STATISTICAL APPROACH TO OPTIMIZATION OF ETHANOL FERMENTATION BY *Saccharomyces cerevisiae* IN THE PRESENCE OF VALFOR® 100 ZEOLITE NAA

**OPTIMIZACIÓN ESTADÍSTICA DE LA FERMENTACIÓN ETANÓLICA DE *Saccharomyces cerevisiae* EN PRESENCIA DE ZEOLITA VALFOR® NAA**


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**Abstract**

The technologies for ethanol production from sugars, starch and lignocellulosic materials for food and biofuel applications are being constantly improved. A number of modifications to increase the production and yield of ethanol have been implemented such as immobilization of cells, genetic modification and use of mixed cultures. In this work, the addition of zeolites to increase the alcohol production of the yeast *Saccharomyces cerevisiae* was studied. The experiments were designed with seven factors for ethanol yield (carbon and nitrogen source, Mg²⁺ and zeolite concentration, temperature, pH and inoculum size) at two levels with an orthogonal array layout of L₈(2⁷) designed to keep the number of experiments to a minimum. Addition of 0.2 g L⁻¹ of Valfor® 100 zeolite NaA resulted in important increases in ethanol production (20%) and yield (25%). An adsorption phenomenon could be observed by SEM between the zeolite particles and the yeast cells. This and the well known effects of toxic cation concentration decrease, pH regulation and ethanol and carbon dioxide adsorption could have caused the improvement in the ethanol production and yield. The optimization study indicated that zeolite concentration was the most significant factor in this increase even though it was used at lower levels compared with other studies, indicating the importance of the optimization studies in bioprocesses.

**Keywords:** zeolite, ethanol, Taguchi optimization, *Saccharomyces cerevisiae*.

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1. **Introduction**

The progressive depletion of the energy resources mainly based on non-renewable fuels and the record-high gasoline prices have shifted the attention to the production of ethanol, the most common renewable fuel produced from sugar or grains (Hahn-Hägerdal *et al.*, 2006; Sánchez and Cardona, 2008; Yang & Wyman, 2007). Ethanol is also an important product for the alcoholic beverage industry including beer,
Table 1. L8 (2^7) orthogonal array for ethanol, YP/S and cell biomass production

<table>
<thead>
<tr>
<th>Run</th>
<th>Sucrose (g L^-1)</th>
<th>(NH_4)_2SO_4 (g L^-1)</th>
<th>MgSO_4 (g L^-1)</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Inoculum (g L^-1)</th>
<th>Zeolite (g L^-1)</th>
<th>Ethanol* (g L^-1)</th>
<th>YP/S* (g ethanol g sucrose^-1)</th>
<th>Biomass* (g L^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200</td>
<td>0.35</td>
<td>0.020</td>
<td>32</td>
<td>4.3</td>
<td>2</td>
<td>200</td>
<td>79.40±3.18</td>
<td>0.484</td>
<td>4.3±0.13</td>
</tr>
<tr>
<td>2</td>
<td>180</td>
<td>0.25</td>
<td>0.020</td>
<td>28</td>
<td>4.3</td>
<td>2</td>
<td>50</td>
<td>71.46±2.86</td>
<td>0.476</td>
<td>5.2±0.16</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>0.25</td>
<td>0.024</td>
<td>32</td>
<td>4.3</td>
<td>3</td>
<td>50</td>
<td>73.84±2.95</td>
<td>0.453</td>
<td>7.4±0.22</td>
</tr>
<tr>
<td>4</td>
<td>200</td>
<td>0.35</td>
<td>0.020</td>
<td>28</td>
<td>4.7</td>
<td>3</td>
<td>50</td>
<td>71.46±2.95</td>
<td>0.493</td>
<td>6.7±0.21</td>
</tr>
<tr>
<td>5</td>
<td>200</td>
<td>0.25</td>
<td>0.24</td>
<td>28</td>
<td>4.7</td>
<td>2</td>
<td>200</td>
<td>87.34±3.50</td>
<td>0.508</td>
<td>7.4±0.24</td>
</tr>
<tr>
<td>6</td>
<td>180</td>
<td>0.25</td>
<td>0.020</td>
<td>32</td>
<td>4.7</td>
<td>3</td>
<td>200</td>
<td>79.40±2.94</td>
<td>0.509</td>
<td>7.1±0.21</td>
</tr>
<tr>
<td>7</td>
<td>180</td>
<td>0.35</td>
<td>0.024</td>
<td>32</td>
<td>4.7</td>
<td>2</td>
<td>50</td>
<td>63.52±2.54</td>
<td>0.467</td>
<td>4.8±0.14</td>
</tr>
<tr>
<td>8</td>
<td>180</td>
<td>0.35</td>
<td>0.024</td>
<td>28</td>
<td>4.3</td>
<td>3</td>
<td>200</td>
<td>83.37±3.33</td>
<td>0.511</td>
<td>6.8±0.20</td>
</tr>
</tbody>
</table>

*Values after 56 h of fermentation

2.2. Optimization of fermentation conditions using the L8-orthogonal array

The design for the Taguchi L8-orthogonal array (2^7) was developed and analyzed using Design Expert 7.0.3 (Stat Ease Inc., Minneapolis, USA) software. Table 1 shows the fermentation conditions tested according to the experimental design used in this study along with the resulting ethanol and biomass concentration after 56 h of incubation. The assayed fermentation conditions were: sucrose (180 and 200 g L^-1), ammonium sulfate (0.25 and 0.35 g L^-1), magnesium sulfate (0.02 and 0.024 g L^-1) and zeolite (50 and 200 mg L^-1) concentrations, incubation temperature (28 and 32°C), initial pH of the medium (4.3 and 4.6) and size of the inoculum (2 and 3 g L^-1). The zeolite utilized was Valfor® 100 (The PQ Corporation, Malvern, USA), a white hydrated zeolite sodium A powder with condensed formula: Na_12 [(Al O_2)_{12} (SiO_2)_{12}]·7 H_2O, an average particle size of 3.6 μm and a nominal pore diameter of 4.2 Å.

2.3. Fermentation

Batch ethanol production was carried out under anaerobic conditions in a 4 L glass vessel bioreactor containing 3 L of medium whose composition varied according to the experimental design shown in Table 1. Cell biomass production was carried out aerobically in 1 L Erlenmeyer flasks containing 150 mL of the above medium on a rotary shaker at 200 rpm. This assay was performed to be able to compare the effect of the zeolite and the medium composition.
in anaerobic and aerobic conditions. Both systems were previously tested and proved to be adequate in our laboratory (data not shown).

2.4. Analysis

Ethanol was obtained by distillation of the fermentation broth and its concentration was determined according to the specific-gravity method 942.06 of the AOAC (AOAC International, 1995). The \( Y_{P:S} \) yield coefficient was calculated according to the following definition (Blanch & Clark, 1997):

\[
Y_{P:S} = \frac{\text{mass product produced}}{\text{mass substrate consumed}}
\]

In this case, ethanol was the product and sucrose the substrate. The decrease in sucrose was measured by the Lane-Eynon method (AOAC methods 920.183b and 923.09) (AOAC International, 1995). Biomass concentration, determined as dry weight, was measured after the sample was vacuum filtrated through a Whatman 5 filter paper and dried at 65°C for 96 h to a constant weight. All the results were expressed as the average of three determinations. An additional run on the ethanol production was performed in order to evaluate the behavior of the yeast in the absence of ethanol. Production was performed in order to evaluate the behavior of the yeast in the absence of ethanol. The yeasts were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2), dehydrated with ethanol, critical point dried, and coated with gold alloy (Bomchil et al., 2003). Observations were performed within an amplification range of 890-3520 X.

2.5. Scanning electron microscopy (SEM)

A Tescan VEGA II LM U Scanning Electron Microscope (Czech Republic) operated at a high vacuum and fitted with a detector of secondary electrons and a voltage acceleration of 10 KV was used to try to observe the interactions between the zeolite and the yeast cells. For SEM, samples are usually required to be completely dry, since the specimen chamber is at a high vacuum. Also, for adequate imaging, specimens must be electrically conductive, at least at the surface, and electrically grounded to prevent the accumulation of electrostatic charge at the surface. Nonconductive specimens, like biological material, tend to charge when scanned by the electron beam, and especially in secondary electron imaging mode, causing scanning faults and other image artifacts. They are therefore usually coated with an ultrathin coating of electrically-conducting material such as gold, deposited on the sample by low vacuum sputter coating. In this case, samples of culture media containing zeolites and yeasts were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h and then postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2), dehydrated with ethanol, critical point dried, and coated with gold alloy (Bomchil et al., 2003). Observations were performed within an amplification range of 890-3520 X.

2.6. Data analysis

Once the data for the three responses (ethanol and biomass concentration after 56 h and \( Y_{P:S} \)) were introduced in the software (Design Expert), the ANOVA for a multiple linear regression model was performed and the significance and determination coefficient \( R^2 \) were calculated. A model was significant \( (p \leq 0.05) \), its coefficients along with their significance were determined. Next, the non significant coefficients were deleted from the model and the effects, sum of squares and percent contribution were calculated for the significant ones. An effect is defined as the change in response as the factor changes from its low to its high level. The sum of squares \( (SS) \) for a term is the amount of information that can be attributed to the term as it changes. The percent contribution is obtained by summing all the term sum of squares and then taking each individual SS and dividing by the total SS and multiplying by 100. When all the terms have the same degrees of freedom (as in this case), the % contribution is used to determine which terms are larger contributors than others. The software also allows the numerical optimization of the models. A desired goal for each factor and response is chosen from the menu. The possible goals are: maximize, minimize, target, within range, none (for responses only) and set to an exact value (factors only).

A minimum and a maximum level must be provided for each parameter included. A weight can be assigned to each goal to adjust the shape of its particular desirability function. The "importance" of each goal can be changed in relation to the other goals. The default is for all goals to be equally important. The goals are combined into an overall desirability function. The program seeks to maximize this function. The goal seeking begins at a random starting point and proceeds up the steepest slope to a maximum. There may be two or more maximums because of curvature in the response surfaces and their combination into the desirability function. By starting from several points in the design space chances improve for finding the "best" local maximum.

3. Results and discussion

Fermentation experiments with the designed experimental conditions showed great variation in the final concentrations of ethanol and cell biomass and in the \( Y_{P:S} \) yield coefficient (Table 1). In the case of ethanol production, the software fitted the data to the following significant \( (p \leq 0.05) \) model:

\[ \text{[Ethanol]} = 80.66 + 0.1787\text{[Sucrose]} - 35.73\text{[Ammonium sulfate]} + 397\text{[Magnesium sulfate]} - 1.0918\text{Temperature} - 3.97\text{pH} + 1.5888\text{[Inoculum]} + 0.082047\text{[Zeolite]} \]

The above model had a determination coefficient \( R^2 = 1.00 \) and all the residuals were zero.
Table 2. Contribution of each fermentation factor to ethanol production and YP/S

<table>
<thead>
<tr>
<th>Factor</th>
<th>% Contribution to ethanol production</th>
<th>% Contribution to YP/S coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Sucrose]</td>
<td>6.27</td>
<td>2.43</td>
</tr>
<tr>
<td>[Ammonium sulfate]</td>
<td>6.27</td>
<td>0.31</td>
</tr>
<tr>
<td>[Magnesium sulfate]</td>
<td>1.24</td>
<td>2.05</td>
</tr>
<tr>
<td>Temperature</td>
<td>9.37</td>
<td>21.84</td>
</tr>
<tr>
<td>pH</td>
<td>1.24</td>
<td>10.90</td>
</tr>
<tr>
<td>[Inoculum]</td>
<td>1.24</td>
<td>3.73</td>
</tr>
<tr>
<td>[Zeolite]</td>
<td>74.38</td>
<td>58.73</td>
</tr>
</tbody>
</table>

For the YP/S, the model, also significant, was as follows:

\[ Y_{PS} = 0.51844 - 3.125 \times 10^{-4} [\text{Sucrose}] + 0.0225 [\text{Ammonium sulfate}] - 1.4375 [\text{Magnesium sulfate}] - 4.688 \times 10^{-3} [\text{Temperature}] + 0.0821 [\text{Inoculum}] + 0.0331 [\text{pH}] + 7.75 \times 10^{-3} [\text{Zeolite}] \]

The R² was also 1.00.

When the numerical optimization of the software was selected, the optimal combination for maximal ethanol concentration and YP/S after 56 h of fermentation was as follows: sucrose 200 g L⁻¹, (NH₄)₂SO₄ 0.25 g L⁻¹, MgSO₄ 0.024 g L⁻¹, temperature 28°C, initial pH 4.7, inoculum size 2 g L⁻¹ and zeolite 200 mg L⁻¹, which are the conditions of run 5. When this combination was used, ethanol concentration produced (P), yield (YP/S) and productivity (Qp) were 87.34 g L⁻¹, 0.508 g ethanol g sucrose⁻¹ and 1.559 g L⁻¹ h⁻¹ respectively. In these conditions, the ethanol production was 20% higher than the production obtained under the conditions of run 5 but in the absence of zeolite (72.8 g L⁻¹). The YP/S value of 0.508 g ethanol g sucrose⁻¹ (run 5) is 21% higher than the value obtained by Laopaiboon et al. (2007) when sweet sorghum juice supplemented with 0.5% ammonium sulfate was used as substrate. The contribution of each fermentation factor to ethanol production and YP/S are shown in Table 2. It can be observed that the concentration of zeolite is the largest positive contributor with 74.38% for ethanol and 58.73% for YP/S. Also, in both cases, temperature is the second main effect, however, it was a negative one (see the negative sign of the coefficient in both models). It is also worth mentioning that although the ammonium ion has been reported as a potential stimulator of ethanol production by *Saccharomyces cerevisiae* (Harding et al., 1984), in this case ammonium sulfate have a positive effect on the yield but not on the production of ethanol.

In the case of the YP/S coefficient, an important increase of 25% with respect to Medium A was obtained, also related to the addition of zeolite. This effect is in agreement with the data of Castellar et al. (1998). They studied the effect of zeolite NaY on ethanol production from glucose by *Saccharomyces bayanus* and found that the addition of 5 g L⁻¹ of zeolites improved the production of ethanol. The highest ethanol concentration (130 g L⁻¹) was obtained from a 350 g L⁻¹ glucose medium which could be used due to the osmotolerance of this yeast. They concluded that the zeolite acted as a buffer keeping a pH value adequate for the yeast viability and metabolic activity. Tosun and Ergun (2008) also found a positive effect of zeolite addition on ethanol production from synthetic molasses by *S. cerevisiae*. They found that the addition of 5 g L⁻¹ of Ca-Montmorillonite and 10 g L⁻¹ of zeolite NaY resulted in increases of 24 and 40% in ethanol production. They concluded that the addition of these compounds decreased the toxic effects of some cations and also acted as a buffer improving in this way the fermentative performance of the yeast. In our case, the addition of only 0.2 g L⁻¹ of the zeolite Valfor 100 NaA had an effect in the same order of 5 g L⁻¹ of the Ca-Montmorillonite in the improvement of ethanol production. It has been shown that some zeolites (NaZSM-5) have a high selectivity for ethanol and that the contact of the fermentation broth with them avoids the inhibition by final product therefore improving the production of the alcohol (Adnadev et al., 2008; Einicke et al., 1991). There are also reports about the capacity of CO₂ absorption of zeolites (Roque-Malherbe et al., 1987), so the possible inhibitory effect of this other final product could be diminished too. It is possible that a combination of all the effects described above is responsible for the important increase in ethanol production by *S. cerevisiae*.

In the case of biomass production, the zeolite addition is not as important as in the case of ethanol since similar concentrations (7.4 g L⁻¹) were achieved in runs 3 and 5 with totally different zeolite concentrations and similar medium composition (see Table 1). No significant model could be fitted to this process response.

SEM images of *Saccharomices cerevisiae*-culture media at 0 and 24 h fermentation time are shown in figs. 1 and 2. It is noteworthy that a complex zeolite-yeast starts to be formed as from initial contact (Fig. 1) and evident adsorption of zeolites onto the surface of the microorganism was observed after 24 h of fermentation time which lead to saturation of the surface with the mineral (Fig. 2). These phenomena have been reported by other authors (Kubota et al., 2008; Roque-Malherbe et al., 1987) as the attachment mechanism of gram-positive bacteria and yeast to zeolites. Also, formation of the complex induces a deformation of the attachment site on the surface of *Saccharomices cerevisiae*. These phenomena are evident and highlighted in Fig. 2. From SEM micrographs three stages could occur during the formation of the zeolite-yeast complex:

a. Initial contact zeolite-yeast
b. Deformation of the attachment site on the surface of the yeast.
c. Saturation of surface of yeast with zeolites.
Conclusions

Culture conditions and medium composition optimization by the Taguchi method of orthogonal array (OA) experimental design led to a significant increase in ethanol production and yield. This method also identified the influence of individual fermentation factors on the process. Zeolite concentration was, by far, the most significant factor in this increase even though it was used at lower levels compared with other studies, indicating the importance of the optimization studies in bioprocesses. Strong adsorption phenomena could be observed by SEM which could indicate that immobilization of the yeast cells along with adsorption of ethanol and carbon dioxide could be important in explaining the increase in ethanol production and yield.

References


Kubota, M., Nakabayashi, T., Matsumoto, Y., Shiomi, T., Yamada, Y., Ino, K.,...


