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ENHANCEMENT OF CANTHAXANTHIN PRODUCTION FROM *Dietzia natronolimnaea* HS-1 IN A FED-BATCH PROCESS USING TRACE ELEMENTS AND STATISTICAL METHODS

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Abstract - Under fed-batch process conditions, the statistical analysis of trace elements was performed by application of Plackett-Burman design (for screening tests) and response surface methodology (for predicting the optimal points) to achieve the highest level of canthaxanthin production from *Dietzia natronolimnaea* HS-1. Plackett-Burman design was conducted on eleven trace elements (*i.e.*, aluminum, boron, cobalt, copper, iron, magnesium, manganese, molybdenum, selenium, vanadium and zinc) to select out elements that significantly enhance the canthaxanthin production of *D. natronolimnaea* HS-1. Plackett-Burman design revealed that Fe^{3+}, Cu^{2+} and Zn^{2+} ions had the highest effect on canthaxanthin production of *D. natronolimnaea* HS-1 (P<0.05). These three elements were used for further optimization. By means of response surface methodology for the fed-batch process, the optimum conditions to achieve the highest level of canthaxanthin (8923±18 μg/L) were determined as follow: Fe^{3+} 30 ppm, Cu^{2+} 28.75 ppm and Zn^{2+} 27 ppm.
Keywords: Canthaxanthin; Fed-batch process; Trace elements; *Dietzia natronolimnaea* HS-1; Statistical designs.

INTRODUCTION

Carotenoids are synthesized *de novo* from isoprene units (*ip*) by a wide range of carotenogenic microbes (bacteria, fungi and yeasts) and photosynthetic organisms (green micro-algae, higher plants and lichens). These metabolites are the most extensively distributed class of natural pigments and more than 700 carotenoid molecules had been identified, characterized and classified up to 1999 (Tao et al., 2007). Carotenoids have essential nutraceutical functions in humans (Rao and Rao, 2007). Among them, canthaxanthin (β, β′-carotene-4, 4′-dione) is one of the most important xanthophylls from a commercial point of view because it is extensively applied in medicine, pharmaceuticals, cosmetics, poultry, fishery and food industries (Perera and Yen, 2007). Canthaxanthin is synthesized by bacteria (*D. natronolimnaea* HS-1 (Khodaiyan et al., 2007), *Dietzia* sp. CQ4 (Tao et al., 2007), *Dietzia natronolimnaios* sp. nov. (Duckworth et al., 1998), *Gordonia jacobaea* (Veiga-Crespo et al., 2005), *Bradyrhizobium* strain ORS278 (Hannibal et al., 2000), *Corynebacterium michiganense* (Saperstein and Starr, 1954), *Micrococus roseus* (Cooney et al., 1996) and *Brevibacterium* sp. KY-4313 (Nelis and De Leenheer, 1989)), green micro-algae (*Chlorococcum* sp. (Zhang and Lee, 2001), *Chlorella zofingiensis* (Li et al., 2006) and *C. pyrenoidosa* (Czygan, 1964)) and a halophilic archaeon *Haloferax alexandrinus* TM^T (Asker and Ohta, 2002). Canthaxanthin pigment can

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be produced biotechnologically by different carotenogenic microbes, but they synthesize relatively low concentrations of canthaxanthin and so cannot compete economically with synthetic canthaxanthin (Nelis and De Leenheer, 1991). Among the above mentioned microbial sources of canthaxanthin, the bacterium *D. natronolimnaea* HS-1 is reported as the one of the most promising sources for microbial production of canthaxanthin (Khodaiyan et al., 2007; Khodaiyan et al., 2008).

The carotenoid productivity of carotenogenic microbes can be improved by application of two strategies, including the supplementation of the culture broth by stimulators and the optimization of culture conditions via statistical experimental designs (Bhosale, 2004).

The effect of several types of stimulants on carotenoid production, *e.g.*, trace elements or bio-elements, has been investigated by researchers (Bhosale, 2004). Trace elements have significant roles in microorganisms and act as cofactors of several enzymes involved in the biosynthetic pathway of valuable metabolites (Goodwin, 1984).

Optimization of culture conditions by use of statistical experimental designs is a fundamental strategy for microbial fermentations to achieve the highest level of valuable metabolites produced by target strains (Parekh et al., 2000). Statistical experimental designs such as Plackett-Burman design (PBD) and response surface methodology (RSM) are powerful tools that are extensively applied in various fields, including microbial processes, to determine the interactive influences of fermentation variables and optimize the significant factors for the target responses (Myers and Montgomery, 2002). Statistical designs have been successfully performed in different fields, including food engineering (Singh et al., 2008), bioprocess engineering (Oddone et al., 2007), medium composition and fermentation conditions (Vohra and Satyanarayana, 2002).

Plackett-Burman design (PBD) is a useful tool for 'screening tests' to identify and select the most effective variables with positive significant effect from others with negative effect on response level. This approach has been extensively used in various fields, including the optimization of culture conditions, the evaluation of culture requirements and food engineering (Soliman et al., 2005). Also, PBD has been successfully applied in the optimization of fermentative mediums for carotenoid production by carotenogenic strains (Liu and Wu, 2007).

On the other hand, response surface methodology (RSM), which includes certain statistical techniques, has been widely used in various fields for designing trials and determining the most significant factors and optimal conditions for the target responses (Harker et al., 2005). Also, this method leads to a better understanding of the effects of variables and, more importantly, the interaction of factors (Myers and Montgomery, 2002).

In this work, we supplemented cultures with trace elements to enhance the canthaxanthin production by *D. natronolimnaea* HS-1. Then, a combination of Plackett-Burman design (PBD) with response surface methodology (RSM) was performed on the fed-batch process to select out the trace elements that significantly increased the canthaxanthin production of *D. natronolimnaea* HS-1 and determine their optimum concentrations.

MATERIAL AND METHODS

Reagents and Chemicals

The media ingredients, trace elements (aluminum as $Al_2(SO_4)_3 \cdot 6H_2O$, iron as $FeCl_3 \cdot 6H_2O$, cobalt as $CoCl_2 \cdot 6H_2O$, magnesium as $MgSO_4 \cdot 7H_2O$, selenium as Na_2SeO_3, manganese as $MnCl_2 \cdot 4H_2O$, molybdenum as $Na_2MoO_4 \cdot 2H_2O$, vanadium as $VOSO_4$, copper as $CuSO_4 \cdot 5H_2O$, boron as H_3BO_3 and zinc as $ZnSO_4 \cdot 7H_2O$), D(+)–glucose, yeast extract, peptone, malt extract, agar and Antifoam 289 were all purchased from the Sigma-Aldrich Chemical Company (Sigma-Aldrich Co., United States). Methanol, dichloromethane and acetonitrile (HPLC grade) were obtained from Merck (Merck Co., Germany). The pure ethanol (99.9%, v/v) was purchased from the Bidestan Company (Qazvin, Iran). The canthaxanthin standard was supplied by Dr. Ehrenstorfer Gmbh (Germany).

Source Microorganism and Culture Conditions

The strain of bacterium *D. natronolimnaea* HS-1 (DSM 44860) used in this work was isolated by Razavi (2004) (Dept. of Food Science & Engineering, University of Tehran), and canthaxanthin was identified as the predominant pigment of this bacterium (Razavi et al., 2006). The culture was maintained on YM (yeast-malt) agar plates containing 10 (g/L) glucose, 5 (g/L) peptone, 5 (g/L) yeast extract, 3 (g/L) malt extract, and 15 (g/L) agar at pH 7.5. The cultures were incubated

for 5 days and transferred to fresh plates every month, and then kept at 4°C.

Preparation of Inoculum

Inoculum was prepared in liquid YM medium as mentioned above but without agar in 500-mL Erlenmeyer flasks, containing 100 mL of YM medium each. 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, United Kingdom) at 180 rpm and 28±1°C and, after 4 days, used to inoculate the bioreactor.

Experiments and Bioreactor Set-Up

For experiments, cultures were prepared with 15 (g/L) glucose, 6 (g/L) yeast extract and 10 (g/L) peptone. To determine the effects of trace elements on canthaxanthin production, they were added to the above mentioned medium according to Table 1 (for screening tests) and Table 2 (for the optimization test). Fed-batch trials were performed in a 3 L bioreactor (model LiFlus, GSBiotron Inc., Korea) according to Table 1 and Table 2. The feeding flow rates were defined using the dynamic optimization techniques to optimize the stepwise feeding flow rate. The initial volume of fermentation medium in the fermentor was 1 L and the total volume of medium in the fermentor was 1.5 L. In all experiments, 500 mL of feeding solution was added to the fed-batch trials between 48 and 120 h (*i.e.*, logarithmic growth phase) using the following flow rates: 48 to 56 h: 2.625 mL/h; 56 to 64 h: 3.75 mL/h; 64 to 72 h: 4.25 mL/h; 72 to 80 h: 5.75 mL/h; 80 to 88 h: 6.875 mL/h; 88 to 96 h: 7.75 mL/h; 96 to 104 h: 8.625 mL/h; 104 to 112: 10.75 mL/h; 112 to 120: 12.125 mL/h; and 128 to 136: 12.125 mL/h. The pH was automatically controlled at 7.5±0.1 by use of 2 M HCl and 2 M NaOH. The temperature was automatically controlled at 28±1°C. The dissolved oxygen concentration was maintained at above 40% of air saturation by controlling air flow-rate and agitation speed (300-900 rpm). Also, Antifoam 289 was used as an antifoam agent to prevent foaming.

Table 1: Plackett-Burman design matrix and experimental results. aluminum (Al, ppm); boron (B, ppm); cobalt (Co, ppm); copper (Cu, ppm); iron (Fe, ppm); magnesium (Mg, ppm); manganese (Mn, ppm); molybdenum (Mo, ppm); selenium (Se, ppm); vanadium (V, ppm); zinc (Zn, ppm); canthaxanthin (CXN, µg/L); total carotenoid (CAR, µg/L); cell mass (CM, g/L)

Design matrix[a]												Experimental results		
Run	Al (x_1)	B (x_2)	Co (x_3)	Cu (x_4)	Fe (x_5)	Mg (x_6)	Mn (x_7)	Mo (x_8)	Se (x_9)	V (x_{10})	Zn (x_{11})	CXN (µg/L)	CAR (µg/L)	CM (g/L)
1	+1	–1	–1	–1	+1	+1	–1	+1	–1	+1	+1	6199	6666	6.99
2	+1	–1	–1	+1	+1	–1	–1	+1	–1	+1	–1	6337	6838	7.40
3	+1	–1	–1	+1	–1	–1	+1	+1	–1	+1	+1	5621	6150	6.60
4	+1	+1	+1	–1	–1	–1	–1	–1	+1	+1	–1	5231	5639	6.21
5	–1	+1	+1	–1	–1	–1	+1	+1	+1	+1	–1	5200	5665	5.96
6	+1	+1	+1	–1	–1	+1	–1	+1	+1	–1	–1	5179	5641	5.98
7	–1	–1	+1	+1	+1	–1	–1	–1	+1	–1	+1	6511	7036	7.54
8	–1	+1	–1	+1	+1	–1	+1	–1	+1	–1	+1	6509	7033	7.61
9	–1	+1	+1	+1	+1	+1	–1	–1	–1	–1	+1	6440	6954	7.45
10	+1	–1	+1	+1	+1	–1	+1	–1	+1	+1	–1	6182	6660	7.11
11	–1	+1	–1	+1	–1	+1	+1	+1	+1	+1	+1	5626	6089	6.66
12	+1	+1	+1	–1	+1	+1	–1	+1	–1	–1	+1	5935	6377	7.00
13	–1	–1	+1	–1	+1	–1	–1	+1	+1	+1	+1	5899	6404	6.86
14	+1	+1	–1	+1	+1	–1	–1	+1	+1	–1	–1	6084	6548	6.97
15	+1	+1	+1	+1	–1	–1	–1	–1	–1	+1	+1	5622	6152	6.65
16	+1	+1	–1	–1	+1	+1	+1	–1	+1	+1	+1	5868	6438	6.85
17	–1	+1	+1	+1	+1	+1	+1	+1	–1	+1	–1	6215	6698	7.30
18	+1	–1	+1	+1	–1	+1	+1	+1	+1	–1	+1	5655	6123	6.64
19	+1	–1	+1	+1	–1	+1	+1	–1	–1	–1	–1	5349	5799	5.93
20	–1	–1	–1	–1	–1	+1	–1	–1	+1	+1	+1	5333	5754	6.28
21	–1	+1	–1	–1	+1	–1	+1	+1	–1	–1	–1	5967	6345	6.93
22	–1	–1	+1	–1	–1	–1	+1	+1	–1	–1	+1	5306	5786	6.26
23	+1	–1	–1	–1	+1	+1	+1	–1	+1	–1	–1	5661	6130	6.74
24	–1	+1	–1	+1	–1	+1	–1	–1	–1	+1	–1	5464	5904	6.25
25	+1	+1	–1	–1	–1	–1	+1	–1	–1	–1	+1	5272	5685	6.06
26	–1	–1	+1	–1	+1	+1	+1	–1	–1	+1	–1	5717	6128	6.55

Continuation Table 1

Design matrix[a]												Experimental results		
Run	Al (x_1)	B (x_2)	Co (x_3)	Cu (x_4)	Fe (x_5)	Mg (x_6)	Mn (x_7)	Mo (x_8)	Se (x_9)	V (x_{10})	Zn (x_{11})	CXN (µg/L)	CAR (µg/L)	CM (g/L)
27	–1	–1	–1	+1	–1	+1	–1	+1	+1	–1	–1	5532	5918	6.60
28	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	5147	5543	6.04
29	0	0	0	0	0	0	0	0	0	0	0	4097	4405	4.92
30	0	0	0	0	0	0	0	0	0	0	0	5059	5444	5.85
31	0	0	0	0	0	0	0	0	0	0	0	5168	5567	6.20
32	0	0	0	0	0	0	0	0	0	0	0	5017	5395	5.94
33	0	0	0	0	0	0	0	0	0	0	0	5048	5431	5.92
34	0	0	0	0	0	0	0	0	0	0	0	5043	5368	6.06
35	0	0	0	0	0	0	0	0	0	0	0	5066	5451	6.02
36	0	0	0	0	0	0	0	0	0	0	0	4798	5148	5.82

[a]Fed-batch runs in the bioreactor.

Table 2: Coded levels and actual values of the variables tested in response surface methodology (RSM): canthaxanthin (CNX, µg/L); total carotenoid (CAR, µg/L); cell mass (CM, g/L)

Design matrix							Experimental results		
	Cu^{2+} (x_4)		Fe^{3+} (x_5)		Zn^{2+} (x_{11})		CXN (µg/L)	CAR (µg/L)	CM (g/L)
Run[a]	Code	Level (ppm)	Code	Level (ppm)	Code	Level (ppm)			
1	–1	10	–1	10	–1	10	5160	5503	5.88
2	–1	10	+1	35	–1	10	6685	7266	7.86
3	+1	35	–1	10	–1	10	6729	7356	7.66
4	+1	35	+1	35	–1	10	8231	8898	8.98
5	–1	10	–1	10	+1	35	6527	7010	7.28
6	–1	10	+1	35	+1	35	7932	8805	8.98
7	+1	35	–1	10	+1	35	7831	8543	8.96
8	+1	35	+1	35	+1	35	8483	9114	9.24
9	0	22.5	–1.682	1.5	0	22.5	6174	6697	6.81
10	0	22.5	+1.682	43.5	0	22.5	8361	9056	9.19
11	–1.682	1.5	0	22.5	0	22.5	6371	6837	7.26
12	+1.682	43.5	0	22.5	0	22.5	8139	8747	8.97
13	0	22.5	0	22.5	–1.682	1.5	6508	6991	7.32
14	0	22.5	0	22.5	+1.682	43.5	8039	8843	8.92
15	0	22.5	0	22.5	0	22.5	8574	9392	9.40
16	0	22.5	0	22.5	0	22.5	8573	9319	9.49
17	0	22.5	0	22.5	0	22.5	8671	9387	9.44
18	0	22.5	0	22.5	0	22.5	8573	9357	9.58
19	0	22.5	0	22.5	0	22.5	8573	9207	9.36
20	0	22.5	0	22.5	0	22.5	8399	9130	9.27

[a]Fed-batch runs by bioreactor.

Analytical Determinations

Extraction and Estimation of Carotenoid

At appropriate time intervals (*i.e.*, during the fermentation process, samples were taken from the bioreactor every 8 h), aliquots (10 mL) of cultures were taken from each fed-batch trial and centrifuged at 10,000 ×g for 5 min at 4°C to remove supernatant. The supernatant was collected to measure the glucose content. Then, cell pellets were washed twice with physiological water (NaCl; 9 g/L in deionized water) and centrifuged again. Then, cells were resuspended in 2 mL of pure ethanol by vortexing for 5 min, and the pellet centrifuged to extract the pigment. Fresh ethanol (3 mL) was added and mixed with the pellet and centrifuged until colorless. Ethanol extracts were collected and subsequently filtered through a 0.2 µm hydrophobic fluorophore membrane (Sigma-Aldrich Co., United States). Total pigments were measured based on the optical density. The absorbance in the spectral region of 300-600 nm of the ethanol extracts was analyzed by using a spectrophotometer (model 2502; BioQuest, United Kingdom) and the absorbance of the total carotenoid content was measured at λ_{max} (474 nm). Total carotenoid was then calculated according to the following equation provided by Schiedt and Liaaen-Jensen (1995):

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

where A_{474} is the absorbance maximum of total carotenoid in ethanol, V_s 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, $A_{1\text{ cm}}^{1\%}$ =2200. The canthaxanthin concentration was determined by high performance liquid chromatography (HPLC) (model Knauer, Germany) using a Symmetry analytical C18 column (150 mm×4.6 mm, 300 Å) with a 3.5 µm sphere diameter (Waters, United States) (Razavi et al., 2007). The ultraviolet (UV) detector (model K-2600, Knauer, Germany) was operated at 420-500 nm and the column temperature was maintained at 35°C. The mobile phase was 1 mL/min of an isocratic acetonitrile-methanol-dichloromethane solvent mixture (71:22:7, v/v/v).

Cell Biomass and Sugar Content Measurement

For biomass dry weight measurement, culture samples (5 mL) were filtered through 0.2 µm-pore-size polyamide membrane filters (Sigma-Aldrich Co., United States) (dried at 65°C for 12 h), washed twice with distilled water, and dried at 105°C to constant weight (48 h).

For glucose content measurement, the supernatant from ethanol extraction of carotenoid pigments was filtered through 0.2 µm filters. Then, glucose content (reducing sugar) was measured via Miller (3, 5-dinitrosalicylic acid) method (Miller, 1959).

Experimental Design and Data Analysis

Plackett-Burman design (for screening tests) and response surface methodology (for predicting the optimal points) were applied to perform the optimization strategy. For the experimental design and statistical calculations, the variables X_k were coded as x_k by means of the following equation:

$$x_k = \frac{X_k - X_k^*}{\Delta X_k} \quad (1)$$

In this equation, x_k is the dimensionless coded value of the variable X_k; X_k^* the value of X_k at the center point; and ΔX_k is the step change.

To evaluate the effect of trace elements on cell mass, total carotenoid and canthaxanthin production by *D. natronolimnaea* HS-1, screening trials were conducted on all eleven variables (n=11) by the use of Plackett-Burman design. This approach resulted in 28 experimental runs and eight center points in the fed-batch process (Table 1). The coded levels and natural values of the eleven trace elements (factors) examined via Plackett-Burman design (screening tests) are shown in Table 3. To identify and select the most effective variables with positive significant effect from others with an insignificant effect on response level, we employed a pareto chart and a normal probability plot at an alpha level of 0.05. To optimize the selected variables (significant factors) from Plackett-Burman design, a response surface methodology was performed according to Table 2 for the fed-batch process. The data obtained by this approach were fit with a second-order polynomial equation by application of a multiple regression method. To predict the optimal points of selected variables, the following quadratic polynomial model was used:

$$Pr = \alpha_0 + \sum_{k=1}^{3} \alpha_k x_i + \sum_{k=1}^{3} \alpha_{k,k} x_k^2 + \sum_{k=1}^{3} \sum_{i<k} \alpha_{k,i} x_k x_i \quad (2)$$

In this model, the subscripts (*i.e.*, k and i) range from 1 to the number of variables, Pr is the predicted response, α_0 is the intercept term, α_k are the linear coefficients, $\alpha_{k,k}$ are the quadratic coefficients, and $\alpha_{k,i}$ are the interaction coefficients. Also, x_k and x_i are the coded independent variables. It should be mentioned here that, to predict the optimal values of the independent factors by means of the model (Equation (2)), the partial derivative of the model response with respect to the individual independent factors was assumed to be equal to zero and the equations obtained were solved (Myers and Montgomery, 2002). To express the quality of the regression model and evaluate the statistical significance, the r^2 value (the coefficient of determination) and the F-test (the statistic test factor) were employed, respectively. Furthermore, the t-test was applied to examine the significance of the regression coefficients. The analysis of variance (ANOVA) and graphical optimization were performed using Statistica 6.0 software (StateSoft, Inc., USA) and Minitab 14 software (Minitab Inc., USA), respectively.

Table 3: Factors to be screened in Plackett-Burman design (screening tests) and actual values for the three levels of the variables

Factors (Variables)	Symbols	Levels					
		Low		Middle		High	
		Coded level	Actual value (ppm)	Coded level	Actual value (ppm)	Coded level	Actual value (ppm)
Aluminum (Al)	x_1	–1	10	0	22.5	+1	35
Boron (B)	x_2	–1	10	0	22.5	+1	35
Cobalt (Co)	x_3	–1	10	0	22.5	+1	35
Copper (Cu)	x_4	–1	10	0	22.5	+1	35
Iron (Fe)	x_5	–1	10	0	22.5	+1	35
Magnesium (Mg)	x_6	–1	10	0	22.5	+1	35
Manganese (Mn)	x_7	–1	10	0	22.5	+1	35
Molybdenum(Mo)	x_8	–1	10	0	22.5	+1	35
Selenium (Se)	x_9	–1	10	0	22.5	+1	35
Vanadium (V)	x_{10}	–1	10	0	22.5	+1	35
Zinc (Zn)	x_{11}	–1	10	0	22.5	+1	35

RESULTS AND DISCUSSION

Screening of the Significant Factors

In statistical experimental designs, screening tests are extensively applied to select the most effective variables with positive significant effect from others with and insignificant effect on target responses (Myers and Montgomery, 2002). In this work, we employed a Plackett-Burman design for the fed-batch process as screening tests to evaluate the influence of eleven trace elements (*i.e.*, aluminum, boron, cobalt, copper, iron, magnesium, manganese, molybdenum, selenium, vanadium and zinc) on canthaxanthin, total carotenoid and cell mass production. The design matrix and experimental results (dependent variables) for canthaxanthin, total carotenoid and cell mass production that resulted from the 36-trial Plackett-Burman test under fed-batch process conditions are shown in Table 1. The data obtained by this approach were subjected to regression analysis and analysis of the variance (ANOVA). The main influences of eleven trace elements (independent variables) were evaluated via the fit of first order models to the experimental data. At the 95% confidence level, the *F*-value was used to determine the significance of the models. On the basis of the data obtained from the analysis of the variance (ANOVA) and the regression coefficients, it is clear that the first order models for canthaxanthin, total carotenoid and cell mass production are satisfactory (Table 4).

Table 4: Summarized data of analysis of variance* (ANOVA) of the second-order model obtained for canthaxanthin production according to the experimental design defined in Table 2

Source of variation			df[a]	Sum of squares	Mean square	F-value	P-value
Canthaxanthin production model (Eq. (3))							
Regression			9	21259555	2362173	165.94	<0.0001
Linear			3	13378144	4459381	313.27	<0.0001
Square			3	7490128	2496709	175.39	<0.0001
Interaction			3	391282	130427	9.16	0.003
Residual Error			10	142350	14235		
Lack-of-Fit			5	103537	20707	2.67	0.153
Pure Error			5	38813	7763		
Total			19	21401905			
Model terms	**Coefficients**	**Values**	**df[a]**	**Standard error**	**t-value**	**P-value**	
Intercept	α_0	8560.60	1	48.66	175.92	<0.0001	
x_4 (Cu^{2+})	α_4	581.65	1	32.29	18.01	<0.0001	
x_5 (Fe^{3+})	α_5	641.64	1	32.29	19.87	<0.0001	
x_{11} (Zn^{2+})	α_{11}	479.14	1	32.29	14.84	<0.0001	
x_4^2 ($Cu^{2+} * Cu^{2+}$)	$\alpha_{4,4}$	–459.37	1	31.43	–14.61	<0.0001	
x_5^2 ($Fe^{3+} * Fe^{3+}$)	$\alpha_{5,5}$	–454.77	1	31.43	–14.47	<0.0001	
x_{11}^2 ($Zn^{2+} * Zn^{2+}$)	$\alpha_{11,11}$	–452.89	1	31.43	–14.41	<0.0001	
x_4x_5 ($Cu^{2+} * Fe^{3+}$)	$\alpha_{4,5}$	–97.04	1	42.18	–2.30	0.044	
x_4x_{11} ($Cu^{2+} * Zn^{2+}$)	$\alpha_{4,11}$	–157.50	1	42.18	–3.73	0.004	
x_5x_{11} ($Fe^{3+} * Zn^{2+}$)	$\alpha_{5,11}$	–121.19	1	42.18	–2.87	0.017	

*r^2=99.3% and adjusted r^2=98.7%.
[a]Degrees of freedom.

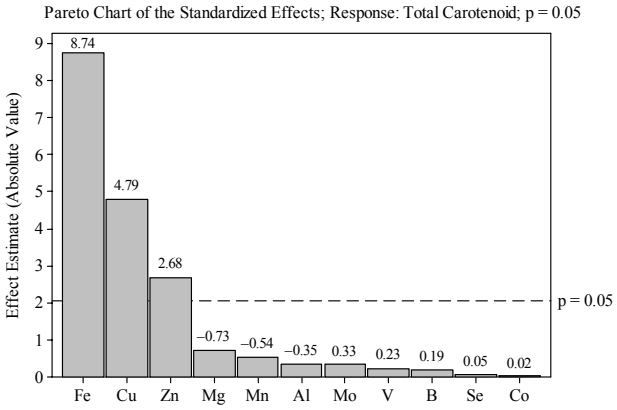


Figure 1: Pareto chart of the main effects for Plackett-Burman design on total carotenoid production. Al (aluminum, ppm); B (boron, ppm); Co (cobalt, ppm); Cu (copper, ppm); Fe (iron, ppm); Mg (magnesium, ppm); Mn (manganese, ppm); Mo (molybdenum, ppm); Se (selenium, ppm); V (vanadium, ppm); Zn (zinc, ppm)

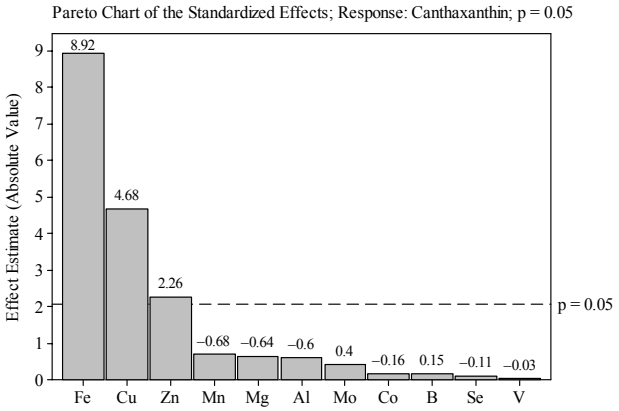


Figure 2: Pareto chart of the main effects for Plackett-Burman design on canthaxanthin production. Al (aluminum, ppm); B (boron, ppm); Co (cobalt, ppm); Cu (copper, ppm); Fe (iron, ppm); Mg (magnesium, ppm); Mn (manganese, ppm); Mo (molybdenum, ppm); Se (selenium, ppm); V (vanadium, ppm); Zn (zinc, ppm)

The pareto chart can be used in statistical experimental design to identify the most important effects of independent variables on the target responses (Haaland, 1989). For this reason, it was employed for determining the significant factors in this work. It should be noted here that the length of each bar on the pareto chart is proportional to the absolute value of its estimated effect or associated regression coefficient (Haaland, 1989) (Fig. 1 and Fig. 2). The pareto chart and the normal probability plot for total carotenoid and canthaxanthin production are shown in Fig. 1 and Fig. 2, respectively. From these figures, it is clear that, of the eleven trace elements tested, only Fe^{3+}, Cu^{2+} and Zn^{2+} ions have a significant effect (P<0.05) on canthaxanthin and total carotenoid production by *D. natronolimnaea* HS-1. For this reason, only these three trace elements were chosen as the significant variables and used in the subsequent optimization trials.

Optimization of Canthaxanthin Production

Because more than 90% of the total carotenoid produced by *D. natronolimnaea* HS-1 is canthaxanthin, we focused on the optimization of canthaxanthin production in this work.

Under fed-batch process conditions, the optimum concentration of the trace elements (*i.e.*, Fe^{3+}, Cu^{2+} and Zn^{2+}) selected from Plackett-Burman design to achieve the highest level of canthaxanthin was determined by application of a response surface methodology (RSM). The optimization process was performed according to Table 2. Ferric, zinc and copper ions, each at five levels and six replicates at the center point were used in the fed-batch process. It should be noted here that the use of center points is necessary to account for pure internal error in optimization processes (Myers and Montgomery, 2002) (Table 4). The design matrix and experimental results for canthaxanthin, total carotenoid and cell mass production that resulted from the 20-trial response surface test for the fed-batch process are shown in Table 2.

The data obtained by RSM (Table 2) were fit with a second-order polynomial equation (Equation 2), which created the second-order response surface model in the form of analysis of variance (ANOVA) (Table 4). According to ANOVA, the model obtained was significant because the lack-of-fit test, which measures the fitness of the model, did not result in a significant P-value (P=0.153), and only 0.70% of the total variance was not explained by the model (r^2=99.3%). The value of the adjusted determination coefficient, which is a measure of fitness of the regressed model, was also high (r^2_{adj}=98.7%), suggesting that the experimental data are in good agreement with predicted values and that higher-order terms are not necessary (Myers and Montgomery, 2002). As a result, the polynomial model describing the correlation between canthaxanthin synthesized by *D. natronolimnaea* HS-1 and the three variables (x_4=Cu^{2+}, x_5=Fe^{3+} and x_{11}=Zn^{2+}) can be represented according to the following equation:

$$\text{Canthaxanthin production} = 8560.60 + 581.65x_4 + 641.64x_5 + 479.14x_{11} - 459.37x_4^2 - 454.77x_5^2 - 452.89x_{11}^2 - 97.04x_4x_5 - 157.5x_4x_{11} - 121.19x_5x_{11} \quad (3)$$

Where the x_k are the coded independent factors (Table 2 and Table 4).

The contour plots (two-dimensional plots) were established from the quadratic model (Equation 3) summarized in Table 4 and applied to show the influence of the three independent variables (Cu^{2+}, Fe^{3+}, and Zn^{2+} ions) on the dependent variable (canthaxanthin production) and the interaction of the independent variables. In these plots, two variables alter in the range of the experimental design (Table 2), while the third factor remains constant at its optimal value (Fig. 3). The contour plots provide useful information on the process conditions necessary to achieve the desired value of the response. All contour plots were convex, containing the maximum response inside the design boundary (Fig. 3).

Fig. 3(a) shows that canthaxanthin synthesized by *D. natronolimnaea* HS-1 is promoted by increasing Fe^{3+} or Cu^{2+} concentration up to around 30 and 28 ppm, respectively. Also, when the concentration of Fe^{3+} or Cu^{2+} stayed steady, canthaxanthin synthesis reduced with increasing level of Fe^{3+} or Cu^{2+} above 30 and 28 ppm, respectively. Fig. 3(b) shows the response arising from the interaction between Fe^{3+} and Zn^{2+} concentrations. On the basis of this plot, canthaxanthin synthesis is promoted by enhancing both the variables (Fe^{3+} and Zn^{2+}) up to around 30 and 27 ppm, respectively. From this plot, it is clear that a further increase in Fe^{3+} and Zn^{2+} concentration resulted in a reduction in canthaxanthin synthesis by *D. natronolimnaea* HS-1. The interaction of Cu^{2+} and Zn^{2+} is shown in Fig. 3(c). In this graph, canthaxanthin synthesis is promoted by increasing Cu^{2+} and Zn^{2+} concentration up to around 28 and 27 ppm, respectively. However, canthaxanthin synthesis is reduced at higher concentrations of mentioned variables.

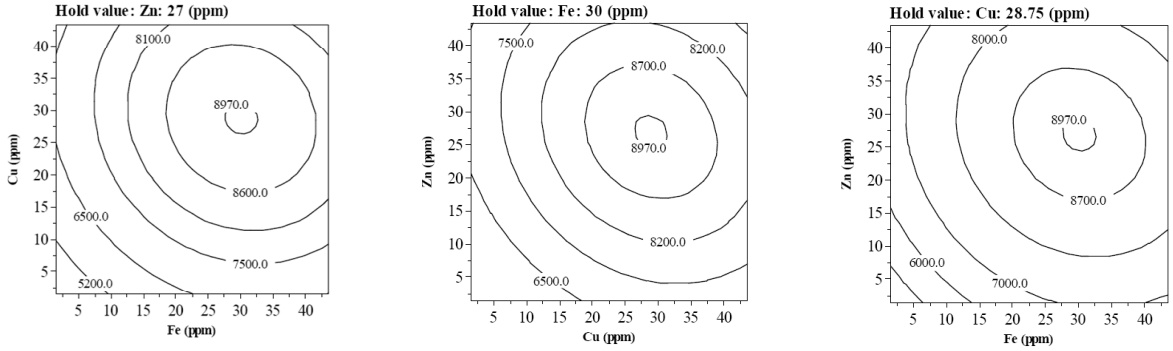
The results in Table 4 and Eq. (3) reveal that the interactions between variables are significant (P<0.05). In other words, the quadratic factors (Fe^{3+} * Fe^{3+}, Cu^{2+} * Cu^{2+}, and Zn^{2+} * Zn^{2+}) and the interaction between variables (Fe^{3+} * Cu^{2+}, Fe^{3+} * Zn^{2+}, and Cu^{2+} * Zn^{2+}) showed the more pronounced stimulatory influences on canthaxanthin synthesis (response) (P<0.05).

On the basis of the above mentioned results, the optimal concentrations Fe^{3+}, Cu^{2+} and Zn^{2+} ions to achieve the highest level of canthaxanthin are around 30, 28 and 27 ppm, respectively.

Equation 3 was solved and the optimum point for the achievement of maximal canthaxanthin production was determined to be at x_4=0.50, x_5=0.60, and x_{11}=0.36, corresponding to the actual values of Cu^{2+} 28.75 ppm, Fe^{3+} 30 ppm, and Zn^{2+} 27 ppm, respectively. Under optimal conditions, the model predicts that the highest level of canthaxanthin production is 8988 μg/L.

Validation of the Optimal Conditions and Model

Under the optimal conditions, an additional series of fed-batch experiments in three replicates (confirmatory experiments) was carried out to assess the validity of the quadratic model. Accordingly, the maximal content of canthaxanthin resulting from the fed-batch experiments was 8923±18 μg/L on the 7th day of fermentation. This result is very near to the canthaxanthin content predicted by application of response surface methodology under optimal conditions (8988 μg/L). This suggests that there is a good agreement between the values predicted by the model and experimental data, and confirms that the model is a good predictor of the experimental results.



(a) (b) (c)

Figure 3: Contour plots (two-dimensional surface plots) of the model equation fitted to the data. (a) interaction of Fe^{3+} and Cu^{2+} concentration, (b) interaction of Fe^{3+} and Zn^{2+} concentration, (c) interaction of Cu^{2+} and Zn^{2+} concentration. (Cu^{2+} (copper, ppm); Fe^{3+} (iron, ppm); Zn^{2+} (zinc, ppm))

The time courses of cell mass, canthaxanthin and total carotenoid production are shown in Fig. 4. For the optimal condition test, the experiments were carried out for 240h, as in Figure 4, and the feed solution was added until 120 h. The samples for analysis were collected from the bioreactor every 8 h. This plot shows that, by using the optimal conditions for the fed-batch process, the maximal content of cell mass (9.11±0.12 g/L) was obtained on the 6th day of fermentation, while the maximal content of canthaxanthin (8923±18 μg/L) and total carotenoid (9684±22 μg/L) were achieved on the 7th day of fermentation. This means that the maximal content of canthaxanthin synthesized by *D. natronolimnaea* HS-1 occurs at the end of the exponential growth phase. It should be mentioned here that carotenoid molecules are synthesized from isoprene units (*ip*) produced via the mevalonate biosynthetic pathway (MVA), which is one of the most important secondary metabolic pathways in carotenogenic microbes (Bailey and Ollis, 1986). Canthaxanthin synthesized by *D. natronolimnaea* HS-1 is a partially growth-associated product because it is produced during the growth phases and its accumulation occurs at the end of the exponential growth phase (Khodaiyan et al., 2008). A similar pattern has also been observed in some other carotenogenic strains such as *Phaffia rhodozyma* (Vazquez et al., 1997), *Chlorococcum* sp. (Zhang and Lee, 2001), *Rhodotorula mucilaginosa* (Aksu and Eren, 2005), *Gordonia jacobaea* (Veiga-Crespo et al., 2005), and *Sporobolomyces ruberrimus* H110 (Razavi et al., 2007).

In this work, our results revealed that ferric, zinc and copper ions have the greatest stimulatory effect on the canthaxanthin biosynthesis of *D. natronolimnaea* HS-1. In previously work, without the use of trace elements, Khodaiyan et al. (2007) showed that the maximum canthaxanthin production was 5.29 mg/L. Table 5 shows the comparison between microbial sources of canthaxanthin.

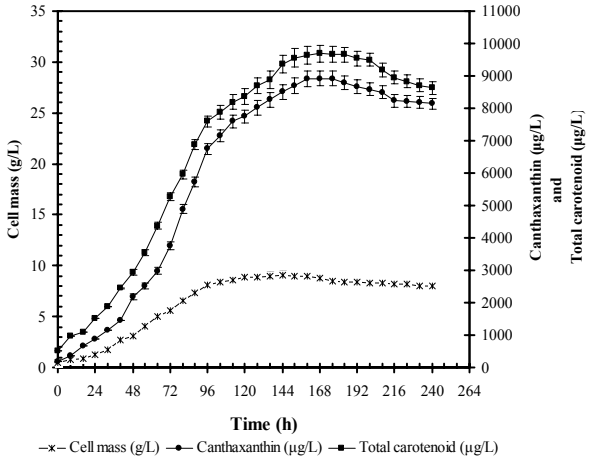


Figure 4: Time-course of canthaxanthin, total carotenoid and cell mass production by *Dietzia natronolimnaea* HS-1 in the fed-batch process under the optimum conditions (*i.e.*, Fe^{3+} 30 ppm, Cu^{2+} 28.75 ppm and Zn^{2+} 27 ppm)

Table 5: A comparison between important canthaxanthin-producing strains

Microbial source	Microorganism type	Canthaxanthin (μg/L)	Canthaxanthin (μg/g)	Reference
Bradyrhizobium strain ORS278 *	bacterium	780	1340	Hannibal et al. (2000)
Brevibacterium KY-4313 *	bacterium	7200	---	Nelis and De Leenheer (1989)
Chlorella emersonii *	green mico-alga	600	---	Arad et al. (1993)
Chlorella zofingiensis *	green mico-alga	---	2970	Pelah et al. (2004)
Chlorococcum sp. strain MA-1 *	green mico-alga	---	1950	Zhang and Lee (2001)
Dictyococcus cinnabarinus *	green mico-alga	---	1000	Gribanovski-Sassu (1973)
Dietzia natronolimnaea HS-1[a] *	bacterium	5320	700	Khodaiyan et al. (2007)
Dietzia natronolimnaea HS-1[b] *	bacterium	5290	---	Khodaiyan et al. (2007)
Dietzia natronolimnaea HS-1 **	bacterium	8923	---	This work
Gordonia jacobaea MV-1 *	bacterium	3200	227	De Miguel et al. (2000)
Haloferax alexandrinus TM^T *	archaea	2190	690	Asker and Ohta (2002)
Micrococcus roseus *	bacterium	1700	---	Cooney et al. (1966)
Xanthophyllomyces dendrorhous *	yeast	2950	---	Vazquez et al. (1997)

[a]Results obtained from Shake-flask culture.
[b]Results obtained from batch fermenter system.
*without trace elements.
**with trace elements.

It was found that carotenogenesis in other carotenogenic strains was induced in the presence of trace elements. For example, Kobayashi et al. (1992) supplemented the medium of *Haematococcus pluvialis* with ferric salts and showed the improvement of astaxanthin synthesized by this green micro-alga (Kobayashi et al., 1992). Also, Mahattanatavee and Kulprecha (1991) reported that the supplementation of the growth medium with iron, zinc and copper ions improved the carotenoid production of *Rhodotorula* strains (Mahattanatavee and Kulprecha, 1991). Bhosale and Gadre (2001) showed that iron, zinc, and calcium ions had the highest stimulatory influence on both the cellular accumulation (mg/g) and volumetric production (mg/L) of carotenoids by the yeast *Rhodotorula glutinis*. Furthermore, it was reported that the carotenogenesis of mated *Blakeslea trispora* was enhanced in the presence of copper, iron and magnesium ions, a several-fold increase being observed in the final yield. In other research, copper, cobalt, calcium, and barium ions stimulated the carotenogenesis of the pink yeast *Rhodotorula rubra* (Atamanyuk and Razumorskii, 1974; Daushvili and Elisashvili, 1990; Gammal and Rizk, 1989). Komemushi et al. (1994) demonstrated that divalent cations (such as barium) act as inducers for growth of *Rhodotorula glutinis* (Komemushi et al., 1994). Furthermore, statistical analysis of trace elements by means of response surface methodology revealed that Co^{2+} and Mn^{2+} ions had the highest effect on total carotenoid production of the red yeast *Rhodotorula graminis* (Buzzini et al., 2005).

It has been reported that heavy metals such as cobalt promote carotenoid production from the cyanobacterium *Spirulina platensis* at very low concentrations. Similarly, Wang et al. (1999) reported that heavy metal ions such as lanthanum, cerium and neodymium induce the carotenoid synthesis of the yeast *Xanthophyllomyces dendrorhous*. For the stimulatory effect of heavy metal ions on carotenogenesis of carotenogenic microbes, it has been reasoned that these ions are commonly associated with uptake systems and intracellular binding sites, which is turn enhance carotenoid synthesis (Bhosale, 2004). In contrast, in our study cobalt had no significant effect on canthaxanthin production of *D. natronolimnaea* HS-1 (Fig. 2). This suggests a difference between the various species of carotenogenic microbes in carotenogenesis.

Although the above mentioned reports and their data confirm the stimulatory effect of trace elements on carotenogenesis, bio-elements have not yet been assigned a definite function in carotenoid synthesis and no detailed study or research has been performed on the optimum concentration of trace elements to ensure high carotenoid synthesis by carotenogenic microbes.

The stimulatory effect of trace elements on carotenogenesis of the carotenogenic strains can be explained according to the following hypotheses. It can be assumed that trace elements affect the enzymes involved in carotenogenesis (Goodwin, 1980). For example, some trace elements such as Fe^{2+}, Zn^{2+}, Mg^{2+} and Mn^{2+} ions are known to act as cofactors for enzymes involved in the carotenoid biosynthetic pathway and, therefore, they enhance carotenoid production at certain concentrations (Sandmann, 1994; Sandmann, 2001). Another suggested hypothesis is that the generation of active oxygen radicals (*e.g.*, $^1O_2$ and $HO^\bullet$) in the culture broth stimulates carotenoid synthesis. On the basis of this hypothesis, the stimulatory effect of ferrous ions on carotenogenesis might be due to the hydroxyl radical generated via the 'Fenton reaction' ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + HO^- + HO^\bullet$), which would in turn increase the biosynthesis of carotenoids (Tjahjono et al., 1994).

Compared to the concentration of canthaxanthin produced by other microbial sources of this pigment, such as various bacteria, including *Dietzia* sp. CQ4 (Tao et al., 2007), *D. natronolimnaios* sp. nov. (Duckworth et al., 1998), *Brevibacterium* KY-4313 (Nelis and De Leenheer, 1989), *Bradyrhizobium* strain ORS278 (Hannibal et al., 2000), and *Gordonia jacobaea* MV-1 (De Miguel et al., 2000); the halophilic archaeon *Haloferax alexandrinus* TM^T (Asker and Ohta, 2002) and various green micro-algae, including *Chlorella pyrenoidosa* (Czygan, 1964), *Chlorococcum* sp. strain MA-1 (Zhang and Lee, 2001), and *Chlorella zofingiensis* (Li et al., 2006), there is clear potential for employing *D. natronolimnaea* HS-1 to synthesize canthaxanthin. Table 5 shows a comparative study between microbial sources of canthaxanthin.

CONCLUSION

In this work, a model describing the relationship between trace element concentration and carotenoid production by *D. natronolimnaea* HS-1 is developed and shown to be in good agreement with experimental data. The high R^2 values for the equations expressing the different responses strongly support our model, making it a valuable tool for optimizing the production of canthaxanthin by adjusting selected culture conditions.

NOMENCLATURE

A_{474}	the absorbance maximum of total carotenoid in ethanol	
$A_{1\ cm}^{1\%}$	the specific absorption coefficient of total carotenoid for a 1% solution in a 1 cm cell	
ANOVA	analysis of variance	
Al	Aluminum	x_1, ppm
B	Boron	x_2, ppm
Co	Cobalt	x_3, ppm
Cu	Copper	x_4, ppm
Fe	Iron	x_5, ppm
Mg	Magnesium	x_6, ppm
Mn	Manganese	x_7, ppm
Mo	Molybdenum	x_8, ppm
Se	Selenium	x_9, ppm
V	Vanadium	x_{10}, ppm
Zn	Zinc	x_{11}, ppm
$HO^{\bullet}$	hydroxyl radical	
$^1O_2$	singlet oxygen	
Pr	predicted response	
PBD	Plackett-Burman design	μg/L
RSM	response surface methodology	
r^2_{adj}	adjusted determination coefficient	
r^2	the coefficient of determination	
V_s	the volume of sample solution	mL
x_k	the dimensionless coded value of the variable X_k	
X_k^*	the value of the X_k at the center point	
α_0	the intercept term	
α_k	the linear coefficients	
$\alpha_{k,\,k}$	the quadratic coefficients	
$\alpha_{k,\,i}$	the interaction coefficients	
ΔX_k	the step change	

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