Research Paper

Canthaxanthin biosynthesis by *Dietzia natronolimnaea* HS-1: effects of inoculation and aeration rate

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Abstract

The interest in production of natural colorants by microbial fermentation has been currently increased. The effects of D-glucose concentration (3.18-36.82 g/L), inoculum size (12.5 x 10^9-49.5 x 10^9 cfu cells/mL) and air-flow rate (1.95-12.05 L/L min) on the biomass, total carotenoid and canthaxanthin (CTX) accumulation of *Dietzia natronolimnaea* HS-1 in a batch bioreactor was scrutinized using a response surface methodology-central composite rotatable design (RSM-CCRD). Second-order polynomial models with high R^2 values ranging from 0.978 to 0.990 were developed for the studied responses using multiple linear regression analysis. The models showed the maximum cumulative amounts of biomass (7.85 g/L), total carotenoid (5.48 mg/L) and CTX (4.99 mg/L) could be achieved at 23.38 g/L of D-glucose, 31.2 x 10^9 cfu cells/mL of inoculation intensity and air-flow rate of 7.85 L/L min. The predicted values for optimum conditions were in good agreement with experimental data.

Key words: Dietzia, microbial canthaxanthin, batch bioreactor, response surface methodology, modeling.

Introduction

Recently, penetration of the fermentation-derived components into the various industries, especially food industry, is increasing in throughout the world. Carotenoids possess significant biological activities physiological functions in the prevention of cancer and heart diseases by quenching free radicals or singlet oxygen, enhancing in vitro antibody production, as well as providing visual systems with precursors of vitamin A (Veiga-Crespo *et al.*, 2005; Gharibzahedi *et al.*, 2014a, 2014b). They as an important class of bioactive compounds are responsible for bright yellow/orange colors of different plants, microorganisms and animals (Asker and Ohta, 1999). Production of carotenoids from microorganisms because of the selec-

tivity of the biological reactions is preferred to those produced by chemical synthesis (Gharibzahedi *et al.*, 2013a). Canthaxanthin (CTX, β-β-carotene-4,4-dione) is a ubiquitous keto-carotene that is of substantial industrial interest because of its widespread applications in nutraceutical, cosmetic, food and feed industries (Veiga-Crespo *et al.*, 2005). The microbial sources of CTX production has been reviewed by Nasri Nasrabadi and Razavi (2010b). Among the introduced sources such as *Corynebacterium michiganense*, *Micrococcus roseus*, *Brevibacterium* sp. KY-4313, *Bradyrhizobium* strain ORS278, *Gordonia jacobaea*, green micro-algae (*Chlorococcum* sp. strain MA-1, *Chlorella zofingiensis* and *Chlorella emersonii*) and an extremely halophilic *archaeon* (*Haloferax*

alexandrinus), the *Dietzia natronolimnaea* HS-1 is recognized as a promising producer of natural CTX (Khodaiyan *et al.*, 2007; Kumar *et al.*, 2011). This bacterium is Gram positive, catalase positive, and oxidase negative with orange colonies that isolated during a routine screening of pigmented microorganisms (Duckworth *et al.*, 1998). This CTX-synthesizing bacterium is aerobe and the aeration rate in the culture is a necessary parameter to assimilate the substrate as well as for growth rate, cell mass and carotenoids synthesis (Razavi *et al.*, 2007). Moreover, increasing inoculation rates alleviates fermentation problems caused by limited nitrogen (O'Connor-Cox and Ingledew, 1991). This factor can also increase the bacterial growth rate and can provide high-yield synthesis of carotenoid pigments (O'Connor-Cox and Ingledew, 1991).

Khodaiyan *et al.* (2007) studied the effects of different culture parameters including carbon sources, temperature, initial pH of culture, NaCl concentration, and light on the growth and CTX production by *D. natronolimnaea* HS-1. They reported that numerical optimization of pH, whey-lactose concentration and yeast extract can considerably increase CTX production by this bacterium in shake flask cultures (Khodaiyan *et al.*, 2008). To the best of our knowledge, there is no specific study on the effect of various rates of aeration and inoculation on CTX production by *D. natronolimnaea* HS-1. However, influence of aeration on the production of carotenoid pigments by *Sporidiobolus salmonicolor* (CBS 2636) and *Rhodotorula rubra-lactobacillus casei* subsp. *casei* co-cultures in a bioreactor were previously reported (Simova *et al.*, 2003; Valduga *et al.*, 2011).

With response surface methodology (RSM), several variables are examined simultaneously with a minimum number of trials, according to experimental designs, which enables to find interactions between variables (Myers and Montgomery, 2002). RSM also has the advantage of being less expensive and time-consuming than the classical methods. This methodology has been generally discussed in the literature for optimizing different fermentation processes (Kumar *et al.*, 2011; Yaakob *et al.*, 2011; Lee *et al.*, 2012).

Therefore, the aim of this work was to optimize the effects of air-flow rate, inoculation intensity and glucose concentration on the improvement of biomass, total carotenoid, CTX production from *D. natronolimnaea* HS-1 in a batch bioreactor.

Materials and Methods

Reagents and materials

D-glucose, yeast extract, peptone, malt extract, agar, iron as $FeCl_3 6H_2O$, and Antifoam 204 were purchased from Sigma-Aldrich (Sigma-Aldrich Co., United States). The pure ethanol (99.9%, v/v) and CTX standard were provided by the Bidestan Company (Qazvin, Iran) and the Bio-

process Engineering Laboratory (BPEL) (University of Tehran, Iran), respectively. Methanol and acetonitrile (HPLC grade) were supplied from Merck (Darmstadt, Germany).

Source microorganism and culture conditions

The strain of bacterium *D. natronolimnaea* HS-1 (DSM 44860) used in this work was obtained from Bioprocess Engineering Laboratory (BPEL), University of Tehran, Iran. It was kept on yeast/malt agar (YMA) plates containing 10 (g/L) glucose, 5 (g/L) yeast extract, 5 (g/L) peptone, 3 (g/L) malt extract and 15 (g/L) agar at pH 7.2. Every month, single colonies were transferred to a fresh plate, incubated for 4 days, and then maintained under refrigeration at 0 to 4 °C (Nasri Nasrabadi and Razavi, 2010a).

Inoculum preparation

Pure cultures of the strain of *D. natronolimnaea* HS-1 from the YMA were transferred into 500-mL Erlenmeyer flasks containing 100 mL of a GPY medium (per liter: 10 g glucose, 10 g peptone, 6 g yeast extract) as previously described by Khodaiyan *et al.* (2007). The flasks were inoculated with a loopful of the bacterium *D. natronolimnaea* HS-1 from an agar plate and incubated in an orbital incubator (model Stuart S150; Staffordshire, UK) at 180 rpm and 28 ± 2 °C. After 72 h, they applied to inoculate in a batch fermentation bioreactor.

Cultivation medium

Preliminary investigation demonstrated that the strain of *D. natronolimnaea* HS-1 was able to use D-glucose as an ideal carbon source for the CTX production. 10.0 mL of the prepared inoculum into 500-mL Erlenmeyer flasks containing 100 mL growth medium with 10 g/L pepton, 6 g/L yeast extract, 30 ppm Fe^{3+} and D-glucose as carbon source at various concentration (3.18, 10, 20, 30 and 36.82 g/L) was added. Then, they were incubated in a rotary shaker (180 rpm) under constant illumination (600 ± 50 lux) provided by cool white fluorescent lamps (30W, Resell-Shark, Switzerland) (Razavi *et al.*, 2007). Subsamples were periodically harvested during a 7-day period.

Khodaiyan *et al.* (2007) determined the optimum conditions (pH, 7; temperature, 31 °C; illumination, 600 ± 50 luxs) for CTX production by the wild type of this bacterium. According to these conditions, a Bioflo III (New Brunswick Scientific, USA) containing 2.2 L of the above mentioned GPY medium was used as a batch bioreactor for CTX production. The pH-value during the fermentation culture was controlled at 7 using 2 M NaOH and 2 M HCl solutions. The bioreactor was operated isothermally at 31 °C with a stirring rate of 350 rpm. Foam was controlled by the addition of 25% Antifoam 204. Treatments of aeration rate and inoculation size in the bioreactor were conducted according to the optimization procedure based on a three-

factor central composite rotatable design (CCRD, Tables 1 and 2). Samples during certain times were withdrawn from the batch fermenter to evaluate the growth rate and level of CTX production.

Analytical methods

Biomass dry weight and glucose content determination

In order to determine biomass dry weight (BDW), a concentrated cell suspension was diluted with a suitable amount of the synthetic mineral medium to give an optical density (OD) ranging from 0.1 to 1.0 when measured at 600 nm. A portion (10 mL) of each dilution was filtered through a marked, dried (at 65 °C for 12 h) and pre-weighed membrane filter (Sigma-Aldrich Co., pore diameter 0.2 µm, USA). All filters were then dried at 105 °C to constant weight (48 h) and placed in a desiccator to cool down. The biomass dry weight was calculated as the difference of the filter weight before and after the procedure. Finally, the calibration curve was constructed as the dependence of the OD on the BDW concentration (Gharibzahedi *et al.*, 2012b). Glucose concentration was also assayed by the Miller method using 3,5-dinitrosalisylic acid (DNS) on cell-free supernatants filtered through 0.2 µm filters (Miller, 1959).

Carotenoid extraction and pigment analysis

Aliquots (10-mL) of cultures at the appropriate times during the fermentation process were taken from the bioreactor and centrifuged at 7500 *g* (2-4 °C) for 7.5 min. The supernatant to measure the glucose content was collected. Then, the cell pellets were washed twice with physiological water (NaCl; 9 g/L in deionized water) and centrifuged again. These cells were resuspended three times in 3 mL of pure ethanol by vortexing for 5 min and centrifuged again to extract the pigment. A water bath (45 °C) was also used to completely extract the pigments. The carotenoid extracts were subsequently filtered through a 0.2 µm hydrophobic fluorophore membrane (Sigma-Aldrich Co., USA). The absorbance of the total carotenoid content was determined using a UV-visible spectrophotometer (U-5100 model, Hitachi, Japan) at 474 nm. Total carotenoid content was calculated by using the formula (Eq. 1) recommended by Nasri Nasrabadi and Razavi (2010b):

$$\text{Total carotenoid } (\mu g / L) = \frac{A_{474} \times V_s \times 10^9}{A_{1 \text{ cm}}^{1\%} \times 100} \quad (1)$$

where A_{474}, V_s and $A_{1 \text{ cm}}^{1\%}$ are the absorbance maximum of total carotenoid in ethanol, the volume of sample solution, and the specific absorption coefficient of total carotenoid for a 1% solution in a 1 cm cell (in ethanol, $A_{1 \text{ cm}}^{1\%}$), respectively.

A Knauer (Berlin, Germany) HPLC system including a k-1001 HPLC pump, a k-1001 solvent organizer, an on-line degasser, a dynamic mixing chamber and a UV-visible detector (K-2600, Knauer, Germany) was used for the determination of individual carotenoids according to the modified method of Razavi *et al.* (2006). The separation was performed on a Lichrospher 100 RP-18 silica column (5.0 mm, 250 x 4 mm) at 35 °C. The isocratic mobile phase used was acetonitrile/methanol (80:20, v/v) at a flow rate of 2 mL/min.

Experimental design and statistical analysis

The Design Expert (Trial Version 7.1.3, Stat-Ease Inc., Minneapolis, MN, USA) was used for regression and graphical analyzes of the data obtained. RSM was chosen as the method for the software to calculate the optimum value in the software. The effects of three parameters of glucose concentration (10-30 g/L, X_1), inoculation intensity (20 x 10^9-42 x 10^9 cfu cells/mL, X_2) and aeration rate (4-10 L/L min, X_3) on the biomass (Y_1), total carotenoid (Y_2) and CTX (Y_3) of produced by *D. natronolimnaea* HS-1 were evaluated. Twenty treatments were conducted based on the central composite rotatable design (CCRD), each at five coded levels -1.68, -1, 0, 1 and 1.68 (Table 1). Experiments were randomized in order to minimize the effects of unexplained variability in the actual responses due to extraneous factors. The center point was repeated six times to calculate the repeatability of the method (Myers and Montgomery, 2002; Gharibzahedi *et al.*, 2013c). The applied design was integrated to (i) determine a reasonable relationship between three independent variables and each response, and (ii) to find the optimum level of the independent variables resulting in the desirable objectives. Multiple regression coefficients were determined by employing the least-squares technique to predict linear and polynomial models for the response variable (Myers and Montgomery, 2002). The behaviour of the response surface was evaluated for the response function (Y_i, the predicted response) using the regression polynomial equation. The generalized polynomial model proposed for predicting the response variables is given as:

$$Y = \beta_{k0} + \sum_{i=1}^{4} \beta_{ki} x_i + \sum_{i=1}^{4} \beta_{kii} x_i^2 + \sum_{i<j=2}^{4} \beta_{kij} x_i x_j \quad (2)$$

where Y is the predicted response; β_{k0}, β_{ki}, β_{kii} and β_{kij} represent regression coefficients; and x_i, x_j are the coded independent factors.

The suitability of the fit of the polynomial model equation was tested by the R^2 (coefficient of determination), adjusted-R^2 (R^2-adj), coefficient of variation (CV), the prediction error sum of squares (PRESS) and adequate precision (ADP). The *PRESS* statistic and ADP are calculated as were previously reported by Gharibzahedi *et al.* (2013b).

A reduced model was obtained by analyzing the coefficients regression model using ANOVA (p < 0.05) and removing the non-significant coefficients from the initial model. Surface plots were generated by assigning constant

Table 1 - Experimental domain of central composite rotatable design (CCRD).

Independent variables	Unit	Symbol		Coded variables levels				
		Uncodified	Codified	-1.682 (-α)	-1	0	+1	+1.682 (+α)
Glucose concentration	g/L	X_1	x_1	3.18	10	20	30	36.82
Inoculation intensity	x 10^9 cfu cells/mL	X_2	x_2	12.5	20	31	42	49.5
Aeration intensity	L/L min	X_3	x_3	1.95	4	7	10	12.05

(zero) values to two of the three variables and solving the fitted equations as a quadratic equation in the remaining one variable. Subsequently, five additional experiments were conducted to verify the validity of the statistical experimental strategies.

Results and Discussion

Model fitting

The levels of factors (glucose concentration (X_1), inoculation intensity (X_2) and aeration intensity (X_3)) and the effect of their interactions on the production of biomass, total carotenoid and CTX were determined through the RSM-CCRD. Twenty experiments were performed at different combinations of the factors (Table 2). Regression analysis and ANOVA were applied for fitting the models

and to evaluate the statistical significance of the terms. The estimated regression coefficients of the polynomial models for the response variables, along with the corresponding R^2, R^2-adj, CV, PRESS and ADP are illustrated in Table 3. The mathematical models for the biomass (Y_1), total carotenoid (Y_2), and CTX (Y_3) levels with the coefficients in actual values were expressed as follows:

$$Y_1 = 7.71 + 0.54X_1 + 0.33X_3 - 0.84X_1^2 - 0.35X_2^2 - 0.87X_3^2 + 0.25X_1X_2 + 0.29X_1X_3 \tag{3}$$

$$Y_2 = 5.28 + 0.50X_1 + 0.15X_2 + 0.12 X_3 - 0.66X_1^2 - 0.45X_2^2 - 0.33X_3^2 - 0.25X_2X_3 \tag{4}$$

$$Y_3 = 4.91 + 0.38X_1 + 0.21X_3 - 0.70X_1^2 - 0.45X_2^2 - 0.40X_3^2 - 0.13X_1X_2 - 0.11X_2X_3 \tag{5}$$

Table 2 - Experimental design with the observed responses and predicted values for the production of biomass, carotenoid and CTX using RSM-CCRD.

Run	Independent variables			Biomass (Y_1, g/L)		Total carotenoid (Y_2, mg/L)		CTX (Y_3, mg/L)	
	X_1	X_2	X_3	Experimental*	Predicted	Experimental*	Predicted	Experimental*	Predicted
1	-1	-1	-1	5.17	5.08	2.75	2.67	2.47	2.48
2	+1	-1	-1	5.41	5.31	3.89	3.90	3.50	3.51
3	-1	+1	-1	5.31	5.08	3.68	3.60	3.31	3.22
4	+1	+1	-1	5.79	5.83	4.71	4.59	3.48	3.41
5	-1	-1	+1	5.73	5.74	3.44	3.59	3.03	3.12
6	+1	-1	+1	6.37	6.66	4.36	4.47	4.05	4.16
7	-1	+1	+1	4.42	4.57	3.37	3.39	3.08	3.09
8	+1	+1	+1	6.84	6.99	4.15	4.26	3.90	3.91
9	0	0	0	7.81	7.59	5.12	5.22	4.81	4.82
10	0	0	0	7.75	7.59	5.26	5.22	4.89	4.82
11	0	0	0	7.89	7.83	5.33	5.28	4.95	4.98
12	0	0	0	7.61	7.83	5.42	5.28	5.04	4.98
13	-1.68	0	0	4.29	4.41	2.64	2.64	2.32	2.32
14	1.68	0	0	6.42	6.22	4.36	4.31	3.63	3.61
15	0	-1.68	0	6.82	6.78	3.91	3.81	3.71	3.59
16	0	1.68	0	6.69	6.65	4.26	4.32	3.65	3.74
17	0	0	-1.68	4.42	4.68	4.03	4.20	3.36	3.45
18	0	0	1.68	6.13	5.80	4.81	4.59	4.27	4.15
19	0	0	0	7.51	7.69	5.12	5.34	4.76	4.93
20	0	0	0	7.65	7.69	5.42	5.34	5.02	4.93

* Mean of triplicate determinations.

The statistical analysis showed that the polynomial models were adequate (p < 0.0001), showing no significant lack-of-fit (p > 0.05) with very satisfactory values of R^2 for all responses. The R^2 values for biomass, total carotenoid and CTX were 0.97, 0.98 and 0.99, respectively (Table 3); indicating that a high percentage of response variations were explained by the response surface models. It should be noted that adding a variable to the model will always increase R^2, regardless of whether the additional variable is statistically significant or not. A high value of R^2 does not always imply the adequacy of the model. Therefore, it is more suitable to apply an adj-R^2 of over 90% to evaluate the model adequacy (Gharibzahedi *et al.*, 2012a, 2012b). The adj-R^2 values were found to be 0.95, 0.96 and 0.98 for biomass, total carotenoid and CTX, respectively (Table 3). The low PRESS (1.27-7.10) values propose for the adequacy of the fitted quadratic models for predictive applications (Table 3). The CV values were 4.34, 4.13 and 3.24 for biomass, total carotenoid and CTX, respectively (Table 3). ADP measures the signal-to-noise ratio. A ratio greater than four is desirable (Gharibzahedi *et al.*, 2012b). The ADP amounts for the proposed models were between 16.16 and 27.51. The comparison between the actual response values obtained from experimental data and the predicted response values based on the quadratic regression models is depicted in Figure 1a-c. This figure demonstrates that the models cover the experimental range of studies sufficiently.

Biomass production

The results illustrated in Table 3 show that linear D-glucose concentration and aeration rate had significant effects on the biomass (p < 0.0001; p < 0.01). Quadratic effects of all independent variables were also significant (Table 3). Moreover, the interactions of D-glucose concentration-inoculum size and D-glucose concentration-aeration rate were significant (Figures 2a and b). Based on the sum of squares, the importance of the independent variables on the biomass production by *D. natronolimnaea* HS-1 could be ranked in the following order: aeration rate > D-glucose concentration > inoculation intensity. Since *D. natronolimnaea* HS-1 is a strictly aerobic microorganism, the high aeration supplied better homogeneity in the culture medium during submerged fermentation, and more availability of nutrients and oxygen, leading to higher production of biomass (Frengova and Beshkova, 2009; Valduga *et*

Table 3 - ANOVA and regression coefficients of the second-order polynomial models for the response variables.

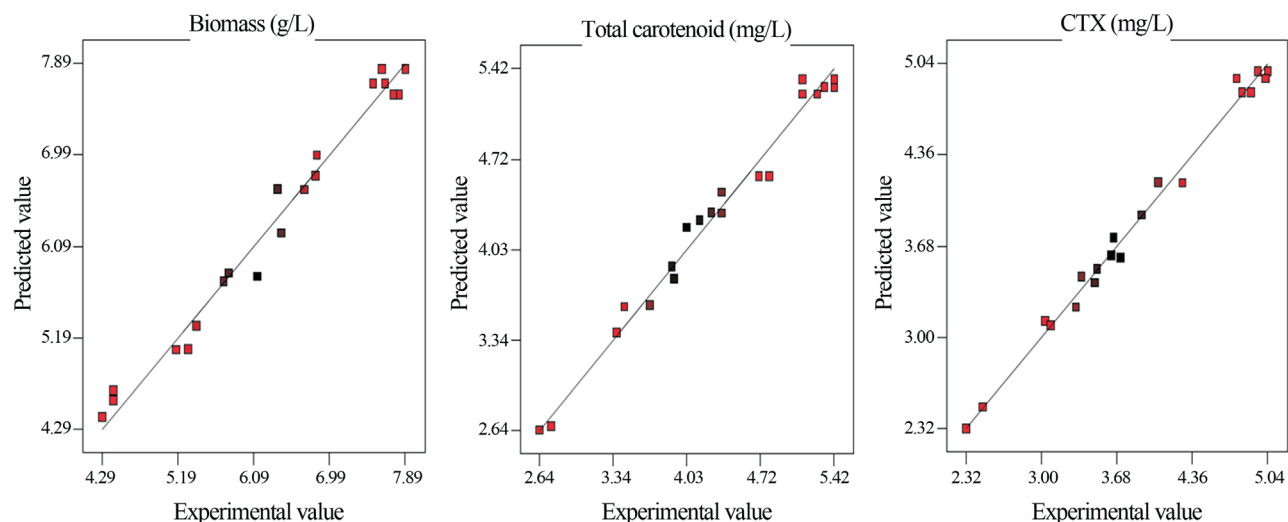| Source	DF	Biomass (Y_1, g/L)			Total carotenoid (Y_2, mg/L)			CTX (Y_2, mg/L)		
		Coefficient	Sum of squares	p-value	Coefficient	Sum of squares	p-value	Coefficient	Sum of squares	p-value
Model	9	7.71	26.59	< 0.0001	5.28	13.57	< 0.0001	4.91	13.25	< 0.0001
Linear										
β_1	1	0.54	3.97	< 0.0001	0.50	3.35	< 0.0001	0.38	2.01	< 0.0001
β_2	1	-	0.02	ns	0.15	0.31	0.01	-	0.03	ns
β_3	1	0.33	1.52	0.002	0.12	0.19	0.04	0.21	0.59	0.0003
Quadratic										
β_{11}	1	-0.84	10.17	< 0.0001	-0.66	6.23	< 0.0001	-0.70	6.97	< 0.0001
β_{22}	1	-0.35	1.72	0.001	-0.45	2.93	< 0.0001	-0.45	2.87	< 0.0001
β_{33}	1	-0.87	10.87	< 0.0001	-0.33	1.59	0.0001	-0.40	2.29	< 0.0001
Interaction										
β_{12}	1	0.25	0.51	0.03	-	0.007	ns	-0.13	0.14	0.01
β_{13}	1	0.29	0.68	0.01	-	0.02	ns	-	0.05	ns
β_{23}	1	-	0.23	ns	-0.25	0.52	0.003	-0.11	0.11	0.03
Residual	8		0.60			0.25			0.13	
Lack- of-fit	5		0.55	0.077		0.19	0.305		0.08	0.460
Pure error	3		0.05			0.06			0.04	
Total	19		27.42			13.84			13.45	
R^2		0.978			0.981			0.990		
Adj-R^2		0.953			0.961			0.980		
CV (%)		4.34			4.13			3.24		
PRESS		7.10			2.68			1.27		
ADP		16.16			19.57			27.51		

Figure 1 - Linear correlation between observed and predicted values for biomass, total carotenoid and CTX produced by *D. natronolimnaea* HS-1.

al., 2011). The carbon substrate provides the energy necessary for product formation through intermediary metabolism. Thus, an increase in sugar concentration will result in a proportional increase in biomass (Gharibzahedi *et al.*, 2012a, 2012b). However, the reduction of biomass at concentrations higher than 23 g/l may be explained by the fact that the substrate inhibition (Figures 2a and b). The individual optimum condition indicated that maximum biomass production (7.85 g/L) by *D. natronolimnaea* HS-1 in a batch bioreactor would be obtained at D-glucose concentartion of 23.67 g/L, inoculation intensity of 31.16×10^9 cfu cells/mL and aeration rate of 7.76 L/L min using response surface plots and response optimizer.

Carotenoid production

Table 3 indicates that all linear and quadratic terms of independent variables have significant effect ($p < 0.05$; $p < 0.001$; $p < 0.0001$) on the total carotenoid. As shown in Table 3, the quadratic effect of D-glucose concentartion followed by the linear effect of D-glucose concentartion demonstrateed the most significant ($p < 0.05$) effect on the changes of total carotenoid value. The bacterium rapidly consumed D-glucose for providing the required ATP for cell growth. The glucose concentration is one of the most important variables in fermentation of *Blakeslea trispora* for the biosynthesis of β-carotene (Choudari and Singhal, 2008). Aksu and Eren (2007) also reported that an increase in the rates of carotenoids formation by *R. glutinis* from 14.2 to 69 mg/L by increasing the glucose concentration from 2.5 to 20 g/L. It showed only a significant interaction effect between inoculation intensity and aeration rate at $p < 0.01$ with a negative effect (Table 3). However, the carotenoids biosynthesis could be improved with an enhancement in quantities of these factors up to a specified level (Figure 2c). *D. natronolimnaea* HS-1 is a microorganism that depends on oxygen supply, showing that the determi-

nation of optimum conditions of aeration rate has a vital importance due to improvements in the characteristics of the mass transfer related to glucose, carotenoid and oxygen. Aksu and Eren (2007) also obtained an increase in specific growth rates and carotenoids formation (112.8 mg/L) by *R. mucilaginosa* using optimal rates of aeration (0-2.4 vvm). Cho *et al.* (2002) demonstrated that a high aeration rate (3.5 vvm) gave rise to significantly improved red pigment production by *Paecilomyces sinclairii* in a batch bioreactor. They pointed out that this production increase may be related to the result from oxidation reaction of the pigment produced thereby increasing the optical density. The obtained results in this work also agreed with some reports on enhanced carotenoid formation in *Rhodotorula* sp. by intensive aeration rates (Sakaki *et al.*, 1999; Simova *et al.*, 2003; Malisorn and Suntornsuk, 2008). However, Nasri Nasrabadi and Razavi (2011) found that when the value of temperature remained steady, β-carotene biosynthesis by a mutant strain from *R. acheniorum* was increased with an increasing level of aeration rate. This discrepancy might be attributed to differences in the used microorganism type, the chemical composition of the medium, the aerobic growth system, and, generally, the conditions under which the fermentation takes place (Frengova and Beshkova, 2009). It seems that after inoculation of desirable numbers of the microorganisms in active state (32×10^9 cfu cells/mL), the cells entered exponential growth immediately without a lag phase, and the cell density enhanced gradually until it reached the maximum by about 6 to 7 days of the fermentation. The growth of cells was ended upon reaching the maximum cell density. In next step, they entered stationary phase and steadily converted to carotenoid pigment. The reduction of the initial phase time by increasing inoculation rate has been previously reported by several authors (Augustin *et al.*, 2000; N'Guessan *et al.*, 2008). As considered in Figure 2c, the carotenoid levels were de-
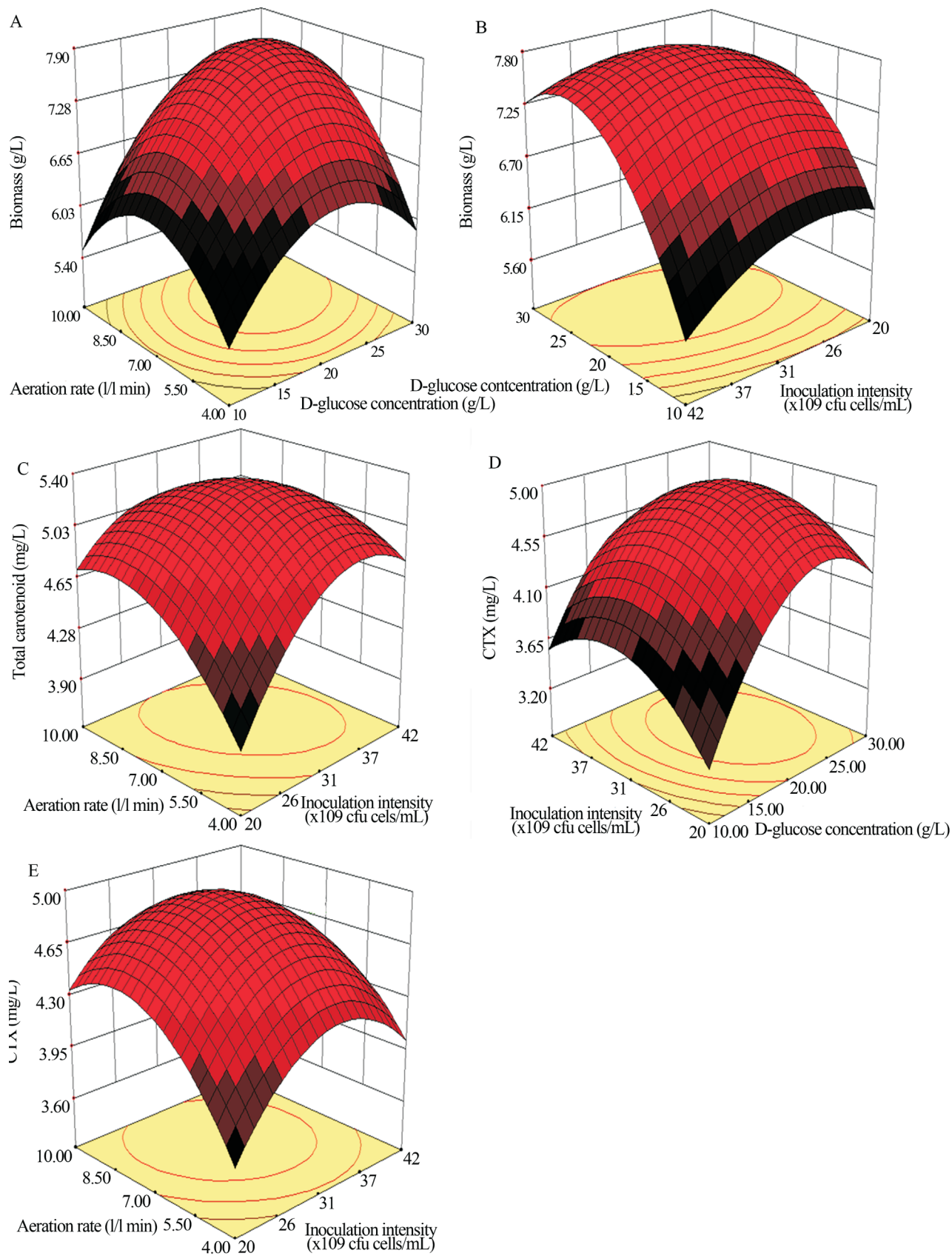
Figure 2 - 3D surface plots showing the significant (p < 0.05) interaction effects on the variation of the biomass (a and b), total carotenoid (c) and CTX (d and e) synthesized by *D. natronolimnaea* HS-1.

creased by increasing inoculum size from 32×10^9 to 42×10^9 cfu cells/mL. Since glucose was utilized as carbon and energy sources by *D. natronolimnaea* HS-1 during the fermentation, the consumption rate of glucose is related to the cells concentration. Therefore, the decrease of carotenoid pigments in the high inoculation intensity can be probably attributed to the reduction of glucose concentration. Wu *et al.* (2003) also stated that a reasonable explanation for the reducing mycelial growth by increasing inoculum size could be the yield limitation by the amount of nutrients available in the medium. According to the analysis by the "Design-expert" software, the optimal values of the three key factors for maximum production of carotenoid (5.39 mg/L) by *D. natronolimnaea* HS-1 were D-glucose concentration of 23.69 g/L, inoculum size of 32.41×10^9 cfu cells/mL, and air-flow rate of 7.28 L/L min.

CTX production

The results of ANOVA and response surface plots showed that the linear effect of D-glucose concentration and aeration rate on the CTX produced by *D. natronolimnaea* HS-1 was significant ($p < 0.0001$; $p < 0.001$). Moreover, the quadratic effects of all parameters are significant were highly significant ($p < 0.0001$). The findings clearly revealed that the mutual interactions of D-glucose content and inoculation intensity and, aeration rate and inoculation intensity were significant on the CTX (Table 3; Figure 2d and e). The variables with the largest effect were the quadratic term of D-glucose concentration followed by the quadratic terms of inoculation intensity and aeration rate. The effect of inoculation intensity and D-glucose concentration on the CTX production is also presented in Figure 1d. It shows a linear effect of these factors. Increasing D-glucose content up to a certain value (23 g/L) increases the CTX yield but which then decreases at increased concentrations. The CTX enhancement is probably due to the stimulation of precursors of the carotenoid pathway, such as mevalonic acid or related substances by the suitable quantities of glucose present in the media which could have enhanced the synthesis and accumulation of polyprenoid-derived carotenoids especially CTX (De Miguel *et al.*, 2000). Khodaiyan *et al.* (2007) by studying the effects of various carbon sources on cell growth and CTX production observed that glucose as an inexpensive energy source had the highest effect on the total carotenoid and CTX formation by *D. natronolimnaea* HS-1. The use of glucose for optimal growth and pigmentation medium for CTX production by *Gordonia jacobaea* mutants as *G. jacobaea* MV-1 sp. nov. have previously been reported (De Miguel *et al.*, 2000; De Miguel *et al.*, 2001). Okagbue and Lewis (1985) found that the range of glucose concentration at 10-20 g/L appeared to be critical for high extractability of astaxanthin (AX) in a single stage mixed culture of *Xanthophyllomyces dendrorhous* and *Bacillus circulans.* Fang and Chiou (1996) also reported that the total caro-

tenoid (μg/mL), total volumetric AX (μg/mL), and BDW (g/L) produced by a mutant of the red yeast namely *Phaffia rhodozyma* NCHU-FS501 were increased at concentrations of glucose below 35 g/L. The results of this work indicated a higher glucose concentration than 23 g/L in culture medium of *D. natronolimnaea* HS-1 can lead to decrease the pigment production due to the substrate inhibition effect. Fang and Chiou (1996) claimed that the fermentative growth and AX concentration by the mutant strain of *P. rhodozyma* was reduced with increasing concentrations of glucose from 35 to 45 g/L. It also seems that glucose concentrations more than 23 g/L by inducing the high viscosity in culture medium most likely limited oxygen transfer, resulting in lowered yields of carotenoid pigment (Gharibzahedi *et al.*, 2012b). The increase in aeration from 2 to 8 L/L min caused a significant increase in the dissolved oxygen among high-cell-density cultures, favoring aerobic metabolism and the substrate consumption (Figure 2e). As considered in Figure 2e, the simultaneous increase of aeration rate and inoculum size up to a certain quantity can lead to enhance CTX by *D. natronolimnaea* HS-1 in a batch bioreactor. Asker and Ohta (1999) also found that the growth and CTX production by halophilic bacteria were significantly increased with increasing the rate of dissolved oxygen in high-inoculum density.

According to the individual optimization data, when optimum values of independent variables (D-glucose content of 22.95 g/L, inoculum size of 31.2×10^9 cfu cells/mL and aeration rate of 7.88 l/l min) were incorporated into the regression equation, 5.00 mg/L CTX concentration was obtained.

Optimization and verification of the models

Numerical and graphical optimization procedures were performed for predicting the optimum level of independent variables to obtain maximum biomass, total carotenoid and CTX. The RSM package's response optimizer determined the overall optimum region to be at 23.38 g/L of D-glucose concentration, 31.2×10^9 cfu cells/mL and 7.85 L/L min of air flow rate (Desirability = 0.98). The corresponding response values for biomass, total carotenoid and CTX predicted under the recommended optimum condition were 7.85 g/L, 5.48 mg/L and 4.99 mg/L, respectively. After verifying by five experimental tests with the predicted values, it was demonstrated that no significant ($p > 0.05$) difference between the experimental and predicted values was observed (Table 4). The findings also revealed a close correspondence between those values. Thus, the experimental values were found to be in agreement with the predicted ones.

Conclusion

In this study, RSM-CCRD was successfully used for optimization of the biosynthesis of biomass, carotenoid and CTX from *D. natronolimnaea* HS-1 in a batch bioreactor. We concluded that the investigated responses were more

Table 4 - Predicted and experimental values of the responses obtained at optimum conditions.

Independent variables	Optimum condition	Response variables	Optimum condition	
			Experimental*	Predicted
Glucose concentration	23.38 g/L	Biomass yield	7.91 ± 0.23	7.85 g/L
Inoculation intensity	31.2 x 10^9 cfu cells/mL	Carotenoid yield	5.63 ± 0.22	5.48 mg/L
Aeration intensity	7.85 L/L min	CTX yield	5.07 ± 0.07	4.99 mg/L

*Mean ± standard deviation (n = 5).

influenced by carbon substrate and air flow rate than by inoculum size. However, all quadratic effects of the independent variables on the production biomass, carotenoid and CTX by this bacterium were highly significant. The optimum set of the independent variables was predicted graphically in order to obtain the desired levels of biomass (7.85 g/L), total carotenoid (5.48 mg/L) and CTX (4.99 mg/L). The optimal experimental biomass, total carotenoid and CTX were respectively obtained 7.91 mg/L, 5.63 g/l and 5.07 g/L, when the optimum conditions of bioproduction were D-glucose concentration of 23.38 g/L, inoculation intensity of 31.2 x 10^9 cfu cells/mL and air-flow rate of 7.85 L/L min. The obtained results in this work can be seen as an effective contribution to the development of more efficient bioprocesses for industrial synthesis of CTX. The CTX isolated from *D. natronolimnaea* HS-1 may be used as a natural antioxidant for possible production of healthy-functional foods in the future. However, the batch cultures applied in this study have some limitations, mainly related to high sugar concentrations and CTX yields. The techniques of fed-batch and continuous cultures are recommended for overcoming these problems.

Acknowledgments

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