The growth of *Synechococcus* sp RDB001 at temperature of 30 ± 5 °C and 50 ± 5 °C: a comparison study of cell density and chlorophyll content

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**Abstract.** Research on comparison of cell density and chlorophyll content of *Synechococcus* sp. RDB001 grown at a temperature of 30 ± 5 °C and 50 ± 5 °C has been carried out. *Synechococcus* sp. RDB001 was isolated from hot spring water samples in the area of Rawa Danau-Banten with a water temperature of 50 °C which grown in MA medium (pH 6). The research aims to determine the comparison of the average cell density and chlorophyll content of *Synechococcus* sp. RDB001 grown at a temperature of 30 ± 5 °C and 50 ± 5 °C in incubation cabinets. The research results are useful for understanding the physiological tolerance limits and adaptation of *Synechococcus* sp. RDB001 ex-situ. Culturing of *Synechococcus* sp. RDB001 was carried out in cabinets with the incubation temperature of 30 ± 5 °C and 50 ± 5 °C for 16 days, from day 0 (t) until day 16 (t). Each treatment was done in 16 replicates. Non-parametric statistical analysis using the Mann Whitney test (α = 0.05) and the Spearman test (α = 0.01). The results showed there were significant differences (α = 0.05) in cell density of *Synechococcus* sp. RDB001 grown at a temperature of 30 ± 5 °C and 50 ± 5 °C. The highest average of cell density of *Synechococcus* sp. RDB001 at a temperature of 30 ± 5 °C occurred at t. (24.2075 ± 5.33926 x 10⁹ cells.mL⁻¹), while at a temperature of 50 ± 5 °C occurred at t. (1.21313 ± 2.92573 x 10⁹ cells.mL⁻¹). There was no correlation (α = 0.01) between cell density and chlorophyll content of *Synechococcus* sp. RDB001 grown at a temperature of 30 ± 5 °C and 50 ± 5 °C. The research showed that increasing the cell density of *Synechococcus* sp. RDB001 is not always followed by an increase in chlorophyll content of *Synechococcus* sp. RDB001.

**Keywords:** *Synechococcus*, cyanobacteria, hot spring, growth temperature

1. Introduction

Cyanobacteria are a group of photosynthetic prokaryotic algae [1]. The members of cyanobacteria are not only found in aquatic habitats but also found in terrestrial habitats and extreme habitats such as in hot springs, deserts, frozen lakes, and snow fields so are called as cosmopolites [2]. Several species of cyanobacteria are capable to grow and adapt in an extreme environment, for example *Synechococcus* sp. This microorganism is known to be able to adapt to high temperatures [1]. Temperature is one of the environmental factors that affect the biological processes of cyanobacteria. This will result in a slightly different appearance of morphological and cell size (slightly smaller) than freshwater cyanobacteria [3].

As it is known, that based on resistance to ambient temperature, cyanobacteria can be grouped into psychrophilic, mesophilic, thermophilic, and hyperthermophilic [4]. *Synechococcus* sp. is the dominant species found in high temperature environments (± 24 °C – 90 °C). This microorganism which is lived in hot springs can survive in the 50 °C - 73 °C temperature range [1]. Another study of the effects of high temperature (± 72 °C) on thermophilic cyanobacteria *Synechococcus vulcanus* have
been performed in Japan [5]. Nevertheless, knowledge of hot spring cyanobacteria, especially *Synechococcus* in Indonesia has not been done well, especially in cell density and chlorophyll content of strain of *Synechococcus* sp., which is predominantly grown in the Rawa Danau Banten hot spring (*Synechococcus* sp. RDB001). It is not known whether this cocoid cyanobacteria is able to grow at a maximum of 30 ± 5 °C and 50 ± 5 °C in artificial incubation cabinets.

The aim of this research is to know the comparison of cell density and chlorophyll content of *Synechococcus* sp. RDB001 which were grown at 30 ± 5 °C and 50 ± 5 °C in incubation cabinets. This can be one as the bases for further research, such as DNA level research or bioprospective research. The research has benefits for an understanding of physiological tolerance and adaptation limits on *Synechococcus* sp. RDB001 ex situ. By knowing this, it can be seen whether *Synechococcus* belongs to the group of psychrophilic, mesophilic, thermophilic, and hyperthermophilic growth temperatures.

2. Materials and methods

2.1. Microorganisms, medium, locations and time of study

The *Synechococcus* used in the study was isolated from hot spring water sample at Rawa Danau Banten with water temperature 50 °C. The isolate was coded RDB001 (Rawa Danau Banten isolate number 001) or HS-8 (Hot Spring number 8) [6]. Medium used for the culture was MA [7]. The research was conducted at Department of Biology FMIPA UI especially in Laboratory of Plant Taxonomy, and at CoE Laboratory IBR-GS FMIPA UI, Depok.

2.2. Treatment and research design

The treatments were given to *Synechococcus* sp. RDB001 for 16 days and using two temperatures (30 ± 5 °C and 50 ± 5 °C) with 16 replications each. The amount of treatment and replication was made based on the Frederer formula, i.e. (t-1) (n-1) ≥15, where t is the number of treatments and n is the number of replications. The research was descriptive using non-parametric statistical analysis of Mann Whitney test with real level (α) 0.05 [8]. The Mann Whitney test aims to compare the average of cell density of *Synechococcus* sp. RDB001 (cells/mL) which was grown at temperature of 30 ± 5 °C and 50 ± 5 °C for 16 days of observation. In addition to Mann Whitney test analysis, Spearman test was also used with α = 0.05 [8]. Spearman test aims to test the correlation hypothesis between the cell density (cells/mL) with the chlorophyll content (mg/L) of *Synechococcus* sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C for 8 days of observation (t, t, t, t, t, t, t, t).

2.3. Working culture *Synechococcus* sp. RDB001 as starter

*Synechococcus* sp. RDB001 for working culture was rejuvenated by inserting the cultures with cell densities of 0.25 x 10⁶ cells/mL into MA medium in a 500 mL Erlenmeyer flask. The combined medium and culture results were 400 mL. After rejuvenation, purification was carried out with a dilution method using a microplate. The purified cultures *Synechococcus* sp. RDB001 starter was made into a ready-made and free of contaminants. The starter was made by inserting the cell density cultures of 0.15 x 10⁶ cell/mL into MA medium in the Erlenmeyer flask. 1000 mL until the combined medium and culture result was 400 mL. The starter was then grown in an incubation cabinet at a temperature of 50 ± 5 °C with a light intensity of 2,500 lux for 16 days. The photoperiodicity (light/dark) was 14 light hours/10 dark hours set with a timer (timer). The inoculum was 0.15 x 10⁶ cells/mL in 172 mL MA. Temperature measuring instrument was a thermometer temperature 100 °C.

2.4. Calculation of cell density of *Synechococcus* sp. RDB001

The calculation of cell density of strain RDB001 was performed by the Improved Neubauer method (Improved Neubauer Assistant Germany) [9]. Microscopic observation was continued every 24 hours from the day 0 (t) to day 16 (t) regularly and time constant. Observation of the first day (t) was calculated after inoculation. The cell number data obtained was used to calculate the cell density in 1.0 mL. The calculated cell was a cell located in 4 large compartments located at the outer corner of the Improved Neubauer chamber with a W (white) mark [9]. The cell density in 1.0 mL of the sample was calculated by the formula k = n x p x 2500, with k = cell density of cyanobacteria (sel/mL), n = total number of individual cells on all four compartment counting chambers, and p is the level of dilution used. Calculations were carried out in 16 replications of each treatment. The growing curve is the relationship between cell densities of *Synechococcus* sp. RDB001 (Y axis) at specified intervals (X axis).
2.5. Measurement of chlorophyll content Synechococcus sp. RDB001

Measurement of the chlorophyll content of Synechococcus sp. RDB001 was carried out not every day such as the calculation of cell density. The measurement of the chlorophyll content of Synechococcus sp. RDB001 follows the phases of the growth of the microorganisms, i.e. the first day (t), second (t), third (t), seventh (t), ninth (t), fourteenth (t), and sixteenth (t). Each measurement was carried out at a constant time. The first step of measuring chlorophyll content was weighing a 15 mL empty centrifugation tube. After that, 4 mL of the test culture was put into a centrifugation tube, and then centrifuged for 25 minutes at 4000 rpm.

The formed supernatants were decanted, and then weigh the fresh weight of the culture (pellet and centrifugation tube). After weighing, 0.5 mL of 85 % acetone was added as an organic solvent into the centrifugation tube, and then mixed between acetone and pellets using the pipetting method until homogeneous. The homogeneous mixture was transferred into a 1.5 mL Eppendorf tube containing 25 sterile glass beads. The mixture in the Eppendorf tube was homogenized using vortex for ± 5 minutes, then the Eppendorf tube was centrifuged for 5 minutes at 13,000 rpm using micro-centrifugation. The formed supernatant was taken as much as 4 µL, then dropped into a hole of UV Vis spectrophotometer NANODROP 1000 and measured the chlorophyll concentration at a wavelength of 645 nm and 663 nm [10].

3. Results and discussion

3.1. Cell density and growth curve of Synechococcus sp. RDB001

The difference in average cell density of Synechococcus sp. RDB001 between Synechococcus sp. RDB001 grown at a temperature of 30 ± 5 °C and grown at a temperature of 50 ± 5 °C was presented in figure 1. The average cell density of Synechococcus sp. RDB001 on the first day of observation (t) which was grown at temperatures of 30 ± 5 °C and 50 ± 5 °C decreased, when compared with the number of initial inoculums (t). After that, on the second day of observation (t), the average cell density of Synechococcus sp. RDB001 grown at a temperature of 30 ± 5 °C and 50 ± 5 °C increased. The average cell density at t was not so different from the average cell density at t.

The average cell density of Synechococcus sp. RDB001 on the third day of observation (t) grown at temperatures of 30 ± 5 °C and 50 ± 5 °C also increased, although the two temperature treatments resulted in differences in the average number of cell densities. The average cell density of Synechococcus sp. RDB001 grown at a temperature of 30 ± 5 °C started to increase at t, whereas in Synechococcus sp. RDB001 which grown at 50 ± 5 °C occurred at t. Based on figure 1, it could be concluded that the possibility of Synechococcus sp. RDB001 grown at a temperature of 30 ± 5 °C through a phase of adaptation for 3 days (t - t), while Synechococcus sp. RDB001 grown at a temperature of 50 ± 5 °C through a phase of adaptation for 8 days (t1 - t8).

The difference in the adaptation phase of Synechococcus sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C, probably due to Synechococcus sp. grown at a temperature of 50 ± 5 °C experiences stress due to heat exposure derived from incandescent light 4x100 Watt and 2x75 Watt. The stress of heat exposure resulted in the reduction of fluidity of the cell membranes of Synechococcus sp. cells [5]. It may also be experienced in cell of Synechococcus sp. RDB001 grown at a temperature of 50 ± 5 °C, resulting in cells of Synechococcus sp. RDB001 took more time to adapt to the environment. In addition to the reduction of fluidity of the thylakoid membrane, the possibility of the increased temperature causes the cells of Synechococcus sp. RDB001 to synthesize heat shock proteins (HPSs) and photosynthetic pigment proteins (phycobilin and caroten) that reduce heat exposure [3].

After passing the adaptation phase, the cell Synechococcus sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C experienced a significant increase in cell density, when compared with observations at t. The average cell density of Synechococcus sp. RDB001 grown at a temperature of 30 ± 5 °C was increased high enough, which is ± 60 times, from t of 0.73750 ± 0.40944 x 10^6 cells.mL^-1 to t, which was equal to 24.2075 ± 5.33926 x 10^6 cells.mL^-1. The highest cell density rate of Synechococcus sp. RDB001 grown at a temperature of 30 ± 5 °C occurred at t, that was 24.2075 ± 5.33926 x 10^6 cells.mL^-1.

In contrast, Synechococcus sp. RDB001 grown at a temperature of 50 ± 5 °C had an increase in the average cell density which was not so high. The average increase in cell density was only ± 6 times, from t by 0.23975 ± 0.18798 x 10^6 cells.mL^-1 to t, that was 1.21313 ± 2.92573 x 10^6 cells.mL^-1. The highest cell density rate of Synechococcus sp. RDB001 grown at 50 ± 5 °C occurred at t, that was 1.21313 ± 2.92573 x 10^6 cells.mL^-1.
Figure 1. Growth curve of *Synechococcus* sp. RDB001 based on cell density at temperature (a) 30 ± 5 °C and (b) 50 ± 5 °C

Based on figure 1, it could be concluded that the possibility of *Synechococcus* sp. RDB001 grown at a temperature of 30 ± 5 °C experienced a log phase for 9 days (t - t.), while *Synechococcus* sp. RDB001 grown at a temperature of 50 ± 5 °C experienced a log phase for 6 days (t - t.). The speed of the log phase in *Synechococcus* sp. RDB001 which grown at 50 ± 5 °C (6 days) compared to 30 ± 5 °C (9 days) was not followed by a higher average increase in cell density (only 6 times) compared to 30 ± 5 °C (60 times). The difference in the old log phase that occurs in *Synechococcus* sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C may be affected by cell metabolism and cell resistance to high temperatures. This was in accordance with the statement of Fogg and Thake [11], which stated that the log phase is the phase when the microorganisms require more energy for growth than the other phases. In the log phase, microorganisms experience the highest growth and increase in the number of cells. In addition, in the log phase, the condition of microorganisms is sensitive to environmental conditions.

After the log phase, observation of *Synechococcus* sp. cells. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C still showed an increase and decrease in cell density (fluctuation). *Synechococcus* sp. RDB001 grown at a temperature of 30 ± 5 °C continued to fluctuate in the average cell density at t. to t.. Meanwhile, *Synechococcus* sp. RDB001 grown at 50 ± 5 °C fluctuated in the average of cell density at t. to t.. Fluctuations in the average of cell density of *Synechococcus* sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C may be caused by death of the cells. However, organic matter from the dead cells causes nutrients in the growth medium to increase, so the nutrients are used by living cells for increasing the subsequent cell division. After the average cell density fluctuation occurs on the 16-day observation at both treatment temperatures, the cell will enter the stationary phase and then it will enter the phase of death. The growth curve of *Synechococcus* sp. RDB001 for 16 days observation at both new temperature treatments can be seen in 2 phases, that was phase of adaptation and logarithmic phase (log). Meanwhile, the stationary phase and the death phase were not observed in the study.

3.2. Macroscopic observation of *Synechococcus* sp. RDB001 culture

The results of macroscopic observation of cultures during 16 days of observation showed significant
Figure 2. Macroscopic appearance of Synechococcus sp. RDB001 culture at temperature 30 ± 5 °C and 50 ± 5 °C on (a) first day, (b) seventh day and (c) sixteenth day

color differences between cultures of Synechococcus sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C (figure 2). Macroscopic observations of the cultures of Synechococcus sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C on the first day (t) still showed a clear color and no color difference at both temperature treatments, although the average cell density of the cultures of Synechococcus sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C were different. Possibly, it was because the cells have not metabolized to the maximum because it is still adjusting to the environment.

The macroscopic color difference of culture began to look different on the 7th day observation. The macroscopic observations of the cultures of Synechococcus sp. RDB001 grown at 30 ± 5 °C appeared slightly cloudy compared to the macroscopic cultures of Synechococcus sp. RDB001 grown at 50 ± 5 °C which still looked clear. It supports that the average cell density of Synechococcus sp. RDB001 grown at 30 ± 5 °C was higher than Synechococcus sp. RDB001 grown at 50 ± 5 °C. The significant macroscopic color difference of cultures was seen at day 16 of observations. Macroscopic appearance of the cultures of Synechococcus sp. RDB001 grown at 30 ± 5 °C in leaf green (112), while the macroscopic appearance of culture Synechococcus sp. RDB001 grown at 50 ± 5 °C remains clear. The macroscopic appearance of the culture color indicates the average number of cell densities in the culture. The more viscous the macroscopic color of culture, the higher the cell density.

Macroscopic appearance of the cultures of Synechococcus sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C were significantly different in 16 days of observation. It is probably related to the photoinhibition and photobleaching on cells grown at a temperature of 50 ± 5 °C. Photobleaching causes damage to chlorophyll, so the macroscopic color of the culture does not look green. High temperatures above 48 °C can trigger photobleaching [12].

The result of the statistical analysis [8] concludes that there was a difference in average cell density of Synechococcus sp. RDB001 grown at a temperature of 30 ± 5 °C to the one grown at 50 ± 5 °C, at α = 0.05. The average cell density of Synechococcus sp. RDB001 grown at 50 ± 5 °C was lower than the average cell density grown at 30 ± 5 °C, although the isolate originates from the Rawa Danau Banten hot spring at 50 °C. It was possible that Synechococcus sp. RDB001 was a thermophilic facultative species. The thermophilic facultative nature is a tolerant property with a wider range of temperatures compared with other thermophilic Synechococcus properties. Bergey stated that the thermophilic facultative is a cell capable of growing at room temperature (20 °C) and its maximum
temperature at 60 °C [13]. Inoue et al. [5] concluded that the temperature sensitivity of Photosystem II determines the growth limits at high temperatures and low temperatures in photosynthetic organisms. Therefore, the possibility is the Synechococcus sp. RDB001 grows optimally at a temperature of 30±5 °C.

In addition to thermophilic facultative properties and temperature sensitivity in Photosystem II, the possibility of temperature tolerance to the cell growth becomes one of the factors causing the low average cell density of Synechococcus sp. RDB001 grown at 50 ± 5 °C. The optimum temperature for growth and photosynthesis of Synechococcus sp. from Hunter-Oregon's hot spring ranges from 63 to 67 °C, lower than the average temperature of its original habitat 70 °C [14]. The results of laboratory tests suggested that the cyanobacteria experience a chronic stress in nature [14]. Possibility of Synechococcus sp. RDB001 also experiences chronic temperature stress in nature. In addition, there may be also microhabitat factors that were not fulfilled in the incubation cabinet. Therefore, Synechococcus sp. RDB001 grown optimally at a temperature of 30 ± 5 °C, which was lower than the water temperature in Rawa Danau Banten (50 °C) as the temperature of its original habitat.

Alternatively, Synechococcus sp. RDB001 had an optimum temperature of 30 ± 5 °C. If the temperature given was above the optimum temperature, then the cell density will decrease. This is supported by the research of Meeks and Castenholz [15] which shows that the cell density of Synechococcus sp. decreases gradually when the temperature is above the optimal (63 to 67 °C) before it reaches the upper limit temperature. The evolution of the temperature response to photosynthesis in thermophilic Synechococcus sp. has shown a different response than that proposed by Brock, which states that high temperatures in Synechococcus sp. can optimize growth and photosynthesis as long as the temperature is below the upper limit (50 – 75 °C) [15].

In addition to temperature tolerance related, the possibility of heat shock protein (HSPs) response are also the one factor in the growth of cells Synechococcus sp. RDB001. Research conducted by Borbély et al. [12] on the Synechococcus sp. PCC 6301 strains showed that the response of cells during a heat shock at a temperature of 47 °C resulted in the decrease cell growth. It was shown that the increase in temperature from 39 to 47 °C in cultures that grew logarithm was followed quickly by
the changes in protein synthesis (HSP). At 47 °C, protein synthesis is greatly reduced compared to at 39 °C. *Synechococcus* sp. RDB001 responds to shifting the growth temperature increase (47 °C) with a temporary induction of a set of specific polypeptides. This phenomenon increases the cell level quickly and markedly as in growth under normal temperature conditions. Based on these studies, the possibility of *Synechococcus* sp. RDB001 also experiences the same response when grown at a temperature of 50 ± 5 °C. *Synechococcus* sp. RDB001 which was grown at a temperature of 50 ± 5 °C decreased, so that the average of cell density of *Synechococcus* sp. RDB001 which was grown at a temperature of 50 ± 5 °C was lower than the average cell density of *Synechococcus* sp. RDB001 which was grown at a temperature of 30 ± 5 °C.

### 3.3. Correlation between cell density and chlorophyll content of *Synechococcus* sp. RDB001

The increase of cell density, generally followed by an increase in chlorophyll content. Chlorophyll is a pigment that plays an important role in photosynthesis that produces carbohydrates, which will then be used in cell metabolism to support cell growth and multiplication [16,17]. Based on the research, the correlation between the average cell density and average of chlorophyll content of *Synechococcus* sp. RDB001 grown at 30 ± 5 °C and 50 ± 5 °C for 8 days of observation (t, t, t, t, t, t, t, and t.) showed different patterns (figure 3). An increase in the average cell density is not always followed by the increase in average chlorophyll content. *Synechococcus* sp. RDB001 grown at 30 ± 5 °C, at t, t, t, and t, had the same pattern of increase in cell density averages and chlorophyll content (table 1). However, *Synechococcus* sp. RDB001 grown at 50 ± 5 °C occurs only in t, (table 1). Differences in the pattern of increasing, probably due to the degradation of phycobilin at high temperatures. Fork et al. [18] found that high temperatures (72 °C) caused phycobiliprotein to degrade. In addition, the difference in average cell density and chlorophyll content may be due to chlorophyll content having a percentage of only 1.5% of the dry weight of an organic material or equal to 1 to 67 weight of biomass [19]. Therefore, in the study of the increasing cell density of *Synechococcus* sp. RDB001 is not always followed by increasing of chlorophyll content of *Synechococcus* sp. RDB001 both grown at a temperature of 30 ± 5 °C and 50 ± 5 °C.

The result of the statistical analysis [8] concludes that there was no correlation between the average cell density and the content of chlorophyll *Synechococcus* sp. RDB001 both grown at a temperature of 30 ± 5 °C and at a temperature of 50 ± 5 °C. Increasing of the average cell density of *Synechococcus* sp. RDB001 had no effect on the average increase of chlorophyll content.

### 4. Conclusions

From this research the following conclusions can be drawn, *Synechococcus* sp. RDB001 which was grown at 50 ± 5 °C did not experience an optimum growth with parameters of the average cell density and chlorophyll content. In addition, the average cell density and chlorophyll content of *Synechococcus* sp. RDB001 grown at 30 ± 5 °C was higher than *Synechococcus* sp. RDB001 grown at 50 ± 5 °C.

### Table 1. Comparison between average of cell density and chlorophyll content of *Synechococcus* sp. RDB001 at 30 ± 5 °C and 50±5 °C

| Age days | Average of cell density (x10⁶ cells.mL⁻¹) | Average of chlorophyll content (mg.L⁻¹) |
|----------|------------------------------------------|----------------------------------------|
| 30 ± 5 ºC | 50±5 °C                                  |                                        |
| t₀       | 0,15000 ± 0                              | 0,15000 ± 0                            |
| t₁       | 0,13515 ± 0,00455                        | 0,13219 ± 0,0087                       |
| t₂       | 0,28313 ± 0,06724                        | 0,15638 ± 0,02708                      |
| t₃       | 0,41563 ± 0,09041                        | 0,17944 ± 0,04939                      |
| t₇       | 5,94000 ± 1,54237                        | 0,17813 ± 0,06948                      |
| t₉       | 13,2444 ± 3,22954                        | 0,23975 ± 0,18798                      |
| t₁₄      | 19,0838 ± 4,90953                        | 0,46350 ± 0,11532                      |
| t₁₆      | 24,6056 ± 7,46444                        | 0,13875 ± 3,14631                      |

Based on the research, the following conclusions can be drawn: *Synechococcus* sp. RDB001 which was grown at a temperature of 50 ± 5 °C did not experience an optimum growth with parameters of the average cell density and chlorophyll content. In addition, the average cell density and chlorophyll content of *Synechococcus* sp. RDB001 grown at 30 ± 5 °C was higher than *Synechococcus* sp. RDB001 grown at 50 ± 5 °C.
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