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ORIGINAL ARTICLE

Optimization of citric acid production from a carrot juice-based medium by *Yarrowia lipolytica* using response surface methodology

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Abstract In this study, diluted and fortified carrot juice was used for modelling and optimization of citric acid production by a new mutant strain, *Yarrowia lipolytica* K-168. Protein concentrate obtained from fine flour -a byproduct of semolina production- was used as a nitrogen source in the fermentation medium. Interactive effects of selected independent variables, initial total sugar concentration, initial pH, initial concentration of protein concentrate obtained from fine flour of semolina and temperature, on the growth and citric acid production of the yeast were investigated. An experimental design including 30 experiments was conducted by using the method of central composite design. Modelling the effects of these independent variables on maximum citric acid concentration, maximum citric acid production rate, citric acid yield, the ratio of maximum citric acid concentration to maximum isocitric acid concentration and specific growth rate were performed by response surface methodology. The variations of all of the responses with the independent variables were defined by a quadratic model. Numeric optimization was performed by using the desireability function. The conditions with 190.83 g/L initial sugar concentration, 5.90 initial pH, 0.07 g/L initial concentration of fine flour protein concentrate and 27.86 °C were determined as optimal conditions for citric acid production. The maximum citric acid concentration reached to 80.53 g/L in optimal conditions.

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Introduction

Citric acid is one of the most commonly used organic acids in the food and pharmaceutical industries. The food industry is the largest consumer of citric acid, using almost 70 % of the total production (Darouneh et al. 2009). It was reported that in recent years, the consumption of citric acid and its salt, trisodium citrate, has reached 1.4 million tonnes with a growth of 5 % per year (Kamzolova et al. 2011). The world's existing demand for citric acid is almost entirely met by fermentative process (Dhillon et al. 2013). Traditionally, different strains of *Aspergillus niger* have been used in the commercial production of citric acid from molasses, sucrose, or glucose. Alternatively, there is a great interest in various strains belonging to *Yarrowia lipolytica*, which are capable of citric acid production from various carbon sources such as glucose, ethanol, methanol, glycerol and hydrocarbons (Kamzolova et al. 2011).

The production of citric acid is strongly dependent on the type of the strain, the fermentation medium and the cultural conditions employed (Haq et al. 2003). The search for inexpensive substrates as an alternative to high cost substrates is reported to be vital to reduce the production cost of citric acid. In recent years, considerable interest has been focused on the use of agro-industrial wastes or raw materials for citric acid production (Dhillon et al. 2011). Molasses, apple pomace, grape pomace, carob pod (Kumar et al. 2003), olive-mill waste water-based medium (Papanikolau et al. 2008a), and raw glycerol were reported to be used as natural substrates for citric acid production. There are many reports, especially about the use of raw glycerol, for citric acid biosynthesis and various aspects in this field were studied, including the effects of different kinds of raw glycerol, different cultivation systems and operating conditions (Papanikolau et al. 2008b; Rymowicz et al. 2010; Rywińska et al. 2011, 2012). Carrot is cultivated worldwide (Ozcan and Chalchat 2007). It is one of the cheap vegetables in Turkey, and it was reported that carrot production rose around 10.2 percent in recent years (Hurriyetdailynews.com 2012). It is regarded as a healthy food item because of its high vitamin and fiber content (Ozcan and Chalchat 2007). Carrot juice contains high amounts of vitamin A in addition to glucose, fructose, saccharose, aminoacids and fatty acids (Cazor et al. 2006; Puchooa and Ramborn 2004). Although it is not an agro-industrial waste, carrot juice could be considered as a raw material for citric acid production because of its low cost, easy handling, and high nutritional value.

Optimization of fermentation conditions by the traditional approach using the "one factor at a time" method involves changing one independent variable and keeping the other factors constant (Dhillon et al. 2011). This technique is not only time consuming, but also often easily misses the alternative effects between components (Imandi et al. 2007). In fermentation processes, the operational variables interact and influence each other's effect on the response. In order to determine the true optimum, the optimization technique should account for the interactions among the variables. Response surface methodology (RSM) can be used to evaluate the combined effects of all the factors in a fermentation process (Dhillon et al. 2011).

The objective of this study is to investigate the interactive effects of fermentation conditions on citric acid production by a mutant strain of *Yarrowia lipolytica* in fortified carrot juice medium, in order to determine optimum fermentation conditions.

Materials and methods

Microorganism

The mutant strain *Yarrowia lipoytica* K-168 was used in this study. This strain was obtained by chemical mutagenesis in a previous study by using ethyl methane sulfonate (Karasu Yalcin 2012). The yeast culture was kept at 4 °C on Yeast Extract Malt Extract (YM) agar consisting of (g/L): yeast extract, 3; malt extract, 3; pepton, 5; glucose, 10; and agar, 15. Cultures stored in YM agar were activated in the same medium by maintaining consecutive transfers.

Protein concentrate obtained from a by-product of semolina

Protein concentrate obtained from fine flour (particle size under 150 μm) -a by-product of semolina production- was kindly provided by Dr. E. Yalcin, Cereal Technology Laboratory, Department of Food Engineering, Abant Izzet Baysal University, Turkey. Total nitrogen content of fine flour protein concentrate was given as 14.1 %.

Growth medium

The inocula used in the experiments were prepared by incubation of the cultures at 28 °C for 24 h in a modified growth medium containing (g/L): glucose, 30; yeast extract, 2; NH_4Cl, 2; KH_2PO_4, 0.5; and $MgSO_4 \cdot 7H_2O$, 1 (Rane and Sims 1996).

Preparation of fermentation medium

Carrots were obtained from a certain market and carrot juice was produced by using a squeezer (Philips HR 1854). Carrot juice was divided into appropriately sized aliquots and stored at −20 °C until use. Fermentation medium was prepared by using 50 % diluted carrot juice containing 40 g/L $CaCO_3$ as buffering agent. The medium was supplemented by glucose and FPC.

Equipment and culture conditions

Experiments were carried out in water bath shakers (Nuve ST-402) using 300 mL cotton-plugged flasks containing 100 mL of fermentation medium. The yeast was inoculated to fermentation media at an inoculum volume fraction of 5 % . Experiments were carried out with a shaking rate of 100 strokes per min.

Measuring microbial growth

Microbial growth was measured by determining yeast cell number. During fermentation, samples were taken from the media at specific time intervals. Cultures were spreaded on YM agar after preparing serial dilutions of the culture, and incubated at 28 °C for 48 hours. Yeast count was determined in terms of CFU/mL.

Analytical methods

Concentration of citric acid was measured spectrophotometrically by the pyridine-acetic anhydride method (Marier and Boulet 1958; Kumar et al. 2003). Isocitric acid, glucose, fructose and mannitol concentration were determined enzymatically (Megazyme Assay Kits, Megazyme International Ireland Limited, Wicklow, Ireland). Total nitrogen content of carrot juice was detected by the Kjeldahl method (Chang 1998). Total dry solid content (Bradley 1998) and titratable acidity (Sadler and Murphy 1998) were also analysed in carrot juice.

Experimental design

A central composite design (CCD) with four factors at five levels (−2, −1, 0, +1, +2) was performed to determine the effects of initial total sugar concentration (X_1), initial pH (X_2), initial FPC concentration (X_3) and temperature (X_4) on citric acid production by *Y. lipolytica* K-168. Total number of design points were determined on the basis of Eq. 1, where N is the number of experiments, n is the number of factors and n_o is the number of center points. The total number of experimets was 30 with six replications in the center point.

$$N = 2^n + 2n + n_o \quad (1)$$

The chosen independent variables used in this experiment were coded according to Eq. 2, where x_c is the coded value, x_i is the actual value of the variable, x_o is the value of the variable at the center point, and Δx is the step change. Design of experiments was performed by using Design Expert 8.0.7.1. (trial version, Statease Inc., Minneapolis, USA).

$$x_c = \frac{X_i - X_o}{\Delta x} \quad (2)$$

Modelling and optimization

Effects of the chosen independent variables on five responses were explained by using a quadratic model shown in Eq. 3, where y is the response, β_o is the constant coefficient, β_j is the linear effects, β_{jj} is the quadratic and β_{ij} is the interaction term.

$$y = \beta_o + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum \sum_{i<j} \beta_{ij} X_i X_j \quad (3)$$

The maximum citric acid concentration (Y_1), productivity (Y_2), citric acid yield (Y_3), the ratio of citric acid concentration to isocitric acid concentration ($C_{s\ m}/C_{i\ m}$; Y_4) and specific growth rate (Y_5) were chosen as dependent variables (responses). Data were processed for Eq. 3 using the Design Expert 8.0.7.1. (trial version) including ANOVA to obtain the interaction between the process variables and the response. The quality of fit of the model was expressed by the coefficient of determination (R^2), and its statistical significance was checked by the F-test in the same program.

Numeric optimization was performed by using the desireability function in Design Expert 8.0.7.1. (trial version). The aims for maximum citric acid concentration, citric acid yield and productivity were selected as "maximum". The ratio of maximum citric acid concentration to maximum isocitric acid concentration and specific growth rate were used as "in range". The most suitable conditions were selected among the solutions according to the desireability function.

Results and discussion

Modelling the effects of independent variables on responses in carrot juice-based medium

The chemical composition of the carrot juice used in this study is given in Table 1. Having very low amounts of glucose, fructose and mannitol; carrot juice also contained citric acid. The amount of citric acid in carrot juice was taken into account in calculations during the experiments. The pH of the carrot juice was determined as 6.58. The glucose and fructose concentrations were also determined after sterilization in an autoclave as 8.62 and 8.83 g/L, respectively. The initial total sugar in the 50 % diluted carrot juice was adjusted by the addition of glucose.

In Table 2, the calculated and predicted values of maximum citric acid concentration (Y_1) and productivity (Y_2) are given with the central composite design matrix. The quadratic model predicted for the effects of X_1, X_2 X_3 and X_4 on maximum citric acid concentration (Y_1) is shown in Eq. 4.

$$\begin{aligned} Y_1 = &-192.565 + 0.89517X_1 + 7.23955X_2 + 1.21263X_3 + 8.90744X_4 \\ &+0.042175X_1X_2 - 0.055807X_1X_3 - 0.030801X_1X_4 - 0.027750X_2X_3 \\ &-0.11843X_2X_4 - 0.23562X_3X_4 + 6.21730x10^{-4}X_1^2 - 0.65873X_2^2 \\ &+1.03612X_3^2 - 0.65655X_4^2 \end{aligned} \quad (4)$$

According to the ANOVA results in Table 3, the model was found as significant. The linear effects of initial substrate concentration and FPC concentration were found to be very significant for maximum citric acid concentration. In addition, the interactions between these variables as well as the interactions between initial sugar concentration and temperature were also found to affect Y_1 significantly. The linear and quadratic effects of initial pH on Y_1 and interactions of pH with other variables were found to be insignificant ($P>0.05$).

The response surface graphics showing the effects of independent variables on maximum citric acid concentrartion (Y_1) are given in Fig. 1. For an individual graphic showing the effects of two parameters, the other two parameters were used in the center points. It can be observed that maximum citric acid concentration was positively affected from the high initial

Table 1 Composition of the used carrot juice

Component	Concentration
Glucose (g/L)	8.22±0.156
Fructose (g/L)	8.69±0.021
Mannitol (g/L)	1.47±0.071
Citric acid (g/L)	0.58±0.042
Titratable acidity (malic acid, %)	0.11±0.014
Total solids (%)	8.03±0.226
Total nitrogen (%)	0.12±0.028

Table 2 Central composite design matrix showing the real values of independent variables and the calculated and predicted values of Y_1 and Y_2

Experiment	Independent variables				Some Responses			
	X_1 (g/L)	X_2	X_3 (g/L)	X_4 (°C)	Obtained Y_1 (g/L)	Predicted Y_1 (g/L)	Obtained Y_2 (g/L.h)	Predicted Y_2 (g/L.h)
1	140.00	6.25	8.00	28.50	9.89	16.10	0.059	0.051
2	140.00	6.25	4.00	28.50	15.98	20.43	0.129	0.114
3	260.00	6.25	4.00	28.50	45.82	57.19	0.265	0.281
4	80.00	5.00	6.00	32.00	13.71	11.18	0.024	0.018
5	80.00	7.50	6.00	32.00	12.45	7.24	0.027	0.029
6	140.00	6.25	4.00	28.50	18.92	20.43	0.111	0.114
7	140.00	3.75	4.00	28.50	6.81	12.75	0.022	0.067
8	200.00	7.50	2.00	25.00	65.76	69.29	0.304	0.323
9	200.00	7.50	6.00	32.00	11.88	15.05	0.060	0.088
10	140.00	6.25	4.00	21.50	20.10	23.04	0.121	0.132
11	20.00	6.25	4.00	28.50	0.21	1.56	0.007	0.023
12	200.00	5.00	2.00	32.00	41.37	43.80	0.172	0.193
13	140.00	6.25	4.00	28.50	20.56	20.43	0.112	0.114
14	80.00	7.50	2.00	25.00	11.12	8.81	0.126	0.118
15	200.00	5.00	6.00	32.00	17.77	6.34	0.107	0.071
16	140.00	6.25	0.00	28.50	51.39	57.91	0.235	0.275
17	80.00	5.00	2.00	25.00	12.58	10.41	0.125	0.109
18	140.00	6.25	4.00	28.50	37.61	20.43	0.128	0.114
19	80.00	7.50	6.00	25.00	5.92	4.48	0.026	0.018
20	200.00	7.50	6.00	25.00	47.69	38.17	0.114	0.113
21	200.00	7.50	2.00	32.00	68.14	52.79	0.294	0.245
22	140.00	6.25	4.00	35.50	1.59	11.37	0.010	0.030
23	80.00	5.00	6.00	25.00	4.74	6.35	0.037	0.041
24	200.00	5.00	6.00	25.00	27.19	27.38	0.129	0.130
25	80.00	7.50	2.00	32.00	17.39	18.19	0.064	0.075
26	140.00	6.25	4.00	28.50	15.30	20.43	0.089	0.114
27	200.00	5.00	2.00	25.00	66.75	58.23	0.354	0.307
28	140.00	8.75	4.00	28.50	13.08	19.86	0.108	0.095
29	80.00	5.00	2.00	32.00	26.07	21.86	0.074	0.031
30	140.00	6.25	4.00	28.50	14.19	20.43	0.112	0.114

sugar concentration and high pH values, but adversely affected by high values of initial FPC concentration (Fig. 1).

Effects of X_1, X_2 X_3 and X_4 on productivity (Y_2) were estimated with the quadratic model shown in Eq. 5.

$$\begin{aligned} Y_2 = {} & -0.16012 + 2.31341\,x10^{-3}X_1 + 0.022512X_2 - 0.05509X_3 + 0.015392X_4 \\ & +2.25x10^{-5}X_1X_2 - 2.28646\,x10^{-4}X_1X_3 - 4.25595\,x10^{-5}X_1X_4 - 3.325\,x10^{-3}X_2X_3 \\ & +2.04286\,x10^{-3}X_2X_4 + 1.9375\,x10^{-3}X_3X_4 + 2.67072\,x10^{-6}X_1^2 - 5.20667x10^{-3}X_2^2 \\ & +3.09115\,x10^{-3}X_3^2 - 6.53912\,x10^{-3}X_4^2 \end{aligned} \qquad (5)$$

According to the ANOVA results in Table 4, the model was found to be very significant ($P<0.01$) which had a high R^2 value (0.933). The linear effects of initial total sugar concentration, FPC concentration and temperature on productivity were determined as significant. The interactive effects of initial sugar concentration and FPC concentration were also found to be significant, while interactive effects of other parameters were found to be insignificant ($P>0.05$). It was

Table 3 ANOVA results for the model predicted for maximum citric acid concentration

Source	Degree of freedom	Mean squares	*F*-value	R^2
Model	14	702.68	7.50**	0.875
X_1	1	4642.32	49.56**	
X_2	1	76.01	0.81	
X_3	1	2621.49	27.99**	
X_4	1	204.11	2.18	
X_1X_2	1	160.09	1.71	
X_1X_3	1	717.57	7.66*	
X_1X_4	1	669.39	7.15*	
X_2X_3	1	0.08	8.221×10^{-4}	
X_2X_4	1	4.30	0.046	
X_3X_4	1	43.86	0.47	
X_1^2	1	137.41	1.47	
X_2^2	1	29.06	0.31	
X_3^2	1	471.13	5.03*	
X_4^2	1	17.74	0.19	
Lack of fit	10	102.25	1.34	
Pure error	5	76.50		
Total	29			

*$P<0.05$, ** $P<0.01$

determined that linear effects of pH and quadratic effects of all parameters on this response were insignificant.

Equation 6 was derived for the expression of the effects of the four independent variables on citric acid yield (Y_3). According to the ANOVA results, the model was found to be significant ($P<0.01$) with a R^2 of 0.788.

$$Y_3 = -87.72284-0.5436X_1 + 1.73483X_2-5.34874X_3 + 5.02242X_4 +0.028558X_1X_2-0.016016X_1X_3-0.021854X_1X_4 + 0.23025X_2X_3 -0.14500X_2X_4-0.14545X_3X_4 + 7.20197x10^{-5}X_1^2-0.15567X_2^2 +0.85388X_3^2-0.010876X_4^2 \quad (6)$$

The linear effects of initial sugar concentration and FPC concentration on citric acid yield was found as significant, similar to the results obtained for maximum citric acid concentration and productivity (data not shown). Additionally, the quadratic effect of FPC concentration was also found to be significant on this response. ANOVA results also indicated that the interaction between initial sugar concentration and temperature significantly affected citric acid yield. The linear and quadratic effect of pH and its interaction with other variables were found to be insignificant ($P>0.05$).

The quadratic model describing the change of the ratio of maximum citric acid concentration to maximum isocitric acid concentration (Y_4) by independent variables is given in Eq. 7. The model was found to be significant ($R^2=0.867$).

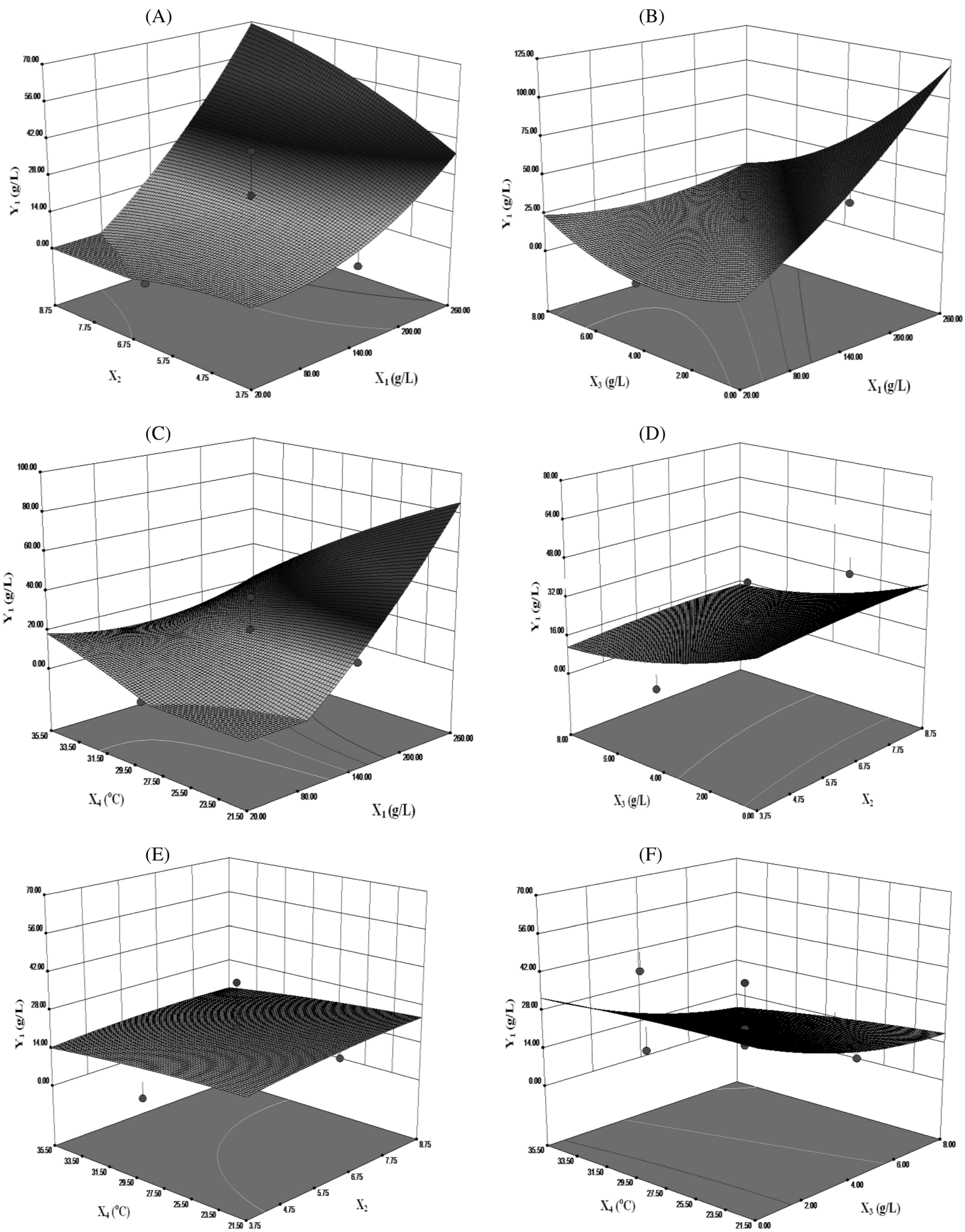
$$Y_4 = -2101.92404 + 1.83475X_1 + 132.12719X_2 + 29.77598X_3 + 110.30689X_4 -2.63333x10^{-3}X_1X_2-0.021365X_1X_3-8.48810x10^{-3}X_1X_4 + 1.643300X_2X_3 -0.57971X_2X_4-0.15571X_3X_4-5.11343x10^{-3}X_1^2-9.30773X_2^2 -3.70333X_3^2-1.85344X_4^2 \quad (7)$$

The linear effects of initial sugar concentration, initial pH, initial FPC concentration and temperature and their interactions were determined to be insignificant ($P>0.05$). Only quadratic effects of the four independent variables on Y_4 were found to be very significant ($P<0.01$, data not shown).

The response of specific growth rate (Y_5) was estimated by Eq. 8. ANOVA results of the quadratic model indicated that the model equation derived by response surface methodology could adequately be used to describe the specific growth rate under a wide range of operating conditions (Table 5).

$$Y_5 = -0.014914-9.6422x10^{-5}X_1 + 0.012819X_2-1.21694x10^{-3}X_3 +8.772114x10^{-4}X_4 + 1.05x10^{-5}X_1X_2 + 1.77083x10^{-6}X_1X_3 +2.38095x10^{-6}X_1X_4 + 8.1x10^{-4}X_2X_3-3.97143x10^{-4}X_2X_4 -1.23214x10^{-4}X_3X_4-1.69560x10^{-7}X_1^2-3.66667x10^{-4}X_2^2 +9.42708x10^{-5}X_3^2 + 1.70068x10^{-7}X_4^2 \quad (8)$$

It can be seen in Table 5 that, the linear effect of initial total sugar concentration on Y_5 was insignificant ($P>0.05$), although it was significant for all of the responses related to citric acid production. Additionally, the linear effect of initial pH was significant ($P<0.05$) for the specific growth rate although it was insignificant for the other responses. Another distinct result was obtained for the significant interaction of FPC concentration and pH, which was insignificant for the other responses. The quadratic effects of all of the parameters on the specific growth rate were insignificant. The response surface plots showing the effects of independent variables on specific growth rate were represented in Fig. 2. When X_3 and X_4 were used in the center points, increasing initial pH in the studied range caused an increase in specific growth rate at a certain initial sugar concentration. However, this increase was more obvious at high initial sugar concentrations. It can be seen from the plots that increasing initial


(A)
Y1 (g/L)
X2
X1 (g/L)
(B)
Y1 (g/L)
X3 (g/L)
X1 (g/L)
(C)
Y1 (g/L)
X4 (°C)
X1 (g/L)
(D)
Y1 (g/L)
X3 (g/L)
X2
(E)
Y1 (g/L)
X4 (°C)
X2
(F)
Y1 (g/L)
X4 (°C)
X3 (g/L)

◀ **Fig. 1** Response surface plots for the effects of independent variables on maximum citric acid concentration (**a**: effects of initial total sugar concentration and pH, **b**: effects of initial total sugar and FPC concentration, **c**: effects of initial total sugar concentration and temperature, **d**: effects of initial FPC concentration and pH, **e**: effects of pH and temperature, **f**: effects of initial FPC concentration and temperature)

FPC concentration made a positive effect on the specific growth rate. When X_2 and X_3 were used in the center points, high temperature values adversely affected specific growth rate at all studied initial sugar concentrations. In the studied temperature range, the insignificant effect of initial sugar concentration on specific growth rate can also be seen from Fig. 2.

In this study, the most important parameter for citric acid production was found to be initial total sugar concentration. This parameter was very effective especially on maximum citric acid concentration and productivity. This outcome is in agreement with the results obtained by Imandi et al. (2007) in raw glycerol. In the related study, initial substrate concentration, initial yeast extract concentration and salt concentration were chosen as independent variables in citric acid production by *Y. lipolytica* from raw glycerol. The effect of initial substrate concentration on maximum citric acid concentration was found as very significant. In another study, the effect of initial saccharose concentration on citric acid production by *A. niger* was reported as very significant (Aghaie et al. 2009). In the present study, the responses related to citric acid production were generally higher in the media having high initial sugar concentrations. But a different effect was observed for the ratio $C_{s\ m}/C_{i\ m}$. This response was very low when total sugar concentration was used between 20–80 g/L and 200–260 g/L, probably due to the the low levels of citric acid concentration at low sugar concentrations and the high levels of isocitric acid concentration at high sugar concentrations (data not shown).

Table 4 ANOVA results for the model predicted for productivity

Source	Degree of freedom	Mean square	*F*-value	R^2
Model	14	0.016	14.84**	0.933
X_1	1	0.100	93.27**	
X_2	1	1.134×10^{-3}	1.06	
X_3	1	0.075	70.08**	
X_4	1	0.016	14.74**	
X_1X_2	1	4.556×10^{-5}	0.04	
X_1X_3	1	0.012	11.27**	
X_1X_4	1	1.278×10^{-3}	1.20	
X_2X_3	1	1.106×10^{-3}	1.03	
X_2X_4	1	1.278×10^{-3}	1.20	
X_3X_4	1	2.943×10^{-3}	2.75	
X_1^2	1	2.536×10^{-3}	2.37	
X_2^2	1	1.815×10^{-3}	1.70	
X_3^2	1	4.193×10^{-3}	3.92	
X_4^2	1	1.760×10^{-3}	1.65	
Lack of fit	10	1.498×10^{-3}	7.05*	
Pure error	5	2.123×10^{-4}		
Total	29			

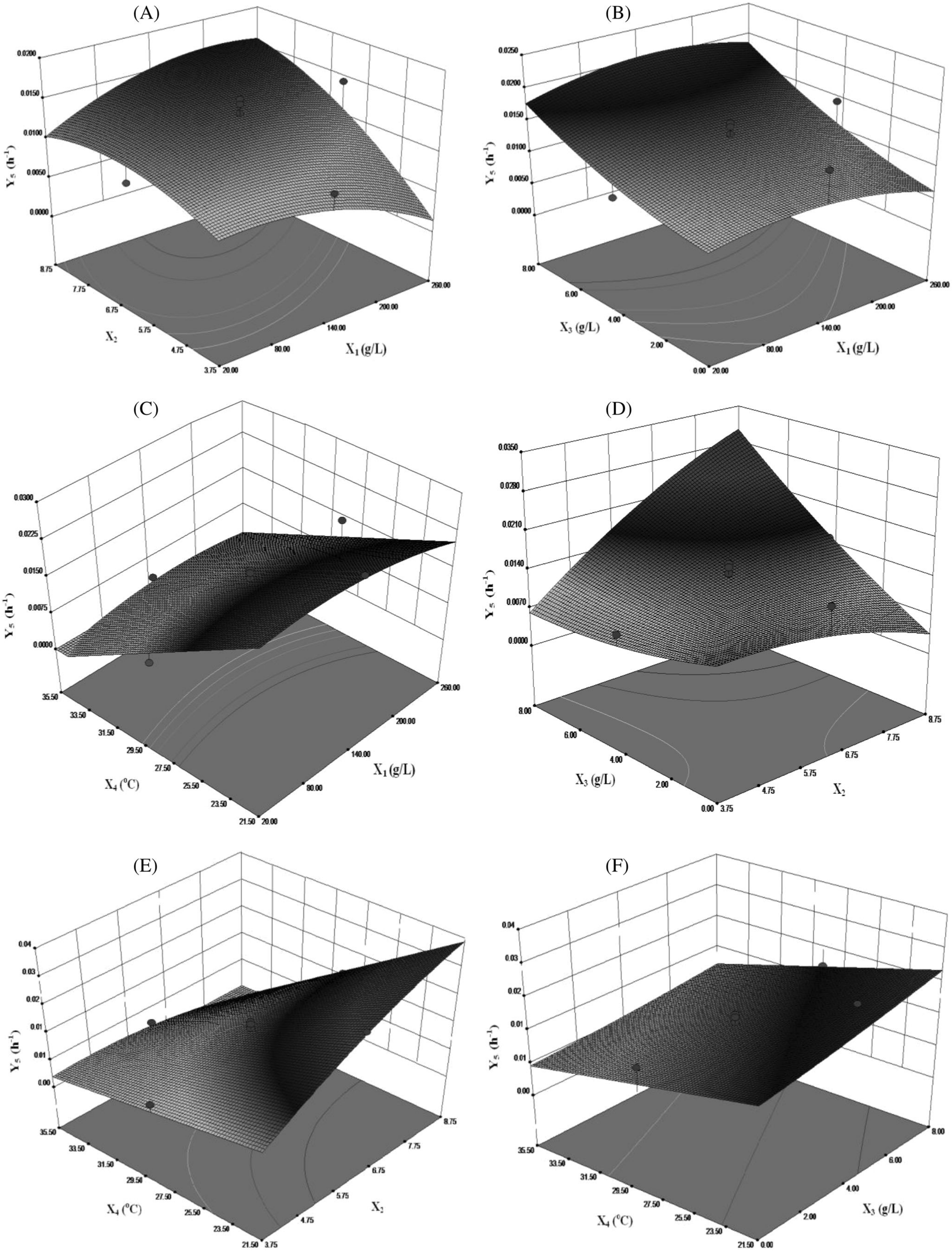
*$P<0.05$, ** $P<0.01$

Table 5 ANOVA results for the model predicted for specific growth rate

Source	Degree of freedom	Mean square	*F*-value	R^2
Model	4	8.865×10^{-5}	7.38*	0.873
X_1	1	9.600×10^{-7}	0.080	
X_2	1	9.923×10^{-5}	8.26*	
X_3	1	1.707×10^{-4}	14.21*	
X_4	1	8.050×10^{-4}	67.05*	
X_1X_2	1	9.923×10^{-6}	0.83	
X_1X_3	1	7.225×10^{-7}	0.060	
X_1X_4	1	4.000×10^{-6}	0.33	
X_2X_3	1	6.561×10^{-5}	5.46*	
X_2X_4	1	4.830×10^{-5}	4.02	
X_3X_4	1	1.190×10^{-5}	0.99	
X_1^2	1	1.022×10^{-5}	0.85	
X_2^2	1	9.003×10^{-6}	0.75	
X_3^2	1	3.900×10^{-6}	0.32	
X_4^2	1	1.190×10^{-10}	9.915×10^{-6}	
Lack of fit	20	1.682×10^{-5}	7.07*	
Pure error	5	2.378×10^{-6}		
Total	29			

*$P<0.05$, ** $P<0.01$

The used carrot juice-based medium supplied 0.06 % total nitrogen, and this component was increased by addition of the FPC which contained 14.1 % total nitrogen. But, at high initial sugar concentrations, high FPC concentrations adversely affected citric acid production. When FPC concentration was low, high levels for maximum citric acid concentration, productivity and citric acid yield were obtained, while specific growth rate was adversely affected. It was concluded that enrichment of the carrot juice with FPC supported the yeast growth very well. It is known that nitrogen source and its concentration is a critical factor in citric acid production. It is reported that citric acid production increases when nitrogen limitation occurs in the medium at the end of the logarithmic phase (McKay et al. 1994; Antonucci et al. 2001). It is thought that, high concentrations of nitrogen compounds deccelarated the initiation of citric acid production in carrot juice medium. However, this effect of FPC concentration was only observed at high sugar concentrations. Significant interaction of initial sugar concentration and FPC concentration for Y_1 and Y_2 was another apparent result in this study. Anastassiadis et al. (2002) reported that citric acid formation in various yeasts occurred under limiting nitrogen conditions with an excess of carbon source. A balance between nitrogen concentration and


(A)
Y5 (h-1)
X2
X1 (g/L)
(B)
Y5 (h-1)
X3 (g/L)
X1 (g/L)
(C)
Y5 (h-1)
X4 (0C)
X1 (g/L)
(D)
Y5 (h-1)
X3 (g/L)
X2
(E)
Y5 (h-1)
X4 (0C)
X2
(F)
Y5 (h-1)
X4 (0C)
X3 (g/L)

◀ **Fig. 2** Response surface plots for the effects of independent variables on specific growth rate (**a**: effects of initial total sugar concentration and pH, **b**: effects of initial total sugar and FPC concentration, **c**: effects of initial total sugar concentration and temperature, **d**: effects of initial FPC concentration and pH, **e**: effects of pH and temperature, **f**: effects of initial FPC concentration and temperature)

other nutrients was also reported to be essential for optimum citrate excretion in yeasts. In the present study, the adverse effect at high sugar concentrations may be due to a wrong ratio of carbon to nitrogen (Anastassiadis et al. 2002; Anastassiadis and Rehm 2006).

Temperature was also found to be an important operational parameter affecting growth and citric acid production of *Y. lipolytica* K-168. Temperature effect was very significant on productivity, and its effect on maximum citric acid concentration and productivity was more apparent at high sugar concentrations. Both responses were adversely affected from temperatures above 30 °C. The best results for $C_{s\ m}/C_{i\ m}$ were obtained between 27.5-31.5 °C (data not shown). Temperature effect was also important for specific growth rate, but it was not highly dependent to initial total sugar concentration as observed for the other responses. The best results for growth were obtained at temperatures near 21.5 °C. Barrington et al. (2009) studied citric acid production by *A. niger* grown on peat moss and aeration rate, bed depth and temperature were selected as independent variables. It was reported that the linear effect of temperature and its interaction with aeration rate were found to be very significant. It was also indicated that increasing the temperature between the studied range of 22-32 °C made for a positive effect on maximum citric acid concentration and productivity.

Optimization

After modelling the responses, optimization of citric acid production by *Y. lipolytica* K-168 was performed in the diluted carrot juice medium by using the desireability function. In the optimization, maximum citric acid concentration, productivity and citric acid yield were chosen as significant parameters and the goal was selected to maximize them. The goals for the responses Y_4 and Y_5 were selected as "in range". The main reason for this selection is that there is not a direct correlation between citric acid production properties and these responses. In some cases, the ratio of maximum citric acid concentration to maximum isocitric acid concentration could be very high although citric acid production is low. The same event can be seen for specific growth rate, since citric acid is a seconder metabolite and the optimum conditions for growth and its production can be different (Dhillon et al. 2011).

The optimum conditions were chosen among 39 solutions suggested by the program, considering the value of desireability function as 1, and maximizing the important parameters (Y_1, Y_2 and Y_3). The optimum conditions in diluted carrot juice were determined as 190.83 g/L initial total sugar concentration, initial pH of 5.90, 0.07 g/L initial FPC concentration and 27.86 °C. In these conditions, maximum citric acid concentration was estimated to be 81.09 g/L. The values for productivity and citric acid yield were predicted as 0.382 g/L·h and 46.48 %, respectively. Y_4 was estimated to be 10.76, and Y_5 0.01 h^{-1} in optimum conditions for citric acid production.

In this study, an experiment was performed by using the optimal conditions in diluted carrot juice in order to understand the accuracy of the predicted responses. Variations of citric acid concentration, total sugar concentration and yeast count with time in optimal conditions are given in Fig. 3. In the experiment, the maximum citric acid concentration was found to be 80.53 g/L, which was very similar to the predicted value. The results for the productivity and citric acid yield were also satisfactory which were calculated as 0.374 g/L·h and 42.21 %, respectively. The ratio of $C_{s\ m}/C_{i\ m}$ was calculated as 9.04, while specific growth rate reached to 0.0170 h^{-1}.

Carrot juice was first suggested to be a natural fermentation medium in citric acid production by Karasu Yalcin (2012). In the present study, it was concluded that high citric acid production was obtained in carrot juice-based medium by optimization and this raw material could be used in a possible citric acid production process. Imandi et al. (2007) reported that citric acid production by *Y. lipolytica* enhanced by optimization and 77.39 g/L citric acid was obtained under optimum conditions. In another study, maximum citric acid concentration was given as 73.12 g/L, produced by *A. niger* in a medium containing kaolin under optimum conditions (Aghaie et al. 2009). In a study by Kumar and Jain (2008), citric acid production of *A. niger* was investigated in sugarcane bagasse and 41.56 % citric acid yield was obtained while productivity and specific growth rate were given as 0.064 g/100 g and 0.043 h^{-1}, respectively. Another study was performed by Rymowicz et al. (2010) with an acetate-negative mutant of

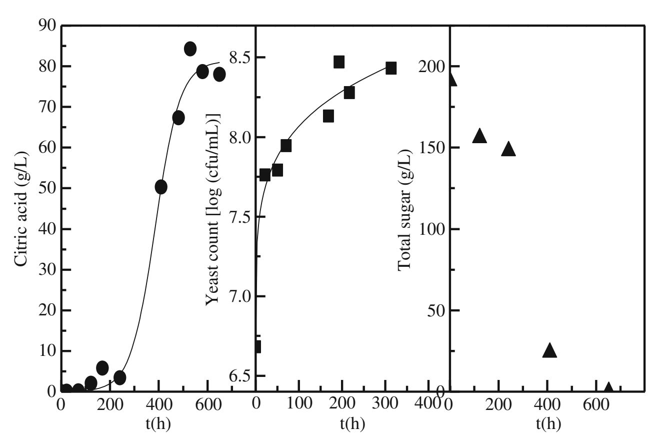


Fig. 3 Variations of citric acid concentration, yeast count and total sugar concentration with time in optimal conditions

Y. lipolytica and 112 g/L citric acid was obtained from raw glycerol with 60 % yield and 0.71 g/L·h productivity. Javed et al. (2010) reported that 67.72 mg/mL was obtained under optimum conditions by *A. niger* in a medium containing banana peels.

Specific growth rate values (0.003-0.026 h^{-1}) obtained in carrot juice-based medium were relatively low in comparison to those reported in glucose or glycerol medium (data not shown). The maximum specific growth rate reported for the use of raw or pure glycerol ranged between 0.10-0.41 h^{-1} (Papanikolaou and Aggelis 2003; Papanikolau et al. 2008b; Rywińska et al. 2010), while it was in the range of 0.055-0.23 h^{-1} in glucose medium (Papanikolau et al. 2006; Rywińska et al. 2006, 2010; Moeller et al. 2007). Being a complex medium, carrot juice differs from the defined fermentation media, it probably serves a source of inhibitory factors in addition to nutritional components, leading to a decceleration in specific growth rate.

Using natural media containing fermentable substrates or supplemented with them does not always give positive results for citric acid production. The most important reason for this effect is the complex composition of these media (Roukas and Kotzekidou 1997; Dhillon et al. 2011). It is known that high vitamin and mineral content, especially heavy metal ions adversely affects citric acid production. Therefore, removal of metal ions are usually suggested for these natural media by deionization or chelating agents (Dhillon et al. 2011). In the present study, high levels of citric acid production could be achieved in fortified carrot juice without any pretreatment. It is reported that carrot juice contains various minerals such as iron, calcium, magnesium, zinc and selenium in addition to carbohydrates, aminoacids, fatty acids, purins, primidines and a number of vitamins (Puchooa and Ramborn 2004; Bergqvist et al. 2006). Some modifications could also be done in the composition of carrot juice, i.e., deionization, in order to enhance citric acid production as a further study.

By optimization, it was concluded that the diluted carrot juice should be supplemented with a low amount of FPC (0.07 g/L). FPC is a product obtained from fine flour of semolina, which is one of the most important by-products of the pasta industry. The protein concentrate produced from fine flour was used as a nitrogen source of fermentation medium for the first time in this study. Besides supporting citric acid production at low concentrations, its positive effect on yeast growth was very obvious. This outcome may be promising for evaluation of this by-product in fermentation processes.

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