CELL WALL DEGRADATION OF *Chlorella vulgaris* UNDER DARKNESS AND ULTRAVIOLET IRRADIATION-B (UVB) CONDITIONS FOR LIPID AND CARBOHYDRATE RECOVERY

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Abstract. The production of microalgae biofuel is still not economically viable due to the high cost of downstream processing, which is attributed to cell harvesting and disruption processes. In this study, the biochemical and structural changes of *Chlorella vulgaris* UPSI JRM01 that was cultivated under darkness for 13 days and ultraviolet irradiation-B (UVB) (0, 15, 30, 60, 120, and 180 min) stress conditions to induce cell wall degradation were investigated. Under the condition of darkness, *C. vulgaris* invested their fixed carbon towards the accumulation of carbohydrates (35 %) instead of lipids (25 %). Meanwhile, the highest lipid and carbohydrate content obtained under UVB were 34.4 % and 47.5 %, respectively. The cell wall thickness of culture stained with calcofluor white (CFW) observed under fluorescence microscopy revealed that degradation of cell wall occurred under darkness on the seventh day (0.076 ± 0.037 μm) and 30 min of UVB irradiation (0.091 ± 0.044 μm). The transmission electron microscopy (TEM) micrographs further confirm the cell wall thinning under both conditions. Cell wall polysaccharides content measured under darkness and UVB conditions were 4.3 % and 6.6 %, as compared to control 11.3 % and 10.2 %, respectively. These results show that darkness and UVB stress are feasible means to induce cell wall degradation for lipid and carbohydrate recovery.

Keywords: Microalgae, cell wall, degradation, darkness, UVB

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Introduction

Several factors have led to the exploration of alternative energy sources, including the inadequate stock of fossil-derived fuel resources and the growing demands for energy due to rapid industrialization and population growth. The combustion of petroleum-derived fuel contributed to the emission of greenhouse gases, particularly carbon dioxide, thus leading to the global climate change phenomenon. Microalgae are known to produce high biomass and lipid production compared to other biomass sources, thus creating interest in biofuel feedstock production from these microorganisms. Although lipids and carbohydrates accumulated in microalgae cells are a promising feedstock for biofuel production, harvesting, cell disruption and extraction of intracellular lipids and carbohydrates are the primary obstacles impeding their commercial application. Both macromolecules are the main feedstock for the production of bioethanol and biodiesel. Extraction of a product, whether intracellular lipids, free fatty acids, or a heterologous product, has proven to be a very expensive procedure, accounting for roughly 60 % of the whole process cost [1].

Triacylglycerol (TAG) and starch granules are found in the microalgae intracellular and can only be recovered through the process of degradation of the cell wall. However, the microalgae cell wall layer, which is thick and rigid with high mechanical strength and chemical resistance, makes the extraction of biomolecules challenging [2]. The *Chlorella* species possess two cell wall layers. The outer cell wall layer is highly resistant, consisting of a trilaminar structure with the presence or absence of algaenan. The inner cell wall is a rigid fibrillary structure with high cellulose content [3]. Therefore, an appropriate cell disruption method is necessary to ensure efficient recovery of the biomolecules. To date, various methods through mechanical, thermal, chemical, and biological approaches have been employed to assist cell disruption in microalgae. The commonly used methods are bead beating, microwave, ultrasonication, chemical hydrolysis, osmotic shock, subcritical water hydrolysis, and enzyme hydrolysis [2,4]. However, mechanical methods such as bead beating and ultrasonication were found to exhibit the highest energy consumption. On the other hand, the energy consumption of nonmechanical method was found to be dependent on treatment time, temperature, and stirring [5].

Among the potential cell disruption method are biological approaches through autolysis. Autolysis is a self-digestion process that causes the destruction of organelle and cell wall structures by an endogenous enzyme [6]. Light regulation such as cultivation under darkness and UVB exposure not only affect the biomass productivity and composition of energy-rich biomolecules such as lipid and carbohydrate, but they also alter the cell wall structures of microalgae and induce autolysis [7]. Ruptured cell walls caused by autolysis were observed in *Chlorella vulgaris* SDEC-3M after 12 hours dark cycle [8]. Thermally coupled dark-anoxia conditions were reported to be able to induce an autolytic fermentation pathway in *Nannochloropsis* [9]. From the perspective of industrial bioprocess, cell autolysis requires an inductor to activate the cell digestion and release all intracellular material into broth culture. Therefore, in this study, cultivation of *C. vulgaris* under darkness and UVB irradiation stress conditions is suggested. Said factors could weaken cell wall structure and thus make it more susceptible to be applied as an economic means of cell disruption in microalgae for recovery of lipids and carbohydrate molecules.

Hence, this study is devised as a direct response to the aforementioned issues, aiming to investigate cell wall thinning in *Chlorella vulgaris* UPSI-JRM01 induced by darkness and UVB irradiation conditions. The biochemical composition changes and cell wall thickness

were characterized as a function of darkness and UVB irradiation periods. The findings are prominently important to advance economical microalgae biofuel production, particularly for cell disruption process for lipid and carbohydrate recovery.

Materials and Methods

Microalgae Growth Condition

The culture was initially grown in an Erlenmeyer flask to the middle of the logarithmic phase (day 6 to 7) in blue-green 11 medium (BG11). The pre-cultured cells were collected by centrifugation (4000 rpm x 5 min) and resuspended in nitrogen-free BG11 to completely remove the nitrogen. The pre-cultured microalgae containing 5.5 x 10^6 cells mL^{-1} was inoculated into a 250 mL Erlenmeyer flask containing 100 mL of BG11. Two-stage cultivation technique was used in this study. In the first stage, microalgae were cultivated photo-autotrophically under optimized conditions [10], with the following growth parameters: 500 mgL^{-1} NO_3^-, 28 °C, 10 500 lux, and pH 8. In the second stage, the harvested cells from the first stage were cultivated independently under darkness and UVB irradiation.

Cultivation in Darkness

The culture was grown in darkness for 13 days until the cell's viability is less than 40 %. The control culture was cultivated under 12:12 hours (Light: Dark) photoperiod.

Exposure to UVB Irradiation

Microalgae cultures were exposed to UVB irradiation (UV dose 2 W^{-2}) at different lengths of exposure (15, 30, 60, 120, and 180 min). After the UVB irradiation, the cultures were grown under the optimized growth parameter with cultivation period of 0, 5, 24, 48, 72, and 96 hours. The control culture was cultivated under the same cultivation condition but without exposure to UVB irradiation.

Sample Collections

Samples were collected daily for biochemical, total cell wall polysaccharide and cell wall measurement.

Total Lipid

The total lipid analysis was performed using a modified Bligh and Dyer method [11].

Total Carbohydrate.

The total carbohydrate analysis was performed using a modified phenol-sulphuric acid method [12].

Total Cell Wall Polysaccharides

Cell wall polysaccharides were hydrolyzed by 0.5 mL of 80 % H_2SO_4 at 0 – 4 °C for 20 hours. 6.0 mL ice-cold Milli-Q water was added to the hydrolysate to dilute it to 1 mol

H_2SO_4 before it was filtered (GF/ C). The phenol-sulphuric acid method was used to examine the cell wall hydrolysate separately [13]. The mixture was quickly agitated with a glass rod or vortexed after adding 0.5 mL 3 % aqueous phenol and 5 mL concentrated H_2SO_4 to 2 mL sample (containing 5 – 100 µg carbohydrate) in a test tube. The tube was let to stand for 30 min before being cooled under running water. Spectrophotometric absorbance was measured in a 1 cm cell at 485 nm and sample absorbance (0.1 – 1.0) was corrected for blanks (< 0.01) [13].

Calcofluor White (CFW) Staining and Cell Wall Thickness

CFW, an optical brightener, has a high affinity for cellulose and β-linked glucans. All samples were stained for 5 min with a 0.01 % CFW solution (Sigma-Aldrich, Inc., Saint Louis, USA), to observe the effect of darkness and UV irradiation on cell walls. The cells were then centrifuged at 10000 rpm for 1 min before being washed twice with phosphate-buffered saline (PBS). A fluorescence microscope (Nikon Eclipse TE 2000-U, UK) was used to capture the images. BV-2A filter with an excitation filter (400 – 440 nm) was used to view microalgae stained with CFW. The cell wall thickness was measured daily using the NIS-elements software. The measurement was taken from the inner side of the cell wall to the outer side of the cell. A total of 10 – 15 cells were measured for each condition where each cell was measured at 2 – 3 different points depending on the cell wall clarity.

Visualization of Microalgae Ultrastructure

TEM was used to observe the ultrastructure of *C. vulgaris* using a modified method according to Nordin et al. [10]. The cells were pelleted at 4000 rpm, fixed in 4 % glutaraldehyde for 24 hours, and washed three times with 0.1 M sodium cacodylate buffer for TEM imaging. The cells were then immersed in cacodylate buffer after being fixed for 2 hours using a 1: 1 ratio of osmium tetroxide: cacodylate buffer. The cells were rinsed and dehydrated in a graduated ethanol series ranging from 35 % to 100 % (vv^{-1}) before being embedded in epoxy resin. The samples were stained with toluidine blue to determine the proper area for ultrathin sectioning. The EM UC6 ultramicrotome (Leica, Austria) was used for ultrathin sectioning. Uranyl acetate and lead citrate were then used to stain the sample. TEM Microscope Talos L120C was used to acquire micrographs of the specimen (Thermo Fisher, USA).

Statistical Analysis

Independent-samples *t*-test was used to statistically evaluate the significant differences between the means in control condition and under darkness. Meanwhile, one-way analysis of variance (ANOVA) and Tukey's test were performed to statistically compare means of different lengths of UVB irradiation and evaluate the *p*-value. In all cases, comparisons that showed a *p*-value of ≤ 0.05 were considered significant. All analyses were conducted using the SPSS software package (IBM statistics Version 26).

Results and Discussion

Lipid and Carbohydrate Changes Under Darkness and UVB Conditions

Figure 1 shows the total lipid and carbohydrate yield of *C. vulgaris* throughout the cultivation period until the death phase where the viability reached below 50 % under darkness. The results show that the highest lipid yield (32 %) was from the control condition on day 6. Meanwhile, the highest lipid yield achieved in the darkness was 25 % on day 7. Independent-samples *t*-test shows that there is a significant difference between the total lipid yield under darkness (19 ± 2.2) and the control condition (22.92 ± 4.68); t (24) =2.67, p-value = 0.013. The total lipid yield under the control condition was significantly higher than in the darkness because the photosynthesis process was interrupted without the presence of light. Sforza et al. [14] stated that under strong continuous light, the lipid accumulation of microalgae will be stimulated. This is the opposite of what is being conducted in this study, hence the reason why the total lipid yield achieved in darkness is low. Darkness also creates unsuitable conditions for lipid and carbohydrate production in microalgae because, when the light-dependent process of photosynthesis is hindered, adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), which are required for fatty acid and lipid synthesis cannot be produced [15]. Morales et al. stated that while extending the dark period increases the lipid content, an excess of dark time results in a decrease in total lipid content, which is likely to be re-used within the cell and eventually dissipates via respiration [16]. This is the reason why the result of total lipid yield in this study fluctuated throughout the 13 days of the cultivation period.

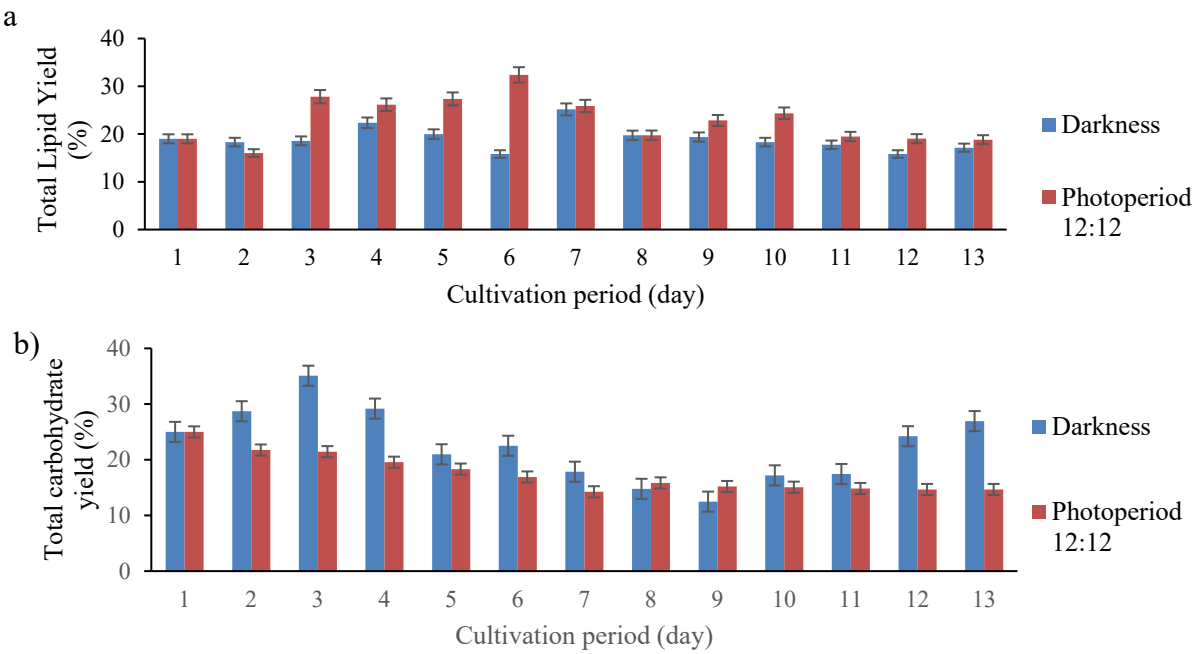

Figure 1: (a) Total lipid yield (%) and (b) total carbohydrate yield (%) of *C. vulgaris*

The initial value of total carbohydrate yield was 25 % for both conditions. The total carbohydrate yield in the darkness increased from day 1 to day 3 which accumulates up to 35 %, and the value started to decrease until day 9 (12 %) before it increased again until day 13 (27 %). The increase in carbohydrate content from day 10 to day 13 might be related to the nitrogen deprivation in the media which induces carbohydrate accumulation. A similar trend was also reported in the study by Jia et al. [17]. Meanwhile, under the control condition, the

total carbohydrate yield decreased after day 1. The results under darkness show that initially, the carbohydrate yield was higher compared to the lipid yield before the carbohydrate yield started to decrease. Independent-sample *t*-test shows that there is a significant difference between the total carbohydrate yield under darkness (19 ± 2.2) and the control condition (22.38 ± 6.5); t (24) =2.98, p-value = 0.031. These results show that darkness has a significant effect on the total carbohydrate yield of *C. vulgaris.* Debnath et al. [18] reported that light energy is a critical factor affecting the growth and carbohydrate accumulation of microalgal strains where the quality (color/ wavelength), quantity (light intensity), and photoperiod (light: dark cycles) affect the growth and carbohydrate accumulation. Previous studies reported that under stress circumstances, carbon and energy fixed by photosynthesis are shifted from carbohydrate synthesis to lipid synthesis [19,20] thus explaining the overall diminution of total carbohydrate yield and the increase in total lipid yield under the control condition from day 1 to day 6.

UVB irradiation had been reported to cause damage to microalgae ultrastructure mainly chloroplast [21]. The effect of UVB irradiation on the total lipid and carbohydrate yield was investigated from the irradiation period of 0 to 180 min with a cultivation period of up to 96 hours. Figure 2 shows the total lipid and carbohydrate yield of *C. vulgaris* under different exposure to UVB irradiation. The highest total lipid yield of 34.4 % was obtained from 180 min of UVB irradiation after 48 hours cultivation period. The results show that at 24 hours of cultivation period and above, the total lipid yield for the cell that had been exposed to UVB irradiation increased for the duration of 120 and 180 min. One-way ANOVA test shows that there is a statistically significant difference in the means of UVB irradiations between at least two groups (F-value (5, 30) = [3.869], p-value = 0.008). Post-hoc comparisons using the Tukey HSD test show that the total lipid yield after 48 hours of cultivation from the culture exposed to 30 min of UVB irradiation is the most significant (p-value = 0.024, 95 % Confidence Interval = -17.81, - 8.57).

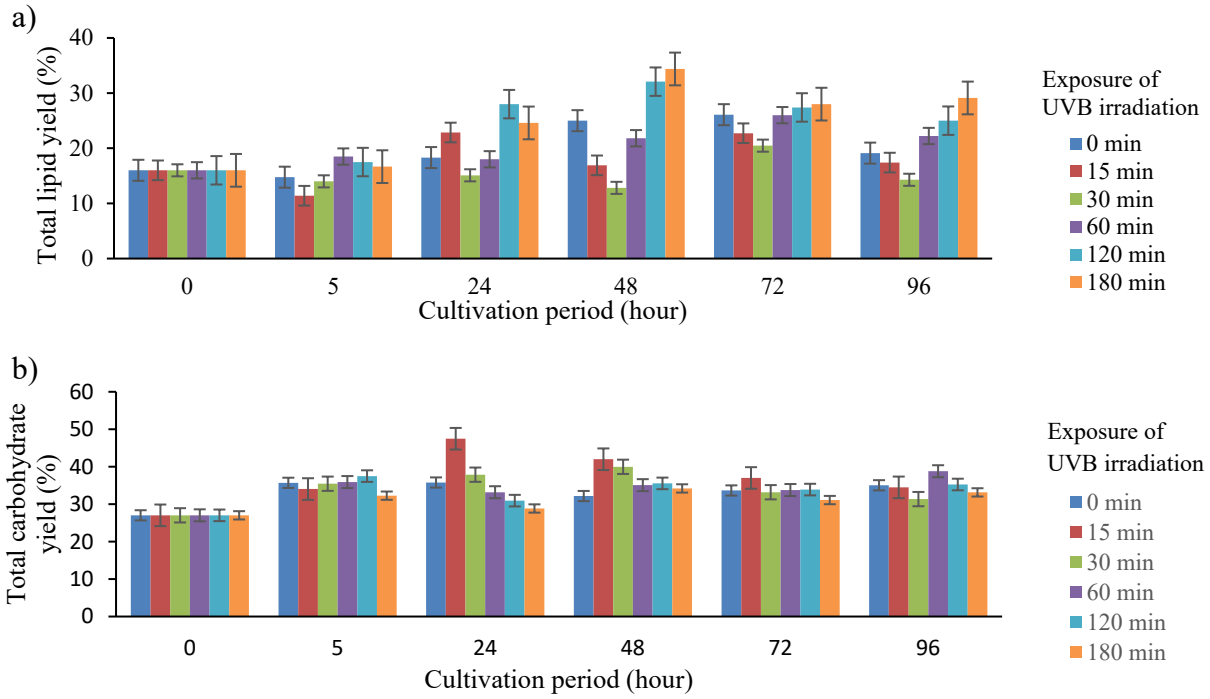

Figure 2: (a) Total lipid yield and (b) total carbohydrate yield of *C. vulgaris* under different exposure of UVB irradiation

The initial total carbohydrate yield of microalgae was 27 % and the value increased after UVB irradiation treatment was given. The highest total carbohydrate yield of 47.5 % was obtained after 24 hours of cultivation period from the culture that has been exposed to 15 min of UVB irradiation. However, within the same cultivation period, the total carbohydrate yield decreased as the length of UVB exposure extended from 30 to 180 min. One-way ANOVA test shows that there is a statistically significant difference in the means of UVB irradiations between at least two groups (F-value (5, 35) = [3.869], p-value = 0.001). Post-hoc comparisons using the Tukey HSD test show that the total carbohydrate yield after 24 hours of cultivation from the culture exposed to 30 min of UVB irradiation is the most significant (p = 0.02, 95 % Confidence Interval = 2.68, 14.66). A significant increase in carbohydrate content was also observed in the study by El-Sheekh et al. [22] where the increase of carbohydrate content was due to the adaptation strategy of microalgae towards environmental stress including UVB irradiation. A study by Zaparoli et al. [23] found that UV radiation (up to 6 min) was important for the accumulation of carbohydrates in *Spirulina platensis*, while the current study found that the carbohydrate accumulation of *C. vulgaris* is triggered by UVB exposure up to 15 min. UVB exposure higher than 15 min caused the total carbohydrate yield to be reduced. Ganapathy et al. [24] demonstrated that *C. vulgaris* is resistant to UVB radiation damage at shorter exposure times (10 min). Additionally, the potential negative effect of increased UVB radiation on microalgae growth may have been successfully caused by a rise in UV-absorbing compounds induced by UVB radiation stress.

Cell Wall Degradation Under Darkness and UVB Conditions

Figure 3(a) shows the total amount of cell wall polysaccharides produced under darkness from day 0 to day 13. On day 0 the total cell wall polysaccharide was 12.3 % with a cell wall measurement of 0.174 ± 0.032 µm under darkness and the control condition. The result shows that the lowest total cell wall polysaccharides occur at day 7 (4.3 %) with a cell wall thickness of 0.076 ± 0.037 µm (Table 1). In this study, the thinning of the cell wall was in accordance with the cell wall polysaccharide content. The total cell wall polysaccharides and cell wall thickness began to increase in darkness after day 8, which might be related to nitrogen depletion in the media since no nitrogen sources were added during the experiment. This trend was also observed in a study conducted by Halim et al. [8] in which it was reported that the cell wall doubled in thickness when it was harvested under a nitrogen deprivation condition. There is a significant difference between the total cell wall polysaccharides under darkness (10 ± 4.4) and the control condition (13.49 ± 1.228); t (24) =2.21, p-value = 0.036. These results show that the darkness condition has a significant effect on the total cell wall polysaccharides of *C. vulgaris*.

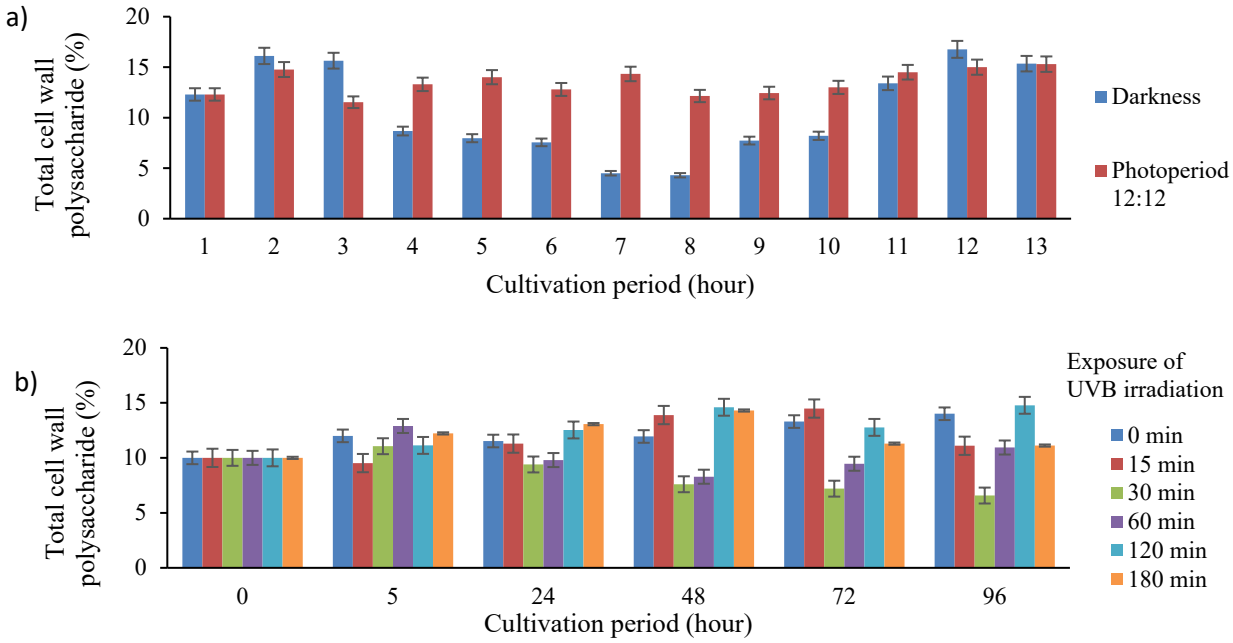

Figure 3: (a) Cell wall polysaccharides of *C. vulgaris* under darkness and (b) different exposure of UVB irradiation

The results of the study show that the viability of *C. vulgaris* cell were lower than 45 % after being exposed to UVB radiation for an extended period. After being exposed to UVB irradiation for 180 min, the cell's viability decreased immediately, reaching a maximum of 45 %, 5 hours later. Figure 3(b) depicts the total amount of polysaccharide in the cell wall after it is subjected to different types of exposure to UVB radiation. The results show that UVB irradiation for 30 min has the greatest effect on the cell wall thinning of the microalgae that have been cultured for more than 24 hours, whereas longer exposure (60 – 180 min) causes the cell wall to thicken. The cell wall thickness of *C. vulgaris* taken under different lengths of UVB irradiation are shown in Table 2, which correspond to the results of total cell wall polysaccharides content. The lowest percentage of cell wall polysaccharides was 6.6 %, with a cell wall thickness of 0.091 ± 0.044 µm after 24 hours culture period under 30 min of UVB irradiation.

Table 1: Cell wall measurement of *C. vulgaris* under darkness

Day	Darkness	Control	Day	Darkness	Control
	Cell wall thickness (mean ± sd) (µm)			Cell wall thickness (mean ± sd) (µm)	
1	0.174 ± 0.032	0.174 ± 0.032	8	0.124 ± 0.057	0.192 ± 0.061
2	0.206 ± 0.028	0.203 ± 0.081	9	0.141 ± 0.032	0.173 ± 0.047
3	0.191 ± 0.038	0.174 ± 0.027	10	0.187 ± 0.024	0.162 ± 0.048
4	0.127 ± 0.043	0.188 ± 0.036	11	0.218 ± 0.034	0.218 ± 0.023
5	0.125 ± 0.041	0.179 ± 0.034	12	0.233 ± 0.054	0.197 ± 0.068
6	0.096 ± 0.045	0.182 ± 0.027	13	0.228 ± 0.047	0.199 ± 0.053
7	0.076 ± 0.037	0.187 ± 0.052			

Table 2: Cell wall measurement of *C. vulgaris* under UVB irradiation

Cultivation period (hour)	0	5	24	48	72	96
Irradiation Period (min)	Cell wall thickness (mean ± sd) (µm)					
0	0.184 ± 0.052	0.142 ± 0.042	0.199 ± 0.037	0.217 ± 0.031	0.221 ± 0.036	0.231 ± 0.032
15	0.187 ± 0.038	0.141 ± 0.048	0.187 ± 0.025	0.201 ± 0.035	0.235 ± 0.024	0.207 ± 0.026
30	0.183 ± 0.043	0.152 ± 0.051	0.126 ± 0.074	0.119 ± 0.028	0.091 ± 0.035	0.095 ± 0.034
60	0.178 ± 0.037	0.184 ± 0.025	0.109 ± 0.044	0.115 ± 0.037	0.142 ± 0.024	0.194 ± 0.054
120	0.182 ± 0.062	0.161 ± 0.041	0.216 ± 0.056	0.206 ± 0.032	0.219 ± 0.063	0.222 ± 0.028
180	0.179 ± 0.047	0.176 ± 0.068	0.192 ± 0.033	0.226 ± 0.036	0.255 ± 0.035	0.263 ± 0.046

CFW is a fluorescent dye that binds β-glucans, such as cellulose, xyloglucans, callose, and chitin, and was used to observe cell walls of plant, fungi, and microalgae [25]. The cell wall of *C. vulgaris* was coated with distinct blue-white fluorescence after being treated with CFW for 30 min (Figure 4). The results show that the cell wall of *C. vulgaris* was thinner on the seventh day of darkness (Figure 4(b)) as compared to day 0 (Figure 4(a)). Figures 4(c) and 4(d) show that a significant amount of cell debris was present, indicating that the cells had broken apart due to UVB irradiation. The microscopy analysis proves that cell wall thinning occurs under both conditions and in accordance with the result obtained from total cell wall polysaccharides and cell wall thickness.

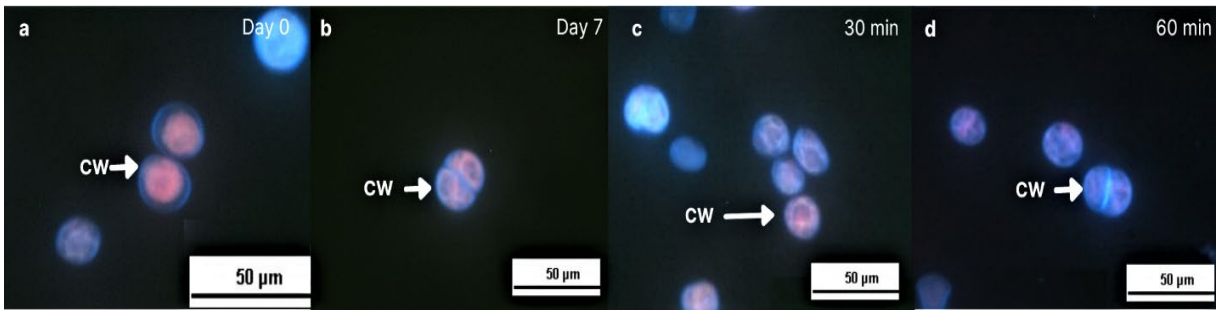

Figure 4: Fluorescence microscopy images of *C. vulgaris* UPSI-JRM01 stained with calcofluor white. (a) cultured under darkness on day 0, (b) cultured under darkness on day 7, (c) 30 min UVB irradiation and (d) 60 min UVB irradiation. CW: Cell wall. Thick cell wall observed on day 0 (a)

Meanwhile, Figure 5 shows the ultrastructure of *C. vulgaris* under TEM microscope. The TEM images show the thinning of the cell wall under stress conditions (Figure 5(b) to (e)) as compared to the control condition (Figure 5(a)). The TEM micrograph under the control condition shows a well-defined cell shape with smooth cell wall surfaces (Figure 5(a)). Meanwhile, under stress conditions, the cell wall was disintegrated and found in an irregular shape. In addition, irregular chloroplast ultrastructure was observed in Figure 5(b) and (c). The plastoglobuli that function as plastid lipid storage were found under longer UVB irradiation (60 min). A similar finding was reported in a study by Schmidt et al. [26] where the chloroplast was destructed and the number of plastoglobuli increased under intense UVB irradiation in *Gracilia domingensis*. In this study, blebbing of the cell membrane which is associated with programmed cell death (PCD) was also observed (Figure 5(e)). During PCD, cell autolysis which is triggered by environmental factors was believed to occur. Thus,

enhanced cell rupture is desirable for the recovery of lipids and carbohydrates under both stress conditions.

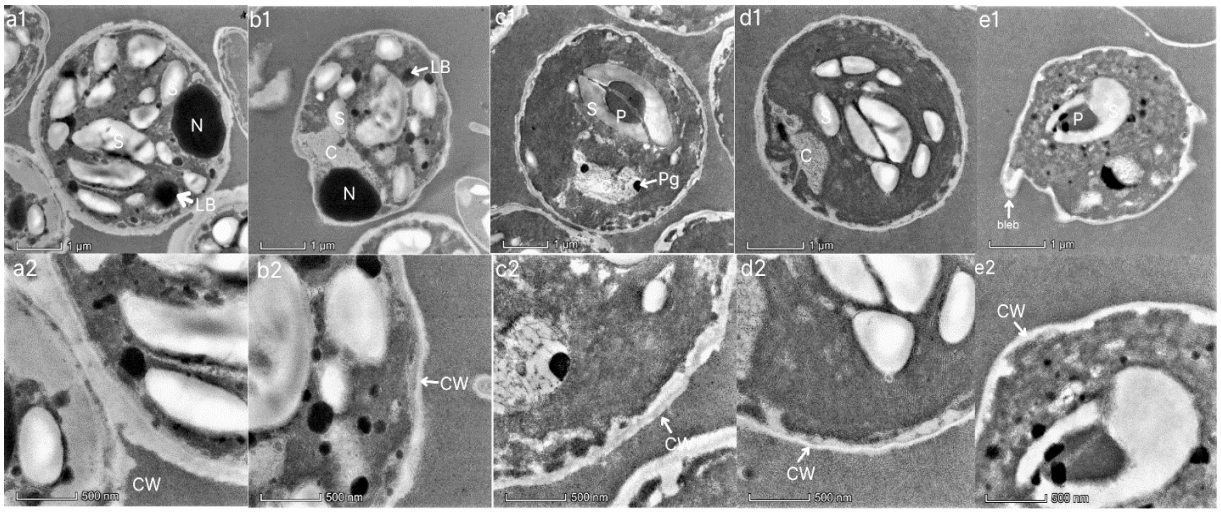

Figure 5: Ultrastructure of *C. vulgaris* UPSI-JRM01 cultured in different conditions. (a) control, (b) 30 min UVB irradiation, (c) 60 min UVB irradiation, (d) darkness on day 6 and (e) darkness on day 7. (a1- e1) cell section showing the whole cell, (a2- e2) detail showing part of the cell wall. C: chloroplast, CW: cell wall, LB: lipid body, N: nucleus, P: pyrenoid, Pg: plastoglobuli, S: starch granule

Conclusions

In this study, it was found that UVB irradiation produced higher lipid and carbohydrate yields than in the condition of darkness. Induction of cell wall thinning in *C. vulgaris* was feasible under both darkness and UVB irradiation, particularly on day seven of darkness and 30 min of UVB irradiation. The thinning of the cell wall was believed to be caused by autolysis triggered by stress factors. These findings are of prominence and are especially important for a cost-effective method of cell disruption in microalgae to extract lipids and carbohydrate biomolecules to be utilized as a biofuel feedstockage.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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