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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, Mg^{2+} and zeolite concentration, temperature, pH and inoculum size) at two levels with an orthogonal array layout of L8 (2^7) 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.*

Resumen

En la actualidad existe gran interés en mejorar las tecnologías para producir etanol a partir de azúcares, almidón y materiales lignocelulósicos para aplicaciones en alimentos y como biocombustibles. Se han introducido varias modificaciones para incrementar la producción y el rendimiento de etanol como son la inmovilización de células, la modificación genética y el uso de cultivos mixtos. En este trabajo se estudió la adición de zeolitas para incrementar la producción de etanol de la levadura *Saccharomyces cerevisiae*. El diseño experimental se planeó con siete factores importantes para la producción de etanol (concentración de las fuentes de carbono y nitrógeno, Mg^{+2} y zeolita, temperatura, pH y tamaño de inóculo) a dos niveles con una matriz de diseño ortogonal L8 (2^7) diseñada para un número mínimo de experimentos. La adición de 0.2 g L^{-1} de la zeolita Valfor® 100 resultó en incrementos importantes en la producción (20%) y rendimiento (25%) de etanol. Se pudo observar el fenómeno de adsorción entre las partículas de zeolita y las células de levadura por microscopía electrónica de barrido. Esto y los efectos conocidos de adsorción de cationes tóxicos, regulación de pH y adsorción de etanol y CO_2 podrían ser responsables de la mejora en la producción y rendimiento de alcohol. El estudio de optimización indicó que la concentración de zeolita fue el factor más importante para este aumento, sobretodo porque se usaron niveles más bajos que en otros estudios, indicando la importancia de los estudios de optimización en bioprocesos.

Palabras clave: zeolita, etanol, optimización de Taguchi, *Saccharomyces cerevisiae.*

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,

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Table 1. L_8 (2^7) orthogonal array for ethanol, $Y_{P/S}$ and cell biomass production

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

*Values after 56 h of fermentation

wine and spirits; the perennial choice for the production of ethanol has been the yeast *Saccharomyces cerevisiae*, a microorganism able to ferment glucose to ethanol efficiently (Jeffries, 2005). The ethyl alcohol production depends on different factors such as nitrogen source, temperature, presence of metal cations and pH. Different approaches have been used to improve the production of ethanol fermentation including the use of recombinant strains of *S. cerevisiae* (Jeffries, 2005) and the addition of different kinds of zeolites (Castellar *et al.,* 1998; Tosun and Ergun, 2008). Zeolites are crystalline aluminosilicate materials with nanoporous structures (pores with diameters of 1 nm) with different applications like catalysis, purification systems and now biotechnological processes (Chmelka, 2006). Currently, Mexico produces only around 60 million liters of ethanol per year, mainly from sugar cane. However, this country will start producing ethanol to be used as biofuel in 2010 with a goal production of 800 million liters for 2012. Zeolite addition is one of the new technologies that could be implemented for this goal to be reached. This paper presents the application of Taguchi method of orthogonal array (OA) experimental design for the optimization of ethanol production by the fermentation process. Taguchi approach has been previously shown to have potential use in bioprocess optimization (Prasad *et al.,* 2005). The experiments were designed with seven factors for ethanol yield (carbon and nitrogen source, Mg^{2+} and zeolite concentration, temperature, pH and inoculum size) at two levels with an OA layout of L8 (2^7) designed to keep the number of experiments to a minimum.

2. Materials and methods

2.1. Microorganism and media

Saccharomyces cerevisiae yeast was obtained from the collection of the Biochemical Engineering Department (Escuela Nacional de Ciencias Biológicas, IPN, México City, Mexico). The yeast was maintained on potato dextrose agar (PDA) slants and stored at 4ºC with periodic (1 month) subculturing. For the production of the inoculum, a loopful of cells from a slant was suspended in 10 mL of a medium (A) containing (g L^{-1}): sucrose (Dibico,México), 6; $MgSO_4 \cdot 7H_2O$ (Alyt, México), 0.024; $(NH_4)_2SO_4$ (Alyt, México), 0.3; KH_2PO_4 (Alyt, México), 0.24. The pH of the medium was adjusted to 4.5 with 1N sulfuric acid (J.T. Baker, México) after sterilization in an autoclave for 15 min at 121ºC. The cell suspension was inoculated in 150 mL of sterile medium A in a 1000 mL Erlenmeyer flask, and fermentation was carried out incubating at 28ºC with agitation at 200 rpm for 24 h.

2.2. Optimization of fermentation conditions using the L_8-orthogonal array

The design for the Taguchi L_8-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 32ºC), 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} $[(Al\ O_2)_{12}$ $(SiO_2)_{12}] \cdot 27\ H_2O$, 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 65ºC 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 zeolite and under optimal conditions. The zeolite-free medium contained (g L^{-1}): sucrose 200, ammonium sulfate 0.3 and magnesium sulfate 0.024 and was incubated at 28ºC.

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. When 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 ($p < 0.05$) model:

[Ethanol] = 80.66 + 0.1787[Sucrose] – 35.73[Ammonium sulfate] + 397[Magnesium sulfate] – 1.0918Temperature – 3.97pH + 1.5888[Inoculum] + 0.082047[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 $Y_{P/S}$

Factor	% Contribution to ethanol production	% Contribution to $Y_{P/S}$ coefficient
[Sucrose]	6.27	2.43
[Ammonium sulfate]	6.27	0.31
[Magnesium sulfate]	1.24	2.05
Temperature	9.37	21.84
pH	1.24	10.90
[Inoculum]	1.24	3.73
[Zeolite]	74.38	58.73

For the $Y_{P/S}$, the model, also significant, was as follows:

$$\mathbf{Y_{P/S} = 0.51844 - 3.125X10^{-4}[Sucrose] + 0.0225[Ammonium\ sulfate] - 1.4375[Magnesium\ sulfate] - 4.688X10^{-3}Temperature + 0.0331pH + 7.75X10^{-3}[Inoculum] + 0.0821[Zeolite]}$$

The R^2 was also 1.00.

When the numerical optimization of the software was selected, the optimal combination for maximal ethanol concentration and $Y_{P/S}$ after 56 h of fermentation was as follows: sucrose 200 g L^{-1}, $(NH_4)_2SO_4$ 0.25 g L^{-1}, $MgSO_4$ 0.024 g L^{-1}, temperature 28ºC, 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 ($Y_{P/S}$) and productivity (Q_p) 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 $Y_{P/S}$ 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 $Y_{P/S}$ 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 $Y_{P/S}$. 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 $Y_{P/S}$ 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 (Ađnađević *et al.,* 2008; Einicke *et al.,* 1991). There are also reports about the capacity of CO_2 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.

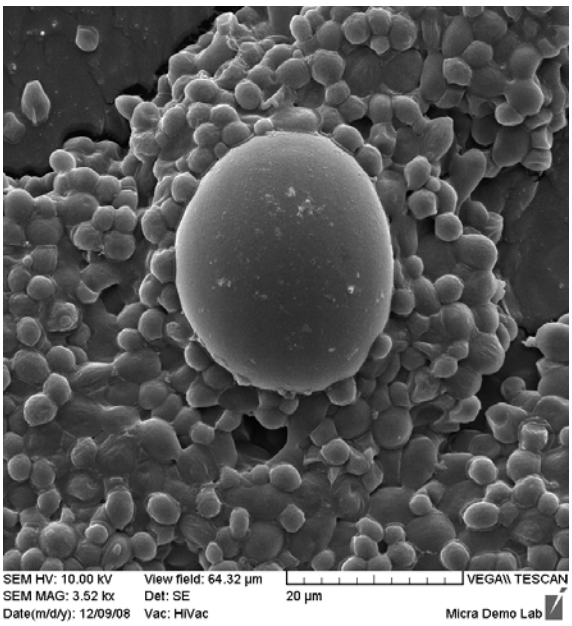

Fig. 1. Cell of *Saccharomyces cerevisiae* in the optimized medium (200 mg L^{-1} of zeolite) at the start of the fermentation (t = 0 h) surrounded by the smaller zeolite particles. Magnification 3520 X.

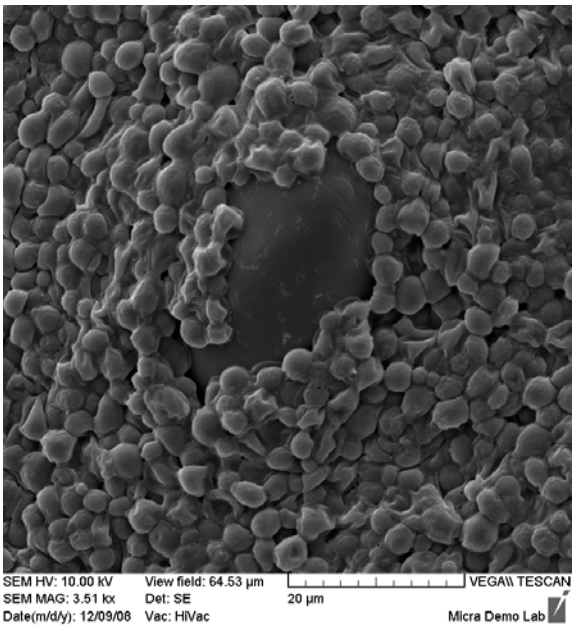

Fig. 2. Cell of *Saccharomyces cerevisiae* in the optimized medium (200 mg $L^{-1}$of zeolite) after 24 h of fermentation surrounded by the smaller zeolite particles. Magnification 3510 X.

These images could indicate that an immobilization of the yeast cells is occurring in the reactor which might have influence on the ethanol production (Shindo *et al.,* 2001).

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.

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