STATISTICAL EVALUATION OF NUTRITIONAL COMPONENTS IMPACTING PHYCOCYANIN PRODUCTION IN *SYNECHOCYSTIS* SP.

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ABSTRACT

Alkaliphilic cyanobacterial cultures were isolated from Lonar lake (MS, India). Among the set of cultures, *Synechocystis* sp, was studied for phycocyanin production. A maximum yield was obtained in BG-11 medium at optimized conditions (pH 10 and 16 h light). In order to increase the phycocyanin yield media optimization based on the eight media components a Plackett-Burman design of the 12 experimental trials was used. As per the analysis $CaCl_2.2H_2O$ and Na_2CO_3 have been found to be the most influencing media components at 95% significance. Further the optimum concentrations of these components were estimated following a Box Wilson Central Composite Design (CCD) with four star points and five replicates at the center points for each of two factors was adopted for optimization of these two media components. The results indicated that there was an interlinked influence of $CaCl_2.2H_2O$ and Na_2CO_3 on 98% significance. The maximum yield of phycocyanin (12% of dry wt) could be obtained at 0.058 g/l and 0.115 g/l of $CaCl_2.2H_2O$ and Na_2CO_3, respectively.

Key words: *Synechocystis,* Lonar lake, Phycocyanin, Plackett-Burman, Central composite design

INTRODUCTION

The alkaline lake of Lonar (Dist. Buldhana, MS, India, lat 19° 58', lag 76° 34') is one few alkaline lakes on the earth. Meteoritic impact around 50-60 thousand years ago formed this crater. This is a unique crater in basaltic rock (9). Craters formed by such hypervelocity impact are considered to be a unique ecological habitat (14). The lake water remains alkaline throughout the year owing to the high content of sodium carbonate. Soda lakes are characterized by the presence of large amount of sodium carbonate (or complexes of these salts) formed by evaporative concentration under particular condition of geology, geography, and climate (7). Soda lakes are the most stable and productive naturally occurring alkaline environments in the world, with pH values greater than 10 (9).

Along with *Spirulina, Synechocystis* sp., a unicellular cyanobacterium gives the water a dull blue-green color. Cyanobacteria has four basic types of biliproteins i.e.; phycocyanin, allophycocyanin, phycoerythrin and phycoerythrocyanin (16). These chromophores, called bilins,

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are covalently attached to the cystine residues of the apoproteins. Allophycocyanin and C-phycocyanin have a bilin called as phycocyanobilin, and C-phycoerythrin has a bilin called phycoerythrobilin (15). The absorbance coefficient of phycobiliproteins is markedly higher than those of most commercial dyes (6). This is the main reason that phycobiliproteins are used as highly- fluorescent substance in Fluorescence Activated Cell Sorting (FACS), immunochemistry, detection of reactive oxygen species, and amino acid sequencing (1, 2). Cyanobacterial phycobiliproteins have gained importance in the commercial sector, as they have several applications. The primary potential of these molecules are as natural dyes but a number of investigations have shown their health-promoting properties and broad range of pharmaceutical applications (16). They are used as the colorants in chewing gum, ice sherbets, popsicles, candies, soft drinks, dairy products and cosmetics like lipstick and eyeliners. In addition, phycobiliproteins are widely used in clinical and immunological research laboratories (20).

The viability of product produced from micro-organism depends on cost of production and net yield of the product. Media composition and growth conditions influence culture growth rate and in this case phycocyanin production. In order to avoid conventional optimization, statistical models Fractional Factorial Design (FFD) and Central Composite Design (CCD) have been proved to be effective (8, 22). Here we attempt to increase the production of phycocyanin by *Synechocystis* sp, isolated from Lonar Lake, using methods of FFD and CCD. Media components affecting phycocyanin production were evaluated by the application of two-level factorial Plackett-Burman design (4, 13).

MATERIALS AND METHODS

Organism and Culture Conditions

The unicellular cyanobacterium *Synechocystis* sp. used in this study was isolated from the water sample of Lake Lonar. The identification was done as per Desikachary (5). The cyanobacterium was maintained on BG-11 media (11). The pH of the media was adjusted with NaOH at 10.36 (lake water pH). In 250 ml capacity Erlenmeyer flasks 100 ml culture medium was taken and the initial cell density was maintained at 10^6 cells/ml. The flasks were exposed to a light intensity of 75 µmol photons.$m^{-2}.s^{-1}$ with a light/dark cycle of 16/8 h at 24 ± 2°C. The irradiance was measured under the white fluorescent tube light in the culture racks using a luxmeter.

Extraction of Phycocyanin

After incubation of 12 days the cells were harvested by centrifugation at $5000 \times g$ for 10 min at 5°C (Sorvall, USA, Model RC2). The pellet was washed with distilled water and resuspended in 5 ml of 0.05 M phosphate buffer. The content was sonicated (Sonics and Materials Inc., USA) with 30 Hz frequency at a pulse of 10/5 min. Freezing-thawing of the sonicated content was done at least for two times and cell rupture was confirmed microscopically. The extract was centrifuged at $10000 \times g$ for 10 min at 5°C. The supernatant was collected and the concentration of phycocyanin was measured spectrophotometrically at 615 and 652 nm using following Equation (1) with 0.05 M phosphate buffer as blank (11).

$$\text{Phycocyanin (PC) mg/ml} = \{A_{615} - (0.474 \times A_{652})\}/5.34 \qquad (1)$$

Effect of pH and Light Exposure on Phycocyanin Production

In order to check the effect of pH and light exposure on phycocyanin production two sets of Erlenmeyer flasks containing 100 ml BG-11 medium at varying pH (5 to 12) and light exposure (8L:16D, 12L:12D, 16L:8D and 24L:0D h) were inoculated with *Synechocystis* sp. The pH of the medium was adjusted using 0.1 M sodium hydroxide and incubated at 24 ± 2°C for 12 days. After incubation phycocyanin content was measured.

Statistical Analysis

A 12-run Plackett-Burman design (13) was used to screen

the eight major nutrients in BG-11 growth medium that could influence the production of phycobiliproteins. The level of micronutrients was kept constant in all experiments. A method of least squares was used to analyze the experimental response fit using the following first-order model, Equation (2):

$$\hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_6 X_6 + \beta_7 X_7 + \beta_8 X_8$$

$$(2)$$

where, $\hat{Y}$ was the predicted response (phycobiliproteins yield), β_0, β_1, β_2, β_3, β_4, β_5, β_6, β_7 and β_8 were the regression coefficients, and X_1, X_2, X_3, X_4, X_5, X_6, X_7 and X_8 were the coded levels of the independent variables. Based on the first-order model equation obtained by the Plackett–Burman design, a second-order polynomial model i.e. a central composition design (CCD) with five coded levels was performed. The quadratic model for predicting the optimal point was expressed according to the following Equation (3):

$$\hat{Y} = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{12} X_1 X_2 + \beta_{22} X_2^2 \qquad (3)$$

where, $\hat{Y}$ was the predicted response (phycobiliprotein yield), β_0, β_1, β_2, β_{11}, β_{12} and β_{22} were the regression coefficients, and X_1 and X_2 were the coded levels of the independent variables. All statistical analyses were done using statistical software MINITAB 13.31, USA, Inc.

RESULTS AND DISCUSSION

The alkaliphiles are unique microorganisms, with great potential for microbiology and biotechnological exploitation. In present study cyanobacterial culture, *Synechocystis* sp. was isolated from the soda lake of Lonar, which is a unique habitat in India, rich source of naturally occurring alkaliphilic microbes. Fig. 1 shows the effect of medium pH on phycocyanin production by *Synechocystis* sp. Culture could not grow at pH 5 and 6. Maximum phycocyanin production of 14.5 mg/l was obtained at pH 10. The phycocyanin production by *Synechocystis* sp. was also assessed at varying light exposures (Fig. 2). Light exposure of 16 h was found optimum for phycocyanin production.
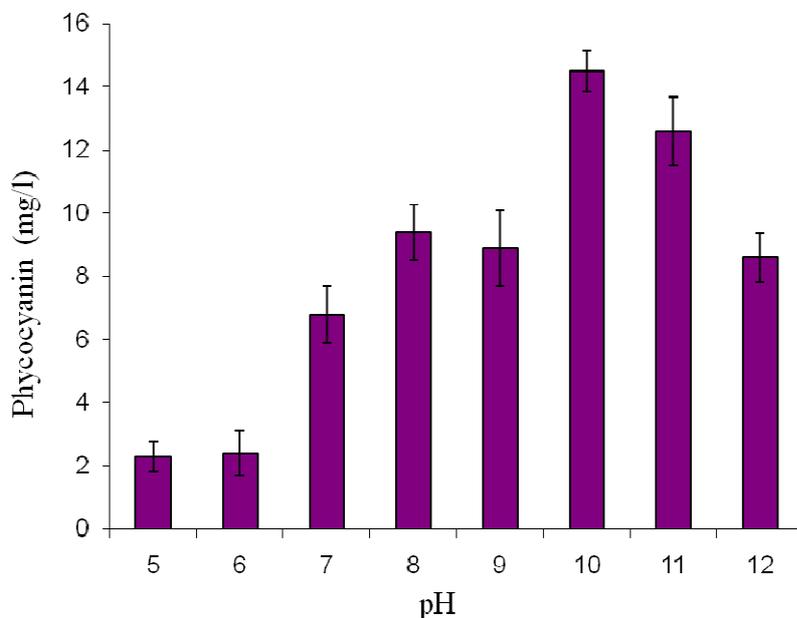

Figure 1. Effect of pH on phycocyanin production by *Synechocystis* sp. grown in BG-11 at varying pH (5 to 12) at $24 \pm 2°C$ with 16 h light exposure of 75 µmol photons.m^{-2}.s^{-1}
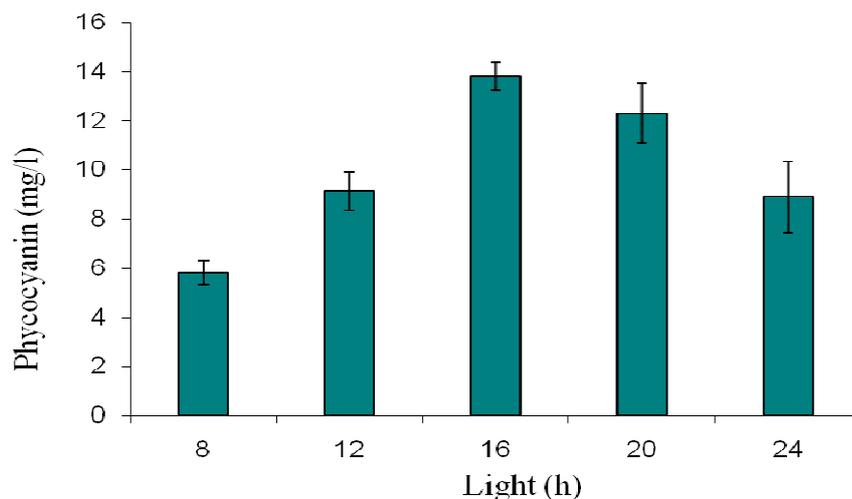
Figure 2. Effect of light exposure on phycocyanin production by *Synechocystis* sp. grown in BG-11 medium at pH 10.36 at 24 ± 2°C with varying light exposure of 8, 12, 16 and 24 h (75 µmol photons.m^{-2}.s^{-1})

Cyanotech is developing micro-algae based technologies and producing phycobiliproteins, currently range in price from US $ 5,000-33,000 g^{-1} (16). While Dainippon Ink Corporations have been the market leaders for many years in production of commercial products from algae and cyanobacteria (23). Statistical design is an aid to reach a statistical valid outcome with minimum number of observations (8,10). Response Surface Methodology (RSM) is a sequential procedure with an initial objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum and since the location of the optimum is unknown prior to running RSM

experiments; it makes sense to have a design that provides equal precision of estimation in all directions employed. Based on the eight media components a Plackett-Burman design of the 12 experimental trials was used as in Table 1. Each row represents the 12 different experiments to evaluate their effect on phycocyanin production and each column represents a different variable. Each independent variable was investigated at a high (+1) and a low (-1) level which represents two different nutrient concentrations. The significant levels of each media variable were determined by using *t* test and regression analysis and are shown in Table 2.

Table 1. The Plackett-Burman experimental design matrix for screening medium composition of BG-11

Trial	Level and concentration of variable (g/l)								PC Yield (mg/l)	
	X_1 NaNO$_3$	X_2 K$_2$HPO$_4$	X_3 MgSO$_4$.7H$_2$O	X_4 CaCl$_2$.2H$_2$O	X_5 Citric acid	X_6 Ferric ammonium citrate	X_7 EDTA	X_8 Na$_2$CO$_3$	Exptl.	Pred.
T$_1$	+1 (2.25)*	-1 (0.02)	+1 (0.1125)	-1 (0.018)	-1 (0.003)	-1 (0.003)	+1 (0.0015)	+1 (0.03)	18.1 [0.20]**	16.8
T$_2$	+1 (2.25)	+1 (0.06)	-1 (0.0375)	+1 (0.054)	-1 (0.003)	-1 (0.003)	-1 (0.0005)	+1 (0.03)	18.9 [0.15]	19.3
T$_3$	-1 (0.75)	+1 (0.06)	+1 (0.1125)	-1 (0.018)	+1 (0.009)	-1 (0.003)	-1 (0.0005)	-1 (0.01)	10.1 [0.15]	8.9
T$_4$	+1 (2.25)	-1 (0.02)	+1 (0.1125)	+1 (0.054)	-1 (0.003)	+1 (0.009)	-1 (0.0005)	-1 (0.01)	15.3 [0.05]	15.9
T$_5$	+1 (2.25)	+1 (0.06)	-1 (0.0375)	+1 (0.054)	+1 (0.009)	-1 (0.003)	+1 (0.0015)	-1 (0.01)	16.6 (0.20)	16.1
T$_6$	+1 (2.25)	+1 (0.06)	+1 (0.1125)	-1 (0.018)	+1 (0.009)	+1 (0.009)	-1 (0.0005)	+1 (0.03)	10.1 [0.20]	11.3
T$_7$	-1 (0.75)	+1 (0.06)	+1 (0.1125)	+1 (0.054)	-1 (0.003)	+1 (0.009)	+1 (0.0015)	-1 (0.01)	17.9 [0.30]	18.2
T$_8$	-1 (0.75)	-1 (0.02)	+1 (0.1125)	+1 (0.054)	+1 (0.009)	-1 (0.003)	+1 (0.0015)	+1 (0.03)	19.3 [0.10]	20.6
T$_9$	-1 (0.75)	-1 (0.02)	-1 (0.0375)	+1 (0.054)	+1 (0.009)	+1 (0.009)	-1 (0.0005)	+1 (0.03)	18.9 [0.20]	17.5
T$_{10}$	+1 (2.25)	-1 (0.02)	-1 (0.0375)	-1 (0.018)	+1 (0.009)	+1 (0.009)	+1 (0.0015)	-1 (0.01)	10.1 [0.15]	10.5
T$_{11}$	-1 (0.75)	+1 (0.06)	-1 (0.0375)	-1 (0.018)	-1 (0.003)	+1 (0.009)	+1 (0.0015)	+1 (0.03)	16.3 [0.05]	15.9
T$_{12}$	-1 (0.75)	-1 (0.02)	-1 (0.0375)	-1 (0.018)	-1 (0.003)	-1 (0.003)	-1 (0.0005)	-1 (0.01)	10.0 [0.11]	11.2

*Values in parentheses are concentrations in g/l of each variable in BG-11; **, Values in rectangle brackets are of standard deviation; Exptl., experimental; Pred., predicted. Level of micronutrients in all experiments was kept constant

Table 2. Estimated effect on phycocyanin (PC) production from the results of Plackett-Burman design

Factor	Effect	Standard error	t- value	p-value	Confidence level (%)
Intercept	$\beta_0 = 15.13$	0.5369	28.19	0	-
X_1	$\beta_1 = -0.57$	0.5369	-0.53	0.634	36.6
X_2	$\beta_2 = -0.30$	0.5369	-0.28	0.798	20.2
X_3	$\beta_3 = 0$	0.5369	0	1.000	0
X_4	$\beta_4 = 5.37$	0.5369	5.00	0.015	98.5
X_5	$\beta_5 = -1.90$	0.5369	-1.77	0.175	82.5
X_6	$\beta_6 = -0.73$	0.5369	-0.68	0.544	45.6
X_7	$\beta_7 = 2.50$	0.5369	2.33	0.102	89.8
X_8	$\beta_8 = 3.60$	0.5369	3.35	0.044	95.6

The experimental yields of phycocyanin at varied levels of medium components as per Plackett-Burman design are given in Table 1. The analysis showed that $CaCl_2.2H_2O$ and Na_2CO_3 had a significant influence (95% significant level) on phycocyanin production, while other factors had insignificant influence on phycocyanin production from *Synechocystis* sp. On the basis of statistical testing and the F-test applied for the analysis of variance (ANOVA) the F-value and p-value were 5.70 and 0.090, respectively. The coefficient of determination (R^2) obtained was 0.938 had indicating that the model explained 93.8%.of variability in the response.

Though the Plackett-Burman design pointed out the factors influencing phycocyanin production one cannot determine the exact optimum values of the individual factors. This has necessitated using a second order experimental design. A central composite design (CCD) was applied. A steepest ascent experiment was performed before the second-order polynomial model. The design and the steepest ascent experiment are shown in Table 3. In CCD the centre point could be taken as a level that had shown maximum response in

the steepest ascent experiment (21). It can be seen from Table 3 that the maximum phycocyanin production was obtained at 0.052 g/l of $CaCl_2.2H_2O$ and 0.10 g/l of Na_2CO_3. On this basis a Box Wilson CCD (22) with four star points and five replicates at the center points for each of two factors was adopted for optimization of these two media components. Table 4 shows the observed and predicted values of phycocyanin yield obtained using CCD Equation (3).

The real values of significant variables X_4 and X_8 used in the Box Wilson model are shown in Table 5. The significant levels of each variable were determined by t-test (Table 6). The factors, $X_4.X_4$ and X_4 were found to be significant with a probability of 99% and 96.3%, respectively. The interaction between X_4 and X_8 had an interlinked influence on phycocyanin production at 98% significance.

The equation for phycocyanin yield for the second order model could be written as:

$$\hat{Y} = 23.6 + 1.574\ X_4 + 0.564\ X_8 - 5.483\ X_4.X_4 + 2.528\ X_4.X_8 - 1.381 X_8.X_8 \qquad (4)$$

Table 3. The steepest ascent experiment for determination of centre point for CCD

Step change value	Coded variable level		Real variable level (g/l)		PC Yield (mg/l)
	X_4	X_8	$CaCl_2.2H_2O$	Na_2CO_3	
Centre point	0	0	0.036	0.02	13.8 [0.15]*
Centre point +1	+1	+1	0.040	0.04	16.1 [0.20]
Centre point +2	+2	+2	0.044	0.06	17.4 [0.15]
Centre point +3	+3	+3	0.048	0.08	19.2 [0.25]
Centre point +4	+4	+4	0.052	0.10	23.1 [0.35]
Centre point +5	+5	+5	0.056	0.12	18.3 [0.40]

* Values in rectangle brackets are of standard deviation

Table 4. Central composite design and the corresponding responses for phycocyanin.

Run	X_4	X_8	PC Yield (mg/l)	
			Exptl.	Pred.
1	-1.000	-1.000	15.6 [0.25]*	17.1
2	1.000	-1.000	12.8 [0.15]	15.2
3	-1.000	1.000	13.0 [0.30]	13.2
4	1.000	1.000	20.3 [0.05]	21.4
5	-1.414	0.000	11.1 [0.30]	10.4
6	1.414	0.000	16.8 [0.25]	14.8
7	0	-1.414	22.3 [0.20]	20.0
8	0	1.414	22.0 [0.20]	21.6
9	0	0	23.8 [0.20]	23.6
10	0	0	23.9 [0.10]	23.6
11	0	0	24.1 [0.15]	23.6
12	0	0	23.8 [0.15]	23.6
13	0	0	22.4 [0.05]	23.6

X_4, $CaCl_2.2H_2O$; X_8, Na_2CO_3; Exptl, Experimental response; Pred, Predicted (values calculated from the fitted second order model), * Values in rectangle brackets are of standard deviation.

Table 5. Real values of the significant factors used in CCD.

Factor	Component	Real values				
		-1.414	-1	0	+1	+1.414
X_4	$CaCl_2.2H_2O$ (g/l)	0.023	0.026	0.052	0.078	0.083
X_8	Na_2CO_3 (g/l)	0.046	0.05	0.1	0.15	0.16

Table 6. Coefficients and *t* values calculated from the central composition experiment

Factor	Coefficient	Standard error	t- value	p-value
Intercept	$\beta_0 = 23.60$	0.769	30.65	<0.0001
X_4	$\beta_1 = 1.57$	0.608	2.579	0.037
X_8	$\beta_2 = 0.56$	0.608	0.919	0.389
$X_4.X_4$	$\beta_{11} = -5.48$	0.653	-8.397	<0.0001
$X_4.X_8$	$\beta_{12} = 2.53$	0.861	2.933	0.022
$X_8.X_8$	$\beta_{22} = -1.38$	0.653	-2.116	0.072

Statistical testing of the experimental data was carried out using Fisher's test for analysis of variance (ANOVA) using MINITAB 13.31. The *F*- and *p*-values were 17.54 and 0.001, respectively. The quality of the fit of the quadratic regression model equation was expressed by R^2, which indicated 92.6% of the variability of the response could be explained by this model. The value of the Adj R^2 (87.3%) also indicated the high significance of the model. Thus these results indicated that the response equation provided a suitable model for the CCD experiment. The response surface plot for the CCD used for phycocyanin production is depicted in Fig. 3. It can be seen that maximum yield of phycocyanin (23.8 mg/l, i.e. 12% of cell dry weight) could be obtained at 0.058 g/l and 0.115 g/l of X_4, $CaCl_2.2H_2O$ and X_8, Na_2CO_3, respectively.

In order to confirm the second order model, *Synechocystis* sp. was grown using optimized media constituents and yielded 22.6 mg/l of phycocyanin. The yield of phycocyanin obtained with *Synechocystis* sp. isolated from Lonar lake was comparable with earlier report on *Synechocystis* sp. PCC 6701 (25 mg/l) (8). Nitrate and phosphate were identified as the most significant factors.

The production of phycocyanin not only depends upon the media components but also some operational factors such as light and pH (8). The pH of the medium also influences the

production of phycobiliproteins. *Gloeotrichia natans*, a nitrogen fixing cyanobacterium has been studied as a possible source of phycobiliproteins (3). Under optimal growth conditions (38°C, pH 8.0, with no carbon enrichment), the specific growth rate of the rice-field isolate of *Gloeotrichia natans* was 0.076 h^{-1}. The pH of the medium (between 6.5 and 9.0) did not influence the growth rate, but it did affect phycobiliprotein content, as reflected by a change of the color of the cultures. At pH 7.0, the culture was green-brown with phycobiliproteins constituting up to 10% of the total protein, while at pH 9.0 the culture was brownish-black and the content of these pigments was as high as 28% of the total protein. In the current study the optimum pH level for the production of phycocyanin was found to be pH 10 (Fig. 1). Phycocyanin production from cyanobacterial cultures has been reported to be dependent on various factors viz. culture conditions, particularly nitrogen and carbon sources. Supply of 3% carbon dioxide and 97% nitrogen in gaseous form supported 20.1% of C-phycocyanin yield in a laboratory culture of *Nostoc* sp. (19). A screening process indicated that the content of C-phycocyanin was 17% of dry weight in some strains of *Anabaena* and *Nostoc* and 10% of in some *Nostoc* sp. (12). A commercial process of open-tank mass cultivation of a marine cyanobacterium *Phormidium valderianum* BDU 30501 was also developed for producing phycocyanin (17). The average pigment yield was 20% on dry weight basis. Optimization of mass cultivation media for the production of biomass and natural colourants from marine cyanobacteria by a mathematical design of experiments were also performed (18). Kathiresan *et al.* (10) reported effect of major media constituents of *Porphyridium spp.* studied using response surface methodology (RSM) on biomass yield, total phycobiliprotein and the production of phycoerythrin. The independent variables such as concentration of NaCl, $MgSO_4$, $NaNO_3$ and K_2HPO_4 influenced the total phycobiliproteins and phycoerythrin production.

The production of phycobiliproteins by cyanobacteria has been observed as a pH dependent phenomenon (8). The production of phycobiliproteins increases with increase in alkaline conditions. The soda lake habitats often have a combination of Ca^{2+} and carbonate levels (7). The *Synechocystis* sp. was isolated from Lonar Lake, a saline alkaline condition. The alkaline conditions are mainly favorable for primary producers such as cyanobacteria in the lake.
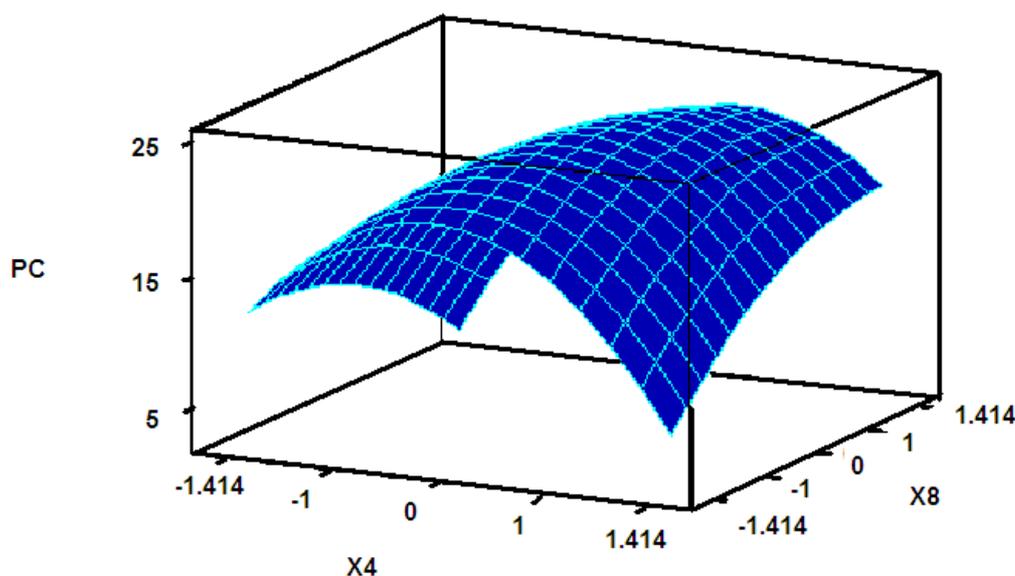

Figure 3. Response surface plot of phycocyanin (PC) production as a function of $CaCl_2.2H_2O$ (X_4) and Na_2CO_3 (X_8). When X_4 = 0.1931 (0.058 g/l of $CaCl_2.2H_2O$) and X_8 = 0.3194 (0.115 g/l of Na_2CO_3) PC will arrive at the maximum point (23.8 mg/l)

REFERENCES

1. Arad, S.M.; Yaron, A. (1992). Natural pigments from red microalgae for use in foods and cosmetics. *Trends Food Sci. Technol.,* 3, 92-97.

2. Bermejo, R.; Talavera, E.M.; DelValle, C.; Alvarez-Pez, J.M. (2000). C-phycocyanin incorporated into reverse micelles: a fluorescence study. *Colloids Surf. B Biointerfaces,* 18, 51-59.

3. Boussiba, S. (1991). Nitrogen fixing cyanobacteria potential uses. *Plant Soil,* 137, 177-180.

4. Deshmukh, D.V.; Puranik, P.R. (2010). Application of Plackett-Burman design to evaluate media components affecting antibacterial activity of alkaliphilic cyanobacteria isolated from Lonar Lake. *Tur. J. Biochem.,* 35, 112-118.

5. Desikachary, T.V. (1959). *Cyanophyta.* Indian Council of Agricultural Research, New Delhi, India.

6. Glazer, A.N. (1999). Phycobiliproteins. *In:* Cohen, Z. (ed). *Chemicals from Microalgae.* Taylor and Francis Ltd, London, p.261-280.

7. Grant, W.D. (2004). Half a lifetime in soda lakes. *In:* Ventosa, A. (ed). *Halophilic Microorganisms.* Springer-Verlag, Berlin, p.17-32.

8. Hong, S.J.; Lee, C.G. (2008). Statistical optimization of culture media for production of phycobiliprotein by *Synechocystis* sp. PCC 6701. *Biotechnol. Bioprocess Eng.,* 13, 491-498.

9. Joshi, A.A.; Kanekar, P.P.; Kelkar, A.S.; Shouche, Y.S.; Wani, A.A.; Borgave, S.B.; Sarnaik, S.S. (2008). Cultivable bacterial diversity of alkaline lonar lake, India. *Microbial Ecology,* 55, 163-172.

10. Kathiresan, S.; Sarada, R.; Battacharya, S.; Ravishankar, G.A. (2006). Culture media optimization for growth and phycoerythrin production from *Porphyridium purpureum. Biotechnol. Bioeng.,* 96, 456-463.

11. Kaushik, B.D. (1987). *Laboratory methods for blue-green algae.* Associated Publication Co., New Delhi, India.

12. Moreno, J.; Rodriquez, M.; Vargas, A.; Rivas, J.; Guerrero, M. (1995). Nitrogen fixing cyanobacteria as a source of phycobiliproteins pigments - composition and growth performance of ten filamentous heterocystous strains. *J. Appl. Phycol.,* 7, 17-23.

13. Plackett, R.L.; Burman, J.P. (1946). The design of optimum multifactorial experiments. *Biometrika,* 33, 305-325.

14. Ranade, D.R.; Thakker, C.D. (2002). An alkalophilic *Methanosarcina* isolated from Lonar crater. *Curr. Sci.,* 82, 455-458.

15. Samsonoff, W.A.; MacColl, R. (2001). Biliproteins and phycobilisomes from cyanobacteria and red algae at the extreme of habitat. *Arch. Microbiol.,* 176, 400-405.

16. Sekar, S.; Chandramohan, M. (2008). Phycobiliproteins as a commodity: trends in applied research, patents and commercialization. *J. Appl. Phycol.,* 20, 113-136.

17. Sekar, S.; Subramanian, G. (1998). A method of mass cultivation of the marine cyanobacterium *Phormidium valderianum* BDU 30501 for the production of blue natural colourant phycocyanin. *In:* Subramanian, G., Kaushik, B.D., Venkataraman, G.S. (eds). *Cyanobacterial Biotechnology.* Science Publishers, New Hampshire.

18. Sekar, S.; Priya, S.L.; Roy, W.P. (2000). Optimization of mass cultivation media for the production of biomass and natural colourants from two marine cyanobacteria by a mathematical design of experiments. *J. Plant Biotechnol.,* 2, 157-163.

19. Silva, H.; Cortinas, T.I.; Ertola, R.J. (1989). Effect of nutritional factors on the culture of *Nostoc* sp. as a source of phycobiliproteins. *Appl. Microbiol. Biotechnol.,* 31, 293-297.

20. Spolaore, P.; Cassan, C.J.; Duran, E.; Isambert, A. (2006). Commercial application of microalgae. *J. Biosci. Bioeng.,* 101, 87-96.

21. Wu, C.F.J.; Hamada, M. (2000). *Experiments: Planning, Analysis, and Parameter Design Optimization.* Wiley, New York.

22. Xiao, Z.J.; Liu, P.H.; Qin, J.Y.; Xu, P. (2007). Statistical optimization of medium components for enhanced acetoin production from molasses and soybean meal hydrolysate. *Appl. Microbiol. Biotechnol.,* 74, 61-68.

23. Yamaguchi, K. (1997) Recent advances in microalgal bioscience in Japan, with special reference to utilization of biomass and metabolites: a review. *J. Appl. Phycol.,* 8, 487-502.