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Optimization of Canthaxanthin Production by *Dietzia natronolimnaea* HS-1 Using Response Surface Methodology

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Abstract: Sequential methodology combining a screening stage by fractional factorial design and an optimization stage by central composite design was applied to enhance canthaxanthin production of *Dietzia natronolimnaea* HS-1 in shake flask cultures. Five variables (pH, luminous intensity, inoculum percent, concentration of glucose and concentration of NaCl) were studied with the first design and the results revealed that three factors (pH, concentration of glucose and concentration of NaCl) had greater influence on the canthaxanthin production ($p<0.01$). A central composite design was then used in the second step to determine the maximum canthaxanthin concentration. The optimum condition for the highest canthaxanthin production (5.32 mg L^{-1}) was a pH of 7.53, glucose concentration of 25.90 g L^{-1} and NaCl concentration of 3.42 g L^{-1}.

Key words: Canthaxanthin, *Dietzia natronolimnaea* HS-1, optimization, response surface methodology

INTRODUCTION

Carotenoids are a group of natural pigments present in a wide variety of bacteria, algae, fungi and plants (Johnson and Schroeder, 1996). Among them, canthaxanthin (4, 4′-diketo-β-carotene) is a ketocarotenoid that is responsible for the orange-red colour of egg yolks and the flesh of many marine animals (Nelis and De leenheer, 1989). Because of its colour and strong antioxidant activity, canthaxanthin is widely applied in medical, pharmaceutical (Bhosale and Bernstein, 2005), cosmetic (Edge *et al*., 1997), poultry, fishery (Nelis and De Leenheer, 1991) and food industries (Gordon and Bauernfeind, 1982). Although the current supply of carotenoids can be met through chemical synthesis, in food and cosmetic industries, the application of such products is restricted due to their possible toxic effects. Therefore, commercial production of the carotenoids using carotenoid-producing microorganisms has received increasing attention (Asker and Ohta, 1999; Bhosale *et al*., 2004).

At present, researchers attempt to find microbial sources for canthaxanthin production. Canthaxanthin has been found in several microorganisms, including the bacteria *Micrococcus roseus* (Cooney and Berry, 1981), *Rhodococcus maris* and *Brevibacterium* KY-4313 (Nelis and Leenheer, 1991), *Bradyrhizobium* strains (Lorquin *et al*., 1997), halophilic bacteria (Asker and Ohta, 1999) and *Gordonia jacobaea* (De Miguel *et al*., 2001); various algae including *Dictyococcus cinnabarinus* (Nelis and Leenheer, 1991), *Chlorella emersonii* (Arad *et al*., 1993) and *Chlorella zofingiensis* (Pelal *et al*., 2004) and the halophilic archaeon *Haloferax alexandrinus* (Asker and Ohta, 2002). However, there are no data reported on the commercial production of canthaxanthin using microorganisms. Furthermore, finding the optimum production conditions for these compounds is also an issue.

Statistical designs are powerful tools that can be used to account for the main as well as the interactive influences of fermentation parameters on the process performance. Among them, response surface methodology (RSM) is a collection of certain statistical techniques for designing experiments, building models, evaluating the effects of the factors and searching for optimal conditions for desirable responses (Myers and Montgomery, 2002). Therefore, during the past decades, RSM has been extensively applied in the optimization of medium composition, fermentation conditions and food manufacturing processes (Vázquez and Martin, 1997; Park *et al*., 2005; Ramirez *et al*., 2001).

The bacterium *Dietzia natronolimnaea* is gram-positive, catalase positive and oxidase negative with orange colonies. *D. natronolimnaea* HS-1 was isolated

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during a routine screening of pigmented microorganisms. In preliminary experiments, the main pigment of this strain was identified canthaxanthin (Razavi, 2004), therefore, the main purpose of the present study was to apply this microorganism as one of the canthaxanthin-producing bacteria and to optimize the effects of different factors of growth medium on its production using statistical experimental design.

MATERIALS AND METHODS

Glucose, peptone, malt extract, yeast extract, sugars, NaCl salt and agar were obtained from Sigma-Aldrich Chemical Company (USA). The pure ethanol (99.9%) was purchased from the Bidestan Company (Iran), the canthaxanthin standard supplied by Dr. Ehrenstorfer GmbH (Germany), the acetonitrile and methanol were of HPLC grade from Merck (Germany).

Microorganism: The strain *D. natronolimnaea* HS-1 (DSM 44860) was isolated in the Laboratory of Chemical Engineering Sciences by Razavi (2004) and maintained on yeast/malt (YM) agar plates containing: 10 g L^{-1} glucose, 5 g L^{-1} peptone, 3 g L^{-1} malt extract, 3 g L^{-1} yeast extract and 15 g L^{-1} agar. Single colonies were transferred to a fresh plate every month, incubated for 4 days and thereafter kept under refrigeration at 4°C (Khodaiyan, 2007).

Preparation of inoculum: A pure culture of *D. natronolimnaea* HS-1 from the YM agar was transferred into 500 mL Erlenmeyer flasks containing 100 mL of a GPY medium (10 g L^{-1} glucose, 10 g L^{-1} peptone, 6 g L^{-1} yeast extract), incubated in a rotary shaker (180 rpm) at 28±1°C and after 72 h used as the inoculum.

Culture conditions: Calculated amounts of inoculum (according to the experimental designs) were transferred into 500 mL Erlenmeyer flasks containing 100 mL growth medium containing 10 g L^{-1} peptone, 6 g L^{-1} yeast extract, glucose and NaCl. Glucose and NaCl concentrations were selected according to the experimental designs. The flasks were incubated in a rotary shaker (180 rpm) for 8 days. The culture conditions varied according to the experimental designs. To study the effect of light on the canthaxanthin production, light was provided by white fluorescent tubes. Some of Erlenmeyer flasks were covered with aluminium foils to make them impermeable to light.

Dry weight: Biomass dry weight was determined by harvesting 5 mL culture samples, filtering the cells through 0.2 μm filter (Sigma-Aldrich Co., USA), washing the cells with distilled water and drying them at 105°C to a constant weight (48 h).

Table 1: Range of variables at different levels for the fractional factorial design

	Independent variables (X_i)	Levels		
		-1	0	+1
X_1	Glucose (g L^{-1})	10	15.00	20.0
X_2	NaCl (g L^{-1})	0	6.00	10.0
X_3	pH	6	7.25	8.5
X_4	Light (lux)	0	500.00	1000.0
X_5	Inoculum (% v/v)	5	7.50	10.0

Table 2: Experimental design matrix and experimental result for the fractional factorial design

	Design matrix					Experimental results			
Run	X_1	X_2	X_3	X_4	X_5	Biomass (g L^{-1})	Carotenoid (mg L^{-1})	Canthaxanthin (mg L^{-1})	Canthaxanthin (μg g^{-1})
1	-1	-1	-1	-1	+1	4.81	3.34	2.97	617
2	-1	+1	+1	+1	-1	3.97	2.89	2.54	640
3	+1	-1	+1	+1	-1	7.32	5.46	4.98	680
4	+1	+1	-1	+1	-1	5.72	4.74	4.06	710
5	-1	-1	+1	+1	+1	5.85	3.65	3.56	609
6	+1	-1	-1	-1	-1	6.59	4.51	4.39	666
7	+1	+1	+1	-1	-1	5.91	4.35	4.10	694
8	-1	-1	+1	-1	-1	5.34	3.65	3.29	616
9	-1	+1	-1	-1	-1	2.96	2.18	1.95	659
10	+1	+1	-1	-1	+1	5.49	4.21	3.87	705
11	-1	-1	-1	+1	-1	5.12	3.67	3.21	627
12	+1	-1	+1	-1	+1	6.98	5.18	4.8	688
13	+1	+1	+1	+1	+1	6.22	4.86	4.49	722
14	+1	-1	-1	+1	+1	6.78	4.98	4.61	680
15	-1	+1	+1	-1	+1	3.77	2.77	2.39	634
16	-1	+1	-1	+1	+1	3.31	2.38	2.11	637
17	0	0	0	0	0	6.19	4.53	4.25	687
18	0	0	0	0	0	6.37	4.57	4.31	677
19	0	0	0	0	0	6.27	4.38	4.12	657
20	0	0	0	0	0	6.02	4.33	4.15	689

Extraction and analysis of carotenoids: Ten milliliter aliquots were centrifuged at 5000 x g for 10 min at 4°C. The pellets were then washed twice with a solution of 9 g L^{-1} NaCl and centrifuged again. Next, the supernatant was resuspended in 3 mL of pure ethanol by vortexing for 5 min and the pellets centrifuged again to extract the pigments. It was repeated three times. Thereafter, the pigments were completely extracted using a water bath (45°C) and the carotenoid extracts subsequently filtered through a 0.2 μm hydrophobic fluoropore membrane (Sigma-Aldrich Co., USA) and analyzed by scanning the absorbance of the wavelength spectra of 300-600 nm using the spectrophotometer (UV-Visible, Cary 300, Varians Co., Germany). The maximum absorbance was determined at a wavelength of 474 nm, which conformed to standard canthaxanthin λ_{max}. The total carotenoid concentration was calculated following the formula provided by An *et al* (1989). Individual carotenoids were determined according to modified method by Razavi *et al.* (2006), using a HPLC (Knauer, Germany) equipped with a UV-visible detector (K-2600, Knauer, Germany) and Pump (K-1001, Knauer, Germany). The chromatographic separation was performed on a Nucleosil 100 C18, 5.0 μm (125×4.0 mm), where the temperature of the column was maintained at room temperature and the mobile phase was acetonitrile: methanol (80:20, v/v) at a flow rate of 1 mL min^{-1}. The eluant was monitored at 480 nm. To protect the column, a pre-column (5×4.0 mm) of the same material was used. The volume of injected solutions was 100 μL.

Experimental design: Screening experiments to select main factors were performed with five factors, by use of a fractional factorial 2^{5-1} resulting in 16 experimental runs and four center points (Table 2). The range and the levels of these five variables, identified on the basis of preliminary experiments (Khodaiyan, 2007) are given in Table 1. A pareto chart was used to exclude insignificant factors at an alpha level of 0.01. The results of the fractional factorial design revealed that three out of the five factors exerted significant effects on canthaxanthin production. A central composite design with five coded levels was used to optimize the values of the 3 factors (Table 3 and Table 4). The experimental results of the central composite design were fitted with a second-order polynomial equation by a multiple regression technique. The quadratic model for predicting the optimal point was expressed as follows:

$$Y = C_0 + \sum_{i=1}^{3} C_i X_i + \sum_{i=1}^{3} C_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j<i} C_{ij} X_i X_j \qquad (1)$$

where Y is response (canthxanthin production), C_0, C_i, C_{ii} and C_{ij} are constant coefficients and X_i, X_j are the coded independent factors. The quality of fit of the second-order model equation was expressed by the coefficient of determination R^2 and its statistical significance was determined by F-value. The significance of the regression

Table 3: Range of variables at different levels for the central-composite design

	Independent variables (X_i)	Levels				
		-α[a]	-1	0	1	α
X_1	Glucose (g L^{-1})	3.18	10	20	30	36.82
X_2	NaCl (g L^{-1})	0	2.54	6.27	10	12.54
X_3	pH	5.15	6	7.25	8.5	9.35

[a] α = 1.682

Table 4: Experimental design matrix and experimental result for the central composite design

	Design matrix			Experimental results			
Run	X_1	X_2	X_3	Biomass (g L^{-1})	Carotenoid (mg L^{-1})	Canthaxanthin (mg L^{-1})	Canthaxanthin (μg g^{-1})
1	+1	+1	+1	6.67	4.99	4.61	691
2	-1	+1	+1	4.07	3.01	2.71	666
3	+1	-1	+1	7.21	5.23	4.79	664
4	+1	+1	-1	6.72	4.98	4.44	661
5	-1	-1	+1	5.84	4.12	3.76	644
6	+1	-1	-1	6.85	4.99	4.69	685
7	-1	+1	-1	3.35	2.33	2.12	633
8	-1	-1	-1	5.19	3.54	3.28	632
9	+α	0	0	6.74	4.94	4.75	705
10	0	+α	0	5.33	4.02	3.60	675
11	0	0	+α	6.22	4.26	4.00	643
12	-α	0	0	2.69	1.86	1.67	621
13	0	-α	0	7.21	5.55	5.20	721
14	0	0	-α	4.84	3.39	3.01	622
15	0	0	0	7.27	5.36	5.12	704
16	0	0	0	7.21	5.11	4.89	678
17	0	0	0	7.31	5.33	5.16	706
18	0	0	0	7.19	5.28	5.01	697
19	0	0	0	6.81	5.01	4.81	706
20	0	0	0	7.01	5.15	4.90	699

coefficients was tested by t-value. The Statistica software (trial version 6.0, StatSoft, USA) and the Minitab 14 software (Minitab Inc., State College, PA, USA) were employed for the regression analyses and the graphical optimization, respectively.

RESULTS

Selection of carbon source and operating temperature: In order to better establish the culture conditions for production of canthaxanthin in optimization process, several preliminary experiments were performed. The best nitrogen source for canthaxanthin production by *D. natronolimnaea* HS-1 was the composition of 10 g L^{-1} peptone and 6 g L^{-1} yeast extract (Razavi, 2004). Also, in investigation of the effects of various carbon sources, the best level of canthaxanthin was observed in the presence of glucose (data not shown).

In the study of effects of various temperatures on growth of *D. natronolimnaea* HS-1, the highest values of biomass, total carotenoid and canthaxanthin production were found at 31°C (Table 5). Therefore, the optimization process was performed using glucose at different concentrations as carbon source at 31°C.

Screening experiments to select main factors: The primary purpose of screening experiments is to select important main effects from less important ones. In this study, a fractional factorial design (2^{5-1}) was used to detect the influence of five factors on canthaxanthin production in shake flasks. The results presented in Table 2 for biomass and canthaxanthin production were subjected to regression analysis and the analysis of variance (ANOVA). First order models were fitted to data to evaluate the main effects of the five factors. After applying the ANOVA statistical test, it was found that the first order models for biomass and canthaxanthin production were satisfactory. The pareto chart, which has been described as a useful tool for identifying the most important effects (Haaland, 1989), was applied to determine the significant factors. In this chart, the length of each bar on a standardized pareto chart is proportional to the absolute value of its associated regression coefficient or estimated effect. The order in which the bars are displayed corresponds to the order of the size of the effects, which allows the most important effects to be identified. The chart includes a vertical line, which corresponds to the 99% confidence limit indicating statistical significance. An effect is therefore significant if it crosses this vertical line. Figure 1 shows pareto charts for biomass and canthaxanthin production. This chart shows that concentration of glucose, NaCl and pH are the most effective factors on biomass and canthaxanthin production by *D. natronolimnaea* HS-1. Hence, only these three factors were used for further optimization experiments.

Table 5: Effects of different temperatures on growth and canthaxanthin production.

Temperature (°C)	$Biomass_{max}$ (g L^{-1})	$Carotenoid_{max}$ (mg L^{-1})	$Canthaxanthin_{max}$ (mg L^{-1})	Productivity $Canthaxanthin_{max}$ (mg L^{-1} h^{-1})
13	5.91	5.86	3.92	0.011
20	6.01	5.24	3.78	0.013
28	5.90	4.31	3.91	0.018
31	6.09	4.48	4.28	0.022
34	5.76	3.82	3.60	0.018
40	1.94	0.75	0.69	0.0004

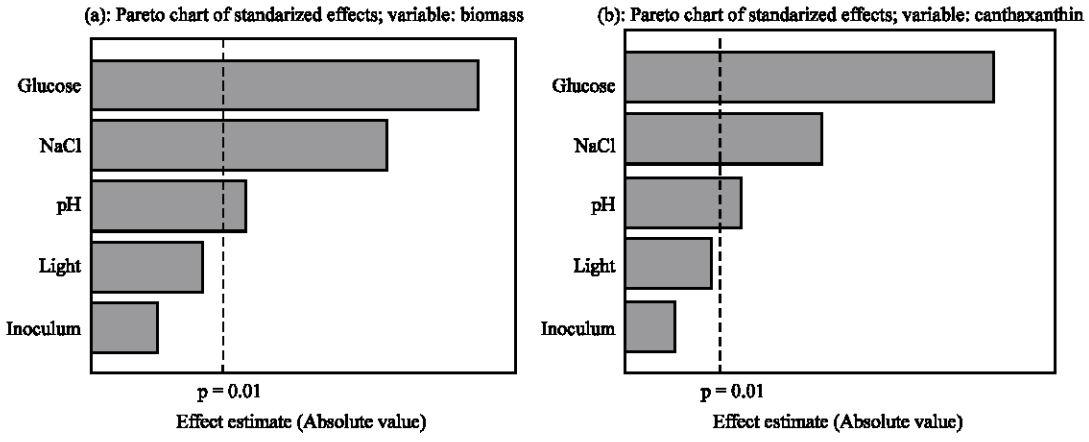

Fig. 1: Pareto chart of main effects for the 25-1 experimental design on: (a) Biomass and (b) Canthaxanthin production

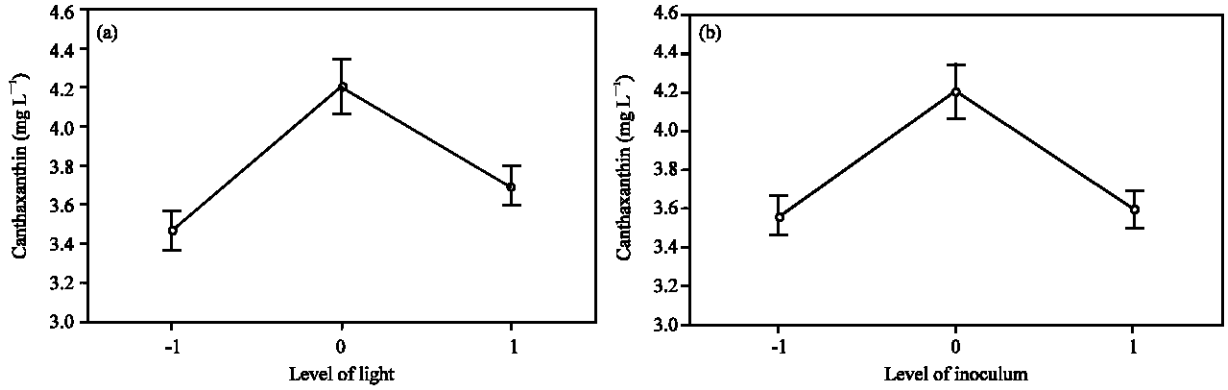

Fig. 2: Effects of factor levels on Canthaxanthin : (a) Light levels and (b): Inoculum levels

Table 6: Analysis of Variance and coefficient estimates for second-order model

SV (Freedom)	df	Sum of Squares	Mean±SE	f-value	p-value
Source of variation					
Regression	7	21.151	3.022	85.91	0.000
Linear	3	13.007	4.336	123.27	0.000
Square	3	7.748	2.583	73.43	0.000
Interaction	1	0.396	0.396	11.26	0.006
Lack of fit	7	0.326	0.047	2.42	0.174
Pure Error	5	0.096	0.019		
Total	19	21.573			
Factors					
Intercept	1	4.98	0.075	65.06	0.000
X_1	1	0.87	0.50	17.08	0.000
X_2	1	-0.39	0.50	-7.69	0.000
X_3	1	0.22	0.050	4.34	0.001
X_1^2	1	-0.59	0.048	-11.94	0.000
X_2^2	1	-0.17	0.048	-3.43	0.005
X_3^2	1	-0.49	0.048	-9.83	0.000
X_1X_2	1	0.22	0.065	3.36	0.006

R^2 = 98.0% and Adjusted R^2 = 96.9%

Optimization of canthaxanthin production: A central composite design for the three factors (pH and concentrations of glucose and NaCl), each at five levels and six replicates at the center (to account for pure internal error), was applied for optimizing canthaxanthin production in shake flasks. The design matrix for these factors in the optimization runs is noted in Table 4. In view of the results of the screening experiments, in this stage, the luminous intensity and inoculum concentration were set at 500 lux and 7.5%, respectively, because the highest value of canthaxanthin production was obtained under condition of middle point of these factors (Fig. 2a and b). We also extended the range of glucose concentration from 10-20 to 10-30 g L^{-1}, because the highest value of biomass and canthaxanthin production in screening experiments was obtained at 20 g L^{-1} (Table 2).

The results of the second-order response surface model in the form of analysis of variance (ANOVA) are given in Table 6. The ANOVA results indicated that the quadratic regression to produce the second-order model was significant. The lack-of-fit test was insignificant ($p = 0.174$) and only 2% of the total variations was not explained by the model (R^2 = 98%). The value of the adjusted determination coefficient (adjusted R^2 = 96.9.0%) was also high to advocate a high significance of the model (Myers and Montgomery, 2002). This suggested that the model accurately represents the data in the experimental region. This also indicated that second-order terms were sufficient and higher-order terms were not necessary.

Table 6 shows that main, quadratic factors and interaction of glucose, NaCl have the most pronounced effects on the response ($p<0.05$). The model was simplified by omitting all terms that were statistically non significant ($p>0.05$). The reduced equation was expressed as follows:

$$Y = 4.98 + 0.87X_1 - 0.39X_2 + 0.22X_3 - 0.59X_1^2 - 0.17X_2^2 - 0.49X_3^2 + 0.22X_1X_2 \quad (2)$$

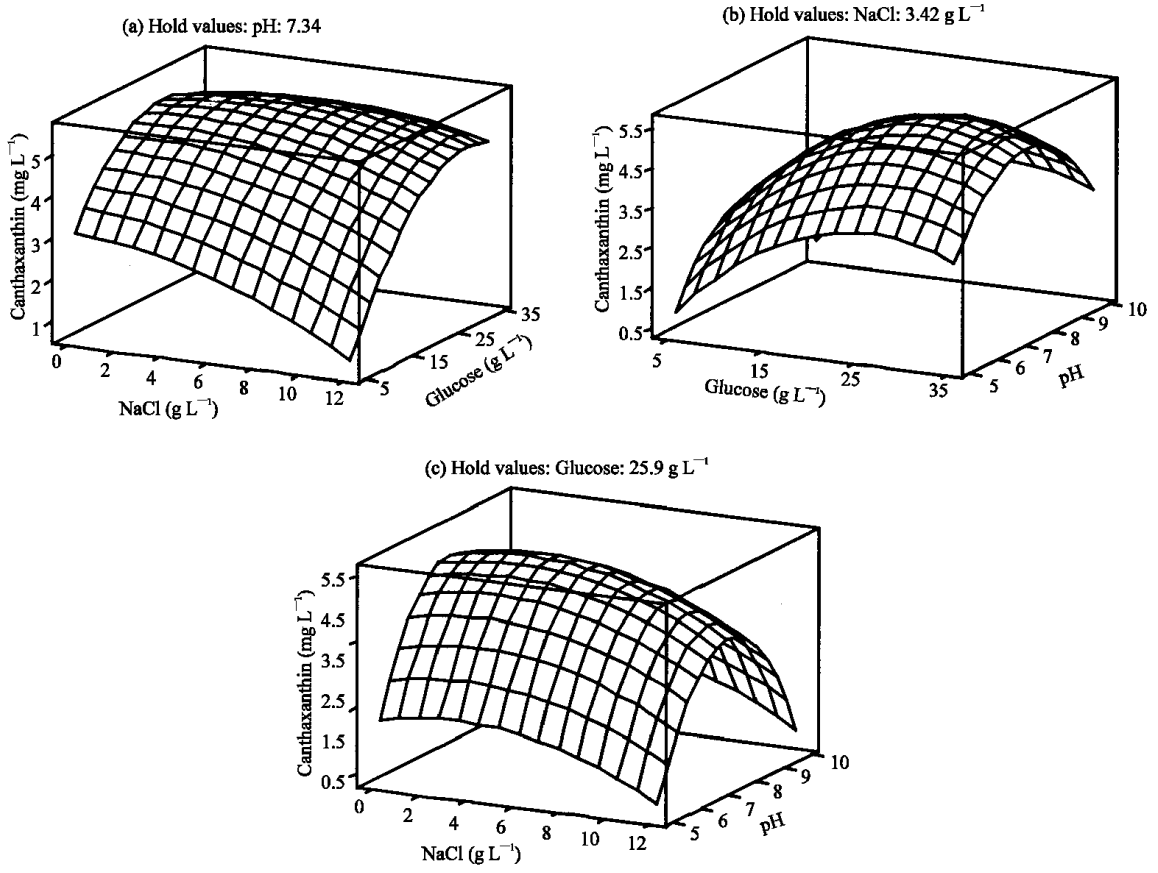

Fig. 3: Surface plats of the model equation fitted to the data of the central composite design. (a) Interaction of glucose concentration and NaCl concentration, (b) Interaction of glucose concentration and pH, (c) Interaction of NaCl concentration and pH

where Y is the experimental response and X_i is the coded independent factors (X_1 = glucose concentration, X_2 = NaCl concentration and X_3 = pH).

The relation between factors and response can best be understood by examining surface plots as a function of two factors at a time and holding all other factors at fixed levels. Figure 3 shows surface plots of calculated response surface. Figure 3a and c indicates that high level of NaCl concentration have negative effect on conthaxanthin production. The surface plots (Fig. 3a and b) also show that the optimal glucose concentration and pH are around 25 g L^{-1} and 7.5, respectively. The optimum values of these factors were obtained by solving mentioned model. The optimal values of the factors are as follows: Glucose = 25.90 g L^{-1}, NaCl = 3.42 g L^{-1} and pH = 7.53. Under these culture conditions canthaxanthin production was 5.41 mg L^{-1}.

To validate the optimum point of the factors, experimental rechecking was carried out using conditions representing these optimal factors in three replicate. Figure 4 shows the time course profile of the production

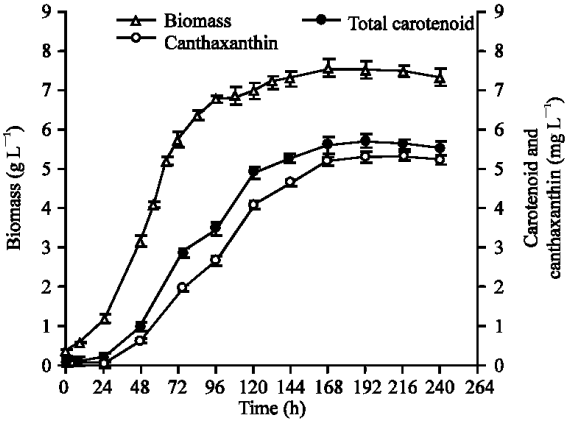

Fig. 4: Time-course profile of the production of biomass, total carotenoid and canthaxanthin by *D. natronolimnaea* HS-1 in Erlenmeyer flask system at the optimum conditions

of carotenoids and canthaxanthin by *D. natronolimnaea* HS-1. The highest concentration of carotenoid and

canthaxanthin obtained after 192 h of cultivation were 5.72 and 5.32 mg L^{-1}, respectively. It indicates that there is high fit degree between the observed value in experiment and the value predicted by model.

DISCUSSION

Ketocarotenoids like astaxanthin and canthaxanthin have been extensively used in many of industries owing to their colorant and strong antioxidant properties (Bhosale and Bernstein, 2005). The high commercial demand for them has long been supplied through synthetic way. However despite the availability of chemically synthetic ketocarotenoids, it may contain undesirable compounds such as unnatural configuration during the processing. Therefore, their production from biological sources has developed to an area of extensive research since the last decade (Ausich, 1997). In this study, optimization of canthaxanthin production by *D. natronolimnaea* HS-1 investigated using statistical experimental designs.

In developing an optimal process for biological production of canthaxanthin, two major aspects are usually considered for improvement. One is the effect of environment conditions and the other is the selection of a suitable nutrient medium. In this work, the effect of both on canthaxanthin production was investigated.

Temperature is one of the most important environmental factors affecting the growth of microorganisms. It causes changes in many biosynthetic pathways, such as carotenoid biosynthesis (Bhosale, 2004). In the present study, *D. natronolimnaea* HS-1 grew at temperatures between 13 to 40°C. The highest value of volumetric production and productivity cantaxanthin was observed at 31°C. This observation is in accordance with obtained results for *Gordonia jacobaea* MV-1 (a canthaxanthin producer) (De Miguel *et al.* 2000) and *Rodotorula mucilaginosa* (Aksu and Tugba Eren, 2005). Hence, temperature of 31°C was used at the all of experiments of optimization process.

In the optimization stage, two-step was employed to optimize canthaxanthin production. In the first step, a fractional factorial design was used for screening of the principal factors implicated in this process. Fractional factorial designs are capable of identifying important factors and determining interaction effects, using a smaller number of experimental runs than a full factorial design without a loss of information on main factor effects and their interactions (Montgomery, 1996). Among five investigated factors in this design, pH and concentrations of glucose and NaCl were determined as the most important factors based on their statistically significant effects ($p<0.01$) on the production of canthaxathin.

Concentration of glucose was the most effective factor on canthaxanthin production. The optimum concentration of glucose in growth media of bacteria is different, depending on the composition of the media and characteristics of bacteria (Gejian and Huazhong, 2004). Concentration of NaCl was the second most important factor. Inorganic salts play important roles in the synthesis of metabolites (Hanhu, 2002). Among the elements, some are highly important for enzyme activities and some such as Na^+ are essential for microbial cells to maintain a proper osmotic pressure (Gejian and Huazhong, 2004). To prevent from lysing or plasmolysing, cells release or accumulate certain low molecular weight osmolytes to respond to osmotic down-or up-ward changes by using such cations as Na^+ (Zimmerman and Trach, 1991; Wood *et al.*, 2001). Hence, proper concentration of Na^+ is essential for careotenoid synthesis by *D. natronolimnaea* HS-1. The pH of growth medium was another important factor, affecting the biomass and cantaxanthin production by *D. natronolimnaea* HS-1. It has been reported that pH significantly influences cell growth and carotenoid production in a lot of microorganism (Ramirez *et al.*, 2001).

In the second step, the central composite design was applied to find the optimal level of those factors in fermentation condition in order to improve canthaxanthin production. A second-order polynomial model was used to identify the relationship between the three factors and canthaxanthin yield. The model estimated that, a maximal yield of canthaxanthin (5.41 mg L^{-1}) could be obtained when the pH value, concentrations of glucose and NaCl were set at 7.53, 25.90 and 3.42 g L^{-1}, respectively. The maximum yield was demonstrated by confirmatory

Table 7: Microbial sources of canthaxanthin

Microorganism	Biomass (g L^{-1})	Canthaxanthin (mg L^{-1})	Canthaxanthin (mg g^{-1})	References
Gordonia jacobaea MV-1	3.2	0.73	0.227	De Miguel *et al.* (2000)
Chlorella emersonii	-	0.60	-	Arad *et al.* (1993)
Haloferax alexandrinus TM[a]	3.17	2.19	0.69	Asker and Ohta (2002)
Haloferax alexandrinus TM[b]	3.12	2.16	0.69	Asker and Ohta (2002)
Micrococcus roseus	-	1.70	-	Cooney and Berry (1981)
Bradyrhizobium sp	0.58	0.78	1.34	Lorquin (1997)
Brevibacterium KY-4313	3.00-3.5	1-2	0.29-0.67	Nelis *et al.* (1989)
Dietzia natronolimnaea HS-1	7.56	5.32	0.70	This work

[a]: The cultivation in Batch fermenter system and [b]: The cultivation in Erlenmeyer flask system

experiment of the optimal medium in 8 day fermentation, that the predict values agreed with the experimental values well.

In conclusion, a comparing this wild strain with other important wilds canthaxanthin-producing strains (Table 7) revealed that *D. natronolimnaea* HS-1, had the highest canthaxanthin production, making it a very promising source for the mass production of canthaxanthin. However, extended studies of different mutants with higher productivity and the genes of enzymes associated with canthaxanthin biosynthetic pathway are also needed to improve canthaxanthin production of *D. natronolimnaea* HS-1.

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