Media optimization for C-phycocyanin production in *Plectonema* sp. using response surface methodology and central composite design

Arbab Husain\(^1\), Fahad Khan\(^2\), Khwaja Osama\(^3\), Sadaf Mahfooz\(^1\), Adeeba Shamim\(^1\), Saheem Ahmad\(^4\), Alvina Farooqui\(^*3\)

\(^1\)Department of Biosciences, Integral University, Kursi Road, Lucknow, Uttar Pradesh-226026 India
\(^2\)Department of Biotechnology, Noida Institute of Engineering and Technology, Greater Noida-201306 India
\(^3\)Department of Bioengineering, Integral University, Kursi Road, Lucknow, Uttar Pradesh-226026 India
\(^4\)Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, University of Hail, Hail-682507 Saudi Arabia

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**Abstract**
Cyanobacteria represent the richest sources of phycobiliproteins with especial reference to C-phycocyanin (C-PC), which in turn holds exhaustive therapeutic implications. Screening of several cyanobacterial strains namely Anabaena sp., Nostoc muscorum, *Cylindrospermum* sp., *Plectonema* sp., *Syctonema* sp., *Spirulina* sp., *Synechococcus* sp. and *Tolypothrix* sp. was carried out for their C-PC producing capacity, however the produced quantity of C-PC varies greatly among different strains. Owing to the crucial role of different media constituents on productivity of C-PC the current study was designed to optimize most appropriate media composition for augmented C-PC production by selected superior producer. 36 factorial central composite design (CCD) dependent response surface methodology (RSM) was utilized to estimate the important medium components attributed with influencing C-PC productivity. RSM analysis of five independent coded factors including Na\(_2\)CO\(_3\), K\(_2\)HPO\(_4\), NaNO\(_3\), citric acid and EDTA were analyzed preceded by recognition of efficient variables for algal components production by *Plectonema* sp. Investigation of results revealed that the eminent medium components were Na\(_2\)CO\(_3\) (0.4 g/L); NaNO\(_3\) (0.5 g/L); K\(_2\)HPO\(_4\) (2.8 g/L); citric acid (0.08 g/L) and EDTA (0.01 g/L) respectively. The optimized combination yielded 0.5536 mg/ml of C-PC. The increment of C-PC yield is R-Sq = 88.2%. Thus, our study led to the recognition of critical nutritional component that can be used further for enhanced productivity of C-PC.

*Corresponding Author
Name: Alvina Farooqui
Phone: +91-9044020079
Email: alvina@iul.ac.in

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in various parts of the world such as Mexico, China, North America, India, Japan, Peru, and others. These Cyanobacteria have been earlier documented for their association with greater morphological, physiological and structural diversity which is a prerequisite for adaptation in a wide range of environment (Mundt et al., 2001). Within cyanobacterial phyla photosynthesis not only serves to be an efficient phenomenon for reducing atmospheric carbon dioxide (Yun et al., 1997) but is also related with production of metabolites having significant economic and scientific value (Lim et al., 2005). Their habitat extends throughout the fresh, brackish marine atmosphere followed by in soil as well on moist surfaces. The immense nutritional significance for the production of cyanobacterial biomass is by the virtue of its high phycobiliproteins (PBPs) content, better digestibility and equilibrium of few essential amino acids.

The biomass production by cyanobacteria varies greatly with the culture medium employed and external environment conditions. Most importantly, manipulation of culture conditions has been shown to regulate biosynthesis of various metabolic compounds which can be further regulated to deflate productional costs of the same (Patel et al., 2018). Phycobiliproteins (PBPs), a water soluble complex pivotal for its role in harvesting light via antenna found in eukaryotic rhodophytes prokaryotic cyanobacteria, and cryptophytes drives photosynthetic electron transport by transferring absorbed energy exploited by chlorophyll to photosynthetic reaction centers. On the basis of spectral properties PBPs are divided into phycoerythrin (λmax 540-570 nm), allophycocyanin (λmax 650-655 nm) and C-phycocyanin (λmax 610-620 nm) (Sonani et al., 2016). The intense color exhibited by PBPs is due to the presence of chromophores, called bilins (Glazer, 1994). Among the PBPs, C-phycocyanin because of its potential in application has been the subject of research as a natural pigment (Eriksen, 2008) the therapeutic properties include antioxidant, anti-inflammatory and anti-cancer activities (Romay et al., 2003; Kronick, 1986).

Alterations in the composition of media components affect the overall biochemistry of microalgae that might be exploited for the production of valuable products like lipids, carbohydrates, proteins and pigments (Mandalam and Palsson, 1998). Therefore, it is the need of hour to optimize media composition so that higher yield of microalgae derived C-phycocyanin can be achieved. For standard available growth conditions such as light, pH, temperature, salinity and agitation speed affect the C-phycocyanin content (Juneja et al., 2013; Demirel et al., 2015). Thus, to enhance the yield of desired microalgae component product optimization of the conditions need to be taken into account.

Keeping in mind the nutritional benefits of cyanobacteria, our study focused on optimization of various nutritional parameters of cyanobacterial culture medium which is further associated with elevated biomass content methods finally leading to production of useful metabolites. Amongst several methods available for enhanced production of biomass and metabolites, medium optimization is the most easy and cheap. The present study aimed to select maximum C-phycocyanin producing cyanobacterium and optimization of the medium composition to achieve the goal by applying response surface methodology (RSM). Central composite design (CCD) to evaluate optimum medium composition was applied to improve crude C-phycocyanin content from cyanobacterial strain.

**MATERIALS AND METHODS**

**Experimental cyanobacteria**

*Anabaena sp.*, *Nostoc muscorum*, *Cylindrospermum sp.*, *Plectonema sp.*, *Scytonema sp.*, *Spirulina sp.*, *Synechococcus sp.* and *Tolyphothrix sp.* were used as selected cyanobacteria in the present study obtained through the courtesy of Dr S. M. Prasad, Department of Botany, University of Allahabad.

**Culture conditions for cyanobacteria**

The pure cultures of cyanobacteria were maintained in the culture room at 27 ± 2°C. For regular experiments, cultures were grown in blue-green (BG-11) medium under 14-h:10-h light and dark photo period, the light intensity of 2400 Lux and at pH=7.0 (Hamouda et al., 2017; Mahfooz et al., 2017).

**Crude extraction and spectrophotometric estimation of C-phycocyanin**

Approximately two week log phase culture were homogenized culture was centrifuged (5,000 rpm, 10 min, 4°C). Finally the resultant pellet was washed using phosphate buffer (10 mM; pH 7.4) having 50 mM NaCl and 0.002 M NaN₃. The pellet was again resuspended in the same buffer with the addition of fine glass powder and then frozen at -20°C. Repeated freeze thawing of tubes containing pallet was done to accumulate phycobiliproteins in phosphate buffer. After freeze thaw, crude C-phycocyanin was obtained as blue supernatant by centrifugation at 12,000 rpm for 15 min at 4°C. To prevent denaturation of C-phycocyanin, 1 µl/ml of PMSF (Phenyl methyl sulphonyl Fluoride)
(100 μg/ml) was added. Amount of C-phycocyanin was measured and purity was determined by using as described earlier (Bennett and Bogorad, 1973; Silveira et al., 2007) using the formulae: Purity = $A_{620}/A_{280}$.

**Crude C-phycocyanin extraction from different experimental strains**

Screening of various experimental cyanobacterial strains was performed to assess maximum C-phycocyanin yielding strain. *Anabaena* sp., *Nostoc muscorum*, *Cylindrospermum* sp., *Plectonema* sp., *Scytonema* sp., *Spirulina* sp., *Synechococcus* sp. and *Tolypothrix* sp. were screened. All the strains were grown in the BG-11 medium under distinctive physiological conditions.

**Optimization of the C-phycocyanin production based on Central Composite Design (CCD) and Response Surface Methodology (RSM)**

To optimize culture media five levels and five variables CCD were applied to enhance C-phycocyanin yield and to evaluate its relationship between dependent (C-phycocyanin) and independent variables (C1-Na$_2$CO$_3$, C2-NaNO$_3$, C3-K$_2$HPO$_4$, C4-Citric Acid and C5-EDTA). Table 1, the CCD experimental procedure suggested by Box and Wilson was used. The central values (zero level) chosen for CCD were Citric Acid, Sodium carbonate, dipotassium phosphate, Sodium nitrate and EDTA.

Total 36 experiments that include eighteen cube points (runs 1-18), six star points (runs 19-24) and six replicas of the central points (runs 25-36) were required to fit the second order polynomial model. The concentrations of test variables (Citric Acid, Sodium carbonate, dipotassium phosphate, Sodium nitrate and EDTA) in the production medium were varied according to CCD (Table 2). To optimize carbon (Na$_2$CO$_3$), nitrogen (NaNO$_3$), phosphate (K$_2$HPO$_4$), Citric Acid and EDTA sources, a Central Composite Design consisting of a set of 36 experiments with six replicates at central point was conducted.

All the experiments were carried out in triplicate in 250 ml Erlenmeyer flask containing 100 ml of media for the period of two weeks. Statistical software ‘MINITAB’ (trial version 17, Pennsylvania, USA) was used to perform regression and graphical analysis of the results obtained from CCD. A second order polynomial response Equation (1). comprising linear, quadratic and interaction terms was obtained.

$$Y = X_0 + X_1C_1 + X_2C_2 + X_3C_3 + X_4C_4 + X_5C_5 + X_1^2C_1^2 + X_2^2C_2^2 + X_3^2C_3^2 + X_4^2C_4^2 + X_5^2C_5^2 + X_12C_1C_2 + X_13C_1C_3 + X_14C_1C_4 + X_15C_1C_5 + X_23C_2C_3 + X_24C_2C_4 + X_25C_2C_5 + X_34C_3C_4 + X_35C_3C_5 + X_45C_4C_5$$

Where Y is compound yield in mg/ml, X0 is the intercept, X1 is the coefficient for linear direct effect, X1$^2$ is the coefficient of quadratic effect and is responsible for curvatures in the model, and X12 is the coefficient for interaction effect a positive or negative significant value implies possible interaction between the medium constituents.

RSM is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously (Pathak et al., 2015).

The statistical significance of the polynomial equation was checked by P test and variance (ANOVA) analysis for response surface quadratic model.

**RESULTS AND DISCUSSION**

The primary requirement for any process development is to search for a high yielding strain and to identify the variables which influence the yield (Minocha et al., 2007). Number of different studies has reported the application of phycobiliproteins with special emphasis on C-phycocyanin in culturing microalgae and cyanobacteria (Harun et al., 2010; DeVree et al., 2015) along with production of bioactive compounds (Meiser et al., 2004). Screening of nine cyanobacterial strains were done to evaluate maximum crude C-phycocyanin producing strain and results obtained is depicted in Figure 1. *Plectonema* sp. was found to be the most C-phycocyanin producing strain and thus this strain would use for further optimization of the culture constituents of composition.

**Statistical analysis of predicted model**

The results obtained from CCD experiments data represented in Table 2 revealed a wide variation in total crude C-phycocyanin concentrations. The highest crude C-phycocyanin production was found in experimental trial no. 34 (0.371 mg/ml), whereas lowest concentration of C-phycocyanin was in trial no. 36 (0.023 mg/ml).

Statistical results of five tested medium variables in case of the C-phycocyanin production revealed that Na$_2$CO$_3$, Citric acid, K$_2$HPO$_4$ had a negative effect, other variables NaNO$_3$ and EDTA had a positive effect and in general there was no significant effect between medium variables on C-phycocyanin production by *Plectonema* sp. At $P \leq 0.05$ (Table 3).
Table 1: High, zero and low levels of media variable constituents in gm/100mL of total media composition involved in Central Composite Design

| Independent Variable | Range     | Levels of Variables |
|----------------------|-----------|---------------------|
|                      | -2        | -1                  | 0     | +1    | +2     |
| Na2CO3               | 0.04-0.17 | 0.04                | 0.07  | 0.1   | 0.13   | 0.17   |
| Citric Acid          | 0.008-0.024 | 0.008              | 0.012 | 0.016 | 0.02   | 0.024  |
| K2HPO4               | 0.12-0.28 | 0.12                | 0.16  | 0.2   | 0.24   | 0.28   |
| NaN3                 | 0.05-0.25 | 0.05                | 0.10  | 0.15  | 0.20   | 0.25   |
| EDTA                 | 0.001-0.009 | 0.001              | 0.003 | 0.005 | 0.007  | 0.009  |

Figure 1: Different concentration of C-phycocyanin from selected cyanobacterial strains

The CCD experiments results for observing the effect of five independent variables Na₂CO₃, NaNO₃, K₂HPO₄, citric acid and EDTA on C-phycocyanin content are represented in Table 2 together with observed and predicted values of thirty six experiments. The regression equation coefficients were calculated and put in second-order polynomial equation for C-phycocyanin content. The response of C-phycocyanin production (Y₁) by Plactonema sp. can be expressed by Equation (2) in term of coded values:

\[ C \text{-phycocyanin mg/ml (Y₁)} = 0.283653 - 0.021917C + 0.002167C² + 0.0235C³ - 0.0295C⁴ - 0.023C⁵ - 0.012229C²C - 0.032979C³C - 0.030104C⁴C - 0.024979C⁵C + 0.003375C₁C₂ - 0.034875C₁C₃ + 0.005C₁C₄ + 0.008875C₁C₅ - 0.045625C₂C₃ - 0.0015C₂C₄ + 0.031625C₂C₅ - 0.006C₃C₄ + 0.035125C₃C₅ + 0.02725C₄C₅ \ldots \]  \hspace{1cm} \text{(2)}

Where, \( Y \) is predicted response i.e. C-phycocyanin concentration in mg/ml, and C₁, C₂, C₃, C₄ and C₅, are the coded independent variables Na₂CO₃, NaNO₃, K₂HPO₄, citric acid and EDTA respectively.

\[ S = 0.04728 \quad R^2(\text{Calculated}) = 0.882 \ (88.2\%) \quad R^2(\text{adjusted}) = 0.725 \ (72.5\%) \]

Here, \( Y \) is the predicted response, i.e. C-phycocyanin mg/ml (Y₁ and C₁, C₂, C₃, C₄ and C₅, are the coded values of the test variables Na₂CO₃, NaNO₃, K₂HPO₄, citric acid and EDTA, respectively. The statistical significance of the polynomial equation was verified by \( P \) test and analysis of variance (ANOVA) for response surface quadratic model. The \( P \) value coefficient for C₁C₃, C₂C₃, C₂C₅, C₃C₅ and C₄C₅ was 0.010, 0.002, 0.017, 0.010 and 0.036 respectively, indicating the significant interaction among three variables C₁ and C₃, C₂ and C₃, C₂ and C₅, C₃ and C₅, C₄ and C₅. The estimated coefficient of C-phycocyanin response (C₆) was represented for protein yield in Table 3. The significance of each coefficient was determined by student’s \( T \)-test and \( P \) values. The larger the magnitude of \( T \)-value and smaller the \( P \) value, the more significant is the corresponding coefficient. The \( T \)-value for C₂C₅, C₃C₅ and C₄C₅ was 2.675, 2.971 and 2.305 respectively. The \( P \) value coefficient for C₁C₃, C₂C₃, C₂C₅, C₃C₅ and C₄C₅ was 0.010, 0.002, 0.017, 0.010 and 0.036, respectively. This means that quadratic main effects of C₁C₃, C₂C₃, C₂C₅, C₃C₅ and C₄C₅ are more significant.

The ANOVA and F-test were employed to checks statistical significance of regression equation for the response surface quadratic polynomial model represented in Table 3. The \( F \)-value of 5.62 for C-phycocyanin suggested that model was significant. The \( F \) value of lack of fit 98.23 for C-phycocyanin suggested that it is not significantly relative to the pure experimental error, suggesting well correlation of the model with the experimental values. The coefficient \( R^2 \) was employed to examine the fitness of model which were estimated to be 0.882 for C-phycocyanin, which indicates 88.2% of variability in the response was explained by the model. Furthermore, the adjusted \( R^2 = 0.725 \) of C-phycocyanin which implied that 72.5% of the all variation were...
Table 2: Central composite design matrix for five test variables along with predicted and experimental values of C-phycocyanin content

| S.No. | Na$_2$CO$_3$ | Citric Acid | K$_2$HPO$_4$ | NaNO$_3$ | EDTA | C-phycocyanin (mg/ml) |
|-------|--------------|-------------|---------------|---------|------|------------------------|
|       | Predicted    | Experimental|                |         |      |                        |
| 1     | 0            | 2           | 0             | 0       | 0    | 0.239071               |
| 2     | 1            | -1          | 1             | 1       | -1   | 0.138424               |
| 3     | 1            | 1           | 1             | -1      | -1   | 0.110508               |
| 4     | -1           | -1          | 1             | 1       | 1    | 0.264258               |
| 5     | -1           | 1           | -1            | 1       | 1    | 0.147092               |
| 6     | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 7     | 1            | -1          | -1            | -1      | -1   | 0.240674               |
| 8     | 2            | 0           | 0             | 0       | 0    | 0.215403               |
| 9     | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 10    | 0            | 0           | 0             | 0       | 2    | 0.137737               |
| 11    | 1            | 1           | -1            | 1       | -1   | 0.188258               |
| 12    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 13    | 0            | 0           | 0             | -2      | 0    | 0.222237               |
| 14    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 15    | 1            | -1          | -1            | 1       | 1    | 0.044924               |
| 16    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 17    | 0            | 0           | 0             | 0       | -2   | 0.229737               |
| 18    | 0            | 0           | 0             | 2       | 0    | 0.104237               |
| 19    | -1           | 1           | -1            | -1      | -1   | 0.277842               |
| 20    | -1           | 1           | 1             | -1      | 1    | 0.260342               |
| 21    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 22    | 0            | 0           | 2             | 0       | 0    | 0.198737               |
| 23    | -2           | 0           | 0             | 0       | 0    | 0.303071               |
| 24    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 25    | 0            | 0           | -2            | 0       | 0    | 0.104737               |
| 26    | 1            | 1           | -1            | -1      | 1    | 0.193008               |
| 27    | -1           | -1          | -1            | 1       | -1   | 0.140758               |
| 28    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 29    | -1           | 1           | 1             | -1      | 1    | 0.106592               |
| 30    | 0            | -2          | 0             | 0       | 0    | 0.230403               |
| 31    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 32    | 1            | 1           | 1             | 1       | 1    | 0.151758               |
| 33    | 1            | -1          | 1             | -1      | 1    | 0.175174               |
| 34    | -1           | -1          | 1             | -1      | -1   | 0.399008               |
| 35    | 0            | 0           | 0             | 0       | 0    | 0.283653               |
| 36    | -1           | -1          | -1            | 1       | 1    | -0.002492              |
Table 3: ANOVA analysis for the experimental results of CCD (C-phycocyanin)

| Source            | DF | Adj MS     | Adj SS    | Seq SS    | F value | P value |
|-------------------|----|------------|-----------|-----------|---------|---------|
| Regression        | 20 | 0.012554   | 0.251070  | 0.251070  | 5.62    | 0.001   |
| Linear            | 5  | 0.011695   | 0.058477  | 0.058477  | 5.23    | 0.006   |
| Square            | 5  | 0.017995   | 0.089749  | 0.089749  | 8.03    | 0.001   |
| Interaction       | 10 | 0.010284   | 0.102845  | 0.102845  | 4.60    | 0.004   |
| Residual Error    |    |            |           |           |         |         |
| Lack-of-Fit       | 6  | 0.005505   | 0.033030  | 0.033030  | 98.23   | 0.000   |
| Pure Error        | 9  | 0.000056   | 0.000504  | 0.000504  |         |         |
| Total             | 35 |            |           | 0.284605  |         |         |

Figure 2: (a-d): Interaction effects of C-phycocyanin response surface plot (C6) with coded independent variables. The plots represent interaction between two factors and the third factor is held constant at middle value.
explained by the model and indicates a good agreement between observed and predicted values.

Response surface regression analysis

The optimum level of each variable on C-phycocyanin content was revealed by 3D response surface plots. Figure 2(a-d) describes the interactive effect of Na$_2$CO$_3$, K$_2$HPO$_4$, NaNO$_3$, citric acid and EDTA.

The combined effect of proportional decrease in the concentration of C1 and increase in the concentration of C2 in modified medium to enhance the yield of C-phycocyanin while keeping other variables constant (Figure 2a). The combined effect of proportional increase in concentration of C2 and decrease in the concentration of C3 in modified medium to enhanced the production of C-phycocyanin while keeping other variables constant (Figure 2b).

The dome structural C-phycocyanin response surface plot that indicates combined effect of slight increase in the concentration of C3 and slight decrease in the concentration of C4 (Figure 2c) in modified medium to enhanced the production of C-phycocyanin while keeping other variables constant. The combined effect of proportional increase in the concentration of C1 and optimum increase in the concentration of C5 in modified medium to enhanced the production or yield of C-phycocyanin while keeping other variables constant (Figure 2d).

The C-phycocyanin yield increases with increase in concentration of (NaNO$_3$ and EDTA) and with decrease in concentration of (Na$_2$CO$_3$, Citric acid and K$_2$HPO$_4$). The results were in accordance with (Minocha et al., 2007) who stated that sodium nitrate source of nitrogen are the best source for maximum biomass as well as C-phycocyanin production. Furthermore, neither citric acid nor EDTA alone was capable of enhancing the C-phycocyanin.

However, quite interesting results were obtained when the interaction effect of the components was studied. The optimum concentration of Na$_2$CO$_3$, citric acid, K$_2$HPO$_4$, NaNO$_3$ and EDTA should be 0.4, 0.08, 0.28, 0.5 and 0.01 in gm per litre of media, respectively. According to this optimized combination the yielded of C-phycocyanin is 0.5536 mg/ml. The percentage of increment of yield is R-Sq = 88.2%.

CONCLUSION

Screening of cyanobacterial strains for C-phycocyanin producing strain showed efficacy of Plectonema sp. in productivity of the same. Thus, Plectonema sp. was further exposed for optimization of culture constituents for C-phycocyanin production using customized blue-green medium (BG-11). The relative importance of chemical factors on C-phycocyanin production has been validated by RSM which reveals the five independent factors (Na$_2$CO$_3$, NaNO$_3$, K$_2$HPO$_4$, citric acid and EDTA) to be more effective for the higher yield of C-phycocyanin. Thus, the present study concluded that the high yield of C-phycocyanin is dependent upon the high concentration of NaNO$_3$ and EDTA with low concentration of Na$_2$CO$_3$. Citric acid and K$_2$HPO$_4$. Furthermore, neither citric acid nor EDTA alone was capable of enhancing C-phycocyanin production.

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Conflict of Interest

None.

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