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Research Article

UV Mutagenesis as a Strategy to Enhance Growth and Lipid Productivity of *Chlorella* sp. 042

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ABSTRACT
Microalgae appeared to be an alternative feedstock for renewable biodiesel production due to their capability to accumulate considerable amounts of lipids. In this study, mutagenesis using UVC light with different periods was applied to *Chlorella* sp. 042 to produce a microalgae strain with high lipid productivity of 45, 60, and 75 min. The Nile red fluorescence method was conducted to select a *Chlorella* sp. mutant with high neutral lipid and generated one mutant from every UV mutation period, M45-06, M60-02, and M75-21. All of the mutants have higher growth rates than the wild type. *Chlorella* sp. 042 M60-02 achieved the highest lipid productivity, with 34 mg L^{-1} day^{-1}. Furthermore, as other major biochemical components, carbohydrate and protein contents were determined. Our results showed that all the mutants enhance their carbohydrate and protein contents compared to the wild type. However, mutations for more than 60 min do not intensely change the protein content of mutant microalgae. Gas chromatography-mass spectrophotometry analysis revealed that M60-02 mutant has similar FAME profiles with the wild type, which contain palmitic acid (C16:0), stearic acid (C 18:0), oleic acid (C18:1), and linoleic acid (C18:2). These results demonstrate that the UV mutation of *Chlorella* sp. 042 for 60 min is suitable as a source of biodiesel production.

Keywords: Biodiesel, *Chlorella* sp., Fatty acids, Lipid productivity, UV mutagenesis

INTRODUCTION
The limited availability of fossil energy sources and the negative impact of fossil fuels on the environment forced researchers to explore alternative and renewable energy sources capable of producing low carbon dioxide emissions (Tan *et al.*, 2017). Biodiesel is a mono-alkyl ester with a long-chain fatty acid, which is a derivative of animal fats or wastes cooking oil or vegetable oils (Yusuf & Yaakub, 2010; Balat, 2011; Abbaszaadeh *et al.*, 2012; Faried *et al.*, 2017). The eminences of biodiesel compared to fossil fuels are its renewability, toxic-free, sulfur-free, and have better lubricity (Aransiola *et al.*, 2014; Goh *et al.*, 2019). However, these materials, especially those originating from vegetable oil, cause new problems due to their implications on food security and commodity prices (Goh *et al.*, 2019). Besides, biodiesel production using crops as raw material requires extensive agricultural land and one of the causes of deforestation (Rawat *et al.*, 2013).

Microalgae oil produced can be used as an alternative feedstock for biodiesel in terms of social and economic aspects. Microalgae are capable of accumulating oil with a shorter harvesting time and less volume of water demand and can be done in open land (Chen *et al.*, 2018; Rawat *et al.*, 2013). Oil productivity from algae is twenty times higher than oilseed plants based on a one-hectare area. Therefore, a more viable biodiesel feedstock (Chisti, 2007; Ahmad *et al.*, 2011; Antoni *et al.*, 2007; Feng *et al.*, 2011; Rawat *et al.*, 2013). Some microalgae with high lipid productivity have been identified as candidates for biodiesel production such as *Chlamydomonas* sp., *Scenedesmus* sp., *Nannochloropsis oculata*, *Dunaliella salina*, *Botryococcus braunii*, and *Chlorella* sp. (Chisti, 2007; Hosseini Tafreshi & Shariati, 2009; Van Vooren *et al.*, 2012; Yoo *et al.*, 2010; Sarayloo *et al.*, 2017). Nevertheless, biodiesel

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production from large-scale microalgae is still not economically feasible (Sarayloo *et al.*, 2017). Therefore, it is important to find candidates for microalgae that have high lipid content with high biomass production (Zhang *et al.*, 2010).

To obtain microalgae strain with high lipid content and biomass production, various strategies can be applied. Some of them include performing screening and species characterization of lipid producing microalgae (Vigeolas *et al.*, 2012; Nascimento *et al.*, 2013) followed by optimization of culture condition and cultivation techniques, and via metabolic engineering (Trentacoste *et al.*, 2013) followed by the screening of lipid producing microorganism using lipophilic dye (Vigeolas *et al.*, 2012). Screening and species characterization to derive robust strain require intensive effort, and many times, they fail to provide strain with high biomass and high lipid production. Meanwhile, metabolic engineering requires the elucidation of lipid metabolism processes and other related metabolism pathways as well as the knowledge about carbon flux in cellular processes (Trentacoste *et al.*, 2013). Targeted genetic engineering is limited due to the lack of the genomic sequence and the detailed study about lipid-producing-microalgae that can be manipulated (Vigeolas *et al.*, 2012).

In addition to those approaches, the effort to increase lipid production is also done via mutagenesis, either physically or chemically. This approach is simple and does not require genomic information as required in genetic engineering. Mutagenesis performed physically using UV irradiation is preferred to chemical mutagenesis. The former is considered more benign either for the operator or the environment, faster, and more effective (Fang *et al.*, 2013; Sivaramakrishnan & Incharoensakdi, 2017). UV mutagenesis has been applied to microalgae and other oleaginous microorganisms such as yeast to provide strain with improved lipid production (Tapia *et al.*, 2012; Sharma *et al.*, 2014; Liu *et al.*, 2015; Sivaramakrishnan & Incharoensakdi, 2017).

In this study, *Chlorella vulgaris*, either the mutagenized or wild type, is investigated for its lipid production as well as lipid productivity. This strain is acknowledged for its ability to grow in various conditions and its resistance to invaders (Pauline *et al.*, 2006; Liang *et al.*, 2009; Sarayloo *et al.*, 2018). It also contains lipids mainly as triacylglycerol. Major fatty acids produced by *C. vulgaris* are saturated and monounsaturated fatty acids like palmitic acid, palmitoleic acid, stearic acid, and oleic acid, which are compatible to be employed as biodiesel feedstock (Yeh & Chang, 2011; Sarkar & Shimizu, 2015; Sarayloo *et al.*, 2018). Besides, this species produces valuable metabolites such as protein, β-carotene, astaxanthin, as well as several types of polyunsaturated fatty acids (PUFAs) (Chacon-Lee & Gonzalez-Marino, 2010; Singh & Cu, 2010; Sarayloo *et al.*, 2018).

Previous studies showed that applying mutagenesis to *Chlorella* sp. for 30 min increased its growth rate and lipid productivity to be 0.257 day^{-1} and 11 mg L^{-1} day^{-1} concerning wild type (growth rate was 0.196 day^{-1} and lipid productivity was 9 mg L^{-1} day^{-1}) (Rahman *et al.*, 2020). In this study, we conducted UV mutagenesis to *Chlorella* sp. isolated from East Kalimantan River for 45, 60, and 75 min. We observed and determined the optimum period for UV mutagenesis concerning growth and lipid productivity. We also characterized fatty acid profiles of the mutants.

MATERIALS AND METHODS

Microalgae strain and growth condition

Wild type *Chlorella* sp. 042 was isolated from the Wain River, East Kalimantan, Indonesia. The cells were inoculated into 500 mL photobioreactors containing 400 mL AF6 medium (140 mg $NaNO_3$, 22 mg NH_4NO_3, 30 mg $MgSO_4.7H_2O$, 10 mg KH_2PO_4, 5 mg K_2HPO_5, 10 mg $CaCl_2.2H_2O$, 2 mg Fe-Citrate, 2 mg Citric acid, and 1,000 mL distilled water). The culture condition was maintained at 25 °C under continuous light (800 Lux) with continuous aeration. The growth curves of the microalgae were made by daily measurement of optical density at 750 nm using a UV-Vis spectrophotometer (Shimadzu PharmaSpec UV-1700).

UV mutagenesis

The mutagenized cell was conducted by 5 mL of *Chlorella* sp. 042 in the exponential growth phase. It was placed in the open petri dishes and exposed to the UV irradiation (Germicidal lamp, UVC 30 W, Philips) at a distance of 25 cm for 45, 60, and 75 min. The mutagenized mutants were kept in the darkroom for 24 h, to avoid light induction of cell recovery. The mutagenized cells were grown in AF6 agar medium and incubated under continuous light for 2-3 weeks in advance of single colonies appeared.

Screening of mutants

The single developed colonies on the agar plate were selected and transferred into a sterile 96-wells plate containing 200 µL of AF6 medium. Plates were incubated under constant light with agitation at 150 rpm for 7 days. 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.* (2009) methods by following Nile Red fluorescence in cell suspensions diluted to the concentration of approximately 0.1 at 750 nm.

Determination of cell dry weight and lipid content

The cell dry weight of *Chlorella* sp. 042 was determined gravimetrically. Aliquot of 10 mL culture was transferred to a 15 mL falcon tube of a known mass and centrifuged at 8000 rpm for 10 min. The supernatant was discharged, and the remaining cell pellets were dried at 60 ℃ for 24 h. The dry weight of cell pellets was determined gravimetrically (Rahman *et al.*, 2020).

Total lipid content was determined by performing lipid extraction prior to gravimetric analysis. Lipid was extracted according to Ryckebosch *et al.* (2012) with modification. A solution of chloroform and methanol (1:1, v/v) was added to the lyophilized cell pellet and mixed. Water was added to the homogenized mixture until the final concentration of chloroform-methanol-water of 2:2:1 (v/v) was reached. The upper layer (lipid) was separated by centrifugation (8000 rpm, 10 min) and evaporated at room temperature. The dried lipid layer was then determined gravimetrically (Ryckebosch *et al.*, 2012).

FAME analysis

Transesterification of microalgae lipids was performed before analysis using GC-MS. Derivatization of lipid into its methyl ester was done according to Lewis *et al.* (2000), with modification. Reagent, consisting of methanol: chloroform: chloric acid (10:1:1), was added to a lyophilized microalga cell, into duplicate, screw-capped reaction tubes. The tubes were mixed using vortex and heated at 90 ℃ for 2 h in a water bath. After the reaction was completed, the reaction mixtures were set to cool until it reached 25 ± 2 ℃ and to which the water was added and mixed. To each reaction mixture, hexane was added, mixed, and was left alone until it reached phase separation. The hexane addition step was repeated twice, and the upper layer (organic phase) was being collected and subjected to GC-MS analysis. This step was done for both mutant and wild type lipids.

FAME analysis using GC-MS (Shimadzu QP 2010 Ultra, DB-23 column) was performed qualitatively. The oven temperature was 50 ℃ for 1 min and increased at 25 ℃ min^{-1} up to 180 ℃ for 4 min. An increment of 5 ℃ min^{-1} followed the step until 235 ℃ for 5 min. The temperature of the ion source was set to 230 ℃, with an interface temperature of 250 ℃ and the solvent cut time of 2 min. The peaks derived from GC-MS analysis were assigned according to the reference of FAME mix C14-C22 components.

Determination of Carbohydrate and Protein

Carbohydrate determination was held using the phenol-sulfuric acid method, according to Dubois *et al.* (1956), with modification. Wet biomass was incubated with 2.25 % sulfuric acid at 90 ℃ for 70 min. The sugar solution (0.5 mL) was pipetted to the reaction tube. 5 % phenol and 96 % sulfuric acid were mixed with the sugar solution. The absorbance of the mixed solution was measured using a UV-Vis spectrophotometer at 490 nm.

The determination of protein content was done using the dye-binding assay (Bradford, 1976). Phosphate buffer (pH 7) was added to microalgae wet biomass. The cell was then disrupted using sonication for 15 s and repeated until 10 cycles. Bradford reagent was added to 0.3 mL of supernatant. The solution was determined using the UV-Vis spectrophotometer at 595 nm.

Statistical Analysis

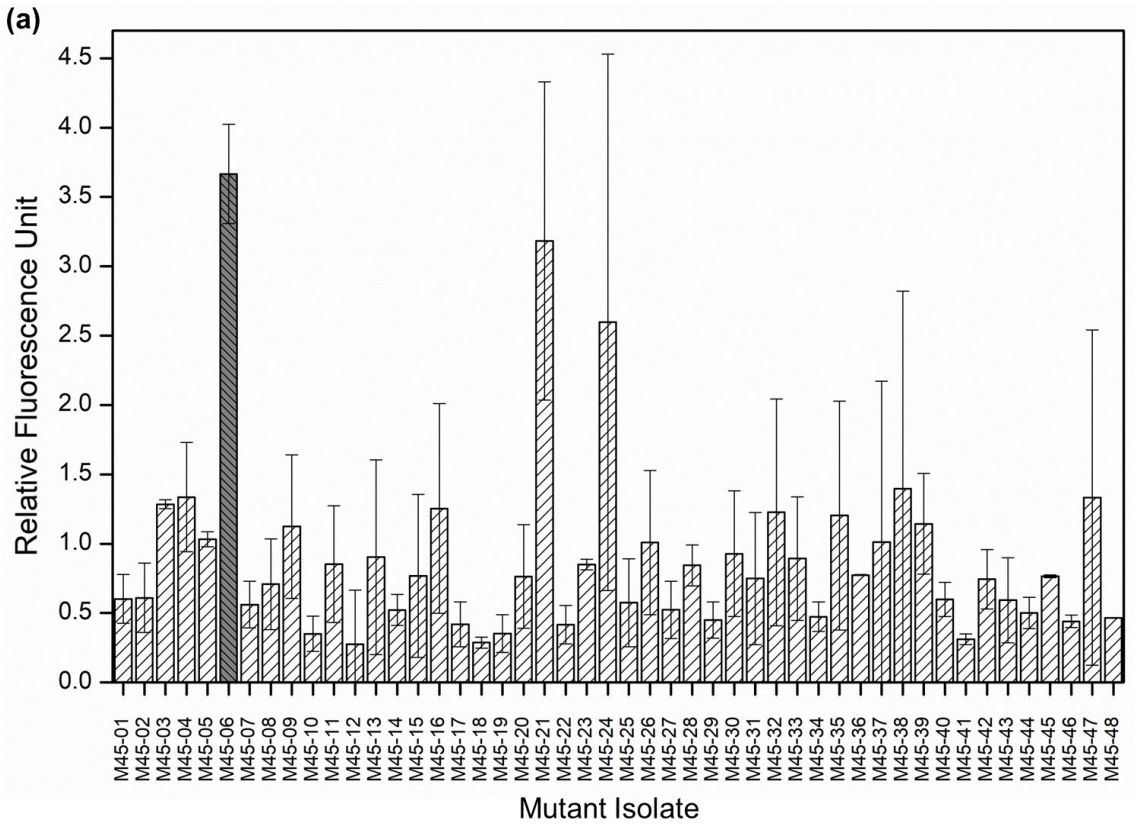
To determine the significant difference among groups ($P< 0,05$), all average values of mutants were analyzed against the control employing one way ANOVA and t-test analysis by using Microsoft Excel Software.

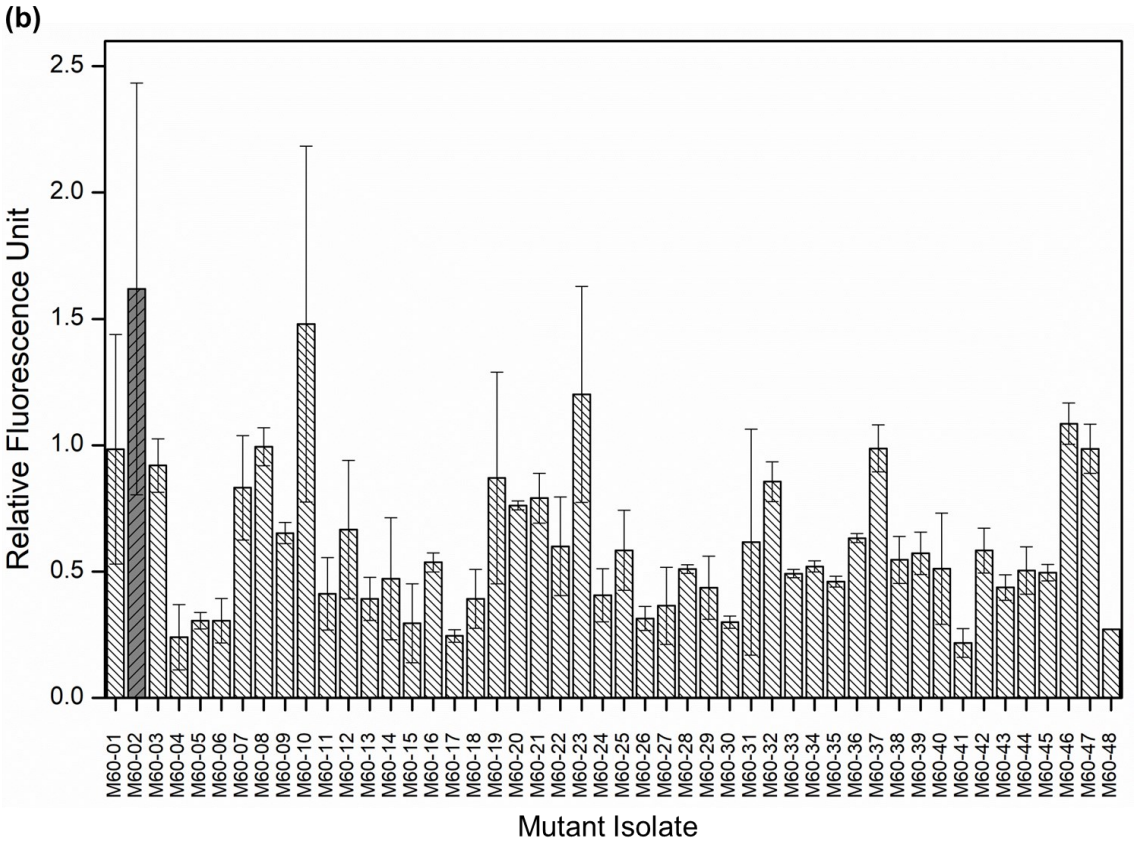
RESULTS AND DISCUSSION

Screening of mutants

Mutagenized green microalgae, *Chlorella vulgaris*, were screened for its lipid content using the Nile red method. Nile red dye has been applied to detect neutral lipid in various oleaginous microorganisms including yeasts (Sitepu *et al.*, 2014; Rostron *et al.*, 2015), bacteria (Alves *et al.*, 2017), and microalgae (Chen *et al.*, 2009; Huang *et al.*, 2009; Satpati & Pal, 2014). This hydrophobic benzophenoxazone dye emits fluorescence as it contacts lipid bodies and organic solvents (Halim & Webley, 2015). Such a characteristic makes it useful to be applied in lipid staining to detect lipid content. In this study, DMSO was used as a carrier for the Nile red dye. The use of DMSO as well as other cell pre-treatments such as cell grounding in liquid nitrogen and the use of methanol, acetone, and ethanol as a solvent were proved to increase fluorescent intensity compared to those without pre-treatment (Chen *et al.*, 2009). The pre-treatments applied to make it possible for the Nile red method to be applied to stain and detect lipid content in green algae are known to have thick and rigid cell walls (Chen *et al.*, 2009). The use of this

Nile Red method also enables high throughput screening, which is difficult to achieve when applied by the conventional gravimetric method. According to fluorescence intensity measured for 48 colonies, generated from 45, 60, and 75 min UV radiation (Figure 1), three colonies showed prominent results, indicating high lipid content. The three colonies are M45-06, M60-02, and M75-21, which originated from 45, 60, and 75 min radiation, respectively. The three colonies were considered as potential candidates for lipid production and subjected to further analysis.





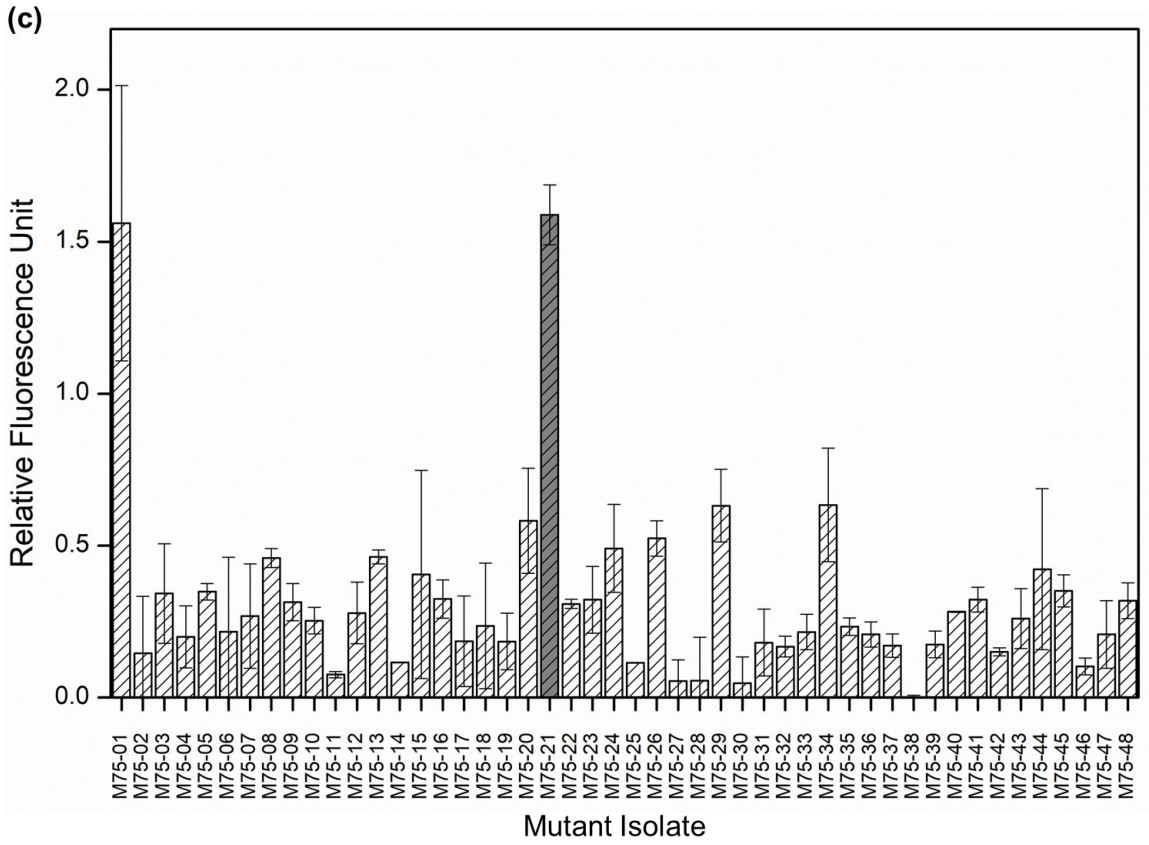


Figure 1. Relative amounts of lipids in *Chlorella* sp. 042 after UV mutagenesis: (a). M45-06 mutant; (b) M60-02 mutant; and (c) M75-21 mutant.

Cell dry weight and lipid contents

The growth rate of *Chlorella* sp. 042 was measured daily using a UV-Vis spectrophotometer. Both wild type and mutants showed an increase in growth rate until the last day of cultivation (Figure 2). The wild type achieved a growth rate of 0.503 day^{-1}, while all mutants exhibited a higher growth rate with 0.641, 0.783, and 0.7369 day^{-1} for M45-06, M60-02, and M75-22, respectively. The mutant M60-02 showed the highest growth rate of all. Previous studies showed that UV mutagenesis to the *Chlorella* sp. increased the biomass productivity and the lipid content, and thus, this strategy suitable for biodiesel production (Liu *et al.*, 2015; Sivaramakrishnan *et al.*, 2017). Our study found a similar result in which all of the mutants have higher biomass productivity (P<0.05) than the wild type (29.95 mg L^{-1} day $^{-1}$). The M60-02 mutant achieved the highest biomass productivity (P<0.05) with a value of 61.8 mg L^{-1} day $^{-1}$. The biomass productivity achieved was also in line with the growth rate in which the mutant M60-02 with the highest biomass productivity also showed the highest growth rate, which increased by two-fold concerning the wild type. The biomass productivity, lipid content, and lipid productivity of *Chlorella* sp. 042 wild type and mutants are provided in Table 1.

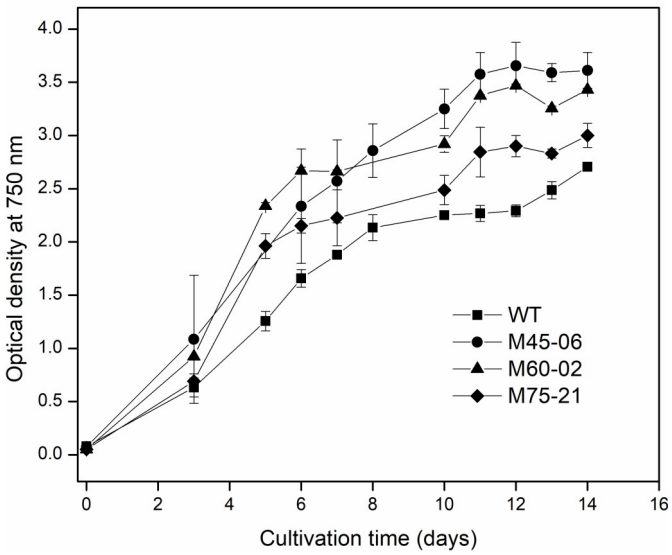


Figure 2. Growth curve of *Chlorella* sp. 042 wild type and mutants.

The lipid content of M45-06, M60-02, and M75-21 showed higher value (P<0.05) than wild type (30.8 %), 60.5 %, 55 %, and 40.6 %, respectively. The lipid content of M60-02 and M75-21 were lower than those of M45-06 (Table 1). However, the biomass productivity of the latter was lower than other mutants. Occasionally, microalgae with a high growth rate have low lipid content, whilst microalgae with a low growth rate have high lipid content (Liu *et al.*, 2015). In microalgae, there

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

Strain	Lipid Content* (%)	Biomass Productivity* (mg L^{-1} day^{-1})	Lipid Productivity* (mg L^{-1} day^{-1})
WT	30.8 ± 1.44[a]	29.95 ± 1.77[a]	9.34 ± 0.97[a]
M45-06	60.5 ± 3.8[b]	50.7 ± 9.92[b]	27.2 ± 0.6[b]
M60-02	55.1 ± 7.2[c]	61.8 ± 3.5[c]	34.0 ± 1.9[c]
M75-21	40.6 ± 4.4[d]	51.1 ± 0.5[d]	20.7 ± 0.2[d]

are three steps responsible for lipid biosynthesis, including fatty acid synthesis, acyl chain prolongation, and lipid formation. Acetyl-CoA carboxylase (ACC) is one of the essential enzymes in fatty acid synthesis. This enzyme catalyzes the biotin -dependent carboxylation of acetyl-CoA to form malonyl-CoA, which is considered a step-in fatty acid synthesis (Sendl *et al.*, 1992; Li & Cronan, 1993; Kim, 1997; Davis *et al.*, 2000; Courchesne *et al.*, 2009). Exposure of UV irradiation to the microalgae may affect the ACC and nutrient fixation. Thus, enhancing the lipids content. High lipid content in UV-mutagenized-*Chlorella* sp. was observed to have higher ACC concentrations than those of the untreated *Chlorella* sp. (Liu *et al.*, 2015).

As shown in Table 1, the lipid productivity for UV mutation microalgae was higher than the value for the wild type (9 mg L^{-1} day^{-1}) (P<0.05). Mutant M60-02 has the highest value of lipid productivity of 34 mg L^{-1} day^{-1} (P<0.05). The total lipid production is proportional to the biomass productivity and the lipid content, which is a significant parameter for large-scale lipid production of microalgae (Fan *et al.*, 2014). Lipid productivity is also a determining factor for the cost-effective production of biodiesel (Rahman *et al.*, 2020). The results suggest that the UV irradiation strategy for 60 min can result in the most favorable lipid productivity (Table 1).

Carbohydrate and protein contents

The major biochemical component of microalgae consisted of proteins, carbohydrates, and lipids, and their composition depends on the strain and culture condition (Behrens & Kyle, 1996; Vigeolas *et al.*, 2012). Since we perform UV mutagenesis, which can alter the metabolism of microalgae, the carbohydrates and protein assay are required to know the effect of the mutation on those compounds. Our study found that the M60-02 and M75-21 (P<0.05) carbohydrates and protein content were higher than those of the wild type after 14 days of cultivation. The results are shown in Figure 3, the carbohydrates content increased along with period times of mutation M60-02, and M75-21 which contained 38 % and 40 %, respectively. The carbohydrate content of M45-06 (34.5 ± 20 %) was not significantly different compared to the wild type (24 ± 0.39 %). The M60-02 and M75-21 mutants showed higher protein content compared to the wild type (4.55 ± 0.2 %). However, the M60-02 and M75-21 mutants exhibited similar results (P>0.05), 6.76 % ± 0.04 % and 6.93 ± 0.16 %, respectively. Further study at the genome level needs to be performed to identify the underlying biochemical process affected

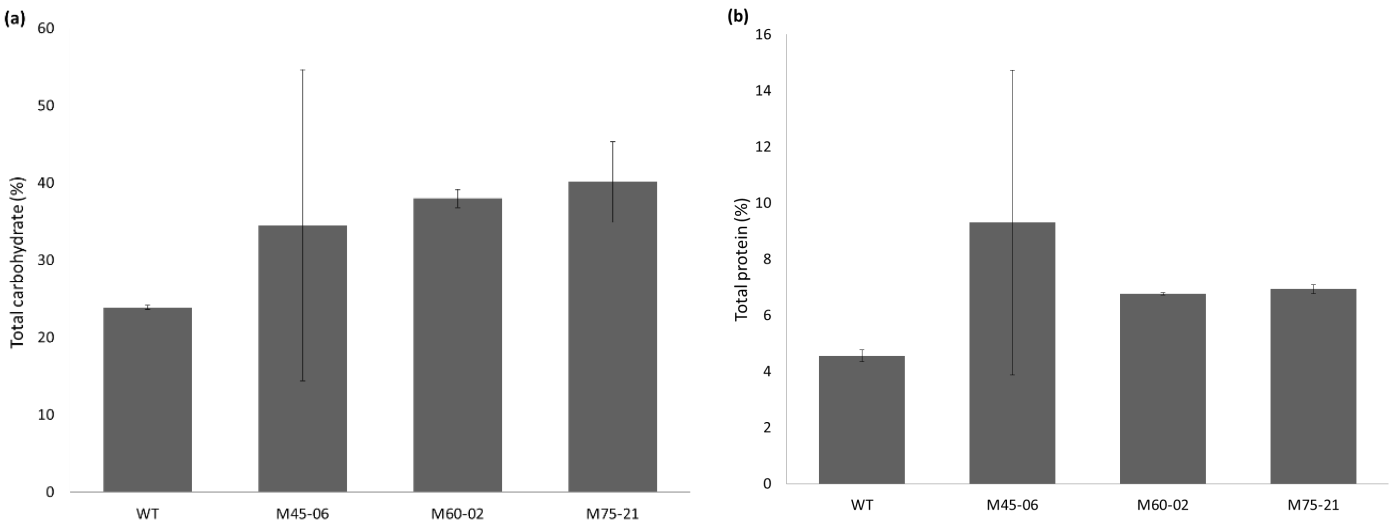


Figure 3. Primary metabolites content of *Chlorella* sp. 042 wild type and mutants: (a) carbohydrate and (b) protein.

by UV mutagenesis.

Fatty acid profiles of mutagenized microalgae

The fatty acid contents of lipids from both mutants and wild type can be inferred by the FAMEs detected by GC-MS analysis. The fatty acids contained in lipids determine the properties of biodiesel (Knothe, 2009). In this study, the analysis performed qualitatively and gave insight into the lipid's fatty acid profile and whether the mutagenesis affects the fatty acid profile of the strain. The result obtained in this study showed that the fatty acid contained in the lipids of both mutant and wild type are similar, with minor differences in which we did not observe oleic acid (C 18:1) in M45-06 and linoleic acid (C18:2) in M75-21. The same result was also shown by the study performed by Vigeolas *et al.* (2012) on *Chlorella sorokiniana* and *Scenedesmus obliquus*. In that study, both mutants and wild type strains produced the same type of fatty acids. However, the composition of certain fatty acids varies in mutant and wild type.

The content of lipid from both strains (Table 2) consists of saturated fatty acid, palmitic acid (C16:0) accompanied by stearic acid (C18:0), and unsaturated fatty acid components, oleic and linoleic acid. The result resembled the fatty acids profile of immobilized *C. vulgaris* obtained in Abu Sepian *et al.* (2017). Lipid from *C. vulgaris* was mainly composed of saturated fatty acids (60%), consisting of palmitic and stearic acid, and the remaining portion (40%) was comprised of unsaturated fatty acid, consisted of oleic, linoleic, and linolenic acid (Abu Sepian *et al.*, 2017). The more significant proportion of saturated fatty acids will contribute to the higher cetane number of the fuel. More specifically, the chain length and degree of unsaturation will affect the cetane number (Knothe, 2010). The higher the cetane number, the shorter the ignition delay time, which is one of the desired properties of a fuel (Knothe, 2009). The presence of palmitic and stearic acid thus may contribute to biodiesel with the desired property. However, further analysis is required to determine the exact composition of each fatty acid. Besides, the FAMEs derived from transesterification are also required to fulfil several other criteria like viscosity, cold flow, oxidative stability, and lubricity as determined in a certain standard of biodiesel (e.g., ASTM D6751, EN 14214) to be eligible for use and commercialization (Knothe, 2009).

CONCLUSION

Based on Nile red fluorescence assay, we found three microalgae mutants from three periods of mutation time, 45 min, 60 min, and 75 min, and there are M45-06, M60-02, and M75-21, respectively. The growth rate for all mutants is higher than the wild type after 14 days of cultivation, so does the lipid productivity. The M60-02 mutant achieved the highest lipid productivity, with 34 mg L^{-1} day^{-1}. Surprisingly, carbohydrate and protein contents of the mutants (M60-02 and M75-21) are also higher than the wild type. We found no difference in FAMEs profile of M60-02 mutant and the wild type that included palmitic acid, stearic acid, oleic acid, and linoleic acid. Finally, our results indicate that random mutation by UV mutagenesis for 60 min is suggested as the best way for generating microalgae mutant with higher growth rate and lipid productivity for environmentally friendly and sustainable energy sources such as biodiesel.

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Table 2. Fatty Acid Methyl Ester (FAMEs) profile of wild type and mutant.

FAMEs	Strains			
	WT	M45-06	M60-02	M75-21
C16:0	**V**	**V**	**V**	**V**
C18:0	**V**	**V**	**V**	**V**
C18:1	**V**	**-**	**V**	**V**
C18:2	**V**	**V**	**V**	**-**

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