Brazilian Journal of Microbiology (2008) 39:325-332
ISSN 1517-8382

# EFFECTS OF PH AND TEMPERATURE ON GROWTH AND GLYCEROL PRODUCTION KINETICS OF TWO INDIGENOUS WINE STRAINS OF *SACCHAROMYCES CEREVISIAE* FROM TURKEY

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Submitted: December 21, 2006; Returned to authors for corrections: November 14, 2007; Approved: April 25, 2008.

## ABSTRACT

The study was performed in a batch system in order to determine the effects of pH and temperature on growth and glycerol production kinetics of two indigenous wine yeast strains *Saccharomyces cerevisiae* Kalecik 1 and Narince 3. The highest values of dry mass and specific growth rate were obtained at pH 4.00 for both of the strains. Maximum specific glycerol production rates were obtained at pH 5.92 and 6.27 for the strains Kalecik 1 and Narince 3, respectively. Kalecik 1 strain produced maximum 8.8 $gL^{-1}$ of glycerol at pH 6.46. Maximum glycerol concentration obtained by the strain Narince 3 was 9.1 $gL^{-1}$ at pH 6.48. Both yeasts reached maximum specific growth rate at 30ºC. Optimum temperature range for glycerol production was determined as 25-30ºC for the strain Kalecik 1. The strain Narince 3 reached maximum specific glycerol production rate at 30ºC. Maximum glycerol concentrations at 30ºC were obtained as 8.5 and 7.6 $gL^{-1}$ for Kalecik 1 and Narince 3, respectively.

**Key-words:** Production kinetics; Glycerol; Growth parameters; *Saccharomyces cerevisiae*

## INTRODUCTION

*Saccharomyces cerevisiae* is reported as the most studied and biochemically best understood species of the yeast domain. It is best known for its domesticated role in the production of fermented products. This yeast converts hexose sugars to ethanol, $CO_2$, and a variety of compounds including alcohols, esters, aldehydes, and acids, that contribute to the sensory attributes of the food and beverage (19). Fermentation of carbohydrates in fruits, grains and other biomass to ethanol by *S. cerevisiae* is the critical process for a wide range of products from fine wines to gasoline additives (3). Glycerol is a sugar alcohol produced as a by-product of the ethanol fermentation process by *Saccharomyces cerevisiae*. It is an economically important alcohol with a slightly sweet taste and with applications in the food, beverage, pharmaceutical, and chemical industries (20). Glycerol can be produced by biochemical methods in which microorganisms are used.

Glycerol is also an important constituent of wine, which does not contribute directly to wine aroma due to its non-volatile nature, but significantly contributes to wine quality by providing sweetness, fullness, and smoothness (14). The concentration of glycerol usually formed by *S. cerevisiae* in wine varies between 1-15 $gL^{-1}$, with avarage values approximately 7 $gL^{-1}$ (15). It is suggested that glycerol production amount of wine yeast *S. cerevisiae* is very important for wine quality in beverage industry. Due to its positive effects on wine's sensory properties, many attemps have been made to increase the glycerol yield during fermentation (4,11,16).

Glycerol production by yeast is influenced by many growth and environmental factors (2,14). Substrate type, initial substrate concentration, pH, temperature, nitrogen source, aeration rate, addition of sulphur dioxide, and inoculation ratio are given among these factors. Several studies have shown that an increase in temperature resulted in higher glycerol production (5,14). It is reported that the optimum temperature for maximum glycerol production by commercial wine yeast strains of *S. cerevisiae* varies between 22-32ºC (15). Glycerol production yield of a wine yeast is also known as one of the most important criteria in strain selection.

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This study was organised to determine the effect of pH and temperature on growth and glycerol production kinetics of two indigenous wine yeast strains *Saccharomyces* cerevisiae Kalecik 1 and *Saccharomyces cerevisiae* Narince 3 in synthetic medium. Another objective was to demonstrate the data which may give preknowledge for the further studies planned to be performed in natural media, such as grape juice.

## MATERIALS AND METHODS

### Yeast strains

Two indigenous wine yeast strains *Saccharomyces cerevisiae* Kalecik 1 and *Saccharomyces cerevisiae* Narince 3 were used in the study. These yeasts were isolated from Kalecik Karasi and Narince grapes cultivated in Turkey, red and white grape varieties, respectively. These grapes are commercially used for the production of wine in Turkey, as well as Kalecik 1 and Narince 3 strains. The strains were obtained from the culture collection of Food Engineering Department, University of Ankara, Turkey. The yeasts were kept as stock culture at 4ºC on yeast extract malt extract glucose (YMG) agar consisting of (in $gL^{-1}$): 10 g yeast extract (Lab M, U.K), 10 g malt extract (Lab M, U.K), 20 g glucose (Merck, Germany), and 15 g agar (Lab M, U.K).

### Preparation of inocula and fermentation medium

Cultures which were stored in YMG agar, were activated in the same medium by maintaining consecutive transfers. In all of the experiments, the inocula were prepared by incubation at 30ºC for 24 h in a medium containing (in $gL^{-1}$): 20 g glucose (Merck, Germany), 1 g yeast extract (Lab M, U.K), 1 g $KH_2PO_4$ (Carlo Erba, Italy), and 0.5 g $MgSO_4.7H_2O$ (Pancreac, Spain). The inocula were added to the fermentation media with a volume percentage of 5% (v/v). The experiments were carried out by using a fermentation medium containing 300 $gL^{-1}$ initial glucose concentration. Other components of the fermentation medium were kept same as the medium used for preparation of inoculum. Medium was sterilized in autoclave at 121ºC for 10 minutes. During the experiments in which effect of temperature was investigated, initial pH of the medium was adjusted to 4.00 by using 0.1 N HCl (Riedel-De Häen, 37% extra pure, Germany).

### Equipment

Experiments were carried out in water bath shakers which have temperature and shaker rate control systems, with 250 ml working volume in suitable flasks. Shaking rate was held constant at 70 strokes/minute during the experiments.

### Biomass determination

Dry mass of the yeasts were 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 certain time intervals, and centrifuged at 5000 rpm for 25 minutes. Precipitate was used for determining dry mass spectrophotometrically at 500 nm (UV 210PC Shimadzu and Bausch & Lomb Spectronic 20). Supernatant was used for glycerol analysis.

### Glycerol analysis

Glycerol concentrations in the fermentation media were determined by periodate – chromotropic acid analysis method (10,20). Supernatant of centrifuged samples were used for glycerol analysis spectrophotometrically at 570 nm.

### pH measurement

Measurement of pH of the fermentation media was done by using Jenway 3010 model pH meter.

### Determining the effects of pH and temperature on growth and glycerol production kinetics of *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3

Effects of pH and temperature were investigated in water bath shakers. The yeasts were inoculated to fermentation media at an inoculation ratio of 5%. In the experiments which effect of pH was investigated, pH range of between 3.48-6.48 was used. Temperature was kept constant at 30ºC. Effect of temperature was investigated between 20-35ºC, and initial pH of the fermentation media were adjusted to 4.00 in these experiments. For each parameter, specific microbial growth rates ($\mu$), specific glycerol production rates ($\upsilon_g$), maximum glycerol concentrations ($P_m$), maximum dry mass ($x_m$), and glycerol yields ($Y_{P/So}$) were calculated. Specific microbial growth rates were determined from the graphs of the changes of dry mass with fermentation time. Specific growth rate values were calculated from the logarithmic plots of the dry mass data versus time. Specific glycerol production rates were calculated from the following relationship by using the changes in glycerol and dry mass concentrations with time.

$$\upsilon_g = \frac{1}{x}\frac{dP}{dt} \qquad (1)$$

Maximum values of the specific glycerol production rates ($\upsilon$) were also determined in the experiments.

### Statistical analysis

Non-linear regression analysis were performed for derivation of equations by using SPSS 10 statistical package program.

## RESULTS

During the incubation time, changes in glycerol concentration and dry mass were determined at specific time

intervals. Specific growth rates (μ) and maximum specific glycerol production rates (υ) were calculated. Temperature and pH values were chosen as independent variables for specific growth and glycerol production rates of the cultures. Second or third-order polynomial models represented the growth data, as well as glycerol production data, reasonably well.

**Effect of pH**

Variations in glycerol concentrations and dry mass of Kalecik 1 and Narince 3 strains during fermentation time are shown in Fig. 1. Related to pH, variations in specific growth and glycerol production rates for both of the strains are stated in Fig. 2. It was found that *S. cerevisiae* Kalecik 1 and the strain Narince 3 reached maximum specific growth rate at pH 4. Maximum specific glycerol production rate was obtained at pH 5.92 and 6.27 for the strains Kalecik 1 and Narince 3, respectively.

The polynomial models were estimated for the experimental data of both specific growth and glycerol production rates related to pH by using non-linear regression method. The model outputs and the experimental values of the specific growth and glycerol production rate as a function of pH are shown in Fig. 2. The equations obtained by non-linear regression method are shown in Eq. 2 and 3 for the strain Kalecik 1, and in Eq. 4 and 5 for Narince 3.

$$\mu = 0.027\,pH^3 - 0.431\,pH^2 + 2.229\,pH - 3.643 \quad (2)$$
$$R^2 = 0.984$$
$$\upsilon = -0.050\,pH^3 + 0.719\,pH^2 - 3.238\,pH + 4.836 \quad (3)$$
$$R^2 = 0.996$$
$$\mu = 0.036\,pH^3 - 0.547\,pH^2 + 2.715\,pH - 4.248 \quad (4)$$
$$R^2 = 0.861$$
$$\upsilon = -0.0107\,pH^2 + 0.1536\,pH - 0.2117 \quad (5)$$
$$R^2 = 0.943$$

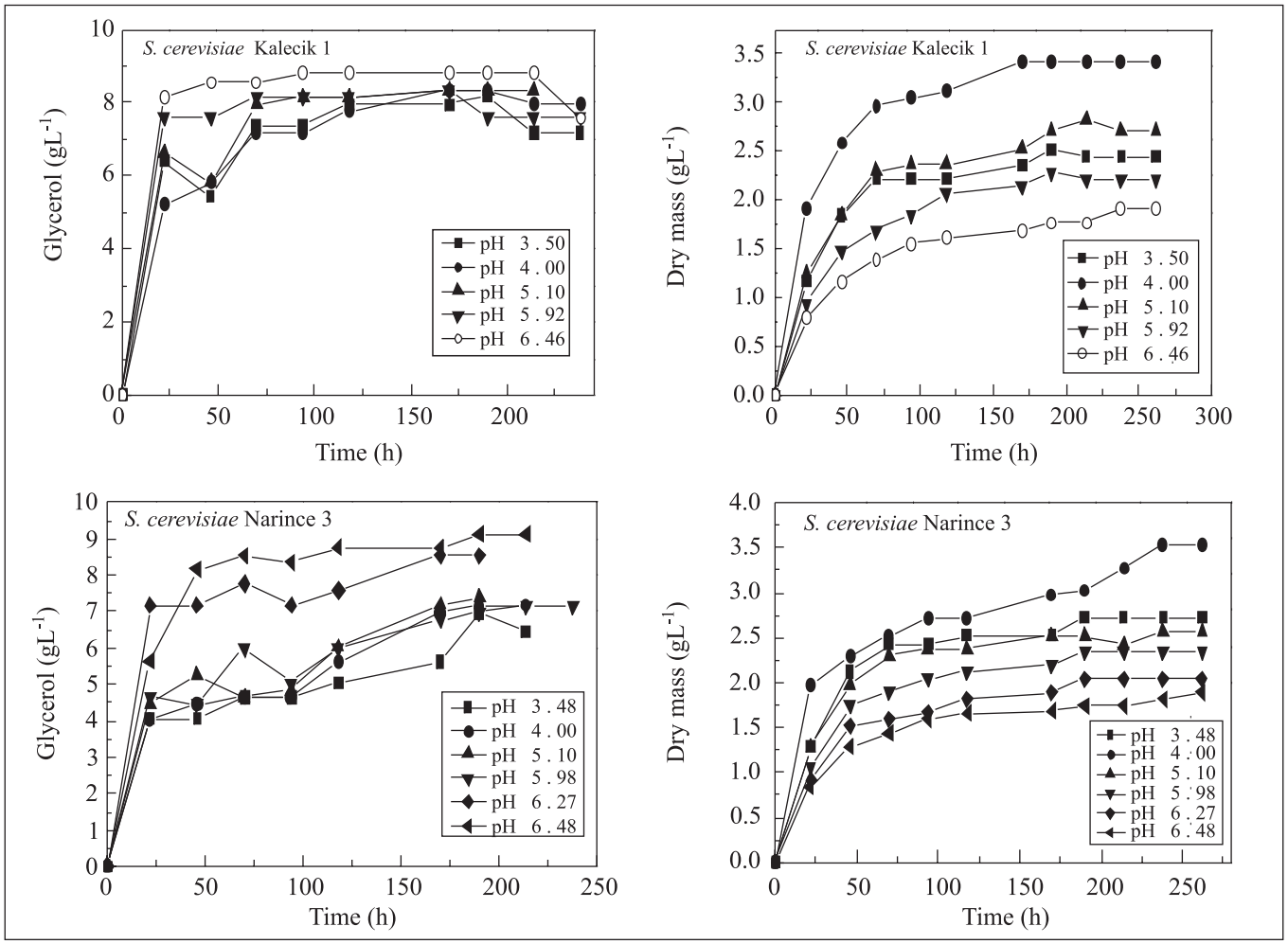


**Figure 1.** Variations in glycerol production and dry mass of *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3 related to pH during fermentation time

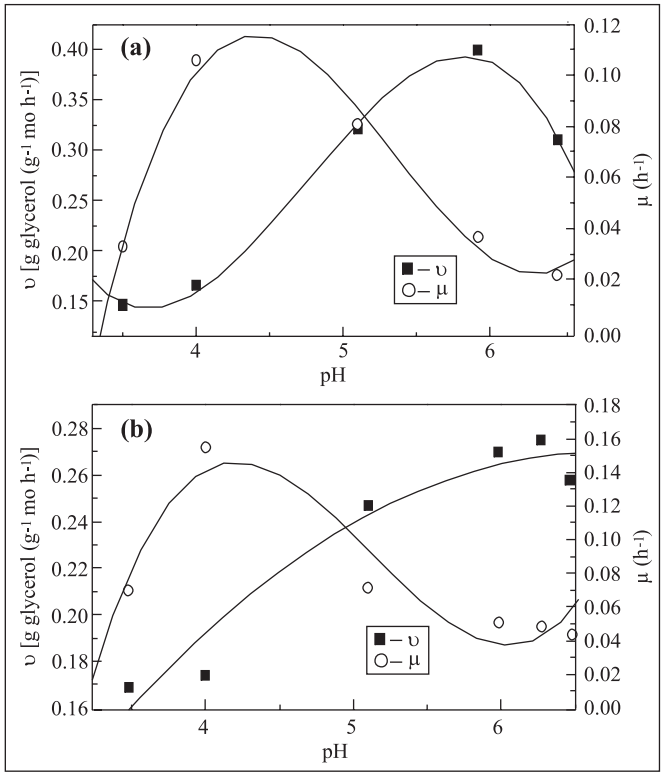


**Figure 2.** Variations in specific growth and glycerol production rates related to pH for *S. cerevisiae* Kalecik 1 (a) and *S. cerevisiae* Narince 3 (b) (μ = specific growth rate, $h^{-1}$; υ = maximum specific glycerol production rate, g glycerol ($g^{-1}$ mo $h^{-1}$); mo = microorganism)

It can be observed from the obtained $R^2$ coefficients that the estimated models adequately fit the experimental data.

Effects of initial pH on maximum glycerol concentrations, maximum dry mass, and glycerol yields are shown in Table 1 for strains Kalecik 1 and Narince 3. Maximum dry mass of 3.40 $gL^{-1}$ was obtained at pH 4.00 for *S. cerevisiae* Kalecik 1. Maximum glycerol concentration was obtained as 8.8 $gL^{-1}$ at pH 6.46 for that strain. Glycerol yield was also at maximum level (2.93%) at pH 6.46. For the strain Narince 3, maximum dry mass of 3.50 $gL^{-1}$ was obtained at pH 4.00. Maximum glycerol concentration and glycerol yield remained constant between pH 4.00-5.98 (Table 1). Maximum glycerol concentration of 9.1 $gL^{-1}$ was obtained for *S. cerevisiae* Narince 3 at pH 6.48, and the maximum glycerol yield (3.03%) was obtained at the same pH level.

**Effect of Temperature**

Variations in glycerol concentrations and dry mass of *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3 during fermentation time are shown in Fig. 3. Maximum specific growth rates were obtained at 30ºC for both of the strains (Fig. 4). For

**Table 1.** Effects of initial pH on maximum glycerol concentration, maximum dry mass, and glycerol yield for *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3.

| pH | $P_m$ ($gL^{-1}$) | $X_m$ ($gL^{-1}$) | $Y_{P/So}$ (%) |
|---|---|---|---|
| *S. cerevisiae* Kalecik 1 | | | |
| 3.50 | 7.5 | 2.45 | 2.50 |
| 4.00 | 8.0 | 3.40 | 2.67 |
| 5.10 | 8.2 | 2.70 | 2.73 |
| 5.92 | 8.6 | 2.25 | 2.87 |
| 6.46 | 8.8 | 1.90 | 2.93 |
| *S. cerevisiae* Narince 3 | | | |
| 3.48 | 6.5 | 2.70 | 2.17 |
| 4.00 | 7.2 | 3.50 | 2.40 |
| 5.10 | 7.2 | 2.50 | 2.40 |
| 5.98 | 7.2 | 2.30 | 2.40 |
| 6.27 | 7.9 | 2.00 | 2.63 |
| 6.48 | 9.1 | 1.85 | 3.03 |

$P_m$ : maximum glycerol concentration, $X_m$ : maximum dry mass, $Y_{P/So}$ : glycerol yield.

the strain Kalecik 1, there was not an important change in specific glycerol production rate between 25-30ºC, which was the optimum range for glycerol production. Narince 3 strain reached maximum specific glycerol production rate at 30ºC (Fig. 4).

The polynomial models were estimated for representation of the experimental data. The equations which represent the relation of specific growth rate and glycerol production rate with temperature were obtained by using non-linear regression analysis. The obtained equations are represented in Eq. 6 and 7 for Kalecik 1, and in Eq. 8 and 9 for Narince 3.

$$\mu = -1.88 \times 10^{-4} T^3 + 0.0147 T^2 - 0.37 T + 3.098 \quad (6)$$
$$R^2 = 1.000$$
$$\upsilon = -0.00085 T^2 + 0.048 T - 0.508 \quad (7)$$
$$R^2 = 0.968$$
$$\mu = -0.000378 T^3 + 0.0300 T^2 - 0.77 T + 6.468 \quad (8)$$
$$R^2 = 1.000$$
$$\upsilon = -0.000061 T^3 + 0.0046 T^2 - 0.110 T + 0.995 \quad (9)$$
$$R^2 = 1.000$$

It is clear from the $R^2$ coefficients of Eq. 6-9 that, the estimated models adequately fit the experimental data. The curve fittings belonging to obtained models are represented in Fig. 4, which also show the validity of the estimated model.

The strain Kalecik 1 reached maximum dry mass of 3.40 $gL^{-1}$ at 30ºC, and produced maximum amount of glycerol (8.5 $gL^{-1}$) at this temperature, as shown in Table 2. Glycerol yield was increased with increasing temperature and reached maximum

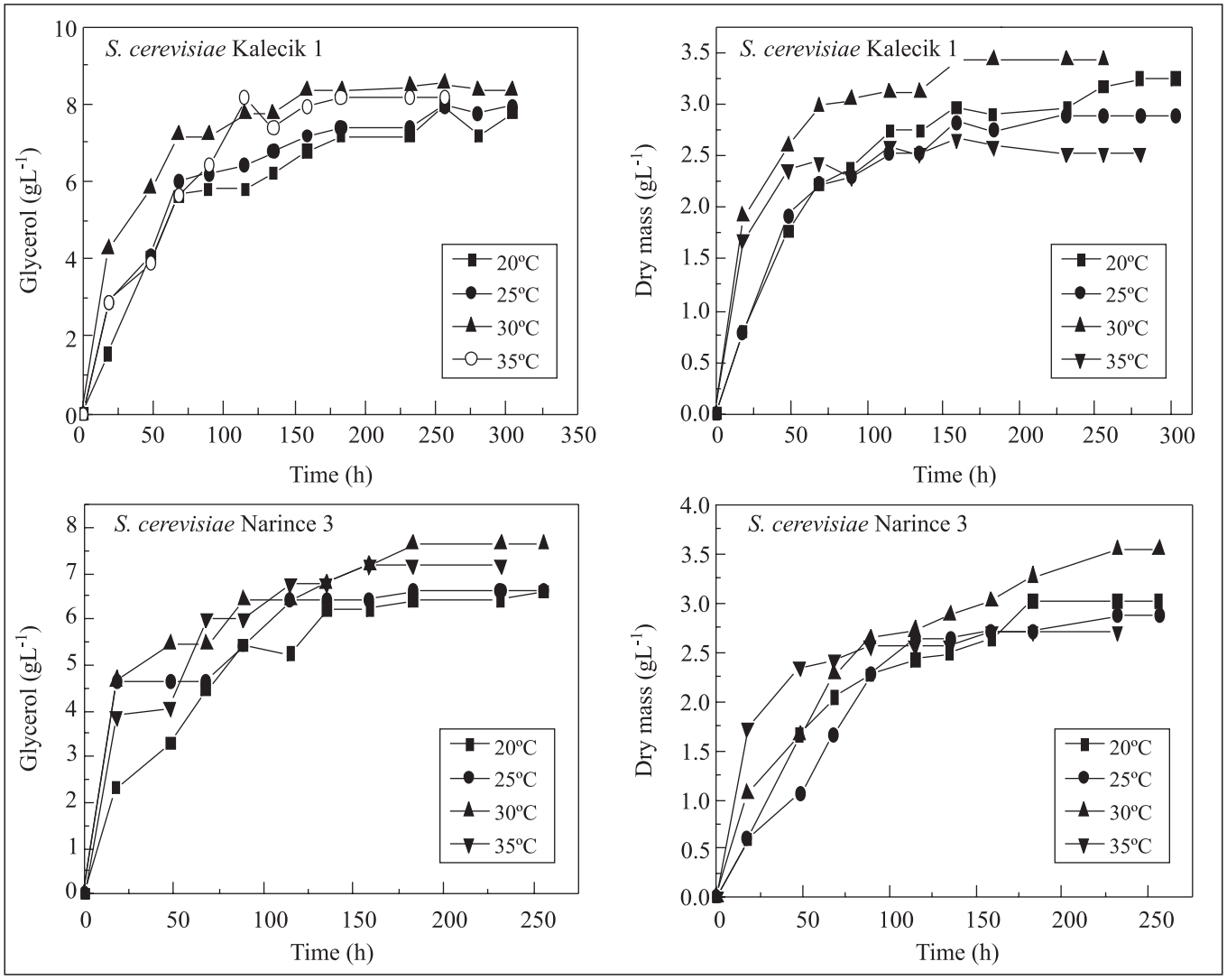


**Figure 3.** Variations in glycerol production and dry mass of *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3 related to temperature during fermentation time

**Table 2.** Effects of temperature on maximum glycerol concentration, maximum dry mass, and glycerol yield for *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3.

| T (ºC) | $P_m$ ($gL^{-1}$) | | $X_m$ ($gL^{-1}$) | | $Y_{P/So}$ (%) | |
|---|---|---|---|---|---|---|
| | *S. cerevisiae* Kalecik 1 | *S. cerevisiae* Narince 3 | *S. cerevisiae* Kalecik 1 | *S. cerevisiae* Narince 3 | *S. cerevisiae* Kalecik 1 | *S. cerevisiae* Narince 3 |
| 20 | 7.1 | 6.5 | 3.10 | 3.0 | 2.36 | 2.17 |
| 25 | 7.8 | 6.5 | 2.85 | 2.9 | 2.60 | 2.17 |
| 30 | 8.5 | 7.6 | 3.40 | 3.5 | 2.83 | 2.53 |
| 35 | 8.4 | 7.0 | 2.50 | 2.7 | 2.80 | 2.33 |

$P_m$ : maximum glycerol concentration, $X_m$ : maximum dry mass, $Y_{P/So}$ : glycerol yield.

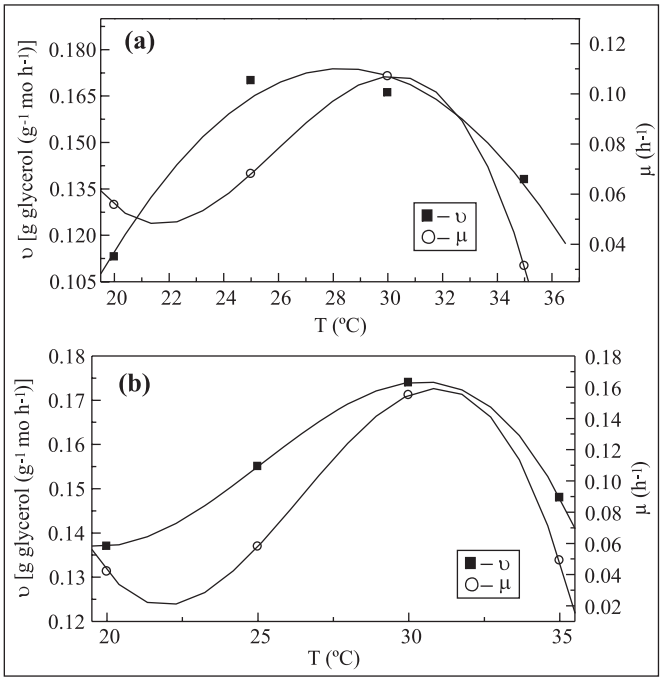


**Figure 4.** Variations in specific growth and glycerol production rates related to temperature for *S. cerevisiae* Kalecik 1 (a) and *S. cerevisiae* Narince 3 (b) (μ = specific growth rate, $h^{-1}$; υ = maximum specific glycerol production rate, g glycerol ($g^{-1}$ mo $h^{-1}$); mo = microorganism)

level of 2.83% at 30ºC. It is shown in Table 2 that Narince 3 strain reached maximum dry mass of 3.50 $gL^{-1}$ at 30ºC, and produced maximum amount of glycerol (7.6 $gL^{-1}$) at this temperature. Glycerol yield was also at maximum level (2.53%) at 30ºC.

## DISCUSSION

In this study, optimum pH value necessary for growth of *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3 was found as 4.00, since maximum values for specific growth rate and dry mass were obtained at this pH. It can be seen from the results that, growth of the yeasts were adversely affected at lower and higher pH values. There were considerable decreases in dry mass especially at high pH levels. It is reported that, most of the yeasts grow very well between pH 4.5-6.5, but nearly all species are able to grow in more acidic or alkaline media (17). Low or high pH values are known to cause chemical stress on yeast cell, which is demonstrated in our study. It is determined that glycerol production by *S. cerevisiae* Kalecik 1 was positively affected by increasing pH. Maximum glycerol concentration and glycerol yield tended to increase with increasing pH, that the best results were obtained at pH 6.46. Specific glycerol production rate reached its maximum value at pH 5.92. For the strain Narince 3, both maximum glycerol concentration and glycerol yield remained constant between pH range of 4.00-5.98, but they considerably increased above pH 5.98. Maximum glycerol concentration was obtained at pH 6.48, while maximum specific glycerol production rate was obtained at pH 6.27. The positive effect of high pH on glycerol production may be explained in relation with the activity of the enzyme aldehyde dehydrogenase. It is reported that, activity of the enzyme aldehyde dehydrogenase is increased at high pH values and acetic acid is produced (18). This oxidation generates a molecule of NADH, which requires reoxidation to maintain the redox balance of the cell. Then, glycerol is formed by the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, and then to glycerol. It is indicated in the studies concerning industrial glycerol production by *S. cerevisiae* that, higher glycerol yields were obtained under alkaline conditions. Thus, alkali-steered process is one of the processes which is used commercially for substantial overproduction of glycerol (16,18). Therefore, increase in glycerol production amount with increasing pH is an expected result in our study. When it is thought that *S. cerevisiae* Kalecik 1 and *S. cerevisiae* Narince 3 are wine yeast strains, glycerol production amount of those strains at pH values of grape must (3.5-4.2) should be also taken in to account. It is reported that the most available pH range for alcohol fermentation in wine production is 3.8-4.2. This pH range provides the conditions for *S. cerevisiae* to be dominant species of the fermentation, and also for inhibition of wild yeasts (18). In the present study, it was determined that in the range of pH values of grape must (3.5-4.2), maximum glycerol concentrations obtained in synthetic medium were 8.0 and 7.2 $gL^{-1}$ for Kalecik 1 and Narince 3 strains, respectively. Since the obtained glycerol levels are similar to those expected for wine, this might give the opinion that the strains could be selected as glycerol producing ones after further investigations.

In the experiments concerning the effects of temperature, optimum yeast growth was observed at 30ºC for both of the strains. Dry mass and specific growth rates were maximum at 30ºC, and with increasing the temperature to 35ºC, these values decreased. Temperature is accepted as one of the most important physical parameters influencing yeast growth. It is known that most laboratory and industrial yeasts generally grow best between 20-30ºC (17). It is reported that wine yeasts grow well between 25-33ºC; temperatures between 25-30ºC is more available for cell reproduction, and 30-37ºC for alcohol production (1,13). In the present study, it can be observed that maximum glycerol concentration was obtained at 30ºC for both of the strains. Specific glycerol production rate did not change significantly between 25-30ºC for the strain Kalecik 1, while maximum glycerol concentration increased. There was not any important change in maximum glycerol concentration between the temperature range of 30-35ºC for Kalecik 1 strain. Maximum concentrations

of glycerol produced by *S. cerevisiae* Narince 3 was not significantly affected between 20-25ºC, while specific glycerol production rate increased. Maximum levels for glycerol concentration, glycerol yield, and specific glycerol production rate were obtained at 30ºC for that strain. It is reported the amount of glycerol formed during fermentation generally increases with increasing temperature, and the optimum temperature for glycerol production by yeasts is apperantly similar to the optimum growth temperature of the organism (15,18). The data obtained in the present study are supported by several studies which indicate that wine yeast strains of *S. cerevisiae* have an optimum temperature between 20ºC and 32ºC for glycerol production (5,15,18). In a study carried out with *S. cerevisiae* and other yeasts in natural grape juice, *S. cerevisiae* was found to dominate in the fermentation at 25ºC and pH 3.0-3.5, that cell population reached to $10^7$-$10^8$ cell $mL^{-1}$ in three days (6).

When the results obtained by the two strains are compared, it can be observed that there are similarities in the responses of the strains to different pH and temperature levels. When it is thought that both of the strains are originated from grape, similar behaviour at various conditions could serve as an expected result. It was determined that glycerol concentrations obtained by the strain Kalecik 1 were slightly higher than those obtained by Narince 3, at almost all conditions used. There are several studies indicating the effect of yeast strain on glycerol production (14,15). The individual growth and glycerol production behaviour of two different strains were also pointed out in the present study.

In conclusion, this study demonstrated growth and glycerol production characteristics of two indigenous wine yeasts at different pH and temperatures. It is thought that the obtained data give idea about some of the important technological properties of the yeasts. It is known that, glycerol production could be controlled by the choice of optimized cultivation conditions and/or selection of appropriate strains. The strains used in the study are originated from wine grapes grown in Turkey and have a potential use in wine production. Maximum glycerol concentrations were obtained between 6.5-9.1 $gL^{-1}$, which were changed between 6.5-8.0 $gL^{-1}$ at pH values similar to pH of grape must. In a study by Remize *et al.* (14), glycerol production of some commercialized wine yeasts were investigated. Maximum glycerol concentrations of the examined strains were reported as 6.4-8.9 $gL^{-1}$ in a synthetic medium, which were similar to our results. The obtained glycerol concentrations demonstrate the potential of the yeasts for improving wine quality. Kinetic properties of glycerol production by these strains were firstly demonstrated in our previous studies investigating some other parameter effects like substrate type, initial substrate concentration and inoculum size (7-9). This study gave novel data by pointing out the effects of pH and temperature, and may lead to further complementary investigations about possible behaviours of the strains during wine production.


## RESUUMO

### Efeito do pH e temperatura na cinética de crescimento e da produção de glicerol de duas linhagens vinícolas selvagens de *Saccharomyces cerevisiae* da Turquia

Este estudo foi conduzido em sistema descontínuo a fim de determinar o efeito do pH e da temperatura na cinética de crescimento celular ede produção de glicerol de duas linhagens selvagens de vinificação, *Saccharomyces cerevisiae* Kalecik 1 e *Saccharomyces cerevisiae* Narince 3. Os maiores valores de massa celular seca e de velocidade específica de crescimento foram obtidos em pH 4,0 para as duas linhagens. A máxima velocidade específica de produção de glicerol foi obtida em pH 5,92 e 6,27 para a linhagem Kalecik 1 e Narince 3, respectivamente. A linhagem Kalecik 1 proporcionou a máxima produção de glicerol, 8,8 $gL^{-1}$ em pH 6,46. A máxima concentração de glicerol obtida pela linhagem Narince 3 foi de 9,1 $gL^{-1}$ no pH 6,48. As duas linhagens atingiram a máxima velocidade específica de crescimento à temperatura de 30ºC. A faixa de temperatura ótima para a produção de glicerol para a linhagem Kalecik 1 variou de 25 a 30ºC. A linhagem Narince 3 atingiu a máxima velocidade específica de produção de glicerol a 30ºC. A máxima concentração de glicerol, obtida a 30ºC, foi de 8,5 e 7,6 $gL^{-1}$ para as linhagens Kalecik 1 e Narince 3, respectivamente.


**Palavras-chave:** cinética de produção; glicerol; parâmetros de crescimento; *Saccharomyces cerevisiae*.

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