Enzymatically Hydrolysed Molasses and Sodium Citrate as New Potentials for the Improvement of Canthaxanthin Batch Synthesis by *Dietzia natronolimnaea* HS-1: A Statistical Media Optimisation

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Abstract

Gharibzahedi S.M.T., Razavi S.H., Mousavi M. (2014): **Enzymatically hydrolysed molasses and sodium citrate as new potentials for the improvement of canthaxanthin batch synthesis by *Dietzia natronolimnaea* HS-1: A statistical media optimisation**. Czech J. Food Sci., **32**: 326–336.

Response surface methodology-central composite rotatable design (RSM-CCRD) was applied to explore the optimum media formulation for maximising canthaxanthin (CTX) biosynthesis by *Dietzia natronolimnaea* HS-1. The effects of three variables of enzymatically hydrolysed molasses (EHM) (16.6–33.4 g/l), sodium (Na)-citrate (21.64–28.36mM), and yeast extract (6.32–9.68 g/l) concentrations on the production of CTX, total carotenoid (TCT), and biomass dry weight (BDW) were appraised. The results showed that the quadratic effects of EHM, yeast extract, and Na-citrate contents in terms of second-order polynomial regression equations (R^2 = 0.968–0.986), respectively, had the most significant effects on the produced TCT and CTX. The predicted maximum accumulation of BDW (8.88 g/l), TCT (7.24 mg/l), and CTX (6.40 mg/l) under the optimum concentrations of the media variables (26.16 g/l EHM, 8.29 g/l yeast extract, and 25.86mM Na-citrate) was very close to the experimental values determined in batch experiments. The high BDW content suggested EHM and Na-citrate as very promising feedstocks for CTX bioproduction by the bacterium studied.

Keywords: enzymatic hydrolysis; sugarbeet molasses hydrolysate; carotenoid pigment; batch submerged fermentation; response surface modelling

Natural carotenoid pigments have been commercially used as food colorants, nutraceuticals, cosmetics and animal feed supplements (Gharibzahedi *et al.* 2012a). It has been reported that carotenoid pigments have potent antioxidant activity and have a key role in delaying or preventing degenerative diseases in humans and animals (Gharibzahedi *et al.* a,b). The carotenoid market is expected to increase to $ 919 million by 2015 with an average annual growth rate (AAGR) of 2.3%. Nevertheless, the AAGR for the worldwide carotenoids market was estimated to be $ 766 million in 2007 (Vachali *et al.* 2012).

Canthaxanthin (CTX, β-β'-carotene-4,4'-dione) is an abundant pigment in marine sources (crustaceans, fishes, and microalgae), plants, fungi, and bacteria (Gharibzahedi *et al.* b). A number of attempts have been made to explore novel natural sources of CTX regarding the rising global concern to avoid the undesirable effects of synthetic food colorants such as allergy, hypersensitivity, intolerance, and childhood hyperactivity (Li *et al.* 2006). *Dietzia natronolimnaea* HS-1 bacterium among all the introduced sources is recognised as a promising producer of natural CTX. It is Gram-positive, catalase positive, and oxidase

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negative with orange colonies that were isolated during a routine screening of pigmented microorganisms (Gharibzahedi *et al.* 2014).

Raw materials and biowastes of agro-industrial origin such as food processing residues have been frequently applied in recent years as low-cost alternative carbon-sources for biotechnological metabolite synthesis, with the view also of minimising the environmental and energetic problems related to their disposal (Mahro & Timm 2007). Molasses is a suitable waste for fermentation processes since it contains a high concentration of sucrose, is cheap and abundant, and requires only a little handling before fermentation. Molasses contains water, sugars (sucrose, glucose, fructose, raffinose), nitrogen compounds, organic acids, amino acids, heavy metals, and others (Goksungur *et al.* 2004).

Several researchers studied the CTX production by *D. natronolimnaea* HS-1 from the different carbon substrates (Khodaiyan *et al*. 2007, 2008; Nasri Nasrabadi & Razavi 2010a,b; Gharibzahedi *et al.* 2012a). Khodaiyan *et al*. (2007) showed that glucose and fructose sugars can lead to increases in the cell biomass and carotenoids, especially in CTX synthesis in a batch bioreactor. The same authors produced low amounts of CTX from this bacterium using cheese whey (Khodaiyan *et al*. 2008). Nasri Nasrabadi and Razavi (2010a,b) also applied sugarbeet molasses for the CTX synthesis from *D. natronolimnaea* HS-1 in a fed-batch fermentor. Since molasses has considerable amounts of sucrose disaccharide (> 50%), enzymatic hydrolysis can cause increased levels of glucose and fructose and can thus improve the CTX bioproduction.

Response surface methodology (RSM) is a collection of mathematical and empirical techniques useful for establishing models, and for optimising processes even in the presence of complex interactions. It not only determines the interactions between parameters, but also reduces the number of experimental trials, development time, and overall cost (Gharibzahedi *et al*. 2014). Therefore, the aim of the present study was to optimise the formulation of the fermentation medium containing enzymatically hydrolysed molasses (EHM, carbon substrate), yeast extract (nitrogen substrate), and sodium citrate for maximising CTX by *D. natronolimnaea* HS-1 in a batch bioprocess.

MATERIAL AND METHODS

Material, reagents, and chemicals. Beet molasses and pure ethanol (99.9%, v/v) were respectively purchased from Bidestan Co. (Qazvin, Iran) and the Marvdasht Sugar Industry (Fars, Iran). The required ingredients for cell growth of *D. natronolimnaea* HS-1 including D-glucose, yeast extract, peptone, malt extract, sodium citrate (Na-citrate), agar and antifoam 204 were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Hydrochloric acid (HCl), sodium hydroxide (NaOH), acetonitrile (HPLC grade), methanol (MeOH, HPLC grade), dichloromethane (HPLC grade), 3,5-dinitrosalicylic acid (DNS, 98%) and invertase enzyme were obtained from Merck Chemical Co. (Darmstadt, Germany). The CTX standard was provided by the Bioprocess Engineering Laboratory (BPEL, University of Tehran, Iran).

Molasses pretreatment and hydrolysis of its solution. The beet molasses was firstly diluted to a desired concentration of total sugars (mainly sucrose) to eliminate the insoluble materials. This solution was then adjusted to pH 3.0 with 0.5M H_2SO_4. The liquid was allowed to stand for 24 h at room temperature and then centrifuged at 10 000 *g* for 10 minutes. The supernatant was treated with activated carbon without heating to avoid sucrose hydrolysis and then filtered using 0.2-µm filter paper (Sigma-Aldrich Co., St. Louis, USA) under vacuum. Prior to the hydrolysis process, the molasses solution was adjusted to pH 4.5. The hydrolysis was performed by invertase enzyme at a ratio of 0.62% (w/v) for 2 h in a water bath (55°C). Various proportions of enzyme were examined and it was found that the proportion of 0.62% (w/v) can achieve more extensive hydrolysis of sucrose. After the hydrolysis, pH of the medium was adjusted to 7.0 with 10M NaOH and the obtained substrate was sterilised at 121°C for 15 min (Goksungur *et al*. 2002).

Microorganism, culture medium, and inoculum preparation. In this study, the strain of bacterium *D. natronolimnaea* HS-1 (DSM 44860) was obtained from the BPEL. It was kept on yeast/malt (YM) agar plates containing 10 g/l D-glucose, 5 g/l yeast extract, 5 g/l peptone, 3 g/l malt extract, 36 ppm Fe^{3+}, and 15 g/l agar. Every month, single colonies were transferred to a fresh plate, incubated for 4 days, and then maintained under refrigeration at 4°C. After preparing the pre-culture in liquid YM medium (i.e., the above formulation without agar added), the inoculum was transferred into Erlenmeyer flasks containing 20 g/l the EHM and 10 g/l yeast extract. Finally, the flasks were incubated in an orbital shaking incubator (Model SI-4000R; Jeio Tech Co., Seoul, Korea) at 180 rpm and 28 ± 1°C for 6 days to inoculate the bioreactor.

Fermenter set-up and growth conditions. A Bioflo III (2.2 l; New Brunswick Scientific Co, Edison,

USA) containing 8 g/l peptone, 36 ppm Fe^{3+}, and various concentrations of EHM (16.6–33.4 g/l), yeast extract (6.32–9.68 g/l), and Na-citrate (21.64–28.36mM) was used as a batch bioreactor for CTX production. The pre-culture was added to the batch bioreactor in a constant proportion of 15% (v/v). The optimum conditions for the cell growth and CTX formation according to the preliminary studies were pH of 7.0, temperature of 31°C, and stirring rate of 130 ± 20 rpm (Khodaiyan *et al*. 2007). The pH-value during the culture fermentation was controlled at 7.0 using 2.0M NaOH and 2.0M HCl solutions. Foam was controlled by the addition of 25% antifoam 204. The samples were withdrawn at certain times from the batch fermenter to evaluate the growth rate and levels of the produced carotenoid and CTX.

Pigment extraction. Ten millilitres of culture medium were taken at appropriate times during the fermentation from the bioreactor and centrifuged at 7500 *g* for 7.5 min at 4°C. After collecting the produced supernatant, the cell pellets were washed twice with physiological solution (NaCl; 9 g/l in deionised water) and centrifuged. The cells were re-suspended three times in 3 ml of pure ethanol by vortexing for 5 min and centrifuged again to extract the carotenoid pigment. A water bath (45 ± 2°C) was also used to completely extract the pigments (Khodaiyan *et al*. 2008). The extracts were subsequently passed through a 0.2 µm hydrophobic fluorophore membrane (Sigma-Aldrich Co., St. Louis, USA) to obtain the culture filtrate.

Analytical methods. The procedure described by Gharibzahedi *et al.* (2012a) was used for the determination of the biomass dry weight (BDW). Briefly, 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. A portion (10 ml) of each dilution was filtered through a dried (at 65°C for 12 h) and pre-weighed membrane filter (pore diameter 0.2 µm; Sigma-Aldrich Co., St. Louis, USA). All filters were then dried at 105°C to achieve constant weight (48 h) and placed in a desiccator to cool down. The BDW was calculated as the difference between the filter weight before and after the procedure. Finally, the calibration curve was constructed as the dependence of the optical density on the concentration of BDW.

The concentration of reducing sugar was determined by the Miller method using DNS on cell-free supernatants filtered through 0.2 µm filters (Miller 1959). Briefly, 1.0 ml sample was centrifuged at 3500 *g* for 5 min, and then 1.0 ml of DNS reagent was added to the supernatant. The samples were heated to boiling for 5 min and placed in an ice-bath. Afterwards, 8.0 ml distillated water was added, and the tubes were shaken for 5 minutes. Sugar concentration was measured by mean of optical density using an UV-visible spectrophotometer (DR/4000U; HACH Co, Loveland, USA) at 575 nm.

The absorbance of the ethanol extracts was measured in the spectral region of 300–600 nm using an UV/Vis spectrophotometer (V-630; Jasco, Tokyo, Japan), and the absorbance of the TCT content (at λ_{max} = 474 nm) was determined. TCT amount was calculated by using the equation recommended by Nasri Nasrabadi and Razavi (2010a). A Knauer GmbH (Berlin, Germany) HPLC system including a k-1001 HPLC pump, a k-1001 solvent organiser, an on-line degasser, a dynamic mixing chamber, and an UV-Vis detector (K-2600; Knauer GmbH, Berlin, Germany) 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. The isocratic mobile phase used was acetonitrile/MeOH/dichloromethane solvent mixture (71 : 22 : 7, v/v/v) at a flow rate of 2 ml/minutes. To protect the column, a pre-column of the same material was used. The volume of the solutions injected was 10 µl.

Experimental methodology. Twenty treatments in terms of RSM-central composite rotatable design (CCRD) were conducted to investigate the effects of three parameters of EHM (16.6–33.4 g/l, X_1), yeast extract (6.32–9.68 g/l, X_2), and Na-citrate (21.64 to 28.36mM, X_3) concentrations on the produced BDW (Y_1), TCT (Y_2), and CTX (Y_3) by *D. natronolimnaea*

Table 1. Experimental domain of CCRD

Independent variables	Symbol		Coded variables levels				
	uncodified	codified	–1.682 (–α)	–1	0	+1	+1.682 (+α)
EHM concentration (g/l)	X_1	x_1	16.59	20	25	30	33.41
Yeast extract concentration (g/l)	X_2	x_2	6.32	7	8	9	9.68
Na-citrate concentration (mM)	X_3	x_3	21.64	23	25	27	28.36

Table 2. Three-factor CCRD used for RSM with experimental (mean ± SD, n = 3) and predicted values for the independent variables

Run	Independent variables			BDW (Y_1, g/l)		TCT (Y_2, mg/l)		CTX (Y_3, mg/l)	
	X_1	X_2	X_3	actual	predicted	actual	predicted	actual	predicted
1	–1	–1	–1	5.42 ± 0.05	5.53	5.57 ± 0.60	5.56	4.84 ± 0.25	4.80
2	1	–1	–1	6.12 ± 0.08	6.19	5.83 ± 0.24	5.86	5.21 ± 0.21	5.25
3	–1	1	–1	6.19 ± 0.21	6.41	5.62 ± 0.35	5.66	4.77 ± 0.10	4.83
4	1	1	–1	6.62 ± 0.41	6.75	5.48 ± 0.23	5.50	5.14 ± 0.15	5.27
5	–1	–1	1	5.97 ± 0.12	5.91	5.44 ± 0.08	5.50	4.84 ± 0.29	4.79
6	1	–1	1	7.41 ± 0.05	7.27	5.71 ± 0.44	5.75	5.11 ± 0.33	5.12
7	–1	1	1	6.21 ± 0.02	6.22	5.89 ± 0.15	5.94	5.25 ± 0.18	5.29
8	1	1	1	7.56 ± 0.54	7.52	6.73 ± 0.66	6.82	5.64 ± 0.22	5.75
9	0	0	0	8.81 ± 0.09	8.66	6.81 ± 0.24	6.81	6.19 ± 0.13	6.13
10	0	0	0	8.65 ± 0.07	8.66	6.95 ± 0.15	6.81	6.21 ± 0.06	6.13
11	0	0	0	8.87 ± 0.19	8.72	7.26 ± 0.03	7.08	6.27 ± 0.02	6.17
12		0	0	8.79 ± 0.22	8.72	7.15 ± 0.01	7.08	6.22 ± 0.17	6.17
13	–1.68	0	0	5.41 ± 0.36	5.27	5.88 ± 0.16	5.83	5.12 ± 0.39	5.15
14	1.68	0	0	6.79 ± 0.48	6.80	6.23 ± 0.19	6.37	5.99 ± 0.14	5.86
15	0	–1.68	0	6.15 ± 0.14	6.19	6.45 ± 0.43	6.19	5.42 ± 0.51	5.48
16	0	1.68	0	7.19 ± 0.66	7.03	6.82 ± 0.27	6.72	6.14 ± 0.23	5.98
17	0	0	–1.68	7.78 ± 0.47	7.49	6.55 ± 0.11	6.53	6.03 ± 0.05	5.95
18	0	0	1.68	8.19 ± 0.18	8.35	7.25 ± 0.21	7.13	6.32 ± 0.27	6.29
19	0	0	0	8.66 ± 0.39	8.94	7.37 ± 0.21	7.52	6.67 ± 0.44	6.74
20	0	0	0	8.88 ± 0.74	8.94	7.31 ± 0.16	7.52	6.49 ± 0.51	6.74

HS-1 (Tables 1 and 2). The Design Expert (Trial Version 7.1.3; Stat-Ease Inc., Minneapolis, USA) was applied for the regression and graphical analyses of the obtained data. The design used was integrated (I) to determine a reasonable relationship between the three independent variables and each response, and (II) to find the optimum levels of the independent variables resulting in the desirable objectives. Our preliminary studies had shown that the selection of the studied levels for different factors resulted in a desirable production of BDW, TCT, and CTX by *D. natronolimnaea* HS-1 in a batch bioreactor. Multiple regression coefficients were determined by employing the least-squares technique to predict the linear and polynomial models for the response variables (Myers & Montgomery 2002). The behaviour of the response surface was evaluated for the response function (Y_i, the predicted response) using the regression second-order polynomial equation. The generalised polynomial model proposed for predicting the response variables is given as (Eq. 1):

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

where: Y – predicted response; β_{k0}, β_{ki}, β_{kii}, β_{kij}– regression coefficients; x_i, x_j – coded independent factors

The suitability of the fit of the polynomial model equations was tested by the coefficient of determination (R^2), adjusted-R^2, coefficient of variation (CV), and adequate precision (AP) as previously explained iny the literature (Gharibzahedi *et al*. 2012). The reduced models were constructed by analysing the regression coefficients using the analysis of variance (ANOVA) and removing the non-significant coefficients from the initial model. Three additional experiments were subsequently conducted to verify the validity of the statistical experimental strategies.

RESULTS AND DISCUSSION

Mathematical modelling and ANOVA analysis. ANOVA analysis was conducted to check the adequacy of the proposed models and identify the significant factors. Based on the experimental results of CCRD (Table 2) and regression analysis (Table 3), quadratic polynomial equations (P < 0.0001) were established to identify the relationship

between the independent and response variables. The obtained models for the production of BDW (Y_1, Eq. 2), TCT (Y_2, Eq. 3), and CTX (Y_3, Eq. 4) in terms of the experimental (uncoded, Table 1) values are given below:

$$Y_1 = 8.78 + 0.46X_1 + 0.25X_2 + 0.26X_3 - 1.03X_1^2 - 0.83X_2^2 - 0.36X_3^2 + 0.21X_1X_3 \quad (2)$$

$$Y_2 = 7.15 + 0.16X_1 + 0.16X_2 + 0.18X_3 - 0.50X_1^2 - 0.38X_2^2 - 0.24X_3^2 + 0.12X_1X_3 + 0.22X_2X_3 \quad (3)$$

$$Y_2 = 6.34 + 0.21X_1 + 0.15X_2 + 0.10X_3 - 0.44X_1^2 - 0.36X_2^2 - 0.22X_3^2 + 0.22X_2X_3 \quad (4)$$

The lack-of-fit test determines the model failure to represent the data in the experimental domain at points which are not included in the regression (Gharibzahedi *et al.* 2012b). In this study, the test implied that the lack-of-fit values were insignificant (Table 3), which suggested the model fit of the experimental data. The R^2 and adjusted-R^2 values respectively were 0.986 and 0.970, 0.976 and 0.949, and 0.968 and 0.932 for BDW, TCT, and CTX synthesised by *D. natronolimnaea* HS-1. Generally, *CV* value should not be greater than 10% (Gharibzahedi *et al*. 2012a,b). The *CV* values for the studied response variables ranged from 2.31 to 3.06. These data represent a high precision and reliability of the conducted experiments. AP also compares the range of the predicted values at the design points to the average prediction error. In other words, it measures the signal-to-noise ratio. A ratio greater than 4 is desirable (Gharibzahedi *et al.* 2012b). AP values calculated for the different proposed models (16.54–21.29) showed a very good signal-to-noise ratio.

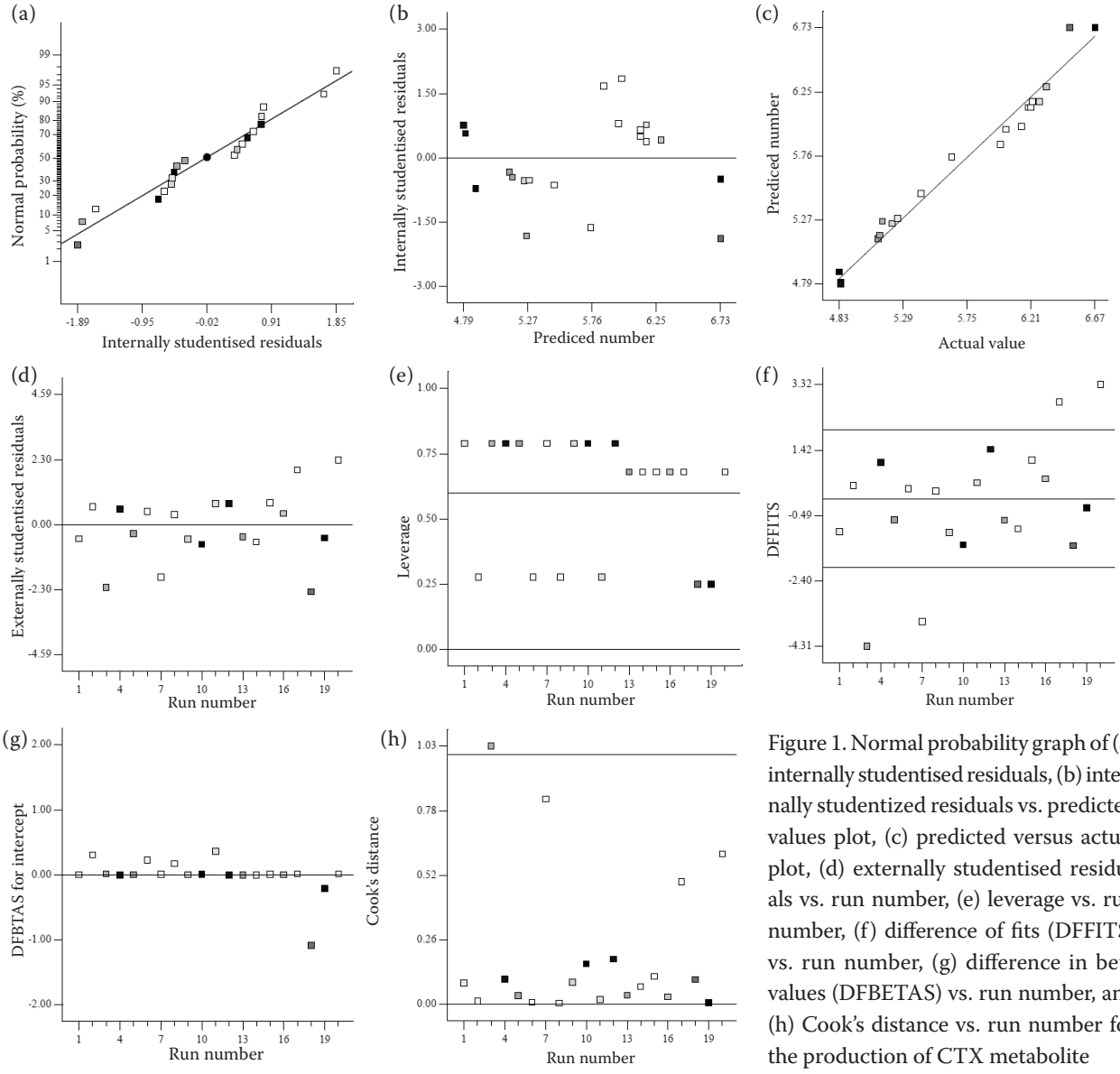

Figure 1. Normal probability graph of (a) internally studentised residuals, (b) internally studentized residuals vs. predicted values plot, (c) predicted versus actual plot, (d) externally studentised residuals vs. run number, (e) leverage vs. run number, (f) difference of fits (DFFITS) vs. run number, (g) difference in beta values (DFBETAS) vs. run number, and (h) Cook's distance vs. run number for the production of CTX metabolite

Diagnostics and influence plots for the CTX model. Figure 1 shows the residual and the influence plots for the CTX experiments as the main metabolite of this study. Figure 1a shows that the normal plot of residuals for the CTX response was normally distributed, as they lie approximately on a straight line and shows no deviation of the variance. The studentised residuals versus the predicted data points are depicted in Figure 1b. Figure 1c reveals the comparison between the actual response value obtained from experimental data and the predicted response value based on the polynomial regression models and proves that the model covers the experimental range of studies sufficiently. As clearly observed in Figure 1d, all the data points lay within the limits. Since all leverage values were less than 1 (Figure 1e), there are no outliers or unexpected errors in the model (Ghasemlou *et al.* 2012). The difference of fits plot (Figure 1f), a measure of the influence of each point on the predicted value, suggested four points (corresponding to runs 3, 7, 17, and 20) that influence the regression equation and the response very disproportionately. However, the difference in beta values plot (Figure 1g) demonstrated no undue influence of any observation on any of the regression coefficients. Since the Cook's distance values are in the determined range (Figure 1h), there is strong evidence for influential observations in these data.

Model interpretation

Production of cell biomass. As considered in Table 3, the linear and quadratic effects of all independent variables were highly significant with the BDW produced by *D. natronolimnaea* HS-1. Among the independent variable effects, the quadratic effects of EHM and yeast extract concentrations followed by the linear effect of EHM content had the most significant ($P < 0.05$) effect on the BDW (Table 3). The results indicated that the interaction between EHM and yeast extract contents, and Na-citrate and yeast extract concentrations had no significant ($P >$

Table 3. ANOVA analysis for the effects of the independent variables on the dependent variables and the regression coefficients of the fitted quadratic equations obtained from experimental results

Source	DF	BDW (Y_1, g/l)			TCT (Y_2, mg/l)			CTX (Y_3, mg/l)		
		coefficient	sum of squares	*P*-value	coefficient	sum of squares	*P*-value	coefficient	sum of squares	*P*-value
Model	9	8.78	28.44	< 0.0001	7.15	7.26	< 0.0001	6.34	5.67	< 0.0001
Linear										
β_1	1	0.46	2.85	< 0.0001	0.16	0.35	0.004	0.21	0.6	0.0009
β_2	1	0.25	0.85	0.0033	0.16	0.34	0.004	0.15	0.3	0.0072
β_3	1	0.26	0.89	0.0028	0.18	0.44	0.002	0.10	0.14	0.0410
Quadratic										
β_{11}	1	–1.03	15.22	< 0.0001	–0.50	3.66	< 0.0001	–0.44	2.75	< 0.0001
β_{22}	1	–0.83	9.84	< 0.0001	–0.38	2.05	< 0.0001	–0.36	1.84	< 0.0001
β_{33}	1	–0.36	1.88	0.0003	–0.24	0.86	0.0002	–0.22	0.68	0.0006
Interaction										
β_{12}	1	–	0.016	ns	–	0.00	ns	–	0.00	ns
β_{13}	1	0.21	0.34	0.0300	0.12	0.12	0.045	–	0.00	ns
β_{23}	1	–	0.09	ns	0.22	0.39	0.0029	0.14	0.15	0.0364
Residual	8		0.04			0.18			0.19	
Lack-of-fit	5		0.36	0.0995^{ns}		0.16	0.0984^{ns}		0.17	0.0915^{ns}
Pure error	3		0.04			0.01			0.01	
Total	19		28.98			9.00			7.30	
R^2		0.986			0.976			0.968		
Adj-R^2		0.970			0.949			0.932		
CV		3.06			2.31			2.67		
AP		21.29			17.66			16.54		

ns – not significant

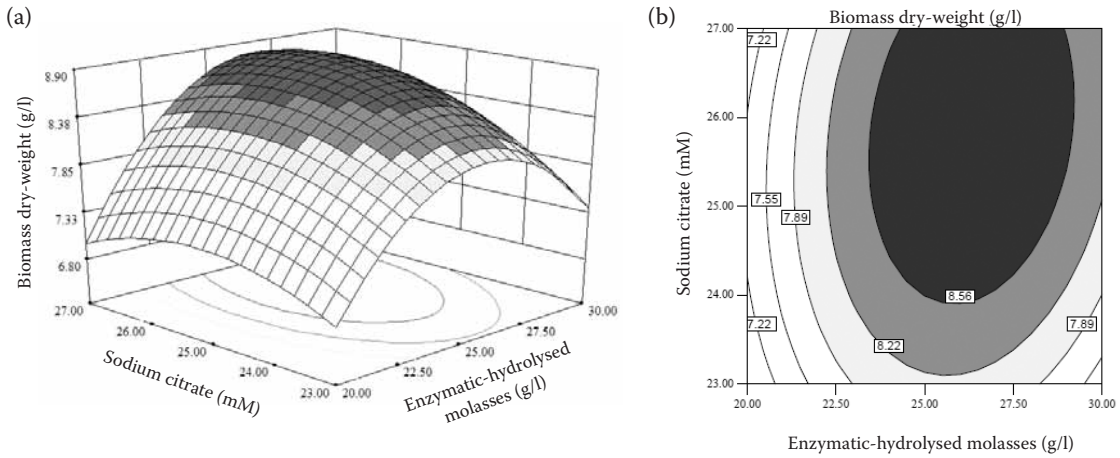

Figure 2. (a) Response surface (3-D) and (b) contour (2-D) plots showing the effect of significant interaction of EHM and Na-citrate concentrations on the biomass produced by *D. natronolimnaea* HS-1

0.05) effect on the BDW (Table 4). Figures 2a and 2b show the surface and contour plots for significant interaction of EHM and Na-citrate concentrations on the biomass produced by *D. natronolimnaea* HS-1. In the culture supplemented with about 26.0 g/l EHM (Figure 2), the BDW increased to 8.56 g/l, but it decreased significantly with further increase in EHM to 30 g/l. Bouallagui *et al*. (2013) stated that the low BDW at higher levels of carbon source can be due to the osmotic stress and/or substrate inhibition effect in the culture media. Park *et al*. (2008) showed that the cell growth reduction of β-Ionone resistant mutant of *Xanthophyllomyces dendrorhous* occurs because of the substrate inhibition effect and length increase of the lag phase at high concentrations of sugar. The high availability of cells to oxygen can stimulate the aerobic metabolism resulting in higher ATP availability for the BDW production. Thus, the existence of high viscosity at high concentrations of EHM can also limit oxygen transfer and decrease the yield of the synthesised biomass. Thus, the low BDW production at reducing sugar concentrations below 26 g/l can be attributed to lower ATP availability for the cells metabolism and growth. An increase in Na-citrate concentration to 25.3mM led to an enhancement of the BDW production (Figure 2). Flores-Cotera *et al.* (2001) and Bohsale (2004) pointed out that the cells stimulation for more extensive growth by citrate up taking into the cytoplasm could probably be due to the greater availability of acetyl-CoA and ATP by activating citrate lyase. However, this factor decreased significantly at higher Na-citrate concentrations. Flores-Cotera *et al.* (2001) also found that the BDW and growth rate of *Phaffia rhodozyma* decreased with increasing citrate concentration from 30mM to 60mM. The growth reducing effect with a high accumulation of citrate in the mitochondria and cytosol has been usually clarified by allosteric inactivation of NAD^+-isocitrate dehydrogenase. Based on this inactivation, the high ATP/AMP and NADH/NAD^+ ratios must reduce the activity of the enzyme (Boulton & Ratledge 1981; Evans *et al*. 1983; Flores-Cotera *et al.* 2001). It was found that the optimum levels of independent variables leading to maximum BDW value (8.89 g/l) were predicted to be achieved by a combined level of 26.10 g/l EHM, 8.16 g/l yeast extract, and 25.34mM Na-citrate. This value was 1.5 times higher than that of the biomass produced by the same bacterium from D-glucose (Khodaiyan *et al.* 2007).

TCT and CTX production. The linear and quadratic effects of all independent variables on the TCT and CTX were significant (Table 3). ANOVA analysis and regression coefficients of the fitted models showed that the quadratic effects of EHM, yeast extract, and Na-citrate, respectively, had the most significant ($P < 0.05$) effect on the synthesised TCT and CTX (Table 3). The influence of EHM and yeast extract contents on the TCT and CTX production was surprisingly insignificant ($P < 0.05$). The results also showed that the interaction effects of Na-citrate content with EHM and yeast extract concentrations on the TCT production by *D. natronolimnaea* HS-1 were significant (Figures 3a–d). Moreover, only significant effect on the CTX synthesis was found on the interaction between the yeast extract and Na-citrate concentrations (Figures 3e and 3f). As the BDW production, the TCT and CTX syntheses were decreased by increasing EHM concentration from 26.2 g/l to 33.4 g/l. This fact could be attributed to the exist-

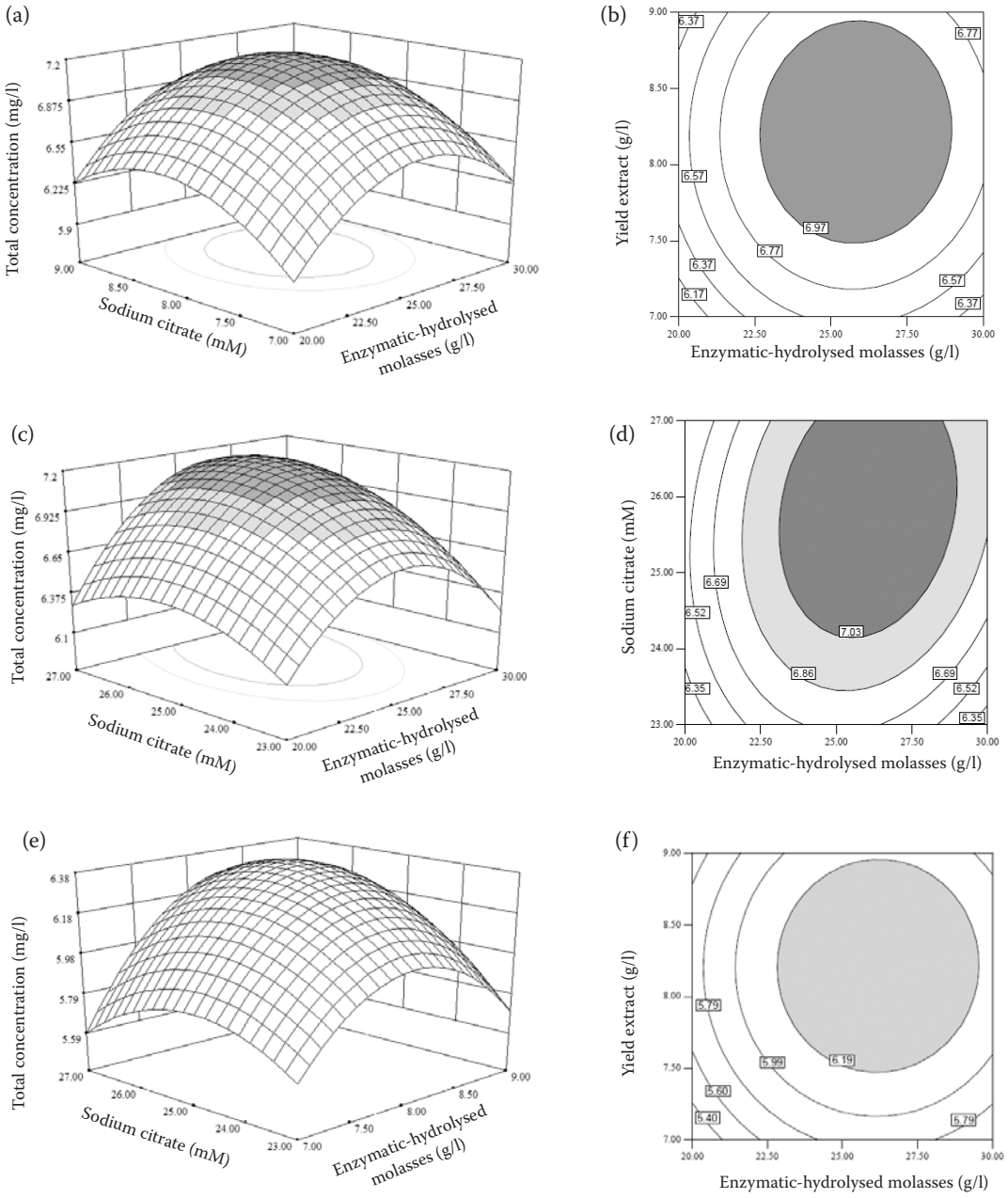

Figure 3. Response surface (a, c, and e) and contour plots (b, d, and f) showing the effects of variables (X_1: EHM concentration; X_2: yeast extract content; X_3: Na-citrate concentration) on the TCT (a–d) and CTX (e–f) produced by *D. natronolimnaea* HS-1

ence of anaerobic and viscous conditions and ethanol synthesis because of the Crabtree effect at high concentrations of EHM under a batch process (Avci *et al.* 2014). A similar finding for astaxanthin (AST) production by *Ph. rhodozyma* cultivation at high contents of glucose was also reported by Reynders *et al.* (1997). Avci *et al.* (2014) also demonstrated that the by-products formed during the anaerobic fermentation can reduce the 1,3-propanediol production by *Klebsiella pneumonia* via inhibition of the enzymes activity involved in its biosynthesis pathway. In general, a decrease in the produced pigment level was observed after 156 h of fermentation at all the concentrations EHM used (data not shown). It probably might be due to the long-time exposure of the dissolved carotenoids to oxygen, light, and microorganisms in the environment which leads to the chance increase of oxidation or degradation of the dissolved carotenoids. In addition, the depletion of carbon in YM medium at these times might have caused the decreased production of carotenoids (Gharibzahedi *et al.* 2012a). The results showed that the increase of

the yeast extract concentration to 8.3 g/l due to the accurate pH-adjustment increased the TCT and CTX biosyntheses in a batch bioreactor. Yeast extract has a buffering capacity, and this might contribute to the control of culture pH at the required level during the cultivation (Gaudreau *et al*. 1997). Moreover, the use of peptone in combination with the yeast extract would further enrich the medium with peptides, B-complex vitamins, and carbohydrates and thus increase the cell growth and product formation (Janssen *et al*. 2000). Khodaiyan *et al.* (2008) by studying various inorganic and organic nitrogen sources proposed that the highest CTX production level by *D. natronolimnaea* HS-1 was obtained in the presence of yeast extract and peptone. Similar findings were previously reported for *Ph. rhodozyma* (Kim *et al*. 2003, 2006) and the mutant of *Rhodotorula glutinis* (Bhosale & Gadre 2002). Kim *et al.* (2005) also showed that the increase of the yeast extract and glucose to optimum values led to a significant enhancement in AST synthesis (36.06 mg/l) by *X. dendrorhous*. Moreover, optimum content of Na-citrate in tricarboxylic acid cycle metabolic pathway can enhance the cell ATP and increase the pigment formation. The optimum conditions for maximising the TCT (7.25 mg/l) was 26.21 g/l EHM, 8.40 g/l yeast extract, and 26.22mM Na-citrate. The highest CTX value (6.40 mg/l) could also be obtained by formulating the culture media with 26.21 g/l EHM, 8.27 g/l yeast extract, and 25.62mM Na-citrate. Khodaiyan *et al.* (2007) reported that 2.87 mg/l TCT and 2.45 g/l CTX were produced by *D. natronolimnaea* HS-1 from 10 g/l molasses and 6 g/l yeast extract. It is well known that the sucrose uptake is lower than that of glucose; therefore, high pigment amounts attained are probably due to a higher carbon availability in media containing EHM. These researchers also showed that glucose and fructose sugars were the best carbon sources for the growth and biomass production by *D. natronolimnaea* HS-1 (Khodaiyan *et al.* 2007).

Optimisation and validation of the reduced response models. A numerical optimisation technique using the desirability approach was employed to develop a new media formulation with the desired responses from *D. natronolimnaea* HS-1. The fermentation process would be considered optimum production if the criteria applied for the optimisation resulted in the highest BDW, TCT, and CTX. The optimisation results indicated that the overall optimum region was predicted to be at the combined level of 26.16 g/l, 8.29 g/l, and 25.86mM of EHM, yeast extract and Na-citrate, respectively. The corresponding response values for BDW, TCT, and CTX predicted under

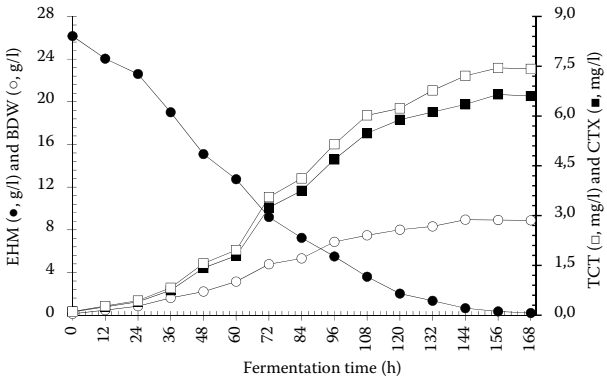

Figure 4. Time profile of batch cultivation of *D. natronolimnaea* HS-1 with 26.16 g/l initial EHM concentration, 8.29 g/l yeast extract, and 25.86mM Na-citrate

the recommended optimum condition were 8.88 g/l, 7.24 mg/l, and 6.40 mg/l, respectively. The adequacy of these predicted models was examined through additional experiments under the suggested optimal production conditions. The actual experimental values for BDW, TCT and CTX, respectively, were 8.95 ± 0.11 g/l, 7.45 ± 0.23 mg/l, and 6.65 ± 0.16 mg/l. The values of the experimental responses were in agreement with the predicted values, as no significant difference ($P > 0.05$) was found between the experimental and predicted values. The time-course of CTX, TCT and cell mass production, and EHM consumption by *D. natronolimnaea* HS-1 in a batch process under optimum conditions is shown in Figure 4. This figure clearly demonstrates that the BDW and CTX concentrations respectively increased as the fermentation proceeded up to 144 and 156 hours. The maximum TCT and CTX syntheses were reached one day after the beginning of the stationary phase. Similar behaviour has also been reported with other microorganisms, such as *Gordonia jacobaea* (Veiga-Crespo *et al*. 2005), *Flavobacterium* sp. (Masetto *et al.* 2001), and *Rh. glutinis* (Bhosale & Gadre 2001). About 1.2-fold increase was observed as compared to the previous report by Khodaiyan *et al.* (2007). They reported that the BDW and CTX production by *D. natronolimnaea* HS-1 from pure glucose were respectively 7.25 g/l and 5.29 mg/l in a batch fermentation process. This fact showed that the response surface optimisation of EHM concentration as a renewable carbon source and Na-citrate content as a tricarboxylic acid cycle intermediate can practically increase the production yield in a batch bioreactor.

CONCLUSIONS

The purpose of this study was to obtain better understanding of the influence of EHM, yeast extract,

and Na-citrate concentrations on the BDW, TCT, and CTX produced by *D. natronolimnaea* HS-1. Using the surface and contour plots in RSM proved to be successfully effective for determining the effects of the three independent variables. The models assumed that the highest BDW (8.88 g/l), TCT (7.24 mg/l), and CTX (6.40 mg/l) could be achieved at 26.16 g/l EHM, 8.29 g/l yeast extract, and 25.86mM Na-citrate. The predicted values were justified by verification tests. Surprisingly, the experimental values under optimum conditions were higher than the predicted values. The synthesis of 6.65 mg/l CTX from EHM by the bacterium studied was very near to its production based on D-glucose in the previous studies. Thus, this investigation can be finally considered as an effective contribution to the development of more efficient bioprocesses for industrial synthesis of CTX.

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