A comparative study of the effects of glycerol and mannitol on citric acid production by two Yarrowia lipolytica strains

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Abstract
In this study, Y. lipolytica NBRC 1658 and a novel domestic strain Y. lipolytica 57 were used for citric acid production from two different substrates; glycerol and mannitol, in a batch system. In each substrate media, the growth was defined by the non-competitive substrate inhibition model for both of the strains. Maximum citric acid concentration obtained by the domestic strain was higher than that of NBRC 1658 in glycerol medium. The best results for productivity and maximum specific citric acid production rate were obtained at 120 g/L initial glycerol concentration for NBRC 1658, and at 160 g/L initial glycerol concentration for the domestic strain. In the medium containing mannitol, maximum citric acid concentration and productivity were obtained at 120 g/L initial mannitol concentration for both of the strains. The study gave promising data for gaining a novel citric acid producer, Y. lipolytica 57, determining substrate profiles of the used strains for citric acid production.

Keywords: citric acid, yeast, Yarrowia lipolytica, glycerol, mannitol.

Introduction
Citric acid is widely used in a great number of applications, ranging from the food processing to the pharmaceutical industry and to the other areas of chemical industry [1]. Due to its distinctive properties as an acidifier, flavouring agent and antioxidant, citric acid is used mainly in food and beverage industry [2].

Citric acid is an intermediate of tricarboxylic acid cycle and holds a key position in the metabolism of each microbial cell. However, under certain conditions of fermentation, fungi and yeasts produce citric acid in excessive amounts. Traditionally, the different strains of fungus, mostly belonging to Aspergillus niger, have been used in the commercial production of citric acid from molasses, sucrose or glucose. It is reported that production of citric acid by fungi is associated with the accumulation of significant amounts of solid and liquid wastes [2]. Alternatively, there is a great interest in the use of yeasts for the industrial production of citric acid. A number of different strains, mostly belonging to the Candida (Yarrowia) genus (C. lipolytica, C. guilliermondii, C. oleophila, C. intermedia, etc.) have been used for citric
acid production, mainly in conventional batch processes, but also in continuous culture, and with immobilized cells [1].

Some yeasts are known to be able to produce citric acid from a wider range of carbon sources than fungi do [1]. Typical carbon sources used for this process are glucose, glucose syrups, industrial ethanol, n-hydrocarbons, crude rapeseed oil, canola oil, mixtures of industrial saturated fatty acids of animal origin, and raw glycerol. It is reported that there is a considerable amounts of data on citric acid production from sugars by *Y. lipolytica* yeast, but a few reports are available on the use of pure or raw glycerol [3]. Citric acid production rates and yields are highly dependent on the type of microorganism, the type of substrate and culture conditions [1]. There have been many attempts for altering the growth media in order to increase the production of citric acid [4]. Besides development and selection of new strains, the study of novel substrates is also gaining importance.

The aim of this study was to determine growth conditions and citric acid production kinetics of two *Y. lipolytica* strains at different initial concentrations of glycerol or mannitol as substrates. The study gives a novel approach for possible substrate profiles of the yeast strains for the citric acid production process. This is also a growth kinetics research, which demonstrates citric acid production characteristics of a novel domestic *Y. lipolytica* strain in comparison with a citric acid producer.

**Materials and Methods**

**Yeast strains and selection**

*Y. lipolytica* NBRC 1658 was obtained from the National Institute of Technology and Evaluation (NITE) Biological Resource Centre, Japan. The strain NBRC 1658 was defined as a “citric acid producer” by the culture collection. *Y. lipolytica* IFO 1195 was obtained from the same culture collection in Japan. 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 stated as “unknown” by the culture collection. The selection of the strain was performed with a preliminary study (data not shown) in which citric acid production abilities of 30 domestic strains belonging to different species such as *Candida guillermondii*, *Candida pelliculosa*, *Candida intermedia*, *Candida parapsilosis*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, *Pichia anomala* and *Y. lipolytica*, as well as the strains *Y. lipolytica* NBRC 1658 and *Y. lipolytica* IFO 1195 were investigated. Of the tested strains, the highest citric acid production was obtained by the domestic *Y. lipolytica* 57, which was 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. For genetic identification of the strains PCR-RFLP method was used (Dlauchy et al., 1999). 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: *MspI*, *HaeIII*, *AluI*, *RsaI* and *Scprl* (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): yeast extract, 3; malt extract, 3; peptone, 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 inoculum used in these experiments were prepared by incubation of the cultures at 28°C for 24 hours in a modified growth medium containing (in g/L): glucose, 30; yeast extract, 3; malt extract, 3; peptone, 3; yeast extract, 3; malt extract, 3; peptone, 3; glucose, 10 and agar, 15. Cultures stored in YM agar were activated in the same medium by maintaining consecutive transfers.
extract, 2; NH₄Cl, 2; KH₂PO₄, 0.5 and MgSO₄.7H₂O, 1 [5]. The experiments were carried out in a fermentation medium containing (in g/L): glycerol 0-160 or mannitol 0-160; NH₄Cl, 2; KH₂PO₄, 1; MgSO₄.7H₂O, 1; yeast extract, 1; CaCO₃, 40 [6]. The media were sterilized at 121°C for 15 min. Initial pH of the fermentation medium was adjusted to 5.2 using 1 M HCl.

**Equipment and fermentation conditions**

Fermentation experiments 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 percentage of 5%. Experiments were carried out at constant temperature of 30°C, with a shaking rate of 100 strokes/min. All experiments were performed in duplicate.

**Biomass determination**

Dry mass (x) of the yeast was determined spectrophotometrically using wet mass-absorbance and wet-dry mass calibration curves which had been prepared before. For this purpose, the growth medium was inoculated with one of the yeast culture, and incubated in a water bath shaker at 30°C with using a shaking rate of 100 strokes/min. Inoculum percentage was 10% (v/v). After 72 hours of incubation, samples were taken from the fermentation medium and then centrifuged at 5000 rpm for 25 minutes. The sediment was used for determination of wet cell mass. The wet biomass was suspended in a constant volume of distilled water. Absorbance of the suspension was measured at 660 nm by a spectrophotometer (UV 210PC Shimadzu and Bausch & Lomb Spectronic 20). Using the data, a wet mass-absorbance calibration curve was then prepared. For plotting the wet mass-dry mass calibration curve, samples were prepared as stated above, and their precipitates were dried at 100°C for 2 hours after determining wet cell mass. During the citric acid fermentation experiments, dry mass concentration of the yeast was obtained using these two curves. In the runs, samples were taken from the fermentation media at specific time intervals and 6N HCl was added into the samples in order to dissolve CaCO₃ [6]. Samples were centrifuged at 5000 rpm for 25 min. The precipitate was suspended in a constant volume of distilled water in order to determine wet cell mass. Absorbance of the suspension was measured at 660 nm, and then the dry mass (dm) of yeast was determined from the wet mass-dry mass calibration curve.

**Citric acid analysis**

Concentration of citric acid was measured spectrophotometrically by pyridine-acetic anhydride method [7, 8].

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

Initial concentrations of glycerol or mannitol (S₆G₉ and S₆M₉) in the fermentation media were changed between 0-160 g/L. For both strains, specific microbial growth rates (μ), citric acid productivities (rₑ), maximum specific citric acid production rates (υₘ), maximum citric acid concentrations (Cₑₘ), maximum dry mass (xₑₘ), citric acid yields (Yₚ/So) and cell yields (Yₓ/So) were calculated. Specific microbial growth rate for the exponential growth phase was calculated from the semi-logarithmic plot of the dry mass data versus time. Specific citric acid production rates (qₑ) were calculated by the following relationship using the changes in citric acid concentrations and dry mass with time.

\[
qₑ = \frac{1}{x} \frac{dP}{dt}
\]  

(1)
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Maximum values of the specific citric acid production rates \( (\nu_m) \) were also determined. Citric acid yields were calculated according to Eq. 2, where \( S_o \) is the initial substrate concentration of the medium.

\[
Y_{P:S_o} = \frac{C_{c,m}}{S_o} \times 100
\]

(2)

Cell yields were calculated according to Eq. 3.

\[
Y_{x:S_o} = \frac{\frac{X_m}{S_o} - \frac{X_o}{S_o}}{S_o}
\]

(3)

**Statistical analysis**

Kinetic constants and rates were calculated using Origin 6.0 (Microcal Software, Inc., Northampton, MA01060, USA) program package. Equations expressing the changes of specific microbial growth and citric acid production rates with initial glycerol or mannitol concentration were derived from non-linear regression analysis using the same package program.

**Results**

During the fermentation period, variations in citric acid concentrations and dry mass of the strains were determined at specific time intervals for both substrates. Rates of the specific growth and maximum specific citric acid production were expressed as a function of initial glycerol or mannitol concentration. It was found that the non-competitive substrate inhibition model was suitable for definition of the growth data (Eq.4).

\[
\mu = \frac{\mu_{\text{max}} S_o}{K_s + S_o + \frac{S_o^2}{K_I}}
\]

(4)

In Eq. 4, \( \mu_{\text{max}} \) represents the maximum specific growth rate, \( S_o \) is the initial substrate concentration, \( K_s \) is the saturation constant of the Monod model, and \( K_I \) is the substrate dissociation constant.

For *Y. lipolytica* NBRC 1658 and the domestic strain *Y. lipolytica* 57, changes of microorganism dry mass \( (dm) \) and citric acid concentration with time at different initial glycerol concentrations are represented in Fig. 1. Maximum value for dry cell mass was obtained at 40 g/L initial glycerol concentration for the strain NBRC 1658. The best results for cell growth were obtained at 80 g/L initial glycerol concentration for the domestic strain. Citric acid production reached the maximum level \( (C_{c,m} = 21.5 \text{ g/L}) \) at 120 g/L initial glycerol concentration for NBRC 1658 strain. The maximum citric acid concentration decreased when the initial glycerol concentration was higher than 120 g/L. For the domestic strain, the maximum citric acid production of 32.80 g/L was obtained at 160 g/L initial glycerol concentration.
In Fig. 2, were represented the changes in citric acid productivity with time and calculated specific growth and citric acid production rates at different initial glycerol concentrations for both of the strains. For NBRC 1658 strain, the best results for productivity and maximum specific citric acid production rate were obtained at 120 g/L initial glycerol concentration. The domestic strain reached the maximum productivity and specific citric acid production rate at 160 g/L initial glycerol concentration. Maximum specific growth rate was calculated using 40 g/L initial glycerol concentration for both strains.

Changes in specific growth rate of the strain NBRC 1658 with initial glycerol concentration was defined with non-competitive substrate inhibition model, and the relationship was represented in Eq. 5. It can be observed from the high $R^2$ value that the derived equation adequately fit the experimental data.

\[
\mu = \frac{0.089 \times S_{GlO}}{20.11 + S_{GlO} + \frac{S_{GlO}^2}{60.71}} \quad (5)
\]

$R^2 = 0.96$
According to Eq. 5, maximum specific growth rate ($\mu_{max}$) was found as 0.089 h$^{-1}$, while saturation constant ($K_s$) and substrate dissociation constant ($K_i$) were determined as 20.11 g/L and 60.71 g/L, respectively.

Variation of maximum specific citric acid production rate with initial glycerol concentration for NBRC 1658 strain is shown in Eq. 6.

$$v_m = -7.95 \times 10^{-4} + 1.249 \times 10^{-4} S_{Glo} - 2.979 \times 10^{-7} S_{Glo}^2$$

$$R^2 = 0.90$$

**Figure 2.** Variations in productivity with time at different initial glycerol concentrations and changes of maximum specific citric acid production rate and specific growth rate with initial glycerol concentration for *Y. lipolytica* NBRC 1658 (a) and *Y. lipolytica* 57 (b)

The equation derived from non-linear regression analysis and expressing the non-competitive substrate inhibition model for growth data of the domestic strain in glycerol medium is given in Eq. 7.
From Eq. 7, \( \mu_{\text{max}} \) was found as 0.102 h\(^{-1}\), while \( K_s \) and \( K_I \) were obtained as 12.30 g/L and 22.25 g/L, respectively.

For the domestic strain, the variation of the maximum specific citric acid production rate with initial glycerol concentration was defined with the model equation represented in Eq. 8.

\[
\mu = \frac{0.102 \times S_{\text{Glo}}}{12.30 + S_{\text{Glo}} + \frac{S_{\text{Glo}}}{22.25}} \tag{7}
\]

\[
\nu_m = \frac{0.0254}{1 + e^{-0.06073(S_{\text{Glo}} - 84.257)}} \tag{8}
\]

The calculated values of maximum citric acid concentration, maximum dry mass, product yield and maximum productivity at different initial glycerol concentrations are shown in Table 1. The highest values for maximum citric acid concentration, product yield and productivity were obtained with 120 g/L initial glycerol concentration for NBRC 1658 strain. While the maximum citric acid productivity was obtained at 160 g/L initial concentration for the domestic strain, the highest citric acid concentration and product yield were obtained at 120 g/L initial glycerol concentration. Cell yield was calculated as 0.032 and 0.039 g dm/g glycerol for NBRC 1658 and the domestic strain, respectively.

<table>
<thead>
<tr>
<th>S_{\text{Glo}} (g/L)</th>
<th>C_{\text{C m}} (g/L)</th>
<th>x_m (g/L)</th>
<th>Y_{P/So} (%)</th>
<th>( r_{\text{C m}} ) [g citric acid/(L.h)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y. lipolytica NBRC 1658</td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>0.00</td>
<td>0.45</td>
<td>0.45</td>
<td>0.000</td>
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<tr>
<td>20</td>
<td>0.03</td>
<td>0.45</td>
<td>0.45</td>
<td>0.015 (t = 44 h)</td>
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<tr>
<td>40</td>
<td>0.03</td>
<td>0.45</td>
<td>0.45</td>
<td>0.022 (t = 254 h)</td>
</tr>
<tr>
<td>80</td>
<td>0.03</td>
<td>0.45</td>
<td>0.45</td>
<td>0.110 (t = 418 h)</td>
</tr>
<tr>
<td>120</td>
<td>0.03</td>
<td>0.45</td>
<td>0.45</td>
<td>0.197 (t = 418 h)</td>
</tr>
<tr>
<td>160</td>
<td>0.03</td>
<td>0.45</td>
<td>0.45</td>
<td>0.202 (t = 418 h)</td>
</tr>
</tbody>
</table>

The effects of initial mannitol concentration on cell growth and citric acid production are represented in Fig. 3. In the medium without mannitol, citric acid was not produced by the strains. Maximum dry mass and citric acid concentrations were obtained with 120 g/L initial mannitol concentration for both strains. An inhibition effect was observed on the citric acid production of both strains when the initial mannitol concentration was 160 g/L. This effect was obviously recognized especially for the domestic strain.
Variations in productivity with time and changes in specific growth and maximum specific citric acid production rate with initial mannitol concentration are represented in Fig. 4. Maximum productivity was obtained at 120 g/L initial mannitol concentration for both strains. While specific growth rate reached the maximum level when the initial mannitol concentration was 40-80 g/L, the highest value of the maximum specific citric acid production rate was obtained at 160 g/L initial mannitol concentration for NBRC 1658 strain. In addition, the maximum values for specific growth and citric acid production rates were obtained at 120 g/L initial mannitol concentration for the domestic strain.

The relationship between specific growth rate of NBRC 1658 and initial mannitol concentration was defined with non-competitive substrate inhibition model, and expressed by...
Eq. 9. For NBRC 1658 strain, $\mu_{\text{max}}$ was calculated as 0.051 h$^{-1}$ and $K_s$ and $K_I$ were determined as 34.88 g/L and 82.28 g/L, respectively.

$$\mu = \frac{0.051 S_m}{34.88 + S_m + \frac{S_m^2}{82.28}} \quad (9)$$

$R^2 = 0.93$

Eq. 10 defined the variation of the calculated experimental maximum specific citric acid production rate of NBRC 1658 strain with initial mannitol concentration. It can be observed from the $R^2$ value that the model equation is suitable for definition of the experimental data.

$$v_m = \frac{0.00482}{1 + 279.469 x e^{-0.0617 S_m}} \quad (10)$$

$R^2 = 0.99$

**Figure 4.** Variations in productivity with time at different initial mannitol concentrations and changes of maximum specific citric acid production rate and specific growth rate with initial mannitol concentration for *Y. lipolytica* NBRC 1658 (a) and *Y. lipolytica* 57 (b)
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The equation expressing the variation of specific growth rate with initial mannitol concentration for the domestic strain is given in Eq. 11. In mannitol medium, $\mu_{\text{max}}$ was found as 0.079 h$^{-1}$, while $K_s$ and $K_I$ were determined as 37.82 g/L and 130.21 g/L, respectively.

$$
\mu = \frac{0.079 S_{\text{Mo}}}{37.82 + S_{\text{Mo}} + \frac{S_{\text{Mo}}^2}{130.21}}
$$

$R^2 = 0.96$

Eq. 12 expressed variation of maximum specific citric acid production rate with initial mannitol concentration for the domestic strain.

$$
u_m = \frac{0.0055}{1 + 53.767 \times e^{-0.281S_{\text{Mo}}}}
$$

$R^2 = 0.93$

In Table 2 are represented the changes in maximum citric acid concentration, maximum dry mass, product yield and maximum productivity of both of the strains with initial mannitol concentration. The highest values for dry mass, citric acid concentration, citric acid yield and productivity were obtained at 120 g/L initial mannitol concentration for both of the strains. Cell yields of NBRC 1658 and the domestic strain for this substrate were calculated as 0.106 and 0.070 g dm/g mannitol, respectively.

| Table 2. Effects of initial mannitol concentration ($S_{\text{Mo}}$) on maximum citric acid concentration ($C_{\text{cm}}$), maximum dry mass ($x_m$), product yield ($Y_{P/So}$), and maximum productivity ($r_{cm}$) |
|---|---|---|---|---|
| $S_{\text{Mo}}$ (g/L) | $C_{\text{cm}}$ (g/L) | $x_m$ (g/L) | $Y_{P/So}$ (%) | $r_{cm}$ [g citric acid/(L.h)] |
| *Y. lipolytica* NBRC 1658 | | | | |
| 0 | 0.00 | 1.39 | - | 0.000 |
| 20 | 0.17 | 11.84 | 0.85 | 0.002 (t = 133 h) |
| 40 | 0.85 | 20.19 | 2.13 | 0.008 (t = 180 h) |
| 80 | 2.55 | 20.52 | 3.18 | 0.027 (t = 156 h) |
| 120 | 20.25 | 24.50 | 16.88 | 0.065 (t = 228 h) |
| 160 | 14.65 | 20.95 | 9.16 | 0.047 (t = 228 h) |
| *Y. lipolytica* 57 | | | | |
| 0 | 0.00 | 0.45 | - | 0.000 |
| 20 | 1.07 | 3.16 | 5.35 | 0.013 (t = 133 h) |
| 40 | 1.17 | 6.06 | 2.93 | 0.011 (t = 90 h) |
| 80 | 6.86 | 10.26 | 8.58 | 0.049 (t = 180 h) |
| 120 | 21.70 | 13.30 | 18.08 | 0.083 (t = 300 h) |
| 160 | 7.76 | 10.67 | 4.85 | 0.059 (t = 228 h) |

**Discussion**

This research was designed in order to determine the effects of two polyalcohols, glycerol and mannitol, on growth and citric acid production kinetics of two *Y. lipolytica* strains, one of which is a citric acid producer. When glycerol was used as a sole substrate, similar effects were observed on growth of the two strains. Both of the strains reached maximum specific growth rate at 40 g/L initial glycerol concentration. The growth data were defined by the non-competitive substrate inhibition model for both strains. Maximum citric
acid concentration obtained by the domestic strain was higher than that of the strain NBRC 1658 in glycerol medium. In the experiments with the domestic strain, maximum citric acid concentration increased in direct ratio to the initial glycerol concentration. However, an inhibition effect was observed on citric acid production of the strain NBRC 1658 at 160 g/L initial glycerol concentration. Besides the use of pure glycerol, there are several studies concerning the potential use of raw glycerol for citric acid production [3, 9, 10]. It is reported that the number of studies about the use of glycerol as a sole substrate are in restricted amount. In a study carried out with a mutant strain of Y. lipolytica, it was reported that maximum citric acid concentration changed between 75.7-124.5 g/L at 200 g/L initial glycerol concentration [3]. In another study by Papanikolaou & al. [11], raw glycerol was used and it was reported that a Y. lipolytica strain produced citric acid with a maximum concentration of 33.6 and 35.1 g/L when initial glycerol concentration was 80 and 120 g/L, respectively. When our results are compared with those in the literature, citric acid production of the yeast strains can be accepted as satisfactory in the medium containing glycerol as substrate.

In the experiments carried out with mannitol as substrate, similar results were obtained for both strains in the mean of growth and citric acid production. Maximum citric acid concentration, citric acid yield and productivity were obtained at 120 g/L initial mannitol concentration for both strains. There was not a significant difference between maximum citric acid concentrations obtained by the two strains in mannitol media. Although there was a substrate inhibition effect on growth of both yeasts at high initial mannitol concentrations, maximum cell dry mass was obtained at 120 g/L initial mannitol concentration. It can be observed from the results that citric acid production of the domestic strain was better in glycerol media than that in mannitol media, while the citric acid production characteristics of NBRC 1658 in both media was the same. There is not any reported study about the use of mannitol for citric acid production. The obtained results in this study may be valuable for suggesting a novel substrate in order to enhance citric acid production.

The study gives promising results for using both polyalcohols as potential substrates for citric acid production. The kinetic data give an idea about optimizing the culture conditions in a possible scale-up of the citric acid production process. Conversions of glycerol to value-added products are of increasing interest due to the production of glycerol as a by-product of biodiesel production [9]. This study may lead to several further studies in which raw glycerol may be used as a cheaper substrate. The results obtained in mannitol medium may also be adapted to further studies concerning the use of some natural sources of mannitol such as celery by-products. It is reported in a study that celery stalks contains 15.2% mannitol [12] which may serve as a good source of substrate for citric acid production. Another aim of this research was to compare citric acid production characteristics of a novel domestic strain with a citric acid producer. It was found that citric acid production of the domestic strain was similar or higher than that of NBRC 1658. This demonstration is associated with generation of a novel citric acid producing strain, which may be used in a possible commercial citric acid production process.

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