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Influence of Temperature Variations on Growth of *Nostoc* (Cyanobacteria) HS-5 and HS-20 Isolated from Indonesian Hot Springs

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The research aims to know the effect of variation temperature to the growth of *Nostoc* HS (Hot Spring)-5 and HS-20. Strain of *Nostoc* HS-5 was isolated from Ciseeng hot spring which has habitat temperature range of 30-43 °C, and *Nostoc* HS-20 was isolated from Pancar Mountain hot spring which has temperature range of 46-69 °C. The research was done by measuring biomass weight and chlorophyll content on day-1, 2, 3, 4, 7, 10, 14, 17, and 21. The temperatures used were 20 °C, 35 °C, and 50 °C. The growth medium used was Bold Basal Medium (BBM) with pH 6.6. Each treatment was made in four replications. Non-parametric statistical analysis used were the Friedman test (α=0.05) and Spearman test (α=0.01). The result showed there were significant differences on the biomass weight of *Nostoc* HS-5 and HS-20 grown at temperature of 20 °C, 35 °C, and 50 °C. The average amount of biomass highest weight for Nostoc HS-5 and HS-20 occurred in both strains were grown at 35 °C. Besides that, there was no correlation between the weight of biomass and chlorophyll content of *Nostoc* HS-5 and HS-20.

Key words: biomass weight, cyanobacteria, hot spring, *Nostoc*, temperature

Penelitian ini bertujuan untuk mengetahui pengaruh variasi suhu terhadap pertumbuhan *Nostoc* HS (Hot Spring)-5 dan HS-20. Galur *Nostoc* HS-5 diisolasi dari sumber air panas Ciseeng yang memiliki rentang suhu habitat 30-43 °C, dan *Nostoc* HS-20 yang diisolasi dari sumber air panas Pancar Mountain yang memiliki rentang suhu 46-69 °C. Penelitian dilakukan dengan menimbang berat biomassa dan mengukur kandungan klorofil pada hari ke 1, 2, 3, 4, 7, 10, 14, 17, dan 21. Suhu yang digunakan adalah 20 °C, 35 °C, dan 50 °C . Media pertumbuhan yang digunakan adalah Bold Basal Medium (BBM) dengan pH 6,6. Setiap perlakuan dibuat dalam empat ulangan. Analisis statistik non parametrik yang digunakan adalah uji Friedman (α= 0,05) dan uji Spearman (α= 0,01). Berdasarkan hasil kualitatif, ada perbedaan yang signifikan pada berat biomassa *Nostoc* HS-5 dan HS-20 yang ditanam pada suhu 20 °C, 35 °C, dan 50 °C. Jumlah rata-rata berat biomassa yang paling tinggi untuk *Nostoc* HS-5 dan HS-20 terjadi pada kedua strain tersebut yang ditumbuhkan pada suhu 35 °C. Selain itu, tidak ada korelasi antara berat biomassa dengan kadar klorofil *Nostoc* HS-5 dan HS-20.

Kata kunci: berat biomassa, cyanobacteria, *Nostoc*, suhu, sumber air panas

Nostoc is a heterocystous filamentous Cyanobacteria genus from order of Nostocales and family of Nostocaceae (Bold and Wynne 1978), has a chain-like structure and each cell is covered by sheath. Nostoc has a differentiated vegetative cell that called heterocyst and akinete. Heterocyst is a place where nitrogen fixation is occurred and akinete is a place where reproduction is occurred (Lee 2008). The asexual reproduction of Nostoc commonly occurs with production of hormogonia. Hormogonium is a cell which produces by heterocyst or decayed small filaments of Nostoc which is separated from parent cell then forms new colony (Komarek *et al.* 2014).

The genus of Nostoc can be found in many habitats. The Nostoc habitats generally in terrestrial and subaerial, spread in alkaline soil and moist stone. Nostoc can also be found in various temperature areas, i.e. hot spring (Hogg 2005). Nostoc has an adaptation activity to survive in high temperature areas, i.e. making a mat-structure, heat shock proteins (HSPs), and etc.

Environmental is the key factor of microorganism growth. Temperature is one of the environmental factors which affects the growth of cyanobacteria (Olsson-francis 2012). The example of cyanobacteria genus which can grow in hot temperature is Nostoc. Prihantini (2015) reported that two strains of Nostoc

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found in two Indonesia hot springs, i.e. HS-5 strain from Ciseeng hot spring and HS-20 strain from Pancar Mountain hot spring. HS-5 and HS-20 are the isolate codes of both strains, while HS stands for Hot Spring.

The growth of Nostoc is performed by increasing of cells amount on certain time unit which describe in growth curve. The growth phase of Nostoc is generally similar with another microorganism, i.e. adaptation phase (lag phase), exponential phase (log phase), stationer phase, and death phase (Madigan *et al.* 2012). The growth measurement of Nostoc is done by measuring the biomass weight of Nostoc during the treatment days. Research was conducted to determine the growth of Nostoc isolated from hot spring Ciseeng (HS-5 strain) and isolated from the red crater of Pancar mountain (HS-20 strain) on temperature variations.

MATERIALS AND METHODS

Microorganisms and Growth Medium. Two filamentous cyanobacteria strains of *Nostoc*, i.e *Nostoc* HS-5 and HS-20 strains were used in this study. Both strains were isolated by pipette method by Prihantini (Prihantini 2015). The two *Nostoc* strains, HS, were isolated from hot spring in Indonesia, i.e. Ciseeng (*Nostoc* HS-5 strain) and Pancar mountain (*Nostoc* HS-20 strain). All strains were cultured in Bold Basal Medium (BBM).

Culturing of Cyanobacteria Starter and Harvesting of Cultures. The cyanobacteria strains were culturing to obtain an optimal biomass on BBM broth. The biomass was prepared as starter cultures and harvested at logarithmic phase or exponential phase on the 14 d of incubation. Then, 10 mg of starter culture were inoculated to 200 mL of fresh medium in Erlenmeyer flask, and incubated in three separated incubators which has temperature 20 °C, 35 °C, and 50 °C. The treatment was given at 3 temperatures, namely 20 °C, 35 °C, and 50 °C, with 4 replication each. Observation was done for 21 d, and the observed parameters were biomass weight, pH, temperature of the incubators, and cyanobacterial morphology.

Measurement of Biomass Weight of *Nostoc*. Biomass weight measurement of *Nostoc* strain was done by counting the different weights of empty microtube and microtube which contains pellet. Measurement was done by growth data collection every day, which were day of 0, 1, 2, 3, 4, 7, 10, 14, 17, and 21. Growth curve was made by comparing the biomass weight in Y axis and harvesting day in X axis. The growth curve could determine the adaptation phase (lag phase), exponential phase (log phase), stationary phase, and death phase.

Measurement of Chlorophyll Content of *Nostoc*. Chlorophyll content measurement of *Nostoc* strains was done by spectrophotometric method. Chlorophyll was extracted from the biomass with 80% acetone and the chlorophyll content was measured by spectrophotometer (Nanodrop UV-Vis 1000, Thermo Fisher Scientific, USA) at 645 and 663 nm of wavelength. The chlorophyll content was calculated using Arnon formula, i.e. 12.7 (D663 nm) – 2.69 (D645 nm) (Meeks 1974). Measurement of chlorophyll content was carried out by collecting data on days 0, 1, 2, 3, 4, 7, 10, 14, 17, and 21.

RESULTS

Macroscopic and microscopic observations of *Nostoc* HS-5 and HS-20 were performed by observing the color of culture (macroscopic observation), and the shape and diameter of *Nostoc* thallus (microscopic observation). Observation of the culture color of *Nostoc* biomass was done by comparing the culture color on the Faber Castell color indicator table. The inoculum used comes from a starter culture age of 21 days. Based on the color chart, both the color of the starter culture and the color of the culture during observation on *Nostoc* HS-5 were green juniper (juniper green), while the color of the culture on the *Nostoc* HS-20 was light brown (bistre) (Fig 1). In addition to macroscopic observations, microscopic observations were also carried out. The thallus shape of *Nostoc* on starter culture and treatment culture were no different. Based on cell diameter measurements, one *Nostoc* HS-5 had a length of 3.36 μm, while one *Nostoc* HS- 20 had a length of 3.69 μm (Fig 2).

Nostoc HS-5 and HS-20 culture growth was seen in changes in the amount of biomass weight. Measurement of biomass weight of the culture was done on day 0 (t0), day 1 (t1), day 2 (t2), day 3 (t3), day 4 (t4), day 7 (t7), day 10 (t10), day 14 (t14), day 17 (t17), and day 21 (t21). The inoculum used is derived from a 21-day starter culture that was in a stationary phase. The inoculum used was 20 mg for each test culture. The biomass weight data can be seen in Table 1.

In addition to the weight of biomass, the measurement of cyanobacteria growth rate can be done by calculating the chlorophyll content in cyanobacteria cells. Chlorophyll is a green pigment produced by cyanobacteria and has an ability to photosynthesize. The measurement of chlorophyll content is generally

Table 1 The average biomass weight ratio of *Nostoc* HS-5 and *Nostoc* HS-20 at incubation temperature was 20 °C, 35 °C, and 50 °C at observation time for 21 days

Time (day)	Biomass weight (mg mL^{-1})					
	20 °C		35 °C		50 °C	
	HS-5	HS-20	HS-5	HS-20	HS-5	HS-20
t_0	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00	0.10±0.00
t_1	0.20±0.08	0.12±0.05	0.22±0.09	0.12±0.05	0.10±0.08	0.12±0.12
t_2	0.20±0.20	0.10±0.00	0.22±0.15	0.07±0.05	0.07±0.05	0.15±0.10
t_3	0.15±0.06	0.10±0.00	0.18±0.09	0.10±0.17	0.10±0.08	0.10±0.00
t_4	0.20±0.08	0.10±0.08	0.20±0.08	0.12±0.12	0.17±0.22	0.05±0.06
t_7	**0.43±0.17**	**0.30±0.18**	**0.53±0.12**	0.32±0.05	**0.20±0.08**	**0.22±0.19**
t_{10}	0.35±0.13	0.20±0.00	0.38±0.09	0.22±0.05	0.12±0.05	0.15±0.06
t_{14}	0.35±0.13	0.12±0.05	0.32±0.38	**0.40±0.022**	0.10±0.14	0.12±0.08
t_{17}	0.20±0.08	0.10±0.08	0.30±0.27	0.30±0.24	0.07±0.09	0.07±0.09
t_{21}	0.15±0.13	0.07±0.09	0.27±0.09	0.15±0.06	0.05±0.05	0.05±0.05

Table 2 Average of chlorophyll content of *Nostoc* HS-5 and *Nostoc* HS-20 incubated in 20 °C, 35 °C, and 50°C

Time (day)	Average of chlorophyll content (mg L^{-1})					
	20 °C		35 °C		50 °C	
	HS-5	HS-20	HS-5	HS-20	HS-5	HS-20
t0	0.13±0.06	0.11±0.07	0.13±0.03	0.10±0.03	0.15±0.02	0.10±0.04
t1	0.23±0.06	0.13±0.04	**0.40±0.22**	0.17±0.07	**0.21±0.12**	0.18±0.03
t2	0.22±0.06	0.16±0.07	0.28±0.11	0.12±0.04	0.13±0.10	**0.19±0.08**
t3	0.14±0.07	0.17±0.03	0.17±0.05	0.17±0.05	0.15±0.003	0.14±0.03
t4	0.14±0.06	0.11±0.04	0.25±0.10	0.17±0.07	0.14±0.06	0.11±0.04
t7	0.16±0.17	0.12±0.06	0.13±0.02	0.12±0.07	0.10±0.08	0.12±0.07
t10	**0.45±0.30**	0.23±0.08	0.33±0.12	0.21±0.05	0.16±0.03	0.13±0.09
t14	0.30±0.21	0.10±0.04	0.25±0.08	**0.24±0.04**	0.12±0.06	0.14±0.03
t17	0.1±0.04	**0.23±0.02**	0.13±0.23	0.16±0.06	0.13±0.02	0.13±0.03
t21	0.37 ±0.07	0.15±0.09	0.32±0.06	0.11±0.04	0.16±0.05	0.12±0.06

performed on the filament cyanobacteria because it is difficult to calculate the number of cyanobacteria cells.

Calculation of chlorophyll content for *Nostoc* HS-5 and HS-20 culture was performed on the same day with the measurement of culture biomass weight. The average of chlorophyll content data can be seen in Table 2.

DISCUSSION

In both strains (Fig 2), there were no heterocyst and akinete. This might be caused by enough nitrogen content in environmental conditions which affecting heterocyst and akinete production. The heterocyst and akinete production are affected by environmental conditions with limited nitrogen content (Ogawa and Carr 1969). The color of both *Nostoc* HS-5 and HS-20 cells had moss green color.

Based on the macroscopic observation of cultures (Fig 3), noticeable color change was apparent between *Nostoc* HS-5 and HS-20 incubated at 20 °C, 35 °C, and 50 °C. *Nostoc* HS-5 incubated at 20 °C appeared to be moss green color, while *Nostoc* HS-20 was golden brown color. At an incubation temperature of 35 °C, visible color differences were quite markedly compared to at 20 °C. The *Nostoc* HS-5 cultures appeared dark green (sap green) color, while the *Nostoc* HS-20 look dark brown (bistre) color.

In contrast to cultures incubated at 20 °C and 35 °C, the *Nostoc* HS-5 and HS-20 cultures incubated at 50 °C experienced very noticeable color changes. *Nostoc* HS-5 and HS-20 which incubated at 50 °C experienced color change to yellowish white (ivory), with a colorless or translucent culture medium. These color changes could be caused by photobleaching that continued until finally changing the color of the culture to white.

Color changes that occurred in cultures, especially

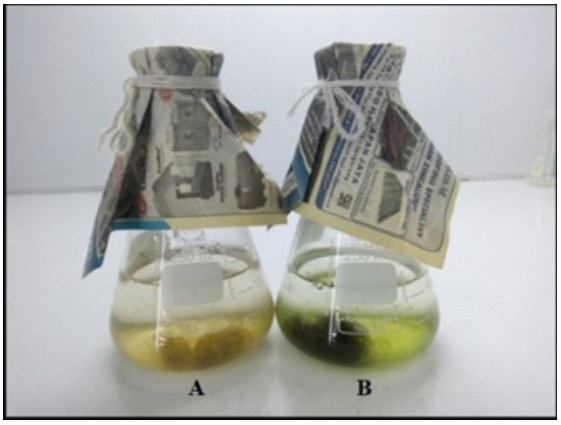

Fig 1 Macroscopic visualization of starter cultures of *Nostoc* HS-5 (A) and *Nostoc* HS-20 (B).

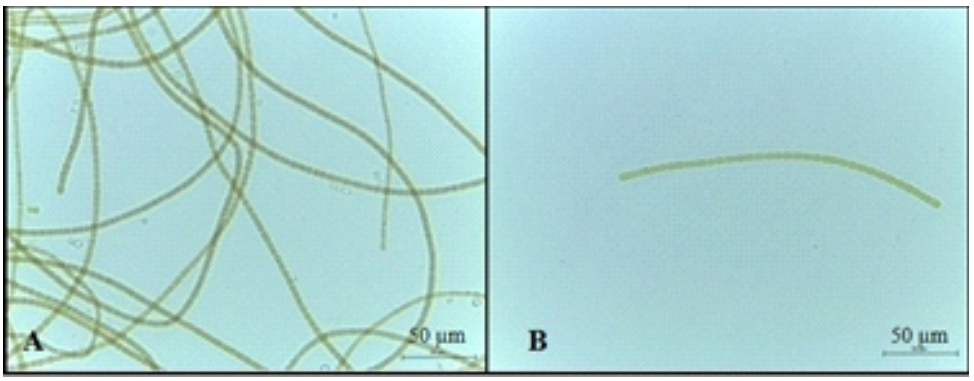

Fig 2 Microscopic characters of *Nostoc* HS-5 (A) and *Nostoc* HS-20 (B) in starter cultures (Bar = 50 µm).

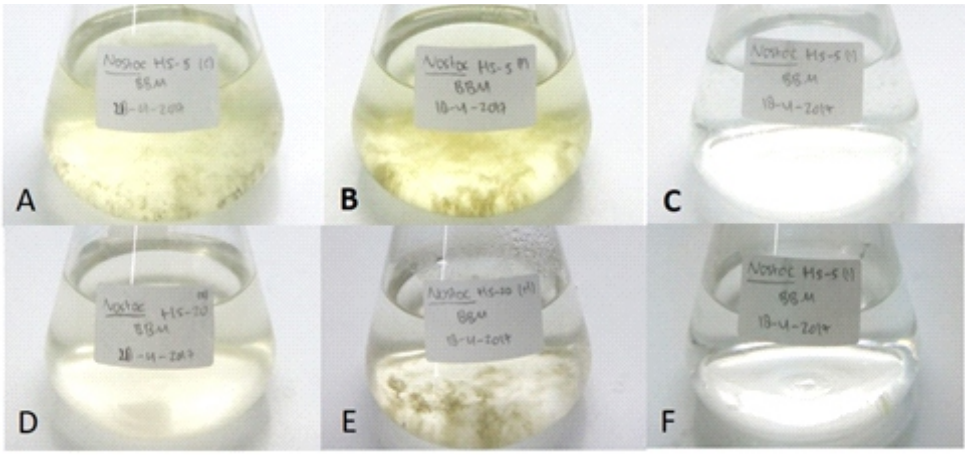

Fig 3 Macroscopic visualization of *Nostoc* HS-5 and HS-20 on day 21. A. HS-5 at a temperature of 20 °C; B. HS-5 at 35 °C; C. HS-5 at 50 °C; D. HS-20 at 20 °C; E. HS-20 at 35 ° C; F. HS-20 at 50 °C.

in cultures of *Nostoc* HS-5 and HS-20 incubated at 50 °C were caused by photobleaching. Photobleaching causes the cell to turn green caused by high temperatures so that the chlorophyll pigment in the cell is damaged (Hsieh *et al*. 2013).

All *Nostoc* strains which were used in this study performed adaptation phase (lag phase), exponential phase (log phase), and death phase, but there's stationer phase in HS-5 incubated in 20 °C and HS-20 incubated in 35 °C in 21 d (Fig 4). All *Nostoc* strains showed a fluctuation on their growth curve. Based on Figure 4, there was any peak difference between the strains.

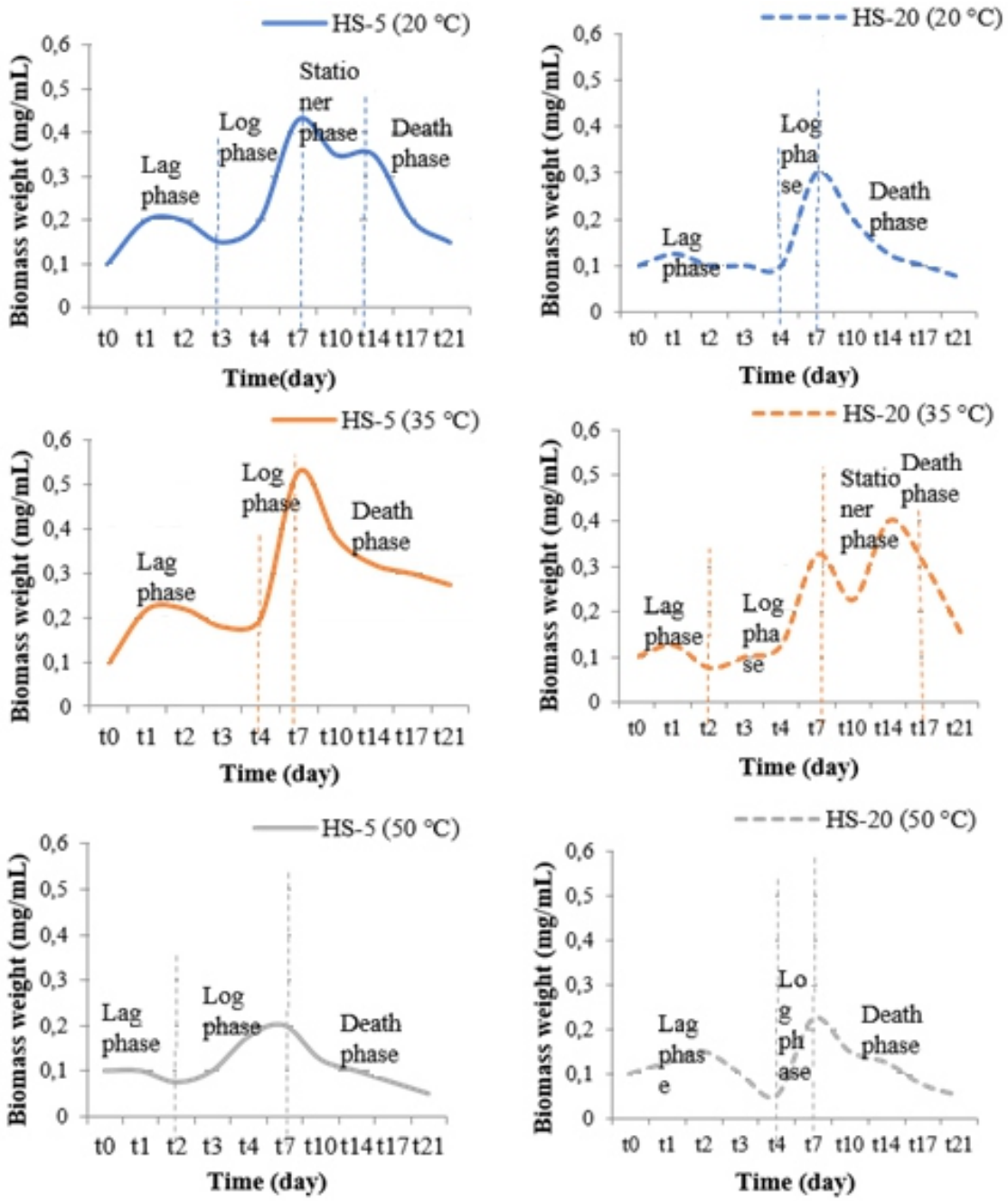

Fig 4 EPS (extracellular polymeric substances) production by an iron-oxidizing indigenous bacterium as represented by emulsifying activity index (EI) over a period of 7 days. Error bars represent standard deviation from the mean (n = 2-3).

Almost all strains performed peak on day-7 (t7), except HS-20 incubated in 35 °C on day-14 (t14). The highest peak occurred in HS-5 incubated in 35 °C that had an average biomass weight of 0.53 mg mL^{-1}. The difference between each strain could be caused by the different metabolism activities between each strain in three kinds of temperatures.

Based on the growth curve of *Nostoc* HS-5 and HS-20 (Fig 4) at the three treatment temperatures, there were a different growth phase between each test culture. The difference in the duration of the adaptation phase could be caused by the ability of each *Nostoc* strain to its new environment. The *Nostoc* HS-5 culture adaptation phase was occurred most rapidly at an incubation temperature of 50 °C, but the amount of weight of the biomass was not apparent. This could be caused because the growth rate of the culture was affected by the high temperature stress founded in the incubator at a temperature of 50 °C so that the weight gain of the biomass was not apparent. Unlike the culture grown at 50 °C, the *Nostoc* HS-5 culture was grown at 35 °C experienced a slower adaptation phase

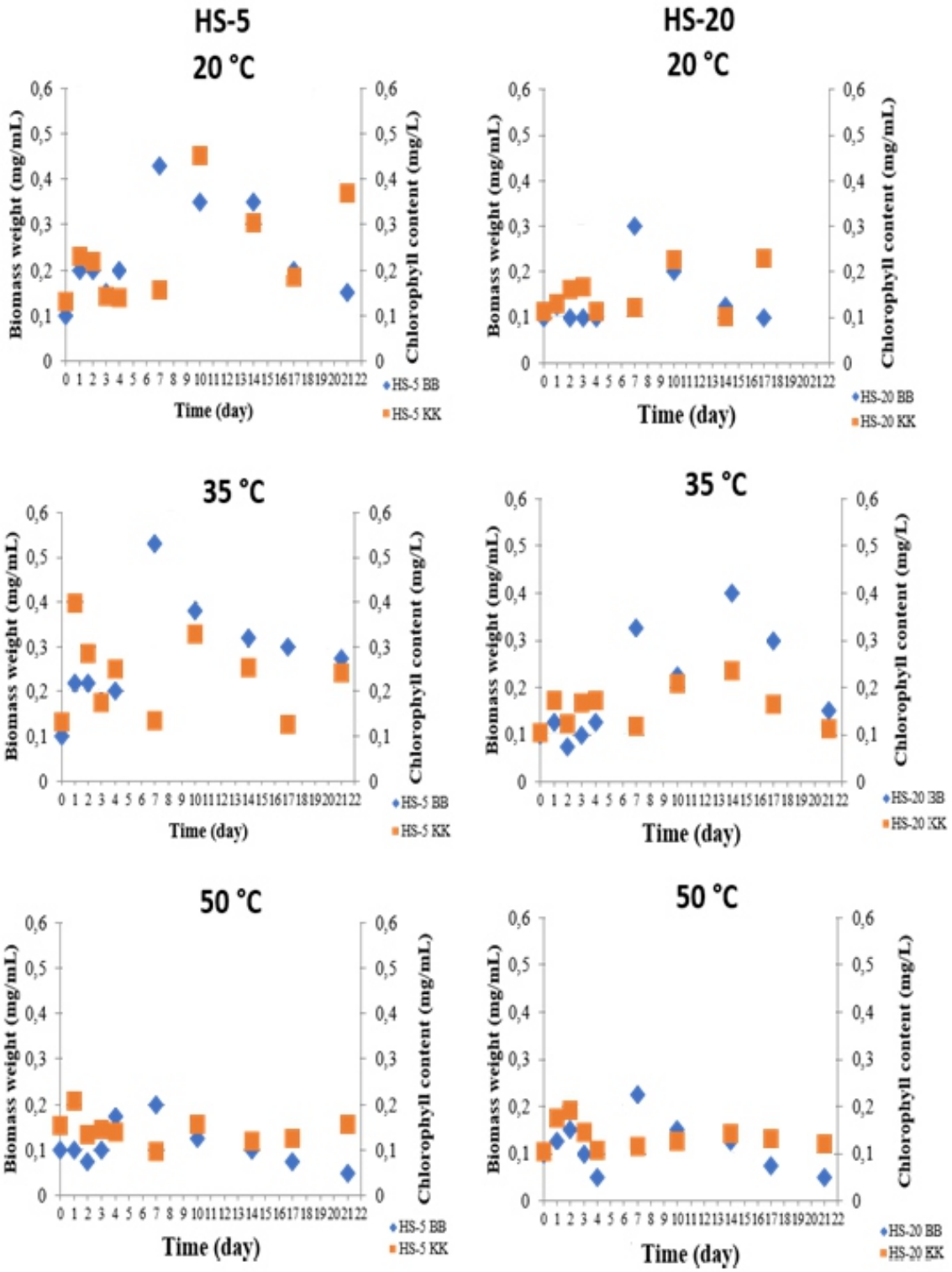

Fig 5 Graphic of correlations between the mean weight of biomass and the chlorophyll content of *Nostoc* HS-5 and *Nostoc* HS-20 incubated in 20 °C, 35 °C, and 50 °C.

but experienced a significant increase in biomass weight. This could be caused by environmental temperature conditions that support the growth of the culture. Unlike *Nostoc* HS-5, the *Nostoc* HS-20 adaptation phase was occurred most rapidly at an incubation temperature of 35 °C. This was supported by the apparent weight gain of biomass compared to 20 ° C and 50 ° C. This could be caused by the *Nostoc* HS-20 culture which was suitable for 35 °C environmental conditions so that the adaptation process at that temperature was quite short.

The difference in the exponential phase of each treatment culture could be caused by the metabolic activity of each culture to the environmental conditions. The difference in metabolic activity was likely to be caused by differences in genetically

different species. The exponential phase in *Nostoc* HS-5 was occurred at an incubation temperature of 50 °C. The culture experienced a weight increase in biomass which was less noticeable compared to 20 °C and 35 °C. *Nostoc* HS-20 culture undergone an exponential phase at an incubation temperature of 35 °C which is seen in the weight gain of biomass which looks quite different.

Based on the graph of the growth curves of both strains (Fig 4), It was seen that *Nostoc* HS-5 cyanobacteria tend to be mesophilic, while *Nostoc* HS-20 is probably a cyanobacteria that tends to be thermophilic. This was shown in the presence of the stationary phase in both strains. The stationary phase in *Nostoc* HS-5 was seen at 20 °C, whereas in *Nostoc* HS-20 there was a stationary phase at 35 °C. In addition, *Nostoc* HS-5 comes from Ciseeng hot springs which have a lower temperature (30-40 °C) than the HS-20 which comes from the hot spring of Pancar mountain (46-69 °C).

Both *Nostoc* HS-5 and HS-20 strains were likely to be thermotolerant. It was based on the growth curve of both strains at 50 °C but the growth was very slow, unlike at 20 °C and 35 °C. In addition, the two strains were found in two Indonesian hot springs which had a high enough temperature. According to Matshushita *et al.* (2015), thermotolerant is a microorganism that can live at high temperatures. Thermotolerant microorganisms are generally found in hot water sources, so that microorganisms have adapted to high temperature environment, but their growth is less optimum (Matshushita *et al.* 2015).

Beside of biomass weight, growth measurement of cyanobacteria can be done by measuring chlorophyll content. Chlorophyll content in *Nostoc* HS-5 and HS-20 had different peaks in each treatment. The highest peak in *Nostoc* HS-5 occurred in 20 °C, although *Nostoc* HS-20 occurred in 35 °C. Based on the average measurement of chlorophyll content, there was no significant difference in the *Nostoc* HS-5 and HS-20 chlorophyll content.

The graphic showed that the increasing of the biomass weight was not followed by the increasing of the chlorophyll content (Fig 5). It could be concluded that there was no correlation between biomass weight and the chlorophyll content in each strain. Besides that, based on the Spearman test conducted for both strains and each incubation temperature, the results obtained were that there was no correlation between the weight of biomass and chlorophyll content.

Temperature variations affected the growth of *Nostoc* HS-5 and HS-20 based on the weight of biomass. *Nostoc* HS-5 and HS-20 grown at 35 °C produced the highest average biomass weight. The highest average chlorophyll content data on both *Nostoc* were different. The highest average chlorophyll content in *Nostoc* HS-5 was found in *Nostoc* grown at 20 °C, while in *Nostoc* HS-20 was found in *Nostoc* grown at 35 °C. There was no correlation between biomass weight and chlorophyll content in *Nostoc* HS-5 and HS-20.

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