Production of Glycerol by Two Endogenic Wine Yeast Strains at Different Inoculum Size

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Summary

In this study, effects of inoculum size on growth and batch glycerol production kinetics of two endogenic wine yeast strains *Saccharomyces cerevisiae* Kalecik 1 and *Saccharomyces cerevisiae* Narince 3 were investigated. Inoculum size was changed between volume ratio of 2.5 and 12.5 %. Maximum glycerol concentration (8.6 g/L) was obtained with 2.5 % inoculum concentration for the strain Kalecik 1. Maximum glycerol concentration was determined as 7.6 g/L for the strain Narince 3 within the range of 2.5–7.5 % inoculum. Maximum dry mass was obtained at 5 % inoculum for both strains. *S. cerevisiae* Kalecik 1 reached specific growth rate and specific glycerol production rate maxima at 10 and 7.5 % inoculum, respectively. Maximum values for both specific growth and glycerol production rates were obtained at 7.5 % inoculum for *S. cerevisiae* Narince 3.

Key words: glycerol, production kinetics, wine yeast, *Saccharomyces cerevisiae*

Introduction

Glycerol, 1,2,3-propantriol, is a widely used sugar alcohol with many commercial applications, presently finding its largest use in the manufacture of foods, beverages, drugs, and oral rinses. In addition, glycerol is used in wrapping and packaging materials, tobacco, cosmetics, detergents, emulsifiers, *etc.* (*1,2*).

Glycerol is a well-known metabolite formed by many microorganisms including bacteria, yeasts, moulds, and algae. Since glycerol often serves the function of an osmolyte balancing external osmotic pressure, osmophilic organisms are of particular interest for glycerol production (*3*). A well-known wine, brewing, and baker's yeast *Saccharomyces cerevisiae* is the most important glycerol producing yeast. In *S. cerevisiae*, glycerol is a by-product of the fermentation of sugar to ethanol in a redox-neutral process (*4*). In this organism, glycerol plays important roles in physiological processes such as combating osmotic stress, managing cytosolic phosphate levels, and maintaining the NAD^+/NADH redox balance (*5*). Be-

sides commercial applications, glycerol production has an additional importance for wine strains of *S. cerevisiae* (*4,6–7*). Glycerol is the most aboundant by-product of wine fermentation after ethanol and carbondioxide. This polyalcohol does not directly contribute to wine aroma due to its nonvolatile nature, but it contributes to sweetness, fullness and smoothness (*4,6,8*). It is reported that glycerol concentration in wine is variable depending on the yeast strain, medium and process conditions. Typical glycerol levels in wine are given as 1–15 g/L, with average values approximately 7 g/L (*4*). Glycerol concentrations are reported to be higher in red wines compared to white wines. It is stated that there is a considerable variation in glycerol concentration in the wines from different regions in the world, even in the same country. Since there is a relationship between wine quality and glycerol levels, producing glycerol at low or high levels is an important characteristic for wine yeasts. It is reported that glycerol concentration in Turkish white wines changes between 6–11.07 g/L, and higher glycerol levels have a positive effect on mouth-feel (*9*). Therefore, increasing

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glycerol production is of concern for wine makers to improve the quality of certain wines (*6,7*). This can be achieved by selecting high glycerol producing strains, or optimizing cultivation conditions.

This is a comparative study determining glycerol production levels of two endogenic wine yeast strains, *Saccharomyces cerevisiae* Kalecik 1 and *Saccharomyces cerevisiae* Narince 3, and demonstrating the influence of inoculum size on growth and glycerol production kinetics of these yeasts.

Materials and Methods

Yeast strains

Two endogenic wine yeast strains *Saccharomyces cerevisiae* Kalecik 1 and *Saccharomyces cerevisiae* Narince 3 were used in the study. These yeasts were isolated from Kalecik Karası and Narince grapes grown 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 University of Ankara, Turkey. The yeasts were kept as stock culture at 4 °C on yeast extract malt extract glucose (YMG) agar consisting of (in g/L): yeast extract 10 (Lab M, UK), malt extract 10 (Lab M, UK), glucose 20 (Merck, Germany), and agar 15 (Lab M, UK).

Preparation of inocula and fermentation medium

Cultures stored in YMG agar were activated in the same medium by maintaining consecutive transfers. The inocula used in the experiments were prepared by incubation at 30 °C for 24 h in a medium containing (in g/L): glucose 20 (Merck, Germany), yeast extract 1 (Lab M, UK), KH_2PO_4 1 (Carlo Erba, Italy), and $MgSO_4·7H_2O$ 0.5 (Pancreac, Spain). The experiments were carried out by using a fermentation medium containing 300 g/L of initial glucose. Other components of the fermentation medium were the same as the medium used for the preparation of inoculum. The medium was sterilized in an autoclave at 121 °C for 10 min. Initial pH of the medium was adjusted to 4 by using 0.1 M HCl (Riedel-De Häen, Germany) in order to obtain a medium with a pH value resembling grape juice.

Equipment and fermentation conditions

Fermentations were run in water bath shakers using cotton-plugged flasks containing 250 mL of fermentation medium. The yeasts were inoculated separately to the fermentation medium at inoculum volume ratio between 2.5–12.5 %. The volume of inoculum that would be transferred was calculated based on the total volume (250 mL) of fermentation medium. Experiments were carried out at constant temperature of 30 °C, with a shaking rate of 70 strokes/min.

Biomass determination

Dry mass 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 medium at specific time intervals, and centrifuged at 5000 rpm for 25 min. 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 concentration in the fermentation medium was determined by periodate-chromotropic acid analysis method (*3,10*). Supernatant of centrifuged samples was used for glycerol analysis spectrophotometrically at 570 nm.

pH measurement

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

Determining the effects of inoculum size on growth and glycerol production kinetics of the strains

Effects of inoculum size (*I*) were investigated in water bath shakers. For both of the strains, specific microbial growth rates (*μ*), specific glycerol production rates (*υ*$_g$), maximum glycerol concentrations (*P*$_m$), maximum dry mass (*X*$_m$), and glycerol yields (*Y*$_{P/S_o}$) 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 (*υ*$_g$) were calculated from the following relationship by using the changes in glycerol concentration and dry mas with time:

$$\upsilon_g = \frac{1}{x} \cdot \frac{dP}{dt} \qquad \text{/1/}$$

Maximum values of the specific glycerol production rates (*υ*) were also determined in the experiments.

Glycerol yields were calculated according to Eq. 2:

$$Y_{P/S_o} = \frac{P_m}{S_o} \cdot 100 \qquad \text{/2/}$$

Statistical analysis

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

Results

During the incubation time, changes in glycerol concentration and dry mass for both of the strains were determined at specific time intervals. Variations in glycerol concentration and dry mass of *S. cerevisiae* Kalecik 1 during fermentation time for each inoculum percentage are shown in Fig. 1. Glycerol concentrations were found to be higher at low inoculum levels. The decrease in glycerol concentration at 12.5 % inoculum can be observed in Fig. 1. There was an apparent increase in cell concentration at 5 % inoculum. It was found that *S. cerevisiae* Kalecik 1 reached maximum specific growth rate at 10 % inoculum volume ratio (Fig. 2). Maximum specific glycerol production rate was obtained in the medium containing 7.5 % inoculum volume ratio (Fig. 2).
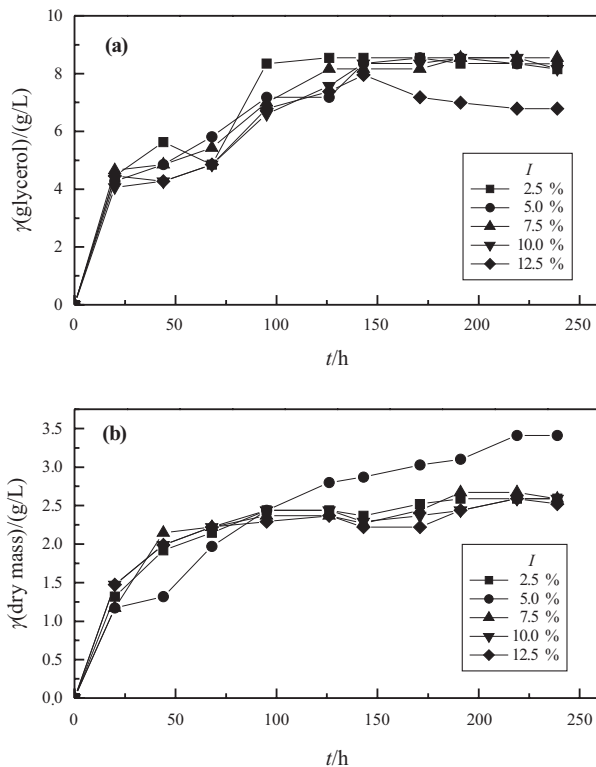
Fig. 1. Variations in glycerol production (a) and dry mass (b) of *S. cerevisiae* Kalecik 1 with different inoculum size
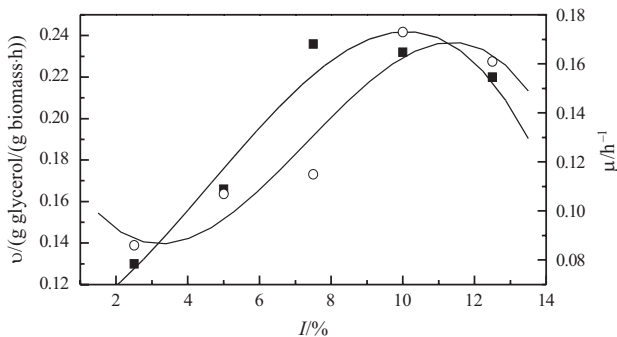

Fig. 2. Variation in specific growth and glycerol production rates related to inoculum size for *S. cerevisiae* Kalecik 1 (μ=specific growth rate (○), 1/h⁻¹; υ=specific glycerol production rate (■), g glycerol/(g biomass·h); I=inoculum size, %)

The polynomial model was estimated for the experimental data of both specific growth and glycerol production rates related to inoculum size by using non-linear regression method. Fig. 2 shows the model outputs and the experimental values of the specific growth and glycerol production rate as a function of inoculum size for *S. cerevisiae* Kalecik 1. The equations obtained by non-linear regression method for the strain Kalecik 1 are shown below:

$$\upsilon = -0.000224 \cdot I^3 + 0.0031 \cdot I^2 + 0.0059 \cdot I + 0.097 \qquad /3/$$
$$R^2 = 0.950$$

$$\mu = -0.00030 \cdot I^3 + 0.0066 \cdot I^2 - 0.033 \cdot I + 0.135 \qquad /4/$$
$$R^2 = 0.913$$

The obtained R^2 coefficients show that the estimated model adequately fits the experimental data.

Effects of inoculum size on maximum glycerol concentration, maximum dry mass, and glycerol yield for the strain Kalecik 1 are shown in Table 1. There was not any important change in glycerol yields ($Y_{P/S}$) and maximum glycerol concentrations (P_m) between inoculum volume ratios of 2.5–7.5 %. Glycerol yield was lower at 12.5 % inoculum than the other inoculum levels. Maximum dry mass (X_m=3.4 g/L) was obtained at 5.0 % inoculum volume ratio for *S. cerevisiae* Kalecik 1.

Table 1. Effects of inoculum size on maximum glycerol concentration, maximum dry mass, and glycerol yield for *S. cerevisiae* Kalecik 1

I	P_m	X_m	Y_{P/S_o}
%	g/L	g/L	%
2.5	8.6	2.6	2.87
5.0	8.5	3.4	2.83
7.5	8.5	2.6	2.83
10.0	8.3	2.5	2.77
12.5	7.5	2.4	2.50

I=inoculum size, P_m=maximum glycerol concentration, X_m=maximum dry mass, Y_{P/S_o}=glycerol yield

For the strain Narince 3, the results of the changes in glycerol concentration and dry mass during fermentation time are shown in Fig. 3. Glycerol concentrations were found to be similar for each inoculum size until the
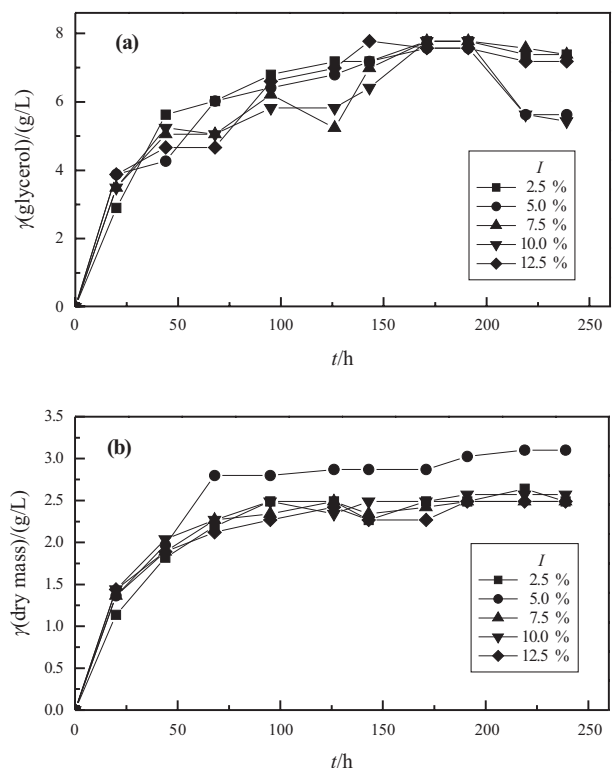

Fig. 3. Variations in glycerol production (a) and dry mass (b) of *S. cerevisiae* Narince 3 at different inoculum size

191th hour. In the media containing 5 and 10 % inoculum, glycerol concentrations decreased after 191 h. It can be seen from Fig. 3 that the best results for cell growth were obtained at 5 % inoculum. Maximum levels for both specific growth and glycerol production rates were obtained in the medium containing 7.5 % inoculum volume ratio (Fig. 4).
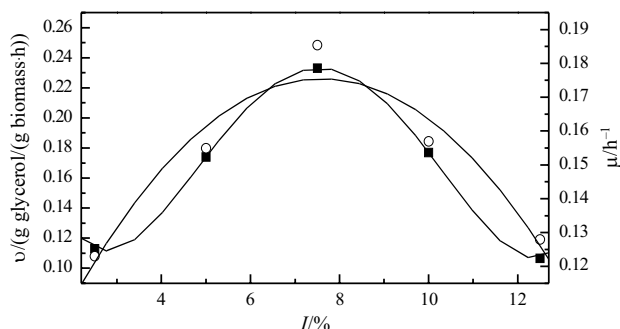

Fig. 4. Variation in specific growth and glycerol production rates related to inoculum size for *S. cerevisiae* Narince 3 (μ=specific growth rate (O), 1/h^{-1}; υ=specific glycerol production rate (■), g glycerol/(g biomass·h); I=inoculum size, %)

The polynomial model was estimated for representation of the experimental data belonging to Narince 3 strain. The equations which represent the relation of specific growth rate and glycerol production rate of *S. cerevisiae* Narince 3 with inoculum size were obtained by using non-linear regression analysis, and are shown below:

$$\upsilon=0.000228 \cdot I^4 - 0.0069 \cdot I^3 + 0.0678 \cdot I^2 - 0.2354 \cdot I + 0.3767 \quad /5/$$
$$R^2 = 1.000$$

$$\mu = 4.27 \cdot 10^{-6} \cdot I^3 - 0.00216 \cdot I^2 + 0.03209 \cdot I + 0.05456 \quad /6/$$
$$R^2 = 0.925$$

It is clear from the R^2 coefficients of Eqs. 5 and 6 that the estimated model adequately fits the experimental data. The curve fittings belonging to the obtained models are represented in Fig. 4, which also shows the validity of the estimated model.

The change in maximum glycerol concentration for *S. cerevisiae* Narince 3 was not found to be important between 2.5–7.5 % inoculum volume ratios, as shown in Table 2. Glycerol yields were nearly constant in the above mentioned inoculum range. Maximum dry mass of 3.1 g/L was obtained at 5 % inoculum volume ratio.

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

I	P_m	X_m	Y_{P/S_o}
%	g/L	g/L	%
2.5	7.6	2.5	2.53
5.0	7.6	3.1	2.53
7.5	7.5	2.4	2.50
10.0	6.5	2.5	2.17
12.5	7.4	2.5	2.47

I=inoculum size, P_m=maximum glycerol concentration, X_m=maximum dry mass, Y_{P/S_o}=glycerol yield

Discussion

Firstly, this study shows that both of the wine yeasts are glycerol producing strains at sufficient amounts. When the results of maximum glycerol concentrations are examined, it can be seen that *S. cerevisiae* Kalecik 1 produces glycerol at higher concentrations than *S. cerevisiae* Narince 3. There was not a considerable change in glycerol concentrations between 2.5–7.5 % inoculum volume ratios for both strains. There was a similarity between the obtained specific glycerol production rate values of the two strains. Maximum specific growth rates were obtained at inoculum volume ratio of 10 % for the strain Kalecik 1, and 7.5 % for the strain Narince 3. When 5 % inoculum volume ratio was used, maximum values for dry mass were obtained for both strains. It is a well-known fact that the density of the used inoculum directly influences the duration of the lag phase, specific growth rate, biomass yield, sporulation, and the quality of the final product in commercial industrial fermentation processes (*11*). It has been reported that high initial cell concentration in the production medium may result in a rapid consumption of oxygen and other nutrients. Consequently, limitation of some nutrients and dissolved oxygen may occur resulting in low growth rate and cell concentration (*12*). Therefore, this can be a reason for the decrease in dry mass and specific growth rate values at high inoculum levels in our study. Our results regarding the glycerol concentrations are in agreement with those obtained in a study performed with the yeast *Candida glycerinogenes* (*3*). In that study, it was reported that different volume ratios of inoculum ranging between 2–8 % did not significantly affect the glycerol concentration. In a study carried out by Vijaikishore and Karanth (*13*), a decrease in glycerol yield was observed when inoculum concentration (dry cell) was increased from 0.312 to 0.937 % (g/100 mL media). It was reported that lower glycerol production levels at higher biomass concentrations can be partly attributed to the limitation of oxygen, causing suboptimal aeration levels. As the inoculum size increases, the demand for oxygen by the cells increases proportionally. Since the oxygen solubility is constant, this increase in oxygen requirement cannot be provided on the shaker, and under such oxygen-starved conditions, the metabolic pathway is switched toward more ethanol formation (*13*). These inadequate aeration levels can trigger anaerobic metabolism and give reduced glycerol yields. So, it is thought that this might be the reason for the decrease in glycerol concentrations, glycerol yields, and specific glycerol production rates at high inoculum concentrations in our study.

It is known that glycerol production could be controlled by the choice of optimized cultivation conditions or selection of appropriate strains. There are several studies indicating the effect of yeast strain on glycerol production (*5,6*). It is demonstrated in a study that obtaining a high glycerol content in wine requires the selection or improvement of yeast strains for high glycerol production rather than the control of growth and cultivation conditions. The difference in the effect of the yeast strains can also be observed from our study. This study pointed out some biochemical characteristics of two different wine yeasts at different inoculum size in a syn-

thetic medium. The obtained data give preknowledge about the possible effects that these yeasts may have during wine production. Glycerol production by those yeasts was also investigated in natural grape juice in our other studies, demonstrating the real behaviour of the strains during wine production. When grape juice was used as fermentation medium, strains Kalecik 1 and Narince 3 produced glycerol at concentrations of 11.8 and 14.1 g/L, respectively (*14,15*). So, these results supported the opinion that the two strains were glycerol enhancing ones during wine production. A more extensive research about this subject is planned for further studies.

Conclusions

This study demonstrated the effect of different inoculum volume ratios on growth and glycerol production characteristics of two endogenic wine yeast strains. With regard to glycerol production, effect of inoculum volume ratio was observed in specific glycerol production rates, rather than maximum glycerol concentrations. It was found that the strains were differently affected by inoculum volume ratio. For each tested inoculum ratio, the strain Kalecik 1 produced higher concentrations of glycerol than those of the Narince 3. Pointing out the effect of inoculum size on glycerol production by two endogenic wine yeast strains, this study may be of concern for wine fermentations.

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