Research Article

Evaluation of Cell-Viability, Intracellular Lipid-Component and Efficiency of Lipid-Extraction of *Chlamydomonas reinhardtii* Cells Treated by UV-C Irradiation Aiming to Use Cell Directly

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Abstract

Aim: We aimed finally to construct a system to utilize intracellular lipids of *Chlamydomonas reinhardtii* by direct cell-use. To realize the system, a system of simple cell-sterilization to avoid environmental contamination without degradation of intracellular lipids was required. Industrially, a simple collecting system of internal lipids was also required.

Methods and results: *C. reinhardtii* cultured in a photo bioreactor under autotrophic condition was irradiated by UV-C. After the irradiation with different time to cells under different culturing conditions, those cells were evaluated for cell-viability by staining with neutral red, intracellular cell components and efficiency of lipid-extraction with GC/FID, respectively. By UV-C irradiation for 10 min, *C. reinhardtii* cells after N-depletion were sterilized. Additionally, although the cells were morphologically crumbled under an optical microscope, the contents and the components of intracellular lipids showed few differences.

Conclusion: *C. reinhardtii* cells were efficiently sterilized by UV-C irradiation and few treated cells leaked the intracellular lipids, indicating that the lipids could not be simply collected by centrifuging direct cell collection.

Significance and impact of study:

In this study, the sterilized cells could gradually leak the intracellular contents, indicating the possibility of direct-use of the cells to utilize lipids produced by *C. reinhardtii*.

Keywords: Green alga; Algal lipids; UV-C treatment; Direct cell-use

Abbreviations

GC/FID: Gas chromatography/flame ionization detector; N-depletion: Nitrogen depletion; OD: Optical Density; PBR: Photo-Bioreactor; SEM: Scanning Electron Microscope; UV: Ultraviolet; vvm: Volume per Volume per Minute

Introduction

In recent years, bio-production by photosynthetic plants has attracted attention because of the effectiveness of plant-derived components such as vitamins and oils, carbon recyclability and low environmental burden [1,2]. Regarding bio-production by plants, the material productivity is calculated by the product of biomass productivity and content of intracellular material [3], resulting in enhanced biomaterial productivity with high biomass productivity. Relating to biomass productivity, several green algal strains show superior biomass productivity with higher carbon fixation efficiency more 10-50 times than popular terrestrial plants [4] for instance, *Chlamydomonas* sp. JSC4 and *Dunaliella salina* exhibited high biomass productivity as 915mgL^{-1}d^{-1} [5] and 540mgL^{-1}d^{-1} [6], respectively. Depending on the high biomass productivity, green algae *Coccomyxa subellipsoidea* and *Haematococcus pluvialis*

demonstrated high material productivity of 232.37mgL^{-1}d^{-1} of lipid [7] and 17.1mgL^{-1}d^{-1} of astaxanthin [8]. Among the green algae, the unicellular green alga *Chlamydomonas reinhardtii* is especially paid attention in industrial use in recent years [9] because of accumulation of the information and technology of culturing condition [10], metabolic flow [11] and safety [12] regarding to this strain so far as a model green algae. Particularly, the proven safety of *C. reinhardtii* is an advantageous property for the use of its metabolites as value-added products in the industrial fields of cosmetics and foods. *C. reinhardtii* is able to accumulate approximate 10~20 % lipid (e.g., linoleic acid, lauric acid, stearic acid) as its cell component [13,14] so those lipids are expected to be used for moisturizing in the cosmetic field [15]. The use of lipids as the cell component, however, faces the problem of cost to extract lipids from the cells because of the robustness of the cell [16]. To extract lipids from the cell, *C. reinhardtii* needs strong crashing systems such as a bead-beater system [17]. Thus, research and development simply to use intracellular lipids could lead to an increase of the value of lipids derived from *C. reinhardtii* as a commercial use. Then, our study aimed to construct the system directly to use the cells for simple utilizing the intracellular lipids without the process of lipid purification based on non-toxicity [12].

The system, however, could be useful for industrial use; the direct use of living cells as the protector of intracellular metabolites could possibly cause contamination in the environment after releasing those cells to the environment [18], requesting a treatment simultaneously for supporting the efficient lipid-extraction and killing the cell. So far, the treatments using Ultraviolet (UV) irradiation have be considered [19], and the report revealed that the treatment using UV-B (280~320 nm) triggered the easy lipid-extraction because of necrosis and apoptosis of *C. reinhardtii*. However, the report focused on the extraction of intracellular lipids not aiming for complete cell death and does not evaluate the extraction efficiency relating nitrogen depletion in detail. Therefore, the treatment using UV irradiation needs more evaluations and improvements simply to use intracellular lipids by direct cell-use. On the other hand, although there are the reports relating the efficiency of sterilizing *C. reinhardtii* cells by the UV-C treatment, those did not refer to the efficiency of lipid-extraction [20,21]. Therefore, the system-construction to use cells directly for the efficient use of intracellular lipids by UV treatment could be drastically meaningful on industrial use.

In this study, *C. reinhardtii* containing lipids was treated by UV-C irradiation, and the cell viability was evaluated by using the staining method with neutral red. Simultaneously, the production and composition of lipids in the treated cells were analyzed to evaluate the effect of UV-C treatment using GC/FID. The use of lipids from the UV-C treated *C. reinhardtii* cells could lead the progression of industrially green-algal use.

Materials and Methods

Microalgal strain and PBR operation

Chlamydomanas reinhardtii strain C-9: NIES-2235 was cultivated in a photobioreactor filled with Modified Bold 3 N medium (MB3N) as described in a previous study [22]. The photobioreactor was equipped with white fluorescent lamps (100µmol photons·m^{-2}·s^{-1}) and a bubbling system of 0.8% CO_2 gas at an aeration rate of 0.05vvm and worked at room temperature (23ºC).

Evaluation of culturing condition

Cell growth was evaluated as cell numbers using value of Optical Density (OD) of 750nm with a spectro-photometer U-2900 (Hitachi, Tokyo, Japan) *via* appropriate calibration curve for OD750 versus cell numbers. The pH of the broth was measured with pH meter FEP20 (Mettler Toledo, Tokyo, Japan) after centrifugation at 5000×g for 1min at 23ºC. Nitrate concentration was measured using an optical method. The broth was centrifuged at 5,000×g for 1 min at 23ºC, and the supernatant was collected and filtered with a 0.45µm filter (Millex®-LCR 13mm, Millipore, Carrigtwohill, Ireland). The flow through was diluted 50-fold with distilled water, and the absorbance of the diluted supernatant was measured at 220nm (i.e., Abs220) using a spectro-photometer U-2900. The residual nitrate content was evaluated using an appropriate calibration curve for Abs220 versus nitrate concentration.

Evaluation of cell viability after UV-C treatment

Cells (5.0×10^5 cells) were irradiated in 2mL of phosphate buffered saline (PBS) (pH=7.4) on a 33mm diameter glass share by using a UV irradiator Etbella (SEVEN BEAUTY Co., Ltd., Tokyo, Japan) equipped with a UV-C lamp (the ultraviolet radiation output:

3.49mWcm^{-2}). One hundred eighty µL of the UV-treated suspension (4.5×10^4 cells) were mixed with 20µL of neutral red (red pigment) (Tokyo Kasei Co., Ltd., Tokyo, Japan) for 5 minutes [23]. The neutral red solution (1.5mgmL^{-1}) was prepared as below, 1.5mg of neutral red was dissolved in 1ml of PBS (pH=7.4); the solution was filtered with 0.22µm filter (Nylon Syringe Filter, Membrane-Solutions). After the staining process, the cells were washed with PBS, and the viability was evaluated by observing the stain-treated cells on cell counter plates (Fukae Kasei Co., Ltd, Kobe, Japan). The life/death of the cells was decided with the criterion: staining/not staining with neutral red.

Evaluation of intercellular contents of chlorophyll a/b after UV-C treatment

Cell-weights were evaluated for constant irradiation of UV-C to the cells. The cell-concentration in the broth was measured by using a calibration curve of OD750-weight, and then cells (0.1-0.5 mg) were collected from the broth by centrifugation at 5,000×g for 3min at 23ºC. After discarding the supernatant, 1mL of methanol was added and vigorously stirred using putiburu MODEL 2330 (Waken B tech Co., Ltd, Kyoto, Japan) at 2,000rpm for 5min at 23ºC. The solution containing chlorophyll a/b was collected by centrifugation at 5,000×g for 3min at 23ºC. To evaluate the concentration of chlorophyll a/b, the values of A665 and A652 of the collected solution were measured after drawing a baseline with A750 using methanol with a spectro-photometer U-2900 and substituted in the formula as below, chlorophyll a (µgmL^{-1}): 16.72 × A665 - 9.16 × A652; chlorophyll b (µgmL^{-1}): 34.09 × A652 - 15.28 × A665 [24]. The contents of chlorophyll a/b in the cells were finally shown using the unit µgmg^{-1}.

Preparation for analysis of lipid-extraction efficiency

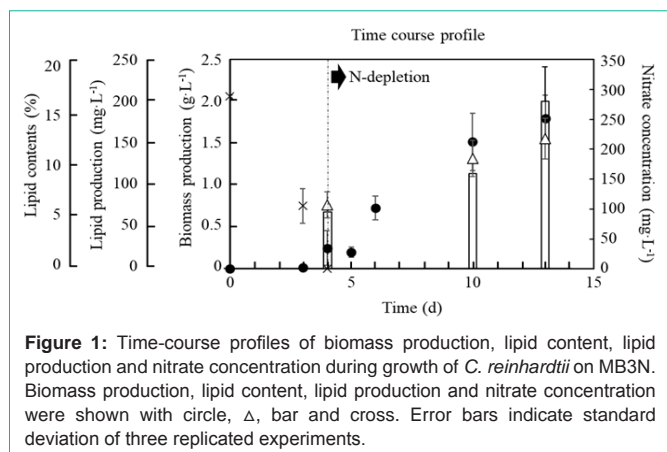One mg of cells was harvested from broth after 3 days of nitrogen depletion by centrifugation (5000×g, 5min, 23ºC) and treated by following 3 steps of cell-breaking processes as below: 1) UV-treatment step, 2) glass beads-adding step, and 3) shaking step. In the 1) step, the cells were UV-irradiated for 60min on a 33mm diameter glass share with a UV irradiator Etbella (SEVEN BEAUTY Co., Ltd.). In the 2) step, the glass beads of 0.5mm were added into the cells. In the 3) step, the cells were vortexed at 23ºC under the condition (2,500rpm, 60min) with putiburu MODEL 2330 (WakenBtech Co., Ltd, Kyoto, Japan). After those processes, the treated cell-components were dried with an evaporator and weighted in detail.

Evaluation of lipid-quantity and profile

The total lipids were esterified and methylated with fatty acid methylation kit (Nacalai Tesque, Kyoto, Japan). The fatty acid methyl esters were identified and quantified using a capillary gas chromatograph GC-2025 (Shimadzu, Kyoto, Japan) equipped with a DB-23 capillary column (60m, 0.25mm internal diameter, 0.15µm film thickness) (Agilent Technologies, CA, United States) with method previously described by Nakanishi et al. [13]. Heptadecanoic acid (Sigma-Aldrich Co., MO, US) was used as an internal standard, and rapeseed oil (Merck KGaA, Darmstadt, Germany) was used as a quantitative standard.

Results and Discussion

This study aimed to construct a practical system that could easily use intracellular lipids by directly using the cells of *C. reinhardtii*. First of all, the time-course profile of *C. reinhardtii* under each

Figure 1: Time-course profiles of biomass production, lipid content, lipid production and nitrate concentration during growth of *C. reinhardtii* on MB3N. Biomass production, lipid content, lipid production and nitrate concentration were shown with circle, Δ, bar and cross. Error bars indicate standard deviation of three replicated experiments.

Figure 2: Cell-viability after UV irradiation for 10min. Time-course profiles of cell-viabilities on 0 day, 6 day and 9 day after nitrogen depletion were shown as circle, triangle and cross, respectively. Error bars indicate standard deviation of three replicated experiments.

culture condition was evaluated (Figure 1). In this study, *C. reinhardtii* was cultured in autotrophic MBBM, resulting that the biomass production was 248 ± 20 mgL^{-1} at 96h (4d) of culture. So far, the abundant information of biomass production of *C. reinhardtii* cultured in various media has shown various data: around 700mgL^{-1} in 4d of culture in a well-growing heterotrophic TAP medium; around 150mg in an autotrophic HSM medium [25], indicating that the autotrophic culturing system in this study works well. Although the nitrogen depletion was shown at 4d, the biomass production constantly increased and finally reached 1.8 ± 0.3gL^{-1} in 13d (9d of nitrogen depletion (9d N-depletion)). Additionally, the results also indicated that the cultural conditions at 4d (0 d N-depletion) and 13d (9 d N-depletion) showed initial and late log-phase in this study, respectively (i.e., log-phase: 4 ~ 13 d).

Increasing lipid contents in the cells were generally triggered by depletions of medium-sources of nitrogen, phosphate and sulphate so a lot of trials of lipid-production by *Chlamydomonas* sp. was performed by N-depletion [26,27]. In this case, 6.2%, 10.5% and 12.4% of lipid contents were shown at N-depletions of 0 d, 6 d and 9 d, indicating that the trend increasing the contents could be derived from nitrogen starvation. Those lipid productions were slightly similar to ones in previous reports [19] so that the generality was certified for lipid-producing ability by *C. reinhardtii* and culturing system as PBR used in this study.

To construct a practical system to apply intracellular lipids by directly using cells, the user-friendly sterilizing system should be developed. In this study, UV-C irradiation, which is widely used for sterilizing microorganisms, was selected. For using the intracellular lipids, the cells after N-depletion should be required since the N-depletion strongly triggers to increase the lipids in the cells of *C. reinhardtii* [13,14]. Then, the cell-condition (e.g., viability and composition) should be different in each culture condition so that the effects of the sterilizing treatment by UV-C irradiation were evaluated on 0 d, 6 d and 9 d of N-depletion (Figure 2). As the results, the viabilities of cells before UV-C irradiation were 82.9%, 9.8% and 1.4% at 0 d, 6 d and 9 d of N-depletion, meaning that extending the culturing days after N-depletion led to decreasing the viabilities. The low cell-viabilities less than 10% at 6 d and 9 d of N-depletion and the effectively UV-irradiating affection to cells in log-phase rather than ones in stationary-phase [19] indicated the possibility effectively
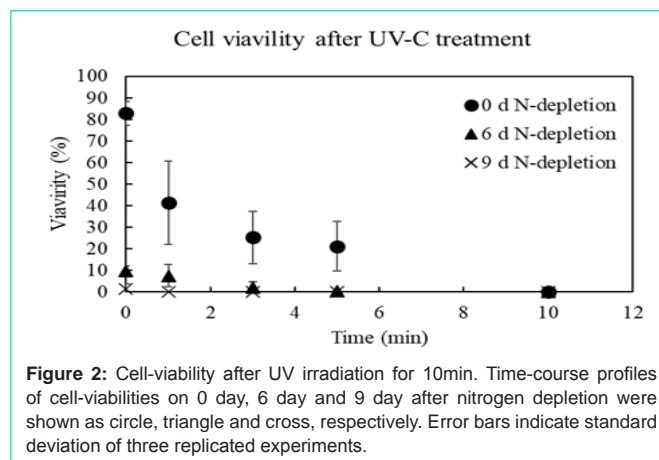
to sterilize the cells in N-depletion by using UV-C irradiation. The UV-C irradiation actually affected those cells as the sterilizer and the ten minutes irradiation completely sterilized cells at all conditions prepared in this study. However, five or less than five minutes irradiation should be required as a sterilizing treatment because of the possibility of adverse effects (e.g., lipid peroxidation) deriving from the high energy of UV-C. The UV-C treatment in 5 min showed 21.2%, less than 1% and 0% of cell-viabilities at 0 d, 6 d and 9 d of N-depletion. The result indicated that the UV-C treatment could lead to the complete death with progressively N-depleting cells. Especially, the one-minute treatment by UV-C was enough to reach 0% of the viability of cells at 9 d N-depletion, showing the effective sterilization. On the other aspect, UV treatment in this study was a useful system since the output of UV was 3.5mWcm^{-2}, meaning that the energy-requirement was lower than 1.5Wcm^{-2} of the output of UV previously reported [19].

The purpose in this study is to use intracellular lipids after UV-C treatment simply sterilizing *C. reinhardtii*. Therefore, the time-course shift of amounts of chlorophyll and the change of lipid composition related to UV-C irradiation were analyzed to evaluate the effect of UV-C in cells (Figure 3 and Table 1). The total amount of intracellular chlorophyll a and b at 0 d N-depletion showed a tendency to increase once at 5 minutes of UV-C irradiation and then decrease at 15 minutes. To date, Nassour et al. reported that short-term UV-C irradiation to *C. reinhardtii* increases the intracellular content of chlorophyll a and b [24]. In particular, UV-C irradiation was known to increase chlorophyll a in *C. reinhardtii* [24; 28] and chlorophyll b in *C. reinhardtii* [24] and *Chlorella* sp. [29]. On the other hand, several reports referred to chlorophyll degradation because of the collapse of chlorophyll on thylakoid membrane by UV-treatment [24,30-32]. The basic knowledge indicated the tendency in the cells at 0 d N-depletion as follows: the increasing the amount of chlorophyll a and b until 5min of UV-C irradiation, i.e., progressing cell-death as shown in Figure 2; the maintaining the amount of chlorophyll a and b to 10min of the irradiation; the decreasing the amount of chlorophyll a and b toward 15min of the irradiation. This study also revealed the higher amount of chlorophyll b than chlorophyll a and the increase of those amounts as response to the UV-C irradiation, which was remarkable in the cells at 0 d N-depletion. A previous report indicated that chlorophyll b could perform not only to transfer the light energy to the reaction centers in photosynthetic
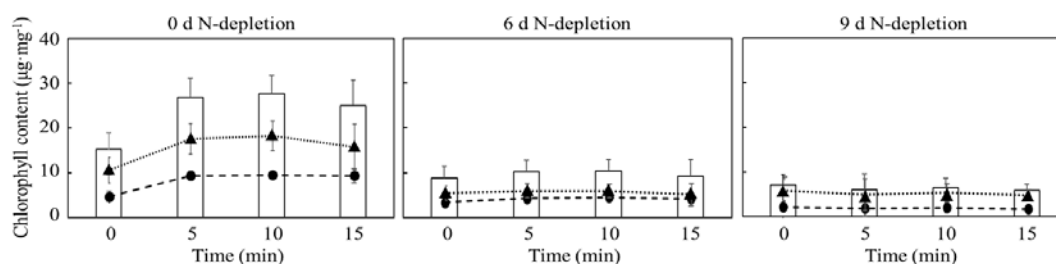
Figure 3: Contents of chlorophyll a, chlorophyll b and chlorophyll a/b in *C. reinhardtii* on 0 d, 6 d and 9 d N-depletion before/after UV irradiation for 5, 10 and 15 min. Contents of chlorophyll a, chlorophyll b and chlorophyll a/b were shown with circle, triangle and bar. Error bars indicate standard deviation of three replicated experiments.
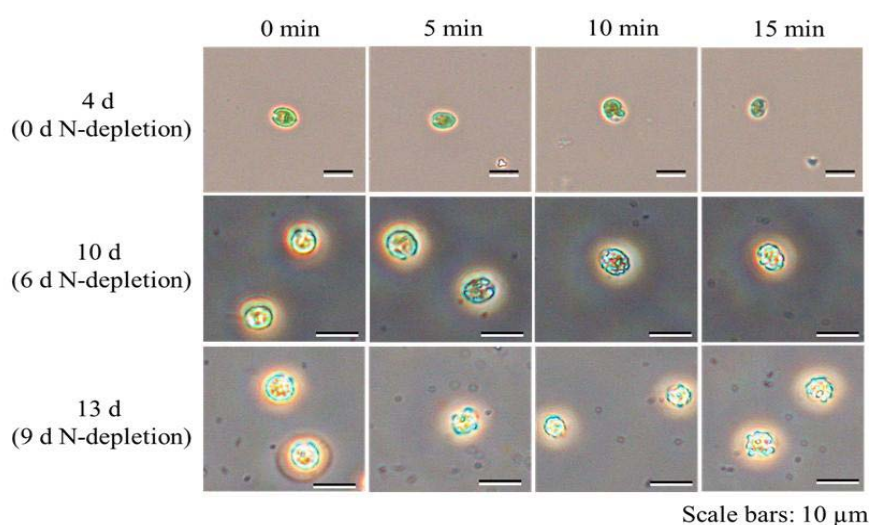
Figure 4: Optical micrographs of *C. reinhardtii* on 0 d, 6 d and 9 d N-depletion before/after UV irradiation for 5 and 10 min.

systems II and I but also to protect chlorophyll a from strong light such as UV [24], supporting the explanation of our results. In cells at 6 d N-depletion, the total amount of intracellular chlorophyll also showed a tendency of increase once at 5 minutes of UV-C irradiation time, and then of decrease at 15 minutes. Although the response of cells at 6 d N-depletion was weak rather than one at 0 d N-depletion, the tendency could be deeply related to the lower cell-viability (9.8%) at 6 d N-depletion than at 0 d N-depletion. In addition, in cells at 9 d N-depletion, the total amount of intracellular chlorophyll could tend to decrease throughout the irradiation time, also relating to the low viability (1.4%). Therefore, those results showed the correlation between the responsiveness of chlorophyll-amount and the viability, and the influence of UV-C toward the intracellular component. As the next step to analyze the UV-C effect to the intracellular, the effect was evaluated by comparing between the ratios of amounts of intracellular lipids before/ after UV-C irradiation (Table 1). After UV-C irradiation, the ratios of linoleic acid (C18:2) and linolenic acid (C18:3) tended to decrease in the cells at 0 d N-depletion; the ratios of unsaturated C18-lipids including oleic acid (C18:1) trendy decreased in the cells at 6 d N-depletion. Although the previous report used UV-B to analyze the effects of UV towards the intracellular of *C. reinhardtii* cells, the report revealed the lipid degradation including C18-lipids [19], corresponding to the results in this study. The UV-C irradiation normally prompts lipid peroxidation [33-35], so that

Table 1: Ratios of change of lipid-compositions by UV-irradiation. The amount of each intracellular lipid after UV-C irradiation was divided with the one before UV-C irradiation. The results were collected from three replicated experiments.

	0 d-N depletion	6 d-N depletion	9 d-N depletion
Lipid Composition (*w/w*)			
Lauric acid (C12:0)	1.08 ± 0.14	1.01 ± 0.01	1.04 ± 0.06
Myristic acid (C14:0)	1.07 ± 0.16	1.03 ± 0.09	1.10 ± 0.21
Palmitic acid (C16:0)	1.05 ± 0.17	0.95 ± 0.03	1.07 ± 0.09
Stearic acid (C18:0)	1.08 ± 0.20	0.98 ± 0.04	1.10 ± 0.14
Oleic acid (C18:1)	1.15 ± 0.66	0.86 ± 0.02	1.08 ± 0.08
Linoleic acid (C18:2)	0.76 ± 0.34	0.74 ± 0.07	1.00 ± 0.12
Linoleic acid (C18:3)	0.80 ± 0.44	0.59 ± 0.08	0.96 ± 0.17

C18-lipids could possibly protect the intracellular from the adverse effects derived by UV-C. On the other hands, after UV-C irradiation, the ratios of the lipid amounts showed a few differences in the cells at 9 d N-depletion. The viability of cells might relate to the UV-C effects toward lipids (e.g., metabolic flow) because of low cell-viability (1.4%) at 9 d N-depletion even before UV-C irradiation.

The disruption of outer cell-structure by UV-C irradiation led to lipid leakage from cells before lipid collection, resulting in loss of lipids. Previous studies reported that *C. reinhardtii* causes necrosis
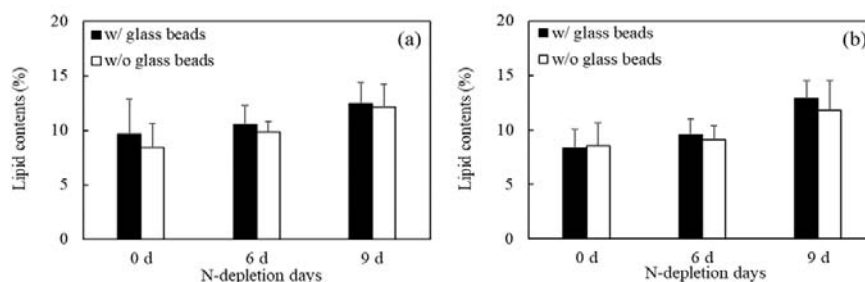
Figure 5: Evaluation of lipid-extraction methods with/without glass beads for cells treated with/without UV-C after nitrogen depletion of 0 d, 6 d and 9 d. Black/white bars meant the evaluation with/without glass beads; (a) and (b) showed before and after UV-C treatment for 10min. Error bars indicate standard deviation of three replicated experiments.

and apoptosis by UV-B irradiation and then the cells collapse [19]; therefore, the observation of outer cell-structure after treatment of UV-C with an optical microscope was significantly important (Figure 4). After 5 minutes of UV-C irradiation, the cells under the N-depletion formed a small grain-like structure in the cells and showed a necrosis-like response. Appearing the grain-like structure in the cells indicated that the UV-C treatment could effectively work for sterilizing cells. In fact, the *C. reinhardtii* necrosis caused by a protease inhibitor showed the multiple small grain-like structures in the cells [36]. In addition, the outline of the cells at 6 d and 9 d N-depletion became unclear after 15min and 5min of UV-C irradiation, respectively. Generally, the cell-outline of *C. reinhardtii* could be unclear after UV irradiation [37], and the knowledge corresponds to our results. In the case that the unclear outline might mean disruption of cells, the cell components could be possibly leaked from the cell. Although there could not be drastic differences in lipid contents in the cells before or after UV-B treatment [19], no research reveals the effect of lipid contents in the cells after UV-C treatment. Thus, the leakage was evaluated by analyzing lipid contents in the cells before and after UV-C irradiation.

In order to clarify the effect of UV-C irradiation on the leakage of lipids from cells and the change in the efficiency of lipid-extraction, the lipid-extraction efficiency was evaluated by analyzing the lipid contents in the cells under the conditions of before/after UV-C treatment and with/without glass beads (Figure 5). First of all, the effects derived from UV-C irradiation were evaluated toward the leakage of intracellular lipids from cells. Under the extraction process with glass beads as a normal process, the lipid contents did not show significant differences even with UV-C irradiation. In this study, the lipid was extracted from UV-C irradiated cells after excluding liquid-phase by centrifugation. Thus, no significant differences of the lipid contents at N-depletion of 0 d, 6 d and 9 d indicated that the UV-C treated cells could not leak the intracellular lipids to liquid-phase. Secondly, the change in the efficiency of lipid-extraction by UV-C irradiation was evaluated by comparing the lipid-contents extracted with/without the glass-beads treating process, resulting in no significant differences. The results indicated that the UV-C irradiated cells could not show the leakage of intracellular lipids, meaning that the lipids could be simply collected by centrifugation. In our research, the UV-C irradiated cells could not leak the lipids from the cells; however, the sterilized cells might gradually promote to leak the intracellular components depending on the progressive cell-collapse. The direct-use of cells could possibly show the simple utilization of intracellular lipids.

Conclusion

This research ultimately aimed to utilize the intracellular lipids simply by the direct use of the green algal cells without environmental contamination. The experimental results revealed that the cells after N-depletion were sterilized by the UV-C irradiation for 10 min and few differences of lipid contents after UV-C irradiation. Although the UV-C irradiated cells could not show the leak of the lipids from the cells, the sterilized cells might promote the leak of the intracellular lipids by the progressive cell-collapse, indicating the possibility of the direct-use of cells to utilize the intracellular lipids.

Acknowledgment

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