



# Box-Behnken Experimental Design for Optimization of Biomass and Ethanol Yield in *Saccharomyces Cerevisiae*

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## Abstract

Recognizing the importance of enhancing yield in industrial fermentation processes, we aimed to optimize biomass and ethanol yields in *Saccharomyces Cerevisiae* using Box-Behnken experimental design, a response surface methodology (RSM) approach. Traditional optimization through the one-variable-at-a-time (OVAT) approach is labor-intensive and often fails to achieve optimal outcomes due to unaccounted interactions between variables. Our study employed a statistically designed RSM approach to evaluate the effects of medium variables interactions for more efficient pathway for industrial enhancement. *S. cerevisiae* strain K88 (CEN.PK122) was used in this study for ethanol production due to its recognized industrial applications. The medium variables were yeast extract, ammonium sulphate, and yeast nitrogen base supplemented with the amino acids: histidine, methionine and tryptophan. Statistical analysis revealed that YNB and amino acid concentrations enforce biomass production and facilitate ethanol production through increased nitrogen uptake. The optimized biomass yield achieved was increased by 16% (to 0.59 g/g), while the ethanol yield was much highly increased by 40% (to 0.42 g/g) over the basal medium. Optimal yield was attained at the following concentrations: yeast (9.4 g/L) extract, of ammonium sulfate (3.7 g/L) and of YNB (5.1 g/L) with amino acids. Surface plots represent the optimal amount for each variable in medium formulation. This study highlights the efficiency of Box-Behnken design in complex fermentation systems for industrial bioprocess applications.

**Keywords:** *Saccharomyces Cerevisiae*, Box-Behnken design, biomass, ethanol, optimization.

## 1. Introduction

An implicit goal in any biotechnological process aiming at the obtention of a given product is the maximization of its production rate and/or yield [1, 2]. Whenever optimization of the fermentation process is to be aimed, the improvement of productivity either through mutation or genetic engineering strain development means or by the nutritional optimization would be the ultimate need [3, 4].

The fermentation medium determines the chemical or nutritional environment and is thus vital for the manufacturing of microbial metabolites. It has always been the critical component of an industrial or commercial fermentation process, directly affecting not only productivity but also process economics [6].

The yeast *Saccharomyces Cerevisiae* has been used for thousands of years in preparation of wine, beer and bread [6, 7]. In industry there is growing demand to optimize not only the yeast ethanol yield but also the biomass yield as well, since ethanol is one of the major bio-energy sources for organic feed stocks and liquid fuels that can be derived from the renewable biomass [8]. On the other hand, yeast biomass can be used as fodder yeast for animal nutrition as well as in baking industry.

Medium improvement by the classical method involves changing one independent variable while fixing the other at a certain level (OVAT) is laborious, time consuming and frequently does not guarantee determination of the optimal conditions as parameters are encountered. Several other techniques have been carried out for medium optimization. A "single omission" technique has been used by Cotaign-Bousquet et al., 1995 [9] to determine the actual nutritional requirements for

sustained growth of *L.lactis*. El-kady and Moubasher 1982 [10] tested a number of nitrogen and carbon sources and amino acids one by one, to get the appropriate medium for the production of verrucarin. Other successful optimization using the single factor approach have been reported [11, 12]. However, such strategies often require considerable work and time. Accordingly, we were encouraged to carry out the optimization making use of the powerful tool of statistical mathematical analysis of the experimental data. An alternative optimization strategy, which became popular specially in industry, is the use of statistically designed experiments that allow the investigator to evaluate more than one independent variable at a time [13].

Several approaches have been carried out in the development of predictive equations to describe the effects of various cultural variables on the behavior of a certain optimal target. Response Surface Methodology (RSM) appears to be particularly promising in the field of microbiology [14]. Response surface methodology (RSM) is a sequential, exploratory empirical modeling technique developed to correlate the relationship between a set of controlled experimental variables and their observed results (or responses). Other than the one- variable- at a time (OVAT) approach, all the possible combinations of the variables are included in the model. This empirical method has been used with success for modeling the effects and interactions of many factors affecting the growth of the yeast [15]. In the present work attempts have been devoted to optimize biomass yield as well as ethanol yield of the experimental strain *Saccharomyces Cerevisiae* CEN.PK122 using the Box-Behnken design as an example of the response-surface type of design [16, 17].

## 2. Materials and Methods

### 2.1 Microorganism

The preculture as well as the fermentation basal media were of the same composition: YNB medium (1L) containing 1.7 g yeast nitrogen base without amino acids and ammonium sulfate (DIFCO), 10 mg histidine, 20 mg methionine, 20 mg tryptophane, 5 g ammonium sulfate, 5 g yeast extract and 10 g glucose monohydrate. The cells were grown in 150 ml aliquots in 500 ml Erlenmeyer flasks at 30°C at agitation of 120 rpm. For the optimization purpose, the basal medium composition was modified according to the tables (1,2) for biomass and ethanol yield optimization, respectively. Trials were done in duplicates, and results presented in this work is the mean of duplicates.

### 2.2 Yeast Strain and Cultivation

*Saccharomyces Cerevisiae* yeasts were cultivated in Yeast Extract-Glucose broth (YG broth), where a yeast suspension was prepared using 500 mL of YPD broth (containing yeast extract 1%, peptone 2%, and glucose 2%). The suspension was agitated at 150 rpm for 48 hours at 30°C. Post-incubation, cells were harvested at 4°C through centrifugation at 4500 g for 5 minutes. The collected cells underwent three sterile distilled water washes, were weighed, and subsequently freeze-dried, storing them at 20°C until required for wall preparation [16].

Table 1: The levels of variables chosen for the biomass yield optimization trials

Coded Units	Yeast Extract g/L	Ammonium Sulfate g/L	YNB+aa
-	1	1	0.57
0	5	5	1.7
+	20	20	5.1

Table 2: The levels of variables chosen for the ethanol yield optimization trials

Coded Units	Yeast Extract g/L	Ammonium Sulfate g/L	YNB+aa
-	1	5	5.1
0	3	7	7.65
+	5	9	10.2

### 2.3 Calculation of fermentation yield

The biomass yield  $Y_{x/s}$  and the ethanol yield  $Y_{p/s}$  were calculated according to the formulae:  $Y_{x/s} = \Delta x / \Delta s$  and  $Y_{p/s} = \Delta p / \Delta s$  whereas the biomass yield coefficient is calculated on the basis of the carbon content for both the dry weight at the fermentation end, and the glucose as well as the yeast extract at the fermentation begin.

### 2.4 Experimental design

The effect of several nutritional requirements influencing the yield such as yeast extract concentration, ammonium sulfate as well as yeast nitrogen base + amino acids in  $g\ l^{-1}$  were investigated and the selected independent variables were designated as  $X_1$ ,  $X_2$  and  $X_3$  respectively. The low, middle and high levels of each variable were designated by -, 0 and + respectively and listed in Table (1, 2). The overall design of experiments is represented in Table (3).

The relationship between the three independent variables  $X_1$ ,  $X_2$  and  $X_3$  and response  $Y$  were represented by the following polynomial model, according to the response surface design (Box and Behnken 1960):

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2$$

where  $Y$  is the predicted response,  $\beta_0$  model constant;  $X_1$ ,  $X_2$  and  $X_3$  independent variables;

$\beta_1$ ,  $\beta_2$  and  $\beta_3$  are linear coefficients;  $\beta_{12}$ ,  $\beta_{13}$  and  $\beta_{23}$  are cross product coefficients and  $\beta_{11}$ ,  $\beta_{22}$  and  $\beta_{33}$  are the quadratic coefficients.

The calculation of the coefficients value were carried out using multiple regression analysis with the help of Microcal Origin 4.01 software, where it is found under the function tools regression. When performing multiple regression analysis, Origin always assumes that the first column in the worksheet contains the independent variable (yield). The worksheet columns that contain the independent variables are highlighted and under the above-mentioned function the calculated coefficients are out putted to the script window. The analysis yields a model developed by fitting the experimental data from the key points to generalized smoothing curve, from which a specific predicted optimized value of the response can be calculated. The maximal yield is calculated mathematically using Microsoft Excel using the Solver function. 3D as well as contour graphs are generated by MapleV-R3 software. The polynomial equation as well as the experimental constrains are introduced into the Maple V-software to generate the three dimensional graphs. The contour plots are also optional in this software.

Table 3: Box-Behnken factorial experimental design

Trial	Yeastextract[X <sub>1</sub> ] g/L	Ammonium Sulfate [X <sub>2</sub> ] g/L	YNB+ a.a [X <sub>3</sub> ] g/L
1	+	+	0
2	+	-	0
3	-	+	0
4	-	-	0
5	+	0	+
6	+	0	-
7	-	0	+
8	-	0	-
9	0	+	+
10	0	+	-
11	0	-	+
12	0	-	-
13	0	0	0

### 3. Results and Discussion

#### 3.1 Optimization of biomass yield

This study aimed to observe the combined effect of three different medium components namely yeast extract, ammonium sulfate and yeast nitrogen base supplied with amino acid mixture of histidine, methionine and tryptophan, that were involved in the batch study. The levels of the independent variables were chosen as shown in Table (4), in which both the calculated biomass and ethanol yield responses are illustrated.

Table 4: Concentration of variables and calculated

Trial	X <sub>1</sub>	X <sub>2</sub>	X <sub>3</sub>	Y <sub>x/s</sub>	Y <sub>p/s</sub>
1	+	+	0	0.38	0.35
2	+	-	0	0.40	0.33
3	-	+	0	0.46	0.30
4	-	-	0	0.41	0.32
5	+	0	+	0.42	0.33
6	+	0	-	0.39	0.33
7	-	0	+	0.49	0.28
8	-	0	-	0.38	0.29
9	0	+	+	0.54	0.32
10	0	+	-	0.53	0.37
11	0	-	+	0.55	0.36
12	0	-	-	0.54	0.30
13	0	0	0	0.51	0.38

\*ND not determined

Minimal value obtained in trial #1 was 0.38 g/g and maximal value was in trial 11 of 0.55 g/g biomass yield. On the other hand ethanol yield coefficient was not that affected under the investigated conditions, where minimal yield was 0.28 g/g and maximal yield was 0.37 g/g.

After calculation, the mathematical expression relating to the fermentation yield with the

variables X<sub>1</sub>, X<sub>2</sub> and X<sub>3</sub> is given below, where Y<sub>x/s</sub> and Y<sub>p/s</sub> are the biomass yield and ethanol

yield, respectively:

$$Y_{x/s} = 3.0003 + 0.03327X_1 + 0.00821X_2 + 0.04265X_3 - 2.29e^{-4}X_1X_2 - 7.9229e^{-4}X_1X_3 - 9.8229e^{-4}X_2X_3 - 0.00151X_1^2 - 1.41e^{-4}X_2^2 - 0.00272X_3^2$$

$$Y_{p/s} = 0.35921 + 0.00889X_1 - 0.01337X_2 - 0.03539X_3 + 7.76e^{-5}X_1X_2 + 2.38e^{-4}X_1X_3 - 5.37e^{-4}X_2X_3 - 3.84e^{-4}X_1^2 + 6.45e^{-4}X_2^2 + 0.00603X_3^2$$

The biomass as well as the ethanol yield predicted from the model are presented in Table (5) in a comparison with the actual experimental data.

Table 5: Comparison between experimental and correlated data derived from the polynomial mathematical model

Trial no	Biomass yield Y <sub>x/s</sub> g/g		Ethanol yield Y <sub>p/s</sub> g/g	
	Experimental	Correlated	Experimental	Correlated
1	0.38	0.38	0.35	0.34
2	0.40	0.40	0.33	0.34
3	0.46	0.46	0.30	0.30
4	0.41	0.41	0.32	0.31
5	0.42	0.42	0.33	0.33
6	0.39	0.39	0.33	0.32
7	0.49	0.49	0.28	0.28
8	0.38	0.39	0.29	0.30
9	0.54	0.54	0.32	0.32
10	0.53	0.52	0.37	0.37
11	0.55	0.55	0.36	0.36
12	0.54	0.54	0.30	0.30
13	0.51	0.51	0.38	0.37

The multiple correlation coefficient ( $R = 0.9938$ ) for the biomass yield, relatively indicates multiple correlation coefficient ( $R = 0.9726$ ) for the ethanol yield reflects more deviation of the model from the experimental data. Moreover, the  $R^2$  value for the biomass yield confirms 97% of the yield. Whereas the  $R^2$  of the ethanol confirms 85% of the yield.

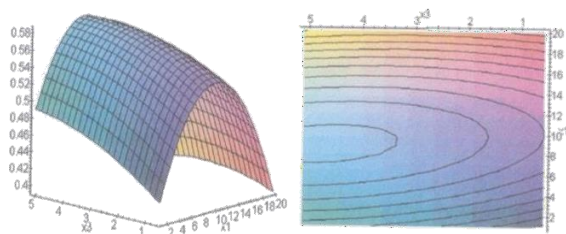


Fig. 1: 3D and contour plots showing the correlation between variables of medium and biomass yield response.

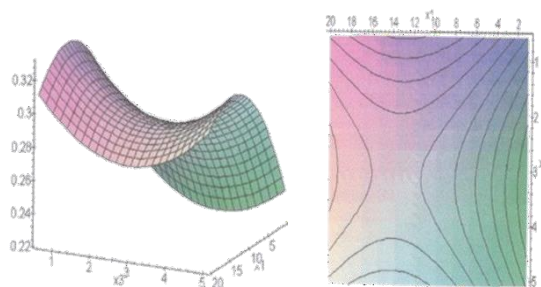


Fig. 2: 3D and contour plots showing the correlation between variables of medium and ethanol yield response.

On the other hand, Figure 1, 2 show the 3D graphs, as well as the contour plots generated by MapleV software and derived for the response surface of the biomass yield show a maximal yield value of 0.59 g/g at the following combination (g/L): yeast extract, 9.4; ammonium sulfate, 3.7 and yeast nitrogen base + amino acids mixture, 5.1. This increase of biomass yield is about 16% increase than the basal medium (the (0, 0, 0) combination). In Figure 1 can also be seen that increasing the concentration of the yeast nitrogen base and amino acid mixture increases the biomass yield, while optimal concentration of yeast extract was found to be 9.4 g/L and increasing the concentration of which over that value decreases the biomass yield. On the other hand, increasing the concentration of the ammonium sulfate over 3.7 g/L results in decreasing the Biomass yield. In spite of that, Figure 2 shows a saddle formation for the ethanol yield giving no theoretical indication of maximal yield, but a minimum instead. This experiment illustrates that only maximal biomass yield was obtained under these conditions, while a "saddle"

form was generated in the ethanol optimization trials in a sign that the medium composition bringing about maximal biomass yield is not necessarily the same one that gives maximum ethanol yield. Maximal biomass yield 0.59 g/g was obtained whenever the ammonium sulfate found in a low concentration in the medium (3.7 g/L) and yeast nitrogen base + amino acids mixture in its highest concentration limit in the trial set (5.1 g/L). It is also worthwhile to state that the presence of high concentration of yeast extract in the medium supports the biomass production because of its carbon content as well as trace elements and vitamins responsible for further encouragement for the conversion of ethanol and acetate bioproduction to biomass [18]. Moreover, according to the mathematical model, additional amounts of ammonium sulphate have a negative effect on the yield optimum that could be due to the presence of the amino acid mixture in the medium composition, where amino acids are more efficient source of nitrogen than the ammonium ions [19]. On the other hand, no theoretical optimum can be obtained for ethanol yield optimization with the same medium, where stationary point is a "saddle" point. In the case of such a saddle shape, statistical techniques can not determine an optimum point for the reaction, but only a series of values corresponding to the highest yield [20].

### 3.2 Optimization of ethanol yield

Another range of independent variables was carried out to find the optimal ethanol yield. The experimental design as well as the ethanol yield responses at each trial is presented in Table (6).

Table 6: Concentration of variables and calculated ethanol yield coefficients

Trial	$X_1$	$X_2$	$X_3$	$Y_{ps}$
1	5	9	7.65	0.342
2	5	5	7.65	0.314
3	1	9	7.65	0.309
4	1	5	7.65	0.375
5	5	7	10.20	0.319
6	5	7	5.10	0.385
7	1	7	10.20	0.396
8	1	7	5.10	0.257
9	3	9	10.20	0.289
10	3	9	5.10	0.328
11	3	5	10.20	0.345
12	3	5	5.10	0.318
13	3	7	7.65	0.42



The coefficients' values are calculated for each independent variable as well as the combination of them and formulated into a single polynomial equation.

The mathematical model describing the relationship between the controllable variables and the response is represented as follows:

$$Y_{p/s} = -0.99431 + 0.0865X_1 + 0.18431$$

$$X_2 + 0.16843X_3 + 0.00588X_1X_2 -$$

$$0.01005X_1X_3 -$$

$$0.00324X_2X_3 - 0.00822X^2 - 0.01303X^2 -$$

$$0.00736X^2$$

The multiple correlation coefficient of this equation is ( $R = 0.9323$ ), so that it explains how much the experimental data are well fitted with the predicted model. According to this model, the optimal medium constituents' concentrations that bring about maximal ethanol yield are calculated with the help of the non-linear optimization routine of Excel software. Maximal ethanol yield coefficient ( $Y_{p/s} = 0.42$  g/g) is obtained under the following medium combination (g/L): yeast extract, 2.65; ammonium sulfate, 6.65 and yeast nitrogen base + amino acids mixture, 8.17.

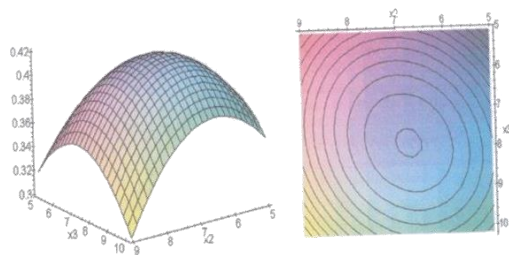


Fig. 3: 3D and contour plots showing the optimum medium variables' concentrations for ethanol yield coefficient optimization.

From Figure 3 it can be concluded that increasing the ammonium sulfate concentration in the medium increases the ethanol yield to reach its maximal value at a corresponding ammonium sulfate concentration of 6.65 g/L, afterwards the ethanol yield decreases with increasing the ammonium sulfate concentration. On the other hand, increasing the yeast extract concentration over 2.65 g/L causes a decrease in the yield. maximal ethanol yield coefficient is attained at 8.17 g/L yeast nitrogen base-amino acids' mixture. By this way, the ethanol yield coefficient is increased by 40% of the basal medium concentrations. It can be said that changing the scale of medium constituents was the solution to find out the ethanol yield optimum. The new set of trials revealed an optimum value (0.42 g/g) where the medium combination is g/L: yeast extract, 2.65; ammonium sulfate, 6.65 and YNB + amino acids, 8.17. It could be concluded that the

experimental strain requires lower amount of yeast extract in comparison with the amount needed to support biomass production (one quarter the amount). Lower concentration of yeast extract in the medium renders to the accumulation of ethanol with lower further conversion rate to biomass as claimed by Bartling 1996 [21, 22].

Whereas about double the amount of ammonium sulfate is needed to support ethanol production. In both cases, either for biomass yield or ethanol yield, the yeast nitrogen base and amino acid mixture is needed in high concentration due to the presence of vitamins, minerals and co-factors in the yeast nitrogen base that promotes enzyme activities. Additionally, the ease of amino acids uptake that relieves the cell of a synthetic carbon demand that would otherwise have to be met by diverting the intermediates sugar metabolism [19, 23]. The present study supports the claims of Bafmcova et al., 1999 [24], that the addition of free amino nitrogen leads to higher final ethanol concentration in the fermented media and amounts of the cell wall accumulation as they have got increasing final ethanol concentration as well as higher ethanol productivity when excess assimilable nitrogen was added.

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