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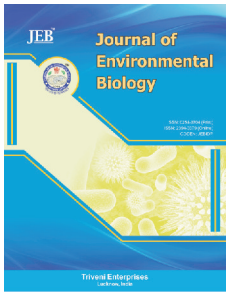

Growth characteristics of shade-acclimated marine *Chlorella vulgaris* under high-cell-density conditions

Authors Info

T. Katayama[1], N. Nagao[2]*, M. Goto[3], F. Md. Yusoff[2], S. Banerjee[2], M. Sato[1], K. Takahashi[1] and K. Furuya[1]

[1]Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo, Tokyo 113-8657, Japan

[2]Laboratory of Marine Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor Darul Ehsan, Malaysia

[3]Faculty of Science and Engineering, Soka University, Hachioji, Tokyo 192-8577, Japan

*Corresponding Author Email : norionagao@gmail.com

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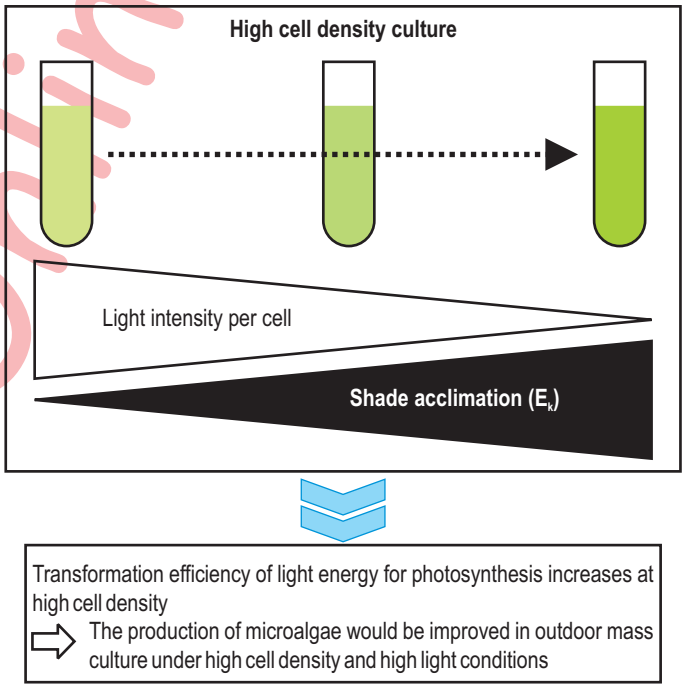
Abstract

Aim : To potentially improve the productivity of microalgae for mass culture, growth characteristics and light-saturation index (E_k) in the marine microalga *Chlorella vulgaris* were investigated under high-cell-density conditions grown in batch cultures.

Methodology : Potential changes in the growth characteristics and E_k in *C. vulgaris* were investigated during 4 weeks of cultivation. The algae was cultivated in a column reactor at 25°C under a surface light intensity of 300 µE m^{-2} s^{-1} in a 12-hr light and 12-hr dark cycle.

Results : Cell density increased and reached 2.7 g-dw l^{-1} by the end of culture with an areal production rate of 3.3 g-dw m^{-2} day^{-1}. The specific growth rate reached a maximum of 0.41 day^{-1} when the light intensity per cell was 7.9 µE g-dw^{-1} s^{-1}. As the light intensity per cell decreased with increasing cell density, E_k decreased gradually during the first half of the culture period.

Interpretation : *Chlorella vulgaris* in this study had the ability to acclimate to shade conditions during high-cell-density cultivation, however, the photosynthetic efficiency decreased. High transformation efficiency of light energy for photosynthesis in shade-acclimated cells will help to exploit the mass culture of microalgae.



Introduction

Mass culture of microalgae are receiving increasing attention because of their potential to yield valuable products (Nistan *et al.,* 1999; Zhu and Jiang, 2008), such as sources for biofuels (Gordon and Polle, 2007). Marine algae are particularly well suited for mass culture because they can utilize the vast supply of seawater unlike freshwater algae. The marine green algae *Chlorella* is recognized as a source of protein and carotenoids for human consumption and animal feeds in aquaculture (Iwamoto, 2004). To exploit these microalgae for large-scale biomass production, it is necessary to obtain a better understanding of the parameters influencing growth and biomass.

Light is a major factor for microalgal growth and must be studied since it provides energy that supports their metabolism. Although algal cells can receive sufficient light energy in low-cell-density conditions, the areal and/or volumetric production rate is lower than those at high densities due to low biomass concentration. On the other hand, denser cell populations enhance production rate. High-cell-density culture has been considered to be one of the effective operational conditions for microalgal production. In high-cell-density cultures, as the algal biomass concentration increases, the penetration of incident light within the algal suspensions gradually decreases due to significant shading effects between the algal cells (Anna *et al*., 2012; Posten, 2009; Sun *et al.,* 2016) and subsequently, the light intensity per cell decreases (Imaizumi *et al.,* 2016).

Shade-acclimated cells can improve the transformation efficiency of light energy for photosynthesis, and therefore it may be possible to improve the algal biomass production by utilizing these cells. However, it is important to understand the mechanisms of shade acclimation during high cell density culture to effectively produce biomass. The ability of physiological acclimations to low light conditions varies among species (Ziegler and Uthicke, 2011). Shade acclimation of microalgae is commonly reported in accordance with its photosynthetic response to light, particularly in terms of light-saturation index E_k (White and Critchley, 1999; Wulff *et al.,* 2008; Katayama *et al*., 2015). Current knowledge on E_k has mainly been obtained through the photosynthetic properties of photosynthesis-irradiance curves based on the end products of photosynthesis assessed by the ^{14}C method (Palmisano and Sullivan, 1982). The variable chlorophyll fluorescence method provides information similar to the E_k values obtained from the properties of electron transport rate of rapid light curve. This method is largely derived from the chlorophyll associated with photosystem II and is an easy experiment to perform (Maxwell and Johnson, 2000). The E_k is considered to be the light intensity that cells use to maintain the balance between photosynthetic energy capture and the capacity of the photosynthetic system to process this energy (Falkowski and Raven, 1997). Shade-acclimation behavior in high-cell-density cultures has not been previously demonstrated in the marine green algae *Chlorella*. In this study, potential changes in the growth characteristics and E_k in marine *Chlorella vulgaris* were investigated during 4 weeks of cultivation.

Materials and Methods

Culture condition and experimental design : *Chlorella vulgaris* was isolated from Port Dickson, Malaysia, and was cultured in a modified Walne's medium (Walne, 1970), which consisted of 0.61 g of $NaNO_3$; 0.10 g of $NaH_2PO_4 \cdot 2H_2O$; 0.23 g of Na_2EDTA; 0.17 g of H_3BO_3; 6.5 mg of $FeCl_3 \cdot 6H_2O$; 1.8 mg of MnCl2·4H_2O; 0.21 mg of $ZnCl_2$, 0.2 mg of $CoCl_2 \cdot 6H_2O$, 0.09 mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.2 mg of $CuSO_4 \cdot 5H_2O$, 0.1 mg of thiamine·HCl, 0.1 mg of cyanocobalamin and 0.002 mg of biotin per liter. The pre-culture was grown in a column reactor at 25°C under a surface light intensity of 300 μE m^{-2} s^{-1} that utilized cool white fluorescent tubes in a 12-hr light and 12-hr dark cycle. The column reactor was made of glass with an inner diameter of 6.0 cm and a length of 53 cm (Fig. 1). The effective volume and the light-receiving area in this reactor were 1.4 l and 2.1 m^2, respectively. An airlift system was installed for water circulation, and the culture was aerated with sterile air supplemented with 0.1-0.2% CO_2, at a rate of 0.2 l min^{-1}. The pH ranged from 8.0 to 6.3 with a pH buffer solution, and the water temperature was maintained at 25°C by circulating thermal-stable water. Triplicate subsamples were collected at 1, 3, 4, 7, 11, 15, 19, 23 and 27 days to measure cell density and variable Chl fluorescence.

Analytical methods : To determine the dry cell weight, a cell suspension sample was filtered through a combusted glass fiber filter with a pore size of 0.7 μm (GF/F, Whatman, USA). The cell pellet was washed twice with distilled water, dried at 60°C for 12 hr

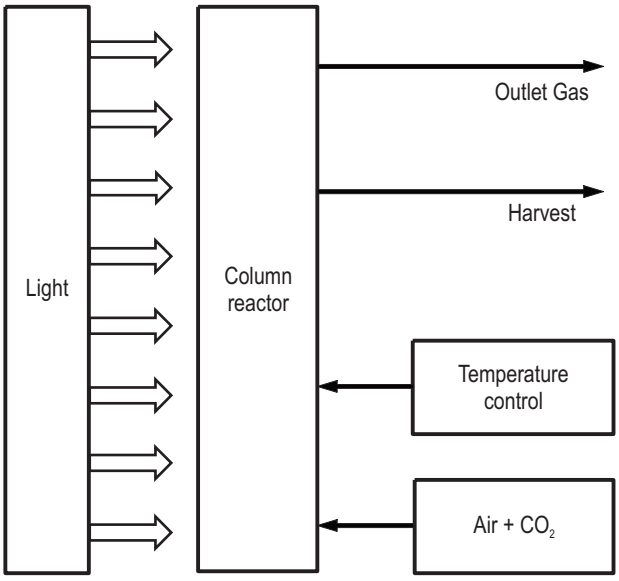


Fig. 1 : Schematic diagram of cultivation system

and subsequently cooled to room temperature in a desiccator before weighing.

The incident light intensity was measured in the range of photosynthetic active radiation (400-700 nm) on the surface of the column reactor using a quantum sensor (QSPL-2100, Biospherical Instruments, USA). The values were in μE m^{-2} sec^{-1} that can be converted to light energy (W m^{-2}) by a factor of 0.218 (1 μE m^{-2} sec^{-1} = 0.218 W m^{-2}) with cool white fluorescent tubes (Thimijan and Heins, 1983).

The light intensity per unit cell biomass (q: μEg-dwt^{-1} sec^{-1}) was calculated as shown below (Yoon *et al.,* 2008; Yoon *et al.,* 2012):

$$q = (2I_0 \times L \times r) / (X \times V) \quad (1)$$

where, I_0 is the incident light intensity on the surface of the column reactor (μE m^{-2} sec^{-1}); l, r and V are the length (m) and the radius of the reactor tube (m) and the culture volume (l), respectively; and X is the cell density (g-dw l^{-1}).

Calculation of parameters : As indices of biomass productivity, the specific growth rate (μ: day^{-1}), the volumetric production rate (P_V: g-dw l^{-1} day^{-1}), the areal production rate (P_A: g-dw m^{-2} day^{-1}), and the photosynthetic efficiency (PE: %) were calculated according to the following equations:

$$\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1) \quad (2)$$

$$P_V = (X_2 - X_1) / (t_2 - t_1) \quad (3)$$

$$P_A = (V \times (X_2 - X_1)) / (A \times (t_2 - t_1)) \quad (4)$$

$$PE = (P_A \times H_b \times 10^3) / E_{in} \times 100 \quad (5)$$

where, X_1 and X_2 are the cell densities (g-dw l^{-1}) at time t_1 and t_2, respectively. A is the light receiving area (m^2). H_b and E_{in} are the enthalpy of dry biomass for *C. vulgaris* (17.44 kJ g-dw: Bhola *et al*., 2011) and the light energy input (W m^{-2}), respectively.

Chl fluorescence : Variable Chl fluorescence was measured with a pulse-amplitude modulated Chl fluorometer (Water-PAM, Walz, Germany). Subsamples for fluorescence analysis were placed in a 15-mm-diameter quartz cuvette that was illuminated by a circular array of 14 red light-emitting diodes with a peak illumination at 655 nm. To measure photosynthetic capacity, maximum quantum yield of PSII (F_v/F_m) was calculated using the following equation (Schreiber *et al.*, 1986):

$$F_v/F_m = (F_m - F_0) / F_m \quad (6)$$

where, F_m and F_0 are the maximum and minimum PSII fluorescence levels of dark-acclimated cells for 30 min at a given time. A saturation pulse of 3,500 μmol photons m^{-2} sec^{-1} was applied for 0.8 sec to determine F_m. *Changes in* F_v/F_m are thought to reflect the photosynthetic health of microalgal cells (Cullen and Davis, 2003).

The electron transport rate (ETR) by PSII versus actinic irradiance curves were performed by applying actinic irradiances from 0 to 786 μmol m^{-2} sec^{-1} using 30 s illumination periods at each irradiance (Perkins *et al.,* 2006). The ETR was obtained using the following equation (Schreiber *et al.,* 1994):

$$ETR = PPFD \times \Delta F/F'_m \quad (7)$$

where, $PPFD$ is the photosynthetic photon flux density of actinic light and $\Delta F/F'_m$ is the PSII operating efficiency for illuminated cells (measured at the end of the 30 s lasting actinic light), which was calculated using the equation of Genty *et al.* (1989). Parameters based on the ETR observation such as the maximum ETR (ETR_{max}) and the initial slope of the curve (α) were estimated (Webb *et al.,* 1974). To examine the shade-acclimation of cells, the light-saturation index (E_k) was calculated using the following equation:

$$E_k = ETR_{max} / \alpha \quad (8)$$

E_k that is proportional to the degree of shade-acclimation decreases when cells acclimate to lower light conditions.

Results and Discussion

Growth characteristics : The cell density increased and stayed at a constant level of 2.0±0.19 g-dw l^{-1} from day 11 to day 19 and then increased again and reached a maximum value of 2.7 g-dw l^{-1} on day 27 (Fig. 2a). The maximum specific growth rate of 0.41 day^{-1} was observed on day 3, and then the specific growth rate decreased by day 19 as the cell density increased (Fig. 2b). The increase in the specific growth rate resumed after day 19, and the specific growth rate reached 0.03 day^{-1} on day 27. The changes in the specific growth rate were observed in a manner similar to the areal production rate, the volumetric production rate and the photosynthetic efficiency (Fig. 2c). The areal production rate is a reliable indicator of algal biomass production, which reached a maximum of 11 g-dw m^{-2} day^{-1} on day 3 even under a light exposure of 300 μE m^{-2} sec^{-1} that is not high light intensity in outdoor culture conditions. After day 3, the areal production rate decreased and reached the lowest level of 1.5 g-dw m^{-2} day^{-1} on day 19, and then increased and stayed at approximately 3 g-dw m^{-2} day^{-1} after 19 days.

Outdoor mass cultures of microalgae in tropical areas are grown under sunlight exposure in the range of 0 to 2000 μE m^{-2} sec^{-1} (Iwamoto, 2004). The daily mean light intensity was approximately 1300 μE m^{-2} sec^{-1}, which is 4.3 times greater than the light intensity of 300 μE m^{-2} sec^{-1} utilized in this study. Microalgal biomass is produced through the transformation process of light energy, and productivity increases as light intensity increases. This study showed an areal production rate of 3.3 g-dw m^{-2} day^{-1} with a high cell density of more than 2 g-dw l^{-1}. It may be possible to improve the areal production rate under a high light irradiance of more than 1000 μE m^{-2} sec^{-1} during outdoor cultivation, even though photo-inhibition may occur.

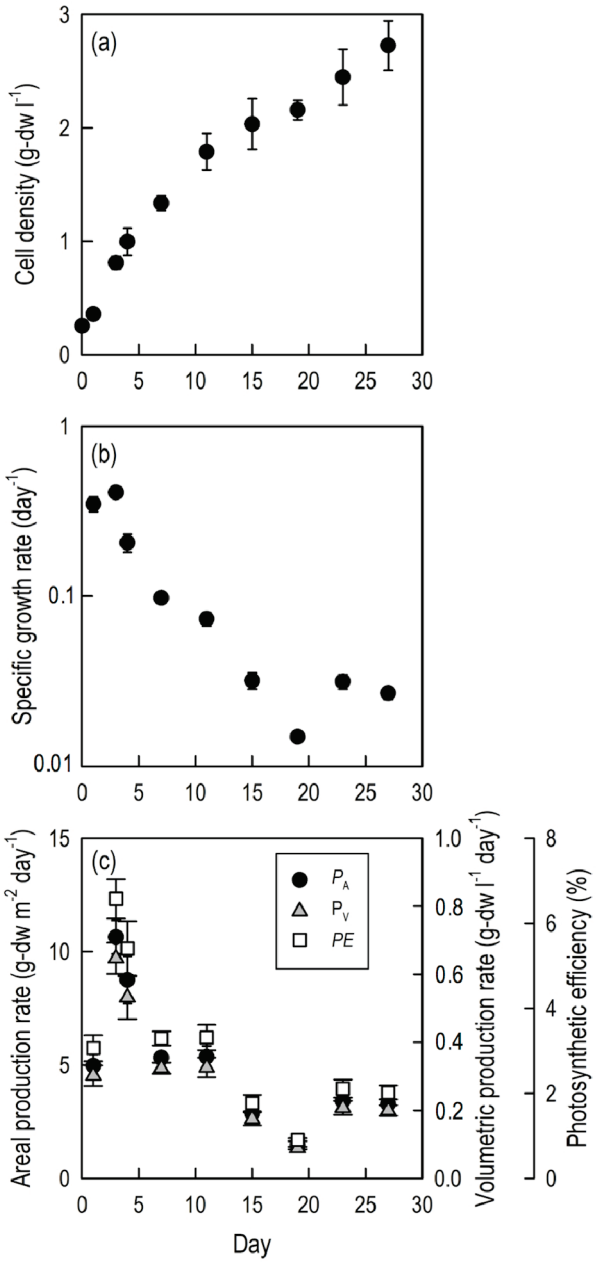


Fig. 2 : Time course of growth characteristics for *Chlorella vulgaris*. (a) Cell density, (b) specific growth rate, and (c) areal production rate (P_A), volumetric production rate (P_B) and photosynthetic efficiency (*PE*). Error bars indicate one standard deviation

Characteristics of Chl fluorescence : The maximum quantum yield of PSII (F_v/F_m) was 0.65 on day 1 and then became stable between 0.46 and 0.55 after day 3 (Fig. 3). Healthy cells have F_v/F_m values between 0.6 and 0.8 (Ting and Owens, 1992; Geel *et al.*, 1997; Casper-Lindley and Björkman, 1998; Lippemeier *et al.*, 2001). Additionally, the F_v/F_m value is relatively constant in non-stressed cultures (White *et al.*, 2011). We hypothesize that the photosynthetic performance of *C. vulgaris* could be maintained in a healthy state during high-cell-density cultivation, even though its F_v/F_m values were lower than 0.6.

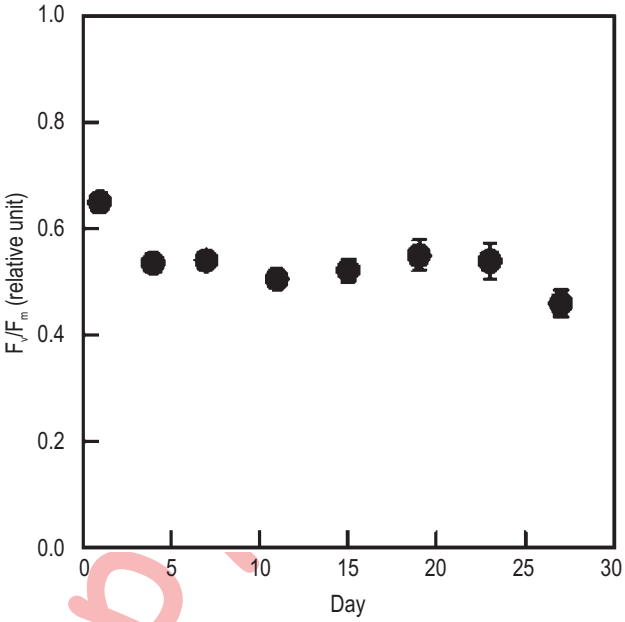


Fig. 3 : Time course of maximum quantum yield of PSII (F_v/F_m) for *Chlorella vulgaris*. Error bars indicate one standard deviation

To evaluate shade-acclimation in the cells, the light-saturation index (E_k) based on the electron transport rate (ETR) is shown in Fig. 4. The E_k on day 1 of 290 µE m^{-2} sec^{-1} coincided with the incident light intensity on the surface of the column reactor (Fig. 4a), indicating that the cells acclimate to ambient irradiance levels. The ETR versus irradiance curve was lower on day 27 than those on day 1 (Fig. 4a). The light-limited slope (α) and the light-saturated rate (ETR_{max}) obtained from the ETR versus the irradiance curve decreased gradually with culture time. Consequently, the E_k values decreased from day 1 to day 15 with culture time and then were relatively constant with an average of 171±22 µE m^{-2} sec^{-1} after day 15 (Fig. 4b). The decrease in the E_k suggests the cells became acclimated to shade. Shade acclimation during the first half period of culture might be due to a decrease in the irradiated light intensity for a single cell as the cell density increases. In the second half of the culture period, the E_k remained at approximately 170 µE m^{-2} sec^{-1}, although the light intensity per cell decreased continuously until the end of the experiment. The slower rate of change in the E_k during the second half of the culture suggests the *C. vulgaris* cells in this study acclimated to a light intensity of approximately 170 µE m^{-2} sec^{-1}.

Relationship between light intensity per cell and growth characteristics : Fig. 5a shows the time course of the light intensity per cell used to evaluate the irradiated light intensity for a single cell at high cell densities. The light intensity per cell

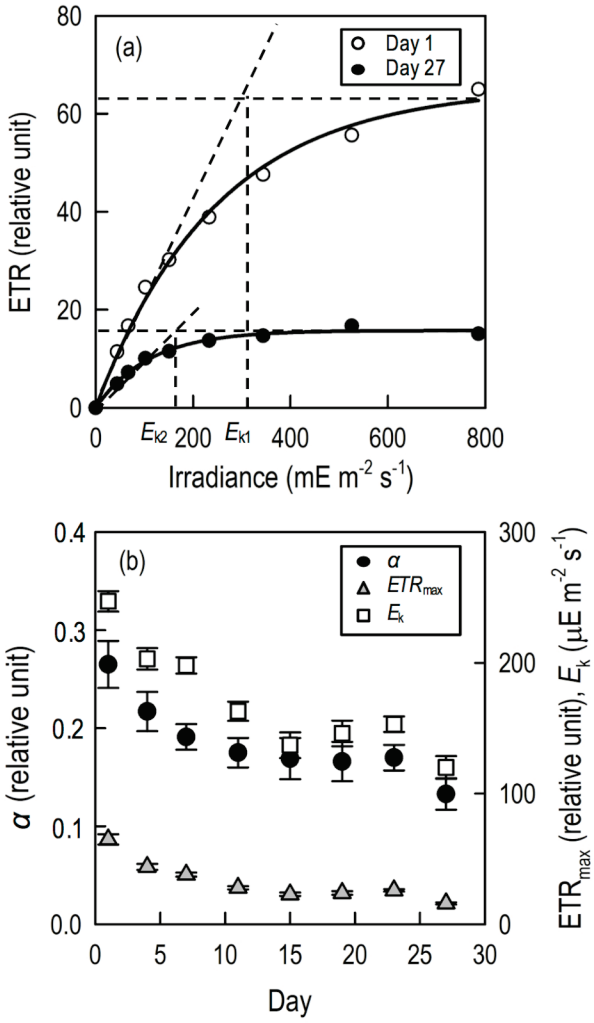


Fig. 4 : (a) Electron transport rate (ETR) versus irradiance curve on day 1 and day 27 and (b) time course of light-limited slope (α), light-saturated rate (ETR_{max}), and light-saturation index (E_k) for *Chlorella vulgaris*. E_{k1} and E_{k2} indicate E_k on day 1 and day 27, respectively. Error bars indicates one standard deviation

decreased exponentially with culture time and fell below 10 μE g-dw^{-1} sec^{-1} after day 3 (Fig. 5a). Imaizumi *et al.* (2014) reported that *Chlorella zofingiensis* had a high specific growth rate (0.5 day^{-1}) when the light intensity per cell was greater than 28 μE g-dw^{-1} sec^{-1}. In this study, the maximum value of the specific growth rate was observed when the light intensity per cell was 7.9 μE g-dw^{-1} sec^{-1}, suggesting the growth rate in *C. vulgaris* could reach relatively high levels even under low light conditions. The increase in the specific growth rate by increasing the light intensity per cell in this study (Fig. 5b) has also been observed in freshwater *C. zofingiensis* (Imaizumi *et al.,* 2014). *Chlorella vulgaris* and *C. zofingiensis* are marine and freshwater algae, respectively, but both have similar specific growth rates under low light intensity per cell. Because *C. zofingiensis* displayed higher values of specific growth under higher light intensity per cell

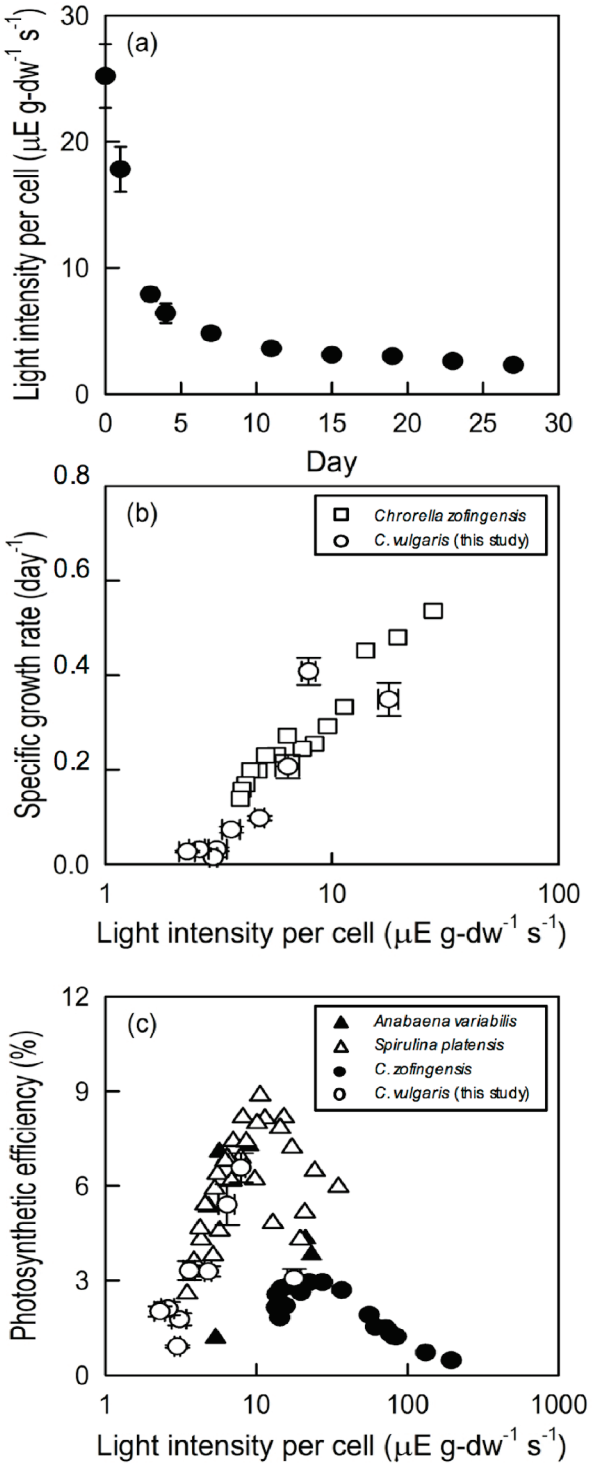


Fig. 5 : (a) Time course of light intensity per cell for *Chlorella vulgaris*; (b) the relationship between light intensity per cell and specific growth rate and (c) the relationship between light intensity per cell and photosynthetic efficiency in this study (open circle) and in the literature (open square, Imaizumi *et al.,* 2014; open triangle, Yoon *et al.,* 2012; solid triangle, Hu *et al.,* 1998; solid circle, Imaizumi *et al.,* 2016). Error bars indicate one standard deviation

(Imaizumi *et al.,* 2014), the higher specific growth rate of *C. vulgaris* may be observed when the cells are exposed to higher light intensity. Further studies regarding the light intensity per cell under sufficient light intensity will help to identify the effective operational conditions for outdoor mass culture to increase biomass productivity.

Species-specific growth characteristics with light intensity for *C. vulgaris*, the relationship between the light intensity per cell and the photosynthetic efficiency is shown in Fig. 5c. The photosynthetic efficiency increased with decreasing light intensity per cell. However, under low light intensity per cell at high cell density, it is likely that the photosynthetic efficiency decreased in this study of *C. vulgaris* and as previously demonstrated in *C. zofingiensis* (Imaizumi *et al.,* 2016), *Spirulina platensis* (Hu *et al*., 1998) and *Anabaena variabilis* (Yoon *et al*., 2012). Other studies suggested that this decrease could be due to the increased viscosity of the culture with increasing cell density, consequently causing the cells at high density to utilize light energy and nutrients less effectively (Lee and Low, 1991) as well as photorespiration rates increasing due to high cell densities (Ugwu *et al*., 2008). Interestingly, these microalgae displayed similar values of the photosynthetic efficiency under low light intensity per cell with the exception of *C. zofingiensis* that displayed a lower photosynthetic efficiency (Imaizumi *et al*., 2016). Imaizumi *et al.* (2016) cultured *C. zofingiensis* in a tubular photobioreactor with extreme high cell density conditions of >10 g-d.w. l^{-1} and high light exposure (>1500 μE m^{-2} sec^{-1}). The lower values of photosynthetic efficiency observed in *C. zofingiensis* (Imaizumi *et al.,* 2016) might be caused by nutrient deficiencies or increased photorespiration.

This study demonstrated that, while the cells of marine green algae *C. vulgaris* acclimate to the low light condition in high-cell-density cultures, the photosynthetic efficiency decreases. This inhibition of algal growth at high cell density correlates with the results from previous studies. To increase the transformation efficiency of light energy for photosynthesis in the shade-acclimated cells at high cell density would be important to further research in order to facilitate effective outdoor mass culture of microalgae.

Acknowledgments

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