Effects of ammonium sulphate concentration on growth and glycerol production kinetics of two endogenic wine yeast strains

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Effects of initial ammonium sulphate concentration on growth and glycerol production kinetics of two endogenic wine yeast strains, *Saccharomyces cerevisiae* Kalecik 1 and Narince 3, were investigated in a batch system. Maximum specific growth rates for Kalecik 1 and Narince 3 were obtained at initial concentrations of 1.0 g/L, and 0.8 g/L ammonium sulphate, respectively. Further, maximum specific glycerol production rates and glycerol concentrations, for both of the strains, were obtained in the medium containing 0.3 g/L ammonium sulphate. In this medium, maximum glycerol concentrations were 6.4 g/L for the strain Kalecik 1, and 6.5 g/L for Narince 3.

**Keywords:** Fermentation, glycerol, growth kinetics, production kinetics, wine, yeast

**Introduction**

Glycerol is a widely used chemical with many commercial applications, presently finding its largest use in manufacture of foods, drugs, and oral care products including toothpaste, mouthwash and oral rinses. In addition, glycerol is used in cosmetics, tobacco, wrapping and packaging materials, cleaning materials, emulsifiers, etc. Glycerol can be produced either by microbial fermentation or by chemical synthesis from petrochemical feedstocks, or can be recovered as a by-product of soap manufacture from fats. Glycerol was produced by fermentation route for the first time during World War I when demand for glycerol in explosive manufacture exceeded the supply from the soap industry.\(^1,3\)

Glycerol is also an important constituent of wine, providing sweetness, smoothness, and fullness. So, it is an important characteristic for wine strains of *S. cerevisiae* to produce high yields of glycerol. The concentration of glycerol usually formed by *S. cerevisiae* in wine varies between 1-15 g/L, with average values approximately 7 g/L.\(^4,5\)

Increased production of some metabolites in a microbial culture can be achieved by optimization of the relationship between the microorganism and the environment. Many environmental factors like type and initial concentration of substrate or nitrogen source, temperature, \(pH\), aeration rate, inoculum ratio are reported to affect glycerol production by yeasts.\(^1,5-7\) Variety and initial concentration of the nitrogen source are also reported to play an important role in glycerol production by yeasts.\(^3\)

This paper reports the influence of initial ammonium sulphate concentration on growth and glycerol production characteristics of two different wine yeast strains, *S. cerevisiae* Kalecik 1, and Narince 3.

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Materials and Methods

Yeast Strains

Two endogenic wine yeast strains *S. cerevisiae* Kalecik 1 and Narince 3 were used in the study. These yeasts had been isolated from Kalecik Karası and Narince grapes, which are commercially used for wine production in Turkey. The strains were kindly provided by Professor F Özçelik (University of Ankara, Department of Food Engineering, Turkey). The yeasts were kept as stock culture at 4°C on Yeast extract malt extract glucose (YMG) agar, which L⁻¹ consists of 10 g yeast extract (Lab M, UK), 10 g malt extract (Lab M, UK), 20 g glucose (Merck, Germany), and 15 g agar (Lab M, UK).

Preparation of Inocula and Fermentation Medium

Cultures stored on YMG agar were activated in the same medium by maintaining consecutive transfers. The inocula to be used in the experiments were prepared by incubation at 30°C for 24 h in a medium containing L⁻¹ 20 g glucose (Merck, Germany), 1 g yeast extract (Lab M, U.K), 1 g KH₂PO₄ (Carlo Erba, Italy), and 0.5 g MgSO₄.7H₂O (Pancreac, Spain). The experiments were carried out by using a fermentation medium containing 300 g/L glucose, 0.1-1.0 g/L ammonium sulphate as initial concentration, and without yeast extract. Other components of the fermentation medium were kept same as the medium used for preparation of inoculum. Medium was sterilized in autoclave at 121°C for 10 min. Initial pH of the medium was adjusted to 4.00 by using 0.1 N HCl (Riedel-De Häen, 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. Temperature was kept constant at 30°C and shaking rate at 70 strokes/min during the experiments.

Biomass Determination

Dry weight of the yeast was determined spectrophotometrically by using wet weight-absorbance and wet weight-dry weight calibration curves, which had been prepared earlier. During the experiments, samples were taken from the fermentation media at certain time intervals and centrifuged at 5000 rpm for 25 min. Cell pellet was used for determining dry weight 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. Supernatant of centrifuged samples were used for glycerol analysis spectrophotometrically at 570 nm.

Effect of Initial Ammonium Sulphate Concentration on Growth and Glycerol Production Kinetics of Strains

Effects of initial ammonium sulphate concentration (S<sub>ao</sub>) was investigated in water bath shakers. The yeasts were inoculated separately to fermentation media at an inoculation ratio of 5%. Initial ammonium sulphate concentrations of the fermentation media were 0.1-1.0 g/L. For both the strains, specific microbial growth rates (µ), specific glycerol production rates (υ<sub>g</sub>), maximum glycerol concentrations (P<sub>m</sub>), maximum dry weights (x<sub>m</sub>), and glycerol yields (Y<sub>P/Sa</sub>) were calculated. Specific microbial growth rates were determined from the graphs of the changes of dry weight with fermentation time. Specific growth rate values were calculated from the logarithmic plots of the dry weight data versus time. Specific glycerol production rates were calculated from the following relationship by using the changes in glycerol and dry weight concentrations with time.

\[
\frac{dP}{dt} = \frac{1}{x} \frac{dP}{dt} \quad \ldots(1)
\]

Maximum values of the specific glycerol production rates (υ<sub>g</sub>) 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 concentrations and dry weights for both of the strains were determined at specific time intervals. Variations in glycerol concentration and dry weight of *S. cerevisiae* Kalecik 1 during fermentation time for each initial ammonium sulphate concentration is shown in (Fig. 1A & B). It was observed that specific
growth rate of the strain Kalecik 1 increased with increasing initial ammonium sulphate concentration in the studied concentration range, reaching its maximum level in the medium with 1.0 g/L initial ammonium sulphate concentration (Fig. 1B).

Maximum specific glycerol production rate was obtained in the medium initially containing 0.3 g/L of ammonium sulphate (Fig. 1A).

The polynomial model was estimated for the experimental data of both specific growth and glycerol production rates related to initial ammonium sulphate concentration 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 ammonium sulphate concentration for Kalecik 1. The equations obtained by non-linear regression method for the strain Kalecik 1 are shown below:

\[
\mu = -0.030 \, S_{Ao}^2 + 0.078 \, S_{Ao} + 0.111 \quad \ldots (2) \\
R^2 = 0.973
\]

\[
\nu = 3.788 \, S_{Ao}^3 - 7.781 \, S_{Ao}^2 + 4.315 \, S_{Ao} + 0.0134 \quad \ldots (3) \\
R^2 = 0.954
\]

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

Effects of initial ammonium sulphate concentration on maximum glycerol concentration, maximum dry weight, and glycerol yield for Kalecik 1 (calculated data) are shown in Table 1. Maximum dry weight of 1.44 g/L was obtained at 1.0 g/L initial ammonium sulphate concentration, while maximum values for glycerol concentration and glycerol yield were obtained in the medium initially containing 0.3 g/L ammonium sulphate.

Changes in glycerol concentration and dry weight of *S. cerevisiae* Narince 3 during fermentation time were represented in Fig. 3A & B. It was found that this strain reached maximum specific growth rate in the medium with 0.8 g/L initial ammonium sulphate concentration (Fig. 3B). In the medium initially containing 0.3 g/L of ammonium sulphate, maximum specific glycerol production rate was obtained for the strain Narince 3 (Fig. 3A).

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 Narince 3 with initial ammonium sulphate concentration were obtained by using non-linear regression analysis, and are shown below:
\[ \mu = -0.3003 S_{Ao}^3 + 0.3678 S_{Ao}^2 - 0.0610 S_{Ao} + 0.1199 \]  
\[ R^2 = 0.976 \]  
\[ \nu = 1.9916 S_{Ao}^3 - 3.8761 S_{Ao}^2 + 2.0841 S_{Ao} + 0.3644 \]  
\[ R^2 = 0.957 \]  

It is clear from the \( R^2 \) coefficients of Eqs 4 and 5 that the estimated model 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.

Calculated data for maximum glycerol concentrations, maximum dry weights, and glycerol yields of \( S. cerevisiae \) obtained in the media with different initial ammonium sulphate concentrations are shown in Table 2. Maximum values for glycerol concentration, dry weight, and glycerol yield were obtained in the medium initially containing 0.3 g/L of ammonium sulphate.

**Discussion**

From the results, it can be inferred that initial ammonium sulphate concentration significantly affected cell growth and glycerol production of both the strains. Growth of \( S. cerevisiae \) Kalecik 1 was positively affected by increasing ammonium sulphate concentration in the studied range and both specific growth rate and dry weight were increased. However, high concentrations of ammonium sulphate did not have the same effect on growth of strain Narince 3. For this strain, a decrease in specific growth rate was determined above 0.8 g/L ammonium sulphate, while dry weight tends to decrease above the concentration of 0.3 g/L. It is known that ammonium salts are a good source of assimilable nitrogen for yeast growth, hence fermentation media are often supplemented with inexpensive inorganic nitrogen forms, such as ammonium sulphate. Ammonium sulphate also

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**Table 1**—Effects of initial ammonium sulphate concentration on maximum glycerol concentration, maximum dry weight, and glycerol yield for \( S. cerevisiae \) Kalecik 1

<table>
<thead>
<tr>
<th>( S_{Ao} ) (g/L)</th>
<th>( P_m ) (g/L)</th>
<th>( X_m ) (g/L)</th>
<th>( Y_{P/So} ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>5.0</td>
<td>1.08</td>
<td>1.67</td>
</tr>
<tr>
<td>0.3</td>
<td>6.4</td>
<td>1.30</td>
<td>2.13</td>
</tr>
<tr>
<td>0.5</td>
<td>5.8</td>
<td>1.40</td>
<td>1.93</td>
</tr>
<tr>
<td>0.8</td>
<td>5.7</td>
<td>1.42</td>
<td>1.90</td>
</tr>
<tr>
<td>1.0</td>
<td>5.7</td>
<td>1.44</td>
<td>1.90</td>
</tr>
</tbody>
</table>

\( S_{Ao} \): initial ammonium sulphate concentration, \( P_m \): maximum glycerol concentration, \( X_m \): maximum dry weight, \( Y_{P/So} \): glycerol yield
provides a source of assimilable sulphur for the yeast. But ammonium salts are not enough alone for the enhancement of yeast growth, since the cell requires growth factors such as amino acids, vitamins, nucleotides, fatty acids, which have specific roles in catalytic or structural reactions. In our previous study in which yeast extract was used in the fermentation medium, dry weight of S. cerevisiae Narince 3 was obtained 2 to 2.5-fold greater than the values obtained in the present study at the same initial glucose concentration. The suppression of cell growth in the present study can be explained by the absence of yeast extract in the fermentation medium, which provides nitrogen and growth factors for the yeasts. It is reported that, when nitrogen content of a fermentation medium is decreased, the sensitivity of S. cerevisiae strains to ethyl alcohol increases. It is thought that this is also a reason for the depression of the cell growth of the studied yeasts.

With regard to glycerol production, similar results were obtained for both the strains. Both produced maximum concentration of glycerol at 0.3 g/L initial ammonium sulphate concentration; the concentrations of glycerol were 6.4 g/L for Kalecik 1 and 6.5 g/L for Narince 3. Specific glycerol production rates and glycerol yields were also at maximum levels at this initial concentration of ammonium sulphate. The obtained values for specific glycerol production rates of Narince 3 were relatively high when compared to the results of our previous study. The same conclusion can be drawn for Kalecik 1 strain. The higher glycerol production rates obtained in the medium lacking proteins can be explained in relation with protein synthesis. It is indicated in several studies that higher glycerol yields are obtained in the media with ammonium sulphate rather than with glutamate, or aminoacid mixtures. It is also reported that protein synthesis in the yeast cell increases in the media having low levels of protein or lack of protein. During protein synthesis, the generated NADH decreases the redox potential in the cell and formation of glycerol by reduction of dihydroxyacetone phosphate is required in order to maintain the redox balance of the cell. So, it could be concluded that increase in glycerol production rate in this study might be a result of the increase in protein synthesis.

Table 2—Effects of initial ammonium sulphate concentration on maximum glycerol concentration, maximum dry weight, and glycerol yield for S. cerevisiae Narince 3

<table>
<thead>
<tr>
<th>S&lt;sub&gt;0&lt;/sub&gt; (g/L)</th>
<th>P&lt;sub&gt;m&lt;/sub&gt; (g/L)</th>
<th>X&lt;sub&gt;m&lt;/sub&gt; (g/L)</th>
<th>Y&lt;sub&gt;PSo&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>4.0</td>
<td>1.20</td>
<td>1.33</td>
</tr>
<tr>
<td>0.3</td>
<td>6.5</td>
<td>1.62</td>
<td>2.17</td>
</tr>
<tr>
<td>0.5</td>
<td>5.5</td>
<td>1.46</td>
<td>1.83</td>
</tr>
<tr>
<td>0.8</td>
<td>5.3</td>
<td>1.52</td>
<td>1.77</td>
</tr>
<tr>
<td>1.0</td>
<td>6.0</td>
<td>1.50</td>
<td>2.00</td>
</tr>
</tbody>
</table>

S<sub>0</sub>: initial ammonium sulphate concentration, P<sub>m</sub>: maximum glycerol concentration, X<sub>m</sub>: maximum dry weight, Y<sub>PSo</sub>: glycerol yield

References