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Research Article

High Nitrate, Phosphate and Alkaline pH Enhances Total Carotenoids Yield in *Leptolyngbya* SP. BTA 287

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ABSTRACT

Carotenoids are commercially used as natural colourant, antioxidant and as a vitamin A precursors. In the present investigation, critical factors for high total carotenoid yield from the effect of stress created by high pH, excess $NaNO_3$ and K_2HPO_4 were investigated by using single factor experiment and later optimization were carried out by using Box–Behnken experimental design from *Leptolyngbya* sp. BTA 287 belonging to Indo-Burma Biodiversity hot spot. This strain was found to produce maximum total carotenoids at pH 9, $NaNO_3$ concentration of 7.50 mg/ml and K_2HPO_4 concentration at 0.20 mg ml^{-1} in BG-11 medium, yielding highest total carotenoids of 40.88 µg/mg dry wt. with 62% more from the control condition, inferring that, carotenogenesis were induced greater than reduced nutrient. The ANOVA showed a significant R^2 value (0.9980), model *F*-value (380.07) and probability (0.0001), with insignificant lack of fit. The results suggested that there was no significant interactions between pH, nitrates and phosphate at alkaliphilic excess nutrients for the production of high yield of carotenoids and appears more to be additive by each factors. Ability to endure nutrient rich and alkaline medium and produce total carotenoid by *Leptolyngbya* sp. may be applicable for sewage water carotenoid production.

Keywords: Box–Behnken design, Carotenoids, cyanobacteria, *Leptolyngbya* sp., nitrate, phosphate

1. INTRODUCTION

Carotenoids are tetraterpenoids that has 40 carbon atoms chains which are obtained in the outer membrane and in the thylakoids [1, 2]. Carotenoids are the accessory photosynthetic pigment of cyanobacteria [3]. In cyanobacteria, β-carotene predominates in the thylakoid membranes whereas xanthophyll lies in inner and outer membrane [4, 5, 6]. Carotenoids in cyanobacteria

have two major functions: they are light harvesting for photosynthesis and prevention from photo oxidative stress [7]. Carotenoids are utilized as natural colorants and value addition for juices as well as in animal feeds like poultry and fish [8]. Carotenoids applications have also reached its arm in cosmetic industries [9]. The carotenoids have nutritional and therapeutic properties by acting as vitamin-A precursors [10, 11]. Carotenoids have also intrinsic anti-inflammatory and anti-cancer properties [12].

Genus *Leptolyngbya* of order oscillatoriales are non-heterocystous cyanobacteria with thin filamentous cell of 0.5 to 3.5 μm wide with parietal thylakoids [13]. It was originally designated as "LPP−group B" by bacteriologists [14] and were recently supported and justified by molecular analyses [15]. They are cosmopolitan in distribution and flourished even in extreme environments like halophilous biotopes, thermal as well as mineral springs [16, 17]. They are commonly found in subaerial rocky sites or walls and soils and also dwell in periphyton and metaphyton of freshwater [18]. Some non-heterocystous cyanobacteria that were reported for carotenoid production were *Lyngbya perelegans*, *Phormidium tenue*, *Limnothrix vacuolifera* and *Plectonema notatum* from the north east India falling under Indo-Burma Biodiversity hot spot [19]. In cyanobacteria, non-heterocystous unicellular forms like *Synechocystis* sp. PCC cultivated under sub-optimal salt concentrations were reported to increased carotenoid yield [20]. Lowering of the nitrogen concentration in half strength of BG-11 medium also increases carotenoid yield (85.20 μg/ml) of *Phormidium* sp. which is a non heterocystous filamentous cyanobacteria [21]. Contrary to this, nitrogen starvation have been reported to decreased β-carotene production in the case of *Phormidium laminosum* [22]. Other factors like pH and phosphate ion concentration affect nitrate uptake [23, 24] which also influences carotenogenesis. Till far, effect of carotenoid production under stress created by excess nutrients and high alkalinity as well as optimum condition for carotenoid production of *Leptolyngbya* using Box-Benhken experimental design under two or more combined effects has not been investigated yet.

In the present investigation, our objective is to find the critical factor and optimal condition for total carotenoids production under the combined effects of pH and nutrient on *Leptolyngbya* sp. BTA 287 at high level of pH, K_2HPO_4 and $NaNO_3$ concentration by using Box-Benhken experimental design.

2. METERIALS AND METHODS

2.1. Organisms

The cyanobacteria used for the investigations were isolated by streak plate method [14] by using BG-11 medium [21] from rice field, West Agartala (latitude of N23°51'32.6" and longitude of E091°14'55.6") that lies under North eastern region of India of Indo-Burma Biodiversity Hotspot and deposited to Freshwater Cyanobacterial and Microalgal Repository at IBSD, Imphal, Manipur, India, a national facility created by the Department of Biotechnology, Govt. of India in 2009 with ref. no. BT/PR 11323/PBD/26/171/2008 having culture accession no.: **BTA 287**. The cyanobacteria was identified as *Leptolyngbya* sp. (GenBank accession no.: **KF953498**) by 16S rDNA analysis as well as morphologically by using Komarek and Anagnostidis [11] key book.

2.2. Medium and growth conditions

BG-11 medium [25] with sodium nitrate (+N) broth were prepared by addition of the macronutrients and micronutrients and the volume was made upto 100 ml with distilled water in 250 ml conical flask and sterilized in an autoclave (Calton, NSW-227) at 15 psi for 15 mins at 121ºC. All the single factor experiment as well as optimization experiments were grown in 54-67 μmol photon/m/s of light intensity with 14/10 h light and dark periods at 28±2°C for 15 days.

2.3. Single factor experiment

The single factor experiment for production of total carotenoids was performed with the analysis of the effect of three factors (pH, different concentrations of $NaNO_3$ and K_2HPO_4).

2.3.1. Effect of pH on total carotenoids

100 ml of (+N) BG-11 medium of eight different pH i.e. 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0 were prepared by using bench top pH metre (Thermofisher, USA) with adjustment of acidic pH by 1N HCl and alkaline pH by 1N NaOH in 150 ml conical flask and took pH 7.0 as control.

2.3.2. Effect of $NaNO_3$ concentration on total carotenoids

Six (06) different concentrations of $NaNO_3$ i.e. N/2 (0.75 mg/ml), N (1.50 mg/ml), 2N (3.00 mg /ml), 3N (4.50 mg/ml), 4N (6.00 mg/ml) and 5N (7.50 mg/ml) of (+N) BG-11 medium with pH 7 of 100 ml were prepared in 150 ml conical flask.

2.3.3. Effect of K_2HPO_4 concentration on total carotenoids

Six (06) different concentrations of K_2HPO_4 i.e. N/2 (0.02 mg/ml), N (0.04 mg/ml), 2N (0.08 mg/ml), 3N (0.12 mg/ml), 4N (0.16 mg/ml) and 5N (0.20 mg/ml) of (+N) BG-11 medium with pH 7 of 100 ml were prepared in 150 ml conical flask.

2.4. Determination of dried biomass

Wet biomass were washed with distilled water and harvested by centrifugation in a a centrifuge (Eppendorf) at 5000 X*g* for 10 mins and the pellets were transferred to a clean dried glass petridish. The initial weight of the pellet was taken as fresh weight after taking the weight of the glass petridish. The pellets along with petridish were kept inside hot air oven (NSW, India) and dried at 75ºC for 4 to 6 h. The biomass were cooled and weighed until constant weight was obtained. The difference between the initial and final weight were taken as dry weight biomass.

2.5. Extraction of total carotenoids [26].

10 ml of homogenized algal suspension from the exponential phase were centrifuged by refrigerated centrifuge (5430R, eppendorf, Germany) at 5000 X*g* for 10 mins and the supernatant was discarded. The pellets were dried as mentioned above. 5 mg of the dried biomass was subjected to 3 ml of 85% acetone and subjected to repeated freezing and thawing at 4ºC until the dried biomass becomes colourless. The volumes of the extract were measured and the final volume was made up to 10 ml with 85% acetone.

2.6. Determination of total carotenoids

Carotenoids were estimated by optical density (O.D.) at 450 nm using 85% acetone as blank using spectrophotometer (UV Vis-1800, Shimadzu, Japan) and expressed the total amount of carotenoids in µg/mg dry wt. The total soluble carotenoids were calculated using the formula below:

C= (D x V x f) x 10 /2500, where, D= O.D. at 450 nm; V= Volume of the extract; f= Dilution factor; 2500=average extinction co-efficient of pigment

2.7. Box-Behnken design

The critical factors were identified through single factor experiment and a Box–Behnken design for independent variables was used for optimization. A total of 3 independent variables *viz*. pH (X_1), $NaNO_3$ (X_2) and K_2HPO_4 (X_3) were added at two levels: low (-1) and high (+1). The low and high levels of each factors were taken as pH (8.5 and 9.5), $NaNO_3$ (6.0 mg/ml and 9.0 mg/ml) and K_2HPO_4 (0.16 and 0.24 mg/ml). The Design expert software was used in the experimental design and data analysis. A second-order polynomial equation with four independent variables is designed as:

$$Y= x_0+\Sigma a_i x_i+ \Sigma a_{ii} x_i^2+ \Sigma \Sigma a_{ij} x_i x_j$$

where Y stands for total carotenoids yield, X_0 denotes the model intercept, X_1, X_2, X_3 are the levels of pH, $NaNO_3$ concentration and K_2HPO_4, respectively; $a_{i\ldots\ldots\ldots\ldots\ldots a_{ij}}$ represent regression coefficients calculated from the experimental data. A multiple regression analysis of the data was carried out to define the response in terms of the independent variables. Response surface graphs were obtained to understand the effect of the variables, individually and in combination and to determine the optimum levels for maximum total carotenoids production. All trials were performed

in triplicate, and the average total carotenoids were used as response *Y*. Finally, the model validation was carried out.

2.8. Statistical analysis

All trials were carried out in triplicate and the averages of carotenoids yields were taken as responsive value. One way analysis of variance (ANOVA) and Tukey's tests were used to determine the significant difference in carotenoids yield extracted from all the studied strains under different conditions by SPSS v19 (IBM, USA). A second-order polynomial regression equation was established on the basis of analysis of Box–Behnken experimental data taking 17 runs, and the optimal conditions for carotenoids production were found using the Design Expert version 9 (Stat-Ease, Inc., USA) software.

3. RESULTS

3.1. Effect of pH on total carotenoids

The effect of different pH ranging from acidic to alkaline on the *Leptolyngbya* sp. BTA 287 is shown in Table 1. It produced highest carotenoids in pH 9.0 (p<0.01) i.e. (53.86±2.48 µg/mg). Total carotenoid production decreased significantly from pH 6 to 7.5 and significantly rises at pH 9.

pH	Total carotenoids (µg/mg dry wt.)
6.0	32.62**
6.5	31.60**
7.0	25.38
7.5	31.62**
8.0	33.12**
8.5	16.16**
9.0	53.36**

Probability of error is represented by asterisk (*) for * p<0.05, ** p<0.01 and 'ns' for non-significant respectively superscripted to the data in tables

Table: 1. Effect of pH on total carotenoids yield (µg/mg dry wt.) of *Leptolyngbya* sp. BTA 287

3.2. Effect of nitrate on total carotenoids

The effect of different concentration of sodium nitrate on total carotenoid production is shown in **Table 2.** *Leptolyngbya* sp. 287 produced highest carotenoids in 7.50 mg/ml (5N) i.e. 31.02±1.07 µg/mg (p<0.01). Total carotenoid production increased significantly from 1.50 mg/ml to 4.50 mg/ml and slightly decreased at 6.00 mg/ml.

Concentration of $NaNO_3$ (mg/ml)	Total carotenoids (µg/mg dry wt.)
0.75	26.89**
1.50	25.31
3.00	28.08**
4.50	30.52**
6.00	30.52**
7.50	31.02**

Probability of error is represented by asterisk (*) for * p<0.05, ** p<0.01 and 'ns' for non-significant respectively superscripted to the data in tables

Table: 2. Effect of $NaNO_3$ (mg/ml) on total carotenoids yield (µg/mg dry wt.) of *Leptolyngbya* sp. BTA 287

3.3. Effect of phosphate on total carotenoids

Production of total carotenoids was found to be (Table 3) highest at 0.20 mg/ml (5N) i.e. 38.26±0.01 µg/mg (p<0.01). Total carotenoids production increased significantly from 0.04 mg/ml and 0.12 mg/ml and slightly decreased at 0.16 mg/ml and later significantly increased at 0.20 mg/ml.

Concentration of K_2HPO_4 (mg/ml)	Total carotenoids (µg/mg dry wt.)
0.02	26.51*
0.04	25.28
0.08	29.56*
0.12	36.12**
0.16	34.42**
0.20	38.26**

Probability of error is represented by asterisk (*) for * p<0.05, ** p<0.01 and 'ns' for non-significant respectively superscripted to the data in tables

Table: 3. Effect of K_2HPO_4 (mg/ml) on total carotenoids yield (µg/mg dry wt.) of *Leptolyngbya* sp. BTA 287

3.4. Analysis of Box-Behnken experiment

Optimization of total carotenoids using the Box-Behnken design was carried out with the

significant critical levels obtained from the single factor experiment. The three levels were designated as (X_1), (X_2) and (X_3) for pH, sodium nitrate and potassium phosphate concentrations. The results of the 17 experiments conducted using the Box- Behnken design is presented in Table-4.

Run no.	pH (X_1)	$NaNO_3$ (mg/ml) (X_2)	K_2HPO_4 (mg/ml) (X_3)	Total Carotenoids (µg/mg dry wt.) (Y)
1	9	9.0	0.16	36.73
2	9	9.0	0.24	34.33
3	8.5	7.5	0.24	24.86
4	9.5	9.0	0.20	32.60
5	9	7.5	0.20	40.88
6	9	7.5	0.20	40.51
7	9	7.5	0.20	39.89
8	8.5	7.5	0.16	27.20
9	9	7.5	0.20	40.10
10	9.5	6.0	0.20	34.88
11	8.5	9.0	0.20	25.61
12	9	6.0	0.16	39.00
13	9.5	7.5	0.24	31.79
14	9.5	7.5	0.16	34.23
15	8.5	6.0	0.2	27.89
16	9	6.0	0.24	36.60
17	9	7.5	0.20	39.65

Table: 4. Observed values of Total carotenoids yield obtained by Box–Behnken experiment

The results obtained were submitted to ANOVA using the software of Design Expert version 9 (Stat-Ease, Inc.), for describing a polynomial model correlation between carotenoids yield and the three variables. The equation was as follows:

$Y=40.21+3.49X_1-1.14X_2-1.20X_3-2.25X_1X_2-0.025X_1X_3-10.63X_2X_3-8.55X_1^2-1.41X_2^2-2.13\ X_3^2$

with $R^2= 0.9980$

where, Y is the total carotenoid yield whereas X_1 is pH, X_2 is $NaNO_3$ and X_3 is K_2HPO_4. The established model was found to be significant (P>0.0001) by applying ANOVA for the above mode and hence, it could be used to predict the yield of carotenoids extraction. The carotenoids yields predicted by the above regression were close to the observed ones (R^2= 0.9980). On the basis of the F-test, pH, sodium nitrate and potassium phosphate concentrations had significant quadratic effect on carotenoids yield.

Source	Sum of Squares	d.f.	Mean Square	F value	*P*-value *P* > F
Model	472.57	9	52.51	380.07	< 0.0001
X_1-pH	97.58	1	97.58	706.32	< 0.0001
X_2-$NaNO_3$	10.35	1	10.35	74.93	< 0.0001
X_3-K_2HPO_4	11.47	1	11.47	83.04	< 0.0001
X_1X_2	1.45	1	1.45	1.82	1.0000
X_1X_3	3.79	1	3.79	0.018	0.8968
X_2X_3	0.00	1	0.00	0.00	1.0000
X_1^2	308.02	1	308.02	2229.53	< 0.0001
X_2^2	8.35	1	8.35	60.42	0.0001
X_3^2	19.16	1	19.16	138.66	< 0.0001
Residual	0.97	7	0.14		
Lack of Fit	0.07	3	8.59	1.62	1.0000
Corrected Total	473.53	16			

Table: 5. Analysis of variance (ANOVA) for the regression

The above optimal parameters for carotenoids production were evaluated by non-linear optimization algorithm and a maximal carotenoids yield of 40.88 µg/mg could be achieved at pH 9, $NaNO_3$concentration of 7.50 mg/ml and K_2HPO_4 concentration at 0.20 mg/ml.

It showed 62% increase of total carotenoid production from the control condition. The effect of pH and sodium nitrate on carotenoids yields was shown in Fig.1. The pH increased up to about 9.0 and sodium nitrate concentration

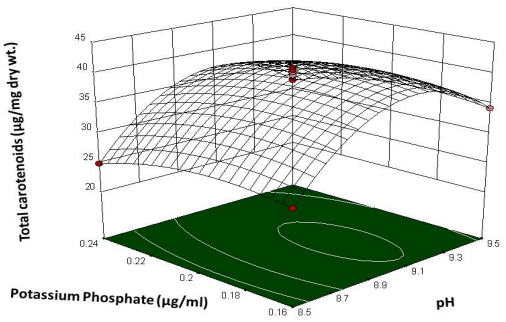

Fig: 1. Effects of Sodium nitrate concentration and pH on total carotenoids yields

was found to be the critical factor for improving carotenoids yield. It could be observed that the optimal pH and sodium nitrate for carotenoids extraction were obtained at pH 9.0 and 7.80 mg /ml. The effect of pH and potassium phosphate concentration on carotenoids yield is illustrated in **Fig.2.** When the potassium phosphate concentration is about 0.20 mg/ml, potassium phosphate becomes the critical factor for improving carotenoids yield. It could be seen from Fig. 2 that the optimal pH and potassium phosphate concentration for carotenoids extraction are about pH 9.0 and 0.20 mg/ml.

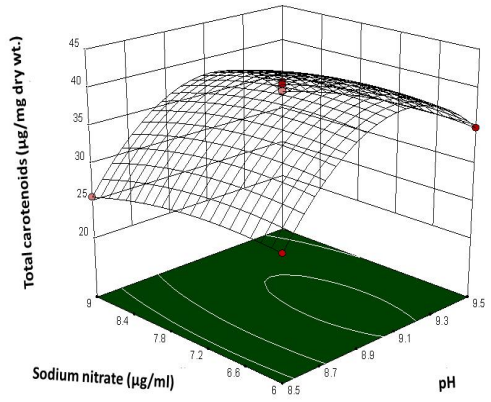

Fig: 2. Effects of Potassium phosphate and pH on total carotenoids yields

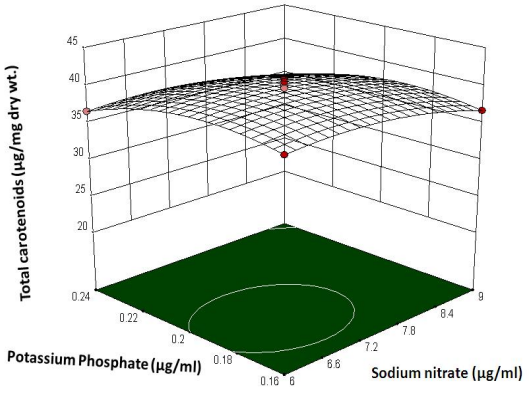

Fig: 3. Effects of Potassium phosphate and Sodium nitrate on total carotenoids yields

The effect of sodium nitrate and potassium phosphate concentration on carotenoids yield is shown in Fig. 3. The potassium phosphate has a significant quadratic effect on carotenoids yield. The yield increased when the potassium phosphate increased from 0.16 to 0.20 mg/ml and continue to increase significantly when the concentration was higher than 0.20 mg/ml. The combined effects of the three factors were foundnot to be significant between the pH, $NaNO_3$ and K_2HPO_4 (Table-5). So, the high yield may be due to collective contribution by each factor.

3.5. Validation of experimental model

Within the scope of the variables investigated in Box–Behnken design, additional experiments with different conditions for total carotenoids extraction were carried out to assess the validity
of the model. Arrangement and result of the confirmatory trials were made for the said optimal condition. It is demonstrated that there is a high fit degree between the values observed in experiment and the values predicted. Our observed yield of total carotenoids extraction (40.88 µg/ml) under the optimal conditions is higher than under all the other conditions, which proved to be necessary to optimize carotenoid yield.

4. DISCUSSION

Single factor experiment showed that total carotenoid production decreased significantly from pH 6 to 7.5 and significantly rises at pH 9. It may be due to the alkaline pH stimulating enhanced accumulation of triacylglyceride (TAG) by high carbonate uptake thereby resulting in inducing of carotenoid formation [27]. The carbonate assimilation by carbonate anhydrase was maximum at pH 9 in alkaliphic cyanobacterium [28]. It can be supported by the fact that *Spirulina maxima* which also belongs to oscillatoriales filamentous non-heterocystous cyanobacteria produces highest beta carotene at pH 9 [29]. The unusual rise of total carotenoid yield under high concentration of sodium nitrate (7.5 mg/ml) in the single factor experiment may be due to increase N/P ratio which results in high carotenoid yield

[30]. High nitrate to low phosphate ratio results in more ferrous ion uptake which generates more hydroxyl radical that stimulates carotenoid synthesis [31]. It may be also due to decreased of nitrate reductase [32] and increase of osmotic stress resulting to production of reactive oxygen species (ROS), H_2O_2 and hydroxyl radical [33] under the increase of exogenous nitrate that stimulates the enhanced carotenoid production. At half strength i.e. N/2, the carotenoid yield increased slightly from the control as supported by the reports that nitrate reduction enhances the carotenoid production [34]. Maximum total carotenoid were also observed at 0.20 mg/ml which may be due to limited supply of nitrate for growth at molar ratio of N:P of merely 0.001 by high phosphate concentration which is infinitely small value less than as mentioned by Balooff and Kavoosi [32]. This may cause nitrate limitation thereby enhancing carotenoid formation [35]. It can be also supported by the fact that enrichment of nutrient media with potassium phosphate induced accumulation of carotenoid pigments [34]. There was slight increase in the carotenoid as the phosphate concentration decreases to half the strength (N/2) as supported by Encarnacao et al. [30].

From the Box-Behnken experiment, we observed the significant optimum total carotenoids production at alkaliphic nutrient rich conditions. It can be collectively inferred that the alkaline pH may increase high carbonate uptake thereby enhancing accumulation of TAG which directed to induce carotenoid formation [27] as supported by the report that carbonate assimilation by carbonate anhydrase was maximum at pH 9 in alkaliphic cyanobacterium [28]. In additions to this, as the nitrate concentration is higher than phosphate concentration it results to high N/P ratio resulting in more ferrous ion uptake [30] thereby enhancing carotenogenesis later. The experiment showed significant individual effect rather than interaction between the factors. So, it can be shown that high total carotenoids production with increase of pH is supported by Otero and Vincenzini [36] that it is related to an inhibition of nitrate uptake which may lead to high carotenoids synthesis. It was earlier reported that the increase of total carotenoids under high nitrate 7.50 mg/ml (5N) concentration might contributed to the N:P ratio [30]. Similar findings were reported by Celekli et al. [40] that the phosphates supply increased biomass and carotenoids production in non-heterocystous cyanobacteria like *Spirulina platensis*. The fluctuation of potassium phosphate could lead to large difference in carotenoids yield. Low nitrate and low pH caused decrease of β-carotene content [38] and it was also reported by Incharoensakdi and Phunpruch [39] that nitrate deficiency in the growth medium did not cause an increased in β-carotene content as in the case of non-heterocystous cyanobacteria.

From the results of this study, *Leptolyngbya* sp. 287 provides a promising new source for total carotenoids production that can produce in nutrient rich and alkaline pH. Further detail study of carotenoid production under nutrient rich and alkaline medium can be made on the basis of carotenoid biosynthetic pathway.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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