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Enhancement of biomass and phycocyanin content of *Spirulina platensis*

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1. ABSTRACT

Response surface methodology (RSM) based on the 2^3 factorial central composite design (CCD) was employed to evaluate optimum culture conditions (temperature, light irradiance and agitation) to enhance biomass and phycocyanin content of *Spirulina platensis*. The predicted maximum biomass and phycocyanin content by RSM was 1.06 g L^{-1} and 107 mg L^{-1}, respectively, whereas maximum biomass and phycocyanin content of 1.32 g L^{-1} and 127 mg L^{-1} was obtained in the validation experiments under optimized conditions after 10 days of cultivation. Further, influence of optimized conditions (temperature 33±2 ºC, light irradiance 44 µmol photons m^{-2} s^{-1} and a flow rate of 2.5 L min^{-1}) on growth and phycocyanin content of *S. platensis* in 7L Panel photobioreactor (PPBR) cultivation was investigated. A 15 days production was carried out and it was observed that a maximum biomass yield of 2.42 g L^{-1} with a specific growth rate 0.202 day^{-1} and phycocyanin content of 228 mg L^{-1} was obtained in the PPBR. The optimum culture conditions obtained through response surface methodology were successfully determined to maximize the biomass and phycocyanin.

2. INTRODUCTION

Cyanobacteria and microalgae have been documented as a source of high-value products such as pigments, fatty acids, polysaccharides, essential minerals and vitamins. Natural pigments (chlorophylls, carotenoids and phycobiliproteins) have an important role in the photosynthetic and pigmentation metabolism of algae (1), and also exhibit a wide range of biological activities like anti-cancer, antioxidant and anti-inflammatory (2). The use of microalgae for the food, pharmaceutical and cosmetic market is increasingly relevant as the natural pigments of microalgae and cyanobacteria are the good alternative to synthetic colorants, have the potential to be competitive with the same components from other sources and the ease of cultivation. Owing to their high content and productivity of valuable metabolites, and the capability to grow under extreme environmental conditions made cyanobacteria and microalgae an ideal candidate for commercial scale production. Currently, the pigments produced by cyanobacteria and microalgae and used commercially are carotenoids (β-carotene) from unicellular alga *Dunaliella*; astaxanthin from green alga *Haematococcus*; phycobiliproteins from Cyanobacteria and red algae.

Phycobiliproteins (PBP), a water soluble light harvesting antenna complexes found in prokaryotic cyanobacteria, eukaryotic rhodophytes and cryptophytes, which absorb energy in portions of visible spectrum that poorly exploited by chlorophyll and transfer to the photosynthetic reaction centers to drive photosynthetic electron transport. Based on their spectral properties PBP's divided into phycoerythrin (λ_{Amax} 540-570 nm), phycocyanin (λ_{Amax} 610-620 nm) and allophycocyanin (λ_{Amax} 650-655 nm) (3). PBP's are intensely colored due to the presence of covalently attached chromophores called bilins (4). Among the PBP's, phycocyanin has been the subject of active researches because of its potential for application as a natural pigment (food colorant, cosmetic) (5), having therapeutic properties including anti-inflammatory, neuroprotective, antioxidant (6), anti-cancer activities and its fluorescence properties (7).

Various factors such as nutrient availability, salinity, pH, temperature, light irradiance and agitation speed affect the growth and pigments accumulation in cyanobacteria and microalgae (8, 9). To increase the effectiveness of production of the desired algal product, optimization of the conditions need to be taken into account. The present study aimed to optimize the culture conditions (temperature, light irradiance and agitation speed) to enhance biomass and phycocyanin content of *S. platensis* by applying response surface methodology (RSM). Central composite design (CCD) was applied to evaluate optimum culture conditions to enhance biomass and phycocyanin contents of the *S. platensis* culture. Further, influence of above optimized conditions on growth and phycocyanin profiles of *S. platensis* was evaluated in panel photobioreactor.

3. MATERIALS AND METHODS

3.1. Cyanobacteria

The cyanobacteria strain (*Spirulina platensis* M2) was obtained from The Microalgae Culture Collection of Ege University, Turkey (EGEMACC 30 - http://www.egemacc.com/en/index.php) and cultured in Zarrouk medium (10). The *S. platensis* was firstly cultured in a 1000 mL Erlenmeyer flask under continuous illumination (35 µmol photons m^{-2} s^{-1}) and cultures were aerated with 2 L min^{-1} at the 22±2 ºC temperature to logarithmic phase to attain robust cells for further inoculation.

3.2. Growth and biomass measurement

Biomass in terms of cell concentration of *S. platensis* was measured using UV–Vis spectrophotometer (GE Healthcare Ultrospec 1100 pro, UK) at an absorbance of 560 nm. The dry weight (DW) was measured by filtering 5 mL of *S. platensis* suspension on pre-weighted GF/C Whatman filter papers (Whatman UK), afterwards, rinsed well with distilled water and DWs (g L^{-1}) were calculated after drying in an oven at 65 ºC to a constant weight.

The specific growth rate (μ) of *S. platensis* was calculated as follows: $\mu = (\ln X_2 - \ln X_1)/t_2 - t_1$

3.3. Spectrophotometric determination of phycocyanin

Phycocyanin was extracted from *S. platensis* by using the following method. 5 mL of culture was centrifuged for 5 min at 10000 rpm. The pellet was suspended in 5 mL of 100 mM Sodium Phosphate Buffer (pH 7). To extract phycocyanin, the cell suspension was sonicated at a frequency of 20 kHz for 2 minutes. The suspension was then centrifuged at 4500 rpm for 5 min and the absorbance of the supernatant was recorded at 615 and 652 nm respectively. The phycocyanin content in the extract was calculated as follows (Eq.1) (11).

$$\text{C-PC (mg mL}^{-1}) = \frac{A_{615} - (0.474\ A_{652})}{5.34} \quad (1)$$

Where PC is the phycocyanin concentration (mg mL^{-1}), A_{615} and A_{652} were optical density of the sample at 615 and 652 nm, respectively

3.4. Experimental design and data analysis

A five level and three variables central composite design was applied to optimize the culture conditions to enhance phycocyanin production and to evaluate relationship between dependent (biomass and phycocyanin) and independent variables (X_1-Temperature, X_2-light irradiance and X_3-agitation speed). The levels of the experimental variables investigated are given in Table 1 CCD with eight factorial points, six replicates about the center point and six axial points at a distance α=1.682 from the design center, making a total of 20 runs given in Table 2 was used for optimization of significant culture conditions (temperature, light irradiance and agitation speed). Experimental design and statistical analyses were performed with the aid of software package Design expert (trial version 7.0.0., Stat-Ease Inc., Minneapolis, MN).

The optimizing culture conditions experiments were carried out triplicate in 300 mL of submerged batch cultures in 500 mL Erlenmeyer flasks for the period of 10 days. A 7 % v/v *S. platensis* inoculum in an exponential growth phase was used to inoculate the flasks. The samples were collected for every two days to monitor growth and phycocyanin content. The experimental results were fitted with second-order polynomial equation (Eq.2) which describes relationship between the responses and the independent variables.

Table 1. Central composite design showing low, center and high values, two additional points at α±1.682 were also included

Independent Variable	Symbol Coded	Range	Levels of Variables				
			-1.682	**-1**	**0**	**+1**	**+1.682**
Temperature (°C)	X_1	15 - 30	9.89	15	22.50	30	35.11
Light Irradiance (µmol photons m^{-2} s^{-1})	X_2	20 - 55	8.08	20	37.50	55	66.93
Agitation speed (rpm)	X_3	100 - 140	86.36	100	120	140	153.64

Table 2. Central composite design matrix for three variables along with predicted and experimental values of biomass yield and phycocyanin content

Run No	Temperature (°C)	Light Irradiance (µmol photons m^{-2} s^{-1})	Agitation (rpm)	Biomass (g L^{-1})		Phycocyanin (mg L^{-1})	
				Experimental	**Predicted**	**Experimental**	**Predicted**
1	22.50	37.50	120.00	0.85	0.85	98	97.04
2	30.00	55.00	140.00	0.64	0.65	70	71.56
3	9.89	37.50	120.00	0.26	0.27	43	45.77
4	15.00	20.00	100.00	0.12	0.12	43	42.44
5	15.00	20.00	140.00	0.13	0.11	30	28.29
6	22.50	37.50	120.00	0.87	0.85	97	97.04
7	22.50	37.50	120.00	0.84	0.85	98	97.04
8	22.50	37.50	120.00	0.85	0.85	94	97.04
9	35.11	37.50	120.00	1.05	1.03	107	102.82
10	22.50	37.50	120.00	0.85	0.85	98	97.04
11	30.00	55.00	100.00	0.8	0.83	80	82.70
12	22.50	8.07	120.00	0.23	0.24	39	38.70
13	15.00	55.00	100.00	0.21	0.21	34	33.78
14	30.00	20.00	140.00	0.39	0.39	46	47.21
15	22.50	66.93	120.00	0.56	0.54	53	51.89
16	22.50	37.50	86.36	0.35	0.34	54	51.93
17	15.00	55.00	140.00	0.29	0.29	33	31.63
18	22.50	37.50	153.64	0.17	0.18	30	30.66
19	30.00	20.00	100.00	0.66	0.66	68	70.36
20	22.50	37.50	120.00	0.83	0.85	97	97.04

$$Y = c_0 + c_1 X_1 + c_2 X_2 + c_3 X_3 + c_{12} X_1 X_2 + c_{13} X_1 X_3 + c_{23} X_2 X_3 + c_{11} X_1^2 + c_{22} X_2^2 + c_{33} X_3^2 \quad (2)$$

Where, Y is the predicted response; X_1, X_2, X_3, coded independent variables; c_0, intercept; c_1, c_2, c_3, linear coefficients; c_{12}, c_{13}, c_{23}, interactive coefficients; c_{11}, c_{22}, c_{33}, quadratic coefficients.

3.5. Panel photobioreactor (PPBR)

A 7 L PPBR made of Plexiglas with the dimension of 34 cm x 14.5 cm x 7.7 cm (height, length and width) with a total working volume of 6.5 L was equipped with an on-line controller (Biosis, Pikolab, Turkey). A combined temperature-dissolved oxygen probe and pH probe were installed at the top of the photobioreactor (Figure 1). The culture pH was controlled at 9.0±0.2, while temperature was maintained at 33±2 ºC in the temperature-controlled incubator and dissolved oxygen was measured by a polarographic oxygen sensor. The culture was mixed by air bubbling at a flow of 2.5 L min^{-1} and the flow rate was controlled using flow meter (RST electronic Ltd, LZM-6T Turkey). Light irradiance (44 µmol photons m^{-2} s^{-1}) was provided by LED lamp and measured on surface of photobioreactor using Light meter (LT Lutron LX 1108).

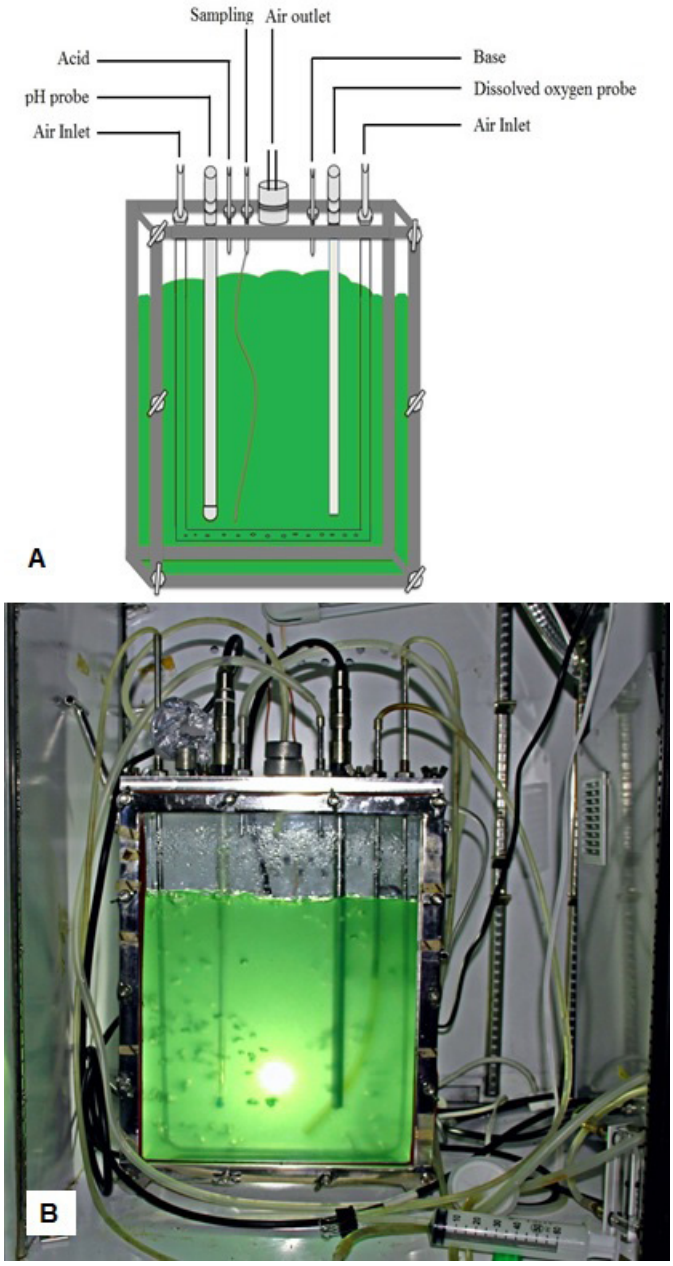

Figure 1. (A) Schematic diagram (B) Experimental set up of the Panel photobioreactor system.

4. RESULTS

4.1. Predicted model and statistical analysis

The results of CCD experiments for studying the effect of three independent variables (temperature, light irradiance and agitation speed) on biomass and phycocyanin content are presented in Table 2 along with the observed and predicted responses of the twenty experiments. The regression equation coefficients were calculated and the data were fitted to a second-order polynomial equation for biomass and phycocyanin content, respectively. The response of biomass production (Y_1) and phycocyanin (Y_2) by *S. platensis* could be expressed by the following second-order polynomial equation (Eq.3 and 4) in term of coded values:

$$\text{Biomass g L}^{-1}\ (Y_1) = 0.85 + 0.22X_1 + 0.088X_2 - 0.047X_3 + 0.018X_1X_2 - 0.065X_1X_3 + 0.023X_2X_3 - 0.070X_1^2 - 0.16X_2^2 - 0.21X_3^2 \quad (3)$$

$$\text{Phycocyanin mg L}^{-1}\ (Y_2) = 97.04 + 16.96X_1 + 3.92X_2 - 6.32X_3 + 5.25X_1X_2 - 2.25X_1X_3 + 3X_2X_3 - 8.04X_1^2 - 18.29X_2^2 - 19.71X_3^2 \quad (4)$$

Table 3. ANOVA for the experimental results of the central composite design (Biomass)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	1.80	9	0.20	539.83	< 0.0001	Significant
X_1-Temperature	0.69	1	0.69	1856.06	< 0.0001	Significant
X_2 - Light	0.10	1	0.10	281.47	< 0.0001	Significant
X_3 - Agitation	0.030	1	0.030	81.42	< 0.0001	Significant
X_1X_2	2.450E-003	1	2.450E-003	6.60	0.0280	Significant
X_1X_3	0.034	1	0.034	90.99	< 0.0001	Significant
X_2X_3	4.050E-003	1	4.050E-003	10.90	0.0080	Significant
X_1^2	0.070	1	0.070	188.82	< 0.0001	Significant
X_2^2	0.38	1	0.38	1014.21	< 0.0001	Significant
X_3^2	0.63	1	0.63	1701.36	< 0.0001	Significant
Residual	3.715E-003	10	3.715E-004			
Lack of Fit	2.832E-003	5	5.663E-004	3.21	0.1134	Not significant
Pure Error	8.833E-004	5	1.767E-004			
Cor Total	1.81	19				

R^2 = 0.9979, adjusted R^2 = 0.9961, predicted R^2 = 0.9872, coefficient of variance = 3.52%

Table 4. ANOVA for the experimental results of the central composite design (Phycocyanin)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	Significance
Model	14811.70	9	1645.74	252.79	< 0.0001	Significant
X_1-Temperature	3928.77	1	3928.77	603.48	< 0.0001	Significant
X_2 - Light	209.94	1	209.94	32.25	0.0002	Significant
X_3 - Agitation	546.14	1	546.14	83.89	< 0.0001	Significant
X_1X_2	220.50	1	220.50	33.87	0.0002	Significant
X_1X_3	40.50	1	40.50	6.22	0.0318	Significant
X_2X_3	72.00	1	72.00	11.06	0.0077	Significant
X_1^2	931.79	1	931.79	143.13	< 0.0001	Significant
X_2^2	4823.02	1	4823.02	740.84	< 0.0001	Significant
X_3^2	5597.53	1	5597.53	859.80	< 0.0001	Significant
Residual	65.10	10	6.51			
Lack of Fit	53.10	5	10.62	4.43	0.0642	Not significant
Pure Error	12.00	5	2.40			
Cor Total	14876.80	19				

R^2 = 0.9956, adjusted R^2 = 0.9917, predicted R^2 = 0.9717, coefficient of variance = 3.89%

Here, Y is the predicted response, i.e. Biomass g L^{-1} (Y_1) and Phycocyanin mg L^{-1} (Y_2), and X_1, X_2, and X_3 are the coded values of the test variables, temperature (°C), light intensity (µmol photons m^{-2} s^{-1}) and agitation speed (rpm), respectively.

The statistical significance of the regression equation was checked by an *F*-test and an ANOVA for the response surface quadratic polynomial model was shown in Table 3 and 4. The Model F-value of 539.83 for biomass and 252.79 for phycocyanin implies that model was significant. There is only a 0.01% chance that a "Model F-Value" could occur because of noise. The *P* (probability)-values were used to check the significance of each coefficient (the smaller the *P*-value, the more significant of the coefficient). Values of $p < 0.05$ indicate that the model terms are significant at 95% confidence level. In this study, the model coefficients, namely, linear (X_1, X_2, X_3), squared (X_1^2, X_2^2, X_3^2) and interaction effects (X_1X_2, X_1X_3, X_2X_3) of the variables significantly affected the yield of biomass and phycocyanin ($p < 0.05$). The F value of lack of fit 3.21 for biomass and 4.43 for phycocyanin implied that it is not significantly relative to the pure

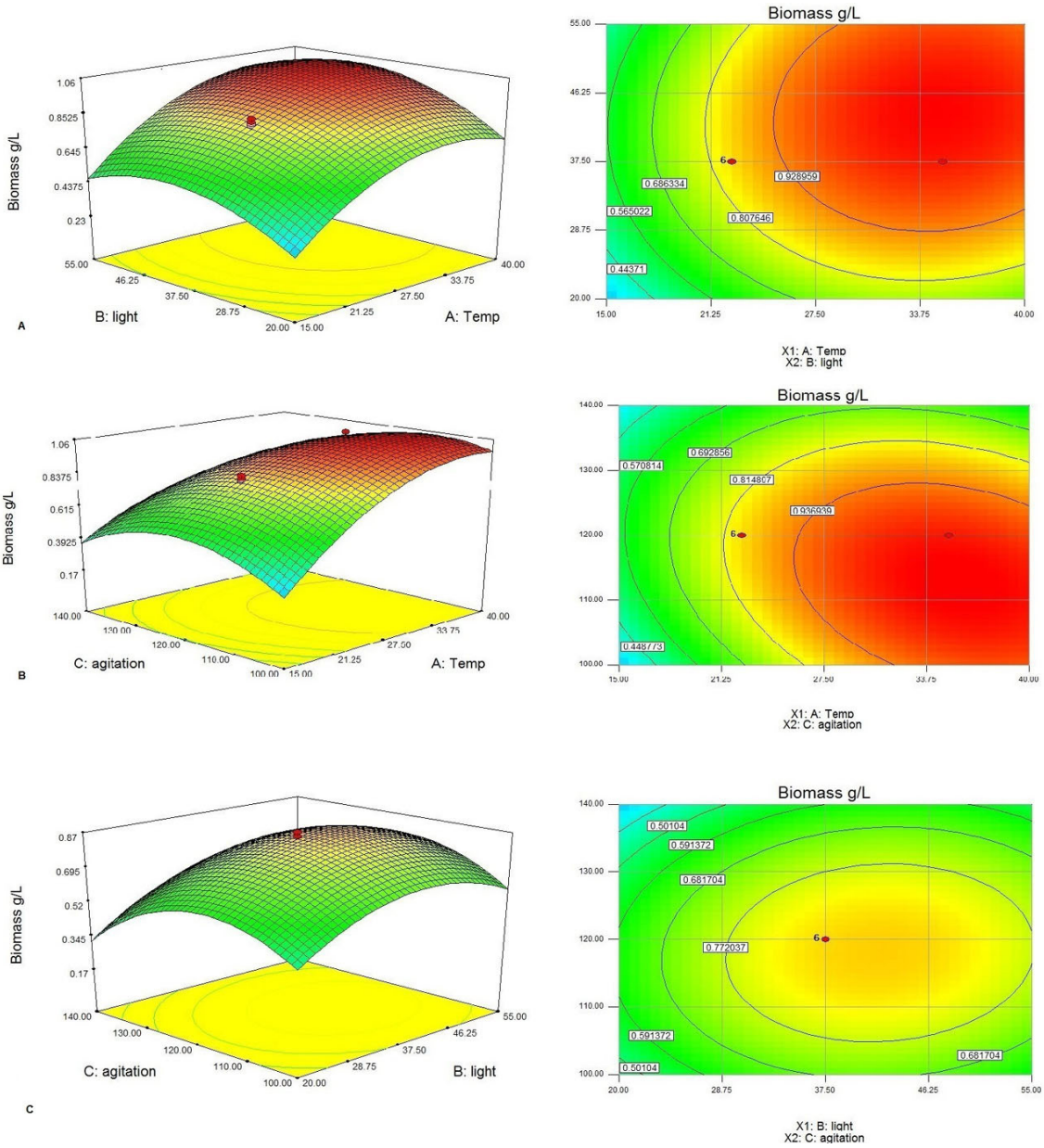

Figure 2. Response surface plots and contour plots for biomass production showing the interaction effects of (A) Temperature and Light; (B) Temperature and Agitation; and (C) Light and Agitation.

experimental error, signifying that the model correlates well with the experimental values. The fitness of the model was examined by determination of coefficient R^2 which were calculated to be 0.9979 for biomass and 0.9956 for phycocyanin, indicating a 99.79% and 99.56% of variability in the response were explained by the model. Furthermore, the adjusted R^2 = 0.9961 and 0.9917 of biomass and phycocyanin which implied that 99.61% and 99.17% of the total variation were explained by the model and signifies a good agreement between observed and predicted values. The predicted determination coefficient of biomass and phycocyanin (Pred. R^2 = 0.9872 and 0.9717) were also reasonable agreement with the adjusted R^2 0.9961 and 0.9917. In this model a relatively lower value of coefficient of variation (CV = 3.52% and 3.89%) for biomass and phycocyanin showed accuracy and reliability of the experiments carried out.

4.2. Analysis of response surface

The optimum level of each variable and their interactive effects on biomass and phycocyanin content were revealed by 3D response surface plots and its contour plots. Figure 2A and 3A describes the interactive effect of temperature and light irradiance on

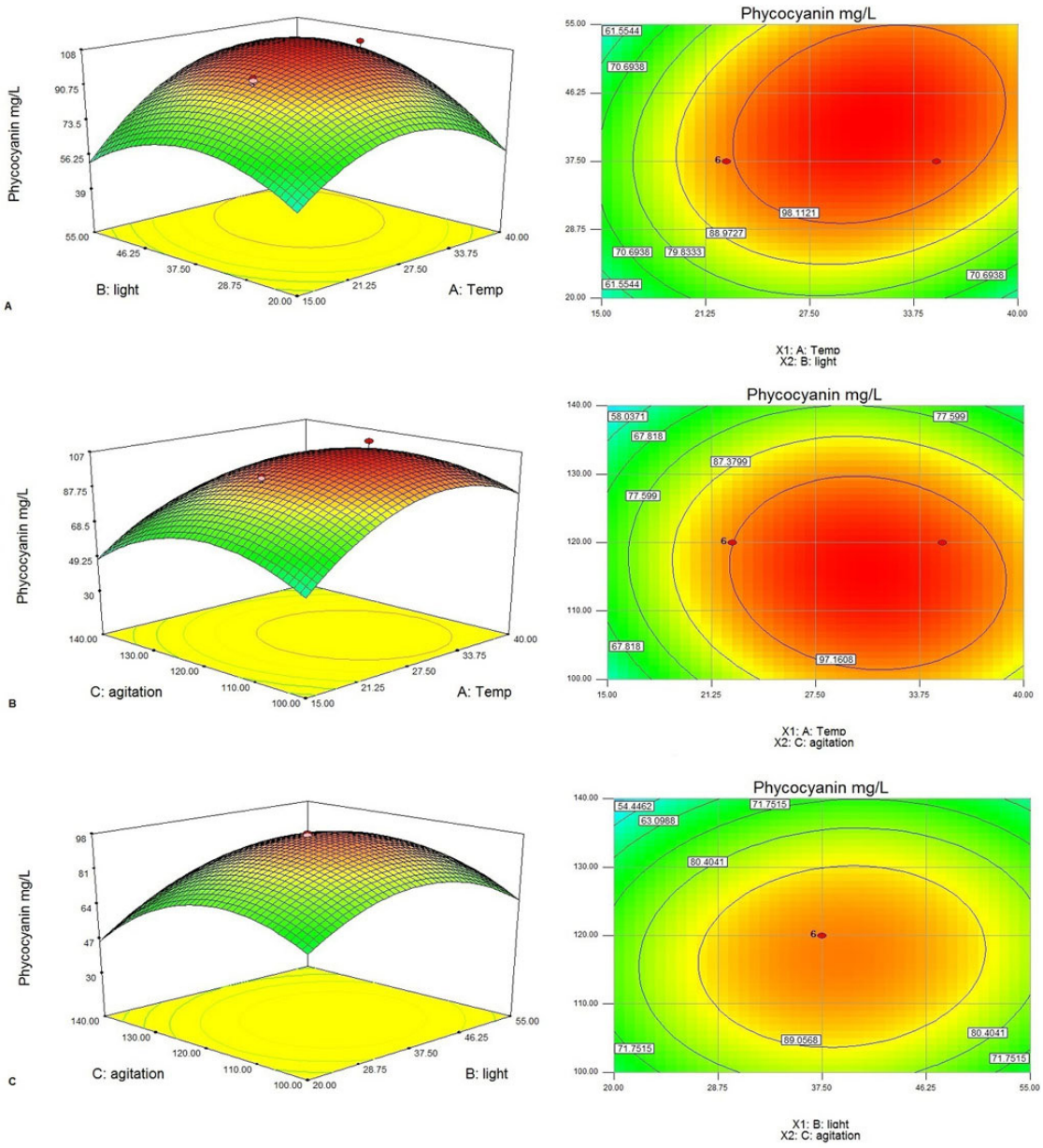

Figure 3. Response surface plots and contour plots for phycocyanin production showing the interaction effects of (A) Temperature and Light; (B) Temperature and Agitation; and (C) Light and Agitation.

biomass and phycocyanin yield keeping the agitation speed constant at its center point of 120 rpm. The biomass and phycocyanin yield increased with the increase of temperature from 15 to 33±2 °C and light irradiance 20 to 44 µmol photons m^{-2} s^{-1} further increase in temperature and light irradiance the trend is reversed. In Figure 2A and 3A contour plots showed elliptical nature which suggests significant interactions between temperature and light irradiance.

The interaction between agitation and temperature were showed in Figure 2B and 3B. It can be noted that increased biomass and phycocyanin production yields can be obtained at temperature and agitation speed lowered from 15 to 33±2 °C and 100 to 120 rpm respectively. Agitation speed and light irradiance interaction represented in Figure 2C and 3C showed that these factors influence biomass and phycocyanin production significantly. It indicated that maximum biomass and phycocyanin yield could be achieved when the agitation speed and light irradiance were 120 rpm and 44 µmol photons m^{-2} s^{-1} respectively.

4.3. Verification of the model

To validate the model obtained from RSM, confirming experiments in shake flasks were

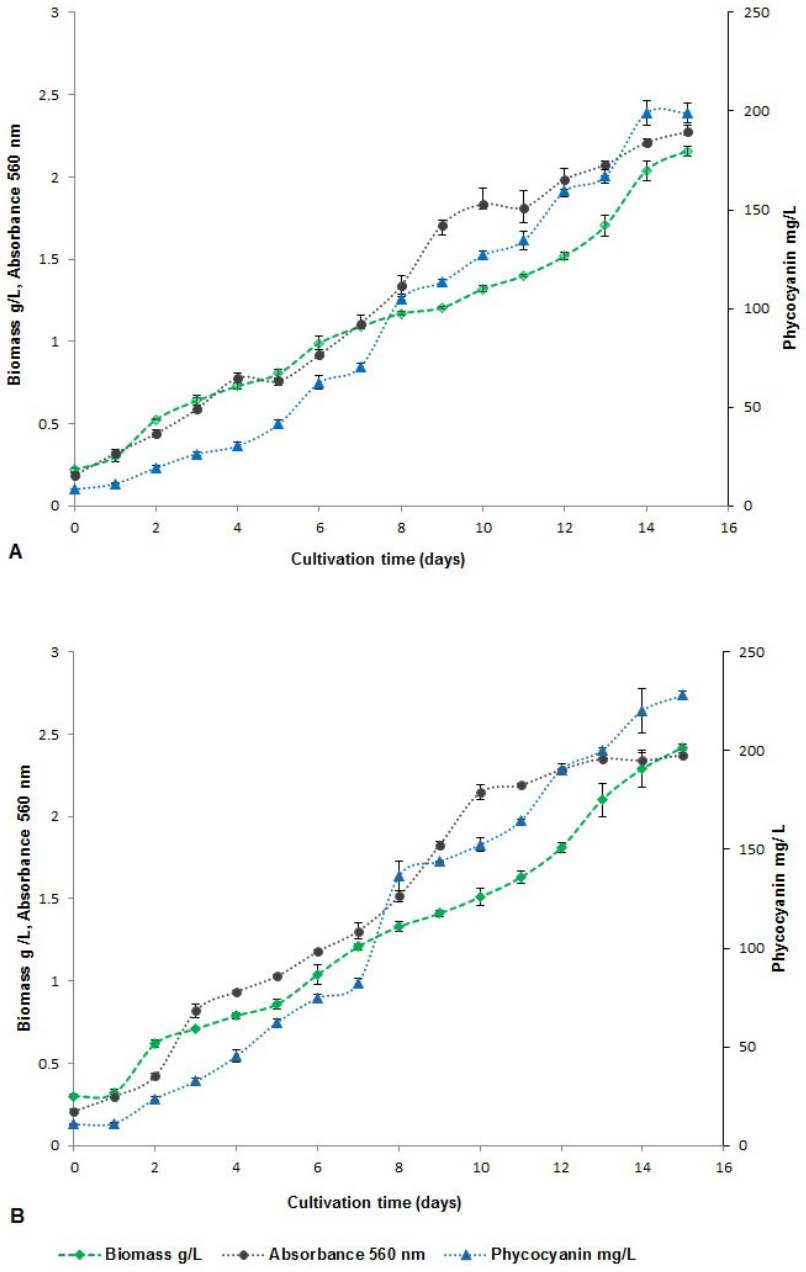

Figure 4. Growth profile and phycocyanin content of *S. platensis* (A) flask cultivation (B) PPBR cultivation under optimized conditions

carried out using the predicted culture conditions (temperature 33±2 °C, light irradiance 44 µmol photons m^{-2} s^{-1} and agitation speed 120 rpm). Under these conditions observed experimental value of biomass yield and phycocyanin content was (1.32 g L^{-1} and 127 mg L^{-1}) after 10 days cultivation, which agreed well with the model predicted value (1.06 g L^{-1} and 107 mg L^{-1}) hence it was concluded that the model was successfully validated. The optimized conditions by RSM were as follows: temperature 33±2 °C, light irradiance 44 µmol photons m^{-2} s^{-1} and agitation speed 120 rpm.

4.4. Influence of optimized conditions on growth profile and phycocyanin content of *S. platensis* in 7L panel photobioreactor

Based on the validation results, the 7L Panel photobioreactor system was used for further cultivation of *Spirulina* under optimized conditions to explore growth profile and phycocyanin production capacity for scale up. Biomass yield, specific growth rate and phycocyanin content profiles of *S. platensis* obtained from photobioreactor studies were shown in Figure 4. The PPBR study revealed that there no significant

influence of optimized conditions on biomass yield, specific growth rate and phycocyanin content of *S. platensis* compare to flask cultivation over the period of 15 days. As illustrated in Figure 4 the maximum biomass yield of 2.42 g L^{-1} and phycocyanin content of 228 mg L^{-1} in PPBR was recorded on 15th day of cultivation, which were close to experimental value of 2.16 g L^{-1} biomass yield and 199 mg L^{-1} of phycocyanin content obtained in flask cultivation respectively.

5. DISCUSSION

Environmental factors strongly influence the photosynthesis, growth, cellular metabolism, chemical composition and pigment production in cyanobacteria and microalgae (12, 13). The effect of environmental factors on growth and phycocyanin content of several species of cyanobacteria have been reported (14 –16). The optimum temperature for biomass yield and PBP synthesis depends on the strain; however, extremely high and low temperatures significantly decrease the quantity of the biomass yield and pigments. In this study, the effect of temperature, light irradiance and agitation speed on *S. platensis* growth and phycocyanin production are stated. The results obtained from CCD experiments (Table 2) showed that the maximum biomass yield of 1.05 g L^{-1} with the phycocyanin content of 107 mg L^{-1} was observed in the flask cultivated under the temperature of 35 °C, light irradiance 37.5 µmol photons m^{-2} s^{-1} and agitation of 120 rpm (run no.9), whereas the lowest biomass yield of 0.12 g L^{-1} was in the flask cultivated under 15 °C temperature (run no.4). The decrease in growth with decreasing temperature beyond the optimum has frequently been reported (17–19). It is accompanied by a decrease in metabolic activities and photosynthetic efficiency. Optimum growth temperature for *Spirulin*a have been stated between 35–37 °C, and temperature below and above 20 - 40 °C retarded the growth (20, 21). In addition to temperature, another important factor studied in this investigation was light irradiance which has a great impact on growth, photosynthetic activity of cyanobacteria and also known to play important role in PBP accumulation. Several studies have reported that low light intensity stimulates phycocyanin production in cyanobacteria (22). Setyoningrum *et al.* reported that the optimum phycocyanin production was recorded under light irradiance of 45 µmol photons m^{-2} s^{-1} under mixotrophic cultivation (16). Similar observations also reported many researchers, Kumar *et al.* observed maximum growth and phycobiliproteins accumulation at 35 °C temperature and 2000 lux light irradiance for *Spirulina*, whereas Chen *et al.* obtained maximum phycocyanin content of *Spirulina* at 4000 lux (23, 24). Agitation of culture media is one of the important factors in algal cultivation since the agitation offer adequate supply of CO_2, continuous removal of oxygen produced in photosynthesis, uniform suspension, and nutrient distribution and prevents sedimentation and clumping of algae (25). However higher agitation (mixing) can lower the biomass production due to cell damage (26). The results presented in Table 2 show that agitation had an effect on biomass and phycocyanin content. In this study, the maximum biomass and phycocyanin content was obtained at the agitation speed of 120 rpm. The results found are similar to those found by Moraes *et al.* where maximum biomass was obtained with 120 rpm (27). The experiments with 153 rpm agitation speed (run no.18) showed lower biomass and phycocyanin content than the cultures grown under the agitation of 86.3 rpm.

Nowadays, a number of studies have been reported the application of PBRs in culturing microalgae and cyanobacteria (28, 29), and for the production of bioactive compounds (30–32). Under similar panel photobioreactor cultivation of *S. platensis* Azgın *et al.* reported the biomass yield of 2.7 g L^{-1} and also a similar trend was reported by Oncel *et al.* with the bubble column and airlift PBRs cultivation of *S. platensis* which is in good agreement with our data (33, 34). Owing to their large surface-to-volume ratio, large illumination surface area, shorter light path length, low shear forces, low oxygen build-up and convenience in scale-up flat-plate PBR's are reported as suitable for large-scale production of cyanobacteria (35).

6. CONCLUSIONS

The optimum culture conditions attained through RSM were successfully determined to maximize the biomass yield and phycocyanin content. The optimum culture conditions obtained from RSM for biomass and phycocyanin production were temperature of 33±2 °C, light irradiance of 44 µmol photons m^{-2} s^{-1} and agitation speed of 120 rpm which gave biomass yield of 1.32 g L^{-1} and phycocyanin content 127 mg L^{-1} over 10 days in flask cultivation. Further, A 15 days PPBR cultivation under optimized culture conditions (temperature 33±2 °C, light irradiance 44 µmol photons m^{-2} s^{-1}) with the flow rate of 2.5 L min^{-1} gave maximum biomass and phycocyanin content of 2.42 g L^{-1} and 228 mg L^{-1} respectively. The optimized culture conditions established in this work, 7L PPBR cultivation studies under optimized conditions and findings from this study could be applied to large scale photobioreactor cultivation of *S. platensis* for phycocyanin production.

7. ACKNOWLEDGEMENTS

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