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Enhancing lipid productivity of *Chlorella vulgaris* using oxidative stress by TiO_2 nanoparticles

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Abstract−Ability to increase the lipid production in microalgae is one of the heavily sought-after ideas to improve the economic feasibility of microalgae-derived transportation fuels for commercial applications. We used the oxidative stress by TiO_2 nanoparticles, a well-known photocatalyst, to induce lipid production in microalgae. *Chlorella vulgaris* UTEX 265 was cultivated under various concentrations of TiO_2 ranging from 0.1 to 5 g/L under UV-A illumination. Maximum specific growth rate was affected in responding to TiO_2 concentrations. In the presence of UV-A, chlorophyll concentration was decreased at the highest concentration of TiO_2 (5 g/L TiO_2) by oxidative stress. The fatty acid methyl ester (FAME) composition analysis suggested that oxidative stress causes the accumulation and decomposition of lipids. The highest FAME productivity was 18.2 g/L/d under low concentrations of TiO_2 (0.1 g/L) and a short induction time (two days). The controlled condition of TiO_2/UV-A inducing oxidative stress (0.1 g/L TiO_2 and two days induction) could be used to increase the lipid productivity of *C. vulgaris* UTEX 265. Our results show the possibility of modulating the lipid induction process through oxidative stress with TiO_2/UV-A.

Keywords: TiO_2, Nanoparticles, Oxidative Stress, *Chlorella vulgaris*, Fatty Acid Methyl Ester

INTRODUCTION

Much of the world is confronted by an energy crisis and the consequent anxiety due to the high levels of dependency on fossil fuels. The exhaustion of fossil fuels has been pointed out continuously, and global warming and climate change are caused by the high consumption of fossil fuels [1]. Under this situation, interest in the development of alternative energy sources such as biofuel, which originates from biomass, has increased. However, first-generation types of biomass, such as soybeans and corn, have problems due to the lack of land use for large-scale cultivation. In addition, there is an ethics issue as to whether or not food materials should be used for biofuels. Second-generation types of biomass, including cellulosic feedstock, are also not ideal materials in that pre-treatment is very difficult [2].

Microalgae, a third-generation type of biomass, are garnering increased research attention, as they are highly advantageous as a reusable biomass. First, they are a ubiquitous eukaryote, growing in fresh water and in sea water. Therefore, it is not necessary to devote a large amount of land for cultivation. Second, cultivation requires less time compared to first- and second-generation types of biomass. Third, regarding environmental problems, microalgae consume CO_2 in the atmosphere. Finally, the most important advantage is that they are good candidates to produce biodiesel as well as value-added products such as food-additives, cosmetics, and industrial materials [3]. However, while there are many advantages of algal biodiesel, major issues remain. Biodiesel originating from microalgae has not been commercialized thus far due to the high cost of the production processes, from cultivation to biodiesel conversion. Therefore, it is necessary to reduce the production cost by devising new methods [4]. Generally, while microalgae grow and increase their cell population under an optimized condition, they rapidly accumulate a large amount of lipids or value-added products under stress conditions. Thus, research on lipid induction processes under stress conditions is valuable to increase lipid productivity. So far, many methods have been developed for enhancing the lipids content and productivity under various stress conditions, such as nitrogen starvation, high amounts of light, and high salt conditions [5-8].

Among the various stress conditions, we focused on the oxidative stress from photocatalyst similar to high light stress condition. Under high light stress, microalgae accumulate a large amount of reactive oxygen species (ROS) including singlet oxygen ($^1O_2$*), superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals, resulting in the direct decomposition of the lipids, protein, and nucleic acids [9]. On the other hand, as a protection mechanism, microalgae have an ROS-scavenging ability. For example, antioxidant enzymes such as glutathione peroxidase and superoxide dismutase are increased and antioxidant molecules such as ascorbate, glutathione, carotenoids and tocopherols are increased as well in response to photo-oxidative stress [9]. Moreover, triacylglycerol (TAG) can be synthesized as an efficient way to reduce photo damage by excess light energy, because TAG biosynthesis requires twice higher energy than carbohydrate synthesis [10].

Normally, ROS can disintegrate organic compounds and inacti-

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‡This paper is dedicated to commemorate Prof. Ji-Won Yang (KAIST) on his retirement.

vate microorganisms. In addition, nanoparticles are responsible for toxicity to the microalgae itself [11,12]. Thus, a nanosized photocatalyst can be used to kill microorganisms and treat algal blooms according to environmental findings [13,14]. Generally, free electrons in TiO_2 are activated by UV from the valence band to the conduction band, and these delocalized electrons induce ROS [15,16]. In this study, to increase lipid content of *Chrolella vulgaris* and understand how an oxidative stress condition relates to lipid productivity, TiO_2 nanoparticles were used as the source of artificial oxidative stress. Here, we report an possible stress condition for high lipid productivity that is modulated by TiO_2 and also introduce a simple lipid induction process that can be further developed as an on/off switch for lipid induction by TiO_2 and UV-A. In addition, our research shows the possibility that it can be applied to various step of biodiesel production from microalgae, because various applications of nanoparticle such as harvesting of microalgae have been conducted.

MATERIALS AND METHODS

1. Methylene Blue Test

To confirm whether or not the intended oxidative stress caused by the photocatalyst arises in a microalgae cultivation system, a methylene blue (MB) degradation experiment was conducted. A solution of 10 mg/L MB (Sigma Aldrich, USA) was used to check the performance of Degussa P25 TiO_2. Degussa P25 TiO_2 0.1 g/L was added to 100 ml of the MB solution. To create the same condition for microalgae cultivation, the reaction was incubated under the following conditions: 25 °C, 120 rpm, and 170 μmol photons/m^2/s of light intensity. To measure the ROS activity, four combinations of conditions were applied: 1) a white fluorescence light, 2) a white fluorescence light+TiO_2, 3) a white fluorescence light+UV-A, and 4) a white fluorescence light+UV-A+TiO_2. The effect of white fluorescent light needed to be tested, because microalgae were phototrophically grown under light condition. The wavelength of the UV-A light (Zeyko, Republic of Korea) ranged from 320 to 400 nm. TiO_2 was removed by a 0.20 μm syringe filter (Sartorius Stedim Biotech, Germany) just before the degree of the decolorizing effect was checked. The degree of producing ROS from TiO_2 was evaluated in terms of the optical density (OD) at 668 nm every hour for seven hours [17]. The decolorizing ratio was calculated by Eq. (1):

$$\text{Decolorizing ratio (\%)} = \frac{(\text{ABS[Initial]} - \text{ABS[Final]})}{\text{ABS[Initial]}} \times 100 \quad (1)$$

Here, ABS[Initial] and ABS[Final] denote the initial OD and the final value after seven hours, respectively.

2. Microalgal Cultivation

Chlorella vulgaris UTEX 265 was purchased from the UTEX culture collection (University of Texas, Austin). All samples were phototrophically cultivated in tris-acetate phosphate (TAP) media [18]. A two-step cultivation process was applied. In the first step, the strain was inoculated to 400 ml of TAP media. *C. vulgaris* UTEX 265 was grown until approximately 0.6 g/L DCW under optimum conditions for four days. Samples were incubated under the condition at 25 °C, 120 rpm, and 170 μmol photons/m^2/s of light intensity. In the second step, various concentrations (0, 0.1, 1, 2.5, and 5 g/L) of TiO_2 were added to 200 ml of *C. vulgaris* UTEX 265 culture. Before the addition of the TiO_2, the TiO_2 stock solution was sonicated for dispersion equally. For the induction stage, UV-A was used when necessary. Then, each sample was incubated for four days until samples were collected for various assays.

3. Chlorophyll Analysis

The chlorophyll concentration was measured by a UV/Vis spectrophotometer (DU 730, Beckman Coulter, USA) after the extraction of chlorophyll using methanol (Merck, Darmstadt, Germany). For harvest, 5 ml of microalgal culture was centrifuged at 7,000 rpm for 10 min. After centrifugation, the supernatant was removed and the remaining pellet was washed twice with deionized water. After the supernatant was removed, 5 ml of methanol was added to the pellet and the cells were resuspended by rapid stirring. The sample was kept in a refrigerator in the dark at 4 °C for 30 min to extract the chlorophyll. After the extraction, the samples were centrifuged at 7,000 rpm for 10 min, and 2 ml of supernatant was centrifuged again at 13,000 rpm for 10 min to obtain a clear extract of chlorophyll. The optical densities of the extracts were measured at 652 nm and 665 nm using the UV/Vis spectrophotometer. The concentrations of chlorophyll a and chlorophyll b were determined by Eqs. (2) and (3), respectively [19]:

$$\text{Chla [μg/ml]} = -8.0962 \times OD_{652} + 16.5169 \times OD_{665} \quad (2)$$

$$\text{Chlb [μg/ml]} = 27.4405 \times OD_{652} - 12.1688 \times OD_{665} \quad (3)$$

4. Measurement of the Specific Growth Rates

Specific growth rates were calculated based on the dry cell weight (DCW). For the DCW, the method to calculate the volatile suspended solid (VSS) was applied [20]. First, Whatman GF/C filter paper washed with deionized water on an aluminum dish was placed in a furnace at 550 °C for 30 min. Five-ml of sample containing TiO_2 was filtered by GF/C filter paper. After drying at 105 °C in an oven overnight, the samples were weighed. After the weight was determined, the GF/C filter papers with the samples were put into a furnace at 550 °C for 30 min to remove the organic materials, that is, the microalgae. After removal of all organic compounds through burning at 550 °C, only the microalgae burned out while inorganic materials such as TiO_2 remained on the GF/C filter paper. Thus, the difference in the mass before and after burning the samples was the DCW of *C. vulgaris* UTEX 265. Based on the calculated biomass, the specific growth rate (μ) was evaluated by Eq. (4):

$$\mu = \frac{\ln(M_2/M_1)}{T_2 - T_1} \quad (4)$$

where M_1 and M_2 are the DCW at T_1 and T_2, respectively. The maximum specific growth rate (μ_{MAX}) was calculated by the DCW on the first and second day after the addition of TiO_2 where cells were in the exponential phase. The average specific growth rate (μ_{AVE}) was calculated by the DCW of the first and last cultivation day after the addition of TiO_2.

5. Fatty Acid Methyl Ester (FAME) Analysis

The total FAME content was determined by gas chromatography (GC) analysis after the transesterification of the extracted lipid according to a modified protocol [21,22]. After cultivation, the samples were harvested and subsequently centrifuged with high-speed centrifuge (Supra-22k, Hanil Science Industrial, Republic of Korea). The samples were centrifuged at 7,000 rpm for 10 min. The *C. vul-*

garis UTEX 265 was cleaned twice with deionized water. The harvested biomass was frozen at −70 °C overnight and then lyophilized with a freeze dryer for three days. The lipid was extracted by a chloroform/methanol solvent mixture (2 : 1, v/v). The solvent mixture was added to 10 mg of biomass and mixed in Teflon-sealed screw-capped Pyrex tubes for 10 min vigorously. After the extraction of the lipid, 0.5 mg of heptadecanoic acid (C17:0) was added as an internal standard, and 1 ml of methanol and 300 μl of sulfuric acid were added to induce the transesterification reaction. The samples were placed in a heating block at 100 °C for 20 min and were then cooled to room temperature. After cooling, 1 ml of 0.3 M NaOH solution was added to the samples to wash residual methanol and sulfuric acid under rapid stirring for 5 min. After centrifuging at 4,000 rpm for 10 min to separate the organic and aqueous phase, the lower layers of the samples were extracted using a syringe and the resulting samples were filtered by a 0.20 μm RC-membrane syringe filter (Sartorius Stedim Biotech, Germany).

Lipid content and composition were analyzed by gas chromatography (HP 6890, Agilent, USA) equipped with a flame ionized detector (FID) and a HP-INNOWax polyethylene glycol column (HP 19091N-213, Agilent, USA). The FAME peak was quantified based on a 37-component FAME standard mix (F.A.M.E. Mix C8-C24, Supelco, USA). The temperature of the GC column was increased from 50 to 250 gradually at 15 °C per min. Finally, the amount of total lipid was calculated by summing up all areas of the FAME peaks except for the solvent peaks, and each composition was classified based on the FAME standard mix in each case.

RESULTS AND DISCUSSION

1. Identification of the Stress Conditions by ROS

To test the production of ROS caused by TiO_2, the degradation of MB was evaluated under the same condition used in the *C. vulgaris* UTEX 265 cultivation system. Fig. 1 shows the production of ROS which was examined by the degradation of MB under four different conditions. The result revealed that under the fluorescent lamp-combined-with-UV-A condition as well as the fluorescent lamp condition in the absence of TiO_2, MB was not degraded significantly, indicating that these two conditions did not produce ROS. As expected, however, the MB was degraded in the presence of TiO_2. Specifically, under the lamp-combined-with-UV-A condition in the presence of TiO_2, the MB was rapidly decolorized within an hour and continuously degraded up to seven hours, showing a decolorizing ratio of approximately 90.5%. In addition, although the decolorizing ratio was lower compared to UV-A illumination, TiO_2 could produce ROS and remove the color of MB under the fluorescent-lamp-only condition, showing a decolorizing ratio of 49.4%. It likely seems that the fluorescence lamp also emitted various wavelengths of light, not only visible light but also UV. This result confirmed that under the UV-A, TiO_2 produced oxidative stress in culture condition.

2. The Effect on *C. vulgaris* UTEX 265 Growth by TiO_2 in the Absence of UV-A

Before the effect of oxidative stress by TiO_2 on microalgae is analyzed, other stress factors generated by TiO_2 nanoparticles should be considered, because it has been reported that TiO_2 nanoparticles are known to cause cytotoxicity to microalgae [23]. We first tested the effects of TiO_2 to *C. vulgaris* UTEX 265 in the absence of UV-A by measuring the concentrations of chlorophylls, specific growth rate, and DCW during the cultivation of *C. vulgaris* UTEX 265. Fig. 2 shows that the total amount of chlorophylls in *C. vulgaris* UTEX 265 grown under various concentrations of TiO_2 (from 0 to 5 g/L) did not change significantly, while the total amount of chlorophyll was increased during the cultivation of *C. vulgaris* UTEX 265 under all conditions. Moreover, the average specific growth rate and DCW of two days and four days after induction did not change

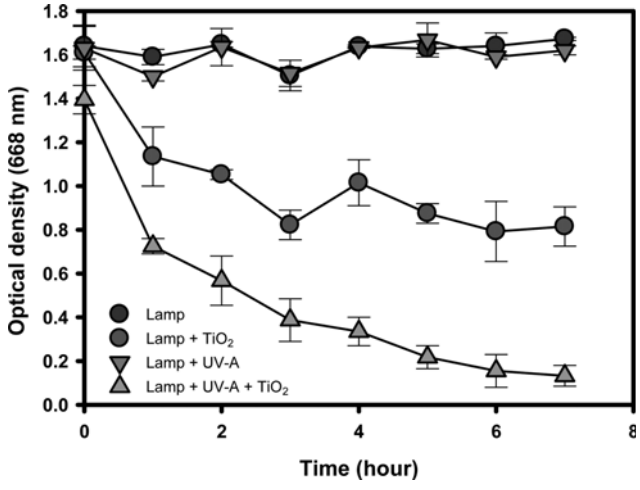

Fig. 1. Degradation curve to check the effect of methylene blue by ROS fromTiO_2 under the different conditions: no TiO_2 with the fluorescent lamp, TiO_2 with the fluorescent lamp, no TiO_2 with the fluorescent lamp and UV-A, and TiO_2 with the fluorescent lamp and UV-A. Samples were incubated with 100 ml of MB solutions at 25 °C, 120 rpm, and 170 μmol photons/m^2/s of a fluorescent lamp and 5.5 μmol photons/m^2/s of UV-A. The data points represent the average of samples and error bars indicate standard error (n=2).

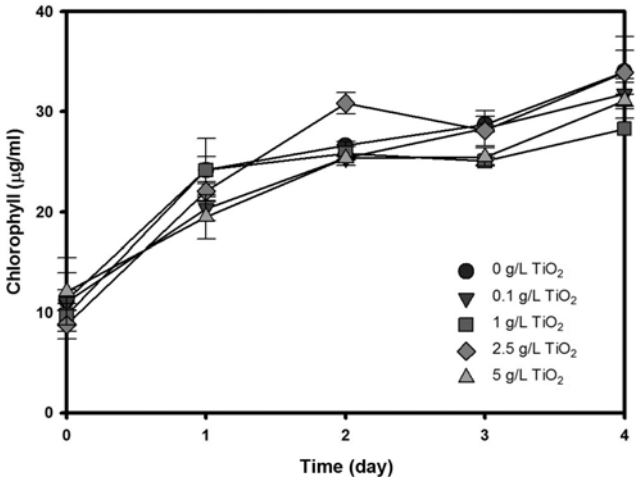

Fig. 2. Growth curve by chlorophyll in the absence of UV-A with the different concentrations of TiO_2: 0 g/L, 0.1 g/L, 1 g/L, 2.5 g/L and 5 g/L. After four days cultivation under the optimized culture condition, TiO_2 was added to 200 ml of culture. The *C. vulgaris* UTEX 265 was cultivated at 25 °C, 120 rpm and 170 μmol photons/m^2/s of a fluorescent lamp in TAP media. The data points represent the average of samples and error bars indicate standard error (n=2).

Table 1. The specific growth rates and DCW values in the absence of UV-A illumination[a]

TiO_2 (g/L)	μ_{max}[b] (d^{-1})	μ_{ave}[c] (d^{-1})	DCW (at 2 day)[d] (g/L)	DCW (at 4 day)[e] (g/L)
0	0.43±0.071	0.15±0.030	1.06±0.040	1.10±0.040
0.1	0.31±0.187	0.11±0.006	0.96±0.020	0.99±0.010
1	0.31±0.026	0.12±0.022	1.00±0.040	1.04±0.060
2.5	0.31±0.036	0.12±0.010	0.95±0.050	1.07±0.010
5	0.22±0.049	0.12±0.007	1.00±0.020	1.07±0.030

[a]The *C. vulgaris* UTEX 265 was cultivated at 25 °C, 120 rpm, and 170 μmol photons/m^2/s of a fluorescent lamp in TAP media with different concentrations of TiO_2 (0, 0.1, 1, 2.5, 5 g/L). The data points represent the average of samples and error bars indicate standard error (n=2)
[b]μ_{max} means maximum specific growth rate based on DCW of 1 days and 2 days after addition of TiO_2
[c]μ_{ave} means average specific growth rate based on DCW of 1 days and 4 days after addition of TiO_2
[d]DCW (at 2 day) means DCW at 2 day after addition of TiO_2
[e]DCW (at 4 day) means DCW at 4 day after addition of TiO_2

significantly under various concentrations of TiO_2 nanoparticle (Table 1). However, specifically, we found that the maximum specific growth rate (μ_{MAX}) decreased by approximately 50% under a high concentration of TiO_2 (5 g/L) compared to the 0 g/L TiO_2 condition (Table 1). This means that although DCW of two days and four days after addition of TiO_2 is similar, DCW reached stationary phase early at 0 g/L TiO_2 compared to 5 g/L TiO_2 condition. These results suggest that the viability was not affected by TiO_2 based on chlorophyll measurement, while a high amount of TiO_2 seemed to affect the instantaneous growth of *C. vulgaris* UTEX 265.

Based on these results, several effects of TiO_2 nanoparticle except for oxidative stress could be considered. First, shading effects of nanoparticles could affect μ_{MAX} due to reduced light penetration by high concentration of TiO_2, resulting in the loss of photosynthetic productivity. Moreover, the aggregation effect was observed during the cultivation. The aggregation phenomena also disturbed the use of light in microalgae, resulting in the inhibition of cell growth [11]. Second, the degree of toxicity by TiO_2 nanoparticle was not significant. Originally, it was reported that nanoparticles also have toxicity compared to bulk-size particles [24]. Besides, ROS induced by metals can cause DNA damage, lipid peroxidation, defective cell growth, and the inactivation of various enzymes in the cell [23]. However, in this experiment, the effect of toxicity did not seem to be enough to reduce the cell viability and growth, because TiO_2 was added in the late exponential phase, where cells are healthy and there was not enough oxidative stress in the absence of UV-A.

3. The Effect on *C. vulgaris* UTEX 265 Growth by TiO_2 in the Presence of UV-A

Based on the results from MB experiments, we assumed that TiO_2 in the presence of UV-A produced ROS (Fig. 1). To understand how oxidative stress induced by TiO_2 in the presence of UV-A affects *C. vulgaris* UTEX 265, we analyzed the chlorophyll, specific growth rate, and DCW in the presence of UV-A.

DCW did not show a significant difference after two days and four days of induction. In addition, the μ_{AVE} was also similar in all conditions (Table 2). Fig. 3 shows that the amount of chlorophyll was increased during the cultivation of *C. vulgaris* UTEX 265.

Even though a high concentration of TiO_2 affects μ_{MAX} of *C. vulgaris* UTEX 265 in the presence of UV-A, this phenomenon was also found in no UV-A condition (Table 2). *C. vulgaris* UTEX 265 reached stationary phase before two days after addition of TiO_2 in all conditions, but *C. vulgaris* UTEX 265 reached stationary phase early under low concentration of TiO_2. However, when we used 5 g/L of TiO_2 in the presence of UV-A, the amount of chlorophyll was lower than that of other conditions (Fig. 3). This result suggests that high concentration of TiO_2 (≥5.0 g/L) in the presence of UV-A can affect the viability as well as the growth rate, whereas the effect of TiO_2 in the absence of UV-A was not significant.

Table 2. The specific growth rates and DCW values in the presence of UV-A illumination[a]

TiO_2 (g/L)	μ_{max}[b] (d^{-1})	μ_{ave}[c] (d^{-1})	DCW (at 2 day)[d] (g/L)	DCW (at 4 day)[e] (g/L)
0	0.89±0.090	0.23±0.030	0.91±0.070	0.98±0.020
0.1	0.88±0.183	0.22±0.041	0.94±0.020	0.97±0.010
1	0.84±0.035	0.26±0.006	0.99±0.010	1.14±0.000
2.5	0.53±0.052	0.19±0.001	0.95±0.010	1.06±0.040
5	0.50±0.015	0.20±0.004	0.92±0.000	1.14±0.060

[a]The *C. vulgaris* UTEX 265 was cultivated at 25 °C, 120 rpm, and 170 μmol photons/m^2/s of a fluorescent lamp and 5.5 μmol photons/m^2/s of UV-A in TAP media with different concentrations of TiO_2 (0, 0.1, 1, 2.5, 5 g/L). The data points represent the average of samples and error bars indicate standard error (n=2)
[b]μ_{max} means maximum specific growth rate based on DCW of 1 days and 2 days after addition of TiO_2
[c]μ_{ave} means average specific growth rate based on DCW of 1 days and 4 days after addition of TiO_2
[d]DCW (at 2 day) means DCW at 2 day after addition of TiO_2
[e]DCW (at 4 day) means DCW at 4 day after addition of TiO_2

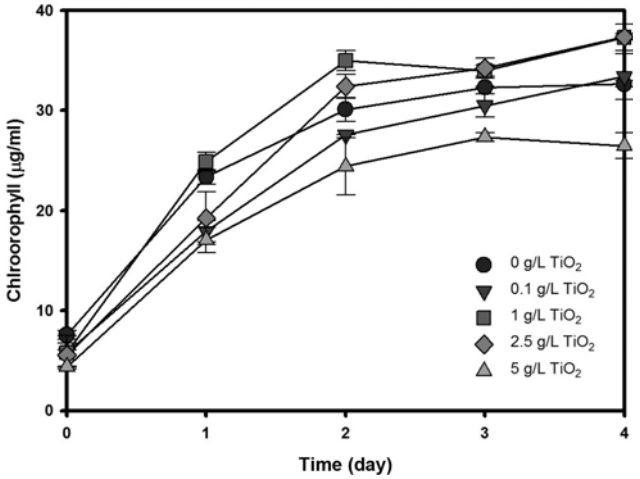

Fig. 3. Growth curve by chlorophyll in the presence of UV-A with the different concentrations of TiO_2: 0 g/L, 0.1 g/L, 1 g/L, 2.5 g/L and 5 g/L. After four days cultivation under the optimized culture condition, TiO_2 was added to 200 ml of culture. The *C. vulgaris* was cultivated at 25 °C, 120 rpm, and 170 μmol photons/m^2/s of a fluorescent lamp and 5.5 μmol photons/m^2/s of UV-A in TAP media. The data points represent the average of samples and error bars indicate standard error (n=2).

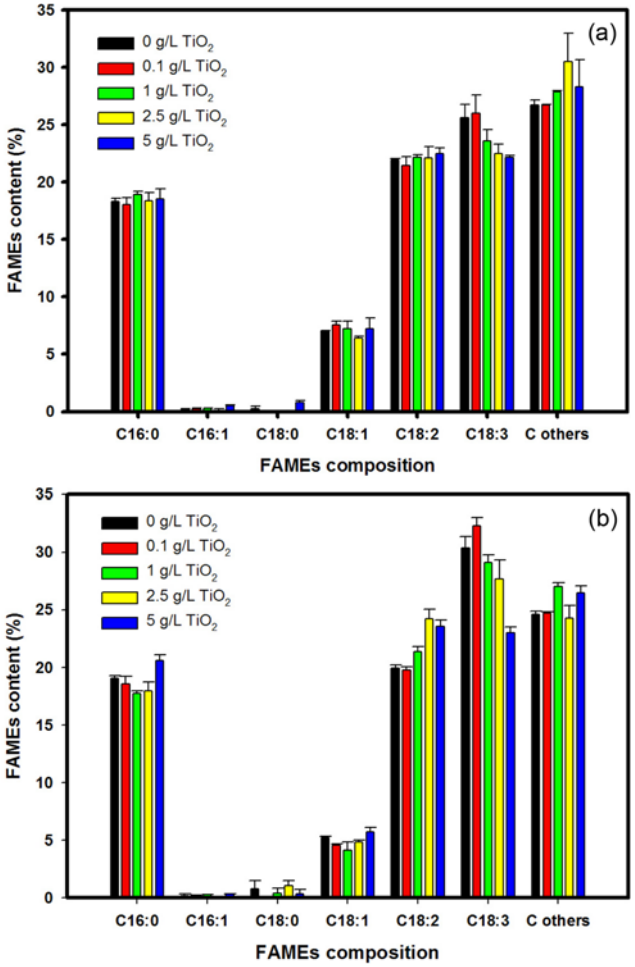

Fig. 4. FAME content according to FAME compositions of *C. vulgaris* UTEX 265 after induction of two days (a) and four days (b) in the presence of UV-A by different concentrations of TiO_2: 0 g/L, 0.1 g/L, 1 g/L, 2.5 g/L and 5 g/L. The data points represent the average of samples and error bars indicate standard error (n=2).

This is consistent with the previous reports that microalgae are affected by ROS, resulting in the accumulation of carotenoid and the reduction of chlorophyll [23,25]. Moreover, note also that we used the weak intensity of UV-A in this experiment, because it has been reported that the weak intensity of UV-A helps to increase biomass and fatty acid content [26]. Similarly, our results revealed that specific growth rate was increased in the presence of UV-A rather than in no UV-A condition, although DCW was not different regardless of UV-A (Table 1, 2).

Consequently, nanosized TiO_2 itself just affected μ_{MAX} (Table 1). On the other hand, ROS in the presence of TiO_2 and UV-A affected both cell viability and maximum specific growth rate (Fig. 3, Table 2). Based on these data, we could predict high lipid productivity under well-balanced conditions, including concentration of TiO_2 and induction time, because biomass was not affected by the stress condition, and lipid production could be increased by oxidative stress produced in the treatment of TiO_2 and UV-A.

4. Analysis of FAME Composition

To further understand the relationship between intracellular lipid production and oxidative stress, we analyzed FAME composition. Fig. 4(a) and 4(b) show FAME compositions of *C. vulgaris* UTEX 265 at two days and four days after induction by oxidative stress, respectively. FAME composition of C18:3 fatty acid (linolenic acid) was highest under condition of 0.1 g/L of TiO_2, and slightly decreased, with increased concentration of TiO_2 (Fig. 4(a)). Similarly, the pattern of the FAME composition at four days corresponded to the pattern at two days (Fig. 4(b)). As induction time went by, C18:3 fatty acid ratio was increased in all conditions. Especially, C18:3 fatty acid ratio was increased more than 5% except for 5 g/L of TiO_2 condition. On the other hand, there was no significant difference at C18:2 fatty acid (linoleic acid) under various concentrations of TiO_2 at two days. When the induction time was extended to four days, C18:2 fatty acid was increased at both 2.5 and 5 g/L of TiO_2 conditions and decreased under conditions of 0 and 0.1 g/L of TiO_2, respectively, when compared to C 18:2 fatty acid at two days. Taken together, these results imply that oxidative stress caused by TiO_2 is involved in the change of FAME composition, depending on the lengths of induction periods and the concentration of TiO_2.

It has been reported that microalgae accumulate high neutral lipid and C18 (octadecanoic acid) fatty acids amounts as a protection mechanism [27]. Under oxidative stress, microalgae produce numerous radicals that play negative roles in various physiological functions, because microalgae cannot control the balance of electrons from the photosynthetic electron transport chain. However, microalgae have a protection mechanism, when they accumulate C18 fatty acid which can consume approximately 24 NADPH derived from the electron transport chain. Therefore, highly accumulated C18 fatty acids under a stress condition make a balance of the over-reduced electrons [5,27]. In contrast, ROS is responsible for lipid peroxidation in microalgae, and this phenomenon arises in relation to the intra- and extra-cellular oxidative stress by ROS [28,29]. As a result,

oxidative stress induces a change in the lipid composition of the microalgae by decomposing the unsaturated fatty acid and repressing TAG accumulation [10,30]. Our result can be explained by this background. Lipid composition analysis indicated that as induction time was extended, the total C18 fatty acids component including saturated and unsaturated forms was slightly increased compared to those of other fatty acids. Especially, C18:3 fatty acid was also increased in all conditions (Figs. 4(a) and (b)). This result suggests that highly accumulated C18 fatty acids play an important role in protection mechanism of *C. vulgaris* UTEX 265 against oxidative stress as one of the ROS scavenging mechanisms. On the other hand, C18:3 fatty acid decreased in response to the increasing TiO_2 concentration simultaneously. This phenomenon became obvious after four days of induction. The decomposed C18:3 fatty acid was also explained in terms of the increase in the C18:2 fatty acid according to the TiO_2 concentrations. This arose because the double bond of the unsaturated fatty acid was decomposed by lipid peroxidation originating from the ROS [31]. Although the exact mechanism how ROS affects microalgae has not been reported thus far, there are many publications that explain the intra- and extra-cellular damage of microalgae by oxidative stress induced by TiO_2 [11,23,32]. In this result, the accumulation and decomposition of the lipid content occurred simultaneously by stress induced by TiO_2, and we hypothesized that there is a threshold level to produce a high lipid content and for the highest level of productivity.

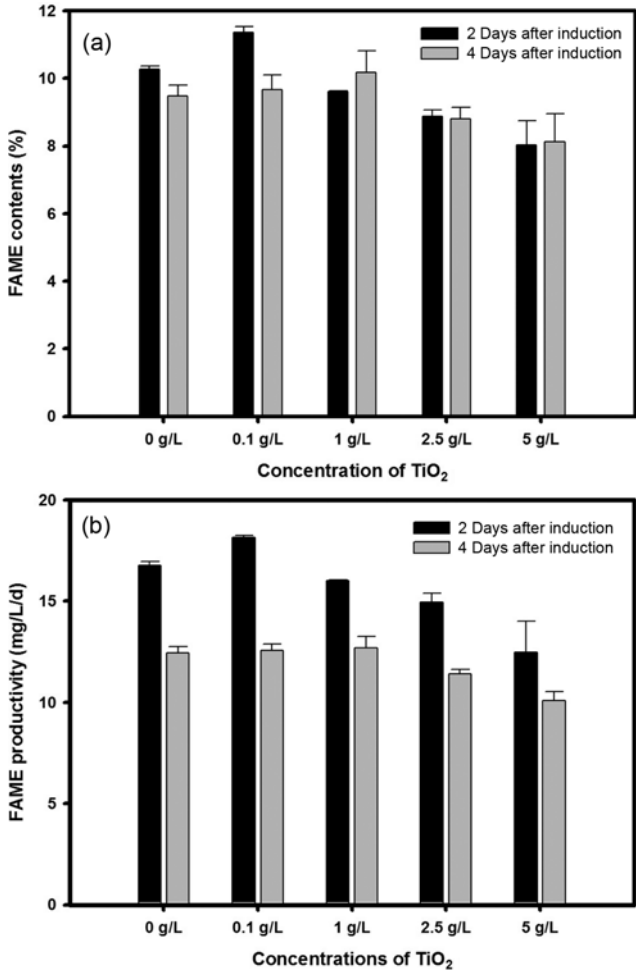

Fig. 5. FAME content (a) and productivity (b) of *C. vulgaris* UTEX 265 after induction of two days and four days by different concentrations of TiO_2 in the presence of UV-A. The FAME content was evaluated based on weight of biomass and the amount of FAME. Productivity was calculated based on total cultivation time including four days of optimized cultivation before induction by TiO_2. Total cultivation time of two days and four days after induction means six days and eight days, respectively. The data points represent the average of samples, and error bars indicate standard error (n=2).

5. Analysis of FAME Content and Productivity under Oxidative Stress Condition by TiO_2

To test our hypothesis, we analyzed FAME content and productivity according to concentrations of TiO_2 (0, 0.1, 1, 2.5, and 5 g/L) at two and four days after induction, respectively. Our analyses revealed that both the content and specific production rate of FAME displayed the highest levels under the 0.1 g/L TiO_2 and induction of two days in the presence of UV-A, while FAME content and productivity was decreased, with increased concentration of TiO_2 (Fig. 4). At four days after induction, the FAME contents were similar in all conditions, although the FAME content at 5 g/L TiO_2 was the lowest. In addition, in terms of FAME productivity, induction of four days was not efficient compared to that at two days. This indicates that the lipids of microalgae can be decomposed with similar levels after a specific threshold, including exposure duration and concentration of TiO_2. Therefore, we emphasize that it is important to balance the conflicting conditions of *de novo* synthesis and degradation of lipid in microalgae, when designing an induction process of biodiesel from microalgae using various stress conditions including TiO_2 nanoparticles. In this experiment, it seems that the protection mechanism occurred most actively at 0.1 g/L TiO_2. On the other hand, lipid peroxidation mechanism was activated at more than 0.1 g/L TiO_2. In addition, lipid was decreased because that extended stress by four days maintained lipid peroxidation mechanism. Thus, the threshold level for high lipid productivity was 0.1 g/L TiO_2 and two days induction. In this condition, the highest lipid content and productivity was 11.35% and 18.16 mg/L/d respectively. As a result, this study suggests the possibility of a simple lipid induction process which has only a switch on/off system. If we could develop this approach with other steps such as harvesting and extraction, a more efficient process of biodiesel production from microalgae could be operated.

CONCLUSIONS

We conducted experiments to enhance the lipid productivity by using artificial oxidative stress from TiO_2. TiO_2 nanoparticles in the absence of UV-A could not inhibit the growth and viability of *C. vulgaris* UTEX 265 when they were added after exponential phase. TiO_2 nanoparticles reduced only the maximum specific growth rate by shading and aggregation effect. However, in the presence of UV-A, oxidative stress by highly concentrated TiO_2 (5 g/L) reduced the chlorophyll. FAME composition analysis revealed that the accumulation and decomposition of the lipid occurred simultaneously by oxidative stress. Finally, we found the possible induction condition by oxidative stress: 0.1 g/L TiO_2 and two days induction. Under this condition, FAME content and productivity were highest. Therefore, we suggest that a lipid induction process can be regulated by artificial oxidative stress, and a similar approach can be also applied

in enhancing lipid productivity as well as production of other valuable products.

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