# Effects of Different Fermentation Conditions on Growth and Citric Acid Production Kinetics of two *Yarrowia lipolytica* Strains

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In this study, effects of initial pH, temperature, initial ammonium chloride concentration and initial concentration of various minerals on growth and citric acid production kinetics of two *Yarrowia lipolytica* strains were investigated in shake flask batch experiments. Specific growth rate and citric acid production rates were correlated with each one of the investigated parameters by using non-linear regression analysis. Maximum citric acid concentration was obtained at initial pH 7.0 and 5.2 for *Y. lipolytica* NBRC 1658 and the domestic strain 57, respectively. The highest citric acid production yield was obtained at 30 °C for both of the strains. Maximum citric acid concentrations and production rates were determined in the medium containing 2 g $L^{-1}$ of ammonium chloride. Inhibition effects of iron and copper salts on citric acid production were found. Addition of manganese salt caused a decrease in citric acid production. Maximum citric acid concentration obtained by *Y. lipolytica* 57 increased from 37.66 g $L^{-1}$ to 41.63 g $L^{-1}$ by addition of 0.008 g $L^{-1}$ of zinc sulphate into the fermentation medium.

*Key words:*
Citric acid, *Yarrowia lipolytica*, batch fermentation, pH, temperature, mineral salts

## Introduction

Citric acid (CA) is a common natural product from citrus fruits that has been industrially produced since the beginning of the last century. It is one of the few bulk chemicals produced by fermentation, and is the most exploited biochemical product.[1] The annual production of citric acid is approximately 1.6 million tons.[2] Citric acid is widely used in food, pharmaceuticals, cosmetics, and beverage industries as an acidifying and flavour-enhancing agent.[3,4] It is used to impart a pleasant, tart flavour to foods and beverages.[5]

Citric acid is a metabolite of energy metabolism, the concentration of which will only rise to appreciable amounts under conditions of metabolic imbalances.[6] *Aspergillus niger* is the traditional producer of citric acid, but during the last 30 years the interest of researchers has been attracted by the use of yeasts as citric acid producers. Specifically yeasts belonging to the species *Yarrowia lipolytica*, *Candida guilliermondii* and *Candida oleophila* have been used for the production of citric acid by using various substrates.[7] *Y. lipolytica* has been the most commonly used yeast species for this purpose.[8–10]

It is reported that citric acid production rates and yields are highly dependent on the type of microorganism, the type of substrate and the culture conditions.[11] Factors that have been shown to exert an effect on citric acid production are the type and concentration of carbon source of the fermentation medium, nitrogen and phosphate limitations, aeration, trace elements, initial pH, and temperature.[6,12] It is reported that initial pH must be very well defined and optimized depending on the microorganism, substrate and production technique. Kamzolova *et al.*[13] reported that citric acid production and ratio of citric to isocitric acid depended on pH of the medium and *Yarrowia lipolytica* produced almost equal amounts of citric and isocitric acids at pH 4.5 while predominantly accumulated co-product isocitric acid at pH 6.0. Effect of temperature on growth and citric acid production of microorganisms was also reported in a number of studies. The optimal level of temperature was indicated as 26–30 °C for both citric acid production and biomass models of *C. lipolytica*.[14] Rane and Sims reported the optimal temperature for growth of *C. lipolytica* as 27 °C.[15] In another study, it was indicated that growth and citrate production of

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*C. lipolytica* was noted even at 35 °C using glucose medium.[16] Citric acid production is directly influenced by the concentration and nature of the nitrogen source and trace metal ions. Divalent metal ions such as zinc, manganese, iron, copper and magnesium have been found to affect citric acid production.[5] Finogenova *et al*. reported that the main factor determining citric acid production in *Y. lipolytica* was growth limitation by nitrogen.[17] In the same study, it was indicated that zinc or iron limited conditions resulted in insignificant cell growth without citric acid production. Addition of zinc into the medium alleviated the zinc deficiency symptoms and increased the production of citric acid. It was also reported in the same study that intensive citric acid production in *Y. lipolytica* required high intracellular iron amounts in the range of 0.2–2.5 mg $g^{-1}$, in conditions of nitrogen limitation growth of cells, grown on ethanol. At an intracellular iron fraction of $w = 7.0$ mg $g^{-1}$, citric acid production was completely inhibited. In a study performed by Anastassiadis and Rehm, the addition of iron was found to enhance biomass formation and to affect continuous citric acid production significantly, for yeast *C. oleophila* growing on glucose.[18]

The aim of this study was to investigate growth and citric acid production kinetics of two different *Y. lipolytica* strains at different fermentation conditions. It was a comparative study which demonstrates effects of pH, temperature, initial ammonium chloride concentration and various inorganic minerals on the growth and citric acid production of the two strains.

## Materials and methods

### Yeast strains and selection

In this study, two strains; *Y. lipolytica* NBRC 1658 and *Y. lipolytica* 57, were used in the fermentation experiments. *Y. lipolytica* NBRC 1658 was obtained from National Institute of Technology and Evaluation (NITE) Biological Resource Center, Japan. The strain NBRC 1658 was defined as a "citric acid producer" by the culture collection. The domestic strain *Y. lipolytica* 57 was obtained from the culture collection of Ankara University, Food Engineering Department, Turkey. Isolation source of the domestic strain is stated as "unknown" by the culture collection. Another strain, *Y. lipolytica* IFO 1195, had been also obtained from NITE Biological Resource Center in Japan. The selection of the yeast strains that would be used for the experiments was performed with a preliminary study in which citric acid production abilities of 30 domestic strains were investigated (data not shown). These domestic strains were belonging to different species such as *Candida guillermondii, Candida pelliculosa, Candida intermedia, Candida parapsilosis, Rhodotorula glutinis, Saccharomyces cerevisiae, Pichia anomala* and *Y. lipolytica*. In this prework, citric acid production of the strains *Y. lipolytica* NBRC 1658 and *Y. lipolytica* IFO 1195 were also evaluated. The domestic yeast strains belonging to the culture collection of Hacettepe University, Food Engineering Department, Turkey, had been isolated from some high-sugar foods (honey, fruit yoghurt, dried apricot, dried fig, date, glucose syrup, etc.) in a previous study performed by Senses-Ergul and Ozbas.[19] Screening of citric acid production capabilities of the strains was performed in a fermentation medium containing (in g $L^{-1}$): glucose, 100; $NH_4Cl$, 2; $KH_2PO_4$, 1; $MgSO_4 \cdot 7H_2O$, 1; yeast extract, 1; $CaCO_3$, 40.[20] Of the tested strains, the highest citric acid production was obtained by the domestic *Y. lipolytica* 57 followed by *Y. lipolytica* NBRC 1658. Therefore, these two strains were chosen for the further studies. At the beginning of the present study, identification of the domestic strain, *Y. lipolytica* 57, and *Y. lipolytica* NBRC 1658 was confirmed with the use of rapid API ID 32C (bioMérieux, France) test system and also molecular methods. PCR-RFLP method was used for genetic identification of the strains.[21] In this method, NS1/ITS2 primer pair was used for the amplification of 18S rDNA with the neighbouring ITS1 region, followed by cutting the amplicon by using five-base cutting restriction enzymes: *Msp*I, *Hae*III, *Alu*I, *Rsa*I and *Scrp*I (data not shown).

The yeast strains were kept as stock cultures at 4 °C on Yeast Extract Malt Extract (YM) agar consisting of (in g $L^{-1}$): 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.

### Growth and fermentation media

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 (in g $L^{-1}$): glucose, 30; yeast extract, 2; $NH_4Cl$, 2; $KH_2PO_4$, 0.5; and $MgSO_4 \cdot 7H_2O$, 1.[22] The experiments were carried out in a fermentation medium containing (in g $L^{-1}$): glucose, 100; $NH_4Cl$, 0–6; $KH_2PO_4$, 1; $MgSO_4 \cdot 7H_2O$, 1; yeast extract, 1; $CaCO_3$, 40.[20] $CaCO_3$ was used in the fermentation medium as a buffering agent to neutralize acids, in order to overcome inhibition of citric acid secretion at lower pH. The substrate (glucose) and its initial concentration were chosen according to our previous studies (unpublished data). The media were sterilized in an autoclave at 121 °C for 15 min. Initial pH of the fermentation media were adjusted by using 1 mol $L^{-1}$ HCl.

### Equipment and fermentation conditions

Fermentations were carried out in water bath shakers using 300 mL cotton-plugged flasks containing 100 mL of fermentation medium. The yeasts were inoculated separately to fermentation media at an inoculum volume fraction of $\varphi = 5$ %. Experiments were carried out with a shaking speed of 100 strokes per min.

In the experiments in which effects of initial pH of the fermentation medium were investigated, a pH range of between 4.2–8.5 was examined. Effects of temperature ($\vartheta$) were investigated between 20–35 °C. Effects of initial ammonium chloride ($NH_4Cl$) concentration ($\gamma_{S,NH_4Cl,0}$) were investigated between the range of 0–6 g $L^{-1}$. Effects of various ions were investigated by addition of different mineral salts to the fermentation media. For this purpose, $FeSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot 4H_2O$ and $ZnSO_4 \cdot 7H_2O$ were added to the fermentation media within the concentration ranges of 0.01–0.1, 0.001–0.02, 0.005–0.05 and 0.002–0.008 g $L^{-1}$, respectively. Temperature was kept constant at 30 °C in the experiments other than the runs concerning the effects of temperature. Effects of temperature, initial $NH_4Cl$ concentration and various mineral salts were determined in the media having an initial pH of 5.2. All of the runs were performed in duplicate.

### Biomass determination

Dry biomass ($\gamma_x$) of the yeast was determined spectrophotometrically by using wet mass-absorbance and wet mass-dry mass calibration curves which had been prepared before. During the experiments, samples were taken from the fermentation media at a time interval of 24–48 hours. 6 mol $L^{-1}$ HCl was added into the samples in order to dissolve $CaCO_3$.[14] Samples were centrifuged at 5000 rpm for 25 min. Precipitate was used for determination of dry mass spectrophotometrically at $\lambda = 660$ nm (UV 210PC Shimadzu and Bausch & Lomb Spectronic 20).

### Analytical methods

Concentration of citric acid was measured spectrophotometrically by pyridine-acetic anhydride method.[3,23]

### Determining kinetic characteristics of cell growth and citric acid production

For each parameter; specific microbial growth rates of the strains ($\mu$), citric acid productivities ($\Gamma_{CA}$), maximum specific citric acid production rates ($q_{CA,max}$), maximum citric acid concentrations ($\gamma_{S,CA,max}$), maximum dry biomass ($\gamma_{x,\,max}$) and citric acid yields ($Y_{P/S_0}$) were calculated. Specific microbial growth rate for the exponential growth phase was calculated from the semi-logarithmic plot of the dry biomass data *vs*. time. Specific citric acid production rates ($q_{CA}$) were calculated from eq. (1) by using the changes in citric acid concentrations and dry biomass with time.[24]

$$q_{CA} = \frac{1}{\gamma_x} \frac{d\gamma_{S,CA}}{dt} \tag{1}$$

where $\gamma_x$ is the dry biomass of the yeast, $\gamma_{S,CA}$ is the citric acid concentration, and $t$ is time.

Maximum values of the specific citric acid production rates ($q_{CA,max}$) were also determined in the experiments.

Citric acid yields were calculated by eq. (2):[24]

$$Y_{CA/S_0} = \frac{\gamma_{S,CA,max}}{\gamma_{S_0} - \gamma_S} \cdot 100 \tag{2}$$

### Statistical analysis

Calculations of kinetic constants and rates were performed by using Origin 6.0 (Microcal Software, Inc., Northampton, MA01060, USA) program package. Equations expressing the changes of specific growth rate and citric acid production rates with pH, temperature, initial ammonium chloride or mineral salt concentration were derived from non-linear regression analysis by using the same program package.

## Results

Changes in citric acid concentration and dry biomass with time were determined during the fermentation. Specific growth rates ($\mu$) and maximum specific citric acid production rates ($q_{CA,max}$) were calculated. Temperature, pH, initial ammonium chloride and mineral salt concentrations were chosen as independent variables for specific growth rates and citric acid productivities of the cultures.

### Effects of pH

Variations in microorganism dry biomass ($m_{DBM}$) and citric acid concentrations with time at different initial pH values of the medium for *Y. lipolytica* NBRC 1658 are represented in Fig. 1. Maximum dry biomass was obtained as 20.20 g $L^{-1}$ at pH 5.2. Citric acid concentrations analyzed were high for the initial pH range of 5.2–7.0, and then decreased at pH 8.5. Citric acid production of the yeast strain was adversely affected in the medium with the initial pH of 4.2. Maximum specific growth rate was obtained when initial pH of the medium was 6.0 (Fig. 1). A relationship between the specific growth rate and initial pH of the me-

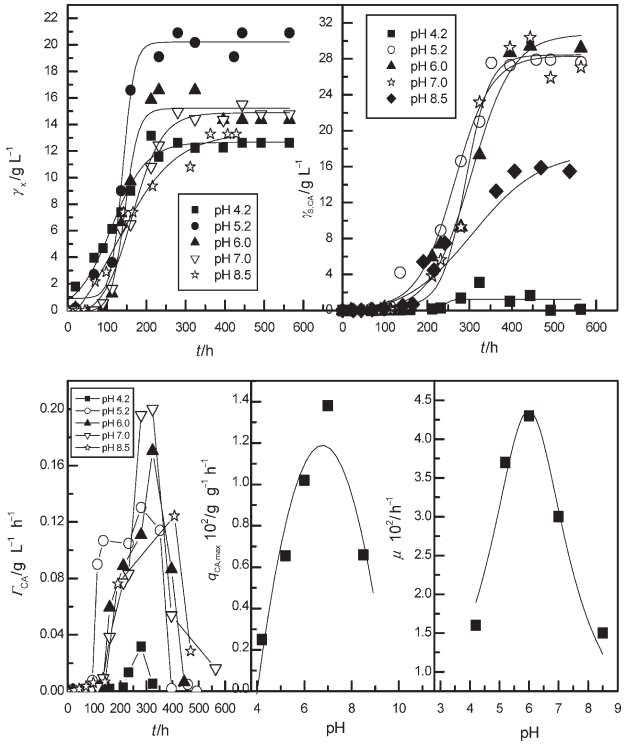



Fig. 1 – *Variations in dry biomass, citric acid concentration and productivity with time at different initial pH values and changes in maximum specific citric acid production rate and specific growth rate with initial pH of the medium for Y. lipolytica NBRC 1658 ($\gamma_{S,Go}$ = 100 g L$^{-1}$, $\vartheta$ = 30 °C, $\gamma_{S,NH_4Cl,0}$ = 2 g L$^{-1}$)*

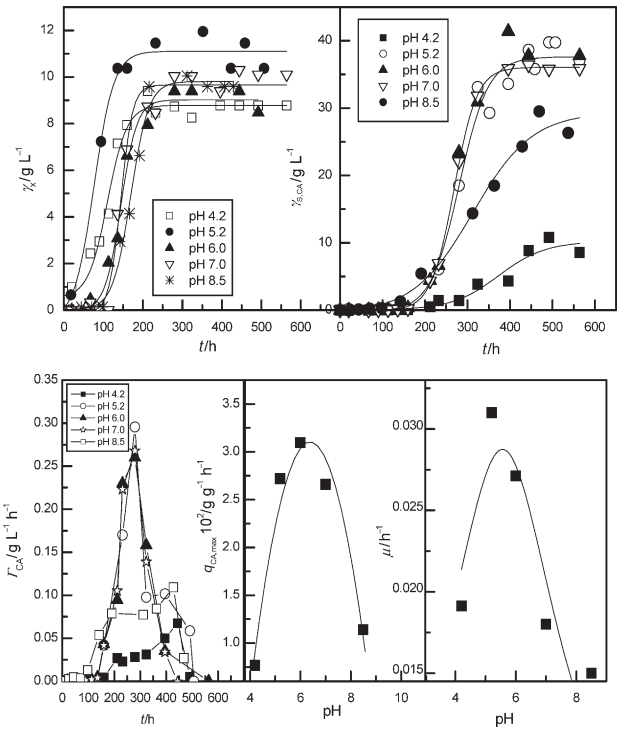



Fig. 2 – *Variations in dry biomass, citric acid concentration and productivity with time at different initial pH values and changes in maximum specific citric acid production rate and specific growth rate with initial pH of the medium for Y. lipolytica 57 ($\gamma_{S,Go}$ = 100 g L$^{-1}$, $\vartheta$ = 30 °C, $\gamma_{S,NH_4Cl,0}$ = 2 g L$^{-1}$)*

dium was derived and shown by eq. (3). It is obvious from the high $r^2$ value that the proposed equation represents the growth data reasonably well.

$$\mu = (356.3412 - 111.31735\ \mathrm{pH} + 9.2914\ \mathrm{pH}^2)^{-1} \quad (3)$$

$$r^2 = 0.964$$

For the strain NBRC 1658, maximum productivity and specific citric acid production rate were obtained at pH 7.0. Variation of maximum specific citric acid production rate with initial pH of the medium was derived by using non-linear regression analysis and expressed by eq. (4).

$$q_{\mathrm{CA,max}} = -0.05947 + 0.02108\ \mathrm{pH} - 0.00156\ \mathrm{pH}^2 \quad (4)$$

$$r^2 = 0.889$$

Effects of initial pH of the fermentation medium on the growth and citric acid production of *Y. lipolytica* 57 are represented in Fig. 2. Maximum dry biomass (11.09 g L$^{-1}$) was obtained at pH 5.2, while citric acid production was high between the initial pH range of 5.2–7.0. The domestic strain 57 reached its maximum specific growth rate in the medium having an initial pH value of 5.2. The model demonstrating the relationship between the specific growth rate and initial pH of the medium for the novel domestic yeast strain 57 are shown in eq. (5):

$$\mu = (236.2144 - 72.4172\ \mathrm{pH} + 6.5064\ \mathrm{pH}^2)^{-1} \quad (5)$$

$$r^2 = 0.793$$

Maximum productivity was obtained at pH 5.2 for the domestic strain 57 (Fig. 2). Calculated productivity values were very low in the media having the initial pH of 4.2 and 8.5. It was found that this strain has a maximum specific citric acid production rate at the initial pH 6.0. A relationship (eq. (6)) between the maximum specific citric acid production rate and initial pH of the medium was also derived for the domestic strain 57.

$$q_{\mathrm{CA,max}} = -0.15639 + 0.0587\ \mathrm{pH} - 0.0046\ \mathrm{pH}^2 \quad (6)$$

$$r^2 = 0.960$$

The effects of initial pH on maximum citric acid concentration, maximum dry biomass, product yield, maximum productivity ($\Gamma_{\mathrm{CA,max}}$), and calculated values of specific growth rate ($\mu_e$) and maximum specific citric acid production rate ($q_{\mathrm{CA,max,e}}$) from the derived equations above are shown in

Table 1 – *Effects of initial pH of the fermentation medium on maximum citric acid concentration ($\gamma_{S,CA,max}$), maximum dry biomass ($\gamma_{x,max}$), product yield ($Y_{P/S_0}$), maximum productivity ($\Gamma_{CA,max}$), and calculated values of specific growth rate ($\mu_e$) and maximum specific citric acid production rate ($q_{CA,max,e}$) from the derived equations for Y. lipolytica NBRC 1658 and Y. lipolytica 57*

| Yeast strain | pH | $\gamma_{S,CA,max}$/ g $L^{-1}$ | $\gamma_{x,max}$/ g $L^{-1}$ | $Y_{P/S_0}$/ % | $\Gamma_{CA,max}$/ g $L^{-1}$ $h^{-1}$ | $\mu_e$/ $h^{-1}$ | $q_{CA,max,e}$/ g $g^{-1}$ $h^{-1}$ |
|---|---|---|---|---|---|---|---|
| | 4.2 | 1.21 | 12.67 | 1.21 | 0.032 | 0.019 | 0.002 |
| | 5.2 | 28.45 | 20.20 | 28.45 | 0.130 | 0.035 | 0.008 |
| *Y. lipolytica* NBRC 1658 | 6.0 | 30.02 | 15.48 | 30.02 | 0.170 | 0.044 | 0.011 |
| | 7.0 | 28.43 | 14.87 | 28.43 | 0.200 | 0.031 | 0.012 |
| | 8.5 | 16.13 | 13.28 | 16.13 | 0.124 | 0.012 | 0.007 |
| | 4.2 | 9.77 | 8.78 | 9.77 | 0.067 | 0.021 | 0.009 |
| | 5.2 | 37.66 | 11.09 | 37.66 | 0.296 | 0.028 | 0.025 |
| *Y. lipolytica* 57 | 6.0 | 38.77 | 9.39 | 38.77 | 0.260 | 0.028 | 0.030 |
| | 7.0 | 36.04 | 9.65 | 36.04 | 0.268 | 0.021 | 0.029 |
| | 8.5 | 27.87 | 9.70 | 27.87 | 0.109 | 0.011 | 0.010 |

Table 1 for both strains. Although the maximum citric acid concentration and yield was obtained at pH 6.0, citric acid production was substantial between pH 5.2–7.0 for both strains. The highest value of the cell dry mass was obtained at pH 5.2 for the yeast strains examined in this study.

## Effects of temperature

Effects of temperature on growth and citric acid production of *Y. lipolytica* NBRC 1658 are shown in Fig. 3. Maximum dry biomass and citric acid concentration were obtained at 20 °C and 30 °C, respectively. Maximum citric acid concentration was $\gamma_{S,CA,\,max} = 28.45$ g $L^{-1}$ at 30 °C. When the temperature was 35 °C, citric acid production and cell growth were adversely affected. Maximum specific growth rate was obtained between 25–30 °C for this strain (Fig. 3). The model equation expressing changes of specific growth rate with temperature was derived and represented in eq. (7):

$$\mu = -0.054 + 0.007\ \vartheta - 1.37 \cdot 10^{-4} \vartheta^2 \qquad (7)$$

$$r^2 = 0.980$$

It can be observed from Fig. 3 that the maximum productivity was obtained at 25 °C and followed by 30 °C for the strain NBRC 1658. Productivities obtained at 35 °C were lower than those obtained at other temperatures investigated. However, maximum specific citric acid production rate was obtained at 35 °C, owing to considerably low dry biomass values at 35 °C. An equation that correlates $q_{CA,max}$ to $\vartheta$ was derived with a high $r^2$ value and given in eq. (8).

$$q_{CA,\,max} = 0.01456 - 0.000981\ \vartheta + 0.0000245\ \vartheta^2 \qquad (8)$$

$$r^2 = 0.977$$

Variations of cell dry biomass and citric acid concentration with time are presented in Fig. 4 for the domestic strain *Y. lipolytica* 57. Maximum dry biomass

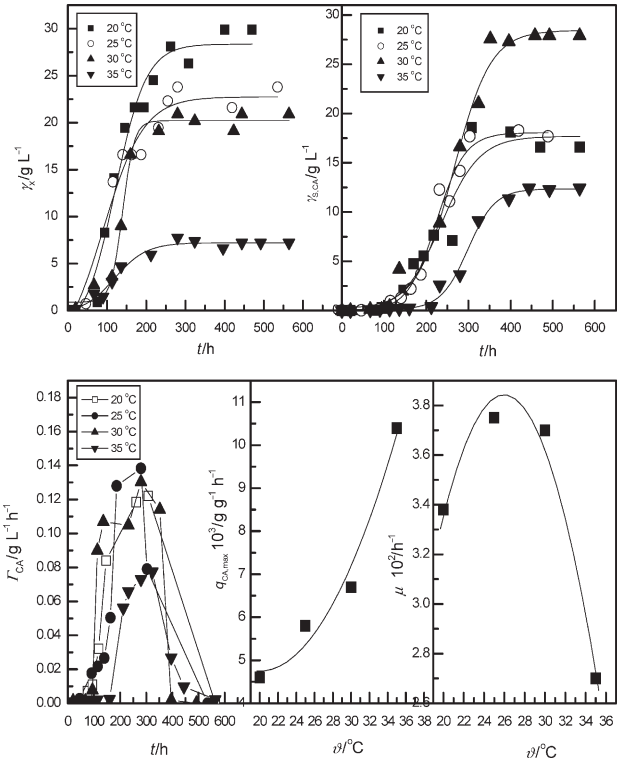



Fig. 3 – *Variations in dry biomass, citric acid concentration and productivity with time at different temperatures and changes in maximum specific citric acid production rate and specific growth rate with temperature for Y. lipolytica NBRC 1658 ($\gamma_{S,Go}$ = 100 g $L^{-1}$, initial pH 5.2, $\gamma_{S,NH_4Cl,0}$ = 2 g $L^{-1}$)*

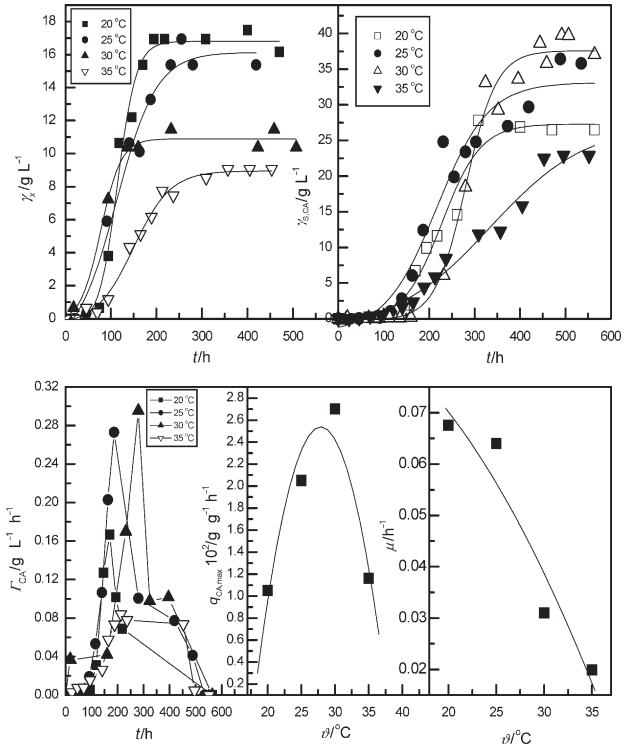



Fig. 4 – *Variations in dry biomass, citric acid concentration and productivity with time at different temperatures and changes in maximum specific citric acid production rate and specific growth rate with temperature for Y. lipolytica 57 ($\gamma_{S,Go}$ = 100 g L$^{-1}$, initial pH 5.2, $\gamma_{S,NH_4Cl,0}$ = 2 g L$^{-1}$)*

and citric acid concentration were obtained at 20 and 30 °C, respectively. Maximum citric acid concentration was obtained as $\gamma_{S,CA,max}$ = 37.66 g L$^{-1}$ at 30 °C. The equation representing the variation of specific growth rate with temperature for the strain 57 is given in eq. (9).

$$\mu = 0.088 + 6.09 \cdot 10^{-4} \vartheta - 7.51 \cdot 10^{-5} \vartheta^2 \quad (9)$$

$$r^2 = 0.922$$

Maximum productivity and specific citric acid production rate were obtained at 30 °C for the domestic strain 57. The relationship between the maximum specific citric acid production rate and temperature was derived and represented by the following equation:

$$q_{CA,max} = -0.172 + 0.0142\ \vartheta - 2.54 \cdot 10^{-4} \vartheta^2 \quad (10)$$

$$r^2 = 0.907$$

The effects of temperature on maximum citric acid concentration, maximum dry biomass, product yield, maximum productivity, and calculated values of specific growth rate ($\mu_e$) and maximum specific citric acid production rate ($q_{CA,max,e}$) from the model equations above are shown in Table 2 for both strains. Citric acid yields of the yeast strains were also maximum at 30 °C.

## Effects of initial $NH_4Cl$ concentration

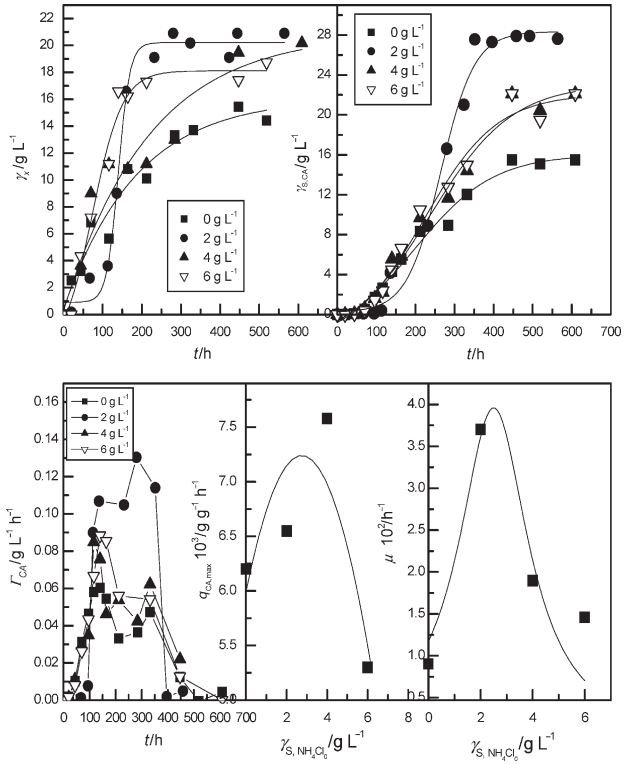
In order to determine the effects of initial $NH_4Cl$ concentration ($\gamma_{S,NH_4Cl,0}$) of the fermentation medium on the growth and citric acid production kinetics of the yeast strains, $\gamma_{S,NH_4Cl,0}$ was changed between 0–6 g L$^{-1}$. It was found that cell dry biomass of *Y. lipolytica* NBRC 1658 was increased with the addition of $NH_4Cl$ to the medium, and reached to a maximum when $\gamma_{S,NH_4Cl,0}$was 2 g L$^{-1}$ (Fig. 5). Cell growth was low in the medium without $NH_4Cl$. Maximum citric acid concentration was obtained in the medium containing $\gamma_{S,NH_4Cl,0}$ = 2 g L$^{-1}$ for this strain. Specific growth rate was correlated with $\gamma_{S,NH_4Cl,0}$ (eq. (11)) and reached to its maximum value at $\gamma_{S,NH_4Cl,0}$ = 2 g L$^{-1}$ as shown in Fig. 5.

$$\mu = (85.18 - 47.89\ \gamma_{S,NH_4Cl,0} + 9.57\ \gamma^2_{S,NH_4Cl,0})^{-1} \quad (11)$$

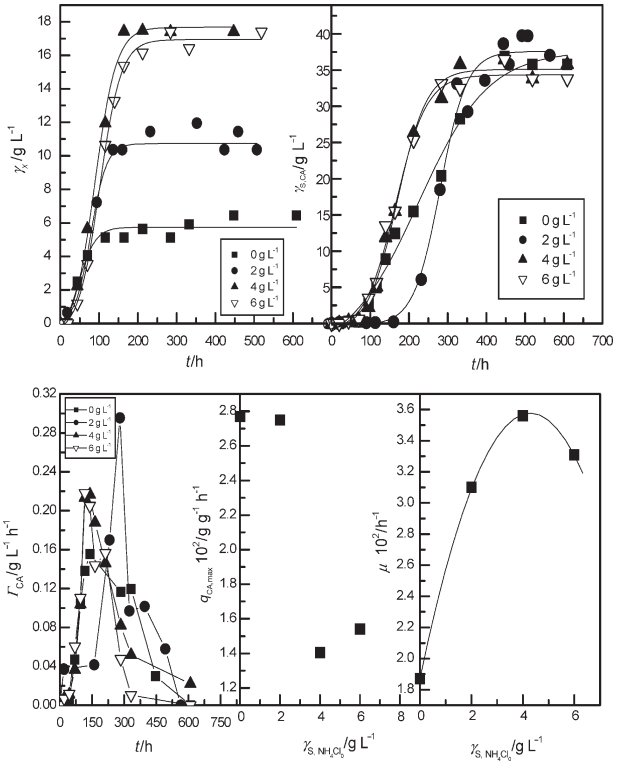
$$r^2 = 0.837$$

Table 2 – *Effects of temperature on maximum citric acid concentration ($\gamma_{S,CA,max}$), maximum biomass concentration ($\gamma_{x,max}$), product yield ($Y_{P/S_0}$), maximum productivity ($\Gamma_{CA,max}$), and calculated values of specific growth rate ($\mu_e$) and maximum specific citric acid productivity ($q_{CA,max,e}$) from the derived equations for Y. lipolytica NBRC 1658 and Y. lipolytica 57*

| Yeast strain | $\vartheta$/°C | $\gamma_{S,CA,max}$/ g L$^{-1}$ | $\gamma_{x,max}$/ g L$^{-1}$ | $Y_{P/S_0}$/ % | $\Gamma_{CA,max}$/ g L$^{-1}$ h$^{-1}$ | $\mu_e$/ h$^{-1}$ | $q_{CA,max,e}$/ g g$^{-1}$ h$^{-1}$ |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* NBRC 1658 | 20 | 17.66 | 28.33 | 17.66 | 0.122 | 0.031 | 0.005 |
| | 25 | 18.03 | 22.72 | 18.03 | 0.138 | 0.035 | 0.005 |
| | 30 | 28.45 | 20.20 | 28.45 | 0.130 | 0.033 | 0.007 |
| | 35 | 12.34 | 7.21 | 12.34 | 0.077 | 0.023 | 0.010 |
| *Y. lipolytica* 57 | 20 | 27.30 | 16.79 | 27.30 | 0.167 | 0.070 | 0.010 |
| | 25 | 33.00 | 16.10 | 33.00 | 0.273 | 0.056 | 0.024 |
| | 30 | 37.66 | 11.09 | 37.66 | 0.296 | 0.039 | 0.025 |
| | 35 | 23.83 | 8.81 | 23.83 | 0.024 | 0.017 | 0.014 |




F i g . 5 – *Variations in dry biomass, citric acid concentration and productivity with time at different initial ammonium chloride concentrations ($\gamma_{S,NH_4Cl,0}$) and changes in maximum specific citric acid production rate and specific growth rate with $\gamma_{S,NH_4Cl,0}$ for Y. lipolytica NBRC 1658 ($\gamma_{S,Go}$ = 100 g L$^{-1}$, initial pH 5.2, $\vartheta$ = 30 °C)*




F i g . 6 – *Variations in dry biomass, citric acid concentration and productivity with time at different initial ammonium chloride concentrations ($\gamma_{S,NH_4Cl,0}$) and changes in maximum specific citric acid production rate and specific growth rate with $\gamma_{S,NH_4Cl,0}$ for Y. lipolytica 57 ($\gamma_{S,Go}$ = 100 g L$^{-1}$, initial pH 5.2, $\vartheta$ = 30 °C)*

Maximum productivity was also obtained in the medium containing $\gamma_{S,NH_4Cl,0} = 2$ g L$^{-1}$ for NBRC 1658 strain. The highest specific citric acid production rate was calculated in the medium containing $\gamma_{S,NH_4Cl,0} = 4$ g L$^{-1}$ (Fig. 5). Variation of maximum specific citric acid production rate with $\gamma_{S,NH_4Cl,0}$ was derived, and given in eq. (12).

$$q_{CA,max} = 0.006 + 9.03 \cdot 10^{-4} \gamma_{S,NH_4Cl,0} - 1.64 \cdot 10^{-4} \gamma^2_{S,NH_4Cl,0} \quad (12)$$

$$r^2 = 0.701$$

Changes of microorganism dry biomass and citric acid concentration with time at different $\gamma_{S,NH_4Cl,0}$ for the domestic strain 57 are presented in Fig. 6. The best result for the cell growth was obtained in the medium containing $\gamma_{S,NH_4Cl,0} = 4$ g L$^{-1}$. Maximum values for yeast dry biomass and specific growth rate were determined in this medium. Maximum citric acid concentrations obtained were approximately the same between the initial $NH_4Cl$ concentration range of 0–6 g L$^{-1}$. Specific growth rate was correlated with the initial $NH_4Cl$ concentration and represented by a second-order polynomial function of $\gamma_{S,NH_4Cl,0}$ (eq. (13)). High $r^2$ value demonstrated that the derived model adequately fits the experimental data.

$$\mu = 0.01873 + 0.00794 \gamma_{S,NH_4Cl\,i,0} - 9.256 \cdot 10^{-4} \gamma^2_{S,NH_4Cl\,i,0} \quad (13)$$

$$r^2 = 0.999$$

For the domestic *Y. lipolytica* strain 57, maximum productivity was obtained in the medium containing $\gamma_{S,NH_4Cl,0} = 2$ g L$^{-1}$ (Fig. 6). Although there was no significant change in maximum specific citric acid production rate between the initial concentration ranges of $\gamma_{S,NH_4Cl,0} = 0$–2 g L$^{-1}$ and 4–6 g L$^{-1}$, a considerable decrease was observed between $\gamma_{S,NH_4Cl,0} = 2$–4 g L$^{-1}$. Changes of maximum citric acid concentration, maximum dry biomass, product yield, maximum productivity, and calculated values of specific growth rate ($\mu_e$) and maximum specific citric acid production rate ($q_{CA,max,e}$) with $\gamma_{S,NH_4Cl,0}$ are shown in Table 3 for both strains. It can be observed that the best results for citric acid production were obtained with the initial $NH_4Cl$ concentration of 2 g L$^{-1}$ in the fermentation medium. The maximum values of the productivity and citric acid yield

Table 3 – *Effects of initial ammonium chloride concentration ($\gamma_{S,NH_4Cl,0}$) on maximum citric acid concentration ($\gamma_{S,CA,max}$), maximum dry biomass ($\gamma_{x,max}$), product yield ($Y_{P/S_0}$), maximum productivity ($\Gamma_{CA,max}$), and calculated values of specific growth rate ($\mu_e$) and maximum specific citric acid production rate ($q_{CA,max,e}$) from the derived equations for Y. lipolytica NBRC 1658 and Y. lipolytica 57*

| Yeast strain | $\gamma_{S,NH_4Cl,0}$/ g $L^{-1}$ | $\gamma_{S,CA,max}$/ g $L^{-1}$ | $\gamma_{x,max}$/ g $L^{-1}$ | $Y_{P/S_0}$/ % | $\Gamma_{CA,max}$/ g $L^{-1}$ $h^{-1}$ | $\mu_e$/ $h^{-1}$ | $q_{CA,max,e}$/ g $g^{-1}$ $h^{-1}$ |
|---|---|---|---|---|---|---|---|
| *Y. lipolytica* NBRC 1658 | 0 | 15.72 | 15.18 | 15.72 | 0.060 | 0.012 | 0.006 |
| | 2 | 28.45 | 20.20 | 28.45 | 0.130 | 0.036 | 0.007 |
| | 4 | 22.26 | 19.69 | 22.26 | 0.085 | 0.021 | 0.007 |
| | 6 | 21.70 | 18.11 | 21.70 | 0.088 | 0.007 | 0.005 |
| *Y. lipolytica* 57 | 0 | 36.99 | 5.74 | 36.99 | 0.155 | 0.019 | [a] |
| | 2 | 37.66 | 11.09 | 37.66 | 0.296 | 0.031 | [a] |
| | 4 | 35.78 | 17.67 | 35.78 | 0.217 | 0.036 | [a] |
| | 6 | 34.34 | 16.93 | 34.34 | 0.218 | 0.033 | [a] |

[a]The experimental data did not fit any model adequately by non-linear regression analysis

were also obtained with the same initial $NH_4Cl$ concentration.

## Effects of different minerals

In order to determine the effects of minerals on the growth and citric acid production kinetics of the yeast strains, some mineral salts were added into the fermentation medium at different concentrations. When $FeSO_4 \cdot 7H_2O$ was used in the medium as a source of iron, an inhibition was observed in citric acid production for both of the strains. Citric acid concentrations were obtained in trace amounts in those media containing the iron salt (data not shown). It was found that growth of the strains was positively affected by addition of the iron salt to the medium. When compared to the media without minerals, shorter lag phases and higher cell dry biomass were obtained in the media containing iron. Specific growth rate of the strains increased by the addition of $FeSO_4 \cdot 7H_2O$ to the fermentation medium (Table 4). There was not a significant change in specific growth rate within 0.01–0.1 g $L^{-1}$ of the initial $FeSO_4 \cdot 7H_2O$ concentration ($\gamma_{S,Fe}$) for the strain NBRC 1658. Maximum specific growth rate was obtained when $\gamma_{S,Fe}$ was 0.05 g $L^{-1}$ for the domestic strain 57.

It was obtained that an insignificant amount of citric acid was produced by both yeast strains when $CuSO_4 \cdot 5H_2O$ was used in the fermentation medium as a mineral salt at different concentrations (data not shown). In all of these runs, a considerable decrease in the time for lag phase was observed for strain NBRC 1658. There was also not a significant change in maximum values of the dry biomass of the yeast. It was observed that the specific growth rate of this strain was not changed between 0–0.001 g $L^{-1}$ of the initial $CuSO_4 \cdot 5H_2O$ concentration ($\gamma_{S,Cu}$) and then decreased above this concentration range (Table 4). An increase was observed in cell dry biomass of *Y. lipolytica* 57, especially in the medium containing $\gamma_{S,Cu} = 0.01$ g $L^{-1}$. However, specific growth rate of this strain decreased by increasing $\gamma_{S,Cu}$.

In the experiments in which $MnSO_4 \cdot 4H_2O$ was used, citric acid production decreased by the addition of the salt into the fermentation media, but maximum citric acid concentrations were higher than those obtained in the media containing iron or copper. The maximum citric acid concentration obtained for the strain NBRC 1658 was 28.45 g $L^{-1}$ in the medium without this salt. It was 11.38, 11.72 and 14.25 g $L^{-1}$ in the media containing 0.005, 0.02 and 0.05 g $L^{-1}$ of manganese sulphate, respectively for the same yeast strain (Fig. 7a). Productivity (Fig. 7c) and specific citric acid production rate were also decreased by the addition of this salt to the medium (Table 4). Maximum specific growth rate of the yeast was obtained in the medium containing 0.02 g $L^{-1}$ manganese sulphate. Changes of specific growth rate of the yeast strain NBRC 1658 with initial manganese sulphate concentration ($\gamma_{S,Mn}$) of the fermentation medium was represented in Fig. 7g, and given by the following equation:

$$\mu = 0.0378 + 1.484\,\gamma_{S,Mn} - 35.313\,\gamma^2_{S,Mn} \quad (14)$$

$$r^2 = 0.995$$

It was determined that citric acid production by *Y. lipolytica* 57 was reduced with the supplement of manganese into the fermentation medium (Fig. 7b). Maximum citric acid concentration, productivity (Fig. 7d) and specific citric acid production rate values obtained for all initial manganese sulphate concentrations examined were lower than that of

Table 4 – *Variations of specific growth rate and maximum specific citric acid production rates of Y. lipolytica NBRC 1658 and Y. lipolytica 57 with initial mineral salt concentration of the fermentation media containing different minerals*

| Mineral salt and concentration | $\mu$/h$^{-1}$ | | $q_{CA,max}$ /g g$^{-1}$ h$^{-1}$ | |
|---|---|---|---|---|
| | *Y. lipolytica* NBRC 1658 | *Y. lipolytica* 57 | *Y. lipolytica* NBRC 1658 | *Y. lipolytica* 57 |
| $FeSO_4$, $\gamma$ /g L$^{-1}$ | | | | |
| 0 | 0.037 | 0.031 | 0.008 | 0.027 |
| 0.01 | 0.048 | 0.032 | – | – |
| 0.05 | 0.047 | 0.044 | – | – |
| 0.10 | 0.047 | 0.034 | – | – |
| $CuSO_4$, $\gamma$ /g L$^{-1}$ | | | | |
| 0 | 0.037 | 0.031 | 0.008 | 0.027 |
| 0.001 | 0.037 | 0.017 | – | – |
| 0.010 | 0.017 | 0.017 | – | – |
| 0.020 | 0.019 | 0.015 | – | – |
| $MnSO_4$, $\gamma$ /g L$^{-1}$ | | | | |
| 0 | 0.037 | 0.031 | 0.008 | 0.027 |
| 0.005 | 0.046 | 0.030 | 0.004 | 0.013 |
| 0.020 | 0.053 | 0.034 | 0.003 | 0.015 |
| 0.050 | 0.024 | 0.020 | 0.003 | 0.014 |
| $ZnSO_4$, $\gamma$ /g L$^{-1}$ | | | | |
| 0 | 0.037 | 0.031 | 0.008 | 0.027 |
| 0.002 | 0.049 | 0.044 | 0.003 | 0.024 |
| 0.004 | 0.049 | 0.045 | 0.004 | 0.024 |
| 0.008 | 0.031 | 0.039 | 0.003 | 0.024 |

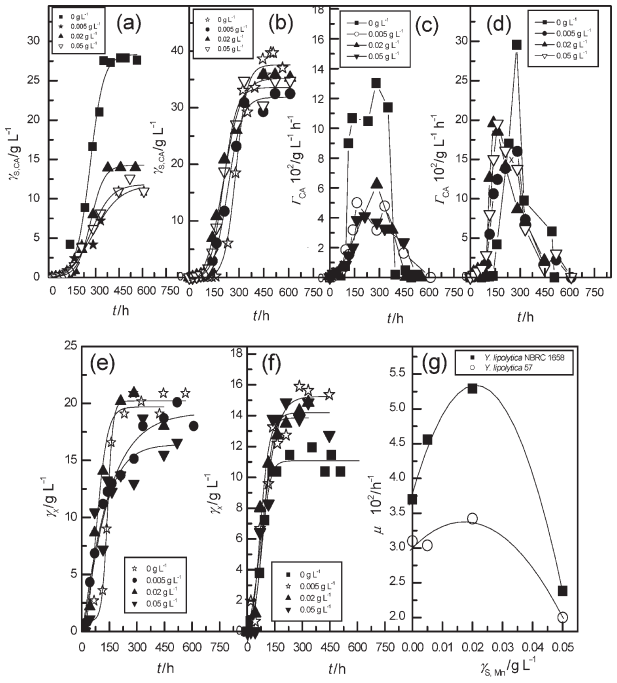


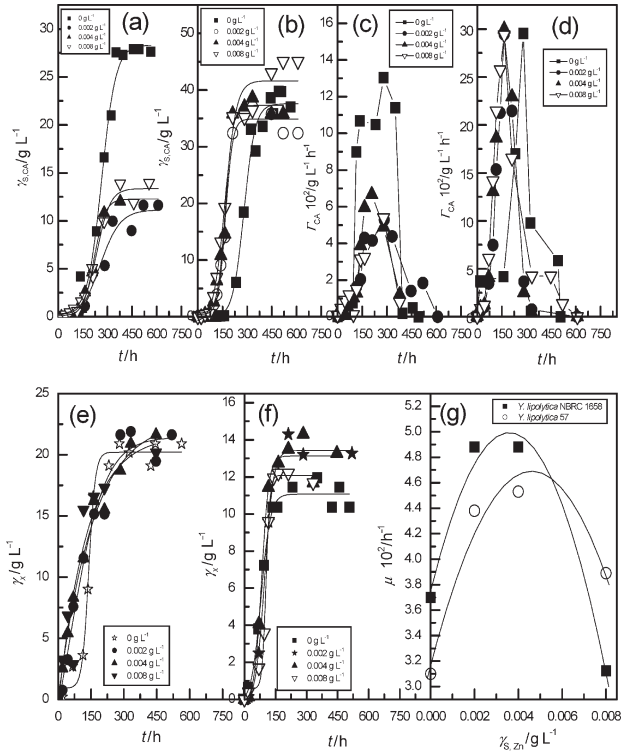

Fig. 7 – *Variations in citric acid concentration, productivity and dry biomass with time at different initial $MnSO_4 \cdot 4H_2O$ concentrations ($\gamma_{S,Mn}$) and changes in specific growth rate with $\gamma_{S,Mn}$ for the yeast strains (a, c, e, g: Y. lipolytica NBRC 1658, b, d, f, g: Y. lipolytica 57, $\gamma_{S,Go} = 100$ g L$^{-1}$, initial pH 5.2, $\vartheta = 30$ °C, $\gamma_{S,NH_4Cl,0} = 2$ g L$^{-1}$)*

the results obtained in the medium without minerals (Table 4). Growth of strain 57 was positively affected by the addition of $MnSO_4$ to the fermentation medium (Fig. 7f). Maximum cell dry biomass values obtained in the media containing this salt were higher than that of in the medium without the salt. Maximum specific growth rate was obtained in the medium containing $\gamma_{S,Mn} = 0.02$ g L$^{-1}$. Specific growth rate was derived as a function of $\gamma_{S,Mn}$ by non-linear regression analysis (eq. (15)).

$$\mu = 0.0299 + 0.4397\ \gamma_{S,Mn} - 12.74\ \gamma^2_{S,Mn} \quad (15)$$

$$r^2 = 0.969$$

For determining the effects of zinc on growth and citric acid production of the yeast strains, $ZnSO_4 \cdot 7H_2O$ was added to the fermentation media at different concentrations. For *Y. lipolytica* NBRC 1658, no significant change was observed in cell dry biomass by the addition of the salt into the fermentation medium (Fig. 8e). The most important effect of zinc on the growth of this strain was the decrease of the time for the lag phase. Citric acid production by this strain was adversely affected in the media containing $ZnSO_4 \cdot 7H_2O$ (Fig. 8a). Maximum citric acid concentrations were changed be-




F i g . 8 – *Variations in citric acid concentration, productivity and dry biomass with time at different initial $ZnSO_4 \cdot 7H_2O$ concentrations ($\gamma_{S,Zn}$) and changes in specific growth rate with $\gamma_{S,Zn}$ for the yeast strains (a, c, e, g: Y. lipolytica NBRC 1658, b, d, f, g: Y. lipolytica 57, $\gamma_{S,Go}$ = 100 g L$^{-1}$, initial pH 5.2, $\vartheta$ = 30 °C, $\gamma_{S,NH_4Cl,0}$ = 2 g L$^{-1}$)*

tween 11.10–13.36 g L$^{-1}$ in those media. Considerable decreases in citric acid productivity (Fig. 8c) and $q_{CA,max}$ (Table 4) were observed when the medium was supplemented with zinc sulphate. Maximum specific growth rate (Fig. 8g) was obtained between 0.002–0.004 g L$^{-1}$ initial $ZnSO_4 \cdot 7H_2O$ concentration ($\gamma_{S,Zn}$). The derived equation expressing variation of specific growth rate with $\gamma_{S,Zn}$ was given in eq. (16).

$$\mu = 0.0375 + 6.943\ \gamma_{S,Zn} - 967.898\ \gamma^2_{S,Zn} \quad (16)$$

$$r^2 = 0.988$$

It was determined that zinc had a positive effect on both the growth (Figs. 8f and g) and citric acid (Fig. 8b) production of *Y. lipolytica* 57. Cell dry biomass increased by the addition of $ZnSO_4 \cdot 7H_2O$ to the medium. There was not a significant change in maximum citric acid concentration between $\gamma_{S,Zn}$ = 0–0.004 g L$^{-1}$ by this strain. However, an increase in citric acid production was observed in the medium containing $\gamma_{S,Zn}$ = 0.008 g L$^{-1}$. Maximum citric acid concentration obtained in this medium was 41.63 g L$^{-1}$, while it was 37.66 g L$^{-1}$ in the medium without minerals for the domestic strain 57. It can be observed from Fig. 8 that citric acid production in the medium containing zinc sulphate begins approximately 100 h earlier than that in the medium without the mineral salt. In all of the used media, similar maximum productivity values were obtained (Fig. 8d). Specific citric acid production rate decreased by the addition of zinc sulphate to the medium, but did not change between $\gamma_{S,Zn}$ = 2–8 mg L$^{-1}$ (Table 4). The model equation derived for change of specific growth rate of the domestic yeast strain 57 with $\gamma_{S,Zn}$ was represented in eq. (17).

$$\mu = 0.0317 + 6.608\ \gamma_{S,Zn} - 716.477\ \gamma^2_{S,Zn} \quad (17)$$

$$r^2 = 0.956$$

In this research, the maximum citric acid production (41.63 g L$^{-1}$) was determined by the domestic strain in the medium containing 0.008 g L$^{-1}$ of zinc salt. The main disadvantage of citric acid production with *Y. lipolytica* is the simultaneous production of the metabolite isocitric acid, the production of which varies with strain and carbon source.[10] In this study, isocitric acid production of *Y. lipolytica* 57 was also followed (data not shown) in a medium containing glucose, $NH_4Cl$ and zinc sulphate ($\gamma_{S,Zn}$ = 0.008 g L$^{-1}$). In the experiment, optimum values of the nutrient concentrations and culture conditions were used ($\gamma_{S,G0}$ = 150 g L$^{-1}$, $\gamma_{S,NH_4Cl_0}$ = 2 g L$^{-1}$, initial pH of the medium 5.2, and $\vartheta$ = 30 °C), and the ratio of citric acid to isocitric acid was obtained as 13.32.

## Discussion

In this study, growth and citric acid production characteristics of two *Y. lipolytica* strains were investigated comparatively at different fermentation conditions. In the experiments demonstrating the effects of pH, maximum specific growth rate was obtained at pH 6.0 and 5.2 for the NBRC 1658 and domestic 57 strains, respectively. Citric acid production was maximum in the range of pH 5.2–7.0 for both strains. A considerable decrease in citric acid production was observed below or above this range. It is known that hydrogen ion concentration influences specific growth rate as a result of its direct effect on enzyme activities of the cell. It is also reported that optimum pH for growth can be different from the required pH for product formation.[24] When working with yeasts, initial pH values above 5 should be used since citric acid production is adversely affected below pH 5.[25] It is reported that citric acid concentration decreases below pH 5 according to the accumulation of some polyalcohols like erytrithol, arabitol and mannitol, instead of citric acid.[25,26] Adverse effect of low pH is also explained by inhibition of citrate production in the

cell and transport of citrate from cell membrane. In a study performed with *C. oleophila*, the effect of pH on citric acid release by specific active transport system was investigated in a continuous system.[18] It was demonstrated that active citric acid transport system was a pH-dependent mechanism. In the same study, it was also reported that growth of the yeast, composition of biomass and citric acid release were directly affected by pH and maximum citric acid concentration was obtained at pH 5. In a study performed by Rane and Sims,[15] effects of initial pH on growth of the strains of *C. lipolytica, Y. lipolytica* and *C. guilliermondii* were investigated. Over the range of pH 2.4–4.8, it was reported that the amount of biomass produced did not appear to be affected by initial pH, although it was indicated that this effect was strain dependent. In the same study, initial pH of the fermentation medium for citric acid production was adjusted to 5.5. It is known that the effects of medium pH on citric acid production by yeasts and molds are considerably different from each other.[25] In a study performed with *Aspergillus niger*, effect of initial pH on citric acid production was investigated in date syrup medium.[27] It was reported that citric acid concentration, citric acid yield and sugar utilization decreased slightly with an increase in pH up to 4.5. On the other hand, the biomass remained almost constant over the pH range of 3.5–6.5.[27] In another study, citric acid production by *Aspergillus niger* in pretreated beet molasses was investigated.[12] Optimum values of the fermentation parameters; initial pH, aeration flow rate and temperature were determined and optimum pH for citric acid production was reported as 4.0. The effect of the fermentation medium pH on the efficiency of two strains of *A. niger* to utilize whey for the production of citric acid was investigated by El-Samragy *et al*.[28] The maximum citric acid concentration and conversion coefficient were obtained at pH 3.5 after 9 days of fermentation.[28] In the present study, according to the obtained results, initial pH of the fermentation medium was adjusted to 5.2 in the experiments concerning the effects of other fermentation parameters. It should be regarded that fermentation experiments running under pH control would be better to find out the real optimum pH for growth and production, to be done in a fermenter as a further study.

One of the most important operational parameters in a citric acid production process is temperature. Effects of temperature on growth and citric acid production kinetics of the two yeast strains were examined in this study. The highest cell dry biomass was obtained at 20 °C for both strains. Cell growth and citric acid production were both low at 35 °C. Optimum temperature for citric acid production of both the yeast strains was obtained as 30 °C. Specific growth rate and citric acid production rates were modelled as functions of temperature and expressed in polynomial form. It is known that optimum temperatures for growth of cells and product formation may be different in some fermentation processes.[24] Optimum temperature for citric acid production may change relative to the used strain and medium conditions. Temperature reported in various researches on citric acid production by yeasts was between 22–35 °C. It is also quite deliberated that determination of the optimum temperature in a batch system is necessary before beginning large scale productions.[14,29] In a study performed with *C. lipolytica*, optimum temperatures for citric acid production in the media containing glucose, fructose, glycerol, molasses or some other substrates were reported as 28–30 °C,[20] which is similar to the results of our study. In another study of citric acid production by *C. lipolytica*, optimum levels of some fermentation parameters were investigated. It was reported that maximum 9.8 g $L^{-1}$ citric acid was obtained at optimal conditions, among which temperature was 26–30 °C.[14] In a study by Lotfy *et al*., estimated optimum fermentation temperature for the production of citric acid by *A. niger* in pretreated beet molasses was reported as 31.5 °C.[12]

Ammonium chloride was used as a nitrogen source in the fermentation medium for this study. Growth of cell and dry biomass of the yeast strains were low in the medium without $NH_4Cl$. Addition of $NH_4Cl$ into the fermentation medium at certain amounts increased both growth and citric acid production of the both strains. The highest citric acid concentration and productivity were obtained at $\gamma_{S,NH_4Cl_0} = 2$ g $L^{-1}$ for both yeast strains. A decrease in citric acid production was observed especially for *Y. lipolytica* NBRC 1658 above the optimum initial $NH_4Cl$ concentration.

Citric acid is known as a seconder metabolite produced at the end of the logarithmic phase under nutrient-limited conditions.[11,30] It was reported that citric acid production began after depletion of nitrogen source in the medium.[31,32] For this reason, the source of nitrogen and its concentration has a primary role on citric acid production, and high concentrations of nitrogen compounds may have negative effects on citric acid production rate. The decrease in citric acid concentration obtained in this study especially at high initial $NH_4Cl$ concentrations may be caused by the delay in the initiation of citric acid production and decceleration of production rate of the yeast strains. In a research performed by *Candida lipolytica*, effects of different nitrogen sources on citric acid production were investigated and the best results were obtained with $NH_4Cl$.[20] In the same study, it was reported that the

experiments were carried out using 0 to 4 g $L^{-1}$ of $\gamma_{S,NH_4Cl,0}$, and the highest citric acid production was obtained at $\gamma_{S,NH_4Cl,0}$ = 1.5 g $L^{-1}$. In some other studies, 2 g $L^{-1}$ of $NH_4Cl$ was used in the composition of the fermentation media.[36] Rane and Sims reported that a cell yield of 0.20 g $g^{-1}$ was obtained even at 0 g $L^{-1}$ nitrogen concentration, in a fermentation medium containing 100 g $L^{-1}$ initial glucose concentration. In the same research, at 0.070 g $L^{-1}$ and 0.017 g $L^{-1}$ nitrogen concentrations, citric acid yield was obtained as 0.64 and 0.67 g $g^{-1}$, respectively.[22]

When different mineral salts were used in the fermentation medium at different concentrations, growth and citric acid production of the strains were quite affected. It was found that iron and copper inhibited citric acid production for the used concentrations, while cell growth was positively affected by the addition of these minerals. When manganese salt was used, a considerable adverse effect on citric acid production was observed especially for the strain NBRC 1658 (Fig. 7a). By the addition of zinc sulphate to the fermentation medium, there was no significant change in the dry biomass of strain NBRC 1658 (Fig. 8e), while a slight increase was determined in the dry biomass of the domestic strain (Fig. 8f). Citric acid production of the two strains were differently affected by zinc sulphate. Maximum citric acid concentration obtained by using the yeast strain NBRC 1658 was decreased in the media containing $ZnSO_4$ when compared with the medium without the salt. For the domestic strain 57, the highest value of the maximum citric acid concentration was obtained in the medium consisting $\gamma_{S,Zn}$ = 0.008 g $L^{-1}$. It is well known that various trace elements and their concentrations strongly influence cell growth and citric acid biosynthesis in yeasts and fungi.[5,17] It was reported that fermentation medium should contain metal ions especially manganese, iron and zinc at required amounts for inducing cell growth to obtain high citric acid yields.[34] It is reported in several studies that fermentation media used for citric acid production contain one or more mineral salts.[11,35–37] Iron, copper, manganese or zinc salts were added into the fermentation media in these studies. Initial concentrations of the salts were similar to those in this study, and high citric acid yields were reported. It is also reported that there is an elevated production of citric acid only if a rigorous control of the trace elements availability is accomplished, mainly in the submerged process.[5] In a study performed by Finogenova *et al*., in a medium containing ethanol, and without nitrogen limitation, an intracellular iron fraction of $w$ = 0.1 mg $g^{-1}$ limited cell growth; in this case, the level of biomass was low (3.9 g $L^{-1}$) and the synthesis of citric acid and isocitric acid was completely supressed in continuous system.[17] In the same study, it is reported that under zinc limitation, biomass was relatively low (5.5 g $L^{-1}$) and citric acid production was suppressed. Kamzolova *et al*. reported that oxygen requirements for growth and citric acid synthesis of *Y. lipolytica* were found to depend on the iron concentration of the medium.[38] At low iron concentration (0.7 mg $L^{-1}$), the biomass content increased from 2.8 to 6.9 g $L^{-1}$, citric acid production increased from 0 to 18.2 g $L^{-1}$ with increasing dissolved oxygen concentration. It was indicated that yeast growth at low iron concentrations was limited by energy supply and that energy limitation might be reversed by an increase in either oxygen or iron limitation.[38] In another study, the effects of glucose, potassium dihydrogen phosphate, magnesium sulphate and copper sulphate concentrations on citric acid yield of *Y. lipolytica* were analysed by multiple regression techniques.[39] With a low glucose concentration of 50 g $L^{-1}$ and 8 mg $L^{-1}$ of copper ions, the citric acid yield reached its highest value of 0.64 g $g^{-1}$. In the present study, different effects of mineral salts on citric acid production of the strains were obtained. While non of the mineral salts were determined as suitable for citric acid production by NBRC 1658 strain, $ZnSO_4$ was demonstrated as a mineral salt enhancing citric acid production by the domestic strain 57. It was reported that the effects of minerals on citric acid production might be variable according to medium composition and strain.[29,34] The different results in this research can be considered as an outcome of the strain variety.

The kinetic data obtained in the experiments give an idea about the optimization of the culture conditions in a possible citric acid production process for scale-up. Besides the effects of fermentation parameters, this study also demonstrated citric acid production characteristics of a novel domestic *Y. lipolytica* strain in comparison with a citric acid producer. The study also covers a strain screening and an identification step for selection of this strain. It was found that citric acid production of the domestic strain was higher than that of NBRC 1658 in all of the used media. Citric acid production properties of this strain were investigated for the first time in this study, introducing a novel citric acid producer. The results may be adapted to further studies for enhancing citric acid production of *Y. lipolytica* domestic strain 57 in different media and performing large scale productions by using this novel strain.

## Conclusions

A comparative study was performed for determination of growth and citric acid production kinetics of two *Y. lipolytica* strains at different fermentation conditions.

Citric acid production reached a maximum at initial pH between 5.2–7.0, 30 °C and in the medium containing $\gamma_{S,NH_4Cl_0} = 2$ g L$^{-1}$ for both of the strains.

In the media containing iron or copper salts, high cell dry biomass and/or short period of lag phase were obtained, while citric acid production was inhibited for both strains. Citric acid production by the domestic strain 57 increased with the addition of zinc salt to the fermentation medium and maximum citric acid concentration was obtained in the medium containing $\gamma_{S,Zn} = 0.008$ g L$^{-1}$.

This study demonstrated citric acid production characteristics of a novel domestic *Y. lipolytica* strain in comparison with a citric acid producer. Results of this study may serve as new findings to introduce a novel strain for industrial uses.

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## List of symbols

$q_{CA}$ – specific citric acid production rate, g g$^{-1}$ h$^{-1}$
$r^2$ – determination coefficient
$t$ – time, h
$w$ – mass fraction, mg g$^{-1}$
$Y_{P/S_0}$ – citric acid yield, %
$\gamma_S$ – substrate concentration, g L$^{-1}$
$\gamma_{S_0}$ – initial substrate concentration, g L$^{-1}$
$\gamma_{S,CA}$ – citric acid concentration, g L$^{-1}$
$\gamma_{S,CA,max}$ – maximum citric acid concentration, g L$^{-1}$
$\gamma_{S,Cu}$ – initial concentration of copper sulphate, g L$^{-1}$
$\gamma_{S,Fe}$ – initial concentration of iron sulphate, g L$^{-1}$
$\gamma_{S,Go}$ – initial concentration of glucose, g L$^{-1}$
$\gamma_{S,Mn}$ – initial concentration of manganese sulphate, g L$^{-1}$
$\gamma_{S,NH_4Cl_0}$ – initial concentration of ammonium chloride, g L$^{-1}$
$\gamma_{S,Zn}$ – initial concentration of zinc sulphate, g L$^{-1}$
$\gamma_x$ – dry biomass, g L$^{-1}$
$\gamma_{x,max}$ – maximum dry biomass, g L$^{-1}$
$\Gamma_{CA}$ – citric acid productivity, g L$^{-1}$ h$^{-1}$
$\Gamma_{CA,max}$ – maximum productivity, g L$^{-1}$ h$^{-1}$
$\lambda$ – wavelength, nm
$\mu$ – specific growth rate, h$^{-1}$
$\mu_e$ – calculated value of the specific growth rate from the model, h$^{-1}$
$q_{CA}$ – specific citric acid production rate, g g$^{-1}$ h$^{-1}$
$q_{CA,max}$ – maximum specific citric acid production rate, g g$^{-1}$ h$^{-1}$
$q_{CA,max,e}$ – calculated value of the maximum specific citric acid production rate from the model, g g$^{-1}$ h$^{-1}$
$\vartheta$ – temperature, °C
$\varphi$ – volume fraction, %

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