Optimization of algal culture medium for zeaxanthin production by *Dunaliella tertiolecta*: an RSM based approach

S. Priyanka[1,2,*], R. Kirubagaran[1] and J. T. Mary Leema[1]

[1]Department of Marine Biotechnology, National Institute of Ocean Technology, Chennai 600 100, India
[2]Sathyabama Institute of Science and Technology, Chennai 600 119, India

The Chlorophycean microalgae *Dunaliella* have gained commercial interest because of the synthesis of highly valuable products. One among them is zeaxanthin, a xanthophyll carotenoid valued for its nutraceutical potential related to prevention of age-related macular degeneration and cataract, which is the primary cause for blindness. To improve zeaxanthin production by the microalgae *Dunaliella tertiolecta* (NIOT-141), De Walne's medium was optimized to its most favourable nutrient level using response surface methodology (RSM) approach. Plackett–Burman method was employed to screen the most significant nutrients influencing zeaxanthin accumulation which revealed sodium nitrate, trace metals and sodium dihydrogen phosphate as the crucial medium components for increasing zeaxanthin production ($P < 0.05$). Further, RSM was employed to study the interaction between these factors and identify optimum concentration of the significant ingredients for higher zeaxanthin production. The highest zeaxanthin production reached 20.2 ± 1.29 mg l^{-1} under the optimal conditions of 910 mg l^{-1} $NaNO_3$, 40.5 mg l^{-1} NaH_2PO_4 and 0.605 mg l^{-1} trace metals solution. Moreover, validation of optimized medium resulted in a three-fold increase in zeaxanthin production compared to unoptimized Walne's medium (6.72 ± 0.22 mg l^{-1}). The results thus obtained were more analogous to the predicted values. Hence the model is effective for enhancing zeaxanthin production in *D. tertiolecta*.

Keywords: *Dunaliella tertiolecta*, De Walne's medium, nutraceutical, response surface methodology, zeaxanthin.

IN recent decades, the carotenoid industry is an emerging sector because of its high-end applications in healthcare, food and feed supplements[1]. The increasing health awareness in developing countries has enhanced the demand for immunity boosters and natural health supplements[2,3]. Zeaxanthin is a yellow xanthophyll carotenoid produced by many microalgae, certain bacteria and higher plants[4]. It is also involved in cellular photo-protection by scavenging harmful blue light[5]. The strong antioxidant activity of zeaxanthin makes it a suitable nutraceutical for the prevention of cardiovascular diseases, some types of cancer and especially age-related macular degeneration (AMD)[6]. The approval of zeaxanthin and lutein as food additives (E161h for zeaxanthin and E161b for lutein, WHO Technical Report Series No. 940; 2007) has expanded the zeaxanthin market demand. The present market for xanthophyll pigments in USA alone surpasses US$ 250 million per year. Though daily xanthophyll intake of 6.0 mg is advocated, the pigment industry is in the fledgling stage due to low productivity of prospective candidate species. Hence there is greater need for identifying microalgal strains with higher productivity[7].

The conventional source of zeaxanthin and lutein production is marigold (*Tagetes erecta*) flower petals; but it suffers from lower pigment content (ca. 0.03%) and prolonged cultivation period (3–6 months). Shorter lifespan (10–15 days) and higher pigment content (0.5–1.0%) have made microalgae a viable alternative. Earlier studies have reported zeaxanthin production from two Chlorophycean[8] and one Cyanophycean algae[9]. Nevertheless, none of them has focused on the prime problem confronted, viz. lower zeaxanthin production. Hence the present study addresses the challenge of poor production using statistical approach of medium component optimization with respect to zeaxanthin production by microalgae. For this, we chose *Dunaliella tertiolecta* (NIOT-141) isolated from salt pans as a model organism. *Dunaliella* species was preferred due to its inherent ability to grow and accumulate higher carotenoid content (up to 10% of cell dry weight) in high salinity and light intensity[10]. Interestingly, these strains lack rigid cell wall as well, which makes pigment extraction easier than other microalgae[11]. At present, *Dunaliella salina* is a rich source for commercial exploitation of β-carotene and glycerol globally[12]. The Chlorophycean marine microalgae *Dunaliella* is also commercially exploited for several other products like SCP (single-cell protein), minerals and other bioactive compounds[13]. However, the actual content of natural pigments and secondary metabolites in the cell is determined by the culture medium conditions and other physico-chemical and environmental parameters like light intensity, salinity, temperature and pH[14].

*For correspondence. (e-mail: spriyankamsc@ymail.com)

With regard to optimization techniques, the drawback of outdated method of single factor experiment involves bulk experiments for optimizing a large number of parameters (variables), thereby time consuming and it might produce inaccurate conclusions, as relationship between factors are eliminated[15]. Plackett Burman, response surface methodology and central composite design (CCD), are some of the statistical methods which aim at better results in shorter time regimes. Additionally, they are an extensive multi-practical apparatus for building experimental models and breaking down the impacts of various factors and their interaction between the variables and testing significance[16]. This statistical approach can be used for most organisms like microbes and algae, for optimizing the recovery of products like secondary metabolites and enzymes to attain fruitful results[17]. The present study focuses on the optimization of zeaxanthin production by *D. tertiolecta* using statistical methodology by a two-step optimization strategy.

Materials and methods

Microorganism and growth conditions

The chlorophycean microalgae, *D. tertiolecta* (NIOT 141) strain used in this study was isolated from the salt pans of Marakkanam (12°12′050″N; 79°57′40.5″E), Tamil Nadu, India. The unialgal axenic cultures were maintained in De Walne's medium[18], with a photo period of 14 h light : 10 h dark regime under 140 μmol photon m^2 s^{-1} light intensity at 25°C.

Morphological characterization of D. tertiolecta (NIOT 141)

Morphological characteristics representing cell shape and size along with cell constituents like pyrenoid, refractile granules, etc. were examined using a light microscope (Zeiss, Axioskope Plus, Germany). Cell length, cell width and flagella length were also measured using an ocular micrometre. Scanning electron microscope (SEM) of *D. tertiolecta* (NIOT141) was performed according to the method adopted by Takaichi[19]. The initial and final biomass were determined according to the protocol of Zhu and Lee[20]. For biochemical characterization of *Dunaliella* strain, salinity tolerance test was performed by growing the axenic cultures in PES medium amended with a series of NaCl concentrations (0.5–4 M)[21].

Molecular characterization

The genomic DNA of *D. tertiolecta* was isolated using a modified CTAB method[22]. The extracted genomic DNA was quantified and its concentration was determined spectrophotometrically using UV-Vis spectrophotometer (Unicam UV 300, USA) by measuring the OD at 260 nm. PCR was carried out in a thermal cycler (AB Biosystems, USA) using three different primers, namely, internal transcribed spacer region (ITS) (AB28 and TW81), species specific 18S rRNA (MA1 and MA2), and plastid encoded gene for a large subunit of RUBISCO (ribulose-biphosphate carboxylase-rbcl) according to standard procedure[23,24].

Pigment extraction and HPLC quantification

Zeaxanthin was extracted from the algal cells using alkali digestion method in darkness, as zeaxanthin being a photo-oxidative pigment[25]. Pigment extraction and quantification were done according to the procedure described by Priyanka *et al*.[26]. The microalgal biomass was disrupted with solvent mixture of methanol : dichloromethane (3 : 1 v/v). The solvent extract was saponified with alkali containing ascorbic acid as antioxidant (3 ml of 10 M KOH with 2.5% ascorbic acid). The extracted zeaxanthin was analysed using RP-HPLC (Shimadzu-LC 2010, Japan) equipped with LC 2010 low pressure gradient HPLC pump, degasser, auto-sampler and programmable UV-Vis detector and C-18 Phenomenox Luna column (4.6 × 250 mm, 5 μm particle size). The mobile phase used was methanol/dichloromethane/acetonitrile/water (67.5 : 22.5 : 9.5 : 0.5, v/v) and data were acquired using LC solutions® software. The zeaxanthin yield ($P_{\text{zeaxanthin}}$) was calculated using the following equation

$$\text{Zeaxanthin yield (mg/l)} = \frac{\text{Cumulative biomass production (g)} \times \text{Zeaxanthin content(mg/g)}}{\text{Working volume (l)}}. \quad (1)$$

Screening of important media components using Plackett–Burman design

For effective zeaxanthin production, it is mandatory to optimize the culture conditions and media ingredients, which further facilitates suitable economic impact in the commercial sector[27]. PB method was utilized to categorize and optimize the constituents of Walne's medium, which significantly affected zeaxanthin production from *D. tertiolecta*. Six independent factors were successfully screened in 12 experimental trials (Table 1). The concentration of six factors (variables), namely sodium nitrate (A), sodium dihydrogen phosphate (B), iron EDTA (C), manganese chloride solution (0.04%) (D), trace metals stock solution (E) and vitamin solution (F) were studied over two specific levels, maximum (+) and minimum (–)[28].

Table 1. Plackett–Burman design matrix for six nutrient factors (independent variables) with zeaxanthin production as the process response

Run	A: $NaNO_3$ (mg)	B: NaH_2PO_4 (mg)	C: FeEDTA (mg)	D: $MnCl_2 \cdot 4H_2O$ (ml)	E: Trace metals (ml)	F: Vitamin solution (ml)	Zeaxanthin production* (mg l^{-1})
1	10 (–1)	0 (–1)	21 (+1)	0 (–1)	2 (+1)	2 (+1)	0.35 ± 0.12
2	10 (–1)	60 (+1)	21 (+1)	0 (–1)	2 (+1)	2 (+1)	1.95 ± 0.4
3	10 (–1)	0 (–1)	1 (–1)	2 (+1)	0 (–1)	2 (+1)	3.3 ± 0.2
4	1210 (+1)	60 (+1)	1 (–1)	0 (–1)	0 (–1)	2 (+1)	10.86 ± 0.23
5	10 (–1)	0 (–1)	1 (–1)	0 (–1)	0 (–1)	0 (–1)	4.48 ± 0.5
6	1210 (+1)	60 (+1)	21 (+1)	0 (–1)	0 (–1)	0 (–1)	15.86 ± 0.23
7	1210 (+1)	0 (–1)	21 (+1)	2 (+1)	0 (–1)	2 (+1)	7.54 ± 0.18
8	1210 (+1)	0 (–1)	21 (+1)	2 (+1)	2 (+1)	0 (–1)	0.73 ± 0.01
9	10 (–1)	60 (+1)	21 (+1)	2 (+1)	0 (–1)	0 (–1)	7.56 ± 0.25
10	10 (–1)	60 (+1)	1 (–1)	2 (+1)	2 (+1)	0 (–1)	1.62 ± 0.08
11	1210 (+1)	0 (–1)	1 (–1)	0 (–1)	2 (+1)	0 (–1)	0.75 ± 0.02
12	1210 (+1)	60 (+1)	1 (–1)	2 (+1)	2 (+1)	2 (+1)	1.69 ± 0.03

*Values are mean ± SD. Values in brackets indicate coded levels.

The composition of trace metals stock solution[29] was 33.6 g l^{-1} H_3BO_3, 21 g l^{-1} $ZnCl_2$, 20 g l^{-1} $CoCl_2 \cdot 6H_2O$, 9 g l^{-1} $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 20 g l^{-1} $CuSO_4 \cdot 5H_2O$ and the composition of vitamin solution[27] was 200 mg l^{-1} of thiamine HCl, 1 ml l^{-1} of 1% cyanocobalamin and 1 ml l^{-1} of 0.1% biotin.

The PB design for culture medium optimization of *D. tertiolecta* (NIOT-141) was analysed using Design Expert® software, version 9.03.1 (Stat-Ease Inc, Minneapolis, USA). Experimental trials were performed in triplicates, which was essential to evaluate the fluctuation of estimations[30]. The PB experiment which follows the first-order model was calculated based on the following equation

$$Y = \beta_{0+} \sum_{0}^{i} \beta_i X_i, \tag{2}$$

where Y is the response of the dependent variable (zeaxanthin production), X_i is the independent variable, β_i is the linear coefficient and β_0 is the intercept of the model[26]. If $P \leq 0.5$, it is considered significant.

Optimization of significant medium ingredients using RSM

To scrutinize the consolidated impact of various media ingredients (independent variables) on zeaxanthin production by *D. tertiolecta*, a full factorial central composite rotatable experimental design (CCRD)[31] comprising of six replicates of focal (centre) points along with 14 star points prompting a sum of 20 trials was developed (Table 2). RSM was utilized to enhance the three most critical variables, viz. sodium nitrate (A), sodium dihydrogen phosphate (B) and trace elements solution (C), which were identified as significant factors affecting zeaxanthin production using PB design. These independent variables ($NaNO_3$, NaH_2PO_4 and trace metals) were studied at five different coded levels as $-\alpha$, –1, 0, +1 and $+\alpha$ (ref. 32). The value for α (1.68179) was chosen to fulfil the rotatability of the design[33]. RSM follows the second-order model

$$Y = \beta_{0+} \sum_{0}^{i} \beta_i X_i + \sum_{0}^{i} \beta_{ii} X_i^2 + \sum_{0}^{i} \beta_{ij} X_i X_j, \tag{3}$$

where Y is the predicted response (zeaxanthin yield), β_i the linear coefficient, β_0 the regression coefficient, β_{ij} the interaction coefficient, β_{ii} the quadratic coefficient and X_i denotes coded values of independent variables.

$$X_i = X_i - X_0/\delta X, \tag{4}$$

where X_i symbolizes the dimensionless coded value of variable X_i and X_0 denotes the value of X_i at the centre point, while δX signifies step change value. The experimental data thus obtained were used for regression analysis[34]. Table 2 shows the structure and design of the experiments and their respective yields.

Purification of zeaxanthin

The extracted crude zeaxanthin was purified using biphasic separation and quantified with RP-HPLC. For biphasic separation, the extracted zeaxanthin was mixed with 30 ml deionized water and portioned with ethyl acetate (40 ml) and fractioned in a separating funnel. The ethyl acetate fraction alone was concentrated in a rotary evaporator and reconstituted with methanol for further purification using RP-HPLC. The collected fractions were analysed by comparing with the spectral properties of standard zeaxanthin[35]. The percentage of purity of the obtained fraction was confirmed using analytical RP-HPLC at a flow rate of 1 ml min^{-1}. An isocratic mobile

Table 2. Central composite rotatable design matrix of independent variables and their corresponding experimental and predicted yields of zeaxanthin from *Dunaliella tertiolecta* (NIOT 141)

Run	A: $NaNO_3$ (mg l^{-1})	B: NaH_2PO_4 (mg l^{-1})	C: Trace metals (mg l^{-1})	Zeaxanthin production*	
				Predicted value (mg l^{-1})	Actual value (mg l^{-1})
1	910 (+1)	15 (–1)	1.5 (+1)	1.63	1.29 ± 0.02
2	610 (0)	4.78 (–1.68)	1 (0)	3.93	4.41 ± 0.05
3	610 (0)	30 (0)	1 (0)	12.51	11.19 ± 0.42
4	610 (0)	30 (0)	1 (0)	12.51	11.58 ± 0.23
5	610 (0)	30 (0)	1 (0)	12.51	13.29 ± 0.56
6	610 (0)	30 (0)	0.159 (–1.68)	7.92	8.40 ± 0.02
7	310 (–1)	45 (+1)	0.5 (–1)	11.92	11.58 ± 0.1
8	105.46 (–1.68)	30 (0)	1 (0)	10.01	10.49 ± 0.6
9	610 (0)	30 (0)	1 (0)	12.51	13.49 ± 0.73
10	610 (0)	55.27 (1.68)	1 (0)	10.23	10.71 ± 0.02
11	910 (+1)	45 (+1)	1.5 (+1)	13.19	12.85 ± 0.87
12	910 (+1)	45 (+1)	0.5 (–1)	20.20	20.01 ± 0.21
13	610 (0)	30 (0)	1.84 (1.68)	5.72	6.20 ± 0.2
14	610 (0)	30 (0)	1 (0)	12.51	12.31 ± 0.56
15	310 (–1)	45 (+1)	1.5 (+1)	8.86	8.52 ± 0.07
16	910 (+1)	15 (–1)	0.5 (–1)	4.25	3.91 ± 0.03
17	610 (0)	30 (0)	1 (0)	12.51	13.02 ± 0.89
18	310 (–1)	15 (–1)	1.5 (+1)	2.81	2.47 ± 0.09
19	1114.54 (+1.68)	30 (0)	1 (0)	19.17	19.65 ± 0.52
20	310 (–1)	15 (–1)	0.5 (–1)	15.86	15.52 ± 0.4

*Values are mean ± SD. Values in brackets indicate coded levels.

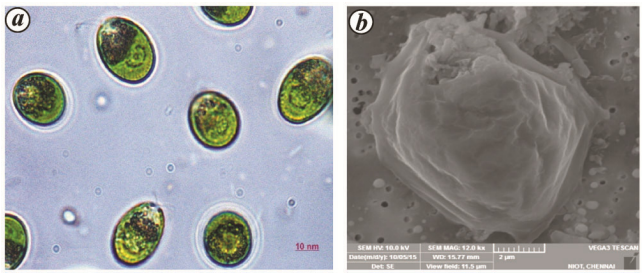

Figure 1. (***a***) Light micrograph and (***b***) scanning electron micrograph showing morphology of strain *Dunaliella tertiolecta* (NIOT-141).

phase of methanol/dichloromethane/acetonitrile/water (67.5 : 22.5 : 9.5 : 0.5 v/v/v/v) was used.

Statistical analysis

The framework of the model was designed and evaluated using Design Expert software version 9.03.1. Data were statistically analysed by two-way ANOVA and expressed as mean ± SD with significant levels held at $P < 0.05$.

Results

Morphological characteristics of D. tertiolecta (NIOT 141)

The algal cells were either ellipsoid or ovoid, but often become spherical during high salinity. Cell micrometry studies revealed a cell length of 11.85 µm, with a range 9–15.17 µm, cell width of 9.49 µm, with a range 6–13.12 µm and two equal apically inserted flagella of 13.75 µm length in *D. tertiolecta*. Light microscopic analysis revealed a cup-shaped chloroplast with a single-centred starch surrounded by a pyrenoid, few vacuoles and the nucleus. Also, some distinctive Golgi bodies, stigma, refractile granules, an eye spot, mitochondria and vacuoles were observed. The algal cells lack a rigid cell wall, but are enclosed by plasma membrane enveloped with a tough mucilaginous surface coat. Figure 1 *a* and *b* shows the light photomicrograph and SEM photomicrograph of 11-days-old culture of *D. tertiolecta* (NIOT-141) respectively. The morphological features observed were characteristic of *D. tertiolecta*, as reported in the literature[36,37].

Molecular characterization and PCR amplification

The amplification of 18S rRNA gene was carried out with MA1 and MA2 primers. Figure 2 *a* shows successful amplification. The PCR product obtained had an approximate size of 1770 bp, which is close to the reported size for *D. teriolecta* by Olmos-Soto *et al.*[38]. For further confirmation of molecular phylogeny based on the more variable ITS gene and conserved *rbcL* gene, PCR amplification was done. The successful amplification of ITS and *rbcL* genes of *D. tertiolecta* (NIOT-141) gave an amplified product of approximately 700 bp on the electropherogram (Figure 2 *b*). The observed product size

closely matched that reported for *D. tertiolecta* for these two genes by Hosseinzadeh Gharajeh *et al.*[37], thus confirming that the Chlorophycean microalgae from the salt pans was in fact *D. teriolecta*.

Evaluating the significant nutrient factors using Plackett–Burman design

PB design (six-factor, two-level) consisting of 12 experimental trials was employed to comprehensively study the influence of Walne's medium components with respect to zeaxanthin production (Table 1). Figure 3 is a graphical illustration of the contribution percentage of independent variables. The factors like NaH_2PO_4, $NaNO_3$ and trace metals have positive effect on zeaxanthin production, whereas other factors like vitamin solution, manganese chloride and FeEDTA have a negative effect. The contribution of the three most significant factors was as follows: trace metal solution – 58.32%, sodium dihydrogen phosphate – 16.16% and sodium nitrate – 10.64% (Figure 3). Hence, the concentration of these three significant factors were further optimized using RSM to obtain maximum response.

Response surface methodology

A sum of 20 tests was structured and experimented in triplicates, with their average mean value of zeaxanthin concentration as the response. Statistical examination of the model was done using analysis of variance (two-way ANOVA). The model was assessed by the coefficient of determination (R^2) and its significance was examined with the help of *P* value and *F*-test (Table 3). Multiple regression analysis was performed and the second-order polynomial equation was utilized to relate the independent factors accomplishing high zeaxanthin production. The response of each variable was determined by the coded factors of the polynomial equation. The second-order model that was constructed after multiple regression analysis is given below

$$\begin{aligned}\text{Zeaxanthin} = {} & + 12.51 + 3.62 * A + 1.87 * B \\ & - 0.65 * C + 3.18 * AB + 0.81 * AC + 0.70 * BC \\ & + 1.27 * A^2 - 1.92 * B^2 - 2.01 * C^2 - 1.80 * ABC \\ & + 1.83A^2B - 2.56\, A^2C - 3.64\, AB^2, \end{aligned} \quad (5)$$

where A = sodium nitrate, B = sodium dihydrogen phosphate and C = trace metals.

In this case A, B, AB, A^2, B^2, C^2, ABC, A^2B, A^2C, AB^2 are significant model terms. Other terms were found to be insignificant (Table 3). Run 12 of CCD design yielded maximum zeaxanthin production of 19.86 ± 0.21 mg l^{-1} under the following condition: $NaNO_3$ – 910 mg l^{-1}, NaH_2PO_4 – 45 mg l^{-1} and trace metals –0.5 ml l^{-1}. As shown in Table 2, the forecasted response varied in the range 1.29–20.01 mg l^{-1}. The model was further validated for its yield to verify the reproducibility of the results obtained.

Based on the results of two-way ANOVA, a high *F*-value (36.91), which is the ratio of mean square regression and mean square residual, and a very low *P*-value (<0.0001), indicate that the model is significant[39]. 'Prob > *F*' less than 0.0500 signifies the reliability of the experiment. 'Adeq Precision' measures the signal-to-noise ratio (adequate precision values ≥4 considered desirable), whereas coefficient of variation (CV) determines the accuracy of the model[40]. In this experimental model, a ratio of 23.05 indicates an adequate signal. Low value of CV (9.97%) indicates good precision and reliability of the experiments. Hence, the experimental model is reliable and reproducible[41].

The observed coefficient of determination (R^2) value of 0.987 demonstrates that 98.70% of the unpredictability in the response value could be elucidated by the mathematical

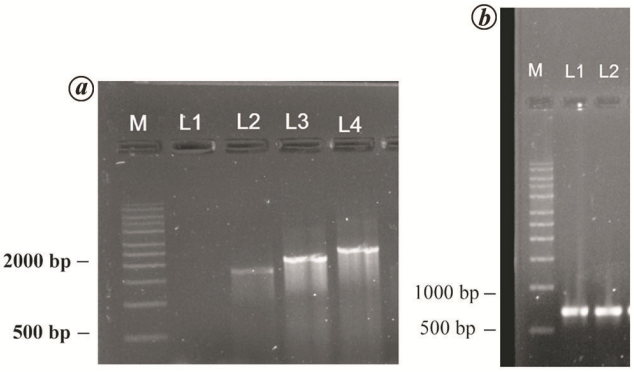

Figure 2. ***a***, Amplification using MA1 and MA2 primers. Lane M, 1 kb marker; lane L1, Negative control; lane L2, *Dunaliella* sp.; lane L3, *D. tertiolecta* (NIOT-141) and lane L4, *Dunaliella salina.* ***b***, Amplification using ITS and rbcL primers. Lane M, 1 kb marker; lane L1, *D. tertiolecta* (NIOT-141) amplified using ITS primers AB28 and TW81 and lane L2, *D. tertiolecta* (NIOT-141) amplified using primers rbcL-F and rbcL-R.

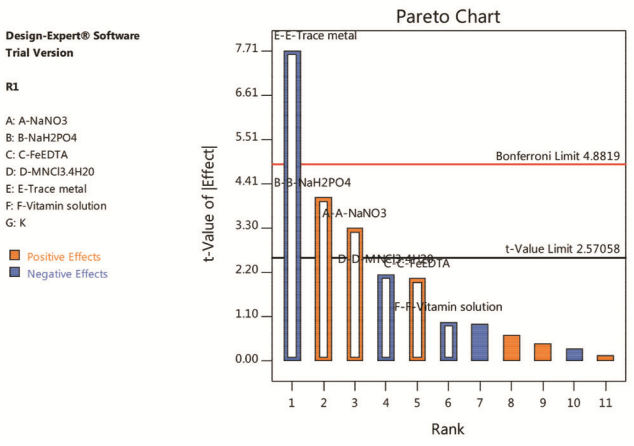

Figure 3. Pareto chart reflecting the significance of De Walne's medium components.

Table 3. Analysis of variance (partial sum of squares – type III) for zeaxanthin concentration from *D. tertiolecta* using response surface methodology (quadratic model)

Source	Sum of squares	d*f*	Mean square	*F* value	*P*-value Prob > *F*
Model	544.29	13	41.87	36.91	0.0001
A-$NaNO_3$	73.93	1	73.93	65.17	0.0002
B-NaH_2PO_4	19.85	1	19.85	17.49	0.0058
C-Trace metal	2.42	1	2.42	2.13	0.1944
AB	80.65	1	80.65	71.09	0.0002
AC	5.25	1	5.25	4.63	0.0750
BC	3.92	1	3.92	3.46	0.1124
A^2	23.08	1	23.08	20.35	0.0041
B^2	53.12	1	53.12	46.83	0.0005
C^2	58.33	1	58.33	51.42	0.0004
ABC	25.85	1	25.85	22.79	0.0031
A^2B	11.09	1	11.09	9.78	0.0204
A^2C	21.78	1	21.78	19.20	0.0047
AB^2	43.85	1	43.85	38.66	0.0008

R^2 = 0.987, Adj R^2 = 0.9609, Pred R^2 = 0.8754, Adeq precision = 23.05, CV = 9.97%.

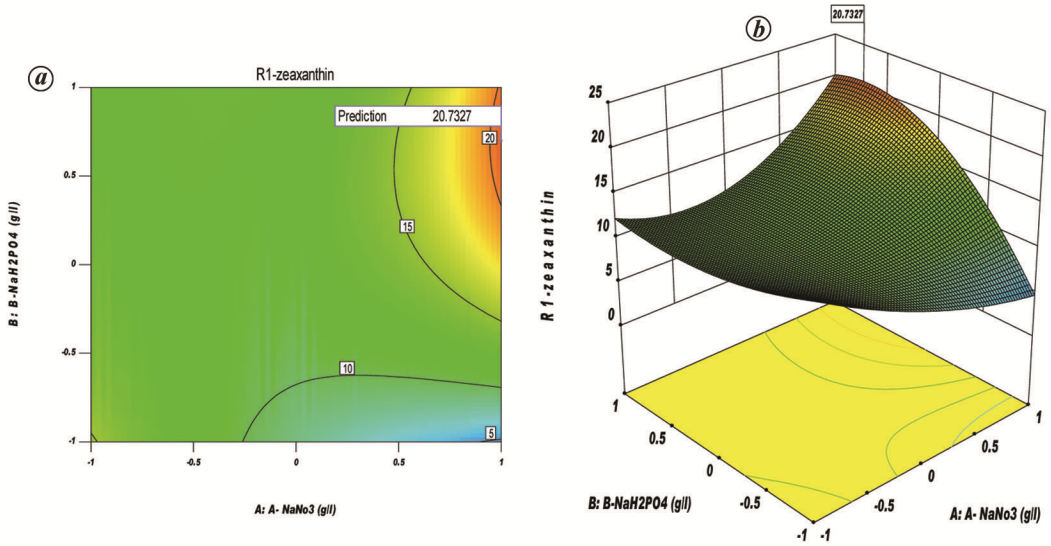

Figure 4. (***a***) Response surface methodology contour plot (***b***) three-dimensional response surface plot for effects of NaH_2PO_4 and $NaNO_3$ concentration and their interaction effect on zeaxanthin production by *D. tertiolecta*. Other variables were set at zero level.

model (Table 3). The 'Pred R^2' of 0.0542 was not much contiguous to 'Adj R^2' of 0.9609, i.e. the difference is above 0.2. This may specify a huge block effect with the experimental model. In order to overcome this defect, the coded factors for $NaNO_3$ were set at +1.00 level, NaH_2PO_4 at +0.70 level and trace metals solution at –0.79 level.

The 3D response surface graph displayed in Figure 4, reveals the mutual interaction effect of the exploratory factors (variables) on the response value (zeaxanthin yield). The regression equation defines the following optimal coded units of test factors: $X_1 = 910$ mg l^{-1}, $X_2 = 40.5$ mg l^{-1} and $X_3 = 0.605$ ml l^{-1} corresponding to the concentration of $NaNO_3$, NaH_2PO_4 and micronutrient solution respectively, for maximal zeaxanthin production of 20.2 ± 1.29 mg l^{-1}, anticipated by the numerical model. However, all experimental models ought to be tested using confirmatory runs to obtain corroborative results.

Validation of the experimental model

In order to authenticate the suitability of the model equations, additional experiments were carried out using the predicted optimized conditions containing the three significant components namely, $NaNO_3$ of 910 mg l^{-1} (+1.00 level), NaH_2PO_4 of 40.5 mg l^{-1} (+0.70 level) and trace elements solution of 0.605 ml l^{-1} (–0.79 level). As a result, zeaxanthin productivity peaked up to 20.2 ± 1.29 mg l^{-1} on utilizing the optimized medium, which is

in good correlation with the estimated value of 20.73 mg l^{-1} by RSM regression study.

An overall three-fold enhancement in zeaxanthin production (20.2 ± 1.29 mg l^{-1}) was obtained due to optimization, when compared with the initial Walne's medium (6.92 ± 0.2 mg l^{-1}). The good correlation between these results confirms that the predicted responses for the model are adequate to reflect the observed optimization; hence the model is reproducible.

Purification of zeaxanthin using RP-HPLC

The obtained zeaxanthin extract from *D. tertiolecta* was purified using analytical RP-HPLC. Figure 5 *a* displays the HPLC chromatogram of authentic zeaxanthin standard in which the major peak was obtained at 13.25 min. Figure 5 *b* and *c* displays the analytical RP-HPLC chromatogram of purified zeaxanthin. Figure 5 *c* shows the major fraction eluted at 13.16 min, characteristic to zeaxanthin, when compared to zeaxanthin standard. The zeaxanthin thus obtained was 97.35% pure.

Discussion

Among the essential macronutrients, nitrogen alone represents almost 10% of the total dry cell weight in microalgae[42]. In several microalgae, N is the most significant supplement in the culture medium as it plays a critical role in influencing growth and controlling cellular metabolism and carotenoid synthesis[43]. Although, there are different nitrogen sources like ammonia, urea, nitrate, peptone, etc. the best N source for *Dunaliella* is nitrate only. In the present study, a higher sodium nitrate concentration of 910 mg l^{-1} shows more zeaxanthin yield. These findings agree well with those of Del Campo *et al.*[44], who demonstrated that *Chlorella zofingiensis* showed an increase in lutein (25 mg l^{-1}) and astaxanthin (18 mg l^{-1}) content with higher nitrogen concentration of 2.5 g l^{-1}. Leesing *et al.*[45] demonstrated that growth and lipid production in *Chlorella* sp. KKU-S2 were highly related to the amount of nitrogen in the culture medium. Shi *et al.*[46] observed that an amalgamation of various nitrogen sources resulted in maximal lutein synthesis in *Chlorella protothecoides*. However, total absence of N source reduces biomass productivity and consequently results in poor zeaxanthin synthesis. Hence, zeaxanthin accumulating ability of algae is mostly related to the amount of N in the culture medium[47]. Therefore, nitrate should be supplied at a moderate level so that growth rate is not hindered.

Hannon *et al.*[48] observed that phosphorus makes up extensively about 1% of absolute algal biomass (up to 0.06%), but it plays a vital role in growth and metabolism of microalgae, thereby promoting carotenoid synthesis[49]. Belotti *et al.*[50], observed that total absence of phosphorus in culture medium could suppress photosynthesis, thereby resulting in poor growth and leading to starvation. In the present study, sodium dihydrogen phosphate was utilized as a PO_4 source, with a range of 15–55.27 mg l^{-1}. In run 12 of CCD, the higher PO_4 concentration of 45 mg l^{-1} yielded maximum amount of zeaxanthin. This result agrees well with that of Celekli *et al.*[51], who observed that phosphate enhanced carotenoid synthesis in the filamentous Cyanophycean microalga, *Spirulina platensis*. In order to achieve higher zeaxanthin content and biomass, a combination of optimum nitrate and phosphate concentrations is essential to increase the zeaxanthin accumulation.

Ben-Amotz and Avron[52] reported that four important micronutrients, namely cobalt, zinc, copper and manganese are essential for sustained growth and metabolite production in *Dunaliella*, which is in line with the present study, that reflected trace metals solution as a significant component for zeaxanthin production. Trace metals also act as cofactors for enzyme synthesis during photosynthesis. Therefore, several ecological stresses along with varied physico-chemical factors could enhance the synthesis of metabolites in a single growth cycle and developing such strategies in large-scale production could be more economical.

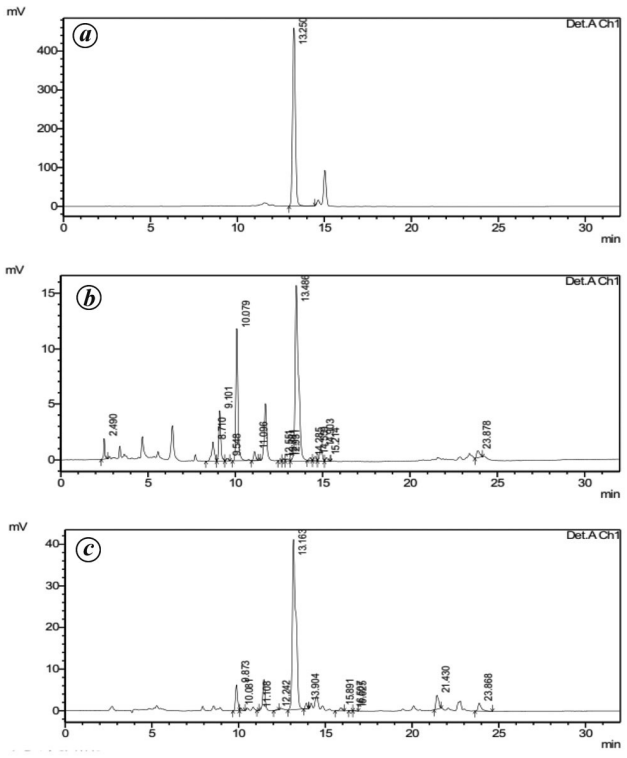

Figure 5. RP-HPLC profile of zexanthin at each step of purification. ***a–c***, HPLC chromatograms of (***a***) zeaxanthin standard. (***b***) crude zeaxanthin and (***c***) partially purified zeaxanthin after biphasic purification.

Conclusion

In the present study, PB and CCD were utilized to improve the suitability of De Walne's medium, thereby

optimizing the conditions for maximum production of zeaxanthin by *D. tertiolecta*. The medium containing 910 mg l^{-1} sodium nitrate, 40.5 mg l^{-1} sodium dihydrogen phosphate and 0.605 mg l^{-1} trace elements solution was considered as optimal, which improved zeaxanthin production by three-fold (20.2 ± 1.29 mg l^{-1}) more than that obtained in the initial medium. Two-way ANOVA demonstrated a high coefficient of determination ($R^2 = 0.987$), thereby proving the reliability and accuracy of the experimental model. It also resulted in the increase of biomass from 2.5 ± 0.26 g l^{-1} to 3.53 ± 0.56 g l^{-1}, which was 1.4-fold higher. Thus, the Chlorophycean halotolerant microalga, *D. tertiolecta* could be a suitable candidate for industrial-scale zeaxanthin production.

1. Sathasivam, R., Ebenezer, V., Guo, R. and Ki, J. S., Physiological and biochemical responses of the freshwater green algae *Closterium ehrenbergii* to the common disinfectant chlorine. *Ecotoxicol. Environ. Saf.*, 2016, **133**, 501–508.
2. Priyanka, S., Kirubagaran, R. and Mary Leema, J. T., Statistical optimization of culture medium composition for enhanced zeaxanthin production by Cyanophycean microalgae *Trichodesmium thiebautii* (NIOT 152). *Ind. J. Sci. Technol.*, 2020, **13**, 4307–4318.
3. Moeller, S. M., Jacques, P. F. and Blumberg, J. B., The potential role of dietary xanthophylls in cataract and age-related macular degeneration. *J. Am. Coll. Nutr.*, 2000, **5**, 522–527.
4. Mulders, K. J., Lamers, P. P., Martens, D. E. and Wijffels, R. H., Phototrophic pigment production with microalgae: biological constraints and opportunities. *J. Phycol.*, 2014, **50**, 229–242.
5. Kiokias, S., Proestos, C. and Oreopoulou. V., Effect of natural food antioxidants against LDL and DNA oxidative changes. *Antioxidants*, 2018, **7**(133), 1–20.
6. Bernstein, P. S., Li, B., Vachali, P. P., Gorusupudi, A., Shyam, R., Henriksen, B. S. and Nolan, J. M., Lutein, zeaxanthin, and meso-zeaxanthin: the basic and clinical science underlying carotenoid-based nutritional interventions against ocular disease. *Prog. Retin. Eye Res.*, 2016, **50**, 34–66.
7. Bhalamurugan, A. L., Valerie, O. and Mark, L., Valuable bioproducts obtained from microalgal biomass and their commercial applications: a review. *Environ. Eng. Res.*, 2018, **23**(3), 29–241.
8. Granado-Lorencio, F., Herrero-Barbudo, C., Acien-Fernández, G., Molina-Grima, E., Fernandez-Sevilla, J., Pérez-Sacristán, B. and Blanco-Navarro, I., *In vitro* bioaccesibility of lutein and zeaxanthin from the microalgae *Scenedesmus almeriensis*. *Food Chem.*, 2009, **114**, 747–752.
9. Chen, F., Li, H. B., Wong, R. N. S., Ji, B. and Jiang, Y., Isolation and purification of the bioactive carotenoid zeaxanthin from the microalga *Microcystis aeruginosa* by high-speed counter-current chromatography. *J. Chromatogr.*, 2005, **1064**, 183–186.
10. Chen, C. R., Hong, S. E., Wang, Y. C., Hsu, S. L., Hsiang, D. and Chang, C. M. J., Preparation of highly pure zeaxanthin particles from sea water-cultivated microalgae using supercritical anti-solvent recrystallization. *Bioresour. Technol.*, 2012, **104**, 828–831.
11. Kim, M., Ahn, J., Jeon, H. and Jeon, E. S., Development of a *Dunaliella tertiolecta* strain with increased zeaxanthin content using random mutagenesis. *Mar. Drugs*, 2017, **15**(189), 1–14.
12. Spolaore, P., Joannis-Cassan, C., Duran, E. and Isambert, A., Commercial applications of microalgae. *J. Biosci. Bioeng.*, 2006, **101**, 87–96.
13. Oren, A., A century years of *Dunaliella* research: 1905–2005. *Saline Syst.*, 2005, **1**(2), 2–14.
14. Barzegari, A., Hejazi, M. A., Hosseinzadeh, N., Eslami, S., Aghdam, E. M. and Hejazi, M. S., *Dunaliella* as an attractive candidate for molecular farming. *Mol. Biol. Rep.*, 2010, **37**, 3427–3430.
15. Desai, K. M., Survase, S. A., Saudagar, P. S., Lele, S. S. and Singhal, R. S., Comparison of artificial neural network (ANN) and response surface methodology (RSM) in fermentation media optimization: case study of fermentative production of scleroglucan. *J. Biochem. Eng.*, 2008, **41**(3), 266–273.
16. Chen, H., Lu, Y. and Jiang, J., Comparative analysis on the key enzymes of the glycerol cycle metabolic pathway in *Dunaliella salina* under osmotic stresses. *PLoS ONE*, 2012, **7**(6), e37578.
17. Sawale, S. D. and Lele, S. S., Increased dextran sucrase production by response surface methodology from *Leuconostoc* species isolated from fermented idli batter. *Global J. Biotechnol. Biochem.*, 2009, **4**(2), 160–167.
18. Walne, P. R., Studies on the food value of nineteen genera of algae to juvenile bivalves of the genera *Ostrea*, *Crassostrea*, *Mercenaria* and *Mytilis*. *Fishery Invest.*, 1970, **26**, 1–62.
19. Takaichi, S., Carotenoids in algae: distributions, biosyntheses and functions. *Mar. Drugs*, 2011, **9**, 1101–1118.
20. Zhu, C. and Lee, Y., Determination of biomass dry weight of marine microalgae. *J. Appl. Phycol.*, 1997, **9**, 189–194.
21. Raja, R., Anbazhagan, C., Ganesan, V. and Rangasamy, R., Effect of *D. salina* in salt refinery effluent treatment. *Asian J. Chem.*, 2004, **16**, 1081–1088.
22. Stewart, C. N. and Via, L. E., A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. *Biotechniques*, 1993, **19**, 394–504.
23. Hejazi, M. A., Barzegari, A., Hosseinzadeh Gharajeh, N. and Hejazi, M. S., Introduction of a novel 18S rDNA gene arrangement along with distinct ITS region in the saline water microalga *Dunaliella. Saline Syst.*, 2010, **6**(1), 4–14.
24. Olmos, J., Paniagua, J. and Contreras, R., Molecular Identification of *Dunaliella* sp. utilizing the 18S rDNA Gene. *Lett. Appl. Microbiol.*, 2000, **30**(1), 80–84.
25. Asker, D., Beppu, T. and Ueda, K., *Zeaxanthinibacter enoshimensis* gen. nov., sp. nov., a novel zeaxanthin-producing marine bacterium of the family Flavobacteriaceae, isolated from seawater off Enoshima Island, Japan. *Int. J. Syst. Evol. Microbiol.*, 2007, **57**(4), 837–843.
26. Priyanka, S., Kirubagaran, R. and Mary Leema, J. T., Statistical optimization of BG11 medium for enhanced zeaxanthin productivity in *Synechococcus marinus* (NIOT-208). *Int. J. Pharm. Biol. Sci.*, 2019, **10**, 58–70.
27. Cheng, K. C., Ren, M. and Ogden, K. L., Statistical optimization of culture media for growth and lipid production of *Chlorella protothecoides* UTEX 250. *Bioresour. Technol.*, 2013, **128**, 44–48.
28. Plackett, R. L. and Burman, J. P., The design of optimum multi factorial experiments. *Biometrika*, 1946, **33**, 305–325.
29. Walne, P. R., *Culture of Bivalve Molluscs, 50 Years Experience at Conway*, Fishing News (Books) Ltd, 1974, pp. 1–173.
30. Mason, R. L., Gunst, R. F. and Hess, J. L., *Statistical Design and Analysis of Experiments*: *With Applications to Engineering and Science*, John Wiley, New York, USA, 2003, 2nd edn.
31. Priyanka, S., Kirubagaran, R. and Mary Leema, J. T., Optimization of process parameters for enzyme assisted ultrasonication extraction of zeaxanthin from *Trichodesmium thiebautii* (NIOT 152). In Proceedings of 2nd International Agricultural, Biological and Life Science Conference (AGBIOL 2020), 1–3 September 2020, Edirne, Turkey, 2020, pp. 1169–1187; ISSN 978-975-374-279-5.
32. Sarada, R., Sila, B., Suvendu, B. and Ravishankar, G. A., A response surface approach for the production of natural pigment astaxanthin from green alga *Haematococcus pluvialis*: effect of sodium acetate, culture age, and sodium chloride. *Food Biotechnol.*, 2002, **16**(2), 107–120.

33. Abdelhafez, A. A., Husseiny, S. M., Abdel-Aziz Ali and Sanad, H. M., Optimization of β-carotene production from agro-industrial by-products by *Serratia marcescens* ATCC 27117 using Plackett–Burman design and central composite design. *Ann. Agric. Sci*., 2016, **61**(1), 87–96.
34. Qin, J. Z., Song, F. F., Qiu, Y. F., Li, X. X. and Guan, X., Optimization of the medium composition of a biphasic production system for mycelial growth and spore production of *Aschersonia placenta* using response surface methodology. *J. Invertebr. Pathol*., 2013, **112**, 108–115.
35. Li, J. *et al.*, Reversed-phase high-performance liquid chromatography for the quantification and optimization for extracting 10 kinds of carotenoids in pepper (*Capsicum annuum* L.) leaves. *J. Agric. Food Chem*., 2017, **65**(38), 8475–8488.
36. Hosseini Tafreshi, A. and Shariati, M., *Dunaliella* biotechnology: methods and applications. *J. Appl. Microbiol*., 2009, **107**(1), 14–35.
37. Hosseinzadeh Gharajeh, N., Hejazi, M. A., Nazeri, S. and Barzegari, A., Characterization of an indigenous isolate, *Dunaliella tertiolecta* ABRIINW-G3, from Gavakhooni salt marsh in Iran based on molecular and some morpho-physiological attributes. *J. Agric. Sci. Tech*., 2012, **14**, 1579–1590.
38. Olmos-Soto, J., Paniagua-Michel, J., Contreras, P. R. and Trujillo, L., Molecular identification of β-carotene hyper-producing strains of *Dunaliella* from saline environments using species-specific Oligonucleotides. *Biotechnol. Lett*., 2002, **24**(5), 365–369.
39. Yang, F., Long, L., Sun, X., Wu, H., Li, T. and Xiang, W., Optimization of medium using response surface methodology for lipid production by *Scenedesmus* sp. *Mar. Drugs*, 2014, **12**, 1245–1257.
40. Kuehl, R. O., *Design of Experiments: Statistical Principles of Research Design and Analysis*, Duxbury Press, Pacific Grove, CA, USA, 2000, 2nd edn.
41. Ahmad, A. L., Ismail, S. and Bhatia, S., Optimization of coagulation–flocculation process for palm oil mill effluent using response surface methodology. *Environ. Sci. Technol*., 2005, **39**, 2828–2834.
42. Wijffels, R. H., Barbosa, M. J. and Eppink, M. H. M., Microalgae for the production of bulk chemicals and biofuels. *Biofuel. Bioprod. Biorefin*., 2010, **4**, 287–295.
43. Griffiths, M. and Harrison, S., Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *J. Appl. Phycol*., 2009, **21**, 493–507.
44. Del Campo, J. A., Rodríguez, H., Moreno, J., Vargas, M. A., Rivas, J. and Guerrero, M. G., Accumulation of astaxanthin and lutein in *Chlorella zofingiensis* (Chlorophyta). *Appl. Microbiol. Biotechnol*., 2004, **64**, 848–854.
45. Leesing, R., Kookkhunthod, S. and Nontaso, N., Microalgal lipid production by microalga *Chlorella* sp. KKU-S2. *Eng. Technol*., 2011, **52**, 499–502.
46. Shi, X. M., Zhang, X. W. and Chen, F., Heterotrophic production of biomass and lutein by *Chlorella protothecoides* on various nitrogen sources. *Enzyme Microb. Technol*., 2000, **1**, 312–318.
47. Del Campo, J. A., Morenom, J., Rodríguezm, H., Vargasm, M. A., Rivasm, J. and Guerrero, M. G., Carotenoid content of chlorophycean microalgae. Factors determining lutein accumulation in *Muriellopsis* sp. (Chlorophyta). *J. Biotechnol*., 2000, **76**, 51–59.
48. Hannon, M., Gimpel, J., Tran, M., Rasala, B. and Mayfield, S., Biofuels from algae: challenges and potential. *Biofuels*, 2010, **1**, 763–784.
49. Fan, J., Cui, Y., Wan, M., Wang, W. and Li, Y., Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. *Biotechnol. Biofuels*, 2014, **7**, 17.
50. Belotti, G., Caprariis, B. D., Filippis, P. D., Scarsella, M. and Verdone, N., Effect of *Chlorella vulgaris* growing conditions on bio-oil production via fast pyrolysis. *Biomass Bioenerg*., 2014, **61**, 187–195.
51. Celekli, A., Yavuzatmaca, M. and Bozkurt, H., Modelling of biomass production by *Spirulina platensis* as function of phosphate concentrations and pH regimes. *Bioresour. Technol*., 2009, **100**, 3625–3629.
52. Ben-Amotz, A. and Avron, M., The biotechnology of mass culturing of *Dunaliella* for products of commercial interest. In *Algal and Cyanobacterial Biotechnology* (eds Cresswell, R. D., Rees, T. A. V. and Shah, N.), Longman Scientific and Technical Press, London, UK, 1989, pp. 90–114.

ACKNOWLEDGEMENTS. We thank the Director, National Institute of Ocean Technology (NIOT), Chennai, and the Ministry of Earth Sciences (MoES), Government of India for funding, infrastructure facilities and support to carry out this research work.

Received 4 September 2019; revised accepted 16 October 2020

doi: 10.18520/cs/v119/i12/1997-2005