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Application of a Statistical Technique to Investigate Calcium, Sodium, and Magnesium Ion Effect in Yeast Fermentation

Dilek Soyuduru · Mübeccel Ergun · Ayşe Tosun

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Abstract In this work, the dependence of the ethanol production using *Saccharomyces cerevisiae* 251TP(3-2) on calcium, sodium, and magnesium ion concentration and interaction effects were studied with the use of a statistical experimental design. The parameters of the ethanol concentration model proposed on the basis of Box–Wilson experimental design method were evaluated with the use of the experimental data. Comparison of the predicted values from the model with the experimentally observed values showed that the model is a good fit. From the analysis of model equation, it was seen that sodium ion concentration has significant main effects on ethanol production, and there is interactive effect only between calcium and magnesium. With the use of developed model, maximum ethanol concentration of 3.73% (*v*/*v*) was obtained when calcium, sodium, and magnesium concentration were 1,515, 930, and 128 mg/L, respectively, for the 10% sugar concentration in synthetic molasses.

Keywords Ethanolic fermentation · Box–Wilson method · Metal ion · *Saccharomyces cerevisiae*

Introduction

The production of ethanol with the method of fermentation has gained great impetus due to the advancement in technology, the rapid increase in the world population, and the run out of petroleum fuel reserves in crust of earth [1].

The *Saccharomyces cerevisiae* is widely used as biocatalyst in bioconversion processes and is suitable for the production of ethanol from molasses under certain conditions [2–5]. This yeast has become a model microorganism for studying metal transporters and their accumulation in the cell. The number of studies of the processes involved in the uptake of metals by the yeast *S. cerevisiae* has increased considerably in recent years [6–9].

D. Soyuduru · M. Ergun · A. Tosun (✉)
Chemical Engineering Department, Faculty of Architecture and Engineering, Gazi University,
06570 Maltepe, Ankara, Turkey
e-mail: ayseyeyolla@gmail.edu.tr

Metal ions can change the rate of glycolysis and, subsequently, the conversion of pyruvate to ethanol. However, the mechanisms underlying the regulation of glycolysis rate and ethanol production by metal ions remain to be elucidated, and it is relevant to know their optimal levels for industrial fermentations [10, 11]. Metal ions are vital for all organisms, play an important role in the cellular metabolism primarily due to their requirements as cofactors for a large number of enzymes [12–15]. However, excess amounts of the metal ions are toxic and can cause damage to the function that they serve [11–13, 16, 17].

Magnesium has been shown to protect against stress conditions such as temperature and ethanol toxicity [18]. Magnesium is essential for yeast growth, metabolism, and fermentation. It is an essential ion in nucleic acid synthesis and is a cofactor of more than 300 enzymes, including hexokinases, phosphofructokinase, phosphoglycerate kinase, pyruvate kinase, and enolase in glycolysis [19]. In contrast, calcium is known to have relatively few specific biochemical functions and many cells actively secrete this element to keep intracellular levels extremely low. The low magnesium–calcium concentration ratios generally suppress the fermentation performance of yeasts in both semisynthetic and complex media [15].

Potassium is mainly involved in osmoregulation, in charge-balancing, and in regulation of divalent ion and phosphate uptake into the yeast cell. Low potassium and high sodium are toxic to yeast cells. One mechanism that has been proposed to explain sodium toxicity in *S. cerevisiae* is inhibition of potassium uptake, leading to potassium depletion in the cell and an increased level of sodium. In *S. cerevisiae,* specific sodium toxicity has been shown to be exerted by intracellular sodium, whose concentration is the net result of the amount of ion entering and leaving the cell. To prevent excessive sodium accumulation, *S. cerevisiae* has the ability to decrease influx and to favor efflux. This yeast does not have a specific sodium uptake system [20, 21].

Experimental design technique is used for the empirical study of relationship between a measured objective on one hand and a number of operating conditions on the other hand [22]. This method is used to find out how a particular objective is affected by a given set of operating conditions over some specified region of interest and to determine the values of operating conditions which will yield a maximum for the specific objective as a result of optimization. The major advantage of applying experimental design is the reduced number of experiments that have to be carried out to get maximum information.

From the view point of industrial ethanol production, the sucrose-based substrate such as sugar cane molasses and sugar beet juices present many advantages including their relative abundance and renewable nature. But many industrial media possess quite high levels of calcium and sodium ions and have relatively low magnesium–calcium concentration ratios. This situation is generally not conducive to efficient fermentation performance by yeast. Molasses, which have been used as the main substrate for the yeast and ethanol production, have most of the necessary microelements, but not in the optimal concentrations.

Some studies exist in the literature concerning the effects of the metal ions on ethanol production [16, 17, 23]. In these studies, experiments were carried out conventionally, i.e., dependency of ethanol concentration on one of metal ions under consideration was investigated for fixed values of the remaining variables. It is useful to have a correlation which will yield information concerning the productivity variations of the processes when the productive process conditions are changed. Therefore, the main objective of this paper is to devise a model in order to study and predict ethanol concentration during fermentation by *S. cerevisiae* as function of metal ions such as calcium, sodium, and magnesium concentration via the Box–Wilson experimental design technique, which has proved very useful in some fields [23], especially in chemical and microorganism reaction conditions [24]. The conditions, which yielded the maximum value of the ethanol concentration productivity for

the selected region of interest and also the response surfaces for the predicted model, are presented.

Materials and methods

Microorganism

S. cerevisiae 251 TP(3-2) was obtained from the Hıfzıssıhha Center in Turkey. It was maintained by transferring to fresh malt agar slants each month and storing at 4 °C. Agar-malt extract contained g/L: malt extract 3.0; yeast extract 3.0; peptone 5.0; glucose 10.0; agar 20.0; and pH 4.5. Fermentation system was inoculated at 1.0 g of cells [dry weight]/L. The growth medium was the same as fermentation medium, and these mediums were sterilized by autoclaving at 121 °C for 15 min.

Synthetic fermentation media

Synthetic molasses composition was determined by the use of molasses composition taken from Ankara Sugar Company in Turkey. Fermentation medium was prepared with pure sucrose diluted in water and addition of chemical substances for nutritional requirements of microorganism. The synthetic molasses medium comprised: sucrose (100 g), $(NH_4)_2SO_4$ (5 g), $ZnSO_4{\cdot}H_2O$ (0.021 g), KH_2PO_4 (25.9 g), $FeSO_4{\cdot}H_2O$ (0.040 g), $MnSO_4{\cdot}H_2O$ (0.017 g), $CuSO_4{\cdot}5H_2O$ (0.00152 g), pepton (2.67 g), H_3BO_3 (0.01 g), pantothenate (0.0010 g), inosit (0.0005 g), biotin (0.000125 g), thamin (0.005 g), pyridoxine (0.006 g), and pure water to 1 L, $MgSO_4{\cdot}7H_2O$, Na_2SO_4, and $CaCl_2$ were used in varying amount depending on the experimental condition given in Table 1. All the chemical substances used are of analytical grade.

Operating Conditions

The yeast was subcultured on agar-malt extract slants at the start of each experiment. This starter culture was grown in an incubator at 30 °C for 24 h. The inoculum from the fresh slant culture was transferred aseptically to previously sterilized 100-mL growth medium; the liquid culture was agitated using a magnetic stirrer at a level that ensures homogeneity. After approximately 19 h corresponding to the mid-exponential growth phase, 5 mL of seed culture was added to 50 mL fermentation medium. Repetition of this procedure in all cases ensured a relative constancy in the initial cell concentrations of the fermentation flasks.

Batch cultures were grown in 250-mL erlenmayer flasks.The initial pH value was adjusted to 4.5 with 1 mol/L H_2SO_4. All the experiments were carried out at 30 °C in a shaking water bath.

Table 1 Real and coded values of independent variables in experimental plan.

Real values (mg/L)	Coded values				
	−1.73	−1	0	+1	+1.73
X_1	69	680	1515	2350	2961
X_2	209	930	1915	2900	3621
X_3	3.3	56	128	200	253

Analytical Techniques

Ethanol in the supernatant fluids was measured after centrifugation and microfiltration using gas–liquid chromatography (Unicam 610 model), equipped with a capillary column (BP20) and flame ionization detector. The temperature of the injector, detector, and oven were kept at 200, 150, and 180 °C, respectively. Hydrogen, nitrogen, and air were used as the carrier gas at a flow rate of 30, 30, and 330 L/min, respectively.

Experimental Design

The Box–Wilson experimental design was used for the determination of dependence of ethanol production on calcium, sodium, and magnesium ion. Calcium ion concentration, X_1; sodium ion concentration, X_2; and magnesium ion concentration, X_3 (mg/L) were chosen as independent factors in the experimental design. Ethanol concentration Y (% *v*/*v*) was the dependent output variable. For convenience, the independent variables in the model are utilized in their coded form. The variables X_i were coded as U_i according to the equation:

$$U_i = \frac{Xi - Xio}{\Delta Xi} \tag{1}$$

where U_i is the coded value of the variable X_i, X_{i0} is the value of X_i at the center point of the investigated area, and ΔX_i is the step size.

In this research, low and high levels of Ca, Na, and Mg were determined by the use of composition taken from different sugar companies in Turkey (Such as Ankara, Çarsamba, Yozgat, Kars, and Elazıg).

To fit a second order model, the Box–Wilson experimental plan with six experiments at star points and with six replicates at the center point with a total number of 20 experiments, was employed where α is a coded value for the star point. Star point is taken as equal to $k^{\frac{1}{2}}$, where k represents the number of variables [25]. So, α is equal to 1.73 for this experimental design. Table 1 represents the real values corresponding to the coded values.

Results and Discussion

Model Fitting

The Box–Wilson experimental designs are a general series of experiments that have been developed to serve as an efficient basis for deriving the mathematical model of a physical process. Their usefulness is enhanced in the study of industrial applications because most physical situations can usually be approximated by a quadratic function over a reasonable range of factors. Therefore, the model of the regression fitted is:

$$Y = b_0 + \sum_i b_i x_i + \sum_{i \le j} \sum b_{ij} x_i x_j \tag{2}$$

where Y is the predicted response, subscript i and j vary from 1 to the number of variables, b_0 is the intercept term, b_i values are linear coefficients, and b_{ij} values are quadratic coefficients. This was a square regression model in terms of coded values. Parameters of this equation were evaluated with the use of a computer program (Design Expert 7.0, Minneapolis, MN, USA) and the experimental results of specific experiments designed to determine their value.

Table 2 ANOVA table of ethanol concentration.

	b_0	b_2	b_{11}	b_{33}	b_{13}	Residual	Corr. total
Sum of squares	4.44	2.45	0.14	1.690	0.24	0.45	4.89
d.f.	4	1	1	1	1	15	19
Mean square	1.11	2.45	0.14	1.69	0.24	0.030	
F value	37.24	82.08	4.72	56.86	8.22		
P value	0.0001	0.0001	0.0463	0.0001	0.0117		

The resultant functional relationship in terms of coded values for predicting ethanol concentration values was:

$$Y = 4.67 + 0.04x_1 + 2.45x_2 + 0.54x_3 + 0.15x_1^2 + 0.01x_2^2 + 1.71x_3^2 + 0.05x_1x_2 + 0.24x_1x_3 + 0.08x_2x_3 \quad (3)$$

This equation includes all the terms regardless of their significance with R value being equal to 0.96. To determine the significance of each coefficient, a statistical analysis was conducted. The decision about the significance is based on the parameter value (p value). The value of α represents the level of significance. If the parameter value is less than the preassigned level α, then the result is satisfactorily significant at level α. The most commonly used level of significance is 0.05. When the significance level is set at 0.05, p value under 0.05 would be significant.

The coefficients of the newly estimated regression equation are given in the equation, which was obtained by the removal of the insignificant coefficients, are given in Table 2 together with the p values.

It can be seen from this table that all of the remaining coefficients are statistically significant because of having lower p values than α, whose value is 0.05. So, the identified statistical model defining ethanol concentration is given below:

$$Y = 4.44 + 2.45x_2 + 0.14x_1^2 + 1.69x_3^2 + 0.24x_1x_3 \quad (4)$$

The analysis of variance indicated that the quadratic response model showed a high coefficient of determination (R=0.91).

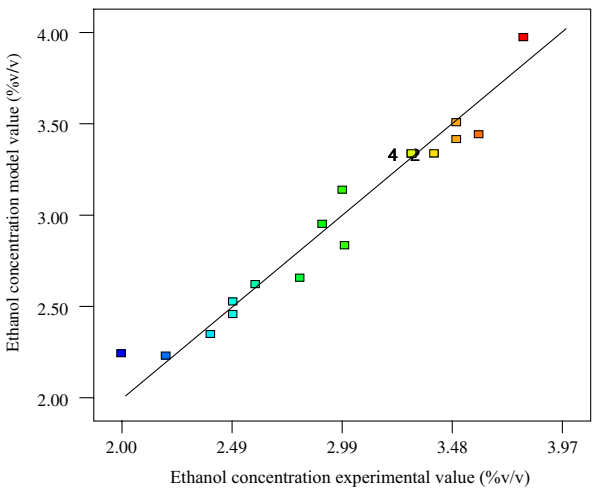

Fig. 1 Ethanol concentration parity plot

The results were shown as a parity plot in Fig. 1.

In this study, from the model equation for ethanol concentration, it was found that sodium ion concentration has significant main effects on ethanol production by *S. cerevisiae* ($p<0.05$), and there is interactive effect only between calcium and magnesium ions ($p>0.05$). The findings of this study supports the results of Chandrasena et al. investigating the main and interactive effects of magnesium, calcium, and potassium ions in yeast fermentation experiments using synthetic molasses [11].

Equation 4 gave the maximum ethanol concentration [3.73% (*v*/*v*)] when the concentration of calcium, sodium, and magnesium are 1,515, 930, and 128 mg/L, respectively. The maximum of the ethanol yield was 75% which is in good agreement with the value give in literature [11, 26, 27]. Experiments were carried out under optimum conditions with synthetic molasses, and experimentally determined value was 3.9% (*v*/*v*), which is very close to the calculated value of 3.73% (*v*/*v*) to check the statistical equation is reconfirmed.

Effect of parameters

Figure 2 shows quadratic response surface for the fitted value of ethanol concentration at varying levels of calcium and magnesium concentration for constant value of sodium concentration of 930 mg/L. At high magnesium values, increasing calcium leads to a reduction in ethanol concentration. But, at low values of magnesium concentration, increase in calcium concentration results in increase in ethanol concentration. So, there is an interactive effect between calcium and magnesium in terms of ethanol concentration. The direction of the effect of calcium depends on the level of magnesium in the fermentation medium. The positive effect of calcium may be explained by the improvement of the flocculation properties of the yeast. Birch et al. demonstrated that low magnesium–calcium concentration ratios generally suppress the fermentation performance of wine yeast in both semisynthetic and complex media [28]. Also, this study indicates that as magnesium–calcium ratio decreases, ethanol concentration decreases for high magnesium concentration values.

This figure showed that optimum calcium concentration was 1515 at 128 mg/L magnesium concentration, and magnesium indicated inhibitory effect on ethanol concentration at higher than its optimum.

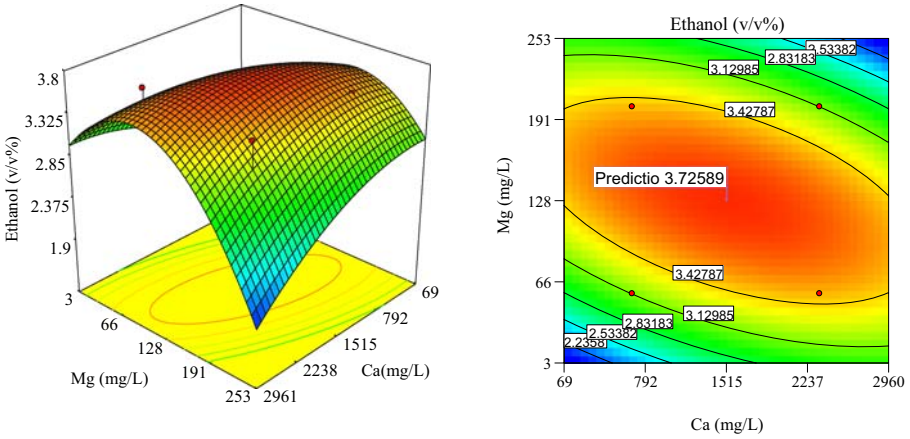

Fig. 2 Quadratic response surface plot and counter plots showing the effect of calcium (X_1) and magnesium (X_3) concentration on ethanol production. Sodium is constant at 930-mg/L levels

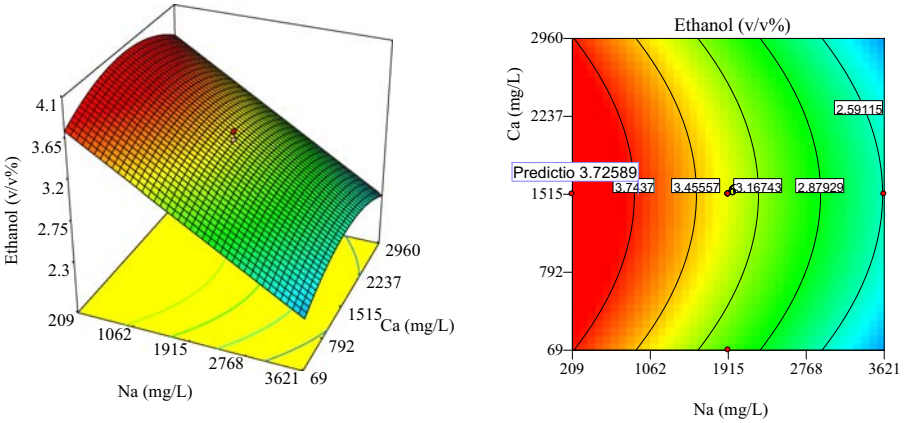

Fig. 3 Quadratic response surface plot and counter plots showing the effect of sodium (X_2) and calcium (X_1) concentration on ethanol production. Magnesium is constant at 128 mg/L

Figure 3 shows quadratic response surface for the fitted value of ethanol concentration at varying levels of sodium and calcium concentration. Magnesium is kept constant at 128-mg/L levels in synthetic molasses.

Sodium ion has negative effect on glycolysis and ethanol production [29]. Low potassium and high sodium is toxic to yeast cells. One mechanism that has been proposed to explain sodium toxicity in *S. cerevisiae* is the competitive inhibition of potassium uptake, leading to potassium depletion in the cell and an increased level of sodium [20, 21]. From the model equation for ethanol concentration, it was found that only sodium ion concentration has significant (p= 0.0001) main effect on ethanol production. The optimum sodium concentration was found to be 930 mg/L. As shown in Fig. 3, increasing sodium levels decrease ethanol concentration at all calcium concentration so there is no interactive effect between calcium and sodium, and calcium concentration seemed to have slight effect on ethanol concentration in comparison to sodium ion.

Conclusion

From the statistical model equation for ethanol concentration, it was found that sodium ion concentration has significant main effect on ethanol production by *S. cerevisiae*, and there is interactive effect only between calcium and magnesium in complex media. It would be useful to study the validity of this type of model for other metal ions known to be effective on ethanol production. Response surface methodology was found to be useful in explaining the effect of parameters.

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