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UV-irradiation mutagenesis of *Chlorella vulgaris* Beijerinck SP17 to enhance lipid production for potential biodiesel application

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ABSTRACT

Microalgae are considered as ideal and renewable feedstock for biofuel production because they do not pose threats to food security and the environment. Moreover, these strains can produce triacylglycerols (TAGs), a precursor in biodiesel production. However, to further improve the lipid accumulation, genetic engineering of these microorganisms is limited due to lack of candidate genes. Hence, induced mutagenesis presents an alternative for strain improvement. This study showed that ultraviolet (UV)-induced mutations which resulted in the reduced starch production of an indigenous strain of *Chlorella vulgaris* Beijerinck SP17 led to an increase in lipid production. Indigenous *C. vulgaris* culture was subjected to induced mutagenesis via UV irradiation. Duration of 10 min UV irradiation was used with the fatality rate of 84% and starch-deficient mutants were selected via negative iodine staining. Selected mutants were grown in upscale and cultured separately in nitrogen-replete (N+) and nitrogen-deplete (N-) conditions to further induce lipid and TAG accumulation. These starch-deficient strains had substantial increase in the production of lipids and TAGs with 58% and 43%, respectively. The N- condition revealed to have induced further synthesis of lipids and TAGs. These demonstrate that connection between lipid and starch biosynthesis pathways may be utilized to improve microalgal strain productivity for biodiesel production.

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KEYWORDS

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INTRODUCTION

By the year 2030, the global energy demand is projected to increase by 50% due to the rapid growth of human population and technological advancements (Maness 2009). The existing natural petroleum can no longer supply the increasing consumption rate, which is already past to what nature can provide (Netravali 2003). Since the use of fossil fuels is harming the environment through greenhouse gas emissions and consequent global warming, there is a great need for renewable

energy that does not cause significant environmental harm (Rittmann 2008; Medipally et al. 2015).

An organism that received considerable interest as a potential feedstock for alternative energy production is microalgae (Slade & Bauen 2013). Microalgae, depending on the species and cultivation conditions, can produce useful quantities of triacylglycerols (TAGs) that serve as feedstock for biofuel production, specifically biodiesel (Slade & Bauen 2013). Biofuels are generally non-toxic, biodegradable, and renewable (Song 2008). Generally, microalgae have rapid growth rate, high photosynthetic efficiency, high lipid production capacity, and the highest CO_2 fixation and O_2 production rates, which make them a more suitable feedstock for biofuel production, particularly biodiesel (Campbell 1997; Chisti 2007). One of the challenges in biodiesel production using microalgae is the fairly low intracellular lipid production per cell when cultivated under normal conditions. As a result, the amount of lipids generated is not enough to meet the standard industrial requirements. To address this problem, new strategies must be applied in order to enhance both growth rate and lipid synthesis simultaneously (Khan et al. 2018).

To create high-lipid producing mutants, the classical genetic approach is site-directed mutagenesis. However, the DNA sequences of lipid-metabolizing genes in algae are understudied; therefore, induced mutation via physical or chemical mutagens are employed (Parekh 2000). Presently, induced mutagenesis presents a substantial advantage compared to genetic engineering because it does not require the wealth of biochemical and genetic knowledge on the organism of choice (Queener 1986), which is currently not available for most microalgal species (Alonso et al. 1996; Ball 2009). An example of a mutagen is UV irradiation. This mutagen may cause different types of mutation, such as transversion, deletion, frameshift, and GC→AT transitions (Parekh 2000). The microbes that survive the mutation in their genetic DNA sequence usually acquire an altered genetic code for reprogrammed metabolic and biosynthetic activity (Parekh 2000).

Biodiesel from microalgae is composed of lipids, particularly, TAGs, preferred for production because of their high content (w/w %) of fatty acids and their absence of other chemical constituents (Breuer et al. 2012). TAGs are neutral lipids that serve as secondary energy storage of microalgae (Zhu et al. 2016). The primary energy storage for microalgae, starch, has the same precursor as TAGs. With TAGs and starch having common C3 precursors, the synthesis of these molecules becomes a competition which results to carbon partitioning (Wang et al. 2009). Hence, by impeding the genes related to starch synthesis, studies revealed that several algal strains produced more lipids (Li et al. 2015; Wang et al. 2009). The genetic modification of inhibiting the pathways that compete with lipid biosynthesis is the approach that has met the most success in the improvement of microalgal strains for biodiesel production, as compared to the transgenic approach (Sirikhachornkit et al. 2016). By utilizing UV irradiation, starch-deficient mutants can easily be obtained without transgenic modification (Sirikhachornkit et al. 2016).

The common freshwater green microalga, *Chlorella vulgaris*, has been considered as a promising candidate for commercial lipid production due to its easy cultivation and faster growth rate (Huntley and Redalje 2006). *C. vulgaris* has shown a great promise as a source of oil (Huntley and Redalje 2006). However, this biofuel feedstock still cannot meet industrial requirements for it to be used globally. Therefore, the present study specifically aimed to (1) perform UV-irradiation mutagenesis on *C. vulgaris* strain SP17, (2) quantify total lipids of *C. vulgaris* cultivated in nitrogen replete (N+) and nitrogen deplete (N-) conditions, (3) quantify TAGs of *C. vulgaris* cultivated in N+ and N- conditions and (4) quantify starch of starch-deficient *C. vulgaris* cultivated in N+ and N- conditions. This study demonstrated that the connection between lipid and starch biosynthesis pathways may be utilized to improve microalgal strain productivity for biodiesel production.

MATERIALS AND METHODS

Microalgal strain and growth conditions

Erlenmeyer flasks with 1L of sterile BG-11 culture broth (Sigma-Aldrich, India) containing the following chemicals: $NaNO_3$ (1.5 g/L), $K_2HPO_4{\cdot}3H_2O$ (0.04 g/L), $MgSO_4{\cdot}7H_2O$ (0.075 g/L), $CaCl_2{\cdot}2H_2O$ (0.036 g/L), Na_2CO_3 (0.02 g/L), citric acid (0.006 g/L), C_6H_5+4yFeNyO_7 (0.006 g/L), EDTA (0.001 g/L), and A5 + Co solution (1 ml/L) that consists of H_3BO_3 (2.86 g/L), $MnCl_2{\cdot}H_2O$ (1.81 g/L), $ZnSO_4{\cdot}7H_2O$ (0.222 g/L), $CuSO_4{\cdot}5H_2O$ (0.079 g/L), $Na_2MoO_4{\cdot}2H_2O$ (0.390 g/L) and $Co(NO_3)_2{\cdot}6H_2O$ (0.049 g/L), were used for the enrichment of *Chlorella vulgaris* strain SP17 cultures previously isolated from Sasmuan, Pampanga, Philippines. The flasks were incubated at 25 (±2)°C under 12:12-h alternate light/dark photoperiod using white fluorescent light at 40 µmol/ m-2s-1 intensity with continuous aeration for 14 days. The setup was placed in an indoor algae cabinet for the whole enrichment period (Tayaban et al. 2017). One *C. vulgaris* mother culture was selected as the main source for the experiment proper. The mother culture was harvested on its log phase and was quantified using cell count method via a hemocytometer for standardization of further experiments.

Experimental design

One mother culture of *C. vulgaris* was selected as the source and served as wild type of the whole experiment. After UV irradiation of cultures with 2 x 10^6 cells, three starch-deficient mutants (sdm) labelled sdm1, sdm2, sdm3 were selected after iodine staining. The lightest spots after iodine staining were selected for upscaling because they have the least starch. This principle for selection is due to carbon partitioning. These mutants were slowly cultivated and upscaled to 1L. After standardization of cell count to 2 x 10^6 via cell count method, the appropriate amount of each mutant culture and wild type was transferred to two separate media—nitrogen-replete (N+) BG-11 and nitrogen-deplete (N-) BG-11. The incubation lasted for five days and each culture was subjected to total lipids, TAG, and starch extraction analyses in triplicates.

UV-irradiation mutagenesis and mutant selection

The experiment was divided into two parts: (1) determining the appropriate duration time of UV exposure; and (2) performing UV irradiation mutagenesis on *C. vulgaris*. Eleven petri plates per trial with 10 mL culture were exposed to a 30-W ultraviolet lamp with wavelength 253.7 nm at 15 cm distance. The distance of 15cm was measured using a standard ruler. After 0, 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 min of exposure duration, the plates were covered with paper and incubated in the dark for 48 h to prevent light-induced DNA repair via photoreactivation. After incubation, the exposure time was established by measuring the fatality rate (FR) of the cells by cell-count method using a hemocytometer (Liu et al. 2015). The formulae used were:

$$\text{viable}\frac{\text{cells}}{\text{mL}} = \frac{(\text{total number of viable cells in 4 squares})}{4} \text{ x DF x } 10^4$$

$$\text{fatality rate (\%)} = \frac{\text{the number of viable cells before mutation}}{\text{the number of viable cells after mutation}} \text{x100}$$

The exposure time with 75-90% fatality rate was chosen. To visualize the fatality rate, 0.1 mL aliquots from each plate were removed from the plate and were spread plated on a BG-11 agar plate. The plates were allowed to grow under light in the algae cabinet for 14 days. After determining the correct exposure time, 20 sterile petri plates with 10 mL culture were exposed to the determined exposure time (10 min) and were then covered with paper and incubated in the dark for 48 h. To select for starch-deficient mutants, 1.5 mL from each of the 20 petri plates and wild type plate was centrifuged at 10000 rpm for 10 min to harvest the cells. An amount of 60µL of the cells was then spot plated on a BG-11 agar plate. Iodine vapor staining by dropping IKI reagent to the spots was employed. The three lightest (yellow to yellow green) spots compared to the wild type and other colonies were selected for upscaling. The light color change was due to the decrease in intracellular starch (Liu et al. 2015; Sirikhachornkit et al. 2016).

The selected mutants were labelled starch-deficient mutants or sdm namely, sdm1, sdm2, and sdm3. The chosen mutants were then upscaled from the petri plate to a test tube diluted with 3mL of BG-11 broth. The tubes were incubated under light conditions for 9 days. Afterwards, the tubes were upscaled to 50 mL on a 250 mL Erlenmeyer flask under light conditions with continuous aeration. The upscaling process ended when the mutant cultures reached 1L. For standardization of cell count 2 x 10^6 cells/mL, the mutant and wild type cultures were quantified using cell count method via a hemocytometer. The appropriate volumes of the mutant cultures were subjected to nitrogen-replete and nitrogen-deplete BG-11 broth for five days.

Extraction and quantification of lipids

To harvest the cells, each flask containing the algae culture was centrifuged at 3000 rpm for 10 min. The supernatant was discarded, and the cell pellet was washed twice with sterile distilled water. The mixture was centrifuged again for 3000 rpm for 15 min. The collected cell pellet was then stored in a -80ºC freezer for 36 h and transferred to a freeze-dryer for 24 h.

A total of 50 mg of lyophilized samples was transferred into a sterile 1.5 mL microcentrifuge tube and dissolved in 2 mL distilled H_2O. The samples were then sonicated for 5 min and dissolved in $CHCl_3$-MeOH (1:1 v/v) with the samples in a proportion of 2:1. The samples were sonicated again for 30 min and were transferred to a separatory funnel and shaken thoroughly. After phase separation, the lipid fraction (heavier phase) was filtered through a syringe filter and was transferred to a vial and was left to dry. The crude lipids obtained from each sample were then measured gravimetrically (Soštarič 2012).

The lipid-recovery yield was determined by reference to the weight of the recovered lipids. The formula used to quantify the lipid content was:

$$\text{Lipid content (\%)} = \frac{\text{Weight of lipid (g)} \times 100}{\text{Dry cell weight (G)}}$$

Nile red fluorescence assay

One mL of the isolated samples was centrifuged at 8000 rpm for 5 min, and the algal pellets were resuspended in 2.5 µL of 100X Nile Red (9-diethylamino -5H-benzo[alpha]phenoxazine-5-one) solution. The samples were incubated for 30 min in the dark, followed by fixation using 250 µL of 8% formaldehyde and incubated for 30 min at room temperature. The tubes were then centrifuged at 8 000 rpm for 10 min and the supernatant was decanted. The resulting pellets were washed twice with 250 µL of 1 X PBS. The stained cells and formation of intracellular lipid droplets were then subjected to fluorescence microscopy (Nikon, Japan) for analysis. Visualization of chlorophyll fluorescence was also utilized by excitation and emission wavelengths at 543 nm and 650nm, respectively. The chlorophyll and Nile red fluorescence images were merged using Adobe Photoshop software (Darunsart 2014).

Extraction and quantification of triacylglycerols (TAGs)

The previously extracted total lipids were dissolved in 250 µL hexane and purified through solid-phase extraction. The column was first washed with hexane thrice and hexane:diethyl ether (87:13) was used as the extracting solvent. This procedure was done twice. After extraction, the obtained TAGs were allowed to dry for 24 h and were measured gravimetrically. The TAG content was calculated using the formula:

$$\text{TAG content (\%)} = \frac{\text{Weight of TAGs (g)} \times 100}{\text{Dry cell weight (G)}}$$

Extraction and quantification of starch

The microalgal biomass was extracted with acetone and boiling 80% (v/v) ethanol. Samples were then hydrolyzed with 1.1% hydrochloric acid at 100°C for 30 min (Oren 1988). Starch was determined colorimetrically by iodine reaction. The absorbance was measured at 595 nm and calibration was carried out simultaneously using glucose as the standard. A calibration curve was obtained and starch content (µg/mL) was calculated (Fernandes et al. 2011).

Data analysis

Statistical analysis was performed using IBM SPSS Statistics 25 software. The means of each of the three replicates were compared with the wild type using one-way analysis of variance (ANOVA). Post-hoc testing was performed for inter-strain comparisons using the Tukey's test. Values were considered significantly different when $p<0.05$.

RESULTS AND DISCUSSION

Many microalgal species are established as candidates for alternative biofuel feedstock. Ease of cultivation and production of high-value products like lipids are among the major considerations. These attributes are possessed by a microalga that has shown potential as biofuel feedstock – *Chlorella vulgaris* (Liu et al. 2015). However, a challenge in biodiesel production using microalgae is the relatively low intracellular lipid production per cell when cultivated under normal conditions. In fact, a study showed that the maximum lipid content of a certain strain of *C. vulgaris* was only 26% under normal nutrition medium (Widjaja et al. 2009). As a result, the amount of lipids generated is not enough to meet the standard industrial requirements (Khan et al. 2018). With this, induced mutagenesis techniques are utilized to improve microalgal strains since they do not require the wealth of biochemical and genetic knowledge of microalgae, which are currently not available for most microalgal species (Ball 2009). Exposure to ultraviolet (UV) rays is the mutagenesis utilized in this study. Upon exposure to UV, the microalgal cells that remained viable usually have acquired an altered genetic code for reprogrammed metabolic and biosynthetic activity (Parekh 2000). This effect was shown in this study by exposing the *C. vulgaris* cultures in increasing durations.

The different exposure durations to the 253.7 nm UV light were utilized to check for the fatality rate of *C. vulgaris* cultures. Results showed that the fatality rate increased from 47 to 97% during the exposure of 1 to 30 min. This shows that the number of viable cells decreased as UV exposure duration increased (Table 1). The fatality rate of 75 to 90% has been recommended as a method to mutagenize algal cells (Liu et al. 2015). With this, the exposure duration of 10 min with fatality rate of 84% was selected as the proper duration of exposure in consideration of the desired mutant, cell activity, and subsequent mass cultures.

Table 1: Fatality rate of *Chlorella vulgaris* strain SP17 after UV irradiation at different time points. Exposure for 10 min is selected as the optimum duration.

	Fatality Rate (%)		
Time point (min)	**TRIAL 1**	**TRIAL 2**	**TRIAL 3**
0			
1	46.33	53	40.35
2	68.36	63	56.14
3	74.58	70.66	62.28
4	76.55	76	67.54
5	80.23	77.33	69.298
10	85.31	88.67	76.32
15	92.65	91.33	82.64
20	94.35	93.33	86.8
25	96.61	95.33	91.23
30	97.195	96.66	93.86

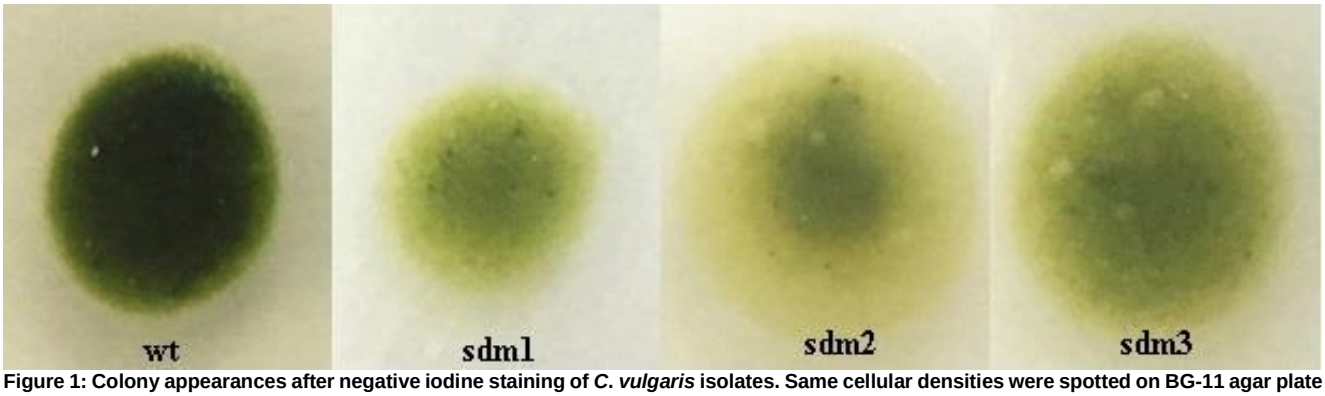

Figure 1: Colony appearances after negative iodine staining of *C. vulgaris* isolates. Same cellular densities were spotted on BG-11 agar plate prior to staining. The three lightest spots compared to the wild type were selected and further upscaled. The light color is indicative of reduction in starch production.

The cultures were then spot plated on BG-11 agar plate and iodine staining was employed. The three lightest spots compared to the wild type were chosen for further experiments since a darker color indicates presence of starch (Liu et al. 2015). The wild type spot appeared as dark green and the other spots turned yellowish or lighter in color. In Figure 1, mutants, namely, sdm (starch-deficient mutant) 1, sdm2, and sdm3 were the lightest of the 20 spots in the experiment. The mutants obtained in this study were screened through iodine staining to select for strains with the least starch. Starch is a primary energy storage compound for several algae. However, algae producing starch as a primary energy storage compound like *C. vulgaris* usually have relatively low lipid content (Li et al. 2015). This low lipid content is due to the metabolism of starch and lipid which both begin with the same initial pool of molecules — glyceraldehyde 3-phosphate (GAP) and 3-phosphoglycerate (3PG) (de Jaeger et al., 2014). Because of these common C3 precursors, the formation of a type of lipid, triacylglycerol (TAG) and the formation of starch becomes a competition that results to carbon partitioning. This partitioning happens such that when the route towards starch formation is inhibited, the pathway towards the formation of TAG molecules is improved (Wang et al. 2009). Moreover, studies on several algal strains revealed that more lipids can be produced by trying to impede the genes related to starch synthesis (Li et al. 2015; Wang et al., 2009). Mutants obtained in this study may be examined further to elucidate physiologic and genetic changes that occur after UV irradiation. The stability of the mutants in the next cell culture generation is also a point of interest for further studies.

When the starch content was extracted and quantified more precisely, only sdm2 and sdm3 in N+ condition showed a significant reduction in starch content compared to the wild type (Figure 2). The mutant sdm1, however, still has lower starch content than the wild type. The possible explanation to this is that these mutants carried different mutations. These different mutations then affected them at different steps of starch synthesis and metabolism resulting to their varying degrees of starch deficiency. For this study, the mutants selected were those with the least starch after iodine staining, which were then confirmed by starch quantification. The selection of these mutants was further reinforced after total lipid and TAG quantification (Figures 4 and 5).

Sirikhachornkit et al. (2016) showed that different genes involved in starch synthesis can be downregulated or altered. In the N- condition, the starch content of all the mutants has not decreased and has no significant difference from the wild type. This can be attributed to the fact that starch is the dominant sink for energy storage (Li et al. 2015) and it is possible that the initial response to nitrogen starvation is to accumulate starch. Several microalgae accumulate carbohydrates during the first days of nitrogen-deplete conditions (Draaisma et al. 2013; Johnson & Alric 2013; Vigeolas et al. 2012). A study showed that *C. zofingiensis* possesses basal levels of starch and lipids under nitrogen-replete conditions and large amounts of starch accumulate initially under nitrogen-deplete conditions (Zhu et al., 2014). The short cultivation period in the nitrogen-deplete conditions may have also caused this because starch degradation and reduction usually occur when the duration of nitrogen starvation is prolonged (Sirikhachornkit et al. 2016).

Microalgae produce lipids at a substantial percentage of their total mass for energy storage (Klyachko- Gurvich 1974; Tornabene et al. 1983; Tonon 2002). Most studies indicate a competitive relationship between starch and lipid synthesis due to their common carbon precursors. The blocking of starch synthesis pathway results in substantially higher lipid accumulation in *Chlamydomonas* and *Chlorella* (Ramazanov 2006). This premise supports the reduction in starch content of the mutants, following their increase in total lipid content compared to the wild type. The highest total lipid content was

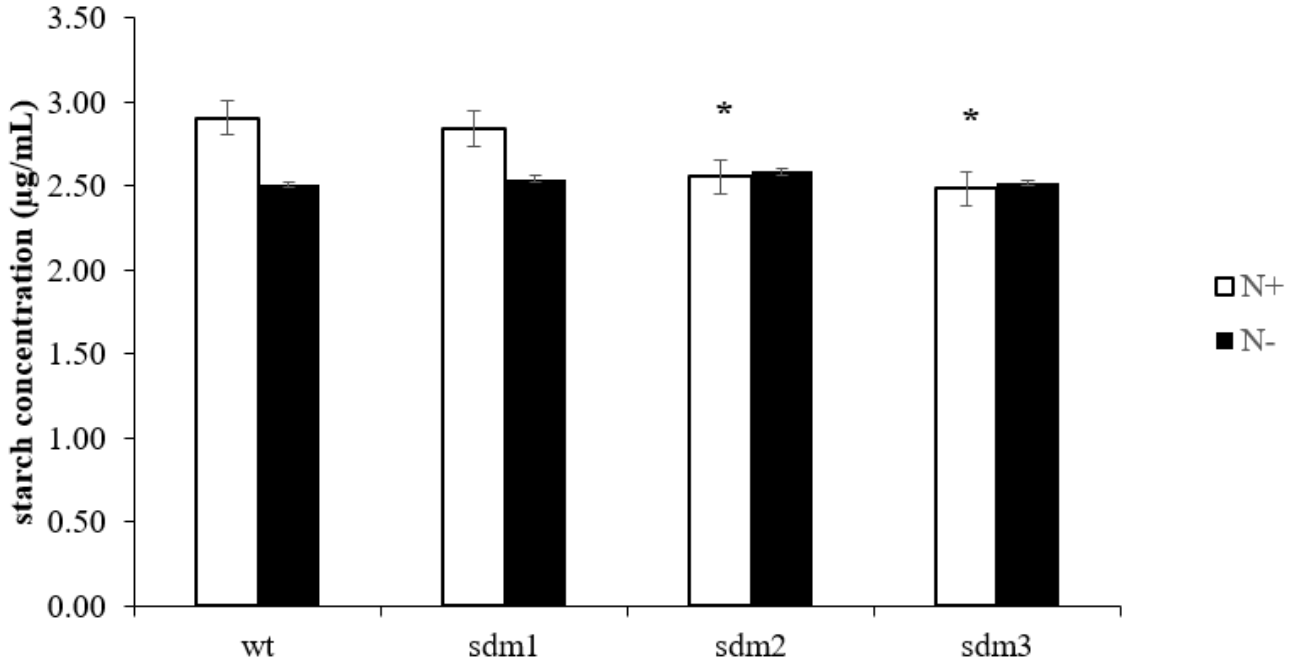

Figure 2: Starch content of each *C. vulgaris* culture – wild-type (wt), sdm1 (starch-deficient mutant 1), sdm2, and sdm3. The sdm cultures were UV-irradiated and selected after iodine staining. The values are from three biological replicates with error bars representing standard deviation. An asterisk (*)indicates a significant difference calculated by one-way ANOVA ($p < 0.05$) between the mutant and the wild type. The light bars are *C. vulagris* cultivated in N-replete (N+) conditions, the dark bars in N-deplete conditions (N-).

Table 2: Amount of frozen (top) and lyophilized biomass of *C. vulgaris* (bottom) obtained under N+ and N- conditions.

Frozen biomass	**N+ (g)**		**N- (g)**
wt	0.75	**wt**	3.77
sdm1	1.09	**sdm1**	3.28
sdm2	1.95	**sdm2**	3.87
sdm3	2.06	**sdm3**	2.24

Lyophilized biomass			
	N+ (g)		**N- (g)**
wt	0.31	**wt**	0.17
sdm1	0.12	**sdm1**	0.18
sdm2	0.14	**sdm2**	0.13
sdm3	0.11	**sdm3**	0.07

found to be 58% and 78% for N+ and N- conditions, respectively. The wild type had the lowest total lipid content of 21% (Table 2). This coincides with the study of Widjaja et al. (2009) which showed that the maximum lipid content of *C. vulgaris* strain was only 26% under normal nutrition medium. The lipid content results are also strongly confirmed by the Nile red fluorescence photos of the mutants in Figure 3. A study by Wong et al. (2007) also reported a significant increase in lipid content in Antarctic *Chlorella* sp. after exposure to UV.

Another experiment by Yu et al. (2013) also showed that low-starch and starch-deficient mutants of *Chlamydomonas* strains produced significantly higher yields of lipid compared with original cultures. Results also showed that the mutant strain contains a defect in the structural gene ADP-glucose pyrophosphorylase (AGPase), which catalyzes starch synthesis (Yu et al. 2013). Similar findings were seen when UV mutagenized starch-deficient mutants of *Scenedesmus obliquus* also showed a significant increase in lipid content compared to its wild type (de Jaeger et al. 2014). The lower starch content of the mutants in the biomass makes the contribution of lipids compared to the dry weight slightly higher, therefore, the relative lipid content would be higher as well (de Jaeger et al. 2014).

Since UV mutagenesis is random and may affect different genes to account for changes (Meireles et al. 2003), the lipid synthesis of microalgae had to be assessed as well. The biosynthesis pathway of lipids in microalgae is made up of three primary steps: (1) fatty acid synthesis, (2) acyl chain prolongation, and (3) lipid formation (Roessler et al. 2009). Among all the enzymes involved in these steps, acetyl-CoA carboxylase (ACC) is considered as the most important enzyme (Liu et al. 2015). ACC is a multifunctional enzyme that catalyzes acetyl CoA to form malonyl CoA, which is regarded as the rate-limiting step in the fatty acid synthesis (Hu et al. 2008; Liu et al. 2015). Although microalgae have different mechanisms against UV irradiation, the overexpression of ACC is common when exposed to UV irradiation as revealed in previous studies (Dunahay et al. 1995; Liu et al., 2015). Anthony et al. (2014) utilized UV to induce mutagenesis on *C. vulgaris* and revealed that there was a replacement of leucine by a serine residue in the ACC gene after mutagenesis. The *C. vulgaris* mutants showed higher lipid production compared to the wild type. This suggests that the reason behind lipid enhancement in the mutant is the mutation in the ACC gene (Anthony et al. 2014), but further studies are needed to prove this. In this study, Figure 4 reveals that the total lipid content of mutants in the N+ condition is higher compared to the wild type. Although the genes altered

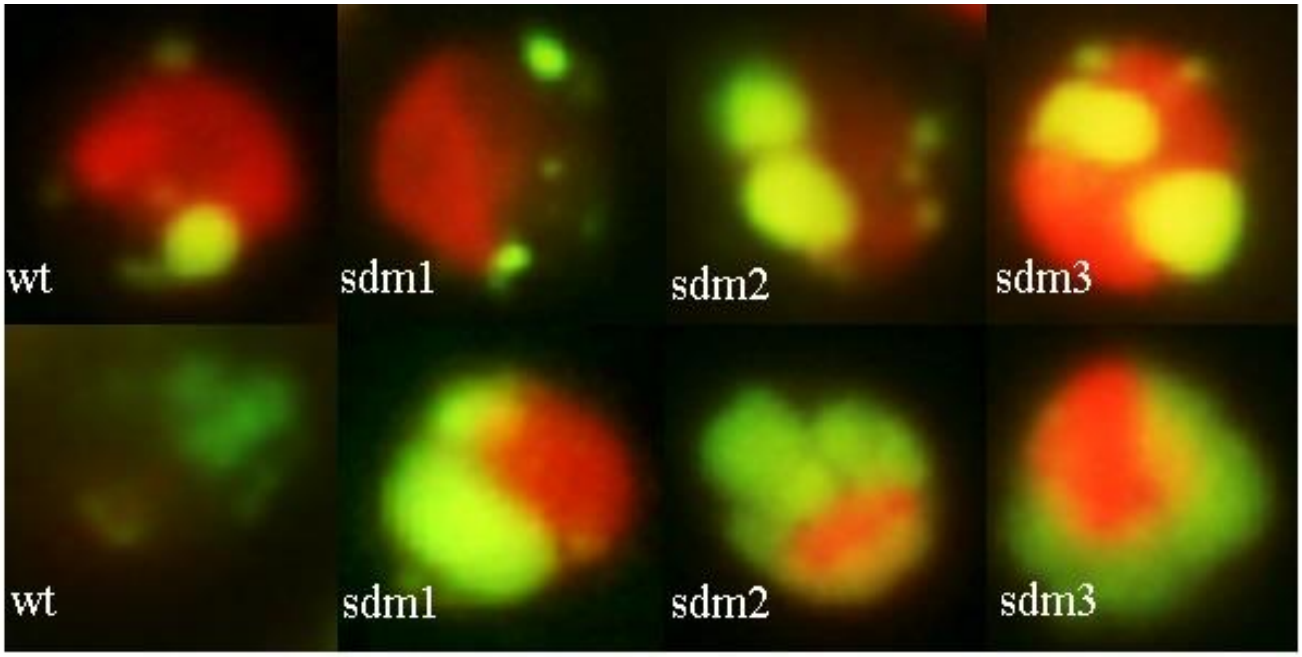

Figure 3: Subcellular lipid bodies in the wild type (wt) and starch-deficient mutants (sdm1, sdm2, sdm3) of *C. vulgaris* cultivated in nitrogen-replete (top) and nitrogen-deplete (bottom) conditions. Shown are merged photos of chlorophyll autofluorescence (red) and Nile red fluorescence (green), which indicate lipids. The cells photographed are representative of the general population.

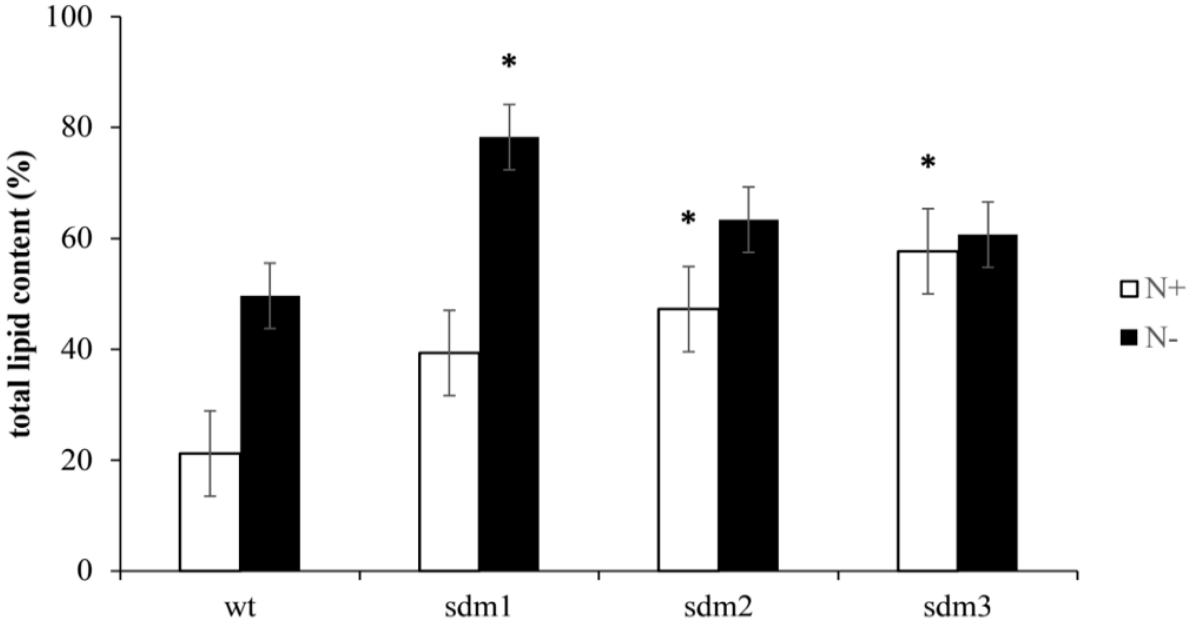

Figure 4: Total lipid content of each *C. vulgaris* culture – wild-type (wt), sdm1 (starch-deficient mutant 1), sdm2, and sdm3. The sdm cultures were UV-irradiated and selected after iodine staining. The values are from three biological replicates and error bars represent standard deviation. An asterisk (*) indicates a significant difference calculated by one-way ANOVA ($p<0.05$) between the mutant and the wild type. The light bars are *C. vulgaris* cultivated in N-replete (N+) conditions, the dark bars in N-deplete conditions (N-).

were not distinguished, the overexpression of ACC may have also enhanced the lipid synthesis.

For the mutants in N- condition, there was no significant difference in the starch content compared to the wild type. However, there was still an increase in total lipid content. Other than the possible starch synthesis blockage and mutation in the ACC gene, the deficiency in nitrogen may have caused further increase in the total lipid content of the mutants. This is supported by Figures 4 and 5 as the total lipid content and TAGs of the mutants in N- condition were higher than that of the mutants in N+ condition. Nitrogen starvation has induced the increase in lipid content. Many studies have shown that nitrogen deficiency can enhance the lipid accumulation of several microalgae (Dunahay et al. 1995; Liu et al., 2015). It is also confirmed that *Chlorella* reacts to nitrogen starvation by accumulating lipids and carbohydrates like starch (Illman et al. 2000; Converti et al. 2009). This can explain the fact that there was no significant difference in starch content in N- mutants. The increase in lipid content under nitrogen-deplete conditions was attributed to the inhibition of cell division resulting from environmental stresses (Sukenik & Livne 1991).

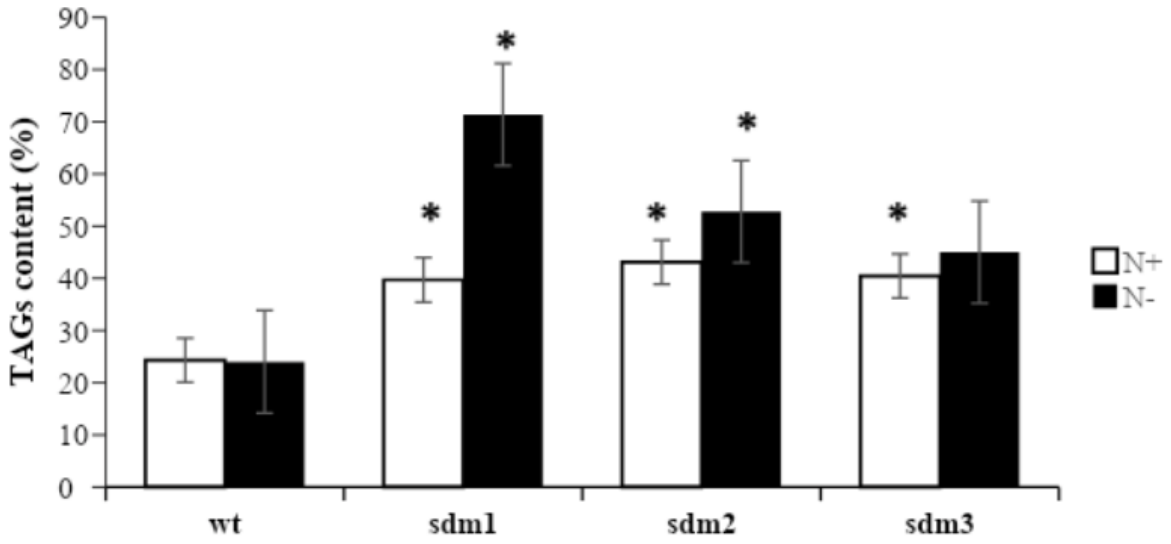

Figure 5: Triacylglycerol (TAG) content of each *C. vulgaris* culture – wild-type (wt), sdm1 (starch-deficient mutant 1), sdm2, and sdm3. The sdm cultures were UV-irradiated and selected after iodine staining. The values are from three biological replicates and error bars are standard errors. An asterisk (*) indicates a significant difference calculated by one-way ANOVA ($p<0.05$) between the mutant and the wild type. The light bars are cultures cultivated in N-replete (N+) conditions, the dark bars in N-deplete conditions (N-).

Microalgal lipids have two types: neutral lipids which serve as energy reserves, and polar lipids which are constituents of membranes and organelles. Microalgae accumulate and store neutral lipids in the form of TAGs (Zhu et al. 2016). TAGs constitute about 80% of the total lipids found in the algal cell and is used as feedstock for biofuel (Klyachko- Gurvich 1974; Tornabene et al. 1983; Tonon et al. 2002). TAGs are preferred for biodiesel production because of their high fatty acid content and the absence of other chemical constituents (Breuer et al. 2012). Since TAG is a type of lipid, it exhibits the same relationship with starch. TAGs and starch have common C3 precursors which result to carbon partitioning (Wang et al. 2009). Starch can easily account for over 40% of the newly produced microalgal biomass (Li et al. 2015; Breuer et al. 2012; Zhu et al. 2014), hence, diverting this large carbon flow away from carbohydrates towards TAG that could substantially enhance TAG synthesis (Li et al., 2015). Because both starch and TAG are energy storage compounds in algae and they share common precursors, the blockage of starch biosynthesis may have a more prominent effect on the TAG content than it does on other classes of lipid. Moreover, TAGs are synthesized in the chloroplast and during photosynthesis, large amounts of starch accumulate in the chloroplast which cause reduced functional activity in the organelle (Klyachko-Gurvich 1974).

The wild type in both conditions exhibited only 24% of TAG content, significantly lower than the TAG content of the mutants. This low TAG content is explained by its high starch content since the primary energy storage compound of *C. vulgaris* is starch. It can be inferred that in normal unmutated conditions, starch is the first molecule synthesized and not TAG. The wild type of *C. vulgaris* is focused more on synthesizing its default energy storage (starch) than lipids and TAGs, which are just secondary energy storage compounds (Hu et al. 2008).

All the mutants showed a substantial increase in TAG content, with 43% being the highest, compared to the wild type in both N+ and N- conditions (Figure 5). The results and analysis of the mutated strains in this study confirmed a correlation between hyperaccumulation of TAG and the starchless phenotype. Other studies of starch mutants in other green algal species also support such correlation, with reports of increased TAG accumulation in starchless mutants of *Scenedesmus obliquus* (de Jaeger et al. 2014) and *Chlorella pyrenoidosa* (Ramazanov 2006) when compared to their wild types. A study by de Jaeger et al. (2014) also revealed a significant increase in TAG accumulation of five starchless mutants.

There was also a noticeable difference between the mutants in both conditions. The mutants cultivated in N- condition had higher TAG content compared to those in N+ condition (Figure 5). This may have resulted from the degradation of starch which usually occurs when nitrogen starvation is prolonged, and hence, supports the synthesis of TAG as a long-term energy storage (Fernandes et al. 2011; Breuer et al. 2014; Zhu et al. 2014; Li et al. 2015). Moreover, an accumulation of TAG under stressful conditions such as nitrogen starvation is also suggested to be a mechanism which protects the cells from the damage caused by reactive oxygen species (Hu et al. 2008; Li et al. 2015). The threshold of the culture in terms of TAG production may be elucidated in further studies of next cell culture generation of this strain.

CONCLUSION

Microalgae are potential sources of lipids for biodiesel production. In this study, UV irradiation mutagenesis was utilized to induce mutation on indigenous *Chlorella vulgaris*. Screening was successfully made to select for starch-deficient mutants since starch competes with the synthesis of lipids and triacylglycerols (TAGs), which are the feedstock for biodiesel. *C. vulgaris* mutants were also cultivated in nitrogen-deplete (N-) condition to induce stress and check for any further increase in synthesis. This study was able to isolate *C. vulgaris* strains with significantly lower starch than its wild type. These starch-deficient strains had substantial increase in the production of lipids and TAGs with 58% and 43%, respectively. The N- condition revealed to have induced further synthesis of lipids and TAGs. However, the starch content did not significantly decrease in this condition which is most likely due to the initial response of *C. vulgaris* to nitrogen starvation. Overall, this study has shown that UV mutagenesis can induce mutation on *C. vulgaris* for enhanced lipid and TAG accumulation. It also highlights the potential of using a starchless mutant of *C. vulgaris* for upscaling as a sustainable biodiesel feedstock.

Mutants obtained in this study may be examined further to elucidate physiologic and genetic changes that occur after UV irradiation. Moreover, monitoring of the next cell culture generation of this strain is recommended to elucidate the threshold of the culture in terms of TAG production.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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