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Influence of Salinity on Growth and Phycoerythrin Production of *Rhodomonas salina*

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Abstract

Microalgae is a photoautotroph organism capable of producing various photosynthetic pigments with diverse beneficial properties. *Rhodomonas salina*, a Cryptophyte cell, contains only phycoerythrin as its phycobiliprotein pigment. The effects of salinity on growth and phycoerythrin concentration were investigated. Microalgae *R. salina* were grown in natural sea water with salinity of 33‰ and 50‰.The microalgae was batch-cultured in f/2 medium at light irradiation of 1100 lux, temperature of 24–26 ^{o}C, and photoperiode of 12 h : 12 h. The microalgae cell density was directly calculated using haemacytometer. The concentration of phycoerythrin was determined by spectrophotometric method. The cell density and phycoerythrin concentration were monitored every 4 days for 20 days of cell growth. Results showed that salinity did not affect significantly both on growth and phycoerythrin concentration extracted from *R. salina* biomass ($p>0.05$; $\alpha = 0.05$). At both salinity, maximum phycoerythrin concentration were reached on day 8. There was a positive correlation between cell density and phycoerythrin concentration from day 1 to day 8 of cell growth. Microalgae *R. salina* which was grown in natural seawater with salinity of 33‰ achieved the highest cell density of 8.4 x 10^5 cells/mL and the phycoerythrin concentration of 0.19 μg. 10^{-5} cell on day 8 of the culture. The highest phycoerythrin concentration was obtained on day 16 of the culture i.e 0.27 μg. 10^{-5} cell.

Keywords: cell density, growth media, phycoerythrin, *Rhodomonas salina*, salinity

1. Introduction

Phycoerythrin (PE) is a type of phycobiliprotein, a water-soluble protein-pigment complex. The color of PE pigment is derived from chromophore-containing compounds categorized as phycobilin. Phycoerythrin structure is a combination of linier tetrapyrrole chain compound (phycobilin) and a polypeptide chain that are connected via a thiol bridge to the cysteine residue in the polypeptide chain. A phycoerythrin compound has a combination of two phycobilins which one phycobilin molecule can have five different types of structures depending on the source. Based on its specific spectral characteristics, the type of phycoerythrin is usually distinguished by adding one letter, either a capital or small cap, in front of the word phycoerythrin as a prefix. Types and sources of phycoerythrin are B-phycoerythrin (545-546 nm; 563-565 nm; 498 nm (shoulder); red algae), b-phycoerythrin (545 nm; 563 nm; red algae), R-phycoerythrin (493-498 nm; 534-545 nm; 564-568 nm; red algae), r-phycoerythrin (498 nm; 542 nm; 560 nm; red algae), and C-phycoerythrin (562-565 nm; cyanobacteria)(de Marsac, 2003; Toole & Allnutt, 2003). Phycoerythrin derived from *Rhodomonas salina* is designated as Cryptophyte-Phycoerythrin (Cr-PE) or Cr-PE545 because it has a maximum absorption at 545 nm wavelength (Toole & Allnutt, 2003).

One Cr-PE molecule contains two phycobilins namely phycoerythrobilin (PEB) and 15,16-dihydrobiliverdin (DBV)(Toole & Allnutt, 2003).

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Therefore, Cr-PE absorbs yellow-green color of the color spectrum and shows red to pink color character. When compared to other phycobiliproteins-containing algae, *R. salina* which is classified as Cryptophyte contains only PE as its phycobiliprotein, so its accumulation within cells does not compete with other phycobiliproteins such as phycocyanin and allophycocyanin (Lee, 2008).

According to several studies, PE production from *Rhodomonas* sp. is influenced by nitrate concentration, photon flux density (light intensity), and temperature (Bartual, Lubián, Gálvez, & Niell, 2002; Chaloub, Motta, de Araujo, de Aguiar, & da Silva,, 2015; da Silva, Lourenco, & Chaloub, 2009; Vu et al., 2015). Compared to chlorophyll, other pigments or accessories pigments, i.e. PE concentration extracted from the cultivated microalgae under optimum growth conditions is still very low (Mulders, Lamers, Martens, & Wijffels, 2014). As a result, the accessories pigment production from microalgae will encounter many challenges economically. Therefore, the content of these pigments in microalgae cells should be increased so that their production can compete economically with synthetic pigments. Attempts can be made by culturing them in a particular growth condition, such as the application of adverse growth or extreme environmental conditions, i.e. high salinity, high light intensity, extreme temperature and/or nutrient deprivation (Mulders et al., 2014). This growth condition has been successfully applied on carotene-producing microalgae such as *Dunaliella salina* (Lamers, Janssen, De Vos, Bino, & Wijffels, 2008).

Attempts in using extreme environmental conditions has been conducted on *Rhodomonas* sp.'s culture condition in term of its growth and phycoerythrin production. da Silva et al. (2009) found that there was a rapid decline in N-containing compound such as hydrosoluble proteins and photosynthetic pigments and ultimately caused an almost complete loss of PE in a nitrogen starvation culture. Meanwhile, attempting to grow *R. salina* in high intensity of light (above 300 µmol photon $m^{-2}s^{-1}$) caused a photoinhibition on its growth and a decrease in photosynthetic activities (Bartual et al., 2002). Study on *Rhodomonas* sp. growth and phycoerythrin production in a high salinity environment has not been reported. Although Jepsen et al. (2018) proved that *R. salina* strain K-1487 can be cultivated in a wide range of salinity (0–65‰), they did not observe the photosynthetic pigment content in that range. Jepsen et al. (2018) also showed that salinity influenced the specific growth rate on *R. salina* strain K-1487. The specific growth rate of *R. salina* increased at the salinity of 0–29‰. On the contrary, the rate decreased at the salinity of 29–65‰. Thus, the objective of this study was to observe the growth of *R. salina* in 33 and 50‰ salinity and to estimate the concentration of PE produced by *R. salina* during its growth.

2. Materials and Methods

2.1. Material

Microalgae *R. salina* were obtained from the Institute for Mariculture Research and Fisheries Extension (IMRAFE), Gondol, Bali. The f/2 medium was produced from mineral salts such as $NaNO_3$ (Merck), $NaH_2PO_4 \cdot 2H_2O$ (Merck), $FeCl_3 \cdot 6H_2O$ (Merck), $Na_2EDTA \cdot 2H_2O$ (Merck), $MnCl_2 \cdot 4H_2O$ (Merck), $ZnSO_4 \cdot 7H_2O$ (Merck), $CoCl_2 \cdot 6H_2O$ (Merck), $CuSO_4 \cdot 5H_2O$ (Merck), $Na_2MoO_4 \cdot 2H_2O$ (Merck). This medium also includes vitamin mix which composed of thiamine·HCl (vitamin B1) (Merck) and cyanocobalamin (vitamin B12) (Merck). Water level 1 is of ICP-MS grade.

2.2. Methods

2.2.1. Cultivation of R. salina microalgae

Microalgae were batch-cultured in two culture conditions with a working volume of 300 mL (Table 1). Before starting the experiments, *R. salina* was cultured in a 500-mL erlenmeyer, with 0.2-mM filtered seawater with a modified f/2 medium (Guillard, 1975 in Andersen, Berges, Harrison, & Watanabe, 2005), at optimal growth condition according to Chaloub et al. (2015). In both cultures (containing f/2 medium and inoculum), microalgae were grown in natural seawater except that the salinity of C1 culture was modified. The modification was made by mixing natural sea water and ASW Salt Mix+ so that the salinity reached 50‰. All growth media were sterilized using autoclave at 121°C for 15 min. All cultures were performed in triplicates.

Subsequently, inoculum solution of 60 mL with a cell density of approximately 1.0×10^6 cells/mL was diluted aseptically into culture erlenmeyer containing 240 mL of culture media, so that the initial cell density of microalgae in all cultures was 2.0×10^5 cells/mL.The culture conditions were set up according to optimal condition obtained by Chaloub et al. (2015). In order to avoid contamination, the observation of cell density was performed once every 4 days from a 1-mL culture solution and the cell density calculation was performed manually using a haemocytometer. For each culture, growth rate (m) was calculated using Equation (1)

$$\mu = \frac{\log_e N_t - \log_e N_o}{\Delta t} = \frac{2.3}{\Delta t} (\log_{10} N_t - \log_{10} N_o) \quad (1)$$

Note:

N_o = cell density at the beginning of a time interval;
Nt = cell density at the end of the time interval;
Δt = the length of th time interval (tt - t0).

2.2.2. Analyses of phycoerythrin

The extraction of phycoerythrin (PE) was performed in accordance to Lawrenz, Fedewa, & Richardson (2011). A wet *R. salina* biomass concentrated from one mililitre culture solution was used as sample. The wet biomass was macerated in 0.1 M phosphate buffer solution (pH = 6.0). The solution was subjected to freeze-thawing extraction, freezing for 2 h and refrigeration for 24 h. Phycoerythrin-content supernatant was concentrated in refrigerated sentrifuge (10,000x g; 4 °C; 10 min). The phycoeryhtrin solutions were placed into 96-well plate to measure the absorbance spectrophotometrically at 280, 455, 545, 564, 592, and 750 nm. The estimation of PE concentration was calculated using formula described in Thoisen, Hansen, and Nielsen (2017). The PE concentration was calculated according to Beer and Eshel (as cited *in* Thoisen et al. (2017))(Eq. 2). Phycoerythrin concentration was expressed as $\mu g.10^{-5}$ cell to indicate the amount of phycoerythrin per microalgae cell density.

$$PE\ (\frac{\mu g}{mL}) = \left[\left((A_{564}-A_{592})-(A_{455}-A_{592})\ x\ 0.2\right)\ x\ 0.12\right] x\ 1000 \quad (2)$$

Note:

PE= phycoerythrin

A = absorption at the indicated wavelengths

2.2.3. Statistical analysis

All data measurements are shown as mean +/- standard deviation (± SD) of three independent replicates. Data were tested for normal distribution (Kolmogorov-Smirnoff goodness of fit test) before being analyzed by ANOVA. The results were analyzed by one-way analyses of variance (ANOVA) with $\alpha = 0.05$, using SPSS 15.0. Least Significant Difference (LSD) post hoc test was performed for in depth analysis of the differences between individual treatment.

3. Results and Discussion

3.1. *Rhodomonas salina* Growth under Different Salinity

This study used batch culture type to grow *R. salina* because it is easy to manipulate the growth condition of the microalgae (Wood, Everroad, & Wingard, 2005). The growth of *R. salina* is shown in Figure 1.

Based on the results of *R. salina* growth curve for 20 days (Figure 1), it was observed that treatment in C1 culture produced smaller cell density compared to C2 culture. In both cultures, the growth of *R. salina* were almost at the same point for the first 4 days. However, C1 culture showed different trend, experiencing lag phase before entering the first day.This shows that *R. salina* needs to adapt to high salinity, because it comes from a smaller volume batch with different salinity. Both cultures showed similar growth profile, reaching maximum growth on day 8 and beginning to decrease afterwards.

The maximum cell density in salinity 33‰ (8.4 x 10^5 cells/mL) was 1.3 times higher than the maximum cell density in salinity 50‰ (6.4 x 10^5 cells/mL). The maximum growth rate of *R. salina* in culture C2 (0.2 day^{-1}) was also 1.7 times higher than that of culture C1 (0.12 day^{-1}). Then, the growth was descending of about 2.27 x 10^5 cells/mL and 2.31 x 10^5 cells/mL in margin in cultures C2 and C1, respectively, between day 8 and 12 of growth. However, entering on day 16, the growth showed an increase in cell density in culture C2 (6.08 x 10^5 cells/mL to 6.61 x 10^5 cells/mL) and culture C1 (4.07 x 10^5 cells/mL to 5.05 x 10^5 cells/mL) and the growth dropped eventually to cell death phase at day 20.

In this experiment setup, the nutrient availability in the culture medium is considered as nutrient deficiency culture (Vu et al., 2015). The *R. salina* growth was depending on the nutrient availability since

Table 1. Experimental conditions of *R. salina* culture

Culture	Growth Media	Salinity	Medium
C1	Natural sea water (modified)	50‰	f/2
C2	Natural sea water	33‰	f/2

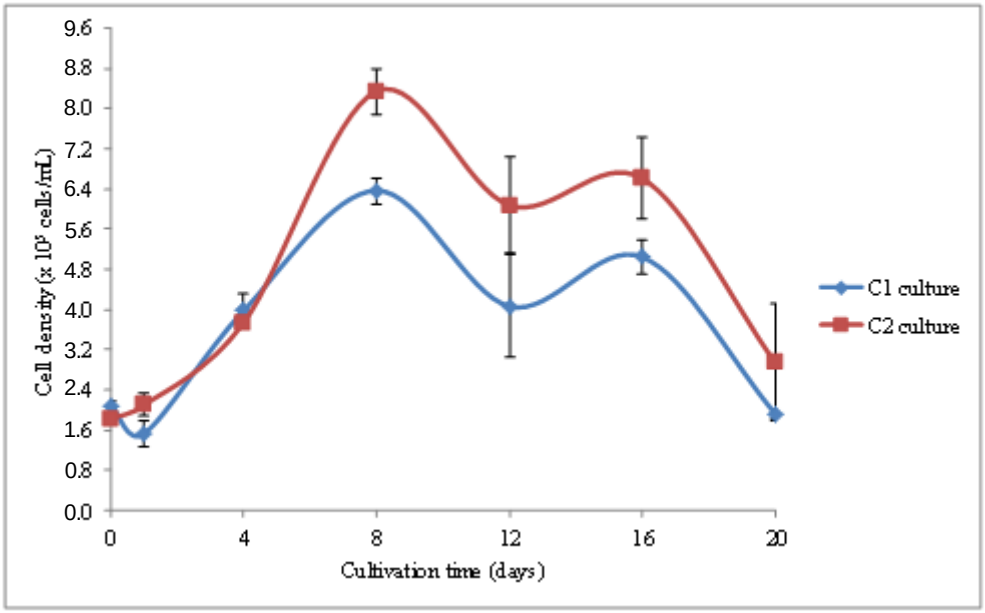

Figure 1. Curve of *R. salina* growth for 20 days. C1 culture (50‰ salinity) is represented in blue(♦) and C2 culture (33‰ salinity) is represented in red(■) . Symbols represent means ± SD (n = 3).

the first day of inoculation. According to previous studies, nutrient availability, and irradiance are two environmental factors responsible for *Rhodomonas* sp. growth (Chaloub et al., 2015; Lafarga-De la Cruz et al.,2006; Vu et al., 2015). Both factors directly influence microalgae growth during the logarithmic phase. Then, beyond the latter phase, the microalgae growth is influenced only by nutrient availability (Lafarga-De la Cruz et al., 2006).

In this experiment, an elevated nitrate concentration of about 1100 μM was added and irradiance of 1100 lux (equal to approximately 14.85 μmol.photon.$m^{-2}s^{-1}$) was applied. According to Chaloub et al. (2015), the optimal nitrate concentration and irradiance for *R. salina*'s growth and phycoeryhtrin production are 1100 μM and 15 μmol.photon.$m^{-2}s^{-1}$, respectively. However, the maximum cell density and maximum growth rate of *R. salina* were reached on the 8th day of culture for culture C2. The maximum growth rate in culture C2 (0.2 day^{-1}) was higher than that of in culture C1 (0.12 day^{-1}), suggesting that salinity influences microalgae growth.

The highest growth rate of the cryptophyte microalgae ever reached was 1.2 day^{-1} by *Pyrenomonas salina* (a taxonomic synonym of *R. salina*)(Lewitus & Caron, 1990). The high growth rate obtained by Lewitus and Caron (1990) is most probably due to the use of ammonium ion as the nitrogen source. While in this study we used nitrate ion as the nitrogen source. Nitrogen is an important macroelement in key molecules such as amino acids for microalgae. When microalgae cell assimilates nitrate ion, it will be reduced into nitrite and ammonium. Eventually the latter will be incorporated into carbon skeletons to form glutamate in an enzymatic process (Sanz-Luque, Chamizo-Ampudia, Llamas, Galvan, & Fernandez, 2015).

3.2. Phycoerythrin Production

We extracted the pigment based on Lawrenz et al. (2011). Figure 2 shows the maximum absorption of phycoeryhtrin at 546 nm. Cryptophyte-phycoerythrin has a maximum absorption in a range of 540–550 nm (Toole & Allnutt, 2003).

Figure 3 shows PE concentration produced by *R. salina* during 20 days of growth. All treated cultures showed the same profile across the elapsed days, although they did not share the same PE concentration. In our study, we obtained the highest PE content on day 16th for all treated cultures, 0.22 μg.10^{-5}cell for culture C1 and 0.27 μg.10^{-5}cell for culture C2 (Figure 3). ANOVA analysis showed that there was no significant difference on phycoerythrin concentration between culture C1 and culture C2 ($p>0.05$; $a = 0.05$). In the previous study, the maximum PE concentration (1.4 μg.10^{-5}cell) was obtained on day 8 with the following culture conditions, i.e. light irradiance of 15 μmol.photon.$m^{-2}s^{-1}$ and culture temperature of 26 ^{o}C (Chaloub et al., 2015).

In accordance to the growth curve (Figure 1), the log phase was reached at day 8, but the PE concentration actually increased until day 16. The biosynthesis of PE also depends on the availability of

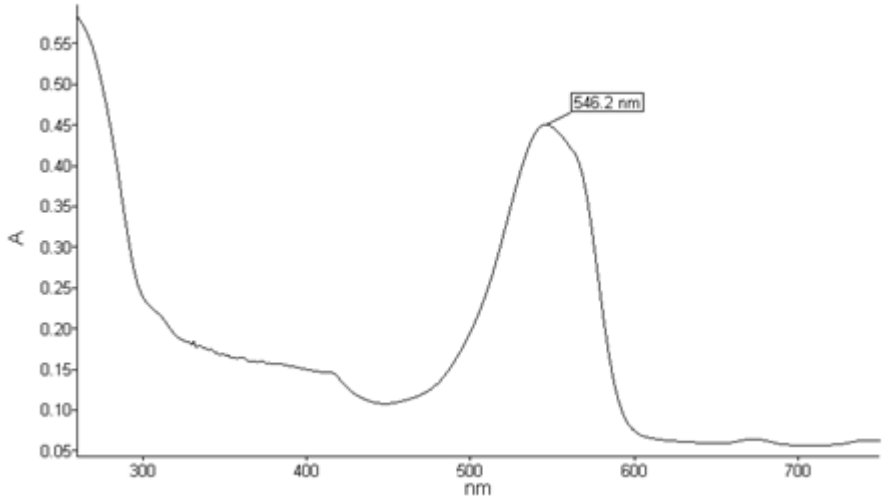

Figure 2. The maximum absorption observed in phycoerythrin crude extract produced by *R. salina*.

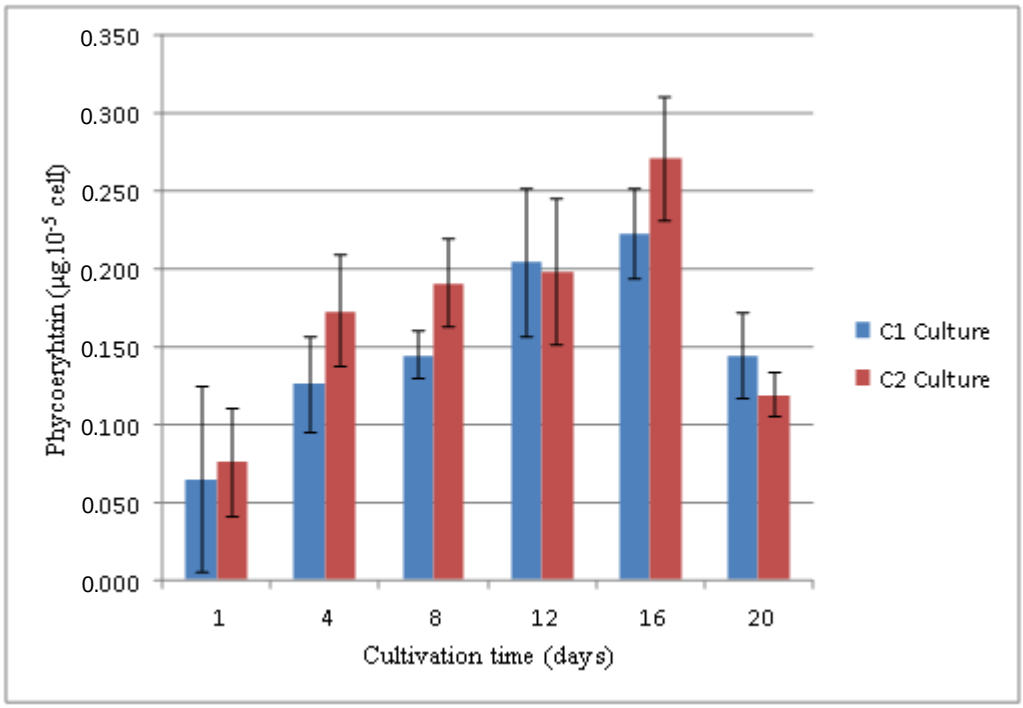

Figure 3. Phycoerythrin concentration observed in *R. salina* culture during 20 days observation. C1 culture in blue bars and C2 culture in red bars. The data represent means of three independent experiments (n = 3).

nitrogen in the growth medium. da Silva et al. (2009) revealed that the initial nitrate ion in *Rhodomonas* sp. culture medium decreased up to 70% during the first 4 days of cultivation. However, the *R. salina* growth did not show a decline in the growth curve after day 4 and/or even between day 12 to day 16 (Figure 1). According to Bartual et al. (2002), it is believed that the PE degradation is a survival mechanism for sustaining cellular growth and/or maintenance in a nutrient short supply medium. At this degradation process, PE is mobilizing its nitrogen to cope nitrogen shortage.

Previous studies have revealed that nutrients which directly affect the content of photosynthetic microalgae pigments are nitrate and phosphate (da Silva et al., 2009; Lewitus & Caron, 1990). In conjunction with its cellular composition, the presence of nitrogen has a role in the biosynthesis of PE (Chaloub et al., 2015). In addition, besides functioning as a light-harvesting apparatus, PE also serves as a source of nitrogen

reserves in *Rhodomonas* sp. cells (da Silva et al., 2009; Lewitus & Caron, 1990). The results of this study reinforce the aforementioned theory. This was observed in the microalgae growth curve as it enters the stationary phase or from day 12 to day 16 (Figure 1) that the cell density is inversely proportional to the concentration of PE (Figure 3).

4. Conclusion

Rhodomonas salina was able to adapt to wide range of saline environment. *R. salina* obtained the cell density in C1 culture of 8.4 x 10^5 cells/mL and PE concentration of 0.19 μg. 10^{-5} cell on day 8, however it reached the highest PE concentration of 0.27 μg.10^{-5} cell on day 16 of the culture. There was no significant difference on growth and phycoerythrin concentration produced by *R. salina* that was cultivated in 33‰ salinity and 50‰ salinity ($p>0.05$; $\alpha = 0.05$).

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