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 selectivity of the biological reactions is preferred to those produced by chemical synthesis (Gharibzahedi et al., 2013a). Canthaxanthin (CTX, \(\beta\)-\(\beta\)-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 jacobaeae*, green micro-algae (*Chlorococcum* sp. strain MA-1, *Chlorella zofingiensis* and *Chlorella emersonii*) and an extremely halophilic archaeon (*Halofex*...
*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 FeCl₂·6H₂O, 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 Nasrabad 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 Fe³⁺ 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-dinitrosalicylic 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 Nasrabad and Razavi (2010b):

\[
\text{Total carotenoid (µg/L)} = \frac{A_{474\text{nm}} \times V_s \times 10^8}{A_{1\text{ cm}}^{\text{1%}} \times 100} \quad (1)
\]

where \(A_{474\text{nm}}\) and \(A_{1\text{ cm}}^{\text{1%}}\) 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, \(A_{1\text{ cm}}^{\text{1%}}\), 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 10^3-42 x 10^3 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:

\[
Y = \beta_{0} + \sum_{i=1}^{4} \beta_{i1} x_{i} + \sum_{i=1}^{4} \sum_{j=2}^{4} \beta_{ij} x_{i} x_{j} + \sum_{i<j=2}^{4} \beta_{kij} x_{i} x_{j} \quad (2)
\]

where \(Y\) is the predicted response; \(\beta_{0}\), \(\beta_{i1}\), \(\beta_{ij}\) and \(\beta_{kij}\) represent regression coefficients; and \(x_{i}\) and \(x_{j}\) 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
(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:

$$Y_1 = 7.71 + 0.54X_1 + 0.33X_3 - 0.84X_1^2 - 0.35X_3^2 - 0.87X_1X_3 + 0.25X_2X_3 + 0.29X_1X_2$$

$$Y_2 = 5.28 + 0.50X_1 + 0.15X_2 + 0.12X_3 - 0.66X_1^2 - 0.45X_2^2 - 0.33X_3^2 - 0.25X_2X_3$$

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

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

| Independent variables | Unit          | Symbol | Uncoded | Codified | Coded variables levels |
|-----------------------|---------------|--------|---------|----------|------------------------|
|                       |               |        | -1.682 (-$\alpha$) | -1 | 0 | +1 | +1.682 (+$\alpha$) |
| Glucose concentration | g/L           | $X_1$  | 3.18    | 10 | 20 | 30 | 36.82   |
| Inoculation intensity | $10^6$ cfu/mL | $X_2$  | 12.5    | 20 | 31 | 42 | 49.5    |
| Aeration intensity    | L/L min       | $X_3$  | 1.95    | 4  | 7  | 10 | 12.05   |

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). 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 ($Y_3$, 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       |    |             |                  |          |             |               |          |             |               |          |
| $\beta_1$    | 1  | 0.54        | 3.97            | < 0.0001 | 0.50        | 3.35          | < 0.0001 | 0.38        | 2.01          | < 0.0001 |
| $\beta_2$    | 1  | -           | 0.02            | ns       | 0.15        | 0.31          | 0.01     | -           | 0.03          | ns       |
| $\beta_3$    | 1  | 0.33        | 1.52            | 0.002    | 0.12        | 0.19          | 0.04     | 0.21        | 0.59          | 0.0003   |
| Quadratic    |    |             |                  |          |             |               |          |             |               |          |
| $\beta_{11}$ | 1  | -0.84       | 10.17           | < 0.0001 | -0.66       | 6.23          | < 0.0001 | -0.70       | 6.97          | < 0.0001 |
| $\beta_{12}$ | 1  | -0.35       | 1.72            | 0.001    | -0.45       | 2.93          | < 0.0001 | -0.45       | 2.87          | < 0.0001 |
| $\beta_{13}$ | 1  | -0.87       | 10.87           | < 0.0001 | -0.33       | 1.59          | 0.0001   | -0.40       | 2.29          | < 0.0001 |
| Interaction  |    |             |                  |          |             |               |          |             |               |          |
| $\beta_{12}$ | 1  | 0.25        | 0.51            | 0.03     | -           | 0.007         | ns       | -0.13       | 0.14          | 0.01     |
| $\beta_{13}$ | 1  | 0.29        | 0.68            | 0.01     | -           | 0.02          | ns       | -           | 0.05          | ns       |
| $\beta_{13}$ | 1  | -           | 0.23            | ns       | -0.25       | 0.52          | 0.003    | -0.11       | 0.11          | 0.03     |
| Residual     | 8  | 0.60        | 0.25            |          |             |               |          |             |               |          |
| Lack- of-fit | 5  | 0.55        | 0.077           |          | 0.19        | 0.305         |          | 0.08        | 0.460         |          |
| Pure error   | 3  | 0.05        | 0.06            |          |             |               |          |             |               |          |
| 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       |                |          |             |               |          |
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 concentration of 23.67 g/L, inoculation intensity of 31.16 x 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 concentration followed by the linear effect of D-glucose concentration demonstrated 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 determination 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 (Frenyova and Beshkova, 2009). It seems that after inoculation of desirable numbers of the microorganisms in active state (32 x 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 $32 \times 10^9$ to $42 \times 10^9$ cfu/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 $32.41 \times 10^9$ cfu/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 ($p < 0.0001$). 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 polyisoprenoid-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). *Okağbue 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 *Xanthophyllozymes dendrorhous* and *Bacillus circulans*. *Fang and Chiou* (1996) also reported that the total carotenoid (µg/mL), total volumetric AX (µg/mL), and BDW (g/L) produced by a mutant of the red yeast namely *Phaffia rhodozyma* NCHU-FSS01 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 (*Gharibzadeh 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 $32.41 \times 10^9$ cfu/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, $32.41 \times 10^9$ cfu/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 10⁹ 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 3.12 x 10⁹ 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.

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References

Aksu Z, Eren AT (2007) Production of carotenoids by isolated yeast of Rhodotorula glutinis. Biochem Eng J 35:107-113.
Aker D, Ohta Y (1999) Production of canthaxanthin by extremely halophilic bacteria. J Biosci Bioeng 88:617-621.
Augustin JC, Brouillaud-Delattre A, Rosso L, Carlier V (2000) Significance of inoculum size in the lag time of Listeria monocytogenes. Appl Environ Microbiol 66:1706-1710.
Cho YJ, Wang HJ, Kim SW, Song CH, Yun JW (2002) Effect of carbon source and aeration rate on broth rheology and fungal morphology during red pigment production by Paecilomyces Sinclairii in a batch bioreactor. J Biotechnol 95:13-23.
Choudari SM, Singhal R (2008) Media optimization for the production of β-carotene by Blakeslea trispora: a statistical approach. Bioreasour Technol 99:722-730.
De Miguel T, Sieiro C, Poza M, Villa TG (2000) Isolation and taxonomic study of a new canthaxanthin-containing bacterium, Gordonia jacobaeae MV-1sp. nov. Int Microbiol 3:107-111.
De Miguel T, Sieiro C, Poza M, Villa TG (2001) Analysis of canthaxanthin and related pigments from Gordonia jacobaeae mutants. J Agric Food Chem 49:1200-1202.
Duckworth AW, Grant S, Grant WD, Jones BE, Meijer D (1998) Dietzia natronolimnaei sp. nov., a new member of the genus Dietzia isolated from an east soda lake. Extremophiles 2:359-366.
Fang TJ, Chiu TY (1996) Batch cultivation and astaxanthin production by a mutant of the red yeast, Phaffia rhodozyma NCHU-FS501. J Ind Microbiol 16:175-181.
Frengova GI, Beshkova DM (2009) Carotenoids from Rhodotorula and Phaffia yeasts of biotechnological importance. J Ind Microbiol Biotechnol 36:163-180.
Gharibzahedi SMT, Mousavi SM, Hamedi M, Khodaiyan F (2013a) Application of response surface modeling to optimize critical structural components of walnut-beverage emulsion with respect to analysis of the physicochemical aspects. Food Bioprocess Technol 6:456-469.
Gharibzahedi SMT, Razavi SH, Mousavi SM (2012a) Developing an emulsion model system containing canthaxanthin biosynthesized by Dietzia natronolimnaea HS-1. Int J Biol Macromol 51:618-626.
Gharibzahedi SMT, Razavi SH, Mousavi SM (2013b) Psyllium husk gum: An attractive carbohydrate biopolymer for the production of stable canthaxanthin emulsions. Carbohydr Polym 92:2002-2011.
Gharibzahedi SMT, Razavi SH, Mousavi SM (2013c) Ultrasound-assisted formation of the canthaxanthin emulsions stabilized by arabic and xanthan gums. Carbohydr Polym 96:21-30.
Gharibzahedi SMT, Razavi SH, Mousavi SM (2014a) Characterizing the natural canthaxanthin/2-hydroxypropyl-β-cyclo-dextrin inclusion complex. Carbohydr Polym 101:1147-1153.
Gharibzahedi SMT, Razavi SH, Mousavi SM (2014b) Feeding strategies for the improved biosynthesis of canthaxanthin from enzymatic hydrolyzed molasses in the fed-batch fermentation of Dietzia natronolimnaea HS-1. Bioreasour Technol 154:51-58.
Gharibzahedi SMT, Razavi SH, Mousavi SM, Moayedi V (2012b) High efficiency canthaxanthin production by a novel mutant isolated from Dietzia natronolimnaea HS-1 using central composite design analysis. Ind Crop Prod 40:345-354.
Khodaiyan F, Razavi SH, Emam-Djomeh Z, Mousavi SMA, Hejazi MA (2007) Effect of culture conditions on
canthaxanthin production by Dietzia natronolimnaea HS-1. J Microbiol Biotechnol 17:195-201.

Khodaiyan F, Razavi SH, Mousavi SM (2008) Optimization of canthaxanthin production by Dietzia natronolimnaea HS-1 from cheese whey using statistical experimental methods. Biochem Eng J 40:415-422.

Kumar S, Sharma HK, Sarkar BC (2011) Effect of substrate and fermentation conditions on pectinase and cellulase production by Aspergillus niger NCIM 548 in submerged (SmF) and solid state fermentation (SSF). Food Sci Biotechnol 20:1289-1298.

Lee YM, Kim JS, Kim WJ (2012) Optimization for the maximum bacteriocin production of Lactobacillus brevis DF01 using response surface methodology. Food Sci Biotechnol 21:653-659.

Malisorn C, Suntornsuk W (2008) Optimization of /beta/-carotene production by Rhodotorula glutinis DM28 in fermented radish brine. Bioresour Technol 99:2281-2287.

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426-428.

Myers RH, Montgomery RC (2002) Response surface methodology, process and product optimization using design experiment. John Wiley & Sons, Inc., New York, USA.

Yaakob H, Abd Malek R, Misson M, Abdul Jalil MF, Sarmidi MR, Aziz R (2011) Optimization of isoflavone production from fermented soybean using response surface methodology. Food Sci Biotechnol 20:1525-1531.

O’Connor-Cox ESC, Ingledew WM (1991) Alleviation of the effects of nitrogen limitation in high gravity worts through increased inoculation rates. J Ind Microbiol 7:89-96.

Okagbue RN, Lewis MJ (1985) Influence of mixed culture conditions on yeast-well hydrolytic activity of Bacillus circulans WL-12 and on extractability of astaxanthin from the yeast Phaffia rhodozyma. J Appl Bacteriol 59:243-255.

Razavi SH, Blanchard F, Marc I (2006) UV-HPLC/APCI MS method for separation and identification of the carotenoids produced by Sporobolomyces ruberrimus H110. Iran J Chem Eng 25:1-10.

Razavi SH, Mousavi SM, Mehrabani Yeganeh H, Marc I (2007) Fatty acid and carotenoid production by Sporobolomyces ruberrimus when using technical glycerol and ammonium sulfate. J Microbiol Biotechnol 17:1591-1597.

Simova ED, Frengova GI, Beshkova DM (2003) Effect of aeration on the production of carotenoid pigments by Rhodotorula rubra-lactobacillus casei subsp. casei co-cultures in whey ultrafiltrate. Zeitschrift für Naturforschung C- J Biosci 58:225-229.

Veiga-Crespo P, Blasco L, Rosa-Dos-Santos F, Poza M, Villa TG (2005) Influence of culture conditions of Gordonia jacobaea MV-26 on canthaxanthin production. Int Microbiol 8:55-58.

Wu JZ, Cheung PCK, Wong KH, Huang NL (2003) Studies on submerged fermentation of Pleurotus tuberregium (Fr.) Singer part D physical and chemical factors affecting the rate of mycelia growth and bioconversion efficiency. Food Chem 81:389-393.