mg; MgSO₄.7H₂O 20 mg; CaCl₂.2H₂O 4 mg; NaHCO₃ 1.68 g; K₂SO₄ 100 mg; FeSO₄.7H₂O 1 mg; NaCl 100 mg; EDTA 8 mg; H₂BO₃ 2.86 mg; ZnSO₄.7H₂O 0.222 mg; MnCl₂.4H₂O 1.81 mg; Na₂MoO₄.2H₂O 0.39 mg; CuSO₄.5H₂O 0.079 mg. For 100 ml of MBM, contained, KNO₃ 25 mg; KH₂PO₄ 17.5 mg; K₂HPO₄ 7.5 mg; MgSO₄.7H₂O 7.5 mg; NaCl 2.5 mg; CaCl₂.2H₂O 1 mg; FeSO₄.7H₂O 0.2 mg; H₂SO₄ 1 drop; citric acid 10 mg; CoCl₂.6H₂O 2 μg; H₂BO₃ 0.572 mg; ZnSO₄.7H₂O 0.362 mg; MnCl₂.4H₂O 0.044 mg; CuSO₄.5H₂O 0.016 mg; Na₂MoO₄.2H₂O 8.4 μg; H₂SO₄ 0.026 μl; H₂BO₃ 2.86 mg; ZnSO₄.7H₂O 0.222 mg; MnCl₂.4H₂O 1.81 mg; Na₂MoO₄.2H₂O 0.39 mg; CuSO₄.5H₂O 0.079 mg; pH 6.0.

2.2. Screening for microalgae candidates and a suitable medium.

Eight strains were cultivated in TAP or AF6 medium before the screening process. The stock culture in liquid medium was used with optical density at wavelength 750 nm (OD₇₅₀) more than 1 and it was preserved for not more than one month. The strains were cultivated in pre-culture medium with the same medium for 2–3 days with initial OD₇₅₀ = 0.1–0.5. The strains culture was collected by
centrifugation at low speed and resuspended with the same media before inoculated into various media, which were AF6, BBM, Chu13, modified Johnson, BG11, TAP, ZM, and MBM, with initial OD\textsubscript{750} = 0.1. The strains were inoculated into a 24-well plate with 1.5 ml medium and the OD\textsubscript{750} were observed for 14 days. Tungsten lamp 2 × 36 watt were used for the light source.

2.3. Optimization of selected medium.
The medium which has the highest biomass and shortest exponential phase was used to be optimized by dilution or concentration. Each composition in the media was modified by 0.5x; 1x; 2x; and 4x from the initial concentration of medium composition. The cells were cultivated in a pre-culture medium for 2-3 days with initial OD\textsubscript{750} = 0.5. The cell culture was collected by centrifugation at low speed and the resuspended with the same medium. The cell culture was inoculated into the various concentration of the medium with initial OD\textsubscript{750} = 0.1. The cells were inoculated into a 24-well plate with 1.5 ml media volume with 2 × 36 watt tungsten lamp and the OD\textsubscript{750} was observed for less than 10 days.

2.4. Biomass production and lipid accumulation.
The strains were cultivated in 1000 ml pre-culture medium for 2–3 days, with initial OD\textsubscript{750} = 0.1–0.5. The cell culture was inoculated with initial OD\textsubscript{750} = 0.1 in 1000 ml TAP medium with aeration using ambient air. For the light source, tungsten lamp 2 × 36 watt were used. The microalgae were cultured for 14 days and in certain days, about 20 ml culture medium were taken as samples and to be analysed for the weight of the biomass and the lipid content by gravimetry [13].

2.5. Starvation condition.
Under starvation condition treatment, the cell culture was cultivated in a pre-culture medium for 2–3 days with initial OD\textsubscript{750} = 0.1–0.5. The cell culture was inoculated with initial OD\textsubscript{750} = 0.1. The microalgae were cultured for 4 days and then centrifugated at 3000 rpm at 10°C for 10 minutes. After that, the cell pellet was resuspended in a tube with 5 ml phosphate buffer containing 0.6 g/l KH\textsubscript{2}PO\textsubscript{4} and 1.4 g/l K\textsubscript{3}HPO\textsubscript{4} as treated condition under nutrient starvation. The cell culture was resuspended back by its supernatant for control. After 3 days, the cell culture was harvested and the cell biomass were taken as sample that would be analysed by Nile red staining for the lipid content.

2.6. Nile red staining.
The staining method that used in these experiments were referred to as Storms et al., 2014 methods with some modifications [14]. About 1 ml cell culture was centrifuged at 3000 rpm at 10°C for 10 minutes. The cell pellet was rinsed by a phosphate buffer and then was resuspended using a 50 µl phosphate buffer. About 10 µl of the concentrated sample reacted with 80 µl of 30% ethanol in water and 10 µl of 10 µg/ml Nile red in ethanol (pro analysis). The sample was put in a 96-well plate and was shaken in an orbital way for 30 seconds at 1200 rpm and then incubated at 40°C for 10 minutes. After the incubation, the 96-well plate was shaken again and the fluorescence intensity was observed at wavelength 535 nm for the extinction and 655 nm for the emission using microplate reader (Spectramax Paradigm). The OD\textsubscript{750} of each sample was measured for loading control. The relative fluorescence intensity is the percentage of the difference between fluorescence intensity of Nile red + sample and sample only and then were divided by OD\textsubscript{750} of each sample and fluorescence intensity of Nile red only.

3. Results and Discussion

3.1. TAP was selected as suitable medium.
The growth of microalgae strains was observed for 14 days. In figure 1, the cell culture was grown in the 24-well plate, which became greener on the 4\textsuperscript{th} day compared to the day of inoculation. From
media screening, the result showed that TAP was a suitable medium for most of the strains, and *C. reinhardtii* was found as the strain with the highest yield (figure 2). Meanwhile, *C. vulgaris* was found from the local collection as the strain with the highest yield (figure 2). From this result, TAP was found the most suitable for culture medium.

**Figure 1.** Growth culture observation of *C. reinhardtii* and *C. vulgaris* at the initial and 4th day of incubation time. The microalgae cells were inoculated at OD<sub>750</sub> = 0.1 at the initial day (D<sub>0</sub>) with 1.5 ml of media volume.

**Figure 2.** Growth curve of *C. reinhardtii* and *C. vulgaris* cultures for 14 days of observation at OD<sub>750</sub> in various microalgae media.

3.2. Higher concentration of TAP composition may lead to the enhancement of biomass production. For eight days, the growth of *C. reinhardtii* and *C. vulgaris* were observed in various concentrations of TAP medium. The result showed that *C. reinhardtii* has grown with high biomass production at four times the concentration of the initial TAP medium. The result might not be significantly different with two times the concentration of the initial TAP medium. Meanwhile, *C. vulgaris* showed higher biomass production at two times the concentration of the initial TAP medium, but it was not significantly different from the initial or with the other concentrations (figure 3). This result showed that the higher concentration of TAP medium increased the biomass yield of *C. reinhardtii*, but not *C. vulgaris*.
Figure 3. Growth curve of *C. reinhardtii* and *C. vulgaris* cultures in TAP medium at concentration 0.5×, 1×, 2×, and 4× of the initial concentration. The microalgae culture was observed for 8 days with initial OD750 = 0.1.

3.3. Biomass production and total lipid accumulation.

The samples were taken in several days of 14 days of cultivation period for measuring the weight of the biomass and the total lipid content of the biomass by gravimetry. *Chlamydomonas reinhardtii* produced about 0.9 g/l biomass with 20–31% lipid content, and *C. vulgaris* produced about 1.7 g/l biomass with 14–27% lipid content (Figure 4). The maximum specific growth and lipid accumulation rates for *C. reinhardtii* were 41.3 mg/l/d and 7.4 mg/l/d, respectively, while in *C. vulgaris* were 106.6 mg/l/d and 17.7 mg/l/d, respectively. From these results, both strains produced lipid content for more than 20% of cell biomass.

Figure 4. Yield of biomass production and lipid accumulation in *C. reinhardtii* (left) and *C. vulgaris* (right) with TAP medium.

3.4. Starvation induced higher lipid content in microalgae cells.

In these experiments, after four days of cultivation, cell culture was collected and centrifuged. For control, the cell pellet was resuspended back in the same medium. Meanwhile, under starvation, the cell pellet was resuspended with phosphate buffer. After three days of incubation, the lipid content from microalgae cells was measured. The result for *C. reinhardtii*, showed that, under phosphate buffer, the lipid content in cells was increased up to 52±25% higher than control, while, in *C. vulgaris*, under phosphate buffer condition, lipid content was increased up to 34±20% higher than control (figure 5). These experiments showed that starvation increased the lipid content in microalgae cells.
This study demonstrated that the TAP medium was suitable for *C. reinhardtii* and *C. vulgaris* cultivation (figure 2). Commonly, TAP is used for culturing *C. reinhardtii*. However, in 1000 ml TAP medium for cultivation with aeration using ambient air, *C. vulgaris* showed higher biomass production than *C. reinhardtii* (figure 4). The same results were demonstrated by Blinová et al [15] and Metsoviti et al [16], who worked with both strains involving aeration for their cultivation. The results showed that *C. vulgaris* was more effective for capturing CO\(_2\) than *C. reinhardtii*, which has been known that in the ambient air, there is CO\(_2\) with a concentration of about 0.03\% [17]. Meanwhile, the biomass that *C. vulgaris* produced in this work, which was about 1.7 g/l, was slightly higher compared to several strains of *C. vulgaris*, which had 0.53–1.55 g/l biomass with BG11 as a medium for cultivation [18].

4. Conclusion
This study revealed that the TAP medium is suitable for the growth of *C. reinhardtii* and *C. vulgaris*. Four times the initial concentration of TAP medium produced the highest biomass for *C. reinhardtii*. Meanwhile, for *C. vulgaris*, the initial concentration was enough to produce the highest biomass yield. Furthermore, nutrient depletion showed the enhancement of lipid production in both microalgae. The nutrient depletion increased the lipid content about 52±25\% and 34±20\% higher than control in *C. reinhardtii* and *C. vulgaris*, respectively.

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