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Interaction Effects of Parameters for Carotenoids Production by *Phormidium* sp.

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Abstract: Carotenoids are used in many industries such as food processing, pharmaceuticals, and cosmetics. Cyanobacteria are recognized as an excellent source of carotenoids. However, carotenoid production by cyanobacterium like *Phormidium* sp. can be influenced by various parameters such as nitrogen source concentration, initial pH, and photoperiod. The objectives of this research are to study the interaction effects between those parameters for the maximum carotenoid production by *Phormidium* sp. and determine the kinetics of the carotenoid production. *Phormidium* sp. was cultured in BG-11 medium at a constant temperature of 25°C and white fluorescent light with an intensity of 266 µmol photons $m^{-2}s^{-1}$. The centered composite of Response Surface Methodology developed in the Design-Expert software was used to study the interaction effects of the parameters on carotenoid production, which are nitrogen source concentrations of 0.0167 to 0.0528 mol/L, pH of 4 to 12, and photoperiod of 12:12 to 24:0. It was found that the pH and nitrogen source concentration highly influenced carotenoids production. The maximum carotenoid yield observed by the software is 0.0557 mg/g at the optimized conditions of initial pH of 8.4, sodium nitrate (nitrogen source) concentration of 0.03 mol/L, and photoperiod of 12:12 (Light/Dark), which results in the maximum specific growth rate (μ_{max}), doubling time (t_d) and growth constant (k) of 0.1050 day day^{-1}, 6.6014 days and 0.1515 day^{-1} respectively. The findings can be used for future application of the cyanobacteria culture system for carotenoid production at a larger scale.

Keywords: Phormidium, Cyanobacteria, Carotenoids, pH, Nitrogen source concentration, Photoperiod

1. Introduction

The rise in the usage of carotenoids in various industrial processes is the reason why carotenoids have been largely synthesized chemically and widely extracted from plants, algae, and animals [1]. Cyanobacteria are recognized as an excellent source of carotenoids [2]. The production of carotenoids by Cyanobacteria can be influenced by different environmental parameters, such as photoperiods, pH, nutrient limitation, nitrogen supplements, salinity, and heavy metals stress [3]. The optimum growth conditions for the maximum carotenoid production from *Phormidium* sp. have not been specified although studies were conducted to study the effects of specific growth parameters on its carotenoid production.

Nitrogen source concentration is an important parameter since it is a major nutrient favoring the development of Cyanobacteria [4]. In a previous research done by Santhose et al. [5] on the production of carotenoids by *Phormidium* sp. in BG11 medium at 24 ± 1ºC, at a photoperiod of 12:12 hours (light: dark), it was found that the carotenoids

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production showed a positive increase in lower nitrogen concentrations. However, the limit as to how low the nitrogen source concentration should be to enhance the carotenoid production by *Phormidium* sp. is not yet identified in earlier research. Most freshwater Cyanobacteria tend to prefer slightly alkaline pH [6]. However, highly specialized acidophilic filamentous Cyanobacteria can survive at pH values as low as 2.8 to 4.5 [7]. In this research, the range of the pH of the culture medium was tested from 4 to 12, to find out how pH below and above neutral affects the carotenoids production in *Phormidium* sp.

On the other hand, the photoperiod was found to influence the carotenoids production as higher yield of carotenoids are obtained at higher photoperiod in the research done by Nhu and Hiep [8] on *Spirulina* sp.. However, Tang and Vincent [9] have proven otherwise by stating that the time of exposure of Cyanobacteria to light had a positive effect on its growth and pigment absorption, but not pigment or carotenoids content. Therefore, in this research, the actual influence of the photoperiod on the carotenoids production by *Phormidium* sp. is tested in the range of 12:12 to 24:0 (L/D). Based on another study [10], the growth kinetics of *Phormidium* sp. in BG-11 medium of pH 9, at the temperature of 29°C and photoperiod of 12:12 (L/D), the species had maximum specific growth rate, μ_{max} of 0.135 day^{-1} and doubling time, t_d of 5.1 days. However, the comparison between the growth kinetics of *Phormidium* sp. at different growth conditions has not been conducted.

The interaction effects between parameters for carotenoids production by *Phormidium* sp. have not been studied. Therefore, this research aims to study the interaction effects between the parameters on carotenoids production which are sodium nitrate (nitrogen source) concentrations of 0.0167 to 0.0528 mol/L, initial pH of 4 to 12, and photoperiod of 12 hours to 24 hours light illumination. This is significant in determining the optimized conditions for carotenoids production. The growth kinetics of the species under the optimized conditions were determined in this research.

2. Materials and Methods

2.1. Cyanobacteria and culture medium

The *Phormidium* sp. used in this study was isolated from the Antarctic and given by University Sains Malaysia (USM) to the Herbarium Unit, Department of Landscape Architecture, Kulliyyah of Architecture, and Environmental Design. *Phormidium* sp. has been chosen for this study since it has great potential to be used in bioprocesses due to its robustness and simple nutritional requirements [11]. The species was cultured in jam jars each filled with 50 mL of BG-11 medium at the constant temperature of 25 °C and white fluorescent light with an intensity of 266 µmol photons $m^{-2}s^{-1}$.

2.2. Experimental setup using Response Surface Methodology

The centered composite Response Surface Methodology developed in the Design-Expert software (version 6) was used to study the interaction effects of the parameters on carotenoids production which are nitrogen source concentrations of 0.0167 to 0.0528 mol/L, initial pH of 4 to 12, and photoperiod of 12:12 to 24:0 (L/D). 17 runs were generated by the software. The two responses which are carotenoids yield and cell number were analyzed using analysis of variance (ANOVA).

2.3. Carotenoids Extraction

The method for the separation and extraction of carotenoids from the culture was performed as described elsewhere [12]. After 4 weeks, the culture was transferred into a sterile tube and centrifuged at 8500 rpm for 3 minutes. Thereafter, the supernatant was discarded, and the fresh weight of the cell biomass was measured. About 5 mL of acetone and methanol mixture (ratio of 7:3) was added and shaken vigorously to release the carotenoids. Under the fume hood, 5 mL of hexane was added to each tube for the extraction process. The mixture was shaken to form two layers, wherein the upper layer consists of carotenoids in hexane that was inserted into the HPLC vial during HPLC analysis.

2.4. HPLC analysis

The carotenoids yield was analyzed using reversed-phase HPLC Agilent model 1200 series. The eluents used are (A) acetonitrile:water (9:1 v/v) and (B) ethyl acetate. The temperature of the column is maintained at 20°C. Detection of individual carotenoids was made at the wavelengths of maximum absorption of the carotenoids in the mobile phase: neoxanthin (438 nm), violaxanthin (441 nm), lutein (447 nm), and β-carotene (454 nm). The identification of the individual carotenoids was done by co-chromatography with standards and by elucidation of their spectral characteristics using a photodiode array detector. The analysis for one injection sample of 200 µL took about 45 minutes. The yield for the total carotenoids present in the sample was calculated by summing up the values of the yield for each individual carotenoids.

2.5. Kinetic study

The kinetic study on the carotenoids production by *Phormidium* sp. was done based on the optimized conditions generated by the software. The species was grown for 30 days in 6 jam jars where each jam jar will be taken every 5 days for sampling. The growth curve was generated by plotting the cell number as the y-axis and time as the x-axis. The exponential phase of the curve was used to generate an exponential trendline in the form of an exponential growth equation as in Equation (1).

$$y = Ae^{Bx} \quad (1)$$

Equation (1) resembles the equation of the growth curve as in Equation (2) :

$$X_f = x_o e^{\mu_{max} t} \quad (2)$$

The constant A is denoted as the initial cell number (X_o) while constant B is the maximum specific growth rate (μ_{max}). The doubling time (t_d) was calculated using Equation (3).

$$t_d = (\ln 2)/\mu_{max} \quad (3)$$

From the doubling time (t_d), the growth constant (k) was calculated using Equation (4).

$$k = 1/t_d \quad (4)$$

3. Results and Discussion

3.1 Quantification of carotenoids from HPLC analysis

The detection of neoxanthin, violaxanthin, lutein, and β-carotene according to their adsorption in the mobile phase was achieved. The individual carotenoids were separated based on their polarity to the mobile phase. The stationary phase used was non-polar silica (ZORBAX Eclipse XDB-C18). Since reversed phase was used, the individual carotenoid with the longest retention time is the most non-polar to the mobile phase. From the peaks generated, it can be observed that β-carotene has the longest retention time out of all four carotenoids detected. This is the result of its properties as a non-polar carotenoid [13]. Carotenoids which have the highest solubility in ethyl acetate and acetonitrile are neoxanthin, followed by violaxanthin and lutein, while β-carotene serves as the least soluble in the mobile phase. The carotenoids that are more polar and have a higher solubility in the mobile phase were able to move fast to the detector since it does not retain itself at the stationary phase.

3.2 Interaction effects between parameters for maximum carotenoids production

The analysis of variance (ANOVA) was performed on the carotenoids yield. The two responses from all 17 runs were investigated as shown in Table 1.

Table 1. The experimental results of carotenoids yield and cell number

Run	Factor *A*: Initial pH	Factor *B*: Nitrogen source concentration (mol/L)	Factor *C*: Photoperiod (Light illumination)	Response 1: Carotenoids yield (mg/g)	Response 2: Cell number (cells/mL)
1	8	0.0353	18	0.0297	35714
2	8	0.0529	18	0.0194	25000
3	4	0.0176	12	0	10000
4	8	0.0176	18	0.0226	36667
5	12	0.0529	24	0	10000
6	4	0.0353	18	0.0002	10000
7	12	0.0176	24	0.0021	10000
8	8	0.0353	12	0.0624	13333
9	8	0.0353	18	0.0291	37143
10	8	0.0353	24	0.0369	38000
11	4	0.0529	24	0.0001	10000
12	12	0.0176	12	0.0028	10000
13	4	0.0529	12	0	10000
14	8	0.0353	18	0.0254	30000
15	12	0.0529	12	0.0008	10000
16	4	0.0176	24	0.0001	10000
17	12	0.0353	18	0.0008	10000

3.2.1. Carotenoids yield and cell number as the responses

Based on the analysis of the responses, the software suggested the quadratic model for both responses. As represented in Table 2, the model F values for carotenoids yield and cell number were 151.74 and 3.94 respectively, indicating that the quadratic models are significant. The P>F values for both responses were lower than 0.05, which indicates that the model terms are significant, and the quadratic functions can be applied for successful prediction of the responses to the carotenoids yield and cell number. The lack of fit F-value for carotenoids yield and cell number were 7.11 and 4.86, respectively, indicating that they are insignificant.

The difference between the predicted R^2 and adjusted R^2 for the carotenoids yield that was not more than 0.2 indicates that they are in reasonable agreement with each other while the same case is not evident for the cell number. The model term A (initial pH) is significant in both of the

responses, while model term B (nitrogen source concentration) is only significant for the carotenoids yield. As for the model term C (photoperiod), it is insignificant for both responses. For the carotenoids yield, the model AB and AC values are both significant while the model BC is insignificant. However, the model terms of AB, AC, and BC are all insignificant for the cell number. The regression model equations for both responses are shown in Equations (5) and (6), where factors A, B, C are pH, nitrogen source concentration (mol/L), and photoperiod, respectively.

Final equation in terms of coded factors:

$$\text{Log10 (Carotenoid production)} = -1.52 + 0.18A - 0.11B - 0.055C - 1.47A^2 - 0.17B^2 + 0.19C^2 - 0.13AB - 0.073AC - 0.032BC \quad (5)$$

$$\text{Cell number} = +31565.37 + 0.000A - 1166.70B + 2466.70C - 19525.14A^2 + 1308.36B^2 - 3858.64C^2 + 0.000AB + 0.000AC + 0.000BC \quad (6)$$

From the regression model equation, the 3-D response surface plot was generated. Figure 1a shows the interaction between initial pH and nitrogen source concentration at a constant photoperiod of 18:6 (L/D). From the plot, the maximum carotenoids yield is found at an optimal initial pH of 8 and nitrogen source concentration of 0.0365 mol/L. The effects of the nitrogen source concentration on the carotenoids yield are not as impactful as the pH. The 3D model in Figure 1b illustrates the response of carotenoids yield corresponding to the interaction of initial pH and photoperiod at nitrogen source concentration of 0.04 mol/L.

Table 2. ANOVA of quadratic model response for carotenoids yield and cell number

A

Source	Sum of Squares	DF	Mean Square	*F* value	Prob>*F*	
Model	1.902 E+009	9	2.113 E+008	3.94	0.0422	Significant
A	0.000	1	0.000	0.000	1.0000	
B	1.361 E+007	1	1.361 E+007	0.25	0.6300	
C	6.085 E+007	1	6.085 E+007	1.13	0.3223	
A^2	1.021 E+009	1	1.021 E+009	19.03	0.0033	
B^2	4.586 E+006	1	4.586 E+006	0.085	0.7785	
C^2	3.989 E+007	1	3.989 E+007	0.74	0.4171	
AB	0.000	1	0.000	0.000	1.0000	
AC	0.000	1	0.000	0.000	1.0000	
BC	0.000	1	0.000	0.000	1.0000	
Residual	3.757 E+008	7	5.367 E+007			
Lack of Fit	3.471 E+008	5	6.942 E+007	4.86	0.1794	Not significant
Pure Error	2.857 E+007	2	1.429 E+007			
Cor. Total	2.278 E+009	16				

R^2 = 0.8351 R^2 *adj* = 0.6230 R^2 *pred* = -0.1680

B

Source	Sum of Squares	DF	Mean Square	*F* value	Prob>*F*	
Model	9.54	9	1.06	151.74	<0.0001	Significant
A	0.34	1	0.34	48.77	0.0002	
B	0.12	1	0.12	16.86	0.0045	
C	0.031	1	0.031	4.39	0.0745	
A^2	5.76	1	5.76	824.67	<0.0001	
B^2	0.075	1	0.075	10.78	0.0134	
C^2	0.092	1	0.092	13.19	0.0084	
AB	0.13	1	0.13	18.65	0.0035	
AC	0.043	1	0.043	6.16	0.0421	
BC	8.385 E-003	1	8.385 E-003	1.20	0.3096	
Residual	0.049	7	6.989 E-003			
Lack of Fit	0.046	5	9.264 E-003	7.11	0.1279	Not significant
Pure Error	2.606 E-003	2	1.303 E-003			
Cor. Total	9.59	16				
R^2 = 0.9949	R^2 *adj* = 0.9883	R^2 *pred* = 0.9486				

From the saddle shape response plot, there is no optimum point for the photoperiod. Figure 1c shows the interaction between the nitrogen source concentration and photoperiod at constant initial pH of 8, where the optimum point for both of the factors cannot be obtained. From this finding, the most significant parameter is pH while photoperiod is the least significant.

Figure 2a shows the interaction between initial pH and nitrogen source concentration at a photoperiod of 18:6. From the response surface plot, the maximum cell number was found at an initial pH of 8. As for the nitrogen source concentration, it seems to not affect the cell number. Another 3D model in Figure 2b illustrates the response of cell number corresponding to the interaction of initial pH and photoperiod at nitrogen source concentration of 0.04 mol/L. Figure 2c shows the interaction between the nitrogen source concentration and photoperiod at constant pH of 8. The maximum cell number is achieved at a photoperiod of 24:0 (L/D) without an obvious optimal value on the nitrogen source. From this finding, the most significant factor for cell growth is initial pH while nitrogen source concentration is the least significant.

3.3 Kinetic study on cell growth

The maximum carotenoids yield observed by the software was 0.0557 mg/g at initial pH 8.4, nitrogen source concentration of 0.03 mol/L, and photoperiod of 12:12 (L/D). Other optimized conditions suggested by the software are the initial pH of 8.23, nitrogen source concentration of 0.03 mol/L, and photoperiod of 24:0 (L/D). It was observed that the highest amount of cell number (42,000 cells/mL) and carotenoids yield (0.045 mg/g) were obtained during day 20 at initial pH 8.4, nitrogen source concentration of 0.03 mol/L, and photoperiod of 12:12 (L/D). Furthermore, there was a rapid increase in the carotenoids yield from 0.000156229 mg/g to the highest yield of 0.045 mg/g at day 20. As for the conditions of initial pH 8.23, nitrogen source concentration of 0.03 mol/L, and photoperiod of 24:00 (L/D), the highest amount of cell number (55,000 cells/mL) was obtained during day 20.

The highest carotenoids yield (0.00029 mg/g) was obtained during day 10, which is lower in yield compared to when the initial pH, nitrogen source concentration, and photoperiod were 8.4, 0.03 mol/L, and 12:12 (L/D), respectively. At higher photoperiod of 24:0, carotenoids yield was much lower compared to the photoperiod of 12:12 (L/D). This finding confirms the observation of the responses for the carotenoids yield obtained from the runs in the Response Surface Methodology. The carotenoids yield obtained from the kinetic study conducted under both optimized conditions were not similar to the values suggested by the software. This would have resulted from the random errors that occurred during the experiment that could be improved by incorporating more accuracy in data measurement such as measuring the dry weight of the cell biomass instead of fresh weight.

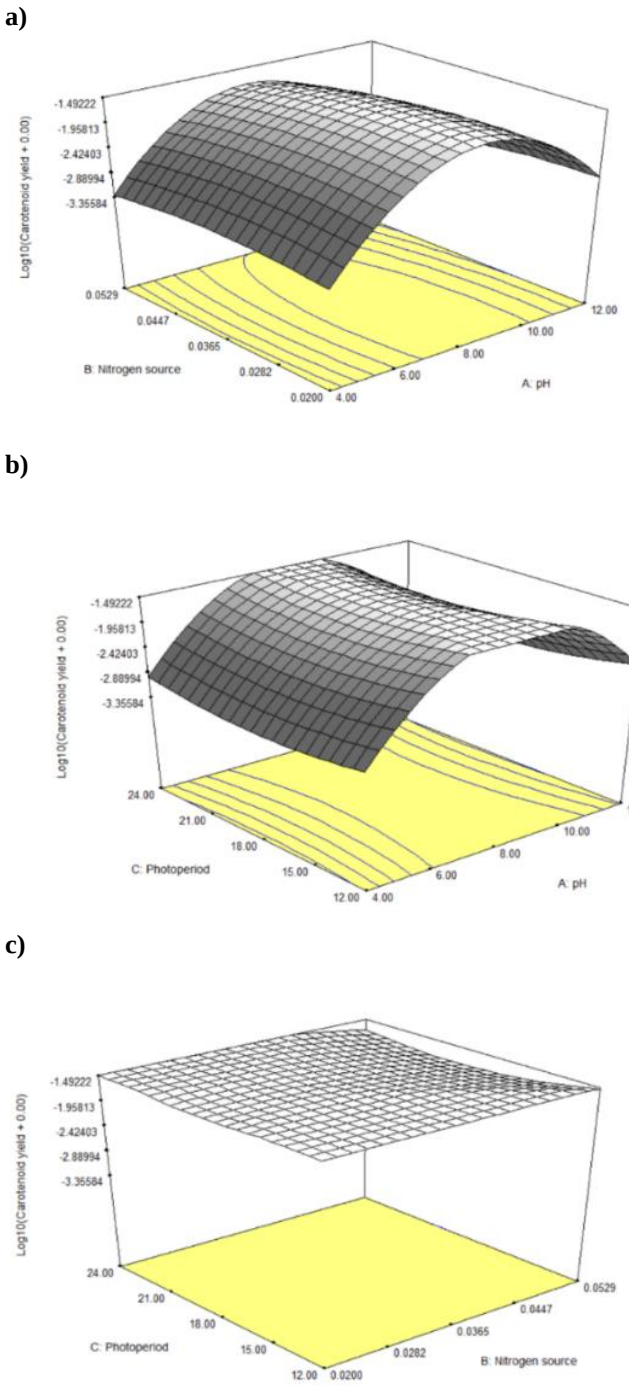

Figure. 1: Response surface curves for carotenoids yield as a function of (a) Initial pH and nitrogen source concentration (b) Initial pH and photoperiod (c) nitrogen source concentration and photoperiod

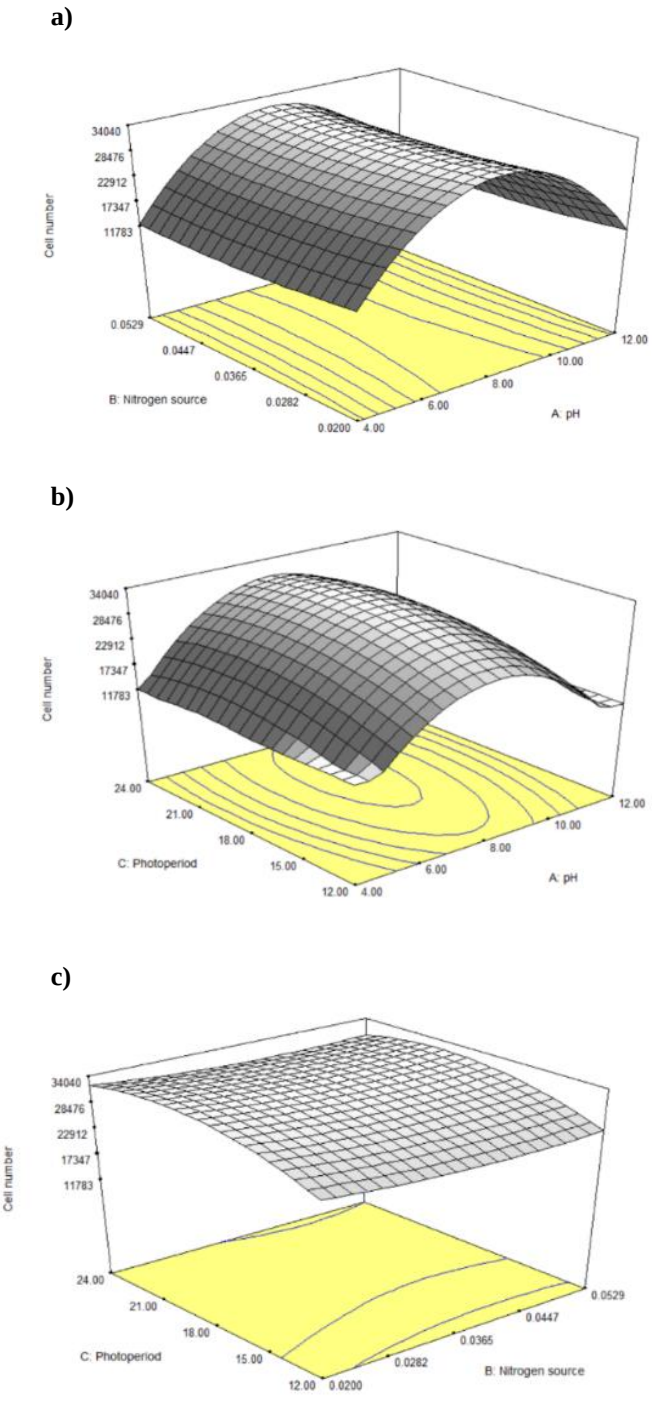

Figure. 2 : Response surface curves for cell number as a function of (a) Initial pH and nitrogen source concentration (b) Initial pH and photoperiod (c) nitrogen source concentration and photoperiod source concentration and photoperiod

Based on the exponential phase curve plotted, the maximum specific growth rate (μ_{max}), doubling time (t_d) and growth constant (k) were obtained. As shown in Table 3, the maximum specific growth rate (μ_{max}) and growth constant (k) when the initial pH is 8.4, nitrogen source concentration is 0.03 mol/L, and photoperiod is 12:12 were lower compared to when the initial pH is 8.23, nitrogen source concentration is 0.03 mol/L and photoperiod is 24:00 (L/D).

Table 3. The comparison between the growth kinetics

Conditions	μ_{max} (day^{-1})	t_d (day)	k (day^{-1})
pH 8.4, nitrogen source concentration 0.03 mol/L and photoperiod 12:12	0.1050	6.6014	0.1515
pH 8.23, nitrogen source concentration 0.03 mol/L and photoperiod 24:00	0.1219	5.6862	0.176

4. Discussion

Based on the responses in Table 1, it was observed that the responses for the carotenoids yield varied in the range of 0-0.0624 mg/g with the highest yield in run 8. However, in the same run, the cell number was 13,333, which is not the highest. The highest cell number was achieved in run 10, under the initial pH of 8, nitrogen source concentration of 0.0353 mg/g, and photoperiod of 24:0 (L/D). The only parameter that differentiates between run 8 with run 10 is the photoperiod, where run 8 and run 10 were conducted at a photoperiod of 12:12 and 24:0 (L/D), respectively. This finding showed that lower photoperiod generates a higher yield of carotenoids while higher photoperiod enhances cell growth. At the maximum carotenoids yield and cell number, the nitrogen source concentration was 0.0353 mol/L. The previous investigation on carotenoids production by *Phormidium sp*. has proven that the carotenoids yield is higher at lower nitrogen source concentrations [5]. The research concludes that during stressful conditions like nitrogen source depletion, more carotenoids are released to stimulate the photosynthetic performance of surface cyanobacterial populations [5].

The highest yield of carotenoids and amount of cell number is obtained at initial pH 8, which makes it the optimal pH for carotenoids production and growth by *Phormidium sp*. This further proved that slightly alkaline pH promotes carotenoids production and growth, which is in agreement with the previous finding on the production of carotenoids by green microalgae *Asterarcys quadricellulare* [14]. This also relates with the finding of the research done by Garcia-Gonzalez et al. [6], which concluded that freshwater Cyanobacteria prefer a slightly alkaline pH. It is proven that the carotenoids yield decreases at higher pH as the light conversion efficiency of the cell was reduced to 8.9% [15]. The finding of the Response Surface Methodology showed that the carotenoids production was enhanced at lower photoperiod, but not the cell number. This finding can be supported by another finding [9], which stated that the time of exposure of Cyanobacteria to light had a positive effect on its growth and pigment absorption, but not pigment content. The less amount of exposure to light at a photoperiod of 12:12 (L/D) has caused the condition to become stressful for the species since the rate of photosynthesis is low and less multiplication of cells is carried out. At the photoperiod of 24:0 (L/D), the cell number was the highest. The same finding was obtained by Touloupakis et al. [16], where the 24:0 (L/D) photoperiod was the best condition for cell growth.

5. Conclusion

From this research, it was found that the pH and nitrogen source serve as the highly significant parameters that influence the carotenoids production by *Phormidium* sp.. The optimum conditions for the carotenoids production were found to be at nitrogen source concentration of 0.03 mol/L, initial pH of 8.4, and photoperiod of 12:12 (L/D). It was found that the interaction effects between the pH and nitrogen source are significant for carotenoids production by *Phormidium* sp.. Through the comparison between the values of specific growth rate, doubling time, and growth kinetics when the photoperiod 12:12 and 24:0 (L/D), it has been proven that the higher photoperiod results in better cell growth instead of the carotenoids production. The findings can be used for future application of the Cyanobacteria culture system for carotenoids production.

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