Citric acid production by a novel autochthonous Candida zeylanoides isolate: optimization of process parameters

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Abstract In this study, citric acid (CA) production by autochthonous Candida zeylanoides 7.12 was investigated and optimized. Response surface methodology (RSM) was used for the analysis of simultaneous effects of the chosen factors and 2 experiment designs were applied. In the first experimental design, the effects of initial pH value (5.5, 6.0 and 6.5), fermentation time (4, 5 and 6 days) and initial glucose concentration (125, 150 and 175 g/L) on CA production were investigated. Initial pH value was adjusted periodically with NaOH. Results of the statistical analysis showed that the model was found to be not applicable sufficiently to the chosen data. A second experimental design was employed at the same levels of glucose concentration and fermentation time by disabling the pH factor. pH level was kept at 6.5 with CaCO₃. Results of the statistical analysis showed that the fit of the model was good and the lack of fit was not significant (P > 0.05). The highest CA concentration of 11.36 g/L was obtained after 6 days of fermentation with an initial glucose concentration of 125 g/L. The results indicated that initial glucose concentration and fermentation time were important parameters for CA production by C. zeylanoides 7.12 and this strain could be used for future studies.

Keywords Autochthonous · Organic acid · Candida zeylanoides · Optimization · Response surface methodology

Abbreviations

Adj MS Adjusted mean square
Adj SS Adjusted sum of square
CA Citric acid
CA/ICA Citric acid/isocitric acid ratio
DF Degrees of freedom
GR Residual glucose concentration
ICA Isocitric acid
PH₀ Initial pH value
qCA Specific citric acid production rate
SCA Selectivity of the bioprocess
SE coef Standard error of the coefficient
Seq SS Sequential sum of squares
t Fermentation time
X Biomass
YCA/glucose Mass yield coefficient
YCA/t Volumetric yield
**Introduction**

CA is formed in the Krebs cycle when carbohydrates are oxidized to carbon dioxide (Abonama et al. 2014). This organic acid is frequently used in the food, pharmaceutical and cosmetic industries due to its properties such as flavoring and buffer agent, antioxidant and preservative. It has also been used as a detergent co-builder in dishwasher cleaners and cross-linker in the production of biodegradable polymers over the past decade (Rzechonek et al. 2019). Worldwide CA production was estimated to be around 2 million tons in 2020 (Fickers et al. 2020). Additionally, it has been emphasized that the global CA market volume will be 3.2 billion dollars by 2023, reaching a Compound Annual Growth Rate (CAGR) of 5.1% (Behera et al. 2021).

The type and amount of carbon source, trace elements used, nitrogen and phosphorus limitations, environmental factors such as aeration, pH and temperature, as well as selection and improvement of species are important factors affecting the microbial CA production (Anastassiadis et al. 2008). However, *Aspergillus niger* is mostly used for industrial CA production, yeasts have been used by many researchers for a long time to investigate and improve the production capacities (Karasu-Yalcin et al. 2010). Nevertheless, compared to CA production by fungi, yeasts have many advantages such as the possibility of using more substrates, less sensitivity to lower dissolved oxygen concentrations and heavy metals, genetic and mechanical stability, and less health hazards (Hesham et al. 2020). For these reasons, the researchers have focused on studies on the CA production capacity of yeasts (Anastassiadis et al. 2002; Papanikolaou et al. 2006; Mafakher et al. 2010; Rywińska et al. 2011; Tomaszewska et al. 2014; Morgunov et al. 2018). On the other hand, it is known that isocitric acid (ICA) can be produced as an undesired by-product in yeast-based CA production processes (Cavallo et al. 2017).

In the literature, studies on CA production by *Candida zeylanoides* have been seen to be very limited. However, in some studies, high concentrations of CA have been obtained. Hattori and Suzuki (1974) reported that 87 g/L of CA was produced by *C. zeylanoides* KY6166 using 10% (w/v) n-alkane in the fermenter (pH 5.5, 100 h). Kim et al. (2015) calculated maximum CA concentration as 91.4 g/L by *C. zeylanoides* ATCC 20367 with submerged fermentation technique where molasses was used as the substrate. These promising results led to the investigation of *C. zeylanoides* strains as an alternative to *Y. lipolytica* in CA production. Furthermore, no previous work has used a statistical approach to the optimization of process parameters in CA production by autochthonous *C. zeylanoides* strains, although there are a few studies on the use of autochthonous strains in CA production (Yalcin et al. 2009a; Justin et al. 2010; Dashen et al. 2013; Adeoye et al. 2015; Adeoye and Lateef 2021).

The aim of this study is to investigate the potential CA production of autochthonous *C. zeylanoides* 7.12. Another important aim is to reveal some optimal conditions for CA production using Response Surface Methodology (RSM). Two experimental designs were applied for this purpose. In the first experiment, initial pH value (5.5–6.5), fermentation time (4–6 days) and initial glucose concentration (125–175 g/L) were selected as factors. In the second experimental design glucose concentration and fermentation time at the same levels with experimental design 1 were used as process parameters to be optimized for CA production while keeping the pH of the medium constant at 6.5 using CaCO₃. In addition, various yield values of the bioprocess were calculated by determining the concentration of ICA, biomass and consumed glucose during fermentation.

**Material and methods**

**Microorganism**

In this study, *Candida zeylanoides* 7.12 which was previously isolated and genetically identified from a Turkish dry-cured meat product pastırma (Kaya et al. 2017) was selected according to its acid production capacity (Sayın Börekçi 2020). Malt Extract Broth (MEB) was used to obtain pre-cultures which were used to inoculate the fermentation media.
Cultivation and fermentation medium

The pre-cultures were obtained in the MEB medium and then transferred to the modified fermentation medium (MFM). The inoculation ratio was 2% (v/v). The MFM medium had the following composition (in grams per liter): glucose, 125, 150 or 175; yeast extract, 0.5; NH₄Cl, 1.5; KH₂PO₄, 1.0; MgSO₄·7H₂O, 1.5; CaCl₂, 0.15; FeCl₃·6H₂O, 0.15; ZnSO₄·7H₂O, 0.02; MnSO₄·7H₂O, 0.06; (NH₄)₂SO₄, 1.0; CuSO₄, 0.02; thiamine, 0.001 (Rane and Sims 1993; Papanikolaou and Aggelis 2002). The CA productions were carried out in shake flasks (50 mL medium/250 mL flask) and the flasks were shaken on a rotary shaker at 28 °C at 180 rpm (JSSI-100, JS Research, Gongju, Korea).

Chemicals

Glucose, yeast extract, KH₂PO₄, CaCl₂, ZnSO₄·7H₂O, MnSO₄·7H₂O, CuSO₄, acetonitrile and malt extract broth were supplied from Merck (Darmstadt, Germany). MgSO₄·7H₂O, FeCl₃·6H₂O, (NH₄)₂SO₄, thiamine, NaOH, CaCO₃, HClO₄, H₂SO₄ and CA-ICA standards were purchased from Sigma-Aldrich Chemical Company (Steinheim, Germany). NH₄Cl was purchased from ISOLAB (Istanbul, Turkey).

Analytical methods

**Determination of CA and ICA concentrations**

When applying the first experimental design, pH of MFM was adjusted between 5 and 6 with aseptically adding 100–400 μL volumes of 5 N NaOH in 18 h after inoculation and every 24 h after this time. Additionally, in the second experimental design, the pH was maintained at 6.5 with 35 g/L of CaCO₃. Cells were removed from the fermentation medium by centrifugation (Hettich Universal-320R, Hettich, Frankenberg, Germany) at 13000×g for 15 min (at 4 °C). Method by Kamzolova and Morgunov (2017) was modified and used for the determination of CA and ICA concentrations. The supernatant obtained was filtered through a 0.45 μm filter and diluted with 8% HClO₄ in an equal volume. Measurements were performed by high performance liquid chromatography (HPLC, Agilent Technologies 1100 Series, Palo Alto, USA) at 210 nm. The column temperature was maintained at 40 °C and the flow rate of the mobile phase was 1 mL/min. 0.01 M H₂SO₄ was used as a mobile phase in reverse-phase column (Intersil ODS-3, 4.6×250 mm, GL Sciences Inc., Tokyo, Japan). CA and ICA standards (Sigma-Aldrich, St. Louis, MO, USA) were used to obtain a calibration curve and to determine quantities. Samples were kept at −20 °C until analysis by HPLC.

**Determination of residual glucose concentration**

Glucose analysis was performed by HPLC with the supernatant obtained after centrifugation of fermentation medium (Shimadzu, Kyoto, Japan). For this purpose, Intersil NH₂ (5 μm, 250×4.6 mm) (GL Sciences Inc., Tokyo, Japan) column was used and flow rate was adjusted as 1.3 mL/min in the system. Mobile phase was prepared with 75% acetonitrile (25% ultra-pure water), and column temperature was adjusted to 40 °C (Anastassiadis and Rehm 2006).

**Determination of biomass and pH level**

Cell growth was evaluated gravimetrically. Cells were separated by the centrifugation of the fermentation medium and dried to a constant weight at 80 °C for 24 h. Biomass (g/L) was calculated as the dry cell weight produced in liters of liquid medium (Papanikolaou and Aggelis 2002). When CaCO₃ was used to control the pH of the fermentation medium, 6 N HCl was used to dissolve residual CaCO₃ in the fermentation medium and biomass concentration was determined (Karasu-Yalcin et al. 2010 et al. 2010). The pH value of fermentation medium was measured with a pH meter (Mettler Toledo Ion S220, Switzerland).

Calculation of fermentation parameters

The following kinetic parameters were calculated in CA fermentation using *Candida zeylanoides* 7.12.

- **Biomass yield:** \( Y_{X/glucose(consumed)} \) (g/g)
- **Mass yield coefficient:** \( Y_{CA/glucose(consumed)} \) (g/g)
- **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)
- **Glucose uptake rate** = Glucose consumed/(X.t) (g/ g h)
Bioprocess selectivity: $S_{CA} = \frac{100 \times CA_T}{CA_T + ICA_T}$

Experimental designs

The statistical analysis of the data was performed using RSM. Table 1(A) and (B) shows the levels of factors used in the experimental design. The levels of the factors were selected after preliminary experiments. Twenty experiments were conducted using a face central composite statistical design ($\alpha = 1$) for the study of 3 factors each at 3 levels (Table 2). Second experimental design contained 13 experiments for the study of 2 factors each at 3 levels (Table 4). The levels were $-1, 0, +1$ where 0 corresponded to the central point.

The second order response function for 3 and 2 quantitative factors are given below. Where $Y$ is the predicted response, $\beta_0$ is model constant; $X_1, X_2$ and $X_3$ are independent variables; $\beta_1, \beta_2$ and $\beta_3$ are linear coefficients; $\beta_{12}, \beta_{13}$ and $\beta_{23}$ are cross product coefficients and $\beta_{11}, \beta_{22}$ and $\beta_{33}$ are the quadratic coefficients.

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

$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{12} X_1 X_2$  \hspace{1cm} (B)

The actual level of the central point of each factor was calculated using the following equation:

Table 1 Levels of factors used in experimental designs, (A) three-factor experimental design, (B) two-factor experimental design

| Factor | Independent variables | Levels |
|--------|-----------------------|--------|
| (A)    |                       | $-1$   | $0$   | $+1$  |
| $X_1$  | 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   |
| (B)    |                       | $-1$   | $0$   | $+1$  |
| $X_1$  | Glucose concentration (g/L) | 125    | 150   | 175   |
| $X_2$  | Fermentation time (days) | 4      | 5     | 6     |

Table 2 Results of CA production by C. zeylanoides 7.12 in three-factor experimental design

| Run | pH0 | Time (days) | Glucose conc. (g/L) | CA (g/L) | ICA (g/L) | X (g/L) | pHF |
|-----|-----|-------------|---------------------|----------|-----------|---------|-----|
| 1   | 6.0 | 5           | 150                 | 1.65 ± 0.84 | 8.64 ± 0.23 | 6.57 ± 0.34 | 5.37 ± 0.06 |
| 2   | 6.5 | 6           | 175                 | 2.25 ± 0.43 | 6.40 ± 1.28 | 7.37 ± 0.37 | 5.31 ± 0.39 |
| 3   | 6.5 | 5           | 150                 | 1.68 ± 0.66 | 6.64 ± 0.48 | 6.12 ± 0.17 | 5.45 ± 0.05 |
| 4   | 5.5 | 5           | 150                 | 1.71 ± 0.59 | 2.66 ± 0.47 | 5.85 ± 0.16 | 5.52 ± 0.31 |
| 5   | 6.0 | 5           | 150                 | 2.17 ± 0.05 | 6.97 ± 1.63 | 6.46 ± 0.15 | 5.45 ± 0.16 |
| 6   | 6.0 | 5           | 150                 | 2.05 ± 0.05 | 7.38 ± 0.18 | 6.79 ± 0.29 | 5.30 ± 0.08 |
| 7   | 5.5 | 6           | 125                 | 2.31 ± 0.57 | 4.21 ± 1.58 | 9.06 ± 0.89 | 7.87 ± 0.30 |
| 8   | 6.0 | 5           | 150                 | 2.11 ± 0.32 | 6.96 ± 0.01 | 5.99 ± 0.04 | 5.68 ± 0.42 |
| 9   | 5.5 | 4           | 125                 | 2.42 ± 0.09 | 8.86 ± 5.72 | 7.11 ± 1.29 | 5.37 ± 0.13 |
| 10  | 6.0 | 4           | 150                 | 2.16 ± 0.14 | 8.73 ± 2.52 | 6.35 ± 0.01 | 5.26 ± 0.13 |
| 11  | 6.5 | 4           | 125                 | 2.56 ± 0.29 | 10.01 ± 0.2 | 7.43 ± 0.47 | 5.37 ± 0.33 |
| 12  | 5.5 | 4           | 175                 | 1.13 ± 0.13 | 3.91 ± 0.44 | 5.23 ± 0.23 | 5.19 ± 0.00 |
| 13  | 5.5 | 6           | 175                 | 1.47 ± 0.50 | 6.99 ± 0.15 | 7.32 ± 0.08 | 5.39 ± 0.08 |
| 14  | 6.0 | 5           | 175                 | 1.71 ± 1.35 | 13.48 ± 4.94 | 7.04 ± 0.76 | 5.45 ± 0.01 |
| 15  | 6.5 | 6           | 125                 | 2.07 ± 0.22 | 2.96 ± 0.19 | 10.08 ± 0.55 | 8.12 ± 0.06 |
| 16  | 6.0 | 5           | 150                 | 2.28 ± 0.12 | 7.25 ± 1.00 | 6.55 ± 0.18 | 5.40 ± 0.01 |
| 17  | 6.5 | 4           | 175                 | 2.18 ± 0.25 | 9.82 ± 0.83 | 6.97 ± 0.04 | 5.19 ± 0.3 |
| 18  | 6.0 | 6           | 150                 | 1.91 ± 0.04 | 6.76 ± 0.77 | 6.53 ± 0.19 | 5.43 ± 0.18 |
| 19  | 6.0 | 5           | 150                 | 2.12 ± 0.00 | 6.80 ± 0.52 | 6.69 ± 0.77 | 5.39 ± 0.02 |
| 20  | 6.0 | 5           | 125                 | 2.32 ± 0.17 | 8.81 ± 0.01 | 7.52 ± 0.25 | 5.77 ± 0.21 |

CA Citric acid, ICA Isocitric acid, GR Residual glucose concentration, pHF Final pH value after fermentation, X Biomass
For determining the extent to which the model obtained corresponds to the experimental data, variance analysis (ANOVA) was used. Fischer’s $F$-test was used for searching the statistical significance (at 95% confidence level) of linear, quadratic and interaction effects of each factor. It is concluded that the model is fit when the error due to lack of fit is insignificant ($P > 0.05$) and $F$-test for regression was significant ($P < 0.05$). All experiments were performed in duplicate. The statistical analysis of the data was performed using Minitab 17.1.0 software (Minitab Inc., State College, PA, USA).

Results and discussion

Results of three-factor experimental design

Our preliminary experiments showed that initial substrate concentration, initial pH and fermentation time influenced CA production by Candida zeylanoides 7.12 in shake flask cultures. Thus, a face centered design was used to determine the optimum levels of the above mentioned variables leading to maximum CA production. As shown in Table 2, CA concentrations varied between 1.13 and 2.56 g/L and ICA which is the byproduct of CA fermentation was produced almost more than CA in all fermentation trials.

$R^2$ is described as the ratio of the explained variation to the total variation and is a measure of the degree of fit (Neşeli et al. 2011). The small value of $R^2$ indicates that the less relevant the dependent variables in the model have in explaining the behavior variation. It is indicated that if $R^2$ approaches unity, the better the response model fits the actual data (Fu et al. 2007). In this situation, it is specified that the less the difference between the predicted and actual values (Chiang 2008). Additionally, if $R^2$ values are higher than 0.8, it means that regression models explained the data well (Sin et al. 2006). ANOVA for the concentration of CA is presented in Table 3. According to the obtained result from the present study, although lack of fit was not significant ($P > 0.05$), the correlation coefficient ($R^2$) value, which shows the fit between the experimental data and the predicted data, was determined to be 66.60% ($R^2_{adj}$). In these conditions, it was decided that the model was not fit and could not adequately explain the variation observed in CA production with the designed levels of the factors.

Results of two-factor experimental design

pH value is one of the most effective factors in CA production by yeasts. When yeasts are used in production, a pH value greater than 5 generally supports production better. Otherwise, this situation may result in the accumulation of some polyalcohols such as erythritol, arabitol and mannitol in the medium. At low pH levels, citrate production in the cell can be inhibited and citrate can be transported from the cell membrane (Yalcin et al. 2010). Considering these reasons, it was determined that the periodic adjustment of the pH value with NaOH in the first experimental design was insufficient and the second experimental design was considered.

A second experimental design was performed to investigate the effect of initial substrate concentration and fermentation time on CA production when pH was kept constant at pH 6.5 using CaCO$_3$. For pH adjustment, CaCO$_3$ at a concentration of 35 g/L was used before sterilization of the fermentation medium.

| Source               | DF | Seq SS  | Adj SS  | Adj MS  | F      | P      |
|----------------------|----|---------|---------|---------|--------|--------|
| Regression           | 9  | 1.96647 | 1.96647 | 0.21850 | 5.21   | 0.008  |
| Linear               | 3  | 1.17272 | 1.17272 | 0.39091 | 9.32   | 0.003  |
| Square               | 3  | 0.14781 | 0.14781 | 0.04927 | 1.17   | 0.368  |
| Interaction          | 3  | 0.64594 | 0.64594 | 0.21531 | 5.13   | 0.021  |
| Residual error       | 10 | 0.41935 | 0.41935 | 0.04194 |        |        |
| Lack of fit          | 5  | 0.18462 | 0.18462 | 0.03692 | 0.79   | 0.601  |
| Pure error           | 5  | 0.23473 | 0.23473 | 0.04695 |        |        |
| Total                | 19 | 2.38582 |         |         |        |        |
The effect of glucose concentration and fermentation time variables, each at 3 levels and their interactions on CA production by *Candida zeylanoides* 7.12 were determined by carrying out 13 experiments given by the model (Experimental design 2). As seen in Table 4, the highest CA concentration (11.36 g/L) was obtained at the 6th day of fermentation with an initial glucose concentration of 125 g/L. The lowest CA concentration (5.63 g/L) was achieved with an initial glucose concentration of 175 g/L after 4 days of fermentation. At all points in experimental design, glucose was consumed by the autochthonous strain. Biomass concentration varied between 12.18–18.50 g/L.

Yalcin et al. (2009b) showed that 44.39 and 37.66 g/L of CA were achieved in 280 h by autochthonous *Y. lipolytica* 57, when 150 and 100 g/L of glucose were used in the fermentation medium, respectively. Çelik et al. (2014) obtained 12 g/L of CA and 1.41 g/L of ICA at the end of 216 h when 100 g/L of glucose was used in the fermentation medium with *Y. lipolytica* TEMYL 3. Although this concentration is very close to the maximum concentration (11.36 g/L) obtained in our study, there is a significant difference in the concentration of ICA and fermentation time. In the present study, it is thought that the CA concentration will increase by extending the fermentation time. Arslan et al. (2016) investigated the effects of different pH values on CA production by *Y. lipolytica* B9 strain and they obtained the maximum CA concentration at pH 5.5 (8.6 g/L).

As seen in Table 5, the highest CA/ICA ratio (568.1), $Y_{X/glucose}$ (0.15), $Y_{CA/glucose}$ (0.9), $Y_{CA/X}$ (0.61) and selectivity of bioprocess (99.82%) were found after 6 days when 125 g/L of glucose was used in the fermentation medium. The formation of very low concentrations of ICA resulted in high values of CA/ICA and high selectivity of the bioprocess. The highest $Y_{CA}$ yield (0.09) was achieved at run number 1 and the highest $q_{CA}$ yield (0.005) was obtained at runs 1, 5 and 6. The highest and lowest glucose uptake rates were calculated as 0.15 and 0.05 at runs 6 and 2, respectively. Kamzolova and Morgunov (2017) reported that *C. zeylanoides* VKM Y-6, VKM Y-14 and VKM Y-2324 produced 3.81, 3.60 and 2.90 g/L of CA, 1.61, 1.91 and 1.91 g/L of ICA, respectively. On the other side, they also determined that the $g_{CA}/g_{cell}$ yields were 1.03, 1.13 and 0.97, respectively. Compared to the results in the literature, higher CA and selectivity were obtained in this study while the $Y_{CA/X}$ were lower (due to high biomass formation).

ANOVA results for the CA concentration are presented in Table 6. $R^2$ was 0.994 indicating that the data was explained 99.4% of the variability in CA concentration. Also, $F$-test for lack of fit (0.270) was not significant ($P > 0.05$). These results show that the model chosen can satisfactorily explain the effects of initial glucose concentration and fermentation time on CA production by *C. zeylanoides* 7.12.

The following model was fitted for CA concentration:

### Table 4 Results of CA production by *C. zeylanoides* 7.12 based on the two factor experimental design

| Run | Glucose conc. (g/L) | Time (days) | CA (g/L) | ICA (g/L) | X (g/L) | $G_R$ (g/L) |
|-----|---------------------|-------------|----------|-----------|---------|-------------|
| 1   | 125                 | 4           | 8.36 ± 0.63 | 0.20 ± 0.21 | 17.02 ± 0.10 | 0.00 ± 0.00 |
| 2   | 125                 | 6           | 11.36 ± 0.21 | 0.02 ± 0.01 | 18.50 ± 0.42 | 0.00 ± 0.00 |
| 3   | 175                 | 5           | 5.77 ± 0.27 | 0.94 ± 0.10 | 12.22 ± 0.37 | 0.00 ± 0.00 |
| 4   | 150                 | 5           | 6.80 ± 0.31 | 0.32 ± 0.02 | 14.79 ± 0.72 | 0.05 ± 0.06 |
| 5   | 150                 | 4           | 6.35 ± 0.61 | 0.52 ± 0.08 | 14.47 ± 2.26 | 0.04 ± 0.02 |
| 6   | 175                 | 4           | 5.63 ± 0.21 | 0.94 ± 0.00 | 12.18 ± 0.32 | 0.13 ± 0.01 |
| 7   | 125                 | 5           | 9.36 ± 0.04 | 0.03 ± 0.02 | 17.85 ± 1.17 | 0.00 ± 0.00 |
| 8   | 150                 | 5           | 6.64 ± 0.57 | 0.48 ± 0.52 | 13.97 ± 0.95 | 0.11 ± 0.06 |
| 9   | 150                 | 5           | 6.82 ± 0.03 | 0.53 ± 0.18 | 15.30 ± 0.13 | 0.17 ± 0.08 |
| 10  | 175                 | 6           | 6.39 ± 0.08 | 0.76 ± 0.01 | 11.87 ± 0.26 | 0.17 ± 0.23 |
| 11  | 150                 | 5           | 6.62 ± 0.11 | 0.33 ± 0.04 | 15.54 ± 0.54 | 0.23 ± 0.01 |
| 12  | 150                 | 6           | 8.34 ± 1.14 | 0.07 ± 0.03 | 15.03 ± 0.95 | 0.44 ± 0.51 |
| 13  | 150                 | 5           | 6.84 ± 0.59 | 0.32 ± 0.04 | 15.08 ± 0.52 | 0.10 ± 0.13 |

CA Citric acid, ICA Isocitric acid, GR Residual glucose concentration, X Biomass

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Estimated regression coefficients for CA concentration are shown in Table 7. Initial glucose concentration had a negative linear effect, and fermentation time had a positive linear effect on CA production ($P < 0.05$). This results shows that with increasing glucose concentration, the CA concentration will decrease, and when the fermentation time progresses, the CA concentration will increase. The glucose concentration had the highest impact on CA production as given by the highest linear coefficient (−1.8817). Initial glucose concentration and fermentation time showed significant positive quadratic effect.

\[ Y = 34.25 - 0.3038X_1 - 0.576X_2 \\
+ 0.001135X_1^2 + 0.4895X_2^2 - 0.02240X_1X_2 \quad (1) \]

\( Y \): CA concentration, \( X_1 \): Initial glucose concentration, \( X_2 \): Fermentation time.

### Table 5: Yields of CA production by C. zeylanoides 7.12

| Run | CA/ICA | \( Y_{X/glucose} \) | \( Y_{CA/glucose} \) | \( Y_{CA/X} \) | \( q_{CA} \) | Glucose uptake rate | \( S_{CA} \) (%) |
|-----|--------|-------------------|-------------------|----------------|-------------|---------------------|-------------|
| 1   | 41.8:1 | 0.14              | 0.07              | 0.49           | 0.09        | 0.005               | 0.08        | 97.66           |
| 2   | 568:1  | 0.15              | 0.09              | 0.61           | 0.08        | 0.004               | 0.05        | 99.82           |
| 3   | 6.1:1  | 0.07              | 0.03              | 0.47           | 0.05        | 0.004               | 0.12        | 85.99           |
| 4   | 21.3:1 | 0.10              | 0.05              | 0.46           | 0.06        | 0.004               | 0.08        | 95.51           |
| 5   | 12.2:1 | 0.10              | 0.04              | 0.44           | 0.07        | 0.005               | 0.11        | 92.43           |
| 6   | 6:1    | 0.07              | 0.03              | 0.46           | 0.06        | 0.005               | 0.15        | 85.69           |
| 7   | 312:1  | 0.14              | 0.07              | 0.52           | 0.08        | 0.004               | 0.06        | 99.68           |
| 8   | 13.8:1 | 0.09              | 0.04              | 0.48           | 0.06        | 0.004               | 0.09        | 93.26           |
| 9   | 12.9:1 | 0.10              | 0.05              | 0.45           | 0.06        | 0.004               | 0.08        | 92.79           |
| 10  | 8.4:1  | 0.07              | 0.04              | 0.54           | 0.04        | 0.004               | 0.10        | 89.37           |
| 11  | 20.1:1 | 0.10              | 0.04              | 0.43           | 0.06        | 0.004               | 0.08        | 95.25           |
| 12  | 119:1  | 0.10              | 0.06              | 0.55           | 0.06        | 0.004               | 0.07        | 99.17           |
| 13  | 21:4:1 | 0.10              | 0.05              | 0.45           | 0.06        | 0.004               | 0.08        | 95.53           |

### Table 6: ANOVA for CA concentration of C. zeylanoides 7.12 based on the second experimental design

| Source          | DF | Seq SS | Adj SS | Adj MS | F      | P     |
|-----------------|----|--------|--------|--------|--------|-------|
| Regression      | 5  | 31.264 | 31.264 | 6.2528 | 406.45 | <0.001|
| Linear          | 2  | 26.754 | 26.754 | 13.3772| 869.56 | <0.001|
| Square          | 2  | 3.2552 | 3.2552 | 1.6276 | 105.80 | <0.001|
| Interaction     | 1  | 1.2544 | 1.2544 | 1.2544 | 81.54  | <0.001|
| Residual error  | 7  | 0.1077 | 0.1077 | 0.0154 |        |       |
| Lack of fit     | 3  | 0.0634 | 0.0634 | 0.0211 | 1.91   | 0.270 |
| Pure error      | 4  | 0.0443 | 0.0443 | 0.0111 |        |       |
| Total           | 12 | 31.3717|        |        |        |       |

### Table 7: Estimated regression coefficients for CA concentration based on the second experimental design

| Term               | Coefficient | SE coefficient | T     | P     |
|--------------------|-------------|----------------|-------|-------|
| Constant           | 6.7759      | 0.515          | 131.57| 0.000 |
| Glucose conc       | −1.8817     | 0.0506         | −37.16| 0.000 |
| Time               | 0.9583      | 0.0506         | 18.93 | 0.000 |
| Glucose conc. × Glucose conc | 0.7095 | 0.0746 | 9.51  | 0.000 |
| Time × Time        | 0.4895      | 0.0746         | 6.56  | 0.000 |
| Glucose conc. x Time | −0.5600 | 0.0620 | −9.03 | 0.000 |
on CA production \((P<0.05)\). The negative interaction between substrate concentration and fermentation time was significant as shown by low \(P\) values \((P<0.05)\) for interactive terms.

In Fig. 1, when one of the factors was kept constant in the center, the effects of other factor levels on the concentration of CA were shown with contour and surface graph.

The fitting of the experimental data to Eq. 1 allowed the determination of the optimum levels of initial sugar concentration \((125\ \text{g/L})\) and fermentation time \((6\ \text{days})\) giving a maximum CA concentration of \(11.37\ \text{g/L}\). The above data optimizes CA production by \(C.\ zeylanoides\ 7.12\) in shake flasks. The final fermentation (validation trial) was performed with the optimized levels and the highest CA concentration \((11.95\ \text{g/L})\) which was close to the value given by the model was obtained.

**Conclusion**

The investigation of new strains for the production of microbial products more economically and efficiently is becoming a considerable issue day by day. The fact that the demand for CA increases every year along with the developing industry makes this organic acid more important, and has increased the interest in the...
development of existing processes. In this study, CA production by autochthonous Candida zeylanoides 7.12 was investigated and RSM was used to determine the effects of initial substrate concentration and fermentation time on CA production. The model generated in this study by RSM satisfied all the necessary arguments for its use in the optimization of CA fermentation. By fitting the experimental data to a second-order polynomial equation, optimum levels of the variables were determined. Using optimum levels of fermentation parameters, a maximum CA concentration of 11.95 g/L was obtained. According to the validation trial, it was shown that the data obtained from the predicted model fitted well with the laboratory results. The kinetic parameters of CA formation by C. zeylanoides 7.12 in shake flasks were also calculated and given in the manuscript. It is considered that this study contributed to the discovery of new strains and to the use of autochthonous isolates in CA production. Even if the autochthonous strains initially have a low CA production capacity, production yields can be increased. From this point of view, it is known that making various genetic manipulations on the strains, optimizing the other process conditions, and reducing the process costs together with the use of waste will provide important gains for a productive CA production. As a result, the current study could allow the improvement of other new processes by considering these issues and the results obtained in the study. Since the ultimate goal of all studies is to help to realize industrial-scale CA production, it is an important requirement for the discovery of new strains and determining the well-adjusted process conditions for improving this industry.

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