

# Photosynthesis, Growth and Dry Biomass Production in *Euglena gracilis* Klebs as Affected by Three Growth Irradiance Levels

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## Authors' contributions

This work was carried out in collaboration among all authors. Authors KG and CKB conceptualized and planned the research. Author CKB carried out the research and conducted the data analysis. The first draft was written by authors CKB and VNK. Both authors CKB and VNK read and approved the final manuscript.

## Article Information

DOI: 10.9734/ajraf/2021/v7i430137

**Editor(s):**

(1) Dr. Christine Wulandari, University of Lampung, Indonesia.

**Reviewers:**

(1) K.A.Narayana, SRM Institute Of Science And Technology, India.

(2) Yagya Dutta Dwivedi, Institute of Aeronautical Engineering, India.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/74869>

Original Research Article

Received 28 July 2021  
Accepted 04 October 2021  
Published 06 October 2021

## ABSTRACT

**Aims:** The research aimed to investigate the shade response of *E. gracilis* Klebs while making the irradiance a crucial factor for photosynthesis based physiological activities and its applicability for industrial level culture conditions.

**Study Design:** *Euglena* was cultured at three different light intensities of 30, 90, and 210 mol m<sup>-2</sup>s<sup>-1</sup> photoautotrophically and axenically in modified Cramer-Meyer medium at 25 °C as batch cultures.

**Methodology:** The photosynthesis O<sub>2</sub> evaluation of *Euglena* cultures was measured under exponential (EP), transitional (TP), and stationary phases (SP). The light compensation point (LCP), light saturation point (LSP), and dark respiration rate (DRR) were obtained. Cell volume and cell number in each culture were measured simultaneously. Cells were collected and obtained dry mass (DM) after drying aliquots at 80°C. Specific growth rate (SGR) and relative growth rate (RGR) were calculated. Tests for homogeneity of variance were performed on all parameters and LSDs

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were used for the mean separation.

**Results:** In the TP, the lowest LCP was achieved in the higher light culture. The values of both the DRR and the LSP were the same as in EP. The DRR, LCP and LSP are lower in lower PFD cultures and decreased with increasing cell titers. The cellular growth levels were lower in lower light culture and decreased as each culture grew. Cellular DM was maintained constant in the EP, where SGR almost equaled RGR. In the EP, SGR was maintained constant in each culture, SGR displayed a saturation phenomenon. In the later TP, SGR became equal to RGR and all the cultures revealed constant DM.

**Conclusion:** *Euglena* photoautotrophic cultures can tolerate low light intensities. With the SGR and RGR behavior under the shade conditions, they can maintain the constant photosynthesis rate and constant dry matter level.

**Keywords:** *Euglena gracilis* Klebs; photosynthesis-irradiance; PFD, nutraceutical; Specific growth rate; relative growth rate.

## 1. INTRODUCTION

Microalgae have gained attention recently as a sustainable food and energy supplement at the industrial level. The algal flagellate *Euglena gracilis* Klebs is one of the most promising microalgae used to produce a vast range of products, such as essential amino acids, polyunsaturated fatty acids and vitamins. Thus it has been utilized in various fields, such as nutraceuticals, pharmaceuticals, foods, food additives and cosmetic [1]. Because of the nutraceutical value, the algal flagellate *Euglena gracilis* Klebs is becoming increasingly popular. Its significance as a source of polysaccharide  $\beta$ -glucan-paramylon which is beneficial in immune recognition and as an immune potentiator [2]. In addition to that, it is important to produce antioxidants such as  $\alpha$  tocopherol,  $\beta$  carotene and vitamin C, and polyunsaturated fatty acids such as DHA and EPA [3]. *Euglena* has gained attention in the recent past as a source for the production of biofuels in an economically effective and environmentally sustainable manner [4]. Nevertheless, sometimes this invaluable alga may present an environmental and social threat making water-blooms on water bodies [5].

Euglenoids grow either photoautotrophically, heterotrophically and mixotrophically, and thus are globally distributed in ponds, lakes and rivers, particularly in the eutrophic region [6]. Biomass concentration and the composition of the cellular products are significantly affected by the cultivation mode [6]. Considerable efforts to optimize the environmental conditions of bioreactors, microalgae straining and efficient nutrient supplements to promote algae growth and biomass accumulation can be occasionally

observed in literature. However, still, there is an issue considering the economic efficiency of large-scale microalgae production systems. The optimum culture conditions for bioreactors to harvest the photosynthesis-derived bioproducts have not been elucidated to date. Because of the relative easiness in culturing and maintaining, *Euglena gracilis* has served as a model laboratory photosynthetic organism for many years. Being a unicellular organism, it has been widely used for studies in physiology and biochemistry, some of which have been published as five volumes of a monograph [7-10]. However, little information is available about its adaptations to light gradients, which is of great importance in ecophysiology.

Compared with land plants, much less is known about algal strategies for coping with environmental changes in light intensities; for example, there are no algal species that are characterized as sun-algae or shade-algae, while many land species have been categorized into either shade-plants (sciophytes) or sun-plants (heliophytes). [11] showed that *E. gracilis* responds to 'end-of-day Far-Red (EOD-FR)'. While the final response was not evaluated as shade-avoidance syndromes, the response has only been documented in the shade-avoidance response of heliophytes; and not yet in any other algae, suggesting that *E. gracilis* may behave as sun-algae. On the other hand, *Euglena* has long been considered as an extreme type of 'sun-alga' [12]. Although [13]'s comprehensive study dealt with growth responses of *Euglena* as affected by light energy supply, yet little is known about how *Euglena gracilis* responds to varying light intensities concerning physiological parameters of population growth and photosynthetic machinery. Therefore, it is not certain that the

alga is a helioalga or a scioalga. Knowledge of shade responses of this alga will surely benefit the commercial cultivation as well. In this context, the research aimed to investigate the shade response of *E. gracilis* Klebs while making the irradiance a crucial factor for photosynthesis based physiological activities and its applicability for industrial level culture conditions that targeting for nutraceutical products.

## 2. MATERIALS AND METHODS

### 2.1 Organism and Culture Conditions

As described previously [14-15], the algal flagellate *Euglena gracilis* Klebs (strain Z) was cultured axenically and photoautotrophically in modified Cramer-Meyer medium at 25 °C as batch cultures. Cultures were continuously irradiated unilaterally by an array of day-white type fluorescent lamps (National FL20SS-N18, Tokyo, Japan) supplying three irradiance levels; 28  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (low light), 84  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (moderate light) and 210  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (high light). All three cultures were magnetically stirred and aerated throughout the experimental period (Plate 1).

### 2.2 Growth Analyses

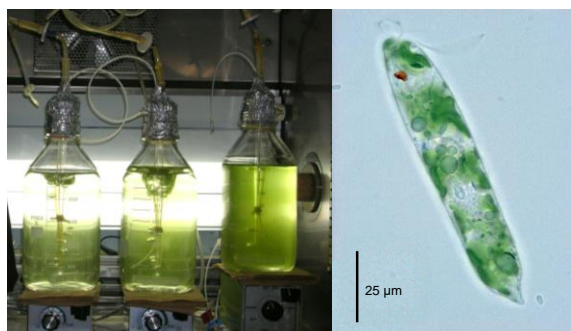
The cell population growth was monitored by progressively counting the cell number in each culture with an electronic particle counter (Coulter Electronics, Inc., Hialeah, FL, USA). A volume of ~5 ml was taken at each sampling point using a fraction collector (SF-2120, Advantec). Cell volume was also measured simultaneously. Specific growth rate (SGR) was calculated according to the following equation;

$\text{SGR} = [\ln (N/ N_0)] / t$ ; where  $N_0$  is the cell count of the previous sampling,  $N$  is the cell count of the current sampling,  $t$  is the time between two samplings (hours).

Cells (not less than  $4 \times 10^5$  cells: the minimum which is required to weigh the cells, with our experience) were collected either by centrifugation at 5000 rpm for 10 min at 4 °C (during the early stages of the experiment) or directly (for dense cultures). Dry mass was obtained after drying aliquots at 80 °C at least for 24 hours until constant weight. Dry mass-based relative growth rate (RGR) was calculated according to the following equation;  $\text{RGR} = [\ln (M/ M_0)] / t$ ; where  $M_0$  is the dry mass of the previous sampling,  $M$  is the dry mass of the current sampling,  $t$  is the time between two samplings (hours).

### 2.3 Measurement of Photosynthesis and Respiration

Photosynthetic  $\text{O}_2$  evolution was measured as a function of light intensity in each culture at exponential, transitional and stationary phases, using an oxygen electrode (Digital Oxygen System, model 10, Rank Brothers, Cambridge, England) and recorded by a chart type recorder (Unicoder U-228, Pantos, Nippon Denshi Kagaku, Japan). For all measurements, a cell density of  $\sim 2 - 2.5 \times 10^5$  cells/ml were used in the chamber. The chamber was irradiated using a xenon light source (LAX-102, Asahi Spectra USA Inc.) of varying intensities. A constant temperature water circulator was used to maintain the chamber temperature at 25 °C. The  $\text{O}_2$  consumption in the darkness (dark respiration) was also recorded.



**Plate 1. (a) Culturing *Euglena gracilis* Klebs as batch cultures under 210  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ; (b) *Euglena gracilis* Klebs growing under 210  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at the exponential phase (Images by C.K. Beneragama)**

## 2.4 Data Analysis

Tests for homogeneity of variance were performed on all parameters and, where applicable, LSDs suitable for comparing means of three replicates were determined.

## 3. RESULTS AND DISCUSSION

Light is considered a vital factor that is important to the growth and development of organisms. In this study, the heterogeneous nature of light was used to induce pressure on the photosynthesis organism as a strategy. Concerning the results, adaptation or acclimation to light gradients, to low light, in particular, can be identified compared to the ground plants, despite the lack of characterization of microalga. *Euglena* seemed to preferentially choose shade-tolerance over shade-avoidance under decreased light availability. Physiological traits, such as reduced growth rate, photosynthetic light saturation point, light compensation point and dark respiration rate are amongst the striking typical shade tolerance responses (see [16] for a review) which we have observed in *Euglena* in the present study. In the following, we discuss the light response of *Euglena gracilis* Klebs. Apart from that, dry matter accumulation and algal population growth are considered here as supportive evidence. Overall, the findings of *Euglena's* light response and growth characteristics can make suggestions for the commercial level bio-product industry.

### 3.1 Photosynthesis, Respiration & Shade Tolerance Response

The plots of net photosynthetic oxygen evolution rate versus PFD in exponential, transitional and stationary cultures of the three different light intensities were established (Fig.1a-c). During the exponential growth phase of each culture (Fig.1a), the values of dark-respiration rate, light-compensation and light-saturation points were remarkably lower in lower light cultures. These differences across cultures were however not maintained until stationary phases, because actual PFD became unparallel to incident PFD due largely to mutual shading; higher incident PFD supports higher cell titers with higher speeds, leading to heavier mutual shading. Thus, in the transitional growth phase (Fig.1b), the lowest light-saturation point was achieved in the alga cultured at the highest incident PFD, whereas the values of both the dark-respiration

rate and the light-compensation point still followed the same as in the exponential cultures; the lower in the lower incident PFD cultures. When they reach the stationary growth phases (Fig.1c), all these variables became indistinguishable from each other culture; all the cultures may have encountered deep shade below a critical level, such that the actual PFD, although physically not the same, were essentially (or biologically) the same for the alga. Despite these complexities, it is obvious that all the three variables, i.e. dark-respiration rate, light-compensation- and light-saturation points are lower in lower PFD cultures and decreased with increasing cell titers, and thus decreasing actual PFD, within each culture.

Photosynthesis and respiration features are well-established characteristics to decide plant light responses. According to this study, decreased light compensation point, light saturation point and dark respiration rate were observed in *Euglena* in exponential culture (Fig. 1). With respect to that, *E. gracilis* Klebs responds to the heterogeneous light levels as a shade tolerant organism [17]. Photosynthesis is the major metabolic function that is responsible for biomass accumulation and the total dry matter content of cell culture.

When thinking about the industrial level culture, this shade tolerance ability can be applied to gain maximum product amount at the end with low-cost for the light source in photoautotrophic culture. As reported previously [18], shade tolerant plants maximize their light-harvesting with several adaptive strategies. After that, the plant efficiently used that captured light in photosynthesis and reduced the respiration level (Fig 1b-c) for essential function using the carbon gain hypothesis [18]. With that point, *E. gracilis* Klebs can gain its carbon and total dry matter content at the reduced light level. This study demonstrated that incident PFD cannot maintain in the cell cultures after the exponential growth phase due largely to mutual shading. In addition, higher incident PFD supports higher cell titer with higher speeds, leading to high mutual shading in a short time. This condition can be expected at an industrial level as well. Shade tolerant nature of *Euglena* can be applied in successful large scale cell cultures. However, *Euglena* was able to maintain the constant level of light compensation point, light saturation point and the dark respiration rate as a response to the incident low light intensity. This tolerance

capacity could be applied for the industry to harvest maximum output using limited light resources by providing enough level of light into the culture in a considerable period.

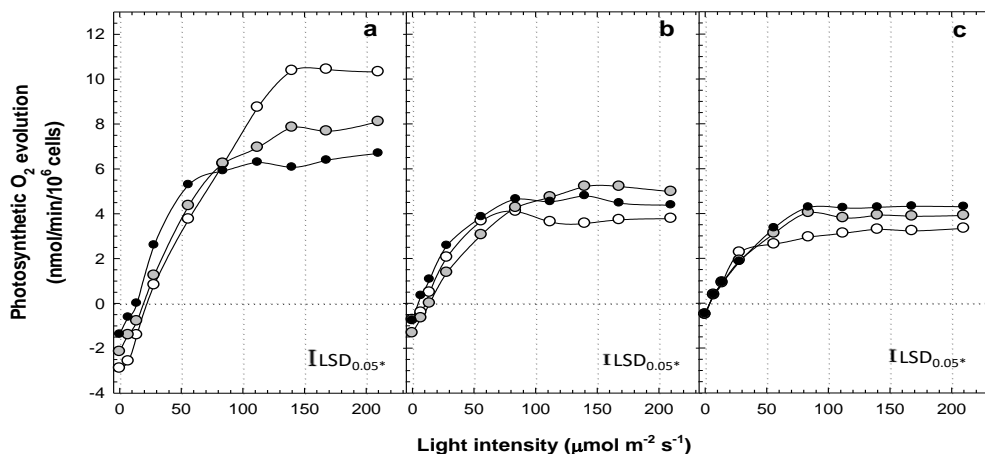
### 3.2 Cellular Growth in the Heterogeneous Light Environment

When considering the *E. gracilis* Klebs commercial level cultures, dry matter accumulation and cellular growth are important to maintain continuous production. The shade response of *Euglena* is important here to identify the pattern of growth. In the previous section, shade tolerance nature is identified with photosynthesis and respiration. According to [17], *E. gracilis* Klebs has been identified as a shade tolerant organism. In shade tolerance, ground plants species are facultatively adapted to shade and are different from species that originated as shade species. *Euglena* can be cultured photoautotrophically, heterotrophically or photoheterotrophically [6]. Bio-products depend on the culture conditions and need to maintain the same condition to collect desirable product with high quality. Some of the specific cell components are only synthesized under inadequate light conditions and the formation of some secondary metabolites enhanced by the light environment [6]; [19].

On a commercial scale, high investments are required for various designs to enhanced light

conditions in photobioreactors. Internal illumination systems, enhanced mixing to shortening the light pathway like methods are applied to improve light transfer into the culture. However, a culture inevitably reaches a linear growth due to light limitation and biomass concentration resulted in limited level [20]. As a shade tolerant organism, it is important to know how relative growth rate (RGR) responses relate to the specific growth rate (SGR: specific growth rate of cell number) of the cell population under the heterogeneous light condition in photoautotrophic culture to maintain a bioreactor's conditions. When RGR is taken into consideration (Fig. 2a), SGR did not completely behave in the same way (Fig. 2b), and the difference may reflect the algal strategy as to what extent it invests in survival, reproduction, or storage for the future sake, depending on the availability of PFDs.

Particularly, the higher cell number of the algal population may make it possible to colonize into more varied environments, but if the higher number is achieved by decreased cellular dry matter (DM) of individual cells, it also means a higher risk of susceptibility to other stresses, chances of starvation, and/or mortality. Thus, it is important to examine the relationships between RGR and SGR (Fig. 2c) for commercial purposes. This relationship can be described as a six-step process with the support of dry matter fluctuation.

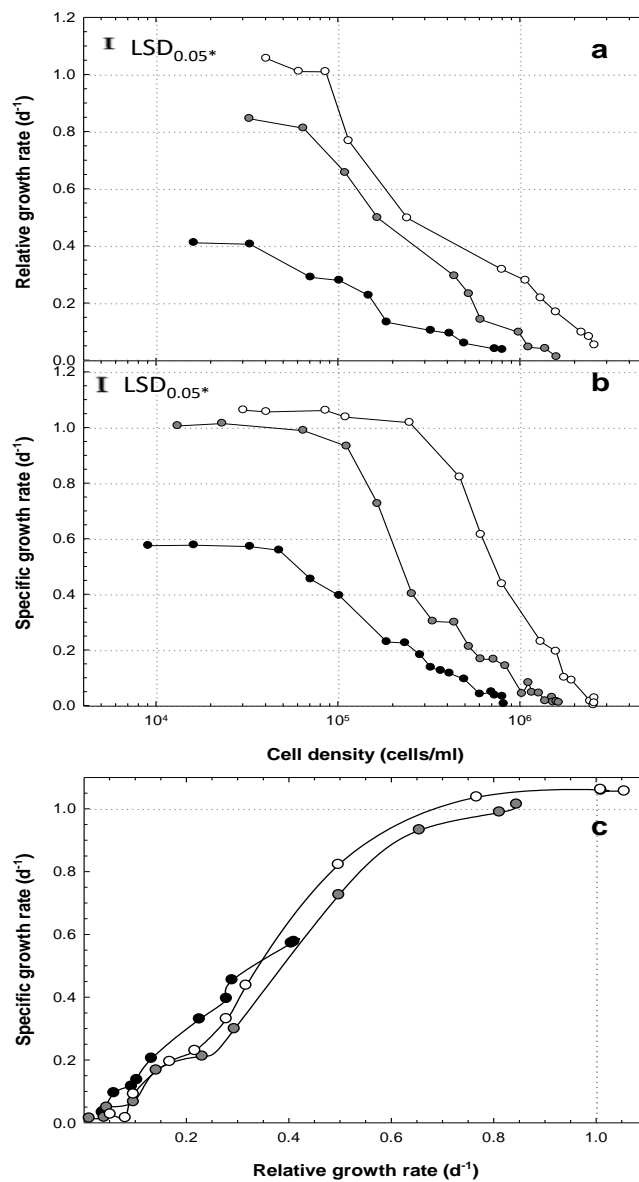


**Fig. 1. Photosynthetic oxygen evolution of photoautotrophic cultures of *Euglena gracilis* as affected by growth PFDs. a) exponential phase, b) transitional phase, c) stationary phase.**

Three light intensities have been used; 28 (black circles), 84 (gray circles) and 210 (open circles)  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . Day-white type fluorescent lamps (National FL 20SS-N18) were used to irradiate the cultures during growth and a xenon light source (LAX-102) was used to irradiate samples in the photosynthetic oxygen evolution measurements

First, the cellular DM levels were lower in lower light cultures and decreased as each culture grew, most probably by mutual shading (Fig. 2c). Thus, it is one of the shade-tolerant responses of *Euglena*. Secondly, in the exponential DM-growth phase (or the optimal conditions in each culture), SGR almost equaled RGR, indicating that cellular DM was maintained constant in this phase in each culture. Thirdly, RGR began to decline earlier than SGR, indicating that cellular

DM declines post the exponential DM-growth modes. Collectively, some portions of the cellular DM seemed a surplus for the alga in the exponential DM-growth modes. The same pattern of dry matter fluctuation and SGR changes were observed by [19]. They demonstrated, sustain exponential growth phase was dependent on the light intensity and dry mass accumulation became limited with high cell density. This condition was observed in both stirred cultures and still cultures.

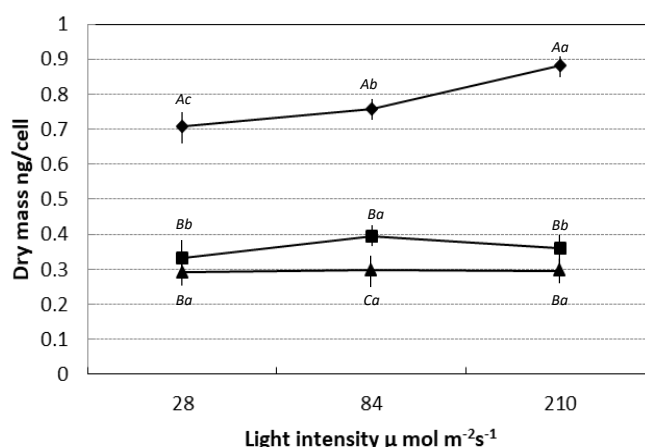


**Fig. 2.** Change in (a) relative growth rate (RGR) and (b) specific growth rate (SGR), and (c) the relationship between RGR and SGR as affected by growth PFDs. Three light intensities have been used; 28 (black circles), 84 (gray circles) and 210 (open circles)  $\mu mol\ m^{-2}\ s^{-1}$

Fourthly, in the exponential modes of cell population growth where SGR was maintained constant in each culture, SGR displayed a saturation phenomenon (Fig. 2c) in which it was no more increased with RGR higher than a critical level; the maximal SGR of  $1.05 \text{ d}^{-1}$  (doubling time = 15.7 h) with RGR higher than  $\sim 0.7 \text{ d}^{-1}$  (doubling time =  $\sim 25 \text{ h}$ ) in the brightest culture, perhaps reflecting the limitation resulting from the algal capacity of the cell-cycle machinery as the length of S + G2 + M is relatively constant  $\sim 8 \text{ h}$  irrespective of incident PFDs [21], at least partly. [20], observed the same behavior of SGR in light of limiting culture by self-shading. From late exponential growth, SGR has not increased higher than critical biomass content and maintain linear increment. Fifthly, in the early transitional phase, the decrease of SGR was smaller than that of RGR, indicating that the cellular DM became increasingly lost. The trade-off between SGR and the cellular DM suggests that the latter is still the surplus and that the alga tends to give priority to reproduction than becoming better physics to look for new better environments when the current environments become more shaded. Sixthly, in the later transitional phase, SGR became equals to RGR; loss of the cellular DM no more occurred, and all the cultures revealed the constant DM of 0.3 ng/cell, despite, before this point, the cellular DM was lower in shaded alga as pointed out at first. In this growth mode, the algal SGR was solely determined by RGR.

So far, only a few evidence can be found about the RGR and SGR pattern in *Euglena* culture after the late transitional phase. However, in this study, we observed that *Euglena* become tolerant to the available light condition in the culture and maintained a constant level of dry matter concentration. Moreover, there is a possibility to maintain this kind of constant dry matter level using a considerably high level of incident PFD than paying more money to the illumination system in bioreactors. Because high light conditions allowed more biomass production in the exponential growth phase and later culture can be able to maintain considerable biomass condition. However, it is important to check the bio-product concentration in cells, because light can be affected by biochemical changes. Especially different light intensities are important for specific fatty acid synthesis in *Euglena* cultures [20]; [22]. The shade tolerance nature of *Euglena* can be applied for cost maintain in commercial level production.

In the present study, we report SGR values in the range of 0.58 (at  $28 \mu\text{mol.m}^{-2}\text{s}^{-1}$ ) to  $1.05 \text{ d}^{-1}$  (at  $210 \mu\text{mol.m}^{-2}\text{s}^{-1}$ ) varying depending on the light intensity of the culture (Fig. 2b). Our results are contradictory to the findings of [20] where they reported an SGR of  $0.90 \text{ d}^{-1}$  during the exponential growth phase, regardless of the light intensity in the range of  $800\text{-}1200 \mu\text{mol.m}^{-2}\text{s}^{-1}$ . This may be because the growth temperature at



**Fig. 3. Dry biomass (ng) per cell in photoautotrophic cultures of *Euglena gracilis* as affected by PFDs ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Three growth phases have been considered; Exponential phase (Diamond), Transitional phase (Square), Stationary phase (Triangles)**

Means with different uppercase letters are significantly different within the same light intensity and means with different lowercase letters are significantly different within the same growth phase ( $P < 0.05$ )

which they reported the aforesaid SGR value was 23 °C whereas our cultures were maintained at 25 °C.

For commercial purposes, the dry mass production is of great importance. In the present study, it was apparent that the alga was able to maintain the cellular dry mass at  $0.88 \pm 0.06 \text{ ng/cell}$  under  $210 \mu\text{mol.m}^{-2}\text{s}^{-1}$  during the exponential growth phase while the same dropped down to  $0.3 \pm 0.02 \text{ ng/cell}$  at the stationary phase regardless of the growth irradiances (Fig. 3). However, if the dry biomass produced per liter of cell culture is considered, within the irradiance range of  $28\text{-}210 \mu\text{mol.m}^{-2}\text{s}^{-1}$ , a harvest of  $0.6 \text{ g.L}^{-1}$  can be obtained at the stationary phase whereas a maximum of  $0.25 \text{ g.L}^{-1}$  dry biomass can be harvested at the exponential phase under  $210 \mu\text{mol.m}^{-2}\text{s}^{-1}$ . Therefore, the culture irradiance condition and the growth phase to be harvested should be decided depending on the purpose of the cultivation of *Euglena*.

As the algal flagellate can tolerate the UV radiation to a greater extent [23]; [24], the massive outdoor commercial cultivation seems to be a possibility which warrants further studies.

#### 4. CONCLUSION

Response to the different light intensities of *Euglena gracilis* Klebs is important for the commercial level nutraceutical and related bioproduct in commercial level cultures. With the findings of this experiment, the photoautotrophic culture of *E. gracilis* Klebs, act as a shade tolerance organism in the heterogeneous light environment. Lower light intensity has resulted in low light compensation point, light saturation point and the low dark respiration rate and reported low level of dry matter accumulation (per cell basis) compared to the high light intensities. *Euglena* was able to maintain the constant level of photosynthesis rate after the transitional phase. When considering the growth responses, SGR and the RGR maintained at a higher level in the exponential phase and start to decline with the increase of shade as a result of culture growth. However, at the end of the transitional growth cultures were able to maintain a constant RGR and SGR under the limited light condition. It can be concluded that *Euglena gracilis* Klebs photoautotrophic cultures can

tolerate under low light intensities, especially the mutual shade conditions which occurred with culture growth. With the SGR and RGR behaviour under the shade conditions, they can maintain the constant photosynthesis rate and constant dry matter level. Findings of this study can be applied to the commercial level bioreactors for nutraceutical production with *Euglena gracilis* Klebs.

#### ACKNOWLEDGEMENTS

The funding received by the Japanese Ministry of Education, Culture, Sports, Science and Technology and the research facilities provided by the Obihiro University of Agriculture and Veterinary Medicine to carry out the research work is gratefully acknowledged.

#### COMPETING INTERESTS

Authors have declared that no competing interests exist.

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