

# Optimisation and kinetic studies on the production of intracellular canthaxanthin in fed-batch cultures of *Dietzia natronolimnaea* HS-1

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## RESEARCH ARTICLE

### Abstract

A critical optimisation and kinetic study on the production improvement of carotenoid pigment extracted from *Dietzia natronolimnaea* HS-1 under fed-batch fermentation was conducted. High pressure liquid chromatography revealed that the most abundant pigment biosynthesised by this bacterium was canthaxanthin. The constant feeding rates (CFRs; 5.59-8.41 ml/h) for different concentrations of enzymatic hydrolysed molasses (EHM; 40.76-49.24 g/l) were optimised to achieve the highest cell biomass, total carotenoid and canthaxanthin amounts using a response surface method. Spectrophotometric and optimisation analysis showed that the maximum cell mass (17.79 g/l), total carotenoid (16.37 mg/l) and canthaxanthin (14.63 mg/l) can be obtained at 45.90 g/l EHM with a CFR of 6.40 ml/h. A very close agreement between experimental and predicted data was found. A substantial increase (123.4%) in production of canthaxanthin was achieved after 152 h fed-batch process under optimal CFR mode as compared with batch process. Moreover, the maximum specific growth rate and, the volumetric productivities and yields of cell mass, total carotenoid and canthaxanthin in fed-batch culture were significantly more than the batch one.

**Keyword:** canthaxanthin pigment, kinetic parameters, microbial carotenoid, response surface methodology, standard methods, statistical optimisation

## 1. Introduction

Canthaxanthin ( $\beta$ - $\beta'$ -carotene-4,4'-dione) is an abundant pigment in marine sources, plants, fungi and bacteria (Gharibzahedi *et al.*, 2014a). *Dietzia natronolimnaea* HS-1 bacterium among the all sources is recognised as a promising producer of natural canthaxanthin. It is Gram positive, catalase positive, and oxidase negative with orange colonies that isolated during a routine screening of pigmented microorganisms (Gharibzahedi *et al.*, 2013c,d).

Molasses is an economic carbon source which is normally used in most fermentation processes. It is a waste of the sugar industry, consisting of approximately 50% (w/w) total sugars, water, crude protein and fat, heavy metals, vitamins and others (Jiang *et al.*, 2009). It has been reported that *D. natronolimnaea* HS-1 could be used for direct fermentation of molasses and its enzymatic hydrolysate

to canthaxanthin, though production decreased greatly with increasing fermenter volume (Gharibzahedi *et al.*, 2014c). However, improvement of production in large-scale fermenters could be achieved via the application of fed-batch fermentation (FBF) techniques (Kim and Hou, 2006; Lee *et al.*, 1999). FBF is a cost-effective bioprocess for the commercial synthesis of value-added products because nutrients during this process were provided to the reactor while cells and products remained in the reactor until the fermentation end point. Thus, development of the fed-batch cultures can extend the productive phase of the batch bioprocess by controlling the nutrient content (Lee *et al.*, 1999). Fed-batch synthesis of the desired metabolite is usually characterised by the relationship between nutrient consumption and cell growth, the dependence of the desired metabolite production dynamics on the feeding nutrient concentration and the enhance in the culture volume (Kim and Hou, 2006).

Owing to the increasing worldwide market, the high cost of synthetic food components and the need of functional products obtained from natural sources at a low cost and high productivity (Gharibzahedi *et al.*, 2013a,b, 2014d), the present study has been focused on its economic production from *D. natronolimnaea* HS-1 by selecting a low-cost fermentation substrate and an excellent strategy for its feeding into a fed-batch bioreactor. On the other hand, the use of statistical experimental design procedures such as response surface methodology (RSM) in development of this bioprocess can result in improved product yields, reduced process variability, and closer conformance of the output responses to nominal and target requirements, thus reducing the development time and the overall costs (Gharibzahedi *et al.*, 2012).

To the best of our knowledge, there are still no reports optimising the effect of feeding rate and enzymatic hydrolysed molasses (EHM) concentration to improve the yield of microbial canthaxanthin in a FBF mode using RSM. This study thus attempted to maximise cell biomass and canthaxanthin synthesised by *D. natronolimnaea* HS-1 using a central composite rotatable design (CCRD). The growth and production kinetic parameters at optimal points determined in the batch and fed-batch bioprocesses were also compared. The findings obtained here may be useful for the industrial production of canthaxanthin.

## 2. Materials and methods

### Raw material

Beet molasses was provided from Marvdasht Sugar Industry (Marvdasht, Iran) during the harvest season of 2012-2013. The dry matter content (DM) was approximately 86%. The contents of sucrose, glucose, fructose and raffinose sugars were  $50.72 \pm 0.42$ ,  $9.65 \pm 0.73$ ,  $4.78 \pm 0.19$  and  $1.25 \pm 0.04\%$  DM, respectively.

### Molasses pre-treatment and its enzymatic hydrolysate preparation

The molasses was diluted to a desired concentration of total sugars in order to discard the insoluble materials. This obtained solution was adjusted to pH=3.0 with 0.5 M H<sub>2</sub>SO<sub>4</sub> (Merck Co., Darmstadt, Germany) and allowed to stand for 24 h at room temperature and then centrifuged at 7,500 rpm for 10 min (Universal model 320R; Hettich, Newport Pagnell, UK). The supernatant was treated using activated carbon without heating to avoid hydrolysis of sucrose and filtered using 0.2 µm filter paper (Sigma-Aldrich Co., St. Louis, MO, USA) under vacuum (Gharibzahedi *et al.*, 2013c). An optimal ratio of 0.62% (w/v) invertase enzyme (Merck Co.) for 2 h in a water bath (35 °C) was used to achieve more hydrolysis of sucrose present in the filtrate. After the enzymatic hydrolysis, pH of the medium was

adjusted to 7.0 with 10 M NaOH and the obtained substrate sterilised at 121 °C for 15 min (Gharibzahedi *et al.*, 2014b).

### Microorganism and media

The strain of bacterium, *D. natronolimnaea* HS-1 (DSM 44860), used in this work was obtained from Bioprocess Engineering Laboratory, University of Tehran, Tehran, Iran. It was maintained at 4 °C on yeast/malt agar plates with composition (per litre) of 10 g glucose, 3 g malt extract, 5 g yeast extract, 5 g peptone and 15 g agar, and transferred monthly. The inoculum medium was composed of 20 g/l EHM and 10 g/l yeast extract. The batch fermentation medium was composed of 26.16 g/l EHM, 8.2 g/l yeast extract, 8.0 g/l peptone, 25.8 mM sodium citrate and 36 mg/kg Fe<sup>3+</sup>. The EHM concentration in the basal media for FBF was varied from 40.76 to 49.24 g/l in various fermentation experiments according to the RSM-CCRD trials (Table 1). All the media components were obtained from Sigma-Aldrich Chemical Co. at analytical grade.

### Experimental bioreactor setup

A 5 l bioreactor (KF-5L; KoBioTech Co. Ltd., Incheon, Republic of Korea) containing 3 l growth medium was used to perform batch and fed-batch fermentations by *D. natronolimnaea* HS-1. The optimum pH, temperature, stirring rate and aeration rate according to the preliminary studies were 7.0, 28 °C, 130 rpm and 3 l/l/min, respectively. The pH-value during the fermentation culture was automatically controlled using additions of 2 M HCl and 2 M NaOH solutions (Merck Co.). Foam was controlled by the addition of 25% antifoam 204 (Sigma-Aldrich Co.). In fed-batch experiments, fermentation was first carried out in the batch mode until the sugar concentration had dropped to a pre-selected value ( $\approx 48$  h), then EHM with a constant rate (5.6-8.4 ml/h) was fed into the fermenter using a computer coupled peristaltic pump.

### Pigment extraction

Aliquots (10 ml) of cultures were taken from bioreactor at the appropriate times during the fermentation process and the supernatant was collected by centrifuging at 6,000 rpm for 7.5 min at 4 °C. The cell pellets were then washed twice with normal saline and centrifuged again. These cells were re-suspended three times in 3 ml of pure ethanol by vortexing and centrifuging for 5 min in order to extract the pigment. A water bath ( $45 \pm 1$  °C) was also used to extract the pigments completely. The extracts subsequently passed through a 0.2 µm hydrophobic fluorophore membrane (Sigma-Aldrich Co.) to get culture filtrate (Gharibzahedi *et al.*, 2013e).

**Table 1.** The amounts of cell mass, total carotenoid and canthaxanthin produced by *Dietzia natronolimnaea* HS-1 in fed-batch fermentation as a function of enzymatic hydrolysed molasses (EHM) concentration and constant feeding rates (CFRs) according to the central composite rotatable design.

Run	Independent variable		Response variables		
	EHM (g/l)	CFR (ml/h)	Cell mass (g/l)	Total carotenoid (mg/l)	Canthaxanthin (mg/l)
1	42.0	6.0	15.83	14.55	13.10
2	42.0	8.0	13.87	12.01	10.80
3	48.0	6.0	15.53	14.13	12.73
4	48.0	8.0	12.14	10.21	9.19
5	45.0	7.0	17.07	15.77	14.24
6	45.0	7.0	17.21	15.78	14.16
7	45.0	7.0	17.20	15.72	14.10
8	45.0	5.59	17.31	16.02	14.38
9	45.0	8.41	12.89	11.52	10.28
10	40.76	7.0	15.43	13.45	12.14
11	49.24	7.0	14.42	11.82	10.78
12	45.0	7.0	17.41	15.96	14.35
13	45.0	7.0	16.93	15.35	13.85
14	45.0	7.0	17.33	15.78	14.15

### Cell biomass determination

A concentrated cell suspension was diluted with a suitable amount of the synthetic mineral medium to give an optical density ranging from 0.1 to 1.0 when measured at 600 nm. 10 ml of each dilution was filtered through a dried (at 65 °C for 12 h) and pre-weighed membrane filter (Sigma-Aldrich Co.). Then, all filters were dried at 105 °C to constant weight (48 h) and placed in a desiccator to cool down. The biomass 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 optical density on the biomass concentration (Shih and Hang, 1996).

### Identification and quantification of the extracted pigments

A Knauer (Berlin, Germany) high-performance liquid chromatography (HPLC) system including a k-1001 HPLC pump, a k-1001 solvent organiser, an on-line degasser, a dynamic mixing chamber and a UV-visible detector (K-2600; Knauer) was used for the determination of individual carotenoids according to the modified method of Razavi *et al.* (2006). According to this method, the separation was performed on a LiChrospher® 100 RP-18 silica column (5.0 mm, 250×4 mm) at 35 °C (LiChrospher®, Berlin, Germany). The used isocratic mobile phase was acetonitrile/MeOH/dichloromethane (71:22:7, v/v/v) solvent mixture (Merck Co.) at a flow rate of 2 ml/min. To protect the column, a pre-column of the same material was used. The volume of solutions injected was 10 µl.

### Total carotenoid determination

A UV-visible spectrophotometer (V-630; Jasco, Tokyo, Japan) in the spectral region of 300-600 nm with a  $\lambda_{\max}=474$  nm was used to determine the absorbance of the ethanol extracts. The content of total carotenoid (µg/l) was calculated by using the equation reported by Schiedt and Liaaen-Jensen (1995):

$$\text{total carotenoid} = \left( A_{474} \times V_s \times 10^7 / A_{1\text{cm}}^{1\%} \right) \quad (1)$$

Where  $A_{474}$  is the absorbance maximum of total carotenoid in ethanol,  $V_s$  is the volume of sample solution, and  $A_{1\text{cm}}^{1\%}$  is the specific absorption coefficient of total carotenoid for a 1% solution in a 1 cm cell (in ethanol,  $A_{1\text{cm}}^{1\%}=2,200$ ).

### Measurement of reducing sugar content

The reducing sugar concentration was measured using 3,5-dinitrosalicylic acid (DNS) on cell-free supernatants filtered through 0.2 µm filters (Miller, 1959). Briefly, 1.0 ml sample was centrifuged at 4,000 rpm for 5 min, and 1.0 ml of DNS reagent was then added to the supernatant. Samples were heated to boiling for 5 min and placed in an ice-bath. In the next step, 8.0 ml distilled water was added, and the tubes were shaken for 5 min. Optical density of glucose concentration was measured using a UV-visible spectrophotometer (V-630; Jasco) at 575 nm.

## Experimental plan and response surface methodology optimisation

The software Design-Expert 7.1.3 Trial (State-Ease Inc., Minneapolis, MN, USA) was applied to accomplish the whole design and regression analysis. A five-level and two-variable of CCRD was used to optimise the constant feeding rate (CFR, 5.59-8.41 ml/h,  $X_1$ ), and feeding EHM concentration (40.76-49.24 g/l,  $X_2$ ). In total, fourteen experiments were designed to perform (Table 1). The cell biomass ( $Y_1$ ), total carotenoid ( $Y_2$ ), and canthaxanthin ( $Y_3$ ) were taken as the response variables. The data obtained in the CCRD experiment (Table 1) were converted into a second-order polynomial equation with three independent variables:

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

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

The analysis of variance (ANOVA) is performed to evaluate the significance and the fitness of the models, as well as the effects of significant individual terms and their interactions on the chosen responses. The quality of the fit of polynomial model was expressed by the coefficient of determination ( $R^2$ ), adjusted  $R^2$  ( $R^2_{adj}$ ), and adequate precision (AP) in Equation 3-6, respectively (Gharibzahedi *et al.*, 2012, 2013f):

$$R^2 = 1 - \frac{SS_{residual}}{SS_{model} + SS_{residual}} \quad (3)$$

$$R^2_{adj} = 1 - \frac{SS_{residual} / DF_{residual}}{(SS_{model} + SS_{residual}) / (DF_{model} + DF_{residual})} \quad (4)$$

$$AP = \frac{\max(\bar{y}) - \min(\bar{y})}{\sqrt{\bar{V}(\bar{y})}} \quad (5)$$

$$\bar{V}(\bar{y}) = \frac{1}{n} \sum_{i=1}^n V(\bar{y}) = \frac{p\sigma^2}{n} \quad (6)$$

In Equations 3-6,  $SS$  is the sum of squares,  $DF$  is the degrees of freedom,  $y_{i,exp}$  is the experimental responses,  $y_{i,per}$  is the predicted responses,  $\bar{y}$  is the predicted value,  $p$  is the number of model parameters,  $\sigma^2$  is the residual mean square from ANOVA table, and  $n$  is the number of experiments.

The coefficient of variation (CV) also used to determine the quality of the fitted models and its statistical significance was checked by an F-test.

## Calculation of kinetic parameters

The specific growth rate ( $\mu_X$ , 1/h) was calculated by derivation of experimental growth curves as previously described by Le Duy and Zajic (1973). Maximum value of  $\mu_X$  found in the exponential growth phase was referred to as  $\mu_{max}$ .

The yield of biomass ( $Y_{X/C}$ , g cells/g C), canthaxanthin ( $Y_{P,CTX/C}$ , mg/g C), and total carotenoid ( $Y_{P,TC/C}$ , mg/g C) on carbon substrate was respectively calculated as the ratio of the produced cell mass, canthaxanthin and total carotenoid to the amount of carbon substrate added to the system (Equations 7-9):

$$Y_{X/C} = \left( V \frac{(X_{max} - X_{in})}{m_C} \right) \quad (7)$$

$$Y_{P,CTX/C} = \left( V \frac{(P,CTX_{max} - P,CTX_{in})}{m_C} \right) \quad (8)$$

$$Y_{P,TC/C} = \left( V \frac{(P,TC_{max} - P,TC_{in})}{m_C} \right) \quad (9)$$

where  $V$  is the reactor working volume (1 l), and  $m_C$  is the total added mass of carbon sources expressed as carbon.  $X_{max}$ ,  $P,CTX_{max}$  and  $P,TC_{max}$  are the maximum values of cell concentration, canthaxanthin and total carotenoid into fed-batch bioreactor.  $X_{in}$ ,  $P,CTX_{in}$  and  $P,TC_{in}$  respectively are concentrations of bacterium cells, canthaxanthin and total carotenoid in the process initial.

The cell ( $Q_X$ , g/l.h), canthaxanthin ( $Q_{CTX}$ , mg/l.h), and total carotenoid ( $Q_{TC}$ , mg/l.h) volumetric productivities were respectively calculated as the ratio of the concentration variation in cell ( $X_{max} - X_{in}$ ), canthaxanthin ( $P,CTX_{max} - P,CTX_{in}$ ) and total carotenoid ( $P,TC_{max} - P,TC_{in}$ ) to the cultivation time (CT, h) (Equations 10-12):

$$Q_X = \left( \frac{(X_{max} - X_{in})}{CT} \right) \quad (10)$$

$$Q_{CTX} = \left( \frac{(P,CTX_{max} - P,CTX_{in})}{CT} \right) \quad (11)$$

$$Q_{TC} = \left( \frac{(P,TC_{max} - P,TC_{in})}{CT} \right) \quad (12)$$

## Statistical analysis

The ANOVA procedure followed by t-student test using the SAS statistical computer package (SAS 9.2 software Institute, Inc., Gary, NC, USA) was applied to determine the significant difference ( $P < 0.05$ ) between means of two

groups in the optimisation (experimental and predicted data) and kinetic (batch and fed-batch) investigations.

### 3. Results and discussion

#### Fitting of response surface methodology models

It can be seen in Table 2 that the quadratic models were very significant at  $P > 0.0001$ . Second-order polynomial models with non-significant lack-of-fit ( $P > 0.05$ ) obtained for each response are given in Equations 13-15:

$$Y_1 = 17.19 - 1.45 X_1 - 0.43 X_2 - 1.21 X_1^2 - 1.30 X_2^2 - 0.36 X_1 X_2 \quad (13)$$

$$Y_2 = 15.73 - 1.60 X_1 - 0.57 X_2 - 1.10 X_1^2 - 1.67 X_2^2 - 0.34 X_1 X_2 \quad (14)$$

$$Y_3 = 14.14 - 1.45 X_1 - 0.49 X_2 - 1.02 X_1^2 - 1.45 X_2^2 - 0.31 X_1 X_2 \quad (15)$$

The  $R^2$  values (0.985-0.991) were reasonably good for all quadratic models. The remaining responses (merely

0.9-1.5%) were therefore, explained by the residues. The values of  $R^2_{adj}$  for the produced biomass, total carotenoid and canthaxanthin were from 0.974, 0.985 and 0.985, respectively. These values showed a good agreement between the experimental and the predicted values and indicated that the models were very reliable. The CV is the ratio of the standard error of estimate to the mean value of observed response expressed as a percentage (Ghasemlou *et al.*, 2012). It is a measure of reproducibility of the models. As a general rule, a model can be considered to reasonably reproducible if its CV is not greater than 10% (Gharibzadeh *et al.*, 2014b). The CV of the models was calculated as 1.74-1.87%. The AP measured the signal to noise ratio, and a ratio of greater than 4 is normally desirable (Ghasemlou *et al.*, 2012). The AP values of 24.80-31.78 indicated that the models could be used to navigate the design space. Comparison between predicted and actual values for the response variables also indicated that the polynomial regression models were suitable to determine optimum conditions for maximising cell mass, total carotenoid and canthaxanthin (Table 3).

**Table 2. ANOVA for response surface quadratic models of the parameters of growth and pigment production by *Dietzia natronolimnaea* HS-1 in fed-batch fermentation.**

Source <sup>1</sup>	DF	Cell mass (g/l)			Total carotenoid (mg/l)			Canthaxanthin (mg/l)		
		Coefficient	Sum of squares	P-value	Coefficient	Sum of squares	P-value	Coefficient	Sum of squares	P-value
Model	5	17.19	40.53	<0.0001	15.73	51.05	<0.0001	14.14	40.84	<0.0001
Linear										
$X_1$ (CFR)	1	-1.45	16.82	<0.0001	-1.60	20.56	<0.0001	-1.45	16.93	<0.0001
$X_2$ (EHM)	1	-0.43	1.50	0.0043	-0.57	2.56	0.0004	-0.49	1.90	0.0004
Quadratic										
$X_{11}$	1	-1.21	10.87	<0.0001	-1.10	8.90	<0.0001	-1.02	7.62	<0.0001
$X_{22}$	1	-1.30	12.50	<0.0001	-1.67	20.48	<0.0001	-1.45	15.54	<0.0001
Interaction										
$X_{12}$	1	-0.36	0.51	0.0455	-0.34	0.48	0.0295	-0.31	0.38	0.0265
Residual	7		0.61			0.45			0.34	
Lack-of-fit	3		0.46	0.0975 <sup>2</sup>		0.25			0.21	
Pure error	4		0.14			0.20			0.14	
Total	13		41.73			51.72			41.37	
$R^2$		0.985			0.991			0.991		
$R^2_{adj}$		0.974			0.985			0.985		
CV		1.87			1.79			1.74		
AP		24.80			31.27			31.78		

<sup>1</sup> AP = adequate precision; CFR = constant feeding rates; CV = coefficient of variation; EHM = enzymatic hydrolysed molasses;  $R^2$  = coefficient of determination;  $R^2_{adj}$  = adjusted  $R^2$ .

<sup>2</sup> Not significant.

**Table 3. Experimental and predicted values for the response variables.<sup>1</sup>**

Run	Cell mass (g/l)			Total carotenoid (mg/l)			Canthaxanthin (mg/l)		
	Y <sub>0</sub>	Y <sub>i</sub>	Y <sub>0</sub> - Y <sub>i</sub>	Y <sub>0</sub>	Y <sub>i</sub>	Y <sub>0</sub> - Y <sub>i</sub>	Y <sub>0</sub>	Y <sub>i</sub>	Y <sub>0</sub> - Y <sub>i</sub>
1	15.83	15.99	-0.16	14.55	14.66	-0.11	13.10	13.19	-0.09
2	13.87	13.81	0.06	12.01	12.14	-0.13	10.80	10.90	-0.10
3	15.53	15.84	-0.31	14.13	14.22	-0.09	12.73	12.83	-0.10
4	12.14	12.23	-0.09	10.21	10.32	-0.11	9.19	9.30	-0.11
5	17.07	16.98	0.09	15.77	15.60	0.17	14.24	14.02	0.22
6	17.21	16.98	0.23	15.78	15.60	0.18	14.16	14.02	0.14
7	17.20	16.98	0.22	15.72	15.60	0.12	14.10	14.02	0.08
8	17.31	17.02	0.29	16.02	15.92	0.10	14.38	14.28	0.10
9	12.89	12.92	-0.03	11.52	11.38	0.14	10.28	10.16	0.12
10	15.43	15.40	0.03	13.45	13.32	0.13	12.14	12.04	0.10
11	14.42	14.18	0.24	11.82	11.72	0.10	10.78	10.66	0.12
12	17.41	17.39	0.02	15.96	15.85	0.11	14.35	14.25	0.10
13	16.93	17.39	-0.46	15.35	15.85	-0.50	13.85	14.25	-0.40
14	17.33	17.39	-0.06	15.78	15.85	-0.07	14.15	14.25	-0.10

<sup>1</sup> Y<sub>0</sub> = experimental value; Y<sub>i</sub> = predicted value; Y<sub>0</sub> - Y<sub>i</sub> = residue.

### Analysis of influence of variables on the cell mass and pigment bioproduction

Table 2 shows that the cell mass of *D. natronolimnaea* HS-1 under FBF were significantly affected by the linear, quadratic and interaction of all the independent variables. An increase in CFR and feeding EHM could lead to a significant decrease in the cell biomass (Figure 1A and 1B). However, the individual optimisation procedure exhibited that the medium containing 44.10 g/l EHM with a CFR of 6.10 ml/h was predicted to provide the highest cell mass (17.81 g/l). The quadratic effect of CFR had the most significant ( $P < 0.05$ ) effect on the changes of cell mass values.

Figure 2 shows a typical HPLC chromatogram of the extracted carotenoids from *D. natronolimnaea* HS-1. Results demonstrated that canthaxanthin composed of more than 90% of the total carotenoid produced by this bacterium. The values of total carotenoid and canthaxanthin production were significantly influenced by the main ( $P < 0.0001$ ;  $P < 0.001$ ), quadratic ( $P < 0.0001$ ) and interaction ( $P < 0.05$ ) effects of EHM and its CFR (Table 2). According to the sum of square of analysed data (Table 2) and model coefficients (Table 2; Equations 10-11), the most significant effect on these parameters was shown to be the quadratic effect of feeding EHM concentration followed by the main effect of CFR of EHM (Figure 1D). Since microbial pigments produced by the studied bacterium is a component of the biomass, a similar trend between the total carotenoid and canthaxanthin, and cell mass as a function of independent variables (Figure 1C and 1D).

An association between cell growth and carotenoid pigments synthesis was previously reported by other researchers (Aksu and Eren, 2005; Tinoi *et al.*, 2005). Aksu and Eren (2005) revealed that the carotenoid synthesis by the yeast *Rhodotorula mucilaginosa* in medium with 15 g/l glucose was extremely correlated with the cell growth. Tinoi *et al.* (2005) also showed that the concentration of carotenoids produced by *Rhodotorula glutinis* was parallel to cell growth. From the optimisation results, a culture medium containing 44.70 g/l with CFR of 6.60 ml/h was predicted to be the individual optimum region that resulted in highest total carotenoid (16.62 mg/l) synthesis. Moreover, the highest canthaxanthin (14.71 mg/l) production was estimated to be at the combined level of 43.50 g/l EHM and CFR of 6.50 ml/h. No significant difference was observed for the values of individual optimisation for three investigated responses.

Bhosale and Gadre (2002) claimed that the feeding rate of carbon substrate compared to its feeding concentration was stronger parameter to significantly enhance the cell growth and  $\beta$ -carotene from *R. glutinis* mutant. In the previous work, the authors had demonstrated that the FBF with CFR strategy was better than exponential feeding rate strategy to enhance the growth and production parameters (Gharibzahedi *et al.*, 2014c). Chen *et al.* (2011) also demonstrated that the fed-batch cultivation at a constant glucose concentration gave better results than the fed-batch cultivation at a constant specific growth rate, since it provided better conditions for cell growth. Therefore, providing the suitable CFR can reasonably maintain the

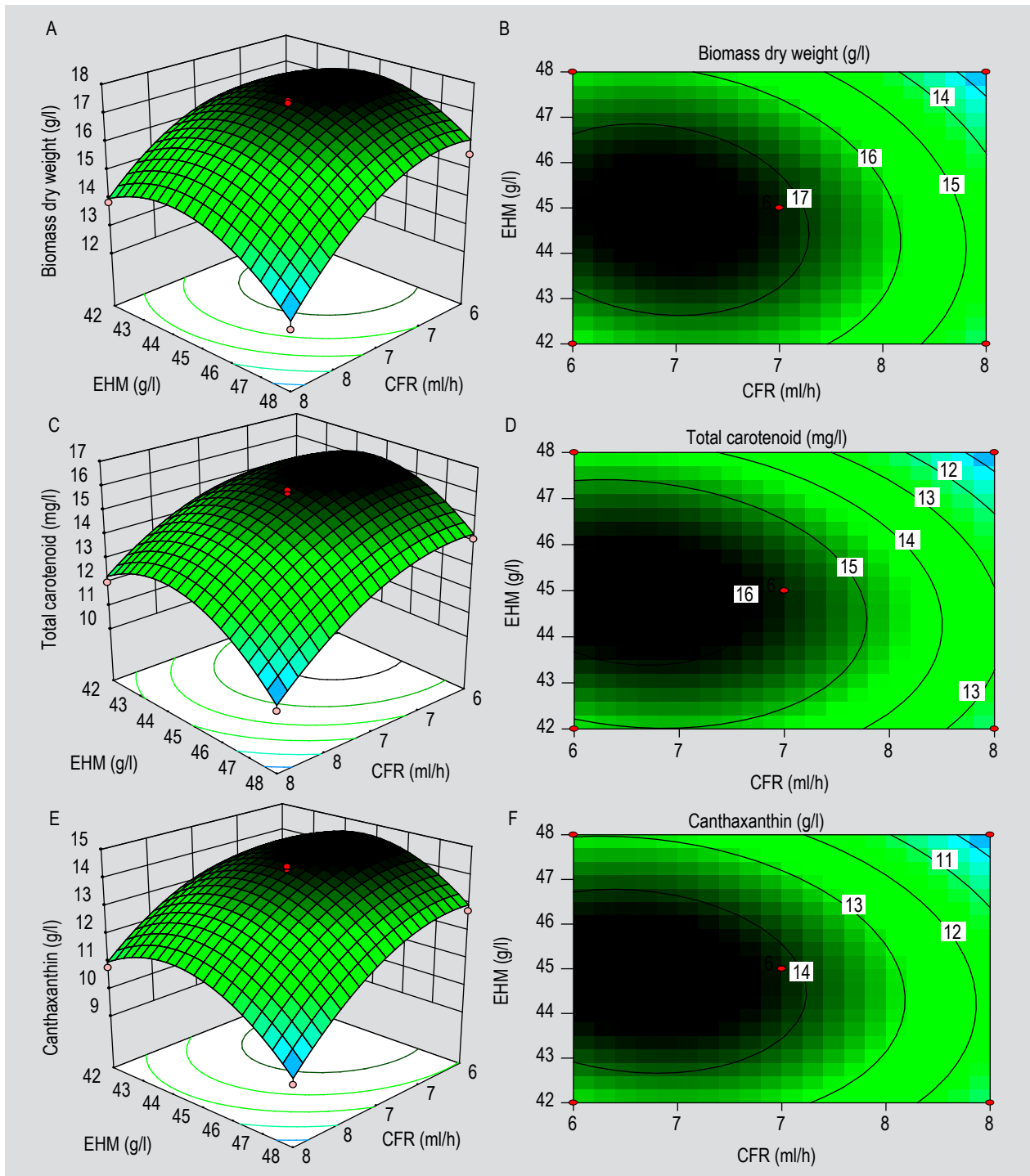


Figure 1. Response surface (A, C, E) and contour (B, D, F) plots showing the significant ( $P < 0.05$ ) interaction effects on the variation of (A, B) cell mass, (C, D) total carotenoid and (E, F) canthaxanthin biosynthesised by *Dietzia natronolimnaea* HS-1 in fed-batch fermentation (CFR = constant feeding rates; EHM = enzymatic hydrolysed molasses).

content of nutrients especially EHM as carbon substrate at an optimum level, increasing yield and productivity of the fermentation process (Yu *et al.*, 2012).

Kim *et al.* (2009) explained this fact with the substrate consumption rate. At the optimal CFR of EHM, inhibitory

effect of the cell growth was avoided because the feeding rate is equal to the consumption rate. Reducing the cell mass and microbial pigment at high CFRs can thus be due to the inhibition effect of accumulated EHM as a result of the more feeding rate of this substrate than its consumption rate. Low amounts of the cell mass and carotenoid pigments at initial

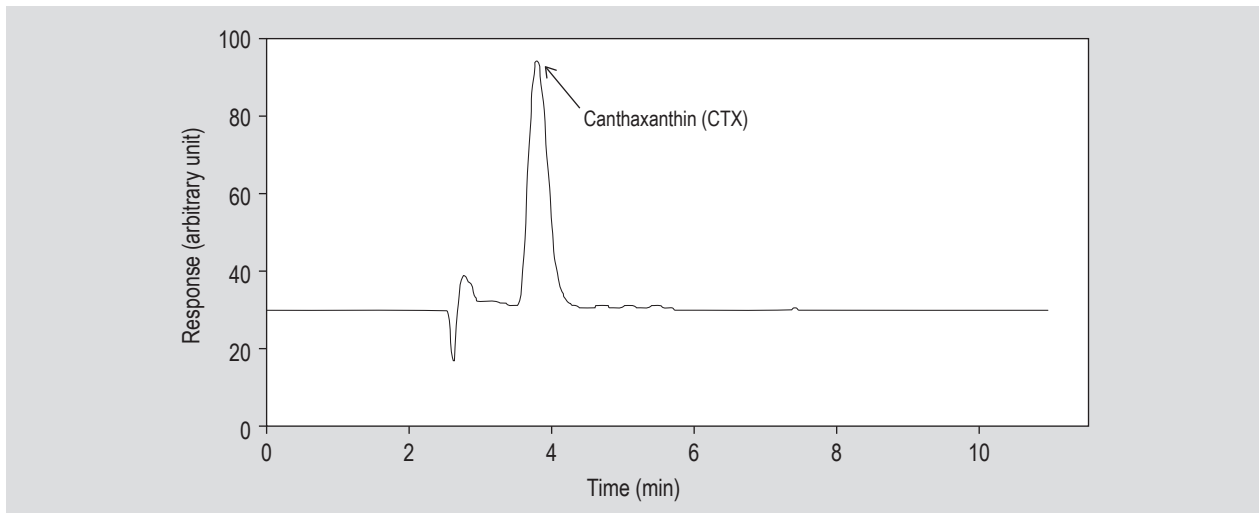


Figure 2. High-performance liquid chromatogram of carotenoid pigments extracted from the studied bacterium, *Dietzia natronolimnaea*.

CFRs of EHM also can be caused by a rapid drop in EHM content and its depletion in the culture medium due to the high consumption rate of simple sugars (Gharibzahedi *et al.*, 2014c). Bence (2011) with comparison of two CFRs of 0.125 and 0.40 l/h showed that increase of dilution rate by the adding fed-batch bioreactor volume at a higher CFR (0.40 l/h) led to a significant improvement in glucose consumption and biosurfactant production by *Bacillus subtilis*. Shankar *et al.* (2014) also found that the use of an ideal dilution rate can significantly supply high amounts of biomass and pullulanase synthesis by *Bacillus flavothermus* KWF-1 under constant feed rate mode.

On the other hand, controlling the nutrient (EHM) concentration in an optimal range is a resourceful mode of cultivating cells to higher concentration. It seems that an increase in EHM concentration from 40.76 to 44.10 g/l (Figure 1E and 1F) can favourably improve the cell mass due to the providing required ATP without any substrate inhibition effect. However, higher EHM concentrations than 44.10 g/l by inducing osmotic stress and/or substrate inhibition effect in culture medium can considerably limit oxygen transfer and decrease the biomass and pigment

production (Gharibzahedi *et al.*, 2013c). The decrease in the amount of produced canthaxanthin at high concentrations of carbon substrate can also be due the inhibition of the enzymes activity involved in its biosynthesis pathway in consequence of the formation of anaerobic fermentation conditions and some the toxic by-products such as organic acids and ethanol (Avci *et al.*, 2014).

#### Overall optimisation and model verification

Multiple response optimisations were carried out to determine the optimum levels of independent variables for achieving the desired response goals. The maximal synthesis of biomass, total carotenoid and canthaxanthin was theoretically obtained at 45.90 g/l EHM with a CFR of 6.40 ml/h (Table 4). The overall closeness between those variables reflected the adequacy of the response surface models employed in predicting the production variation of biomass and carotenoid pigments as a function of EHM concentration and its CFR. Predicted and mean of experimental values for the yields of biomass (17.79 and 17.79±0.97 g/l, respectively), total carotenoid (16.37 and 16.54±1.23 mg/l, respectively) and canthaxanthin (14.63 and

Table 4. Optimum conditions of independent variables (constant feeding rate; CFR, and enzymatic hydrolysed molasses; EHM) and, predicted and experimental values of the responses at these conditions.

Independent variables	Optimum condition	Response variables	Optimum condition	
			Experimental <sup>1</sup>	Predicted
CFR (ml/h)	6.40	Cell mass (g/l)	17.79±0.97	17.79
EHM concentration (g/l)	45.90	Total carotenoid (mg/l)	16.54±1.23	16.37
		Canthaxanthin (mg/l)	14.86±2.16	14.63

<sup>1</sup> Mean ± standard deviation (n=3).

14.86±2.16 g/l, respectively) revealed that the experimental values were very close to the predicted values and were not statistically different at the 5% significance level (Table 4). Therefore, the quadratic models were good predictors for response variables.

### Kinetic study

The authors previously found that the use of 26.16 g/l EHM along with 8.29 g/l yeast extract and 25.86 mM natrium citrate can lead to an optimal production in biomass, total carotenoid and canthaxanthin (Gharibzahedi *et al.*, 2014b). Table 5 shows a statistical comparison between the growth and production parameters for two optimum batch and fed-batch modes. As considered in this table, the maximal biomass after 144 h fermentation in fed-batch system (17.79 g/l) was significantly ( $P<0.001$ ) more than that of the batch system (8.95 g/l). The maximum total carotenoid, canthaxanthin and specific growth rate in fed-batch cultivation system were considerably higher than those of the batch culture ( $P<0.001$ , Table 5). The amounts of  $Y_X/C$ ,  $Y_{P,TC}/C$  and  $Y_{P,CTX}/C$  for the batch and fed-batch modes were 0.346 and 3.53 g cells/g C, 0.253 and 3.12 mg/g C and 0.284 and 3.45 mg/g C, respectively ( $P<0.001$ ). In addition, the quantities of  $Q_X$ ,  $Q_{CTX}$  and  $Q_{TC}$  for the batch and fed-batch cultures of *D. natronolimnaea* HS-1 were 0.061 and 0.172 g/l.h ( $P<0.01$ ), 0.042 and 0.149 mg/l.h ( $P<0.05$ ), and, 0.047 and 0.165 mg/l.h ( $P<0.05$ ), respectively. Overall, the FBF mode was superior to batch processing, and was significantly useful in the improvement of cell biomass and metabolite production. Kim *et al.* (2009) demonstrated that the specific growth rate and volumetric

productivity of  $\beta$ -carotene by recombinant *Escherichia coli* using pH-stat fed-batch cultures with constant feed rate mode can respectively improve 1.7-fold and 2-fold in comparison to the batch cultures. Shi *et al.* (2002) and Wu *et al.* (2007) by evaluating and modelling of lutein production by heterotrophic *Chlorella pyrenoidosa* in batch and fed-batch cultures reported that synthesis of this pigment can noticeably increase under FBF mode. An increase of 20.5% in astaxanthin biosynthesis by *Haematococcus pluvialis* was observed by the applying fed-batch bioreactor (64.36 mg/l) in comparison to the batch bioreactor (53.43 mg/l) (Zhang *et al.*, 1999). Buzzini (2001) by *R. glutinis-Debaryomyces castellii* co-cultures in corn syrup found that the use of fed-batch culture could lead to a volumetric production of 8.2 mg/l total carotenoid that was about 150% of that observed in batch co-cultures. Bhosale *et al.* (2004) also reported a significant increase in the zeaxanthin production by *Flavobacterium multivorum* in fed-batch culture than the batch one. The main advantage of FBF processes can be reduction of the deleterious effects of substrate and/or product inhibitions and also avoidance of catabolite repression on canthaxanthin synthesis as a result of the providing optimal CFR of EHM in the medium (Converti *et al.*, 2003).

### 4. Conclusions

There has been a growing interest in the fermentative production of carotenoid pigments because of their broad applications to chemical, food and pharmaceutical industries. The effects of EHM concentration and CFR of carbon substrate on growth and production characteristics

**Table 5. Comparison of the growth and production kinetic parameters under optimal conditions in two batch and fed-batch bioreactors.<sup>1</sup>**

Kinetic parameter <sup>2</sup>	Cultivation systems		Significant level (P-value)
	Batch	Fed-batch	
$X_{max}$ (g/l, 144 h)	8.95 <sup>b</sup>	17.79 <sup>a</sup>	<0.0001
$P,TC_{max}$ (mg/l, 152 h)	7.45 <sup>b</sup>	16.54 <sup>a</sup>	<0.0001
$P,CTX_{max}$ (mg/l, 152 h)	6.65 <sup>b</sup>	14.86 <sup>a</sup>	<0.0001
$\mu_{max}$ (1/h)	0.072 <sup>b</sup>	0.129 <sup>a</sup>	<0.0001
$Y_X/C$ (g cells/g C)	0.346 <sup>b</sup>	3.53 <sup>a</sup>	<0.0001
$Y_{P,TC}/C$ (mg/g C)	0.284 <sup>b</sup>	3.45 <sup>a</sup>	<0.0001
$Y_{P,CTX}/C$ (mg/g C)	0.253 <sup>b</sup>	3.12 <sup>a</sup>	<0.0001
$Q_X$ (g/l.h)	0.061 <sup>b</sup>	0.172 <sup>a</sup>	<0.01
$Q_{TC}$ (mg/l.h)	0.047 <sup>b</sup>	0.165 <sup>a</sup>	<0.05
$Q_{CTX}$ (mg/l.h)	0.042 <sup>b</sup>	0.149 <sup>a</sup>	<0.05

<sup>1</sup> Different letters in the same row indicate a statistical difference.

<sup>2</sup>  $X_{max}$  = maximum value of cell concentration;  $P,CTX_{max}$  = maximum value of canthaxanthin;  $P,TC_{max}$  = maximum value of total carotenoid;  $\mu_{max}$  = maximum value of the specific growth rate found in the exponential growth phase;  $Y_X/C$  = yield of biomass;  $Y_{P,TC}/C$  = yield of total carotenoid;  $Y_{P,CTX}/C$  = yield of canthaxanthin;  $Q_X$  = cell volumetric productivity;  $Q_{TC}$  = total carotenoid volumetric productivity;  $Q_{CTX}$  = canthaxanthin volumetric productivity.

of *D. natronolimnaea* HS-1 in a fed-batch bioreactor were optimised using RSM procedure. The HPLC analytical procedure initially showed that canthaxanthin was the most abundant pigment produced by *D. natronolimnaea* HS-1. Second-order polynomial models with high  $R^2$  values were developed to predict these properties as a function of the selected variables. A CFR of 6.40 ml/h for 45.90 g/l EHM led to an enhanced production in cell mass, total carotenoid and canthaxanthin. There is a very good agreement between the predicted results by RSM and the measured ones obtained spectrophotometrically, indicating the high accuracy of the models. In addition, applying an optimal fed-batch cultivation system can highly avoid the effects of a continuously falling sugar level during a batch process and therefore can potentially improve the growth and production kinetic parameters.

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