Original Article

Optimization of the Four Most Effective Factors on β-Carotene Production by *Dunaliella salina* Using Response Surface Methodology

Leila Zarandi-Miandoab[a,b], Mohammad-Amin Hejazi[c*], Mohammad-Bagher Bagherieh-Najjar[b] and Nader Chaparzadeh[a]

[a]Department of Biology, Azarbaijan Shahid Madani University, Tabriz, Iran. [b]Department of Biology, Faculty of Sciences, Golestan University, Gorgan, Iran. [c]Department of Food Biotechnology, Branch for Northwest & West region, Agricultural Biotechnology Research Institute of Iran, Agricultural Research, Education and Extension Organization (AREEO), Tabriz, Iran.

Abstract

During recent years, there was growing demand in using microalga valuable products such as β-carotene in health care. β-Carotene has anti-cancer and anti-aging properties for human. In *Dunaliella salina* cells, β-carotene has a major protecting role for biomolecules, when the production of reactive oxygen species is elevated. In the present study, we investigated the influence of the four most effective factors (light intensity, temperature, nitrate and salinity concentration) and their interactions on the β-carotene production and the total chlorophyll/β-carotene ratio in low light adapted *D. salina* cells. Box-Benken design and response surface methodology (RSM) were used for this purpose and optimization of the factor levels. Two models were developed to explain how β-carotene productivity and the total chlorophyll/β-carotene ratio may depend on the stress factors. Among the four stress variables for β-carotene production, light intensity was stronger than the others. Meanwhile, interaction between light intensity and salt concentration exhibited the most important effect on the total chlorophyll/β-carotene ratio. The predicted optimal conditions for maximum β-carotene productivity and minimum total chlorophyll/β-carotene ratio were derived from the fitted model in 200 µmol photons $m^{-2}s^{-1}$ light intensity, 25 ºC, 0.9 mM nitrate and 3.8 M NaCl. When the predicted condition was tested experimentally, the expected results were observed. This suggests that overproduction of β-carotene in *D. salina* under certain conditions depends on used light intensity for preadaptation. The step-wise manner applying of stresses may act as a beneficial strategy to β-carotene overproduction.

Keywords: *Dunaliella salina*; β-Carotene production; Optimization, RSM; Pre adaptation.

Introduction

There has been a growing interest in modelling approaches for optimization of critical metabolite production from microalgae, in recent years

* Corresponding author:
  E-mail: aminhejazi@abrii.ac.ir

(1-3). Carotenoids are important metabolites, with a variety of functions which comprise a large and diverse group in plants and alga and cover more than 700 different biochemical molecules (4, 5). β-Carotene, the most important carotenoid, is considered as an excellent additive for food and cosmetic industries because of its attractive colour and functional properties.

Thereby, universal demands lead to a market value of $261 million in 2010. This market is expected to grow to $334 million by 2018 at a compound annual growth rate of 3.1%. Because of medical effects on vision and heart health and antioxidant, anti-cancer (6), anti-aging, and immunomodulatory properties (7, 8), however; β-carotene have a prominent status in pharmaceutical research. All of these functions depend on the source of β-carotene production. Solely, pure and natural β-carotene is simply digestible and has shown positive effects in the treatment of disorders while synthetic β-carotene not. Natural β-carotene mainly produced by micro-algae and higher plants. *Dunaliella salina* is a unicellular green alga that is known as the only biological source accumulating natural β-carotene approximately 10 - 15% of its body weight. In *D. salina*, β-carotene represent up to 95% of total carotenoids (9). *D. salina* can be adapted to sudden changes in salt concentration, irradiance and nutrient availability in natural habitats (10, 11). The colour of *D. salina* cells changes from green to red under harsh conditions, such as elevated light intensity, high salinity, low nutrient supplies or extreme temperatures, (12, 13). The red *D. salina* cells accumulate more β-carotene in plastid sequestering structures, lipid globules named plastoglobolins (14, 15), in inter thylakoid space of the chloroplast instead of thylakoid membranes (16). Accumulation of β-carotene in plastoglobolins leads to a reduction of the chlorophyll/carotenoid ratio (17). This ability provides *D. salina* as an excellent biological source for commercial development (18). The low chlorophyll content of *D. salina* is an important factor for pure natural β-carotene (with more than 41% 9-cis isomer of β-carotene (19) extraction. The reduction of growth rate by abiotic stresses plays a crucial role in maximizing β-carotene production (20). Most studies regarding to reduction of growth rate and production of β-carotene have been carried out using only one or two factors (light intensity, temperature, nutrient or salt concentrations) at the same time (Table 1).

Table 1. Summary of the studies on the effect of different abiotic factors on β-carotene production on various species of *Dunaliella.*

Variables				Organism	Year	Researchers (Reference)
Light intensity	Temperature	Nutrients	Salinity			
*		*	*	*Dunaliella bardawil*	1983	(Ben-Amotz, Avron 1983) (21)
			*	*Dunaliella salina*	1987	(Al-Hasan, Ghannoum *et al.* 1987) (22)
*				*Dunaliella bardawil*	1990	(Lers, Biener *et al.* 1990) (23)
*				*Dunaliella*	1994	(Vorst, Baard *et al.* 1994) (20)
	*			*Dunaliella salina*	1996	(Mendoza, Jimenez Del Rio *et al.* 1996) (24)
		*	*	*Dunaliella*	1998	(Marin, Morales *et al.* 1998) (25)
*				*Dunaliella viridis*	2001	(Gordillo, Jimenez *et al.* 2001) (26)
*				*Dunaliella salina*	2003	(Hejazi, Wijffels 2003) (27)
*	*	*	*	*Dunaliella*	2005	(Dipak 2005) (28)
*		*	*	*Dunaliella salina*	2008	(Coesel, Baumgartner *et al.* 2008) (29)
		*		*Dunaliella salina*	2010	(Jesus, Rubens Filho 2010) (30)
		*	*	*Dunaliella salina*	2011	(Pasqualetti, Bernini *et al.* 2011) (16)
			*	*Dunaliella tertiolecta*	2011	(Tammam, Fakhry *et al.* 2011) (31)
			*	*Dunaliella salina*	2011	(Narvaez-Zapata, Rojas-Herrera *et al.* 2011) (10)
			*	*Dunaliella sp.*	2011	(Rad, Aksoz *et al.* 2011) (32)
	*			*Dunaliella*	2012	(Ali-zadeh 2012) (33)
		*		*Dunaliella salina*	2013	(Nikookar, Rowhani *et al.* 2013) (34)
*				*Dunaliella salina*	2013	(Fu, Guomundsson *et al.* 2013) (35)
*		*		*Dunaliella salina*	2013	(Dhanam, Dhandayuthapani 2013) (1)
*		*	*	*Dunaliella salina*	2014	(Fu, Paglia *et al.* 2014) (2)

Closed culture systems compared to open ponds potentially produce higher biomass and carotenoid concentration (19). To reduce the growth rate in industrial closed systems, high intensity of light (11), extreme temperatures (28), and high amount of salt (16) or limitation in nutrients (36) in culture medium must be applied. Two problems must be solved for application of high light intensities. First, digital control for stable temperatures is necessary and second, massive power usage is expensive in industry and large scale production systems. Therefore, finding a new strategy for optimization of β-carotene production under relatively low light irradiations could be remarkably economic. In spite of the large number of studies on this subject, finding a feasible model for optimization of β-carotene production is still controversial. Application of mathematical models for optimization of the fermentation process (30) is a relatively new strategy. Mathematical formulation of algal primary productivity was used since 1995 (37, 38). Response surface methodology (RSM) is a proper technique for modelling and optimizing a response affected by several variables. The aim of this study was to optimize the β-carotene production by various factors including light intensity, temperature, nitrate, and salt concentrations in *D. salina*. For this purpose, a statistical experimental design was employed rather than the one-factor-at-a-time approach. As responses of *D. salina* cells, the rate of β-carotene production and the rate of total chlorophylls/ β-carotene ratio have been measured in the mentioned bioprocess.

Experimental

Microalga strain and culture medium

D. salina strain CCAP 19/18 was provided by the branch of Northwest and West region, Agricultural Biotechnology Research of Iran [ABRII NW] (Tabriz). The cultures were grown in modified Johnson medium (27) during the years 2012 and 2013. Different concentrations of NaCl (2, 3 and 4 M) and or KNO_3 (0, 2.5 and 5 mM) were added to the media.

Cultivation conditions

In order to cultivate algal culture, white compact fluorescent lamps with 145W (NamaNor) were selected as light source (21). The experiments were conducted in two steps. First, in order to prephotoadaptation, *D. salina* was cultured at a light intensity of 50 μmol photon $m^{-2}s^{-1}$ and 20 ± 2 ºC for one week (media chemical composition not in limiting rate). Salinity, nitrate concentration, and temperature were selected according to many literatures and this light intensity was selected in order to prephotoadaptation, adapts the cells to low light before exposure to different levels of high lights. Then in a second step, the cells were exposed to combination of stressors for 2 weeks according to RSM designed experiments (Table 3). Nguyen and co-workers also used a two-step method (11). All experiments were done in triplicate in 250 mL Erlenmeyer flasks, containing 150 mL of fresh medium. The average of the initial cell number was 4×10^6 cells.mL^{-1}.

Variables measurement
Cell count

The cell number was determined by direct counting. The cells were immobilized and stained by Lugol´s solution and counted using 0.1 mm deep counting chamber (Neubauer) and light microscope (27).

Pigment analysis

To measure pigment concentrations, the precisely defined spectrophotometric method was applied. In brief, the pigments were extracted from algal pellets in 80% acetone after removal of cell debris by centrifugation at 8000 rpm ($5719 \times g$) for 5 min. Supernatant absorbency was measured at 412, 431, 460, and 480 nm with spectrophotometer (Perkin Elmer precisely-Lambda 35-UV/Vis spectrometer) (39) and pigments content (μg/ml β-carotene) calculated using the following formula. Final data of pigments content present by pg/cell. The suffices Ca, Cb and Cc stand for chlorophyll *a*, chlorophyll *b* and β-carotene, respectively.

$Ca = -1.709A412 + 11.970A431 - 2.998A460 - 5.708A480$

$Cb = -0.171A412 - 0.230A431 + 11.871A460 - 13.248A480$

$Cc = -0.430A412 + 0.251A431 - 4.376A460 + 13.216A480$

Table 2. Process variables and their experimental levels.

Variable	Symbol	Ranges and levels		
		-1	0	+1
Light intensity (μmol photons m^{-2}s^{-1})	X_1	200	600	1000
Temperature (°C)	X_2	25	30	35
Nitrate concentration (mM)	X_3	0	2.5	5
Salt concentration (M)	X_4	2	3	4

All measurements were performed in three replicates. For calculating the **r**ate of **β**-carotene production per **c**ell (RBC) and the **r**ate of **t**otal chlorophylls/β-carotene per **c**ell (RTC) the slope of the regression line for each response (Rate = *dy/dx*) was chosen (supplementary data 1). *dy* is (y_2-y_1), and y_1 is β-carotene content per cell or total chlorophylls/β-carotene per cell at first day and y_2 is β-carotene content per cell or total chlorophylls/β-carotene per cell at 14th days. *dx* is (x_2-x_1), and x_1 is first day of experiments and x_2 is 14th days of experiments. By this way the rates amounts may show positive or negative value.

Experimental design and statistical analysis

Response surface methodology (RSM) was applied to evaluate the effect of the four factors (light intensity, temperature, nitrate, and salt concentration) on β-carotene content of the cells. The factors were studied at the three different levels described in Table 2. Using Box-Behnken design as one of the mostly used response surface methods, a total of 25 experiments were carried out in randomized design (Table 3).

The selection of the ranges was based on several previous studies, indicated in Table 1. This design was considered as the suitable design for exploring quadratic response surfaces and constructing second order polynomial models by using the MINITAB16 software. For predicting the optimum point, a second order polynomial function was fitted to correlate the relationship between independent variables and responses. For 4 factors, the corresponding equation is according to equation (1):

$$(1) \quad Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{44} X^2_4$$

Where Y represents the response variable; β_0 is a regression coefficient (model constant), β_1, β_2, β_3, and β_4 are linear coefficients, and also β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are interaction effect coefficients; β_{11}, β_{22}, β_{33} and β_{44} are quadratic coefficients, and also X_1, X_2, X_3 and X_4 are the coded levels of independent variables. The terms $X_1 X_2$ and X_{2i} (i = 1, 2, 3 or 4) represent the interaction and quadratic terms, respectively. The significance of the regression coefficients was determined by Students *t*-test. The second order model equation was determined by Fishers test. The accuracy of the model was calculated by the regression coefficients R^2 and adjusted R^2 (Adj R^2). To identify the statistically significant terms, the analysis of variance (ANOVA) was employed. These statistical analysis could able us to judge on validity of the model and its reproducibility (40).

All used statistics were based on a confidence level of 95%, so $p \leq 0.05$ was considered to indicate a statistically significant difference and also used to show the power of the significance. For further interpretation of the obtained results, the Pareto analysis was performed. Using this analysis, the percentage effect of each factor on the responses can be calculated according to the following relationship (30):

$$Pi = \left(\frac{bi^2}{\sum bi^2}\right) \times 100 , \quad (i \neq 0)$$

Where b is the related regression coefficient of the factor.

Table 3. Experimental design matrix and responses based on experimental runs proposed by 4-factors Box-Behnken design. RBC is rate of β-carotene production per cell and RTC is rate of total chlorophylls/β-carotene per cell. Rate were calculated by division of changes in β-carotene amount or total chlorophylls/β-carotene per 14 days during origin and end of experiments Rate = dy/dx. Positive and negative amounts show positive or negative rates for each response.

	Independent variables				RBC			RTC		
RUN	X_1	X_2	X_3	X_4	Mean	±	SE	Mean	±	SE
1	200	25	2.5	3	**0.1807**	±	0.0105	-0.0156	±	0.0074
2	1000	25	2.5	3	-0.0909	±	0.0073	0.0042	±	0.0008
3	200	35	2.5	3	0.0368	±	0.0119	0.0087	±	0.0030
4	1000	35	2.5	3	-0.0131	±	0.0021	-0.1260	±	0.0168
5	600	30	0	2	-0.0235	±	0.0047	-0.0660	±	0.0070
6	600	30	5	2	-0.0120	±	0.0106	-0.0558	±	0.0090
7	600	30	0	4	0.0258	±	0.0090	-0.0723	±	0.0074
8	600	30	2.5	3	0.0286	±	0.0041	-0.0654	±	0.0061
9	600	30	5	4	-0.0723	±	0.0023	-0.0591	±	0.0036
10	200	30	2.5	2	0.1410	±	0.0180	0.0597	±	0.0067
11	1000	30	2.5	2	-0.0062	±	0.0012	-0.2179	±	0.0061
12	200	30	2.5	4	0.1324	±	0.0170	-0.0823	±	0.0032
13	1000	30	2.5	4	-0.0144	±	0.0084	0.0566	±	0.0067
14	600	25	0	3	-0.0035	±	0.0006	-0.0280	±	0.0090
15	600	35	0	3	-0.0494	±	0.0046	-0.0886	±	0.0015
16	600	25	5	3	0.0075	±	0.0013	-0.0284	±	0.0053
17	600	35	5	3	-0.0320	±	0.0101	-0.0578	±	0.0058
18	200	30	0	3	0.1243	±	0.0101	0.0378	±	0.0030
19	1000	30	0	3	-0.0095	±	0.0026	-0.1378	±	0.0222
20	200	30	5	3	0.1241	±	0.0114	-0.0429	±	0.0070
21	1000	30	5	3	-0.0404	±	0.0087	-0.0449	±	0.0066
22	600	25	2.5	2	-0.0234	±	0.0045	-0.0373	±	0.0088
23	600	35	2.5	2	-0.0075	±	0.0011	-0.0465	±	0.0017
24	600	25	2.5	4	-0.0110	±	0.0022	-0.0597	±	0.0033
25	600	35	2.5	4	-0.1050	±	0.0072	-0.0519	±	0.0022

X_1: Light intensity (μmol photons m^{-2}s^{-1}) X_2: Temperature (˚C) X_3: Nitrate concentration (mM) X_4: Salt concentration (M)

Results and Discussion

Box-Behnken Model Analysis

For the first time RSM was used to optimize two responses under four independent factors. In the present study, light intensity, temperature, nitrate, and salt concentrations were considered as the independent process variables and their individual and interactive effects on RBC and RTC (as responses) were investigated using the Box-Behnken design approach and the data are presented in Table 3. With respect to data values in Table 3 maximum positive RBC was achieved in experiment 1 while minimum

Table 4. Analysis of variance (ANOVA) for response surface quadratic models.

Source of variance	Response RBC					Response RTC				
	Sum of squares	Degree of freedom	Adjusted mean square	F-value	P	Sum of squares	Degree of freedom	Adjusted mean square	F-value	P
Regression	0.386	14	0.027	**78.76**	**0.000**	0.242	14	0.0173	**38.64**	**0.000**
L	0.208	1	0.208	**595.67**	**0.000**	0.046	1	0.046	**103.67**	**0.000**
T	0.013	1	0.013	**37.61**	**0.000**	0.009	1	0.009	**21.74**	**0.000**
N	0.002	1	0.002	**5.69**	**0.020**	0.001	1	0.001	2.43	0.123
S	0.003	1	0.003	**9.08**	**0.004**	0.002	1	0.002	**5.05**	**0.028**
L²	0.074	1	0.030	**87.62**	**0.000**	0.005	1	0.007	**17.59**	**0.000**
T²	0.016	1	0.026	**75.49**	**0.000**	0.005	1	0.005	**12.75**	**0.001**
N²	0.003	1	0.007	**21.93**	**0.000**	0.000	1	0.000	0.65	0.421
S²	0.009	1	0.009	**25.87**	**0.000**	0.000	1	0.000	1.15	0.288
LT	0.036	1	0.036	**105.25**	**0.000**	0.017	1	0.017	**39.97**	**0.000**
LN	0.000	1	0.000	2.03	0.159	0.022	1	0.022	**50.51**	**0.000**
LS	0.000	1	0.000	0.00	0.983	0.130	1	0.130	**290.55**	**0.000**
TN	0.000	1	0.000	0.09	0.769	0.000	1	0.000	1.63	0.207
TS	0.009	1	0.009	**25.81**	**0.000**	0.000	1	0.000	0.49	0.488
NS	0.009	1	0.009	**25.72**	**0.000**	0.000	1	0.000	0.02	0.902
Residual error	0.023	66	0.000			0.029	66	0.0004		
Pure Error	0.010	56	0.000			0.011	56	0.000		
Total	0.409	80				0.271	80			
R²	94.35%					89.13%				
R² adjusted	93.15%					86.82%				

negative RTC was achieved in experiment 11. The statistical significance of the Box-Behnken models were evaluated by the ANOVA test and the results were illustrated in Table 4 with R^2 and adjusted R^2 amounts. Interaction coefficient of LT, TS, and NS for RBC and alo LT, LN, and LS for RTC are significant at the same confidence level. In order to improve models, the insignificant model terms were omitted from quadratic equation. This resulted in following polynomial equation (2) and (3) based on the coded levels for RBC and RTC.
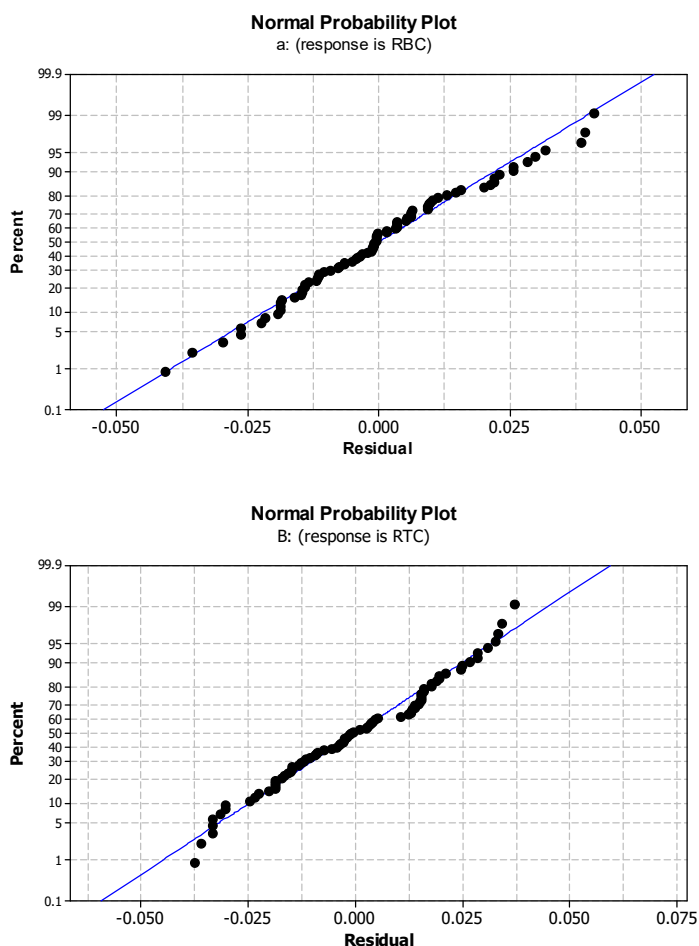
Normal Probability Plot
a: (response is RBC)

Normal Probability Plot
B: (response is RTC)

Figure 1. Normal probability plot for rate of β-carotene production per cell RBC (a) and rate of total chlorophylls/β-carotene per cell RTC (b) Rate were calculated by division of changes in β-carotene amount or total chlorophylls/β-carotene per 14 days during origin and end of experiments Rate = dy/dx.

(2) **RBC** = 0. 032064 -0.076143 L -0.019132 T -0.007439 N -0.009401 S +0.055438 LT -0.027451 TS -0.027406 NS +0.043806 L^2 -0.040659 T^2 -0.021915 N^2 -0.023804 S^2

(3) **RTC** = -0.073514 -0.035914 L -0.016447 T +0.007923 S -0.038627 LT +0.043422 LN +0.104140 LS +0.022192 L^2 +0.018893 T^2

The R^2 value for RBC and RTC models, indicate that the relationship between the variables and responses was good depicted by second order models. R^2 values indicate a high correlation between experimental and predicted values for both responses (Figure 1 a and b). In

a system with different number of independent variables, adjusted R^2 (Adj- R^2) is more suitable for evaluating the model goodness of fit (41). According to the current results Adj- R^2 values (93.15% and 86.82% for RBC and RTC, respectively) were close to the corresponding R^2 values (94.35% and 89.13% for RBC and RTC, respectively).

Screening of Main Effects
To visualize the importance of each factor in full quadratic models and to sort out which effect exerts a significant influence, the Pareto value was calculated and shown in Figure 2 indicating that the most important factor in RBC was
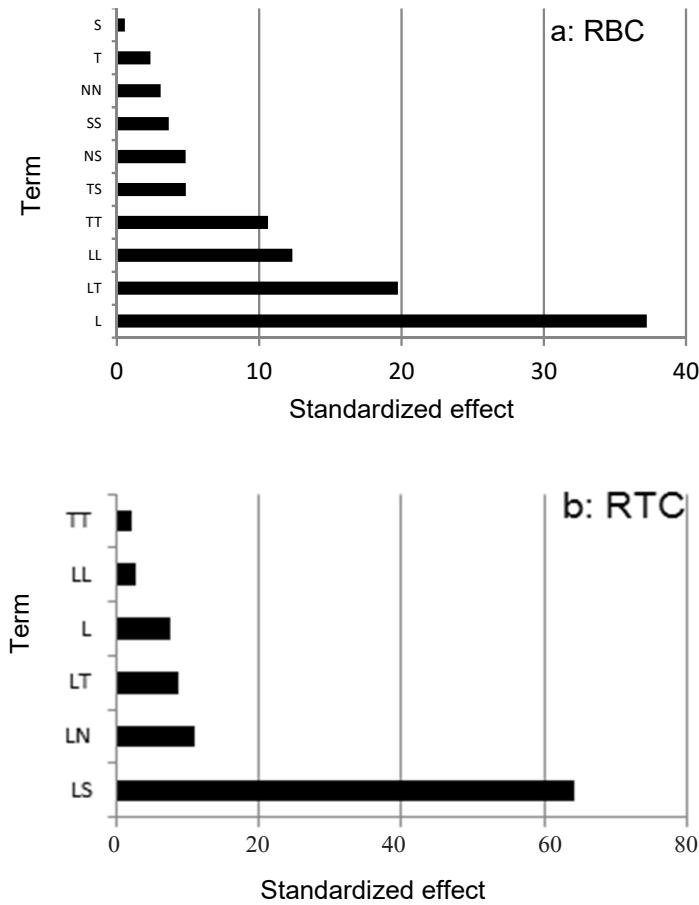
Figure 2. Pareto chart for rate of β-carotene production per cell RBC (a) and rate of total chlorophylls/β-carotene per cell RTC (b) Pareto values calculated using $Pi = \left(\frac{bi^2}{\sum bi^2}\right) \times 100$, (i ≠ 0) Where b is the related regression coefficient of the factor.

light intensity (Pareto amount = 37.24%). For RTC the interaction between light intensity and salt concentration exhibited the most important effect (Pareto amount = 64.34%). These data suggest that light is the most important factor for both RBC and RTC responses.

Effect of Variables on Rate of B-Carotene Production per Cell

Current knowledge about the interaction of salinity, low nutrient levels, high temperatures and high irradiance on β-carotene production by *D. salina* is scared. Then, we tried to optimize pure β-carotene production in this microalga under combined sever conditions, after preadaptation stage for growth. To study the interaction of all four variables on RBC, two dimensional contours were plotted keeping two variables constant at a certain level and the other two variables within the experimental ranges. As seen in Figure 3a, the maximum of RBC occurred when light intensity (200-250 μmol photons $m^{-2}s^{-1}$) and temperature (25-27.5 ºC) were at their minimum levels, while the nitrate (0mM) and salt concentration (4M) was kept at the minimum and maximum level, respectively. Also in the RBC polynomial equation resulted from our experiments, the light intensity and temperature exhibited considerable negative effects on RBC.

ANOVA Table (Table 4) and Pareto chart (Figure 2) confirm the significant impact of these two variables on RBC. Figure 3b indicate that high light intensities can slightly
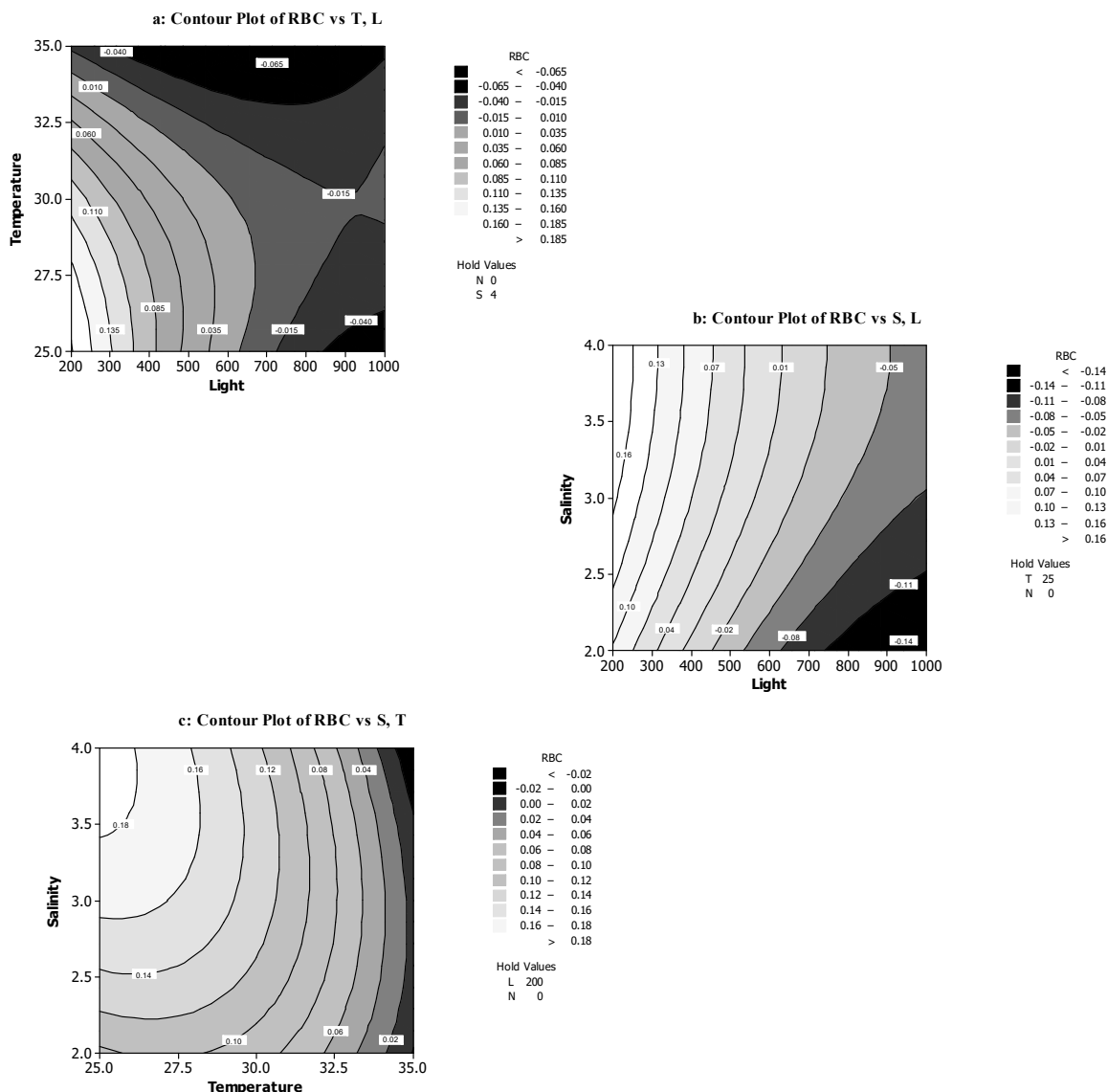
a: Contour Plot of RBC vs T, L

b: Contour Plot of RBC vs S, L

c: Contour Plot of RBC vs S, T

Figure 3. The response surface and contour plots of rate of β-carotene production per cell RBC **(a)** The function of temperature (°C) and light intensity (µmol photons m^{-2}s^{-1}) on RBC. **(b)** The function of light intensity (µmol photons m^{-2}s^{-1}) and salt concentration (M NaCl) on RBC. **(c)** The function of temperature (°C) and salt concentration (M NaCl) on RBC.

increase RBC, while salt concentration was 3-4 M. Figure 3b contour again shows the significant impact of light intensities on RBC. This confirms the results acquired from Figure 3a contour. Moreover, Figure 3c shows the temperature of 25-26 °C and salt concentration of 3.5-4 M enhanced RBC response when light intensity and nitrate concentration were kept at the minimum level (0 mM). In the present study,

salinity showed significant effect on RBC. The results illustrated in Table 4 clearly show this claim. Also, in experiments of 1, 10, 12, 18, and 20 in Table 3 the β-carotene production rate was maximum and varied between 0.12 and 0.18. For example the highest RBC occurred in 200 µmol photons m^{-2}s^{-1} light intensity, 25 °C and 2.5 mM nitrate concentration and 3M salt concentration condition (run1).
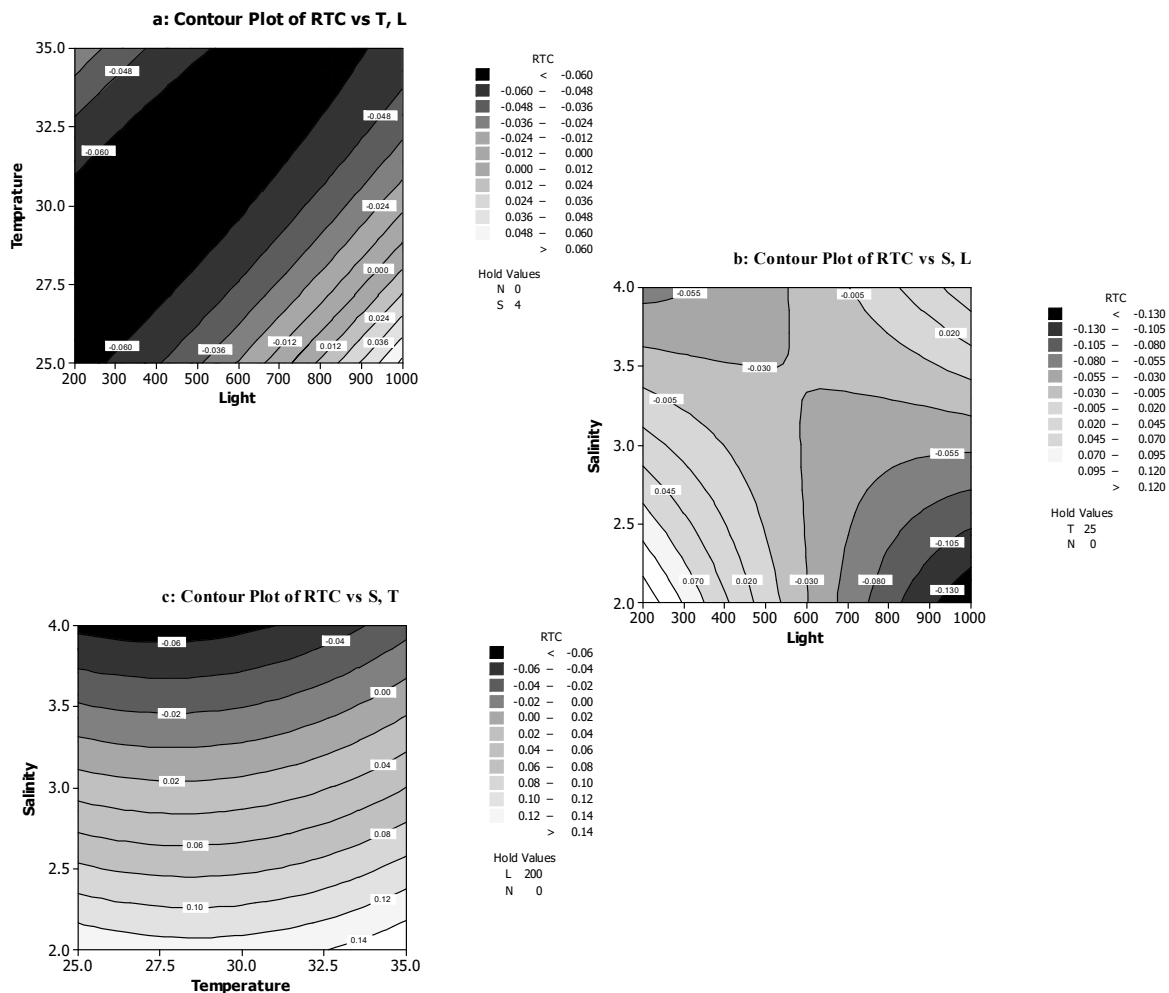
Figure 4. The response surface and contour plots of rate of total chlorophylls/β-carotene per cell RTC **(a)** The function of temperature (°C) and light intensity (μmol photons m⁻²s⁻¹) on RTC. **(b)** The function of light intensity (μmol photons m⁻²s⁻¹) and salt concentration (M NaCl) on RTC. **(c)** The function of temperature (°C) and salt concentration (M NaCl) on RTC.

Furthermore, these data show that the adapted cells to low light intensities (about 50 μmol photons m⁻²s⁻¹) when exposed to relatively high light intensities about 200-250 μmol photons m⁻²s⁻¹ had much more β-carotene production per cell. This finding was confirmed by other scientists (42-45, 28). Of course, it must be mentioned that our results about adopted cells to low light is a little different from previous data. Whereas Xu and co-workers (46) pointed to differences in *Dunaliella* isolates in this case.

Effect of Variables on Rate of Total Chlorophylls/ β -Carotene per Cell

Biosynthesis of carotenoids is a complex process which is coordinated with the biogenesis of chlorophylls and proteins of the photosynthetic apparatus (47). From this point of view, not only over production of β-carotene per cell is very important, but also its purity from other lipophilic molecules such as chlorophylls that could be co-extracted with β-carotene is important too. Hence, in the present study, the rate of total chlorophyll/ β-carotene was calculated.

The influence of the variables on RTC was illustrated in Figure 4 (a-c).

Figure 4a shows the RTC decreases in 200-900 μmol photons m⁻²s⁻¹ light intensity

range and 25-35 ºC temperature range. The polynomial equation of RTC indicated negative effect of light and temperature on RTC. Also ANOVA table confirmed the significance of light and temperature and salt concentration at $p \leq 0.05$ on RTC. Two regions of plot illustrate minimum amounts of RTC (Figure 4b). It was evident that at the first region, high level of salt concentration combined with relatively high level of light intensity was able to reduce RTC. While in the second region, low concentration of salt and very high light irradiation led to a decrease in RTC amounts. Interestingly, in spite of this fact that RTC in second region is smaller than RTC at first region, the authors believe that reaching a minimum amount of RTC by increasing salt concentration in culture medium is better than increasing light intensity. The interaction effect of light and salt concentration has a positive effect on RTC as indicated by the ANOVA analysis and the polynomial equation of RTC. Figure 4c illustrates that high salt concentration at 25-30 ºC can decrease RTC when light intensity was constant at relatively high about 200 µmol photons $m^{-2}s^{-1}$. In all contours of Figure 4 nitrate concentration was kept at low level (0 mM).

Thus, we can say that when algal culture was transferred from low light (50 µmol photons $m^{-2}s^{-1}$) to relatively high light (200 µmol photons $m^{-2}s^{-1}$), β-carotene production key in *D. salina* cell factory turn ON and at the same time chlorophyll degradation increased. Our interpretation is supported by Pirastru and his team believed the changes in the algal physiological state induced by intense conditions (for example 200 µmol photons $m^{-2}s^{-1}$ irradiance) (48) lead to changes in the activity in photosynthetic apparatus. These processes finally lead to the synthesis and accumulation of carotenoids. But, if the cells have to undergo higher light intensities such as 600 or 1000 µmol photons $m^{-2}s^{-1}$ after adaptation to low lights, they need to apply other ways to protect them and save viability except pigment response.

On the other hand, thereby β-carotene is a lipophilic high value compound and the low level of chlorophyll can be essential and very important in β-carotene purification, from the economic and industrial point of view, increasing the β-carotene production has a contrary relationship with total chlorophyll/ β-carotene ratio.

Finding Optimum Conditions for Maximizing RBC and Minimizing RTC

Many investigators have recently turned to find an optimum condition for maximum production using optimization tools. This study aimed to examine this method in the living organism of *D. salina* and the metabolic product of β-carotene. The experimental data were fitted into a full quadratic polynomial model for 4 independent variables. The optimization process consists of finding the combination of input variable settings that jointly optimize the response. Minitab software calculates an optimal solution and draws a plot (Figure 5), which helps to interactively change the input variable settings to perform sensitivity analysis and possibly improve the initial solution.

There are a few reports on the optimization of two related responses. Therefore, we used the quadratic model to predict the optimal conditions for β-carotene maximum production as well as minimum total chlorophyll/ β-carotene ratio. Since maximizing RBC was our priority, we decided to change weight and import values about 9 and 1 for RBC versus RTC, respectively. Surprisingly, when maximizing the RBC and minimizing RTC are considered for optimization, an optimum point of light intensity was introduced at 200 µmol photons $m^{-2}s^{-1}$, 25 ºC and 0.9 mM nitrate concentration in culture medium and 3.8 M of salt concentration. Figure 5, optimality demonstrated the plot to locate optimum factor levels for maximizing RBC and minimizing RTC. Based on this prediction and to confirm the adequacy model, the additional experiments were performed at optimum point and the results were showed in Table 5. These values were according to predicted responses and validate the findings of response surface optimization. Therefore, this observation shows that our models have feasible results.

Conclusion

Traditional optimization tools are very expensive and time consuming, and they cannot

Table 5. Obtained optimum values of the process variables and responses.

	Independent Variables				Response RBC		Response RTC	
	X1	X2	X3	X4	Experimental value	Predicted value	Experimental value	Predicted value
Optimum point	200	25	0.9	3.8	0.190 ± 0.012	0.191	-0.0626±0.0024	-0.0608
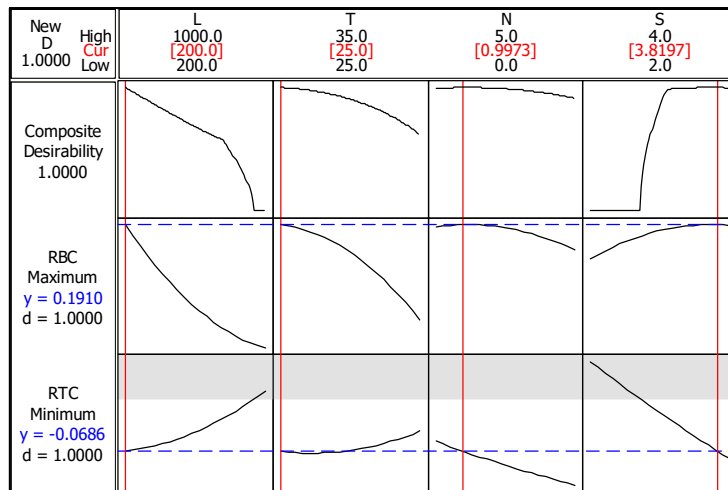
Figure 5. Optimality plot to locate optimum factor levels for maximizing rate of β-carotene production per cell **(RBC)** and minimizing rate of total chlorophylls/β-carotene per cell **(RTC).**

also clarify the factual interactions of the parameters of the experimental data and thus lead to misunderstanding of results that are used to choose the significant factors that influenced the process. A statistical approach in experimental design of biotechnological processes is confirmed to overcome the limitations of conventional optimization process and allows quick identification of the important factors and interactions between them. In the current work, the statistical methodology, the Box-Behnken design under RSM is employed in selecting the statistically significant variables and finding the optimal condition of those variables for maximizing pure natural β-carotene production by *D. salina* in a biological process. The present work is the first to report on the application of Box-Behnken design and Response surface methodology for the optimization of pure

natural β-carotene from *D. salina*. This study recommends a new guideline for applying stress in a step-wise manner in order to acquire rational pure natural β-carotene production. The result of optimization showed a significant increase in β-carotene content per cell whereas chlorophyll content per cell decreased in relatively high light (200 μmol photons m^{-2}s^{-1}) at 25 ºC. It can be due to preadaptation growth stage under low light (50 μmol photons m^{-2}s^{-1}). Thus, to achieve a significant amount of pure β-carotene, we need to use low light intensities for growth and acquire adequate amount of cells and then transfer adapted cells to new culture condition containing high salinity and limited amount of nitrate under relatively high light intensity. These results are considerably different from previous findings. It can be concluded that we can induce light stress without using high light

intensities. Our results indicate the optimized condition might result in a major reduction in the cost of pure natural β-carotene production and extraction. Additionally, the current results indicated that the experimental design worked in this project was a good mathematical tool for optimization of β-carotene production and quality of extracted β-carotene from *D. salina*.

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References

(1)) Dhanam DS and Dhandayuthapani K. Optimization of β-Carotene production by marine microalga-*Dunaliella salina*. *Int. J. Curr. Microbiol. Appl. Sci.* (2013) 2: 37-43.

(2) Fu W, Paglia G, Magnusdottir M, Steinarsdottir EA, Gudmundsson S, Palsson BO, Andresson OS and Brynjolfsson S. Effects of abiotic stressors on lutein production in the green microalga *Dunaliella salina*. *Microb. Cell Fact.* (2014) 13: 3.

(3) Morowvat M H and Ghasemi Y. Culture medium optimization for enhanced β-carotene and biomass production by *Dunaliella salina* in mixotrophic culture. *Biocatal. Agr. Biotechnol.* (2016) 7: 217-223.

(4) Lamers PP, Janssen M, De Vos RC, Bino RJ and Wijffels RH. Exploring and exploiting carotenoid accumulation in *Dunaliella salina* for cell-factory applications. *Trends Biotechnol.* (2008) 26: 631-8.

(5) Ye ZW, Jiang JG and Wu GH. Biosynthesis and regulation of carotenoids in *Dunaliella*: Progresses and prospects. *Biotechnol. Adv.* (2008) 26: 352-60.

(6) Nishino H, Murakoshi M, Ii T, Takemura M, Kuchide M, Kanazawa M, Mou XY, Wada S, Masuda M and Ohsaka Y. Carotenoids in cancer chemoprevention. *Cancer Metast. Rev.* (2002) 21: 257-64.

(7) Mayne ST. Beta-carotene, carotenoids, and disease prevention in humans. *FASEB J.* (1996) 10: 690-701.

(8) Ravi M, De SL, Azharuddin S and Paul SF. The beneficial effects of *Spirulina* focusing on its immunomodulatory and antioxidant properties. *Nutr. Diet. Suppl.* (2010) 2: 73-83.

(9) Jimenez C and Pick U. Differential stereoisomer compositions of β-carotene in thylakoids and in pigment globules in *Dunaliella*. *J. Plant Physiol.* (1994) 143: 257-63.

(10) Narvaez-Zapata JA, Rojas-Herrera R, Lopez-Uc Y and Sanchez-Estudillo L. Different physiological responses influenced by salinity in genetically related *Dunaliella salina* isolates. *Biotechnol. Lett.* (2011) 33:

1021-6.

(11) Nguyen A, Tran D, Ho M, Louime C, Tran H and Tran D. High light stress regimen on *Dunaliella salina* strains for carotenoids induction. *Integr. Food Nutr. Metab.* (2016)3: 347-350.

(12) Lamers PP, Van De Laak CC, Kaasenbrood PS, Lorier J, Janssen M, De Vos RC, Bino RJ and Wijffels RH. Carotenoid and fatty acid metabolism in light stressed *Dunaliella salina*. *Biotechnol. Bioeng.* (2010) 106: 638-48.

(13) Tran D, Doan N, Louime C, Giordano M and Portilla S. Growth, antioxidant capacity and total carotene of *Dunaliella salina* DCCBC15 in a low cost enriched natural seawater medium. World *J. Microbiol. Biotechnol.* (2014) 30: 317-22.

(14) Rabbani S, Beyer P, Lintig JV, Hugueney P and Kleinig H. Induced β-carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. *Plant. Physiol.* (1998) 116: 1239-48.

(15) Bonnefond H, Moelants N, Talec A, Mayzaud P, Bernard O and Sciandra A. Coupling and uncoupling of triglyceride and beta-carotene production by *Dunaliella salina* under nitrogen limitation and starvation. *Biotechnol. Biofuels*. (2017)10: 25.

(16) Pasqualetti M, Bernini R, Carletti L, Crisante F and Tempesta S. Salinity and nitrate concentration on the growth and carotenoids accumulation in a strain of *Dunaliella salina* (Chlorophyta) cultivated under laboratory conditions. *Transit. Water Bull.* (2011) 4: 94-104.

(17) Ben-Amotz A, Shaish A and Avron M. The biotechnology of cultivating *Dunaliella* for production of β-carotene rich algae. *Bioresource Technol*. (1991) 38: 233-5.

(18) Ben-Amotz A, Polle JE and Rao DS. The alga *Dunaliella*: biodiversity, physiology, genomics and biotechnology. Science Publishers Enfield, NH, USA (2009).

(19) Hosseini Tafreshi A and Shariati M. *Dunaliella* biotechnology: methods and applications. *J. Appl. Microbiol.* (2009) 107: 14-35.

(20) Vorst P, Baard RL, Mur LR, Korthals HJ and Van Den Ende H. Effect of growth arrest on carotene accumulation and photosynthesis in *Dunaliella*. *Microbiol.* (1994) 140: 1411-7.

(21) Ben-Amotz A and Avron M. On the factors which determine massive beta-carotene accumulation in the halotolerant alga *Dunaliella bardawil*. *Plant. Physiol.* (1983) 72: 593-7.

(22) Al-Hasan R, Ghannoum M, Sallal A, Abu-Elteen K and Radwan S. Correlative changes of growth, pigmentation and lipid composition of *Dunaliella salina* in response to halostress. *J. Gen. Microbiol.* (1987) 133: 2607-16.

(23) Lers A, Biener Y and Zamir A. Photoinduction of massive β-carotene accumulation by the alga *Dunaliella bardawil* kinetics and dependence on gene activation. *Plant Physiol.* (1990) 93: 389-5.

(24) Mendoza H, Jimenez Del Rio M, Garcia Reina G and Ramazanov Z. Low-temperature-induced *β*-carotene and fatty acid synthesis, and ultrastructural reorganization of the chloroplast in *Dunaliella salina* (Chlorophyta). *Eur. J. Phycol.* (1996) 31: 329-31.

(25) Marin N, Morales F, Lodeiros C and Tamigneaux E. Effect of nitrate concentration on growth and pigment synthesis of *Dunaliella salina* cultivated under low illumination and preadapted to different salinities. *J. Appl. Phycol.* (1998) 10: 405-11.

(26) Gordillo FJ, Jimenez C, Chavarria J and Niell FX. Photosynthetic acclimation to photon irradiance and its relation to chlorophyll fluorescence and carbon assimilation in the halotolerant green alga *Dunaliella viridis*. *Photosynth. Res.* (2001) 68: 225-35.

(27) Hejazi M and Wijffels R. Effect of light intensity on β-carotene production and extraction by *Dunaliella salina* in two-phase bioreactors. *Biomol. Eng.* (2003) 20: 171-5.

(28) Dipak S. Carotenoid production from microalga, *Dunaliella salina*. *Indian J. Biotechnol.* (2005) 4: 476-83

(29) Coesel SN, Baumgartner AC, Teles LM, Ramos AA, Henriques NM, Cancela L and Varela JCS. Nutrient limitation is the main regulatory factor for carotenoid accumulation and for *Psy* and *Pds* steady state transcript levels in *Dunaliella salina* (Chlorophyta) exposed to high light and salt stress. *Mar. Biotechnol.* (2008) 10: 602-11.

(30) Jesus SS and Rubens Filho M. Modeling Growth of Microalgae *Dunaliella Salina* under Different Nutritional Conditions. *American J. Biochem. Biotechnol.* (2010) 6: 279-83.

(31) Tammam AA, Fakhry EM and El-Sheekh M. Effect of salt stress on antioxidant system and the metabolism of the reactive oxygen species in *Dunaliella salina* and *Dunaliella tertiolecta*. *Afr. J. Biotechnol.* (2011) 10: 3795-808.

(32) Rad FA, Aksoz N and Hejazi MA. Effect of salinity on cell growth and β-carotene production in *Dunaliella* sp. isolates from Urmia Lake in northwest of Iran. *Afr. J. Biotechnol.* (2011) 10: 2282-9.

(33) Ali-zadeh G. Low temperature stress increases *Dunaliella* cells population resistance to the effect of chronic dozes of UV-B radiation.*CIBtech. J. Biotechnol.* (2012) 1:36-39.

(34) Nikookar K, Rowhani L, Mohsenzadeh S and Kholdebarin B. Growth and pigment development of *Dunaliella salina* Teod. in response to ammonium nitrate nutrition. *Mol. Biol. Res. Commun.* (2013) 2: 73-9.

(35) Fu W, Guomundsson O, Paglia G, Herjolfsson G, Andresson OS, Palsson BO and Brynjolfsson S. Enhancement of carotenoid biosynthesis in the green microalga *Dunaliella salina* with light-emitting diodes and adaptive laboratory evolution. *Appl. Microbiol. Biot.* (2013) 97:2395-403.

(36) Shaker S, Morowvat M H and Ghasemi Y. Effects of sulfur, iron and manganese starvation on growth, β-carotene production and lipid profile of *Dunaliella salina*. *J. Young Pharm*. (2017) 9: 43-46.

(37) Duarte P. A mechanistic model of the effects of light and temperature on algal primary productivity. *Ecol. Model.* (1995) 82: 151-60.

(38) Steele JH. Environmental control of photosynthesis in the sea. *Limnol. Oceanogr.* (2006) 7: 137-50.

(39) Eijckelhoff C and Dekker JP. A routine method to determine the chlorophyll *a*, pheophytin *a* and β-carotene contents of isolated Photosystem II reaction center complexes. *Photosynth. Res.* (1997) 52: 69-73.

(40) Faller D, Klingmüller U and Timmer J. Simulation methods for optimal experimental design in systems biology. *Simulation*. (2003) 79: 717-25.

(41) Hassani A, Alidokht L, Khataee A, Karaca S. Optimization of comparative removal of two structurally different basic dyes using coal as a low-cost and available adsorbent. *J. Taiwan Inst. Chem. E.* (2014) 45:1597-607.

(42) Gomez-Pinchetti JL, Ramazanov Z, Fontes A and Garcia-Reina G. Photosynthetic characteristics of *Dunaliella salina* (Chlorophyceae, Dunaliellales) in relation to β-carotene content. *J. Appl. Phycol.* (1992) 4: 11-5.

(43) Melis A, Neidhardt J and Benemann JR. *Dunaliella salina* (Chlorophyta) with small chlorophyll antenna sizes exhibit higher photosynthetic productivities and photon use efficiencies than normally pigmented cells. *J. Appl. Phycol*. (1998) 10: 515-25.

(44) Hejazi M, Andrysiewicz E, Tramper J and Wijffels R. Effect of mixing rate on carotene production and extraction by *Dunaliella salina* in two phase bioreactors. *Biotechnol. Bioeng.* (2003) 84: 591-6.

(45) Garcia F, Freile-Pelegrin Y and Robledo D. Physiological characterization of *Dunaliella* sp.(Chlorophyta, Volvocales) from Yucatan, Mexico. *Bioresource Technol*. (2007) 98: 1359-65.

(46) Xu Y, Ibrahim I M, Wosu C I, Ben-Amotz A and Harvey P. Potential of new isolates of *Dunaliella salina* for natural β-carotene production. *Biology* (2018) 7:14.

(47) Bohne F and Linden H. Regulation of carotenoid biosynthesis genes in response to light in *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta*. (2002) 1579: 26-34.

(48) Pirastru L, Darwish M, Chu FL, Perreault F, Sirois L, Sleno L and Popovic R. Carotenoid production and change of photosynthetic functions in *Scenedesmus* sp. exposed to nitrogen limitation and acetate treatment. *J. Appl. Phycol.* (2012) 24: 117-24.

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