Enhancement of lipid production of *Chlorella* sp. 042 by mutagenesis.

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**Abstract.** In this study, the UV mutagenesis was performed to enhance lipid productivity in *Chlorella* sp. 042 isolated from East Kalimantan. One hundred colonies were screened with a method based on Nile Red fluorescence. Growth, biomass, and lipid parameters of the selected mutants (M7 and M22) were grown in a batch system of 400 mL AF6 medium and analyzed in detail. The result showed that the lipid content of M7 and M22 were higher than wild type (30.82%), 35.15%, and 43.85%, respectively. The M22 slightly had higher carbohydrate and protein content than wild type and M7. The overall result indicates that the treatment of UV mutagenesis to *Chlorella* sp. 042 can increase the total lipid production and other biomass content. The mutant could consecutively be used as the lipid feedstock for algal oil production.

**1. Introduction**

Microalgae considered as the alternative feedstock for the future biofuel production [1], as fossil fuel harm the environment (greenhouse gas and global warming) leads the rising interest to establish new fuel feedstock [2]. Microalgae cultivation does not require freshwater and arable land, and the implementation of microalgae biofuel may reduce the need for crop plants for biodiesel production [3]. Furthermore, lipid production from microalgae is higher than the oilseed crops (rape oilseed), put the microalgae a valuable source of biodiesel [4].

Although several studies have suggested that biodiesel production from microalgae is promising, the algal lipid large scale production process is not close to being commercially viable. A suitable microalgae candidate for biodiesel production has to contain high oil content, a high growth rate, and a wide range of environmental tolerances. However, up to now, no known microalgae species can accomplish all requirements [3,5,6].

Several strategies to increase the lipid production in microalgae have been proven, such as optimization of growth medium formulation, optimization of environmental conditions, and improvement of bioreactor design [7,8]. An alternatives strategy to enhance the microalgae metabolite is mutagenesis. The application of the mutagenesis method to increase several metabolite productions is advantageous, as it does not require extensive knowledge of the genetics and biochemistry of the organism under consideration [9]. In the previous study, UV mutagenesis studied have been performed on microalgae to increase their lipid content or modified their fatty acid composition. Mutagenesis of *Nannochloropsis oculata* resulted in a mutant with a 19% increase in biomass and 28% in lipid productivity [10].

In this study, we conduct the UV mutagenesis to enhance lipid content of local strain microalgae isolated from East Kalimantan. We characterize the most promising mutants with respect of growth and biomass composition.
2. Material and methods

2.1. Algal strains, culture conditions and growth rates

The microalgae strain used in this study is *Chlorella* sp. 042 isolated from Wain River, East Kalimantan. The inoculum was cultivated at AF6 medium, which consists of the following per liter of water [11]: 140 mg NaNO₃, 22 mg NH₄NO₃, 30 mg MgSO₄.7H₂O, 10 mg KH₂PO₄, 5 mg K₂HPO₄, 10 mg CaCl₂.2H₂O, 10 mg CaCO₃, 2 mg Fe-Citrate, 2 mg Citric acid. The culture medium pH was adjusted into 6.2 prior autoclaves at 121°C for 15 min. The culture was maintained at room temperature with constant stirring at 150 rpm and continuous light (800 Lux). The growth curves of the algae in the liquid medium were established from the daily measurement of optical density at 750 nm using spectrophotometer UV-Vis.

2.2. UV mutagenesis

5 mL cultures of *Chlorella* sp. 042 in the exponential growth phase were placed in the open petri dish and exposed to UV radiation (Germicidal lamp 30W, Philips) at a distance of 25 cm for 30 min. The mutagenized mutants were kept in the darkroom for 24 h, to avoid cell recovery mechanism by light induction (Modification of Vigeolas et al., 2012 [12]). Mutagenized cell was grown in AF6 agar medium and incubated under constant light for 2-3 weeks until single colonies develop.

2.3. Screening of mutant

Single colonies developing on agar plates after UV irradiation were selected and transferred to sterile 96-well plates containing 200 µL of AF6 medium. Plates were incubated under constant light with agitation at 150 rpm for a week. Cell densities of each culture were measured using Varioscan™ LUX multimode microplate reader (Thermo Fisher Scientific). The amount of neutral lipid was determined using modified Chen et al. [13] methods by following Nile Red fluorescence in cell suspensions diluted to the concentration of approximately 0.1 at OD750 nm.

2.4. Biomass analysis

The biomass analysis was performed on strains grown on 400 mL AF6 medium and collected at the exponential growth phase. Biomass of *Chlorella* sp. 042 from 10 mL culture were harvested using centrifugation at 8000 rpm for 10 min, and the remained pellets were dried at 60°C for at least 24 h.

2.5. Total lipid

Lipid extraction was prepared using a modification of Ryckebosch et al. [14]. The mixture of chloroform and methanol (1:1, v/v) was added to dried microalgae biomass and homogenized for 30 s. Water was added to the mixture until the final concentration reached 2:2:1 (chloroform:methanol:water, v/v/v). The final mixtures were then centrifuged at 8000 rpm for 10 min. Lipid layer was collected and evaporated at room temperature. Lipid weight was determined by weighing the dried lipid after solvent evaporation.

2.6. Carbohydrate and protein content

The carbohydrate content was measured by the phenol sulphuric acid method with glucose as standards adapted from Dubois et al [15]. The protein content was determined using dye-binding assay [16] with bovine serum albumin as the standard and the absorption was measured at 595 nm.

3. Result and discussion

3.1. Screening of mutants

In this study, the cells of *Chlorella* sp. 042 were UV mutagenized, and the colonies developing from the mutagenesis were grown in 96-well plates. The cell suspension was estimated by measuring optical density at 750 nm. After a week of culturing, the wells showing a green color were selected,
and neutral lipid level was estimated by determination of Nile Red fluorescence [13]. One hundred colonies of Chlorella sp. 042 were analyzed and submitted to the Nile Red fluorescence method. Twenty-four colonies of the mutant then further checked by Nile Red fluorescence method and resulting in the relative fluorescence unit (RFU) ranging from 0.5 – 4 (Fig. 1). Based on RFU value, two mutants with the highest RFU value, M7 and M22 were selected for further analysis.

Figure 1. Relative amounts of lipids in Chlorella sp. 042 wild type and mutants

Figure 2. Growth curve of Chlorella sp. 042 wild type and mutants
Although the high Nile Red fluorescence intensity value of mutagenetic cultures indicates the high lipid content in the cell, it is essential to notice that the intensity of Nile Red Fluorescence only informs the relative amount of lipids. Therefore the total lipid content in mutant strains need further analysis [17].

3.2. Growth rates and microscopic overview
The Chlorella sp. 042 wild type and two selected mutants (M7 and M22) were grown in 400 mL working volume of AF6 medium. To determine the growth rate, the number of cells was measured spectrophotometrically from samples collected daily from the batch culture. Fig. 2 showed the growth curve of Chlorella sp. 042 wild type and mutants. The mutant M22 has the highest growth rate of 0.257 day\(^{-1}\), followed by M7 of 0.223 day\(^{-1}\) and wild type of 0.196 day\(^{-1}\). The increasing growth rates in mutant strains may attribute to the fact that the mutation enhanced the metabolic pathway, thereby improving the cell growth [18].

The microscopic overview Chlorella sp. 042 wild type and mutants were conducted to evaluate whether any the physical changes in cell properties after mutagenesis. The cells of wild type and mutants were observed under light microscope (Olympus BX53) (Fig. 3). While the cells of wild type and M7 displayed the same morphology and size. The cells of M22 were slightly bigger than those wild type and M7.

Figure 3. Imaging of different strains of Chlorella sp. 042 wild type (A), M7 (B), and M22 (C). Scale bar: 5 µm
3.3. Lipid contents
At the end of cultivation, the cultures were collected and dried for lipid content analysis. Lipid content was reported as the percentage of dried lipid and dried biomass (% w/w). Lipid content is one of the most critical parameters to assess the lipid accumulation in microalgae. Lipid content and lipid productivity are shown in Table 1. Wild type *Chlorella* sp. 042 contained 30% lipid on a dry weight basis, and mutant M7 and M22 contained approximately 14% and 42% more lipid than the wild type, respectively.

Lipid productivity is also other essential factors for the cost-effective production of biodiesel, its closely related to biomass productivity and lipid content. Lipid productivity could be expressed as gravimetric lipid productivity (mg g⁻¹ day⁻¹) and volumetric lipid productivity (mg L⁻¹ day⁻¹) [19]. The mutants, M7 and M22, have higher lipid productivity compare to the wild type (9.34 ± 0.97 mg L⁻¹ day⁻¹), 11.27 ± 2.02 mg L⁻¹ day⁻¹ and 11.20 ± 0.01 mg L⁻¹ day⁻¹, respectively.

Table 1. Lipid content, biomass productivity and lipid productivity of *Chlorella* sp. 042 wild type and mutants

| Strains | Lipid Content (%) | Biomass Productivity (mg L⁻¹ day⁻¹) | Lipid productivity (mg L⁻¹ day⁻¹) |
|---------|-------------------|-----------------------------------|----------------------------------|
| WT      | 30.82 ± 1.44      | 29.95 ± 1.77                      | 9.34 ± 0.97                      |
| M7      | 35.15 ± 3.75      | 30.80 ± 3.96                      | 11.27 ± 2.02                     |
| M22     | 43.85 ± 3.39      | 25.62 ± 1.98                      | 11.20 ± 0.01                     |

3.4. Carbohydrate and protein content
Microalgae biomass is primarily composed of carbohydrates, proteins, and lipids, with the portion, depends on the strain and culture conditions [20]. The composition of the biomass component was estimated in all of the wild type and mutants of *Chlorella* sp. 042. The results showed in fig. 4. The carbohydrate content of M7 was lower than the wild type, while the M22 have the highest carbohydrate content, about 25.93%. As well as the protein content of M22 is higher than the wild type, approximately 5% and the M7 was lower than the wild type. This result was suggesting that the mutants are affected in the carbon portioning in biomass components by the UV mutagenesis. The same result was found in *Chlorella sorokiniana* and *Scenedesmus obliquus* that mutagenized by UV irradiation [12]. They found that not all of the mutants have significant difference carbohydrate and protein content to the wild type. This may occur as we only determined the main component of the biomass and exclude the other component which may be affected by mutagenesis.

![Carbohydrate and Protein Content](image)

Figure 4. Carbohydrate (left) and protein (right) content of *Chlorella* sp. wild type and mutants
4. Conclusion
In the current study, we exposed Chlorella sp. 042 to UV irradiation for 30 min. Batch cultivation was conducted to determine the ability of selected mutants to produce higher lipid content than wild type. The mutant M22 showed the highest cell growth and lipid content as compared to the wild type, 0.257 day\(^{-1}\), and 43.85\%, respectively. Overall the lipid productivity of the mutant was increased into 11 mg L\(^{-1}\) day\(^{-1}\). The carbohydrate and protein content of mutant M22 also increased compared to wild type, while in mutant M1 those parameters were decreased.

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6. References
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