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Optimization of Mass cultivation Media for the Production of Biomass and Natural Colourants from Two Marine Cyanobacteria by a Mathematical Design of Experiments

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Key words : biomass, design of experiments, marine cyanobacteria, media optimization, natural colourant, orthogonal array.

Abstract

Optimization of chemicals in the large scale sea water medium and inoculum for biomass and natural colourants production in the marine cyanobacteria, *Phormidium tenue* BDU 46241 (phycoerythrin producer) and *P. valderianum* BDU 30501 (phycocyanin producer) was carried out by experiments in L8 orthogonal array. Mathematical analysis revealed the significance of these factors. The factor(s) that critically control the yield varied with the organism and the end-product. Further, the desirable level of these factors between the normal and a higher level tested was identified and improved media were evolved. In both cyanobacteria, higher level of K_2HPO_4, $NaNO_3$ and inoculum with normal level of ferric ammonium citrate was found to be desirable for biomass production and additionally, higher level of $MgSO_4$ for pigment production. The level of other factors varied with the organism and the end-product. Confirmation experiments showed that the clues obtained based on mathematical experimentation are valid. In *P. tenue*, the medium optimized for biomass production increased the yield of biomass by 495% and the medium optimized for phycoerythrin production increased the yield of biomass by 408% with 30% increase in phycoerythrin content of the biomass. Similarly in *P. valderianum*, the medium optimized for biomass production increased the yield of biomass by 224% and the medium optimized for phycocyanin production increased the yield of biomass by 143% with 44% increase in phycocyanin content of the biomass.

Introduction

Increasing attention has recently been paid to the biotechnological potential of converting solar energy and CO_2 into biomass and industrially valuable compounds using marine cyanobacteria. Cyanobacteria are oxygen-evolving photosynthetic prokaryotes found to posses numerous biotechnological applications (Takeyama and Matsunaga, 1998). Although the potential of cyanobacteria as biofertilizers is well known, few commercial-scale processes have been attempted to use them industrially. Some species of *Spirulina* are the only cyanobacteria mass-cultivated industrially, mainly for use as a health food, and in countries like Japan, phycocyanin is extracted from *Spirulina* commercially (Venkataraman, 1989; Borowitzka, 1994a). The presence of phycobiliproteins, the brilliantly coloured accessory pigments as phycobilisomes, is the most striking characteristic of cyanobacteria.

Phycobiliproteins have commercial value as natural pigments for beverages, dry beverage mixtures and natural dyes (Langston and Maing, 1983). They are used in fluorescence microscopy, fluorescence immunoassays and as phycofluors (Glazer and Stryer, 1984; Kronick, 1986). Phycocyanin, the major phycobiliprotein, has also exhibited anti-cancer activity, stimulation of the immune system and ability to treat ulcers and hemorrhoidal bleeding (Richmond, 1990). Phycocyanin is commercially produced from *Spirulina* spp. and used for colouring ice-creams and

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yogurt in addition to its usage in cosmetics, eye shadow, eye liner and lip stick preparations (Venkataraman, 1989; Borowitzka 1994 a, b). A commercial process of open-tank mass cultivation of a marine cyanobacterium, *Phormidium valderianum* BDU 30501 was also developed for producing phycocyanin (Sekar and Subramanian, 1998).

The nature of culture conditions employed, particularly nitrogen and carbon sources, determines the content of phycobiliproteins. Supply of 3% CO_2 and 97% N_2 in gaseous form supported 20.1 % of C-phycocyanin yield in a laboratory culture of *Nostoc* sp. (Silva et al., 1989). In *Anabaena* sp., the yield of C-phycocyanin was 8.3% of cell dry weight (Rodriguez et al., 1989). A screening process indicated that the content of C-phycocyanin was 17% of dry weight in some strains of *Anabaena* and *Nostoc* and C-phycoerythrin was 10% of dry weight in some *Nostoc* sp. (Moreno et al., 1995).

Mass production of such useful strains to obtain valuable products is essential. The development of microalgal production systems requires the solution of many physiological and bioengineering problems. The use of reliable culture medium which can sustain good growth of the selected strain is a necessary pre-requisite. The literature contains many media suitable for cultivating pure species of cyanobacteria. However, they are not optimized for the production of mass cultures. Further, no attempt was made to commercially cultivate and exploit marine cyanobacteria for obtaining biomass and products like natural colourants.

Optimization of media and culture conditions to obtain maximum yield require a large number of experiments. In the classical method, a single factor is varied, while others are kept constant. In the experimental designs method, several factors are simultaneously varied in a planned manner. This is a fast, efficient and precise method for estimating the effects of several factors with minimum experiments (Ross, 1996) and a more balanced alternative to the one-factor-at-a-time approach (Strobel and Sullivan, 1999). This paper deals with the employment of the mathematical tool, Taguchi based design of experiments in L8 Orthogonal Array (OA) to estimate the significance and to choose suitable level of the media chemicals and the amount of inoculum required for mass cultivation and natural colourant production from marine cyanobacteria. Validity of the clues tested by this mathematical experimentation was tested by confirmation experiments.

Materials and Methods

Organism and culturing

Organisms employed in the study, *Phormidium valderianum* BDU 30501 (phycocyanin producer) and *Phormidium tenue* BDU 46241 (phycoerythrin producer), were marine isolates of our laboratory. They were maintained in ASN III synthetic marine medium (Rippka et al., 1979) in controlled conditions with light intensity of 60 μmol $m^{-2}s^{-2}$ and a temperature of 27±2℃. *P. tenue* cultures were scaled up in Haffkine's flasks and *P. valderianum* cultures were raised in mass scale as mentioned earlier (Sekar and Subramanian, 1998). Experiments with *P. tenue* were carried out in 100 mL cultures grown in 250 mL conical flasks provided with 60 μmol $m^{-2}s^{-2}$ of light, temperature of 27±2℃ and 14 h light and 10 h dark period. Experiments with *P. valderianum* were carried out in open outdoor cultures grown in shade using white plastic cups with 500 mL of medium. The conditions prevailed at the study site were 75 μmol $m^{-2}s^{-2}$ of light, atmospheric temperature of 30±2℃ and light dark cycle of approximately 14 : 10 hours respectively. In culturing both organisms, the maintenance of unialgalness was ensured by routine microscopic examinations.

Estimations

The cultures were harvested after 7 days of growth by filtering the culture through 400 mesh nylon filter and draining the medium. To estimate the dry weight, the filtered mass was quickly washed thrice with tap water and finally rinsed with distilled water. It was then dried in oven at 60℃ for 18 hours and weighed. Both phycocyanin and phycoerythrin were extracted from the wet biomass by the large scale method (Padgett and Krogman, 1987) and the resultant dry pigment was weighed.

Experimental design

Experiments in both cyanobacteria were carried out using unsterilised sea water as the basal medium and further added with either normal or higher level of the 6 variables listed in Table 1 as per the plan of experimental design matrix in Table 2. The normal level of the chemical factors (except $NaHCO_3$) was equivalent to their level used in the natural sea water medium, MN (Rippka et al., 1979). $NaHCO_3$ at a concentration of 0.741 g/L was added as the normal level. In higher level, 4 to 6 fold increase was made according to the factor (Table 1). The experiments were conducted in two sets as indicated in Table 3. In each case, columns 3, 5 and 6 were confounded by factors A X B, A X D and B X D respectively. In order to study the influence of all the 6 factors chosen,

Table 1. The concentration of the chemicals and the amount of inoculum used in levels 1 and 2.

Factors	Level 1 (g/L) (Normal)	Level 2 (g/L) (High)	No. of times of increase (Level 2/ Level 1)
$NaHCO_3$	0.741	3.336	4.5
$NaNO_3$	0.750	3.000	4.0
K_2HPO_4	0.040	0.240	6.0
$MgSO_4$	0.038	0.228	6.0
FAC[a]	0.003	0.012	4.0
Inoculum size			
P. valderianum	0.500[b]	2.500[b]	5.0
P. tenue	0.200[b]	1.000[b]	5.0

[a] Ferric Ammonium Citrate.
[b] in fresh weight.

Table 2. The pattern of the two level L8 orthogonal array employed in the study.

	Factors								
	A	B	C	D	E	F	e[a]		
	Column No.[b]								
Trial No.	1	2	3	4	5	6	7	Y_1[c]	Y_2[d]
1	1	1	1	1	1	1	1		
2	1	1	1	2	2	2	2		
3	1	2	2	1	1	2	2		
4	1	2	2	2	2	1	1		
5	2	1	2	1	2	1	2		
6	2	1	2	2	1	2	1		
7	2	2	1	1	2	2	1		
8	2	2	1	2	1	1	2		

[a] error column.
[b] Columns 1 to 6 are meant for assigning factors.
[c] Biomass on dry weight.
[d] Pigment yield on dry weight.
1 and 2 represent normal and high levels of media chemicals respectively.

Table 3. The pattern of factors assigned to each column in the orthogonal array.

Factors	Experiment 1	Experiment 2
$NaHCO_3$	1	3[a]
$NaNO_3$	2	5[a]
$MgSO_4$	3[a]	1
K_2HPO_4	4	6[a]
FAC	5[a]	2
Inoculum size	6[a]	4

[a] columns where confounding occurs.
Numbers denote column no.

the factors assigned to columns 1, 2 and 4 in experiment 1 were placed in columns 3, 5, and 6 in experiment 2 to facilitate the assignment of other factors in columns 1, 2, and 4 in experiment 2.

Calculations

They were based on Taguchi L8 OA method (Ross, 1996). For example, in experiment 1, factors A, B, C, D, E & F were assigned to columns 1, 2, 3, 4, 5, and 6 respectively. As indicated in Table 2, eight trials were performed. In each trial, the level of each parameter was as per the level indications against each column. The seventh column was not assigned with any parameter and used for calculating error variation (SS_e). Analysis of Variation (ANOVA) was calculated starting with the calculation of sums of squares (SS) for each column. For example, SS_A, the sums of squares of factor A (column 1) is,

$$SS_A = \frac{A_1 - A_2^2}{N}$$

A_1 and A_2 were the sums of the data associated respectively with the first and second levels of the factor A. The SS values from all the 7 columns were plotted into ANOVA table. It consisted of column number, SS of each column, degrees of freedom (v) of each column, mean sums of squares (V = SS of each column divided by the degrees of freedom), F values (V divided by the error sums of squares, SS_e) and the confidence level (P) which was obtained by referring to the standard critical values of the F distribution with numerator v value of 1 and denominator v value of 6. The optimum level of each factor was selected based on the sum of level 1 and sum of level 2 in each column. The level that showed higher sum value is the most suitable level. The same method was followed for calculating the data obtained in experiment 2 also. Experiment 1 was used to study the influence of $NaHCO_3$, $NaNO_3$, and K_2HPO_4 and experiment 2 was used to study the influence of the factors, $MgSO_4$, ferric ammonium citrate and size of the inoculum. Finally, the results obtained in both experiments were combined. This analysis was carried out with both cyanobacteria involved in the study.

Results

Analysis of the results obtained with *P. tenue* (Table 4) showed the following trends. Among the 6 parameters studied, $NaHCO_3$, K_2HPO_4, $MgSO_4$ and inoculum size showed higher levels of significance (0.005>P) over the yield of biomass (Table 5). All

Table 4. Yield of biomass and phycoerythrin from *P. tenue* BDU 46241 in eight trials conducted as in Table 2 with experimental set up 1 and 2 of Table 3.

Trial No	Experiment 1		Experiment 2	
	Biomass (mg dry weight / L)	Phycoerythrin (mg dry weight / L)	Biomass (mg dry weight/L)	Phycoerythrin (mg dry weight/L)
1	18.60	1.50	74.15	0.95
2	79.00	7.50	66.15	6.40
3	30.10	2.70	85.95	4.80
4	94.95	9.90	31.80	0.10
5	20.90	0.90	23.85	0.95
6	45.55	5.10	110.65	7.90
7	31.25	0.50	18.30	5.00
8	46.35	3.75	64.25	1.30

Table 5. Confidence level of factors and their suitable level for obtaining maximum yield of biomass and phycoerythrin from *P. tenue* BDU 46241.

Factors	Biomass		Phycoerythrin	
	Confidence level	Concentration in g/L (level)	Confidence level	Concentration in g/L (level)
$NaHCO_3$	0.005>P>0.002	0.741 (n)	0.005>P>0.002	0.741 (n)
$NaNO_3$	0.01>P>0.05	3.000 (h)	P>0.05	3.000 (h)
K_2HPO_4	P>0.001	0.240 (h)	P>0.001	0.240 (h)
$MgSO_4$	P>0.001	0.038 (n)	0.005>P>0.002	0.228 (n)
FAC[b]	0.01>P>0.05	0.003 (n)	P>0.001	0.003 (n)
Inoculum	P>0.001	1.000[a] (h)	0.002>P>0.001	1.000[a] (h)

[a] in fresh weight
[b] Ferric Ammonium Citrate
n = normal level
h = high level

Table 6. Yield of biomass and phycocyanin from *P. valderianum* BDU 30501 in eight trials conducted as in Table 2 with experimental set up 1 and 2 of Table 3.

Trial No	Experiment 1		Experiment 2	
	Biomass (mg dry weight/L)	Phycoerythrin (mg dry weight/L)	Biomass (mg dry weight/L)	Phycoerythrin (mg dry weight/L)
1	207.90	14.70	378.90	24.40
2	512.95	42.75	521.85	47.15
3	365.25	46.25	527.20	20.50
4	453.60	26.90	448.50	25.15
5	304.35	26.55	357.05	24.05
6	674.10	50.22	731.50	59.30
7	506.60	51.66	502.90	30.60
8	497.20	22.85	263.25	37.95

Table 7. Confidence level of factors and their suitable level for obtaining maximum yield of biomass and phycocyanin from *P. valderianum* BDU 30501.

Factors	Biomass		Phycoerythrin	
	Confidence level	Concentration in g/L (level)	Confidence level	Concentration in g/L (level)
$NaHCO_3$	0.1>P>0.005	3.336 (h)	0.02>P>0.01	3.336 (h)
$NaNO_3$	P>0.05	3.000 (h)	0.01>P>0.05	3.000 (h)
K_2HPO_4	0.01>P>0.005	0.240 (h)	0.05>P>0.02	0.240 (h)
$MgSO_4$	P<0.05	0.038 (n)	0.05>P>0.02	0.228 (h)
FAC[b]	P<0.05	0.003 (n)	0.02>P>0.01	0.003 (n)
Inoculum	P<0.5	2.500[a] (h)	P>0.001	2.500[a] (h)

[a] in fresh weight
[b] Ferric Ammonium Citrate
n = normal level
h = high level

parameters, except $NaNO_3$, showed a significant impact (0.005>P) over the yield of phycoerythrin (Table 5). In terms of suitable concentration in the two levels provided, $NaNO_3$, K_2HPO_4 and inoculum size were required at their higher level while $NaHCO_3$, $MgSO_4$ and ferric ammonium citrate were required at the normal level for obtaining maximum biomass (Table 5). For getting maximum yield of the pigment, the requirement was similar to biomass except that $MgSO_4$ was required in its higher level (Table 5).

Results of *P. valderianum* (Table 6) upon mathematical analysis showed that K_2HPO_4 alone have significance (0.01>P>0.005) in determining the biomass yield of this organism (Table 7). However, for phycocyanin production, other than $NaNO_3$, all other parameters showed high level of significance (Table 7). The suitable concentration in the two levels provided in the medium that can support maximum biomass was the higher level in the case of $NaHCO_3$, $NaNO_3$, K_2HPO_4 and inoculum size while it was normal level in the case of $MgSO_4$ and ferric ammonium citrate (Table 7). When compared to *P. tenue*, this organism differed only in its requirement for $NaHCO_3$, as the normal concentration was sufficient for biomass production in *P. tenue*. However, for phycocyanin production when compared to biomass production, $MgSO_4$ was required in its higher level (Table 7) which was as seen in *P. tenue*.

In general, in both organisms, K_2HPO_4, $NaNO_3$, and inoculum requirement were high and iron requirement was normal for both biomass production and pigment production. Moreover, in both organisms, higher level of $MgSO_4$ was required ad-

Table 8. Confirmation results indicating the increase in biomass and phycoerythrin (PE) yield of *P. tenue* BDU 46241 in optimized media designed individually for maximum yield of biomass and pigment.

	Control medium[a]	Optimaized medium	% Of increase
Biomass (g/L)	0.186	1.107	495
PE (mg/L)	15.000	99.000	560
Biomass of PE samples (g/L)	0.186	0.946	408
PE content mg/g of dry biomass	80.645	104.651	30

[a] Where all factors were at their normal level.

Table 9. Confirmation results indicating the increase in biomass and phycocyanin (PC) yield of *P. valderianum* BDU 30501 in the optimized media designed individually for maximum yield of biomass and pigment.

	Control medium[a]	Optimaized medium	% Of increase
Biomass (g/L)	0.416	1.348	224
PE (mg/L)	29.400	103.200	251
Biomass of PE samples (g/L)	0.416	1.013	143
PE content mg/g of dry biomass	70.673	101.876	44

[a] Where all factors were at their normal level.

ditionally for enhanced pigment production.

Confirmation experiments carried out using unsterilised sea water as the basal medium with the chosen optimal level of the six parameters tested for biomass production and pigment yield in both cyanobacteria under study (Tables 8 and 9). It clearly indicated that the optimum level of 6 parameters increased the biomass of *P. tenue* by 495% (Table 8) when compared to the control cultures where the level of all the 6 factors were normal. Interestingly, the optimum level of factors chosen for phycoerythrin production also increased the biomass to 408% and the yield of the pigment increased to 560% when compared to the control cultures. It is clear that the medium that supported maximum phycoerythrin production in turn supported an increase in biomass which is reflected as an increase in phycoerythrin yield. Although the biomass obtained in the medium optimized for phycoerythrin yield was less (408%) when compared to the biomass obtained in the medium optimized for biomass production (495%), the net yield of phycoerythrin per unit biomass has increased by 30% (Table 8).

Confirmation experiments with *P. valderianum* also showed similar patterns of increase as in *P. tenue* (Table 9). The optimum level of 6 parameters increased the biomass of *P. valderianum* to 224%. The optimum level of parameters chosen for phycocyanin production also increased the biomass by 143% and phycocyanin yield to 251% compared to control cultures. As in the case of *P. tenue*, in this cyanobacterium also, the biomass obtained in the medium optimized for phycocyanin yield (143%) was less when compared to the biomass obtained in the medium optimized for biomass production (224%). But, the net phycocyanin yield per unit of biomass increased by 44% (Table 9). It is evident that the medium optimized for phycocyanin production not only increased the yield of biomass but also selectively increased the content of phycocyanin in both cyanobacteria.

Discussion

Bioprocesses are influenced by various factors and the influence becomes more complicated in an industrial dimension. This work indicates that any such process can be suitably optimized by mathematical approaches and design of experiments (DOE) facilitate this. Earlier study indicated that other than the natural components of sea water, amendments with $NaHCO_3$, $NaNO_3$, K_2HPO_4, $MgSO_4$ and ferric ammonium citrate was required for the mass cultivation of the organism used in this study i.e., *P. valderianum* BDU 30501 (Sekar and Subramanian, 1998). The amount of inoculum is a key factor in mass-scale cultivation and its optimum level provides a competitive ability over the possible growth of contaminants (Becker, 1994). So, the study encompasses these 6 factors vitally linked with mass cultivation.

The factors were tested in two levels and are denoted as normal and higher level. The normal level of $NaNO_3$, $MgSO_4$ and ferric ammonium citrate are equivalent to their level in the natural sea water medium, MN (Rippka et al., 1979) while the normal level of K_2HPO_4 is half the level in MN medium as it is known to promote salt precipitation (Tredici et al., 1986). As $NaHCO_3$ is essential to meet carbon requirement of mass cultures, an amount of 0.741 g/L was provided as the normal level. The initial inoculum of *P. tenue* was 0.2 g/L and *P. valderianum* was 0.5 g/L. The elevated level of initial inoculum in *P. valderianum* was to meet the open field conditions in which it was experimented. In higher level, all factors were increased by 4 to 6 times. The increase in each parameter was determined with a view to avoid the formation of precipitates in the medium that may lead to toxicity. The pH of the media employed in the study ranged from 7.5 to 8.5.

Further, this work was carried out using unsterile medium as it is suitable and commonly employed in open-scale mass cultivation of microalgae. Experiments with *P. tenue* was performed in laboratory conditions as it is a slow growing form while *P. valderianum* was grown in field conditions as it is adaptive and its mass cultivation was performed earlier (Sekar and Subramanian, 1998).

In *P. tenue,* biomass production depends on most of the factors tested (4 out of 6 factors) and this may be attributed to the difficulty in mass cultivating this organism. Nitrate is expected as the primary element in determining the yield of phycobiliproteins as phycocyanin is a known N-reserve (Boussiba and Richmond, 1980). However, it was not demonstrated with phycoerythrin and the impact of all factors were significant rather than nitrate over the yield of phycoerythrin. These observations further indicate that the performance of the mass cultivation and pigment production depends on monitoring and maintaining the required level of the significant factors. As many factors operate significantly, it is relatively difficult to mass cultivate this organism. It is interesting that higher level of $MgSO_4$ was required for enhancing phycoerythrin production while all other factors were required at the same level required for biomass production. $MgSO_4$ apart from increasing the biomass yield, it selectively elevated the content of phycoerythrin in cells. Mg ion is a component of chlorophyll and essential for the activity of kinases in general (Stryer, 1995), as well as involved in the aggregation of ribosomes into functional units (Becker, 1994). The sulfur ions are the components of cystein and methionine residues that are present in these phycobiliproteins. Specifically, cysteins are the sites of attachment of bilin chromophores (Betz, 1997). Thus, $MgSO_4$ hold greater importance as a nutrient related to the production of phycobiliproteins.

P. valderianum BDU 30501 is highly adaptive and capable of growing in open scale cultivation system (Sekar and Subramanian, 1998). Among the factors, K_2HPO_4 alone was the determinant of biomass yield. This ability may be attributed to its easiness for mass cultivation. It is also thus essential to monitor and maintain the level of P. Media factors other than $MgSO_4$ and iron were required at higher level for biomass production. As seen in *P. tenue,* this organism also required higher levels of $MgSO_4$ for phycocyanin production.

Conclusively, factors such as $NaHCO_3$, K_2HPO_4, $MgSO_4$ and size of the inoculum are critical over the yield of biomass and additionally, ferric ammonium citrate over phycoerythrin production in *P. tenue.* However, in *P. valderianum,* K_2HPO_4 alone showed significant impact on biomass production, while all other factors except $NaNO_3$ were significant for phycocyanin production. In general, certain features are observed common in both cyanobacteria. As the initial inoculum determines competitive growth, its higher level is always preferred. Ferric-iron was always required in its lower level (3 mg/L) as higher level can lead to heavy metal toxicity. For biomass production, N and P sources were required at higher level as they are the major nutrients supporting growth (Becker, 1994). Furthermore, the number of factors that operate significantly in pigment production was more than in biomass production and their nature also differ. It implies that product oriented optimization is essential and simply mass cultivating the organism is insufficient to get maximum product.

Confirmation experiments carried out based on the chosen optimum level of the 6 parameters amply proves that the clues obtained are acceptable. In both organisms, there was a manifold increase in biomass yield. Interestingly, in the medium optimized for pigment production (it differs only in its requirement for $MgSO_4$) for both cyanobacteria, the biomass increase was not as high as in the previous case. However, there was a net increase in pigment yield and the pigment content increased significantly (30% in *P. tenue* and 44% in *P. valderianum*). The content of phycoerythrin increased from 8% to 10.5% on dry weight basis in *P. tenue* and phycocyanin from 7% to 10.2% in *P. valderianum.* This clearly demonstrates that $MgSO_4$ is a critical factor in determining the yield of phycobiliproteins. Moreover, although the biomass increase is less, the pigment content is more, which will lead to an increase in process productivity and hence the cost of industrial extraction process will get reduced.

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