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Citric Acid Production by *Yarrowia lipolytica* NRRL Y-1094: Optimization of pH, Fermentation Time and Glucose Concentration Using Response Surface Methodology

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Abstract: In this study, three *Yarrowia lipolytica* strains (*Y. lipolytica* NRRL Y-1094, *Y. lipolytica* NRRL YB-423 and *Y. lipolytica* IFP29) were screened for acid-production capacity and the maximum zone-area was formed by *Y. lipolytica* NRRL Y-1094. The strain was then selected as a potential citric-acid (CA) producer for further studies. The CA production by *Y. lipolytica* NRRL Y-1094 was optimized using response surface methodology (RSM) and considering three factors, comprising initial pH-value, fermentation time, and initial glucose-concentration. The highest CA-concentration was 30.31 g/L under optimum conditions (pH 5.5, 6 days, and 125 g/L glucose) in shake flasks. It has been reported that this result gives better results than many productions with shake flasks. According to estimated regression-coefficients for CA concentration, the fermentation time had the greatest impact on CA production, followed by the substrate concentration and initial pH-level, respectively. On the other hand, this study is a fundamental step in solving and optimizing the production mechanism of *Y. lipolytica* NRRL Y-1094, a microorganism that has not yet been used in CA production with a glucose-based medium. The results suggest that future studies can perform higher yields by optimizing other medium constituents and environmental factors.

Keywords: citric acid; *Yarrowia lipolytica*; isocitric acid; response surface methodology

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1. Introduction

Citric acid (CA) is widely used in various industries, including the food industry, for its sour taste, high water-solubility, acidification, chelation, emulsification, preservation, and flavor enhancement [1]. Additionally, it is also used as an acidifier and a chelating agent in the chemical and pharma industries [2]. CA can be produced using synthetic and chemical methods, but these methods are not economically viable, leading to the supply of a large part of the global demand for CA using biotechnological methods [3].

CA is mainly produced by a filamentous fungus *Aspergillus niger*, using media that is based on starch/sucrose (molasses), in submerged fermentation [4]. However, yeasts have been used by many researchers to produce CA since the 1960s. The use of yeasts in production has several advantages. Due to their metabolic versatility and growth, yeasts allow the use of many carbon sources to produce CA [5]. Additionally, it has been stated that the productions made using *Y. lipolytica* have some advantages, compared to the productions by *A. niger*. These advantages can be listed as: higher productivity, easier cultivation, resistance to high substrate-concentrations, easy genetic-manipulation [6], allowing the possibility of using a wide substrate, less sensitivity to metal ions and lower oxygen levels, better genetic and mechanical stability, and fewer health hazards [7]. However, the concomitant production of isocitric acid (ICA) is the major disadvantage in the use of yeasts compared to the use of molds [8].

CA synthesis occurs at a high C/N ratio during the stationary phase, and pH is an effective factor for the production by *Yarrowia lipolytica*. CA production is facilitated by a neutral pH value [9]. Thus, the selection of the producer strain and a detailed examination of other factors affecting production, are important [10]. Although the producer strain is one of the most important factors in production, the production rate and yield of CA vary considerably, according to the substrate type and culture conditions. However, starting CA production after nitrogen depletion at the end of the exponential growth phase is considered to be one of the common aspects of the processes [11].

The method used to improve the performance of the systems in an economic manner and to increase the efficiency of the processes is referred to as "optimization". To determine optimum conditions, other parameters are kept at a constant level with a parameter change (the one-variable-at-a-time technique). The disadvantages of this technique are that it does not include the interactive effects between the variables and that the parameters do not show their effects on the process. In solving this problem, optimization studies can be carried out using response surface methodology (RSM). While RSM provides benefits in the design, development and improvement of new products, it explores the effects of independent variables on processes, either alone or in combination [12]. Process optimization is a good strategy for increasing CA production by *Y. lipolytica*, and there are several studies which have conducted an optimization with RSM [13–19].

Y. lipolytica is an important non-conventional model yeast which has GRAS status and can secrete high concentrations of some industrial metabolites [20]. The use of different strains of *Y. lipolytica* in CA production was investigated in previous studies [21–26]. However, only one study has examined the use of *Y. lipolytica* NRRL Y-1094 in the production of CA, in the literature. In the mentioned study, glycerol was preferred as the substrate [27].

This study aimed to determine the optimum CA-production conditions for *Y. lipolytica* NRRL Y-1094, using RSM for examining glucose concentration, pH, and fermentation time. The original contribution of this study lies in the fact that it is the first optimization research based on initial pH value, glucose concentration and fermentation time in the production of CA by *Y. lipolytica* NRRL Y-1094.

2. Materials and Methods

2.1. Microorganism

Y. lipolytica strains (*Y. lipolytica* NRRL Y-1094 (ATCC 8662), *Y. lipolytica* NRRL YB-423 (ATCC 18942), and *Y. lipolytica* IFP29 (ATCC 20460) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The malt extract broth (MEB) was used for obtaining the pre-cultures. Cells were maintained at −80 °C in MEB, supplemented with additional 50% (*w*/*v*) of sterile glycerol.

2.2. Fermentation Medium

The modified fermentation medium (MFM) was used in CA production. The MFM used contained (g/L): glucose 125, 150, or 175, yeast extract 0.5, NH_4Cl 1.5, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 1.5, $CaCl_2$ 0.15, $FeCl_3.6H_2O$ 0.15, $ZnSO_4.7H_2O$ 0.02, $MnSO_4.7H_2O$ 0.06, $(NH_4)_2SO_4$ 1.0, $CuSO_4$ 0.02, and thiamine 0.001 [28,29].

2.3. Screening of Y. lipolytica Strains for Acid Production Capacities

Three different *Y. lipolytica* strains were screened in petri plates containing MFM with 20 g/L agar and 0.4 g/L bromocresol green [30]. The initial medium-pH was adjusted to 6.0, using 5N NaOH before autoclaving. The amount of organic acid was related to the zone of color change. A total of 50 µL active yeast cultures (activated in 250 mL flasks/50 mL medium) in MEB were added to the wells in petri dishes, separately. At the end of the incubation time (28 °C, 6 days), the yellow-zone diameters of the cultures were measured. The *Y. lipolytica* strain which has the maximum acid-production capacity was used for the CA production.

2.4. Chemicals

Glucose, yeast extract, agar, bromocresol green, KH_2PO_4, $CaCl_2$, $ZnSO_4.7H_2O$, $MnSO_4.7H_2O$, $CuSO_4$, acetonitrile, glycerol and the MEB were supplied by Merck (Darmstadt, Germany). The NH_4Cl was purchased from ISOLAB (Istanbul, Turkey). Standard substances of CA-ICA and other chemicals were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany).

2.5. Cultivation Conditions

Shake-flask fermentations were conducted in Erlenmeyer flasks of 250 mL, filled with 50 mL of fermentation medium, previously sterilized (at 121 °C; for 15 min.) and inoculated with 1 mL of pre-culture containing 1–3 × 10^6 cells. The flask cultures were then incubated in a rotary shaker (JSSI-100, JS Research, Gongju, Korea). After the adjustment of the initial pH value, 5N NaOH was added to the MFM 18 h after inoculation and every 24 h after the first addition, to keep the pH value between 5.0 and 6.0. Only the produced CA concentrations were considered during the optimization.

2.6. Determination of CA and ICA Concentrations

The method proposed by Kamzolova and Morgunov [31] was modified and used in the determination of the CA and ICA concentrations. For this purpose, fermentation broths were centrifuged at 13,000× *g* for 15 min at 4 °C (Hettich Universal-320R, Hettich, Frankenberg, Germany). The supernatant was passed through the syringe-tip filter (0.45 µm), diluted with an equal volume of 8% $HClO_4$ (0.5 mL), and thoroughly mixed. The CA and ICA concentrations were determined using HPLC (Agilent Technologies 1100 Series, Palo Alto, CA, USA). Detection wavelength was set at 210 nm. The column temperature was set to 40 °C, and the flow rate of the mobile phase was adjusted to 1 mL/min. In addition, 0.01 M H_2SO_4 was used as the mobile phase, and the reverse-phase column was used as the column (Inertsil ODS-3, 4.6 × 250 mm, GL Sciences Inc., Tokyo, Japan). The CA and ICA concentrations were determined using a calibration curve that was created using the standards of CA and ICA.

2.7. Determination of Biomass and pH

The biomass was obtained by washing the remaining cells once with distilled water. Then, the fermentation broth was centrifuged and dried at 80 °C for 24 h [29]. Following the fermentation stage, the pH was measured in the fermentation medium using a pH meter (Mettler Toledo Ion S220, Griefensee, Switzerland).

2.8. Yield Calculations

The following kinetic parameters were calculated for CA production by *Y. lipolytica* NRRL Y-1094:

- CA yield on biomass: $Y_{CA/X}$ (g/g);
- Volumetric yield: $Y_{CA/t}$ (g/L h);
- Specific product-formation rate (q_{CA}) = CA/(X.t) (g/g h);
- Bioprocess selectivity: S_{CA} (%) = (100 × CA_T)/(CA_T + ICA_T).

2.9. Experimental Design

The CCD was preferred for allowing the effects of the factors on the response to be above or below the factor levels and providing high-quality predictions of the linear- and quadratic-interaction effects of the parameters affecting the process and was used to provide flexibility to the experimental design. The levels of factors used in the experimental design are provided in Table 1. The actual level of each factor was calculated using the equation following Table 1.

Table 1. Levels of factors used in the experimental design.

		Levels		
Factor	**Independent Variables**	**−1**	**0**	**+1**
X_1	Initial pH-value	5.5	6.0	6.5
X_2	Fermentation time (days)	4	5	6
X_3	Glucose concentration (g/L)	125	150	175

Coded value = (actual level − (high level + low level)/2)/((high level − low level)/2)

Twenty experiments were carried out using a face-centered composite-statistical-design for the study of 3 factors, each at 3 levels (Table 3). The response surface model was fitted to the response variable (CA concentration (g/L)). The second-order response function for the 3 quantitative factors is given by Equation (1):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (1)$$

where X_1, X_2 and X_3 represent the levels of the factors according to Table 1 and β_0, β_1, ... , β_{23} represent coefficient estimates, with β_0 having the role of a scaling constant.

The statistical analyses of the data were performed using the Minitab 17.1.0 software package.

3. Results and Discussion

3.1. Selection of CA Producer Strain

The results showed a higher acid-production capacity for *Y. lipolytica* NRRL Y-1094 than for *Y. lipolytica* IFP29 (Table 2). On the other hand, *Y. lipolytica* YB-423 could not form any zone in these conditions. As can be seen from Figure 1, *Y. lipolytica* NRRL Y-1094 was selected as the producer strain by forming a zone caused by acid formation to cover the entire petri dish, at the end of 6 days.

Table 2. Comparison of acid-production capacities of *Y. lipolytica* strains.

Microorganism	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Y. lipolytica NRRL Y-1094	2.75 ± 0.35	7.50 ± 2.12	18.00 ± 2.83	23.00 ± 1.41	34.00 ± 0.00	35.00 ± 0.00
Y. lipolytica IFP29	1.50 ± 0.71	4.00 ± 0.00	10.00 ± 1.41	19.00 ± 1.41	26.00 ± 1.41	30.00 ± 1.41

Figure 1. Acid-production zone of *Y. lipolytica* NRRL Y-1094 ((**A**): day 0 and (**B**): day 6).

3.2. Results of Experimental Design

The pH value of the fermentation medium is one of the most important factors affecting CA production. It has been reported that the pH value should be above 5 in CA productions

with yeasts [32]. In addition, the type and concentration of the carbon source are important parameters for CA production [33]. Another important point to be noted is that CA production began after the stationary growth-phase, when extracellular nitrogen was a limiting factor for yeast growth [34].

Table 3 shows the results for CA, ICA and biomass concentration. Point 12 yielded the lowest biomass-concentration (8.77 g/L), while point 15 yielded the highest biomass-concentration (13.60 g/L). Points 7 and 17 yielded the highest and lowest CA-concentrations, with concentrations of 30.31 and 8.56 g/L, respectively (Table 2).

Table 3. Results of CA production by *Y. lipolytica* NRRL Y-1094.

Run	pH_0	Time (Days)	Glucose Conc. (g/L)	CA (g/L)	ICA (g/L)	X (g/L)
1	6.0	5	150	16.85 ± 1.00	2.62 ± 1.10	9.88 ± 0.35
2	6.5	6	175	24.30 ± 7.54	2.92 ± 1.34	12.27 ± 1.48
3	6.5	5	150	13.65 ± 0.62	1.47 ± 0.00	9.69 ± 0.06
4	5.5	5	150	16.52 ± 2.25	1.76 ± 0.69	10.20 ± 0.77
5	6.0	5	150	17.93 ± 3.94	2.49 ± 1.24	10.86 ± 1.40
6	6.0	5	150	17.04 ± 3.86	1.68 ± 0.24	9.71 ± 0.45
7	5.5	6	125	30.31 ± 0.47	5.57 ± 1.30	13.42 ± 0.33
8	6.0	5	150	16.27 ± 0.43	3.07 ± 0.32	11.53 ± 1.58
9	5.5	4	125	12.77 ± 0.06	2.52 ± 0.42	10.24 ± 0.86
10	6.0	4	150	8.87 ± 2.06	1.17 ± 0.25	9.25 ± 1.53
11	6.5	4	125	11.64 ± 0.67	2.64 ± 0.25	12.95 ± 2.98
12	5.5	4	175	11.91 ± 2.69	1.73 ± 0.23	8.77 ± 1.58
13	5.5	6	175	26.58 ± 2.06	3.03 ± 0.11	12.42 ± 0.98
14	6.0	5	175	21.40 ± 0.76	3.22 ± 0.03	11.32 ± 1.50
15	6.5	6	125	29.74 ± 1.82	5.38 ± 1.07	13.60 ± 0.63
16	6.0	5	150	16.96 ± 0.00	1.93 ± 0.11	11.83 ± 1.46
17	6.5	4	175	8.56 ± 2.17	1.08 ± 0.25	9.44 ± 0.45
18	6.0	6	150	26.03 ± 6.11	4.54 ± 1.64	12.86 ± 0.40
19	6.0	5	150	17.64 ± 0.01	4.04 ± 2.09	11.80 ± 0.65
20	6.0	5	125	25.36 ± 0.37	4.37 ± 0.66	12.15 ± 1.01

Rane and Sims [28] reported that 13.6 g/L CA was produced by *C. lipolytica* Y 1095 after 27 h, when 50 g/L glucose was present in the medium in a fermenter. However, they found that when the concentration of glucose in the fermentation medium was 150 g/L, the concentration of CA produced increased to 78.5 g/L after 128 h. In a study carried out with *Y. lipolytica* ACA-DC 50109, maximum CA-concentration (42.9 g/L) was obtained after 550 h, with an initial glucose-concentration of 149.5 g/L and a C/N ratio of 500 [35]. Karasu-Yalçın et al. [36] obtained 37.66 g/L CA by *Y. lipolytica* 57, after 280 h of fermentation in a medium containing 100 g/L glucose. When the initial glucose-concentration was adjusted to 150 g/L, the amount of CA produced by the strain increased to 44.39 g/L. However, it was determined that the amount of CA produced in the nutrient media prepared with initial glucose-concentrations of 200 and 235 g/L decreased to 32.54 and 29.19 g/L, respectively. In another study, 49 g/L CA was obtained by the *Y. lipolytica* wt W29 strain for 315 h, in a production medium containing glucose [37]. Lazar et al. [38] reported that *Y. lipolytica* A-101-B56-5 produced 46.25 g/L CA and 0.45 g/L ICA in the glucose-based medium in a fermenter. Çelik et al. [30] obtained a very low CA-concentration (0.69 g/L CA and 0.21 g/L ICA) after 72 h, using *Y. lipolytica* TEMYL3 and 100 g/L glucose. Interestingly,

33.30 g/L CA- and 4.40 g/L ICA-concentrations were achieved after 408 h only by prolonging the fermentation time. Although this result is quite close to the maximum CA-concentration which is obtained from our study, there is a significant difference between the fermentation times. Çarşanba et al. [39] obtained the highest titer (72.3 g/L CA) and productivity (0.04 g /g.h) using a C/N ratio of 367. In our study, however, the highest CA-concentration was obtained with the lowest C/N ratio (Table 3). As can be understood from these results, the C/N molar-ratio plays a significant role in the synthesis of CA by *Y. lipolytica*.

The bioprocess yields at the optimum levels (pH 5.5, 6 days and 125 g/L glucose) were calculated to be as follows: CA/ICA: 5.4:1, $Y_{CA/X}$: 2.26, $Y_{CA/t}$: 0.21 and S_{CA} (%): 84.48. Moeller et al. [40] separately investigated the effects of temperature and pH on CA production, which was performed using a fermenter and *Y. lipolytica* H222. They reported that, after 92 h fermentation, the highest CA-concentration (24.91 g/L) and the highest selectivity (89.87%) were obtained at a pH value of 6.0. In another study examining the effects of different glucose concentrations (50–120 g/L) on CA production, 64.58 g/L CA was produced by *Y. lipolytica* PR32 after 144 h, using 120 g/L glucose in the fermentation medium. In the same study, $Y_{CA/t}$ was calculated to be 0.45 [41]. Kamzolova et al. [31] obtained 17.6 g/L CA and 2.62 g/L ICA by *Y. lipolytica* VKM Y-2373 in flasks containing glucose in the fermentation medium, and $Y_{CA/X}$ efficiency was calculated as 5.30.

In our study, CA production increased with increasing fermentation-time, but the highest CA-concentration was obtained at the lowest substrate-concentration (125 g/L) (Table 3). Although commercial glucose was used in the study, it is thought that the results obtained can provide information about the use and concentration of glucose-based wastes.

3.3. Optimization of CA Production by Y. lipolytica NRRL Y-1094

Analysis of variance (ANOVA) for the concentration of CA is presented in Table 4. The correlation coefficient (R^2) value, which shows the fit between the experimental data and the predicted data, was determined to be 0.989, indicating that the experimental data can be explained with 98.9% accuracy, using the face-centered model. This shows that the model equation, which was obtained using 20 experimental points, could be used with high accuracy in the independent-variable range that was examined for CA production.

Table 4. ANOVA results for CA concentration.

Source	DF	Seq SS	Adj SS	Adj MS	F	*p*
Regression	9	827.773	827.773	91.975	195.70	0.000
Linear	3	731.933	731.933	243.978	519.14	0.000
Square	3	90.158	90.158	30.053	63.95	0.000
Interaction	3	5.682	5.682	1.894	4.03	0.041
Residual error	10	4.700	4.700	0.470		
Lack of fit	5	2.946	2.946	0.589	1.68	0.292
Pure error	5	1.754	1.754	0.351		
Total	19	832.472				

As shown in Table 4, the F-test for the regression was significant at a level of 5% ($p < 0.05$) and the lack of fit was not significant at the 5% level ($p > 0.05$). These results showed that the experimental data fitted well with the model. Additionally, the proposed model can well explain the effects of the initial pH-value, fermentation time, and initial substrate-concentration on CA production by *Y. lipolytica* NRRL Y-1094.

Table 5 shows the results of the ANOVA and estimated regression-coefficients for CA production. The negative coefficient for pH and initial substrate-concentration showed that the CA concentration decreased with increasing initial-pH and substrate-concentration ($p < 0.05$). The smaller *p*-value indicated that the correlation for the coefficient was more im-

portant. The fermentation time had the highest impact on the CA production, considering the highest linear-coefficient (8.321), followed by the substrate concentration (−1.707) and pH level (−1.020), respectively. Moreover, the initial pH value and the fermentation time had a significant negative quadratic-effect on CA production ($p < 0.05$), indicating that the CA concentration of these factors increased and decreased as the level of these parameters increased above certain values. The interaction between the substrate concentration and fermentation time was significant ($p < 0.05$).

Table 5. Estimated regression-coefficients for CA concentration.

Term	Coefficient	SE Coefficient	T	*p*
Constant	17.436	0.236	73.99	<0.001
pH	−1.020	0.217	−4.71	0.001
Time	8.321	0.217	38.38	<0.001
Glucose conc.	−1.707	0.217	−7.87	<0.001
pH × pH	−2.833	0.413	−6.85	<0.001
Time × Time	−0.468	0.413	−1.13	0.284
Glucose conc. × Glucose conc.	5.462	0.413	13.21	<0.001
pH × Time	0.204	0.242	0.84	0.420
pH × Glucose conc.	−0.491	0.242	−2.03	0.070
Time × Glucose conc.	−0.654	0.242	−2.70	0.022

Twenty experiments were carried out from the design and by applying multiple regression analysis on the experimental data; the following second-order polynomial equation was found to explain CA production by *Y. lipolytica* NRRL Y-1094.

$$Y = -267.5 + 137.8\,X_1 + 14.48\,X_2 - 2.323\,X_3 - 11.33\,X_1^2 - 0.468\,X_2^2 + 0.008739\,X_3^2 + 0.407\,X_1X_2 - 0.0393\,X_1X_3 - 0.02615\,X_2X_3 \quad (2)$$

Y: CA concentration, X_1: pH, X_2: fermentation time, X_3: initial substrate-concentration

Figure 2 shows the contour and surface graphs of CA concentration for each pair of factors, keeping the other factor constant at its middle level. It is proved that CA concentration gradually decreased when the glucose concentration exceeded optimal conditions (125 g/L). This point is important in making the whole process economically more feasible and in making it possible for a lower substrate concentration in the potential industrial-application. On the contrary, it can be estimated that CA production would gradually increase when the incubation time exceeded optimal conditions. Finally, it can be stated that on the contour plot, the maximum value predicted by the surface is found in the smallest ellipse.

The second-degree polynomial equation that was created to model the CA concentration was solved, and the optimum conditions for the process were determined. Accordingly, the optimum conditions were as follows: an initial pH-value of 5.97 (X_1), fermentation time of 6 days (X_2), and an initial substrate-concentration of 125 g/L (X_3). These results were determined to be the most suitable levels for maximum CA-production by *Y. lipolytica* NRRL Y-1094. The final fermentation (validation trial) was performed with the optimized levels, and the highest CA concentration was determined as 33.57 g/L, which was close to the 33.12 g/L that was predicted by the model. Validation experiments proved that the model is accurate and reliable.

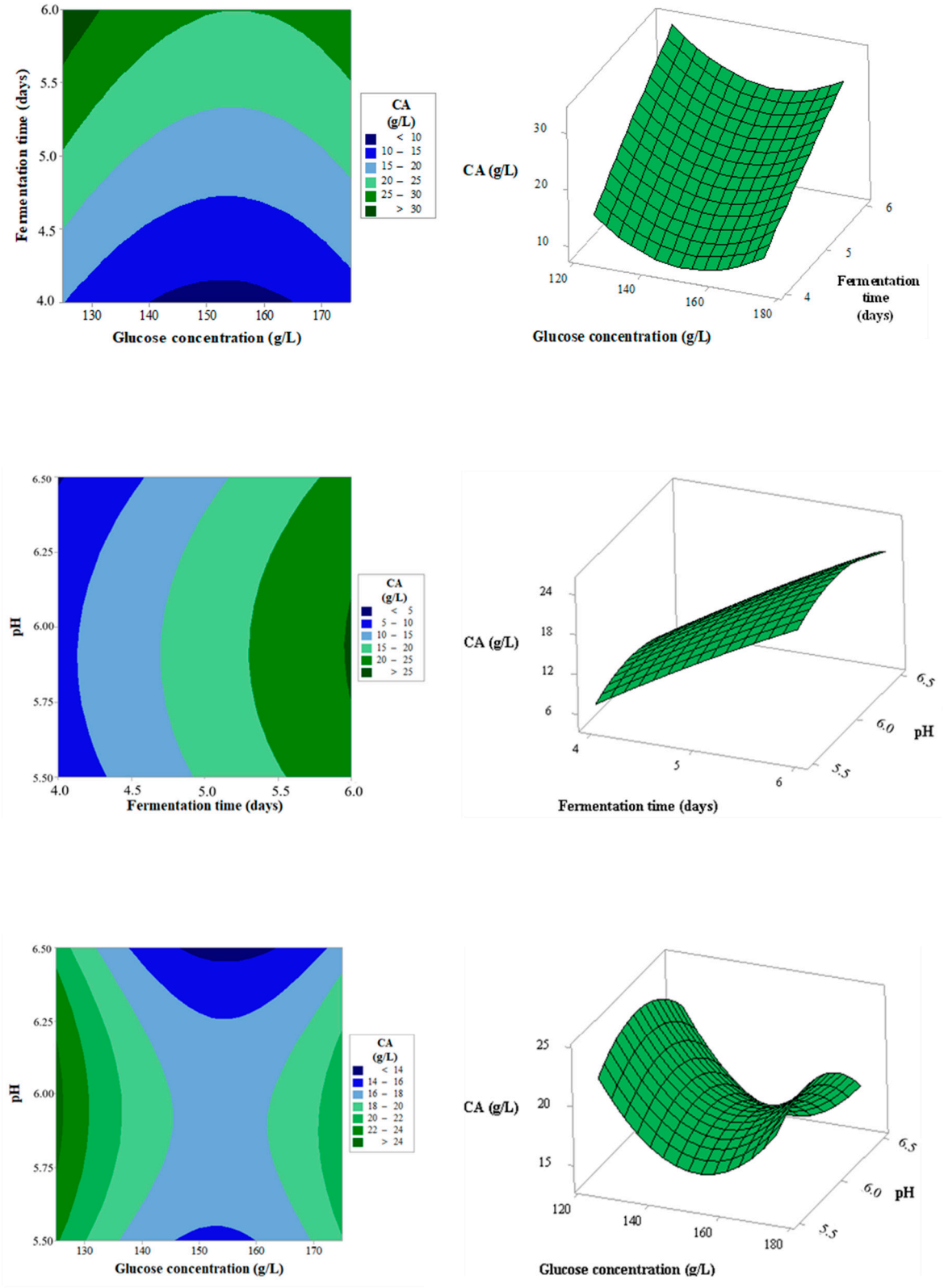

Figure 2. Contour- and surface-graph of glucose concentration and fermentation time at constant pH (pH 6.0), contour- and surface-graphs of fermentation time and pH at constant glucose-concentration (150 g/L), contour- and surface-graph of glucose concentration and pH value at constant fermentation-time (6 days).

4. Conclusions

The CA industry is one of the fastest-growing industries in the world. Therefore, the interest in studies on the microbial production of CA continues. Although CA is produced with high efficiency with many strains of *Y. lipolytica*, screening of new efficient strains and optimization of some process parameters for high CA productivity is necessary. The results of this study showed that *Y. lipolytica* NRRL Y-1094 was a potential strain for the production of CA. The results revealed that a pH value of 5.5, fermentation time of 6 days, and a medium containing 125 g/L glucose, created the best conditions. Additionally, it was concluded that fermentation time is the most effective factor controlling CA synthesis in *Y. lipolytica* NRRL Y-1094, grown on glucose.

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Abbreviations

Adj MS: adjusted mean square; Adj SS: adjusted sum of square; CA: citric acid; DF: degrees of freedom; ICA: isocitric acid; $pH_{0:}$ initial pH value; S_{CA}: selectivity of the bioprocess; SE coefficient: standard error of the coefficient; Seq SS: sequential sum of squares; t: fermentation time; X: biomass

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