Optimization of medium components for production of C-phycocyanin & its purification

Part of this chapter has been published as:

2.1 Introduction

During the past three decades, increasing attention has been paid to cyanobacterial biotechnology owing to its potential to produce foodstuffs, industrial chemicals, compounds with therapeutic applications, and bioremediation solutions (Apt and Behrens, 1999; Sanghvi et al., 2010). Cyanobacterial phycocyanin (C-PC) is one of the potential cyanobacterial products, most commonly used in food, pharmaceutical, therapeutics and others. Recently, there has been renewed interest in the utilization of cyanobacteria as a food, pharmaceuticals and therapeutics. But before wide range of utilization of cyanobacteria or cyanobacterial product/s, it is very necessary to identify the culture potential to produce C-PC. After identification of potential culture to produce high amount of C-PC, further production can be enhanced by manipulation of external environmental parameter. Traditionally, the identification of cyanobacteria has been based on morphological characters such as trichome width, cell size, division planes, shape and arrangement, pigmentation and the presence of characters such as gas vacuoles and a sheath (Baker, 1992, 1991; Komařek & Anagnostidis, 1989, 1986). Beyond the considerable expertise required to identify species by such characters, subjective judgment by operators can lead to errors, resulting in incorrect assignment of isolates. Komařek & Anagnostidis (1989) estimated that more than 50% of the strains in culture collections identified morphologically are misidentified. Moreover, some diagnostic features, such as gas vacuoles or akinetes, can show variations with different environmental or growth conditions and even be lost during cultivation (Lyra et al., 2001; Rudi et al., 1997). To overcome these problems, molecular approaches are useful, especially; 16S rRNA gene sequencing is an established method for identifying cyanobacteria (Nubel et al., 1997). Rudi et al. (1997) demonstrated the cyanobacterial identification by specific amplification of 16S rRNA gene using specific oligonucleotide primers based on 16S rDNA sequencing.

Cyanobacterial phycocyanin (C-PC) is considered a healthy ingredient in cyanobacterial-based foods and health foods as colouring, antioxidant etc (Soni et al., 2008). Recent research and developments in C-PC synthesis and functionality have expanded the potential applications of C-PC in biotechnology, diagnostics, foods and medicine. In view of the multiple uses of C-PC in foods and
pharmaceuticals there is need of increased productivity. Very few strains of cyanobacteria have the ability to produce high quantity of C-PC. There exists a need to look for high yielding strain and improve its production. Besides introducing a productive strain, designing an appropriate growth medium is of crucial importance to improve the efficiency and productivity of phycocyanin because medium composition can significantly affect product concentration, yield, volumetric production, and cost of downstream processing (Kennedy and Krouse, 1999). The statistical experimental design can help to increase the phycocyanin yield at low cost. The conventional and classical method for optimizing medium components for protein production by ‘one variable at a time’ approach involves varying a single independent variable, while maintaining the others at a constant level. This one-dimensional approach is laborious and time consuming especially for large number of variables, and does not consider potential interactions among them.

A statistical experimental design like the response surface methodology (RSM) overcomes the limitations of the classical method (Mohana et al., 2008) and it has been increasingly used for various phases of an optimization process (Prapulla et al., 1992; Mao et al., 2005). It is a collection of mathematical and statistical techniques, involving full factorial search by examining simultaneous, systematic and efficient variation of all complex interactions between variables. The main objective of RSM is to determine the optimum operational condition for the system or to determine a region that satisfies the operating specifications (Myers and Montgomery, 2002; Ravikumar et al., 2006; Liu and Wang, 2007). RSM, which includes factorial design and regression analysis, helps in evaluating the effective factors and in building models to study interaction between the variables and also select optimum conditions of variables for a desirable response.

Pure phycobiliproteins from crude algal extracts are usually obtained by a combination of different and non-scalable methods (Grabowski and Gantt, 1978; Duerring et al., 1991; Ficner et al., 1992; Bermejo et al., 1997; Patil et al., 2006). Various methods have been reported for the purification of phycobiliproteins but all these methods involve the combination of various techniques such as centrifugation, ammonium sulphate precipitation, ion exchange chromatography, gel permeation chromatography, hydroxyapatite chromatography, expanded bed
adsorption chromatography and high performance chromatography (Soni et al., 2006; Glazer and Hixson, 1977; Agnolo et al., 1994; Bermejo et al., 2001, 2002, 2003; Zolla et al., 2001). The major limitations of these methods are non-scalability and the length of time required to complete the process. Apart from these limitations ammonium sulphate precipitation may hinder the future crystallization process because of the difficulty in complete removal of salt by dialysis or even by desalting column (Sirgh et al., 2009). As general information, even a single step increase in chromatography, can reduce about 20% of protein of interest (Soni et al., 2006). We, therefore, looked to develop a method which would be simple, efficient, cheap and easy to perform without compromising the purity and yield, wherein single step chromatography in combination of ultrafiltration cell (UFC) was introduced for the purification of biliproteins. In comparison with earlier methodologies this method avoids product loss, increases yield and reduces both processing time and cost.

In view of the wide industrial application of the phycocyanin, we identified a potential strain of C-PC producing cyanobacteria. Further, we optimize various medium components using RSM for higher production of C-PC. Thereafter, we developed single step method for the purification of C-PC from *Leptolyngbya* sp. N62DM (earlier we considered Phormidium ceylanicum on the basis of morphological identification).

### 2.2 Materials and methods

#### 2.2.1 Chemicals

Diethyl amino ethyl (DEAE) cellulose weak anion exchange resin was purchased from Sigma (St. Louis, Missouri, USA), protein molecular mass standard from Bangalore Genei (Bangalore, Karnataka, India), bis-acrylamide from Himedia (Mumbai, Maharashtra, India), sodium dodecyl sulphate and electrophoresis grade acrylamide from Merck (Darmstadt, Hesse, Germany) and Stirred Ultrafiltration Cell (50 kDa MWCO) from Amicon Bioseparations, Millipore Corporation (Bedford, New York, USA). All other chemicals were ultra pure or molecular biology grade and were used without further purification.
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2.2.2 Isolation and laboratory growth conditions

BG 11 (Waterbury and Stanier, 1981) medium was used for the maintenance and cultivation of Leptolyngbya sp. N62DM, which consists of 1.5 NaNO₃ g/L, 0.040 K₂HPO₄·3H₂O g/L, 0.075 MgSO₄·7H₂O g/L, 0.036 CaCl₂·2H₂O g/L, 10 ml of citric acid stock (composition per 100 ml: 0.06 g of citric acid, 1 mg of EDTA, 0.06 g of ferric ammonium citrate and 0.4 g of anhydrous sodium carbonate) and 1 ml of trace metal mix (composition per 100 ml: 0.286 g of H₃BO₃, 0.181 g of MnCl₂·2H₂O, 0.022 g of ZnSO₄·7H₂O, 0.039 g of Na₂MoO₄·2H₂O, 0.005 g of Co(NO₃)₂·6H₂O and 0.008 g of CuSO₄·5H₂O) and pH 7.4. The culture was incubated in 12h light/12h dark cycles under 36W white fluorescent lamp illumination (130 μmol photon m⁻² s⁻¹) at 27 ± 2°C.

2.2.3 Identification

2.2.3.1 DNA extraction

Genomic DNA extraction from the cultures was performed using a slightly modified method of Wu et al. (2000). Culture pellets were resuspended in 10ml of extraction buffer (100mM Tris-Cl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl) along with 1ml of each 10% Sarkosyl and 10% CTAB at the final concentration of 1% and incubated in water bath at 60°C for 30 minutes. Then it was centrifuged at 10000 × g for 10 minutes at 4°C. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added to aqueous phase and mixed for 20 minutes and then centrifuged at 10000 × g for 10 minutes at 4°C. The pellet was discarded and the supernatant was mixed well with equal volume of chloroform and centrifuged at 10000 × g for 10 minutes at 4°C. Aqueous phase was collected and the DNA was precipitated by adding equal volume of 70% (v/v) chilled ethanol and one tenth volume of 3.0 M ammonium acetate (pH 5.2). The DNA was pelleted down by centrifugation at 10000 × g. Finally the pellets were washed with chilled 70% (v/v) ethanol; air dried and resuspended in 10 mM Tris (pH 8.0). The extracted DNA was quantified and checked for its purity using a Nanodrop spectrophotometer (Implen Version 1.3, Munich, Germany).

2.2.3.2 PCR amplification and sequencing of 16S rRNA gene

The 16S rRNA gene was amplified from the cyanobacterial cultures by PCR amplification. Amplification was carried out in a 30 μl PCR reaction consisting of
1X buffer (10 mM Tris pH 9.0, 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100), 0.33 mM each of dNTPs, 100 ng of template DNA, 0.66 µmoles each of custom synthesized bacteria-specific universal primers 8F (5’-AGA GTT TGA TCC TGG CTC AG-3’) and 1492R (5’-GGT TAC CTT GTT ACG ACT-3’), and 1.5 U of Taq DNA polymerase. Amplification program was performed with initial denaturation step at 94°C for 5 minutes; followed by 30 cycles of 1 minute denaturation step at 94°C, 1 minute annealing step at 55°C, and 1 minute elongation step at 72°C and a final extension step at 72°C for 20 minutes using Biorad iCycler version 4.006 (Biorad, CA, USA). The ~1.5 kb PCR product of culture was sequenced by automated DNA Analyzer 3730xl using BigDye™ Terminator 3.1 sequencing chemistry (Applied Biosystems, Foster City, CA).

2.2.3.3 Comparative sequence analysis
The 16S rRNA gene was sequenced using the original PCR primers with an automated DNA Analyzer 3730xl using BigDye™ Terminator 3.1 sequencing chemistry (Applied Biosystems, Foster City, CA) sequencer. Nucleotide alignments of 16S rRNA gene sequence and several other cyanobacterial taxa were created using CLUSTALX with default gap penalties (Higgins et al., 1988; Higgins et al., 1989; Thompson et al., 1997). Gaps were excluded for phylogenetic analyses. Distance matrix trees were generated by the neighbour-joining algorithm with Kimura 2 parameter distance in MEGA 4.0 software (Tamura et al., 2007). The NJ calculations were subjected to bootstrap analysis (1,000 replicates). Escherichia coli K12 were used as the out-group for all analyses.

2.2.4 Optimization of the media components for increased production of PC
The most important parameter, which affects the efficiency of production of phycocyanin, is the salt concentration. In order to study the individual and combined effect of different salt concentration, statistically designed experiments were performed.

2.2.4.1 Central composite design (CCD)
Response surface methodology was used to optimize the components of the medium for enhanced phycocyanin production (mg/ml) using the central composite design (CCD). RSM is useful for small number of variables (up to five) but is
impractical for large number of variables, due to high number of experimental runs required (Sharma and Satyanarayana, 2006). The concentrations of the four major components of BG-11 medium i.e. NaNO$_3$, CaCl$_2$.2H$_2$O, citric acid stock and trace metal mix were optimized, keeping K$_2$HPO$_4$ and MgSO$_4$.7H$_2$O constant.

According to the design, the total number of treatment combinations is $2^k + 2k + n_0$, where $k$ is the number of independent variables and $n_0$ is the number of repetition of experiments at the central point. A 24 full factorial central composite design with three coded levels was used that lead to 30 sets of experiments. Each factor in the design was studied at five different levels (-$\alpha$, -1, 0, +1, +$\alpha$) as shown in Table 2.1. All variables were set at a central coded value of zero. The minimum and maximum ranges of variables were determined on the basis of our previous experiments. The full experimental plan with respect to their values in actual and coded form is listed in Table 2.2. For statistical calculations, the variables were loaded according to the following equation:

$$x_i = (X_i - X_0) / \delta X$$

(1)

Where $x_i$ is the dimensionless coded value of the variable $X_i$, $X_0$ the value of the $X_i$ at the central point and $\delta X$ is the step change value.

Phycocyanin production (response $Y$), was explained as a second- order response surface model in four independent variables

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i^2 + \sum \beta_{ij} X_i X_j$$

(2)

Where $\beta_0$, $\beta_i$, $\beta_{ij}$ and $\beta_{ij}$ represent, respectively, the constant process effect in total, the linear, quadratic effect of $X_i$ and the interaction effect between $X_i$ and $X_j$ for the production of phycocyanin.
Table 2.1: Experimental range and levels of the independent variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>Components</th>
<th>Range studied</th>
<th>Levels of variable (g/l)</th>
</tr>
</thead>
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<tr>
<td></td>
<td></td>
<td>(g/D)</td>
<td>-α</td>
</tr>
<tr>
<td>$X_1$</td>
<td>NaNO₃ (g/l)</td>
<td>0.50 – 5.00</td>
<td>-1.75</td>
</tr>
<tr>
<td>$X_2$</td>
<td>CaCl₂ (g/l)</td>
<td>0.05 – 0.50</td>
<td>-0.17</td>
</tr>
<tr>
<td>$X_3$</td>
<td>Citric acid stock (ml/l)</td>
<td>3.00 – 30.00</td>
<td>-10.50</td>
</tr>
<tr>
<td>$X_4$</td>
<td>Trace metal mix (ml/l)</td>
<td>0.10 – 1.00</td>
<td>-0.35</td>
</tr>
</tbody>
</table>

2.2.4.2 Software and data analysis
The results of the experimental design were analyzed and interpreted using Design Expert Version 7.0 (Stat-Ease Inc., Minneapolis, Minnesota, USA) statistical software.

2.2.4.3 Growth measurements
Growth of the culture was determined in terms of wet weight (g/L) and by the chlorophyll 'a' content of the cell mass (Marsac and Houmard, 1988), which was measured spectrophotometrically at 665 nm (Hewlett Packard 8452A UV–Vis Diode Array Spectrophotometer, Ramsey, Minnesota, USA).

2.2.4.4 Extraction and estimation of phycocyanin
A set of 250 ml conical flasks with 100 ml medium in each, containing different concentrations of the four variables as mentioned in Table 2.1, were inoculated with a fifteen day old culture [10% (v/v)] and incubated as described in Section 2.2.2. Thirty-two day old cyanobacterial cells from each flask were harvested by centrifugation at 8000 × g for 10 minutes (Kubota 6500, Bunkyo-Ku, Tokyo, Japan) at 20°C. All buffers and solutions were prepared in Milli-Q water supplemented with 0.02% (w/v) sodium azide. The cell pellet was washed with 1 M Tris–Cl buffer (pH 8.1). One volume of the washed cell mass were resuspended in five volume of the same buffer and subjected to repeated freeze-thaw cycle of -30°C and 4°C shocks for the release of phycocyanin. The cell debris
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was removed by centrifugation at 17000 × g for 40 minutes and the supernatant were termed as crude extract. Absorbance of the pooled extracts was recorded at 280, 540, 620 and 650 nm. The amount of C-PC in each sample was calculated using the Bennett and Bogorad (1973) equation:

\[
C-PC (\text{mg ml}^{-1}) = \frac{(\text{OD}_{620} - 0.7\text{OD}_{650})}{7.38}
\]

(3)

2.2.5 Filtration and concentration of crude extract using stirred ultrafiltration cell (UFC)

Fifty ml crude extract was filled in the UFC fitted with a 50 kDa polyethersulphone ultrafiltration membrane (Millipore Corporation, Billerica, Massachusetts, USA). The system was used in conjunction with magnetic stirrer. Nitrogen gas pressure of 5.3 kg/cm² was applied directly to the UFC. The process was carried out at 4°C. After 2 h the volume of the retained solution was 10 ml. Absorbance of this solution was recorded at 280, 540, 620 and 650 nm. The retained solution was again made up to 50 ml with 20 mM Tris–Cl buffer and the same procedure was repeated four times. The protein extract at the end of this step was referred to as the UFC concentrate.

2.2.6 Purification of phycocyanin by a single step ion exchange chromatography

Purification was carried out at 4°C in dark unless specified. All buffers and solutions were prepared in Milli-Q water supplemented with 0.02% (w/v) sodium azide. The UFC concentrate was loaded on a DEAE cellulose weak anion exchange column (60 mm × 12 mm, 5 cm bed height) pre-equilibrated with 20 mM Tris–Cl buffer (pH 8.1). The column was washed with 10 bed volumes of the same buffer and then developed with an elution buffer containing increasing step gradient from 0 to 0.4 M NaCl. Cobalt blue colored fractions was analyzed by UV–Vis absorption spectroscopy and denaturing and nondenaturing gel electrophoresis. Ratio of \( A_{620}/A_{280} \) was taken as a criterion for phycocyanin purity (R) achieved at each stage of purification.
Table 2.2: Full experimental central composite design in coded and actual level of variables and the response functions

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Citric acid stock</th>
<th>Calcium chloride</th>
<th>Sodium Nitrate</th>
<th>Trace metal mix</th>
<th>Biomass yield (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Actual level*</td>
<td>Actual level*</td>
<td>Actual level*</td>
<td>Actual level*</td>
<td>Actual level*</td>
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<tr>
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<td>0.60</td>
<td>0.60</td>
<td>0.60</td>
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</tr>
<tr>
<td>2</td>
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<td>0.50</td>
<td>0.50</td>
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<tr>
<td>3</td>
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<td>0.49</td>
<td>0.49</td>
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<td>6</td>
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<tr>
<td>7</td>
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<td>0.45</td>
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<tr>
<td>8</td>
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<td>9</td>
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<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

*Actual level in (g/L).
"Negative concentrations at the actual level were taken as zero."
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2.2.7 Electrophoresis

2.2.7.1 Non-denaturing gel electrophoresis
Native polyacrylamide gel electrophoresis was carried out in a vertical slab gel apparatus (Mini Protean III, Biorad, Hercules, California, USA). The gel (1.0 mm thick) consisted of 5% stacking and 10% resolving polyacrylamide (25 mM Tris–Cl (pH 8.3) and 250 mM glycine). Samples were prepared in 10 mM Tris–Cl (pH 6.8), 2% (v/v) glycerol and 0.009% (w/v) bromophenol blue. The separation was carried out at 4°C, at 80 V for the initial 30 minutes and then at 100 V till the end of the run. The bands were visualized by silver staining (Garfin, 1990).

2.2.7.2 Denaturing gel electrophoresis
Denaturing gel electrophoresis was also carried out with a vertical slab gel apparatus (Mini Protean III, Biorad, Hercules, California, USA) with a stacking gel 5% and resolving gel of 15% acrylamide concentration. The gel prepared was 1.0 mm thick, containing 0.1% (w/v) SDS. The samples were incubated with 2% (w/v) SDS, 10% (v/v) glycerol, 4.5% (v/v) β-mercaptoethanol, 0.025% (w/v) bromophenol blue and 60 mM Tris–Cl (pH 6.8) for 10 minutes in a boiling water bath. The gels were run at room temperature using a Laemmli buffer system (Laemmli, 1970) at 80 V for initial 30 minutes and then at 100 V till the end of the run. The bands were visualized by silver staining. PMWL 105978 (Bangalore Genei, Karnataka, India) was used as the molecular weight marker [composition-ovalbumin (Mr 43 kDa), carbonic anhydrase (Mr 29 kDa), soyabean trypsin inhibitor (Mr 20.1 kDa), lysozyme (Mr 14.3 kDa), aprotinin (Mr 6 kDa) and insulin (Mr 3.5 kDa)].

2.2.8 Zinc acetate staining
The resolved 10% native and 15% SDS gels were soaked in 20 mM zinc acetate solution for 5 minutes at room temperature. The bilin fluorescence was observed under UV light using AlphaEase FC Imaging System (Alpha Innotech, San Leandro, California, USA).

2.2.9 Molecular weight determination by MALDI
Mass spectral analysis was done on Voyager-De-STR (Applied Biosystems) MALDITOF. A nitrogen laser (337nm) was used for desorption and ionization. Spectra were acquired in the range of 10 kDa to 100 kDa, on linear mode with delayed ion extraction and with an accelerating voltage of 25kV. Low mass ion
gate was set to 4500 Da. All the analysis was done in four replications. The instrument was calibrated with insulin (bovine), ubiquitin, cytochrome C, myoglobin.

2.2.10 Thermal stability of phycocyanin

As the stability of proteins specifically biliproteins are very sensitive to temperature. Four set C-PC sample (one set contains 9 microfuge tube containing 0.5 mg/ml C-PC) was incubated in water bath at 10°C, 30°C, 50°C and 70°C. Each sample was observed for its stability after 1, 2, 5, 15, 30, 60, 120 and 240 minutes. The effect of various temperatures on the stability of C-PC was studied using Hewlett Packard 8452A UV–Vis Diode Array Spectrophotometer of 1 cm path length.
2.3 Results and discussion

2.3.1 16S rRNA gene sequence determination and phylogenetic placement

The full 16S rRNA gene sequences derived from this study were submitted to NCBI Gene Bank under the accession no. FJ410906. Furthermore, sequence was aligned with other 16S rRNA gene sequences available in database. Phylogenetic clusters of strains N62DM along with other closely related cyanobacteria strains was constructed (Fig. 2.1). It was found that N62DM belongs to genus *Leptolyngbya*, hence the identified culture was named *Leptolyngbya* sp. N62DM.

![Fig. 2.1: Phylogenetic tree derived from 16S rRNA gene sequence of N62DM and sequences of closest phylogenetic neighbours obtained by NCBI BLAST(n) analysis. The NJ-tree was constructed using neighbour joining algorithm with Kimura 2 parameter distances in MEGA 4.0 software. *Escherichia coli* K12 strain ATCC00096 has been taken as an out-group. Numbers at nodes indicate percent bootstrap values above 50 supported by more than 1000 replicates. The bar indicates the Jukes–Cantor evolutionary distance.](image)

2.3.2 Optimization of the medium components for increased PC production

The first requirement for any process development is to look for a high yielding strain and to identify the variables which influence on yield and to optimize
conditions for higher yield. Optimization through statistical experimental design is a common practice in biotechnology (Minocha et al., 2007).

2.3.2.1 Central composite design

In the present work, experiments were planned to obtain a quadratic model consisting of $2^4$ trials. The plan includes thirty experiments and two levels of concentration for each factor. In order to study the combined effect of these variables, experiments were performed at different combinations. Table 2.2 summarizes the central composite experimental plan along with the predicted and observed response for each individual experiment. It shows the production of phycocyanin (mg/ml) corresponding to combined effect of four component in the specified ranges. The optimum levels of the selected variables were obtained by solving the regression equation and by analyzing the response surface contour and surface plots (Abdelhay et al., 2008). The regression equation obtained after the analysis of variance (ANOVA) provides an estimate of the level of phycocyanin as a function of NaNO$_3$, CaCl$_2$, citric acid stock and trace metal mix (Table 2.3). The production of phycocyanin may be best predicted by the following model:

$$Y = (0.64) + (0.11X_1) + (-9.625E-003X_2) + (0.075X_3) + (0.041X_4) + (4.838E-003X_1X_2) + (7.625E-004X_1X_3) + (-0.014X_1X_4) + (0.025X_2X_3) + (-4.825E-003X_2X_4) + (7.925E-003X_3X_4) + (-0.084X_1^2) + (-0.039X_2^2) + (-2.765E-003X_3^2) + (-0.023X_4^2)$$  (4)

Where Y is phycocyanin production (mg/ml), $X_1$ is NaNO$_3$ concentration (g/L), $X_2$ is CaCl$_2$ concentration (g/L), $X_3$ is volume of citric acid stock (ml/L) and $X_4$ is volume of trace metal mix (ml/L).

The statistical significance of the second-order model equation was evaluated by F-test analysis of variance which revealed that this regression is statistically highly significant ($p < 0.0001$) for phycocyanin production. Only two ($X_1$ and $X_3$) out of the four linear coefficients and only one quadratic ($X_1^2$) term were significant ($p < 0.0001$) for the production of phycocyanin (Table 2.3).
Table 2.3: ANOVA for Response Surface Quadratic Model of phycocyanin production

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F-value</th>
<th>P-value</th>
<th>Prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>0.71</td>
<td>14</td>
<td>0.051</td>
<td>16.78</td>
<td>&lt;0.0001</td>
<td>Significant</td>
</tr>
<tr>
<td>A-NaNO₃</td>
<td>0.30</td>
<td>1</td>
<td>0.30</td>
<td>97.82</td>
<td>&lt;0.0001</td>
<td>Significant</td>
</tr>
<tr>
<td>B-CaCl₂</td>
<td>2.223E-003</td>
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<td>2.223E-003</td>
<td>0.73</td>
<td>0.4054</td>
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</tr>
<tr>
<td>C-Citric Acid Stock</td>
<td>0.14</td>
<td>1</td>
<td>0.14</td>
<td>44.82</td>
<td>&lt;0.0001</td>
<td>Significant</td>
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<tr>
<td>D-Trace Metal Mix</td>
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<td>3.066E-003</td>
<td>0.9566</td>
<td></td>
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<tr>
<td>AD</td>
<td>3.119E-003</td>
<td>1</td>
<td>3.119E-003</td>
<td>1.03</td>
<td>0.3267</td>
<td></td>
</tr>
<tr>
<td>BC</td>
<td>0.010</td>
<td>1</td>
<td>0.010</td>
<td>3.41</td>
<td>0.0847</td>
<td></td>
</tr>
<tr>
<td>BD</td>
<td>3.725E-004</td>
<td>1</td>
<td>3.725E-004</td>
<td>0.12</td>
<td>0.7309</td>
<td></td>
</tr>
<tr>
<td>CD</td>
<td>1.005E-003</td>
<td>1</td>
<td>1.005E-003</td>
<td>0.33</td>
<td>0.5735</td>
<td></td>
</tr>
<tr>
<td>A²</td>
<td>0.20</td>
<td>1</td>
<td>0.20</td>
<td>64.46</td>
<td>&lt;0.0001</td>
<td>Significant</td>
</tr>
<tr>
<td>B²</td>
<td>0.041</td>
<td>1</td>
<td>0.041</td>
<td>13.62</td>
<td>0.0022</td>
<td></td>
</tr>
<tr>
<td>C²</td>
<td>2.096E-004</td>
<td>1</td>
<td>2.096E-004</td>
<td>0.069</td>
<td>0.7962</td>
<td></td>
</tr>
<tr>
<td>D²</td>
<td>0.014</td>
<td>1</td>
<td>0.014</td>
<td>4.59</td>
<td>0.0490</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>0.046</td>
<td>15</td>
<td>3.034E-003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>0.040</td>
<td>10</td>
<td>3.954E-003</td>
<td>3.31</td>
<td>0.0992</td>
<td>Insignificant</td>
</tr>
<tr>
<td>Pure Error</td>
<td>5.968E-003</td>
<td>5</td>
<td>1.194E-003</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>0.76</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

The coefficient of determination $R^2$ for the above predicting Eq. (4) was 94.00, indicating the good agreement between experimental and predicted values (Table 2.3). Therefore, this equation could be used for predicting response at any combination of four variables in experimental range. The signal to noise ratio of 17.964 indicated an adequate precision (Table 2.3).

The effect of interaction of variables on phycocyanin yield was studied against any two independent variables while keeping the other independent variables at their constant level. These response surface plots or contour plots can be used to predict the optimal values for different test variables. Contour plots showing the effect of interactions of NaNO₃ and citric acid stock and of NaNO₃ and trace metal mix (Figs. 2.2 & 2.3, respectively) are found to be elliptical. The maximum predicted
yield is indicated by the surface confined in the smallest curve of the contour diagram (Ravikumar et al., 2006). The optimal values of the variables are obtained when moving along the major and minor axes of the ellipse and the response at the centre point yields maximum phycocyanin production.

The study of the contour plots revealed that the optimal values of the different variables lie in the following ranges: NaNO$_3$ = 4.0 – 4.5 g/L, CaCl$_2.2$H$_2$O = 0.20 – 0.25 g/L, trace metal mix = 0.90 – 1.0 ml/L and citric acid stock = 28 – 30 ml/L. The optimal values drawn from Figs. 2.2 & 2.3 are in close agreement with the optimizing regression model Eq. (4).
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Fig. 2.2: Contour plot for phycocyanin production showing the effect of interaction between sodium nitrate and citric acid stock. The other two variables were kept at the constant level.

Fig. 2.3: Contour plot of phycocyanin production showing the effect of interaction between sodium nitrate and trace metal mix. The other two variables were kept at the constant level.
2.3.2.2 Growth measurement
Growth was measured in terms of biomass yield (g/L). Citric acid stock had a direct positive effect on growth which was more prominent in presence of higher levels of NaNO₃ and lower levels of trace metal mix (Table 2.2). Increase in the cell mass indirectly helped in phycocyanin production.

2.3.2.3 Extraction and estimation of phycocyanin
The amount of phycocyanin in the crude extract obtained from unoptimized medium was 0.3180 mg/ml (Eq. 3). Under optimized conditions the maximum phycocyanin yield was 0.7314 mg/ml which is 2.3 fold higher than in the unoptimized medium. Amongst the medium components, interaction between NaNO₃ and citric acid stock significantly increased the phycocyanin production. From the industrial point of view, concentration of these interacting components can be changed on the contour plot for the cost-effective production of phycocyanin.

2.3.2.4 Validation of the experimental model
To validate the optimum combination of the process variables, confirmatory experiments were carried out. The selected combinations of the four variables from the contour plots resulted in the production of phycocyanin up to 0.7314 mg/ml.

2.3.3 Filtration and concentration of crude extract using ultrafiltration cell (UFC)
The crude extract was subjected to ultrafiltration in UFC by which solutes above the membrane cut-off were retained in the cell, while water and solutes below the cut-off passed into the filtrate and out of the cell. Continuous stirring conditions maintained the fluid movement during operation, thereby reducing the negative effects of concentration polarization (i.e. the build up of concentrated solutes on the membrane). Nitrogen gas is recommended for pressurizing the cell because use of compressed air can cause large pH shifts due to dissolution of carbon dioxide. Gradual increase in the purity ratio of the retained solution was observed till the process was repeated four times, after which there was no considerable improvement. The final retained solution, termed as UFC concentrate contained C-PC having purity ratio 2.89 (Fig. 2.4) with a yield of 83.58% (Table 2.4). This was
also clearly visible in silver stained gel electrophoresis as the number of unwanted bands was reduced as compared to crude extract (Fig. 2.5). Use of UFC eliminated the steps like ammonium sulphate precipitation and dialysis. Apart from being a time consuming process, ammonium sulphate precipitation may hinder the future crystallization process because of the difficulty in complete removal of salt by dialysis or even by desalting column, thus giving false positive results. A purity ratio of 2.89 at the end of this step indicates the importance and success of filtration and concentration.

2.3.4 Purification of phycocyanin by single step ion exchange chromatography (IEC)

The UFC concentrate was loaded on diethylaminoethyl (DEAE) cellulose weak anion exchange column. After binding, 10 bed volumes of the 20 mM Tris-Cl buffer (pH 8.1) were passed to remove unbound proteins. Step gradient of NaCl was added to elute C-PC. Pure C-PC was eluted at 0.2 M NaCl concentration in buffer. Ten bed volumes, of each 0.05, 0.1, and 0.15 M NaCl, were passed sequentially to remove proteins other than phycocyanin before starting with the 0.2 M elution buffer. Pure C-PC was collected separately and its purity ratio was checked by UV-Vis spectroscopy. The A620/A280 purity ratio of 0.2 M ion exchange elutes was 4.15. Silver staining of denaturing PAGE (Fig. 2.6) proved that there was no protein other than C-PC after the last step of purification, indicating that phycocyanin was purified. The success of the purification protocol was also revealed by the UV-Vis overlay spectra (Fig. 2.4). A sharp peak at 620 nm suggested the high level of C-PC while decrease in absorbance at 280 nm indicated the removal of proteins other than C-PC. No absorbance peak was found at either at 650 nm or 540 nm, which confirmed the absence of phycoerythrin or allophycocyanin in the purified sample. The purity ratio of 4.15 of the protein at the end of the final stage (Table 2.4) is also comparable to that of the reported values (Santiago-Santos et al., 2004; Reis et al., 1998). By the above mentioned purification protocol, we were able to purify C-PC with good purity ratio and high yield from *Leptolyngbya* sp. N62DM.
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Fig. 2.4: UV-Vis absorption overlay spectra of phycocyanin from *Leptolyngbya* sp. N62DM at each step of purification. Steps: (1) crude extract; (2) stirred ultrafiltration cell concentrate; (3) ion exchange chromatography elute.

**Table 2.4:** Data of the purification of C-PC from *Leptolyngbya* sp. N62DM

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total protein content (mg)</th>
<th>Total C-PC content (mg)</th>
<th>C-PC from total protein (mg)</th>
<th>Impurities (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$A_{620}/A_{280}$</th>
<th>Yield of C-PC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>70</td>
<td>292.61</td>
<td>161.52</td>
<td>55.20</td>
<td>44.80</td>
<td>1.05</td>
<td>100.00</td>
</tr>
<tr>
<td>Amicon concentrate</td>
<td>50</td>
<td>192.65</td>
<td>135.00</td>
<td>70.08</td>
<td>29.92</td>
<td>2.89</td>
<td>83.58</td>
</tr>
<tr>
<td>IEC</td>
<td>35</td>
<td>109.20</td>
<td>102.56</td>
<td>93.92</td>
<td>6.08</td>
<td>4.15</td>
<td>63.50</td>
</tr>
</tbody>
</table>

<sup>a</sup>Impurities have been calculated as the difference from the total protein (100%) and the C-PC content in it.
2.3.5 Characterization

2.3.5.1 Non-denaturing gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis of the protein sample obtained from the last stage of purification i.e. IEC sample, showed only one band (Fig. 2.5) indicating the homogeneity of C-PC.

![Image](image_url)

Fig. 2.5: Silver stained 10% Native gel electrophoresis at the last stage of purification of phycocyanin from Leptolyngbya sp. N62DM. Pure phycocyanin shows only one band indicating the homogeneity of C-PC.

2.3.5.2 Denaturing gel electrophoresis

Silver staining of SDS-PAGE (Fig. 2.6) revealed two bands of 18 and 19.5 kDa corresponding to the α and β subunits of C-PC, which is in the range of other reported C-PC subunits (MacColl, 1998; Santiago-Santos et al., 2004; Nield et al., 2003; Duerring et al., 1991; Shah et al., 2001).

2.3.5.3 Zinc acetate staining

Bilins of the protein fluoresce orange in the presence of zinc ions under UV radiation (Brekelman and Lagarias, 1986). To check that both the subunits are linked to bilins, the resolved 15% SDS-PAGE gel was soaked in 20 mM zinc acetate solution for 5 minutes at room temperature and checked for the characteristic orange fluorescence under orange light (Fig. 2.7). Brekelman and Lagarias (1986) suggest the use of zinc-assisted fluorescence enhancement to confirm bilin-containing proteins. It can detect C-PC in quantities as low as 50 ng per gel (Brekelman and Lagarias, 1986). The ease with which the detection is performed along with its specificity for biliprotein made it the method of choice (Brekelman and Lagarias, 1986).
Fig. 2.6: Silver stained 15% SDS gel electrophoresis at each stage of purification of phycoerythrin from *Leptolyngbya* sp. N62DM. Lanes: (1) protein molecular mass standard; (2) crude extract; (3) stirred ultrafiltration cell concentrate; (4) pure phycoerythrin elute from ion exchange chromatography. Pure phycoerythrin showed two bands of 18.0 and 19.5 kDa molecular mass corresponding to characteristic α and β subunits, respectively. Protein concentration was estimated by Lowry (Lowry et al., 1951) assay against bovine serum albumin as standard and 15 μg of protein was loaded in each lane.

Fig. 2.7: Detection of biliproteins of *Leptolyngbya* sp. N62DM by zinc-assisted fluorescence enhancement method at each step of purification as observed under UV light. SDS electrophoresis was run in 17% acrylamide gel. Lanes: (1) protein molecular weight marker; (2) crude extract; (3) amicon concentrate; (4) pure phycoerythrin elute from ion exchange chromatography. There was no biliproteins present on molecular weight marker and so no fluorescence observed indicated the specificity of method for biliproteins detection. The protein loaded was 15 μg of protein was loaded in each lane.
2.3.5.4 Molecular weight determination by MALDI

Mass spectral analysis done on Voyager-De-STR (Applied Biosystems) MALDI-TOF, two peaks having molecular weight 18 and 19.5 kDa (Fig. 2.8) corresponding to α and β subunit respectively. Result obtained by MALDI-TOF validate the data obtained from SDS-PAGE and hence conform the molecular weight of C-PC. There are many low molecular weight proteins integral to the phycobilisome in the mass range of about 10 kDa was also observed which may be most probably represent the linker polypeptides.

Fig. 2.8: MALDI analysis of C-PC purified from Leptolyngbya sp. N62DM.
2.3.5.5 Temperature stability of phycocyanin

As temperature plays an important role in stability and further it affects the crystallization of proteins. Temperature sensitivity of C-PC was calculated on the basis of optical density and percent retained pigment in the solution. It is very well established that purified C-PC from most of the algae is stable for years at 4°C in dark (Soni et al. 2006). It was observed that C-PC purified from *Leptolyngbya* sp. N62DM was also stable at 10°C for long time (several months). (Fig. 2.9) But at 30°C, C-PC degraded slowly and reached to its saturation after four hours, whereas at higher temperature 50°C and 70°C C-PC degraded very rapidly and total biliprotein gets denatured within 3-4 hours of incubation (Fig. 2.9).

![Pigment stability at different temperatures](image.png)

Fig. 2.9: Stability of C-PC purified from *Leptolyngbya* sp. N62DM.
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2.4 Conclusion

In the present study we isolated and further identified the potential C-PC producing cyanobacteria. 16S rRNA gene analysis revealed that *Leptolyngbya* sp. is the nearest neighbour of isolated cyanobacterium. To the best of our knowledge, it is for the first time that the medium components have been statistically optimized for increasing PC production. Predicted and experimental values of the response reflect the accuracy and applicability of RSM to increased production of C-PC. As compared to C-PC production by *Leptolyngbya* sp. N62DM in the unoptimized medium, a 2.3-fold increase was recorded in the RSM optimized shake flasks. Our purification results also indicate the successful development of a simple, cheap and efficient single step method for the purification of C-PC from *Leptolyngbya* sp. N62DM. Purity ratio of 4.15 and percent yield of C-PC about 63.50 reflects that it can be successfully used in the food and pharmaceutical industries. Since, highly purified (4.15) C-PC was obtained without the use of ammonium sulphate, it can be used further for the crystallization work without unnecessary interference of salts.

*How much is the C-PC content in Leptolyngbya?*

*Whether toxicity test done?*