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A STATISTICALLY MOTIVATED CHOICE OF PROCESS PARAMETERS FOR THE IMPROVEMENT OF CANTHAXANTHIN PRODUCTION BY *DIETZIA MARIS* NIT-D (ACCESSION NUMBER: HM151403)

ELECCIÓN DE PARÁMETROS DE PROCESO MEDIANTE ANÁLISIS ESTADÍSTICO PARA MEJORAR LA PRODUCCIÓN DE CANTAXANTINA USANDO *DIETZIA MARIS* NIT-D (NÚMERO DE ACCESO: HM151403)

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Abstract

Dietzia maris NIT-D, a producer of canthaxanthin, is isolated during routine screening of pigment producing bacteria. The effects of process parameters, namely temperature, pH, shaker speed, percentage inoculum, medium volume, and concentration of glucose on the canthaxanthin production are studied by using response surface design methodology. The optimal conditions are temperature = 30°C, pH = 5.9, shaker speed = 125 rpm, inoculum = 1.9 %, volume = 50 mL, and glucose = 15 g L^{-1}, resulting in a canthaxanthin production of 152 mg L^{-1}, which is 25% higher than that of a recently reported study.

Keywords: *Dietzia maris* NIT-D, biomass, canthaxanthin, response surface methodology.

Resumen *Dietzia maris* NIT-D una bacteria productora de cantaxantina, fue aislada durante una selección de cepas. Mediante un diseño de superficie de respuesta fueron estudiados los efectos de los parámetros del proceso para la producción de este pigmento: temperatura, pH, velocidad de agitación, porcentaje de inóculo, volumen del medio de cultivo y concentración de glucosa. Las condiciones optimas fueron 30°C, pH de 5.9, 125 rpm, 1.9 % de inóculo, 50 mL de medio de cultivo y 15 g L^{-1} de glucosa. Como resultado la producción de cantaxantina fue de 152 mg L^{-1}, 25% más que en un reciente estudio.

Palabras clave: *Dietzia maris* NIT-D, biomasa, cantaxantina, metodología de superficie de respuesta.

1 Introduction

Canthaxanthin is a ubiquitous keto-carotenoid in the red wavelength range. It is widely used as a colorant in the food industry and it occurs naturally in a wide variety of living organisms which includes bacteria, microalgae, crustaceans, and fishes (Hannibal *et al.*, 2000). However, microorganisms, as potential canthaxanthin producers, are limited. Similar to mangiferin (Padmapriya *et al.*, 2012), canthaxanthin

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has remarkable possibilities for application as a nutraceutical owing to its anticarcinogenic, tumor suppressant characteristics and antioxidant properties. It displays maturity in animals and protection of tissues (plant and animal) against oxidizing free radicals (Moller *et al.*, 2000). In the present scenario, synthetic canthaxanthin is being produced on an industrial scale since no cheap commercially exploitable plant or animal sources exist. Hence, an increasing demand for safe, naturally occurring edible coloring agents has necessitated the development of promising natural coloring alternatives. Microorganisms provide an excellent system for the large-scale production of carotenoids, due to their ease of manipulation (Veiga-Crespo *et al.*, 2005). Microorganisms have an advantage over other natural sources, as the fermentation process can be easily controlled to achieve higher growth rate and greater cell density without posing any serious production limitations in terms of space and time (Das *et al.*, 2007 and Krupa *et al.*, 2010). Moreover, the potential of selected microbial strains can be fully exploited by optimization of the medium and environmental conditions in microbial fermentations. Therefore, it is important to optimize pigment producing conditions of isolated strains and explore their industrial possibilities.

In a recent study, Goswami *et al.* (2012) reported the process parameters for which the canthaxanthin yield is maximized. They obtained an optimal yield of 121.53 mg L^{-1} when *Dietzia maris* NIT-D was incubated for 120 h at 25°C and 120 rpm, initial pH and percentage inoculum being 5.5 and 2% respectively. Their yield is considerably higher than that reported by other studies; see, for example, Khodaiyan *et al.* (2007a), who reported an optimal yield of 5.32 mg L^{-1}. In a separate study, Khodaiyan *et al.* (2008) have reported a canthaxanthin yield of 2.871 mg L^{-1}, at a pH of 7.66, using whey lactose as the sole source of carbon. In a recent study, Gharibzahedi *et al.* (2013) have reported a high canthaxanthin yield of 6.01 g L^{-1}, when *Dietzia natronolimnaea* HS-1 was cultivated in a batch bioreactor, with waste molasses hydrolysate as the carbon source, at a pH of 7. Previous work with canthaxanthin by several other researchers has reported much lower yield (Ohta, 2002; Cooney and Berry, 1981; de Miguel *et al*., 2000; Khodaiyan *et al*., 2007b; Lorquin *et al*., 1997; Malis *et al*., 1993; Nelis and de Leenheer, 1989; Veiga-Crespo *et al*., 2005). The analysis of Goswami *et al.* (2012) results in a maximum which is attained in the endpoints of their central composite design (CCD). Based on the second partial derivative test, the critical point of their second order model is a saddle point and not a maximum. Consequently, there is a possibility that the process under study attains a maximum beyond the range of their experiments.

Since it was deciphered from our previous experiments that glucose is an important chemical constituent which has an interactive effect with all the other physical parameters, we found it necessary to vary the concentration of glucose along with other physical parameters. Therefore, the study of Goswami *et al.* (2012) is extended by the application of a two stage design. In the present study, the volume of the medium and glucose concentration is also considered besides process parameters namely pH, temperature, shaker speed, and percentage inoculum. First, an extensive screening experiment is considered to explore the canthaxanthin yield for a wide range of parameter values. Based on the screening, a new CCD is constructed which is centred around the maximum canthaxanthin. This assures that the maximum is attained within the interior of the parameter range and hence is guaranteed to be an optimum. A second order statistical model then gives the optimal parameter settings.

2 Material and methods

The details on the microorganism, experimental and analytical methods are described in Goswami *et al.* (2012) and are summarized below.

2.1 Microorganism

Dietzia maris NIT-D was isolated from soil during routine screening of pigment-producing microorganisms. The sequence was submitted to the Gen Bank and an accession number of HM151403 was obtained. The strain was aerobically maintained and aseptically sub-cultured in brain heart infusion agar (BHIA) (Hi-Media, India) slants (Duckworth *et al.*, 1998), every 7 days, by incubation at 25°C for 120 h, and stored at 4°C until further use.

2.2 Experimental methods

2.2.1 Growth Medium

Dietzia maris NIT-D was cultivated in 50 mL of the medium (pH 5.5, prior to sterilization) containing 5 g L^{-1} D-(+)-glucose (Hi-Media, India), 5 g L^{-1}

NaCl (Merck, India), 10 g L^{-1} bacteriological peptone (Hi-Media, India) and 5 g L^{-1} yeast extract (Hi-Media, India). A digital weighing balance (Sartorius, CP225D) was used to weigh all the chemicals. The prepared medium was sterilized by autoclaving at 121°C for 15 min. In order to avoid caramelization, the glucose solution was autoclaved separately and mixed aseptically with the other components under cooling (Don and Shoparwe, 2010).

2.2.2 Preparation of inoculum

In order to prepare the inoculum, pure cultures of *Dietzia maris* NIT-D, initially maintained on BHIA slants, were transferred into 250 mL Erlenmeyer flasks. Each of the Erlenmeyer flasks contained 50 mL of the growth medium. This was followed by incubation at 25°C, with shaking at 120 rpm (Borzenkov *et al.*, 2006), for 48 h. A UV-Vis Spectrophotometer (U-2800, Hitachi) was used to measure the optical density, in order to standardize the inoculum for further experiments.

2.3 Analytical methods

2.3.1 Determination of biomass

Biomass dry weight was determined following the protocol given by Khodaiyan *et al.* (2008) with modifications. 4 mL culture samples were taken in 15-mL centrifuge tubes. Cells were harvested, subsequently filtered through 0.2-μm filter (Pall Corporation, USA) and dried at 65°C for 4 h. The filtered cells were then washed with distilled water and dried, in a hot air oven (OVFU), at 105°C to a constant weight for 48 h. The results are represented as dry weight (g)/1000 mL of culture.

2.3.2 Extraction and analysis of pigment

The pigment was extracted following the protocol described previously by Asker and Ohta (1999) with modifications; 10 mL aliquots of cultures were centrifuged at 7000 g for 30 min using a cooling centrifuge (Eppendorf, 5,810 R). The harvested cells were resuspended in distilled water. Spontaneously, cell lysis occurred. The pigment was then extracted with methanol (HPLC grade; Merck, India) by repeated centrifugation until the cell debris turned colorless and transferred to hexane (Calo *et al.*, 1995). The pigment extracts were subsequently filtered through a 0.45-μm hydrophobic PTFE membrane (Waters) and analyzed by scanning the absorbance in the wavelength region of 350-650 nm using the UV-Vis spectrophotometer (U-2800, Hitachi). The maximum absorbance was determined at a wavelength of 473 nm. The results are represented as pigment yield (mg)/1000 mL of culture.

The pigment was identified by high-performance liquid chromatography (HPLC) (Waters 600, Milford, USA) equipped with a UV-Vis detector (Waters 2489). The pigment extracts were filtered through a 0.45-μm hydrophobic PTFE membrane (Waters). Chromatographic separation was performed on a reverse-phase C_{18} column (250 mm x 4.6 mm, Waters) where the temperature of the column was maintained at room temperature. The mobile phase used was a mixture of methanol and acetonitrile (20:80, V/V) at a flow rate of 1 mL min^{-1}. The pressure was 1.05 kpsi and the injection volume was 20 μL. The peaks were evaluated based on their absorbance at 473 nm. Retention time and concentration of the samples were compared with pure standards of canthaxanthin (Sigma-Aldrich, USA).

2.4 Screening experiments

Experiments are carried out, in the growth medium, for optimizing culture conditions for pigment production by *Dietzia maris* NIT-D. For optimization experiments, the glucose concentration, temperature, pH, shaker speed, and percentage inoculum is varied between specific ranges, as predetermined by the COVT approach. An initial set of experiments is carried out for a wide range of pH (4 - 12.5), temperature (5°C - 55°C), shaker speed (90 rpm - 180 rpm), percentage inoculum (0.5% - 10%), and medium volume (50 mL - 250 mL). The concentration of glucose is fixed at 5 g L^{-1}. Based on results obtained from the above set of experiments, the range of the variables is narrowed down and the next sets of experiments are designed. Glucose concentration is varied from 5 g L^{-1} to 25 g L^{-1}. For individual concentration of glucose, the pH is varied from 5.2 to 8 at two specific temperatures (25°C and 37°C). At optimum pH, the temperature is varied from 15°C to 40°C. The shaker speed is varied from 90 rpm to 180 rpm at 25°C and pH 5.5. Similarly, the percentage inoculum is varied from 0.5% to 4% in batch experiments at 25°C, 120 rpm and pH 5.5. Finally, at optimized conditions of pH, temperature, shaker speed and percentage inoculum, the medium volume is varied from 50 mL to 150 mL in Erlenmeyer flasks having a maximum volume capacity of 250 mL. In total 254 experimental runs are carried out for this screening.

Table 1. Parameter values of the rotatable central composite design

	$-\alpha$	-1	0	$+1$	$+\alpha$
Temperature (°C)	18	22	26	30	34
pH	5	5.4	5.8	6.2	6.6
Shaker speed (rpm)	100	110	120	130	140
Inoculum (%)	0.8	1.4	2	2.6	3.2

2.5 Rotatable central composite design

Based on the analysis of the screening experiments, a rotatable central composite design (RCCD) with 30 design points is constructed (Montgomery, 2001). A RCCD is often used to find the optimal parameter settings, see, for example, Corona-González *et al.* (2013) and Rodriguez-Marín *et al.* (2013). The screening experiment provided sufficient evidence that the maximum canthaxanthin is obtained at a medium volume of 50 mL and a glucose level of 15 g L^{-1}, and hence are fixed accordingly for the RCCD. Each parameter of a RCCD can take on 5 values, usually denoted as $-\alpha, -1, 0, +1$ and $+\alpha$, where α denotes the axial point for rotatability. Table 1 gives the values for each of the four parameters. Note that these differ from those reported by Goswami *et al.* (2012). In addition to the canthaxanthin yield, the biomass is also measured.

2.6 Statistical analysis

A second order statistical model is considered for the analysis of the RCCD. Let X_1 denote inoculum, X_2 shaker speed, X_3 temperature, X_4 pH, and let Y denote the response (Y_B for biomass and Y_C for canthaxanthin), then the second order model (SOM) is given by

$$E(Y) = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j, \quad (1)$$

where $E(Y)$ denotes the expectation of Y. Since the SOM will mainly be used for predictions, the main assumption states that model Eq. (1) is an appropriate response surface of $E(Y)$ as a function of X_1, X_2, X_3, and X_4. This assumption is assessed graphically with residual plots and more formally with the F-test for lack of fit. Since the F-test assumes normality and constant variance of the residuals, these assumptions are examined graphically with residual and QQ-plots, as well as with the Kolmogorov-Smirnov (KS) test for normality and the Breusch-Pagan (BP) test for constancy of error variance (Kutner *et al.*, 2005). All analyses are conducted by using the R statistical software package (R, 2012).

3 Results and discussion

Both growth and pigment production was observed over a broad pH range (5.0-12.0). At both 25°C and 37°C, pigment production increased consistently with the increase in pH from 5 to 5.5 and decreased with the increase in pH beyond 5.5. Minimum pigment content was observed at pH above 10. Canthaxanthin produced by *Dietzia maris* NIT-D, when grown in growth medium at 37°C, was 5.33 mg L^{-1} and 113.53 mg L^{-1} at pH 5 and 5.5 respectively. The pigment yield increased to 5.71 mg L^{-1} and 121.6 mg L^{-1} at pH 5 and pH 5.5 respectively when grown at 25°C. Maximum canthaxanthin production at pH 5.5 is a distinctive characteristic of this strain and has not been reported so far. Pigment production gradually and uniformly decreased with the increase in temperature from 25°C to 37°C. At temperatures over 37°C, low pigment content was observed. Canthaxanthin production did not occur below 10°C and above 45°C. The temperature tolerance range of *Dietzia maris* NIT-D with considerable productivity between 15°C and 40°C reestablished it to be a mesophilic bacterium. The degree of pigmentation was maximum at 120 rpm and minimum at 180 rpm. Only 88.33 mg L^{-1} of canthaxanthin was obtained when the isolate was grown at 180 rpm. Both growth and pigment production decreased with the increase in inoculum from 2% to 10%. Canthaxanthin yield at an optimum percentage inoculum was 121.6 mg L^{-1} which decreased by 23.5% to yield 92.97 mg L^{-1} when 10% inoculum was used. This maximum yield of 121.6 mg L^{-1}, under optimized process parameters, was found to increase to 186.36 mg L^{-1} when glucose concentration in the medium was increased to 15 g L^{-1}. However, with the further increase in glucose concentration to 20 g L^{-1} and 25 g L^{-1}, the pigment yield decreased to 162.45 mg L^{-1} and 128.44 mg L^{-1} respectively. This fact may be explained on the basis of catabolic repression on canthaxanthin synthesis observed at higher concentrations of glucose. Maximum growth and canthaxanthin production occurred in 50 mL medium per 250 mL Erlenmeyer flask. Pigmentation decreased with the increase in medium volume from 50 mL to 250 mL. The pigment yield was 120.65 mg L^{-1} when *Dietzia maris* NIT-D was grown in 50 mL

of the optimal medium. The pigment yield declined by 90.4% to give 11.52 mg L^{-1} of culture when the medium volume increased by five times to 250 mL. Experiments on the effect of medium volume on growth and pigment production by *Dietzia maris* NIT-D have reproduced that both growth and canthaxanthin production decreases with the increase in the volume of the medium with respect to the volume of the Erlenmeyer flasks used for the study. The above set of observations is in accordance with the reports of Asker and Ohta (1999). This may be due to the decrease in the amount of dissolved oxygen with the increase in the medium volume.

Based on these results the ranges of the parameters were selected as given in Table 1 and the corresponding data are given in Table 2. The biomass SOM is given by

$$\begin{aligned}\hat{E}(Y_B) &= -620 + 3.34X_1 + 0.66X_2 + 0.85X_3 \\ &\quad +196X_4 - 0.88X_1^2 - 0.003X_2^2 - 0.019X_3^2 \\ &\quad -16.5X_4^2 + 0.006X_1X_2 + 0.011X_1X_3 \\ &\quad -0.171X_1X_4 + 0.0003X_2X_3 - 0.01X_2X_4 \\ &\quad +0.037X_3X_4. \end{aligned} \quad (2)$$

For canthaxanthin this is

$$\begin{aligned}\hat{E}(Y_C) &= -4896 + 26.4X_1 + 5.240X_2 + 6.69X_3 \\ &\quad +1547X_4 - 6.95X_1^2 - 0.02X_2^2 - 0.15X_3^2 \\ &\quad -130X_4^2 + 0.048X_1X_2 + 0.085X_1X_3 \\ &\quad -1.352X_1X_4 + 0.003X_2X_3 - 0.077X_2X_4 \\ &\quad +0.289X_3X_4. \end{aligned} \quad (3)$$

Table 2. Central composite design and corresponding experimental canthaxanthin and biomass, with X_1: Inoculum (%), X_2: Shaker speed (rpm), X_3: Temperature (°C), and X_4: pH

Run	X_1	X_2	X_3	X_4	Canthaxanthin (mg L^{-1})	Biomass (g L^{-1})
1	1.40	110	22	5.40	98.95	12.54
2	2.60	110	22	5.40	97.04	12.29
3	1.40	130	22	5.40	102.56	12.99
4	2.60	130	22	5.40	101.80	12.90
5	1.40	110	30	5.40	107.01	13.56
6	2.60	110	30	5.40	105.93	13.42
7	1.40	130	30	5.40	111.02	14.07
8	2.60	130	30	5.40	111.09	14.07
9	1.40	110	22	6.20	127.41	16.14
10	2.60	110	22	6.20	124.20	15.74
11	1.40	130	22	6.20	129.77	16.44
12	2.60	130	22	6.20	127.74	16.18
13	1.40	110	30	6.20	137.32	17.40
14	2.60	110	30	6.20	134.94	17.10
15	1.40	130	30	6.20	140.11	17.75
16	2.60	130	30	6.20	138.86	17.59
17	0.80	120	26	5.80	137.68	17.44
18	3.20	120	26	5.80	134.53	17.05
19	2.00	100	26	5.80	134.41	17.03
20	2.00	140	26	5.80	141.94	17.98
21	2.00	120	18	5.80	127.12	16.11
22	2.00	120	34	5.80	146.32	18.54
23	2.00	120	26	5.00	34.81	4.41
24	2.00	120	26	6.60	91.05	11.54
25	2.00	120	26	5.80	146.10	18.51
26	2.00	120	26	5.80	146.10	18.51
27	2.00	120	26	5.80	146.12	18.51
28	2.00	120	26	5.80	146.12	18.51
29	2.00	120	26	5.80	146.14	18.52
30	2.00	120	26	5.80	146.14	18.52

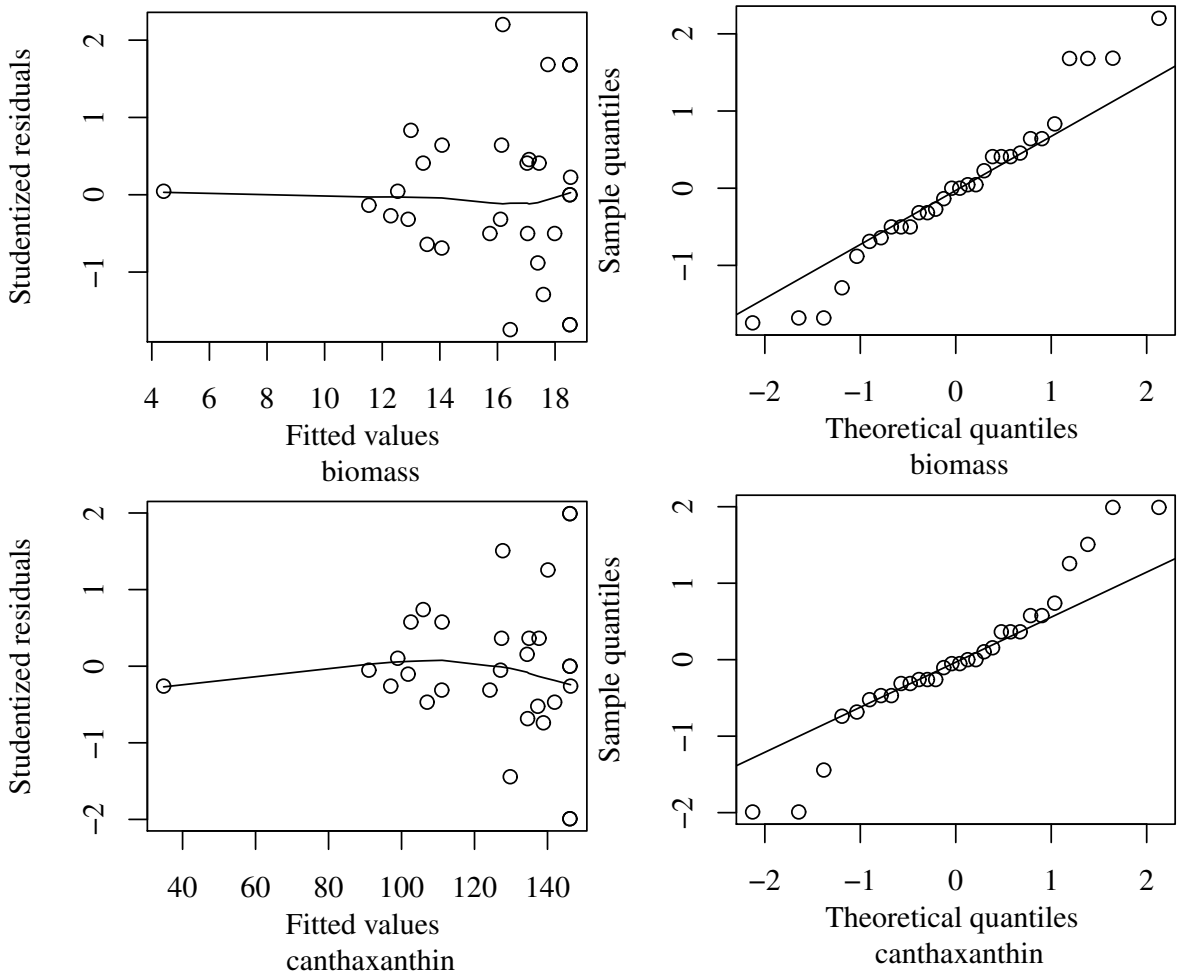

Fig. 1. Residual and normal QQ-plots for the biomass model Eq. (2) and the canthaxanthin model Eq. (3).

For both models all estimated coefficients are significantly different from zero (all $p < 0.00001$). Based on the KS test, there is no evidence against normality: p-values $p = 0.85$ and $p = 0.51$, for models Eq. (2) and Eq. (3), respectively. The QQ-plots, see Fig. 1, show a minor deviation from normality. However, since we use the second order models for deriving optimum values, the standard assumptions of normality and constant variance are not that important. The BP test shows no evidence against constancy of error variances ($p = 0.31$ and $p = 0.24$). The residual plots, see Fig. 1, show some heteroscedasticity. More importantly, the F-test does not reveal evidence of lack-of-fit ($F = 0.39$ with $p = 0.90$ and $F = 0.18$ with $p = 0.99$) and the residual plots, see Fig. 1, support this since the residuals are randomly scattered. The adjusted coefficient of determination, R^2, exceeds 99% for both models.

Based on the partial derivatives, both models obtain an optimum for $X_1 = 1.9$, $X_2 = 125$, $X_3 = 30$, and $X_4 = 5.9$. The second partial derivative test confirms that the corresponding optimum is a maximum and not a saddle point or minimum. The mean biomass for these parameter settings is estimated by 19.206 g L^{-1} (95% prediction interval [19.203,19.210]), while for the mean canthaxanthin this is 151.60 mg L^{-1} ([151.56, 151.62]). These values are respectively 160% and 25% higher than those reported in Goswami *et al.* (2012) (7.4 g L^{-1} for biomass and 121.5 mg L^{-1} for canthaxanthin).

Fig. 2 compares our results with the results of Goswami *et al.* (2012). Theoretically, a five-dimensional plot is needed to show the responses of models Eq. (2) and Eq. (3) as a function of the process parameters. Since this is graphically infeasible, we consider a two-dimensional projection of the response surfaces, where we plot the response as a function

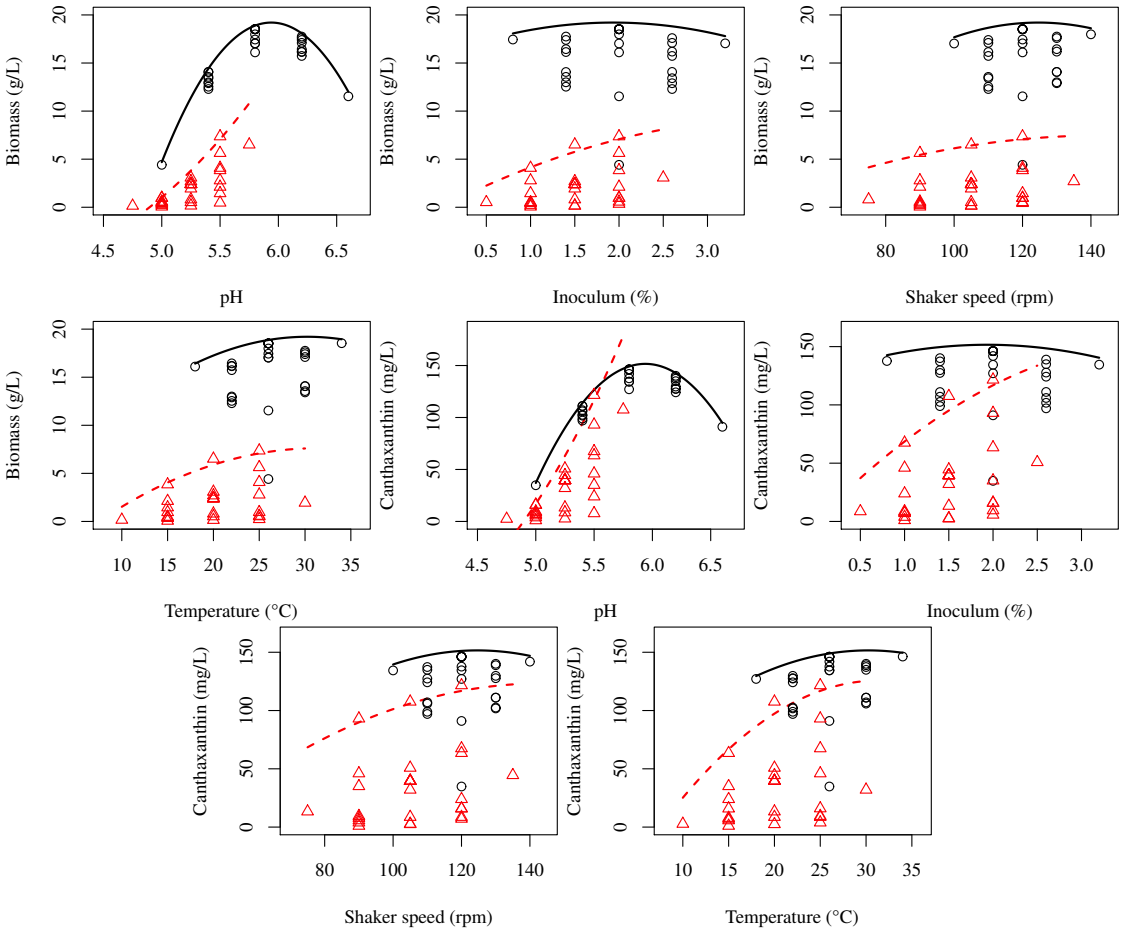

Fig. 2. The biomass and canthaxanthin as a function of the process parameters (pH, percentage inoculum, shaker speed, and temperature). The black dots (○) correspond to the sample observations of our RCCD as given in Table 2. The black solid line (—) is the fit of model (1). The red triangles (△) correspond to the sample observations of the RCCD given by Goswami *et al.* (2012) and the red dashed line (– – –) is the corresponding fit of model (1). The predictors that are not varied are fixed at their optimum.

of one parameter, while keeping the other parameters fixed at their optimum condition. We prefer these plots over three-dimensional plots, since the latter can obscure the assessment of the model fit.

The upper left panel gives the observed biomass as a function of the pH. The fit of the SOM Eq. (1) is shown, for which the other parameters are fixed at their optimal conditions (i.e. $X_1 = 1.9$, $X_2 = 125$, and $X_3 = 30$ for the fit based on our experiments, and $X_1 = 2$, $X_2 = 120$, and $X_3 = 25$ for the fit based on the experiments of Goswami *et al.* (2012)). The plot illustrates that both the observed biomass and the model predictions of our study are higher than those of Goswami *et al.* (2012). Furthermore, our SOM reaches a maximum in the interior of the pH range, where the SOM fitted to the data of Goswami *et al.* (2012) attains a maximum in the right end point. Our model fit is now more consistent with the data. Similar conclusions hold for canthaxanthin and the other process parameters.

Conclusion

The analysis of a two stage design shows that the optimal process parameters for canthaxanthin production by *Dietzia maris* NIT-D are temperature = 30°C, pH = 5.9, shaker speed = 125 rpm, inoculum = 1.9 %, volume = 50 mL, and glucose = 15 g L^{-1}. The corresponding biomass is 19.2 g L^{-1} and the canthaxanthin yield is 152 mg L^{-1}. This is, respectively, 160% and 25% higher than the optimum

reported by Goswami *et al.* (2012). It is shown that this optimum is a global maximum within the experimental range and that the statistical model has an adequate fit.

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