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RESEARCH ARTICLE

Statistical optimization of culture medium composition for enhanced zeaxanthin production by Cyanophycean microalgae *Trichodesmium thiebautii* (NIOT 152)

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Abstract

Background/Objectives: Zeaxanthin is a xanthophyll carotenoid revered for its role in the prevention of age related macular degeneration. The study evaluated the zeaxanthin accumulation of the marine Cyanophycean alga *Trichodesmium thiebautii* (NIOT 152). A sequential statistical technique was applied to optimize the Artificial Sea Water nutrient medium (ASN-III) components for enhancing the zeaxanthin accumulation in *T. thiebautii*.

Methods: A two-level statistical approach involving Plackett-Burman (PB) design to screen the most important nutrients influencing the zeaxanthin accumulation followed by Response surface methodology (RSM) was employed. The results of PB design revealed sodium nitrate, disodium EDTA, magnesium sulphate and sodium carbonate as the crucial medium components for increasing zeaxanthin accumulation. Further, RSM was employed to study the interaction between these factors and identified an optimum concentration of the ingredients for higher zeaxanthin production. **Findings:** The optimized medium components resulted in 2.33 fold increase in zeaxanthin accumulation (4.3 ± 1.29 mg L^{-1}) as compared to ASN III medium (1.84 ± 0.12 mg L^{-1}). **Novelty:** There are only few studies on laboratory cultured *Trichodesmium* and only very few reports are available regarding pigment production from *Trichodesmium* sp. The present study successfully demonstrated the statistical optimization of ASN III medium to improve zeaxanthin accumulation by *Trichodesmium thiebautii*.

Keywords: ASN III medium; zeaxanthin; *Trichodesmium thiebautii*; Plackett-Burman; response surface methodology

1 Introduction

Zeaxanthin is an oxygenated carotenoid which has gained commercial momentum due to its multitude of industrial applications[(1)]. Research has demonstrated efficacy of zeaxanthin as a potential antioxidant, blue light

filter, preventive effect on diseases such as cancer[2], age related macular degeneration etc[3,4]. Industrial applications of zeaxanthin mainly include cosmetics, poultry and pharmaceuticals[5]. Zeaxanthin has fetched the attention of biotechnologists due to renewed public interest on natural pigments. Recently, research on use of marine Cyanophyceans for zeaxanthin production has galvanized, as these carotenoids are major and marker pigments in this group of algae. *Trichodesmium thieubautii*, a marine filamentous cyanobacterium is globally known for its biogeochemical role during N_2 fixation and their blooms are abundant in most subtropical and tropical oceans[6]. Nevertheless, establishing actively growing unialga *T. thiebautii* under laboratory condition has remained problematic[7,8]. The isolation of carotenoid pigments from microalgae like *Haematococcus pluvialis*, *Chlorella vulgaris*, *Chlorella zofingiensis* and *Chlorella pyrenoidosa* have been successfully demonstrated.[9,10].

For effective zeaxanthin production, it is essential to optimize media composition and culture conditions[11]. The first step in the media optimization process is screening of the crucial factors such as nitrate and phosphate concentration affecting the carotenoid of interest[12]. The conventional one-factor-at-a-time approach is time-consuming with little or no focus on the plausible interaction between major factors[13]. Hence, optimization of bioprocess using statistical tools provides better understanding of multi-factorial interactions in the production of algal biomass and product of interest[14]. The present study therefore employed a two-step full factorial optimization strategy which included selection of critical media components and culture conditions using Plackett-Burman Design (PBD) followed by optimization of identified factors using Response Surface Methodology (RSM) with Central Composite Design (CCD). RSM gives the advantage of creating mathematical models relating biomass and zeaxanthin accumulation to independent factors (the concentration of media components or operating parameters) and it also further helps to predict the expected responses and probable levels of independent variables for accomplishing the goal of maximal zeaxanthin accumulation[15,16].

Two-step sequential statistical optimization had been reported by several researchers like Niedz and Evens[17,18] and Terence et al., 2010[19]. To the best of our knowledge the nutritional and culture conditions of laboratory cultured *T. thiebautii* for zeaxanthin production has not been previously reported. The present study was carried out to identify and evaluate the different culture medium components and conditions on biomass and zeaxanthin production of Cyanophycean microalga *T. thiebautii*, identified as a potential zeaxanthin producer using statistical modelling.

2 Materials and Methods

2.1 Microorganism and culture conditions

The Cyanophycean marine microalgae, *Trichodesmium thiebautii* (NIOT 152) was isolated from Andaman & Nicobar Islands, India (93° 55' 55.0" E; 06° 59' 59.2" N) and maintained at the culture collection center of National Institute of Ocean Technology (NIOT). The cultures were maintained in controlled conditions in Artificial Sea Water Nutrient medium (ASN III)[20] with light intensity of 140 μmol photon m^2 s^{-1} under 14:10 light/dark regimes at a temperature of 25 $\pm$ 1°C. The control ASN III media was prepared in filtered sea water (salinity 34.23 % and pH 8.01), which was filtered through 0.22 μm cellulose acetate filter (Millipore) and autoclaved (121°C for 20 min). The growth medium was inoculated with 10% (v/v; average cell concentration of 0.25 g L^{-1} dry weight) of exponentially growing culture under aseptic condition and maintained for a time course of 11 days, after which the algal cells were harvested and lyophilized. The culture flasks were shaken manually twice a day to ensure uniform illumination of the cells. For estimation of biomass and pigments, the algal samples were thoroughly mixed and aliquoted in sterile conditions[21].

2.2 Scanning electron microscopy (SEM) of *T thiebautii* (NIOT 152)

A sample of 5 mL of microalgal cells were harvested during late exponential phase (Day 11) and washed thrice in 0.1 M sodium phosphate buffer (pH 7.2) and then gently filtered using a nucleopore filter (0.45 μM, 47 mm, Whatman). The nucleopore membrane containing the algal cells was fixed in 1000 μL of 2% glutaraldehyde and incubated at 4 °C for 12 h in 2 mL microcentrifuge tube. The cells were washed again with 0.1 M sodium phosphate buffer (pH 7.2) thrice at 4 °C and postfixed for 1 h in 1% osmium tetroxide in the same buffer (4 °C). After a brief wash with 0.1 M sodium phosphate buffer (pH 7.2), the cells were subsequently dehydrated with sequential ethanol series of 50, 70, 80, 90, and 100% (v/v)[22]. The samples were dried in a critical point dryer (E3100, Quorum), mounted on Aluminium stub (12 mm Ø) with double side carbon tape stubs and gold sputtered at a thickness of 200 A° (SC7620 Quorum) before examining under SEM (TESCAN VEGA3-SBU) equipped with secondary electron detector (Everhart-Thornley - YAG Crystal) at an accelerating voltage of 5 -10 kV.

2.3 Pigment extraction and analysis

Zeaxanthin content, biomass, chlorophyll concentration and C-phycocyanin was determined on alternate days for a time course of 11 days and the test samples were aliquoted under aseptic conditions (Laminar Air Flow cabinet) after proper mixing. Post-harvest experiments were performed on freeze dried algal biomass that was lypholized and biomass was determined according to Becker[23]. To evaluate the growth of microalgae *T. thiebautii*, the optical density of the ASN III culture was measured at 560 nm in UV-Vis spectrophotometer (Unicam UV 300, USA) and cell counts were performed using Neubauer cell counting apparatus (HBG, Germany)[24]. The initial and final biomass was monitored according to the method adopted by Zhu and Lee[25]. The algal growth was estimated by plotting the optical density values against biomass using standard calibration curves.

Zeaxanthin, being a photo-oxidative pigment, it is unstable upon exposure to light[26]. Hence, the pigment extraction experiments including HPLC quantification were performed in darkness and the vials containing zeaxanthin pigment were completely wrapped in aluminium foil. Zeaxanthin was extracted from the algal cells using alkali digestion method[27] with 10M solution of potassium hydroxide amended with antioxidant (2.5% ascorbic acid). The alkali extract was heated at 70°C, until the green color changes and finally treated with solvent mixture (methanol: dichloromethane at the ratio of 3:1 v/v) and the extracted zeaxanthin was quantified spectrophotometrically (453 nm) according to Chen et al.[28] and the zeaxanthin content present in the algae was determined as follows:

$$\text{Zeaxanthin } (\mu\text{g/ml}) = \frac{\text{A}_{453}\ \text{X104XV}}{\text{A1 cm1\%X}W} \tag{1}$$

Where,

A1% 1cm = absorption coefficient, which is defined as the theoretical absorbance of a solution of 1% (w/v) concentration (i.e., g in 100 mL) in a cuvette with a path length of 1 cm.

A_{453} = absorbance measured at 453 nm.

V = total extract volume (mL).

W = weight of sample (g).

104 = conversion factor to obtain the concentration in units of μg g^{-1}.

The extracted zeaxanthin was quantified using reverse phase HPLC (Shimadzu, Japan) equipped with an auto sampler (LC 2010 CHT) and quaternary pump (LC 2010) along with programmable UV-Vis detector. Column used was phenomenex Luna C-18 column with a dimension of 4.6 mm x 250 mm and a particle size of 5 μm. LC solutions software was used to retrieve experimental data three-dimensionally, i.e., absorbance-time-wavelength. Mobile phase constituted methanol /dichloromethane/ acetonitrile/ de-ionised water in the ratio 67.5:22.5: 9.5:0.5 (v/v). The flow rate was maintained at 1 mL per minute with injection volume being 0.5 mL and the concentration of zeaxanthin was detected at a wavelength of 453 nm. The zeaxanthin concentration in the microalgal extract was calculated by comparing the peak area with that of authentic zeaxanthin standard (Sigma Chemical Co., St. Louis, MO, USA) using standard calibration curves according to Priyanka et al.[29].

Chlorophyll (Chl-a) was determined using methanol (Merck) by the method of Bennet and Bogorad[30]. The net content of chlorophyll-a pigment present in the methanolic extract was determined spectrophotometrically (750nm, 665nm, 652nm) and calculated as per the following equation.

$$\text{Chlorophyll a } (C_a)\ (\mu\text{g ml}^{-1}) = 16.29\,A_{665} - 9.16\,A_{652} \tag{2}$$

Where, A = optical density (log I_0 /I) at indicated wavelengths, corrected for turbidity by subtraction of a 750 nm reading. The concentration of chlorophyll ~ in the original sample was then calculated using the following equation[31]

$$\text{Chlorophyll } a \text{ in mg m}^{-3}/\text{mgL}^{-1} = \frac{\text{CaX}\,v}{1 \times V} \tag{3}$$

T. thiebautii, is rich in C-phycocyanin, which is another valuable nutraceutical. This phycobiliprotein, a blue color pigment, was extracted using 0.1M phosphate buffer (pH 7.0) in darkness at low temperature and quantified according to the method described by Boussiba and Richmond[32] based on following equation.

$$\text{C} - \text{PC } (\mu\text{g ml}^{-1}) = [\,\text{A}_{620} - 0.474\,(\,\text{A}_{652})]\,/5.34 \tag{4}$$

Where, A=absorbance at indicated wavelengths.

2.4 Screening of nutrient and environmental factors using Plackett -Burman design

The production of carotenoids at an industrial scale requires the selection of suitable strain and optimization of culture conditions for carotenoid formation[33]. The Plackett-Burman (PB) method was employed for screening eleven independent variables namely, sodium nitrate (A), sodium β glycerophosphate (B), FeEDTA (C), initial pH (D), magnesium sulphate (E), calcium chloride (F), potassium chloride (G), sodium carbonate (H), A5 solution (I), EDTA disodium salt (J) and citric acid (K) for their influence on zeaxanthin accumulation. Each variable was tested at two levels, high and low denoted by (+1) and (-1) respectively[34]. An experimental design of 12 runs with 11 factors and 1 dummy variable was formulated. The design for PB was developed and analyzed using "Design Expert® version 9.03.1 (Stat-Ease Inc., Minneapolis, MN, USA)" software. The coded and uncoded levels of each variable are shown in Table 1. All experimental runs were performed in triplicate and the average value of zeaxanthin content was considered as the response.

Table 1. Plackett-Burman design for zeaxanthin accumulation by *T. thiebautii*.

Runs	A	B	C	D	E	F	G	H	I	J	K	Zeaxanthin accumulation
-	g	g	ml	-	g	g	g	mg	ml	mg	ml	(mgL-1)
1	4.5	1.25	0.5	8.5	3.5	0.1	0.01	20	0.1	200	0.1	2.69 ± 0.12
2	0.45	1.25	2.5	6.5	3.5	0.1	0.1	20	0.1	40	1	2.91 ± 0.32
3	4.5	0.25	2.5	8.5	0.35	0.1	0.1	200	0.1	40	0.1	3.73 ± 0.97
4	0.45	1.25	0.5	8.5	3.5	0.01	0.1	200	1	40	0.1	1.79 ± 0.56
5	0.45	0.25	2.5	6.5	3.5	0.1	0.01	200	1	200	0.1	0.98 ± 0.23
6	0.45	0.25	0.5	8.5	0.35	0.1	0.1	20	1	200	1	1.63 ± 0.12
7	4.5	0.25	0.5	6.5	3.5	0.01	0.1	200	0.1	200	1	1.02 ± 0.89
8	4.5	1.25	0.5	6.5	0.35	0.1	0.01	200	1	40	1	1.11 ± 0.49
9	4.5	1.25	2.5	6.5	0.35	0.01	0.1	20	1	200	0.1	0.75 ± 0.44
10	0.45	1.25	2.5	8.5	0.35	0.01	0.01	200	0.1	200	1	0.28 ± 0.01
11	4.5	0.25	2.5	8.5	3.5	0.01	0.01	20	1	40	1	1.28 ± 0.46
12	0.45	0.25	0.5	6.5	0.35	0.01	0.01	20	0.1	40	0.1	1.54 ± 0.11

Where, A= $NaNO_3$,B=Sodium B glycerophosphate, C=Fe EDTA, D= pH, E= $MgSO_4.7H_2O$, F=$CaCl_2.2H_2O$, G=KCl, H=Na_2CO_3,I=A5 solution for AP, J=Na_2EDTA, K=Citric acid.

2.5 Optimization of selected media components using RSM

Response surface methodology was employed to optimize the four most significant medium components (independent variables) viz. sodium nitrate (A), disodium EDTA (B) and magnesium sulphate (C) and sodium carbonate (D) as identified by PB experiments. The four selected independent variables were studied at five different levels coded as $-\alpha$, -1, 0, 1 and $+\alpha$. The value for alpha ($\alpha = 1.68179$) was chosen to fulfill the ratability of the design[35]. To examine the combined effect of four different media components (independent variables) on zeaxanthin production, a full factorial central composite rotatable experimental design (CCRD)[36] of 2^4=16 plus 6 centre points and star points (2 X 4=8) leading to a total of 30 treatments were performed (Table 2). The PBD method represents first order model, while RSM denotes second order model. The second order polynomial coefficients were calculated using the Design Expert Version 9.03.1 to estimate the responses of the dependent variable, which was determined through multiple regression[37]. According to the determination coefficient (R^2) and F-test, the competence of the model was assessed. The RSM statistical model was validated using numerical optimization for zeaxanthin production under the conditions predicted by the model. In order to visualize the relationship between the response and experimental levels of the independent variables, three-dimensional surface plots were constructed according to the quadratic polynomial model equations of coded factors.

Table 2. A full factorial Central Composite Design (CCD) matrix of independent variables forzeaxanthin accumulation (mg L^{-1}) by *T. thiebautii*.

Runs	$NaNO_3$	Na_2EDTA	$MgSO_4.7H_2O$	Na_2CO_3	Zeaxanthin accumulation	
					Predicted value	**Actual value
-	g	mg	g	mg	(mg L^{-1})	(mg L^{-1})
1	1.5 (-1)	100 (-1)	1.25 (-1)	50 (-1)	2.27	2.23 ± 0.6
2	4.5 (+2)	150 (0)	2 (0)	100 (0)	0.71	0.60 ± 0.53
3	2.5 (0)	150 (0)	2 (0)	0 (-2)	2.36	2.56 ± 0.32
4	1.5 (-1)	100 (-1)	1.25 (-1)	150 (+1)	2.37	2.16 ± 0.87
5	1.5 (-1)	200 (+1)	1.25 (-1)	50 (-1)	1.33	1.57 ± 0.25
6	3.5 (+1)	100 (-1)	2.75 (+1)	150 (+1)	2.17	2.67 ± 0.11
7	1.5 (-1)	100 (-1)	2.75 (+1)	50 (-1)	2.46	2.24 ± 0.27
8	2.5 (0)	150 (0)	2 (0)	100 (0)	1.26	1.31 ± 0.9
9	2.5 (0)	150 (0)	2 (0)	100 (0)	1.19	1.09 ± 0.17
10	3.5 (+1)	100 (-1)	2.75 (+1)	50 (-1)	3.72	3.57 ± 0.27
11	2.5 (0)	150 (0)	2 (0)	100 (0)	0.72	0.92 ± 0.67
12	2.5 (0)	250 (+2)	2 (0)	100 (0)	2.25	2.39 ± 0.85
13	2.5 (0)	150 (0)	2 (0)	100 (0)	1.57	1.27 ± 0.67
14	3.5 (+1)	100 (-1)	1.25 (-1)	150 (+1)	1.84	1.64 ± 0.43
15	1.5 (-1)	100 (-1)	2.75 (+1)	150 (+1)	3.9	3.44 ± 0.78
16	2.5 (0)	150 (0)	2 (0)	100 (0)	0.58	0.55 ± 0.52
17	2.5 (0)	150 (0)	0.5 (-2)	100 (0)	2.27	1.98 ± 0.22
18	3.5 (+1)	200 (+1)	1.25 (-1)	150 (+1)	4.28	4.68 ± 0.18
19	0.5 (-2)	150 (0)	2 (0)	100 (0)	1.29	1.31 ± 0.66
20	2.5 (0)	150 (0)	2 (0)	200 (+2)	2.27	2.27 ± 0.53
21	2.5 (0)	50 (-2)	2 (0)	100 (0)	2.27	2.46 ± 0.88
22	1.5 (-1)	200 (+1)	1.25 (-1)	150 (+1)	2.27	2.55 ± 0.34
23	3.5 (+1)	200 (+1)	1.25 (-1)	50 (-1)	2.58	2.62 ± 0.61
24	3.5 (+1)	200 (+1)	2.75 (+1)	150 (+1)	3.43	3.46 ± 0.18
25	3.5 (+1)	200 (+1)	2.75 (+1)	50 (-1)	1.12	0.92 ± 0.73
26	2.5 (0)	150 (0)	2 (0)	100 (0)	1.41	1.80 ± 0.34
27	2.5 (0)	150 (0)	3.5 (+2)	100 (0)	0.83	0.76 ± 0.17
28	3.5 (+1)	100 (-1)	1.25 (-1)	50 (-1)	1.67	1.76 ± 0.89
29	1.5 (-1)	200 (+1)	2.75 (+1)	50 (-1)	1.26	1.04 ± 0.9
30	1.5 (-1)	200 (+1)	2.75 (+1)	150 (+1)	2.27	2.15 ± 0.4

*values in parenthesis indicate coded levels. **Results are mean ± S.D

2.6 Data analysis

All experiments were carried out in triplicates, and the average value along with standard deviations were reported. The obtained data were analyzed statistically and calculations were made using EXCEL (Microsoft Office Enterprise, 2017), analysis of variance (ANOVA) and F test was performed using Design Expert version 9.03.1 (Stat-Ease Inc., Minneapolis, MN, USA)" software, wherever applicable. Significant levels for all analyses were set to $p < 0.05$.

3 Results and Discussion

3.1 Morphological traits of *Trichodesmium thiebautii* (NIOT 152)

The light micrograph and SEM depict of *T. thiebautii* (NIOT 152) was shown in Figure 1A and Figure 1B. The morphology of *T. thiebautii* cells were clearly seen with numerous non-constricted trichomes. These non-heterocystous diazotrophic

cyanobacteria contained rope like fusiform colonies as well. In the present work, the morphological traits of the microalgae *T. thiebautii*, thus observed were similar to those reported by Bergman et al.[38] and more recently by Carpenter et al.[39], as evident from SEM.

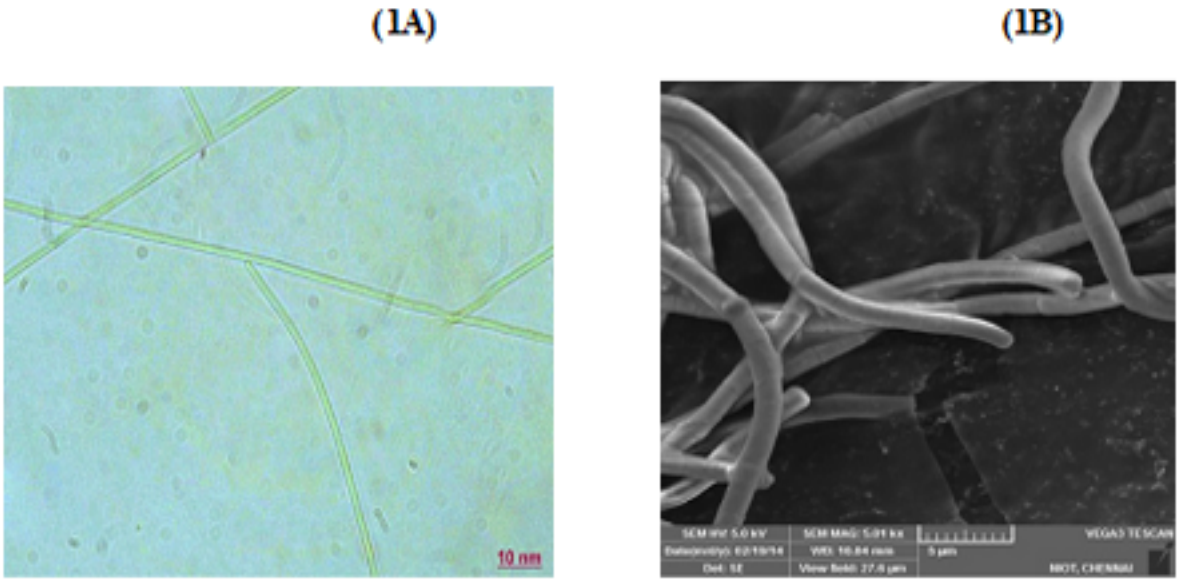

Fig 1. Light micrograph and SEM of *Trichodesmium thiebautii* (NIOT 152)

3.2 Optimization of nutrient concentration using Plackett -Burman design

The Plackett–Burman design was employed to evaluate the influence of significant factors affecting the response value – zeaxanthin accumulation. Table 1 shows the actual levels of various factors and the observed and predicted response for zeaxanthin accumulation. The results of PB experiment showed a wide variation in zeaxanthin and biomass production. This variation reflected the importance of optimization of media for attaining higher zeaxanthin content. The relative levels of significance and the percentage contribution of each variable is represented in Pareto chart (Figure 2). In order to check the fit of the model, R^2 and F-value were calculated. The results were analyzed using two-way ANOVA, (i.e.) analysis of variance suitable for the experimental design. Moreover, the model F-value of 445.69 demonstrated that the model was significant, as revealed by low p-value (0.0022), which further supported the adequacy and ambiguity of the model. Hence, one major and three minor nutrients were considered for further optimization using response surface methodology.

Optimal concentration of major and minor nutrient constituents of culture medium along with standard abiotic factors are the effective determinants of microalgal growth and production of growth associated products like carotenoids. Major culture medium components (nitrate, phosphate, magnesium and potassium) are crucial for cell formation and metabolism, while minor nutrients or trace metals (iron, copper, manganese, zinc, cobalt and molybdenum) mediate as different cofactors for enzymes involved in carotenoid biosynthesis[40]. With regard to zeaxanthin accumulation, it was observed that four out of the eleven factors tested viz. EDTA disodium salt, sodium nitrate, magnesium sulphate heptahydrate and sodium bicarbonate had significant effect on the response ($P < 0.05$). Among the essential factors affecting the zeaxanthin production by *T. thiebautii*, EDTA disodium salt gave the highest negative percentage (44.47%). The negative effect implies that EDTA disodium salt at low concentrations can enhance the zeaxanthin accumulation but can have deleterious effect at higher concentrations. It also explains the significant contribution of EDTA on zeaxanthin content of *T. thiebautii*. Paerl et al.[41] observed that EDTA plays an important role in extending the longevity and growth of *Trichodesmium* collected from natural seawater and maintained under laboratory conditions. EDTA has also been reported to play the dual role as a chelator alleviating the toxic stress of heavy metals like copper, zinc etc and favoring the availability of iron. An optimized concentration of chelating agent has also been indicated in improving iron and phosphate uptake and trace metal availability[42]. EDTA has also been reported to improve biomass and pigment production of *Chlorococcum* sp. by Satpadi et al.[43]. Since, iron is indirectly involved in pigment biosynthesis, improvement of iron uptake might have also contributed to zeaxanthin accumulation of Cyanophycean microalgae *T. thiebautii*. Burns et al.[44] have reported EDTA concentration of 5-200 μM to improve the growth rate of

laboratory maintained *Trichodesmium* sp. Hence, the optimized EDTA concentration of 200 mg L^{-1} is justified to augment zeaxanthin production in *T. thiebautii*.

Sodium nitrate showed a positive effect towards zeaxanthin production with a percentage contribution of 29.78%, which suggests that increase in sodium nitrate concentration from very low to higher value, can augment zeaxanthin content. Nitrate is the most important media component for carotenoid production. According to Rodier et al [45] *Trichodesmium* spp. contributes nearly 5% of the total nitrogen requirement of phytoplanktons in their habitat. Sanchez et al. [46] reported that the nitrogen concentration in the culture played a crucial role in biomass production and xanthophyll carotenoid biosynthesis of microalgae *Scenedesmus almeriensis*. Highest nitrate concentration tested (4.5 g L^{-1}) showed a decrease in zeaxanthin production (0.60 mg L^{-1}). $NaNO_3$ used as a nitrogen source in this study had a significant quadratic effect which implies higher concentration of $NaNO_3$ can be lethal to zeaxanthin accumulation. Nevertheless, total absence of N source also reduced biomass production and consequently led to poor carotenoid synthesis [47], hence nitrate should be supplied at a level where growth rate is not affected. Therefore, $NaNO_3$ concentration optimized (3.5 g L^{-1}) for zeaxanthin production in *T. thiebautii* is justified.

Third factor which displayed significant negative impact and a percentage contribution of 8.73% (Table 3 ; Figure 2) was magnesium sulphate heptahydrate. The negative impact of magnesium sulphate attributes at concentrations higher than optimum resulting in decreased zeaxanthin content. In the present study, magnesium sulphate did not have a very significant effect on zeaxanthin production on its own, nevertheless, there was a significant interaction effect of magnesium sulphate and sodium carbonate. This implies that the optimal concentration of sodium carbonate is highly impacted by magnesium sulphate. In the statistically formulated medium, an optimum concentration of 1.25 g L^{-1} of $MgSO_4$ resulted in higher zeaxanthin production. In concurrence with the present study, Shinde et al. [48] have reported increased lutein yield in the microalga *Auxenochlorella protothecoides* (5 fold) at $MgSO_4.7H_2O$ concentration of 0.8 g L^{-1}. Similarly, Maldonade et al. [49] have reported a negative impact of magnesium sulphate on carotenoid production in yeast *Rhodotorula mucilaginosa*.

Fourth variable which had a significant impact on zeaxanthin production was sodium carbonate, which showed a positive contribution of 6.5%. Na_2CO_3 has been reported to increase the specific growth rate, photosynthetic activity and carbonic anhydrase enzyme activity at optimal concentrations. It plays a significant role in maintaining the alkaline pH of the culture medium. *Trichodesmium thiebautii* prefers alkaline pH for its growth and carotenoid biosynthesis [50]. Similarly, White et al. [51] reported enhanced carotenoid and lipid production in the microalgae *Tetraselmis suecica* and *Nannochloropsis salina* when supplemented with 2 g L^{-1} sodium carbonate, and 1 g L^{-1} bicarbonate. Therefore, our results agree with the previous suggestions according to Fangfang et al. [52] and hence an optimum concentration of 150 mg L^{-1} of Na_2CO_3 for obtaining higher zeaxanthin production (4.68 mg L^{-1}) is justified.

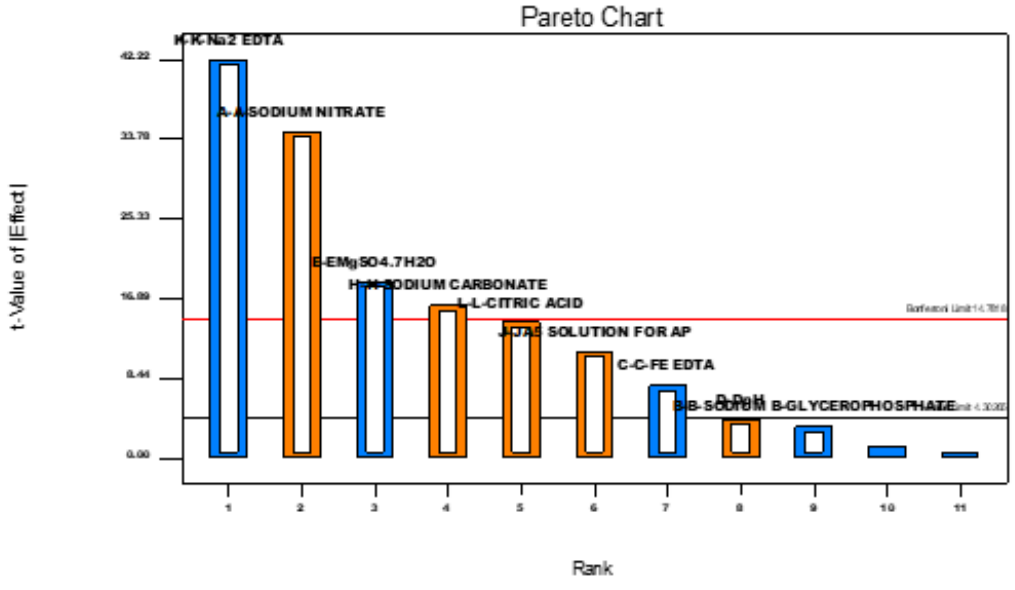

Fig 2. Pareto chart for optimization of zeaxanthin production from *T. thiebautii*.

Table 3. Statistical analysis of the Plackett-Burman experiment design.

Source	Sum of Squares	Df	Mean Square	Contri -bution %	F Value	p-value Prob > F
Model	10.98	9	1.22	-	445.69	0.0022
Sodium nitrate	3.27	1	3.27	29.78	1195.2	0.0008
Sodium B-glycerophosphate	0.031	1	0.031	0.28	11.23	0.0787
Fe EDTA	0.16	1	0.16	1.46	58.69	0.0166
pH	0.047	1	0.047	0.43	17.24	0.0534
Magnesium sulphate	0.96	1	0.96	8.73	350.21	0.0028
Sodium carbonate	0.71	1	0.71	6.5	260.89	0.0038
A5 solution for AP	0.35	1	0.35	3.2	128.22	0.0077
Disodium EDTA	4.88	1	4.88	44.42	1782.71	0.0006
Citric acid	0.57	1	0.57	5.15	206.79	0.0048

R^2 = 0.9838, Adjacent R^2 =0.9554

3.3 Response surface methodology

The significant culture medium components identified by PB design were further optimized using RSM. The optimum concentrations of these four variables, namely, $NaNO_3$, Na_2EDTA, $MgSO_4.7H_2O$ and Na_2CO_3 were identified using RSM design to maximize zeaxanthin production. Zeaxanthin accumulation ranged from 0.60 mg L^{-1} to 4.68 mg L^{-1}. By applying multiple regression analysis on the obtained data, the second order polynomial equation for zeaxanthin content was established as follows:

$$\text{Zeaxanthin (Yi)} = +2.27 + 0.18 * X_1(\text{A}) + 0.37 * X_2(\text{B}) + 0.12 * X_3(\text{C}) + 2.333\text{E} - 003 * X_4(\text{D}) + 0.5 * X_1X_2 - 0.11 * X_1X_3 - 0.72 * X_1X_4 + 0.049 * X_2X_3 - 0.40 * X_2X_4 + 0.24 * X_3X_4 - 0.33 * X_1^2 + 0.18 * X_2^2 - 0.21 * X_3^2 + 0.024 * X_4^2$$

Where, X_1 = concentration of sodium nitrate (g L^{-1}; A), X_2 = concentration of disodium EDTA (mg L^{-1}; B), X_3 = concentration of magnesium sulphate (g L^{-1}; C), X_4= concentration of sodium carbonate (mg L^{-1}; D).

The statistical significance was evaluated by F-test and analysis of variance which revealed that the model was statistically significant ($p < 0.0001$) for zeaxanthin accumulation. The fit of the model was also expressed by the coefficient of determination R^2, which was 0.9447, indicating the significance of the design (Table 4). The ANOVA of the quadratic regression model suggested that the model terms are significant as was evident from the Fisher's F test.

Among the quadratic coefficients $NaNO_3$ displayed significant influence on zeaxanthin accumulation. The quadratic effect of three variables (concentration of $NaNO_3$, Na_2EDTA and $MgSO_4.7H_2O$ had significant impact on zeaxanthin accumulation ($P < 0.01$).

While investigating the interaction effect of the four significant medium components, $NaNO_3$ *Vs* Na_2EDTA and $NaNO_3$ *Vs* Na_2CO_3 were found to have substantial impact on zeaxanthin accumulation ($p < 0.0001$; Table 4). Among the different interactions $NaNO_3$ Vs Na_2EDTA concentration had the maximal interaction effect on zeaxanthin volumetric productivity ($P< 0.01$). This underlines the fact that effect of $NaNO_3$ on zeaxanthin accumulation is dependent on Na_2EDTA and other cofactors and trace metals (Table 4). Three dimensional response surface and contour plots were plotted to evaluate the interactions among the variables and to ascertain the optimum concentration of each factor for obtaining maximum zeaxanthin content (Figure 3). It was also evident that Na_2EDTA had a very pivotal role in zeaxanthin content, while $NaNO_3$ and Na_2CO_3 had a significant interaction effect on zeaxanthin accumulation. In congruence with this study, Fae Neto et al.[53] obtained a zeaxanthin content of 8.1 μg L^{-1} in *Nannochloropsis oculata* using a low cost media. Basu et al.[54] observed that *Trichodesmium* forms a mutual interaction with associated bacteria to acquire iron from dust using siderophores thereby makes a direct contribution for iron assimilation by other phytoplanktons.

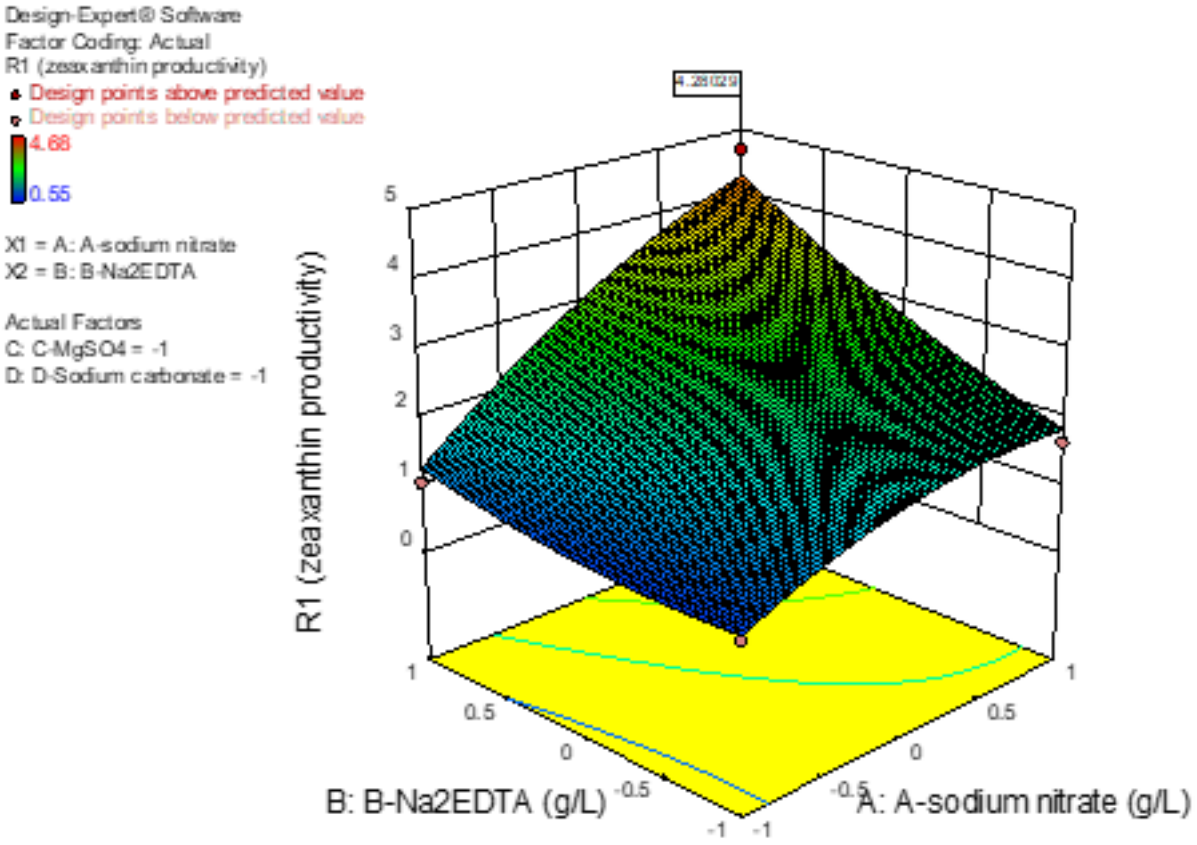

Fig 3. Three dimensional surface plot for zeaxanthin production by *T. thiebautii*.

Table 4. Analysis of variance (ANOVA)- [partial sum of squares – Type III] for the experimental results of the central-composite design (Quadratic Model)

	Sum of	Df	Mean	F	p-value	
Source	Squares		Square	Value	Prob > F	
Model	25.97	14	1.86	18.29	< 0.0001	Significant
A-$NaNO_3$	0.76	1	0.76	7.49	0.0153	
B-Na_2EDTA	3.26	1	3.26	32.13	< 0.0001	
C-$MgSO_4.7H_2O$	0.33	1	0.33	3.29	0.0896	
D-Na_2CO_3	1.31E-04	1	1.31E-04	1.29E-03	0.9718	
AB	4.06	1	4.06	40.06	< 0.0001	
AC	0.21	1	0.21	2.03	0.1745	
AD	8.19	1	8.19	80.69	< 0.0001	
BC	0.038	1	0.038	0.37	0.5516	
BD	2.52	1	2.52	24.89	0.0002	
CD	0.94	1	0.94	9.29	0.0081	
A2	3.07	1	3.07	30.23	< 0.0001	
B2	0.87	1	0.87	8.62	0.0102	
C2	1.22	1	1.22	12.01	0.0035	
D2	0.015	1	0.015	0.15	0.7044	

R^2 = 0.9447, Adj R^2 =0.8930, Pred R^2 =0.7148, C.V %= 15.94, Adeq Precision= 16.431

3.4 Validation of the model

Our model predicts the maximum zeaxanthin production of 4.28 mg L^{-1} in the statistically optimized media. The final optimized ASN-III medium was as follows: sodium nitrate – 3.5 g L^{-1}, magnesium sulphate heptahydrate – 1.25 g L^{-1}, disodium EDTA- 200 mg L^{-1}, sodium carbonate- 150 mg L^{-1}, allother constituents of ASN-III medium were retained in their original level. The growth medium was prepared in sea water with 35% salinity. The predictive ability of the model was evaluated by conducting three separate validation experiments that were compared with the original ASN-III medium. Under the optimized condition, the biomass and chlorophyll content of *T. thiebautii* were 3.1 $\pm$ 0.56 g L^{-1} and 3.67 $\pm$ 0.63 mg L^{-1}, which showed an increase of 2.23 and 1.58 fold more than the un-optimized medium (1.39 $\pm$ 0.26 g L^{-1} and 2.32 $\pm$ 0.18 mg L^{-1}) respectively. Similarly, C-phycocyanin content increased up to 1.64 folds (89.3 mg L^{-1}) than the unoptimized control (54.17 $\pm$ 0.56 mg L^{-1}). The observed zeaxanthin accumulation in the validation experiments were 4.3 mg L^{-1}, agreeing well with the predicted value 4.28 mg L^{-1}, indicating the predicting ability of the model. The optimized medium resulted in 2.33 fold increase in the overall zeaxanthin content. The results demonstrated that the percentage error between predicted and actual observed values were less than 0.4%. This further illustrated the precision of two-step statistical approach for improving zeaxanthin accumulation in *T. thiebauti*.

The marine Cyanophycean algae *Trichodesmium* has gained widespread attention due to its vital role in nitrogen fixation in ocean.[55]. Maintaining viable cultures of this alga for long time under lab conditions still remains as a challenge[44]. Hence, based on the above results, use of *Trichodesmium thiebautii* in the present study is thus justified. This study successfully illustrated an optimized medium for improving the growth and zeaxanthin production of *T. thiebautii* (NIOT 152).

4 Conclusion

In this research, a two-step sequential statistical technique was employed to optimize the ASN III medium components for maximal zeaxanthin production from the diazotroph *T. thiebautii*. However, components of ASN-III medium $NaNO_3$, Na_2EDTA, $MgSO_4$ and Na_2CO_3 had a significant effect on zeaxanthin accumulation. Among the variables Na_2EDTA had the significant linear effect on zeaxanthin accumulation and $NaNO_3$ had significant quadratic effect on zeaxanthin production. The optimized medium improved zeaxanthin production (4.3 $\pm$ 0.29 mg L^{-1}) by 2.33 fold more than that obtained in the initial medium (1.84 $\pm$ 0.12 mg L^{-1}). The optimized culture medium obtained from this experiment can be adopted for scaled up production of zeaxanthin from marine Cyanophycean alga *T. thiebautii*. Moreover, these results proved that the response surface methodology was fairly accurate in predictive modeling and media optimization.

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