

## Response Surface Methodology (Rsm) - Statistical Analysis for Invertase Production by *S. Cerevisiae* MK

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**Abstract:** Invertase producing yeast *Saccharomyces cerevisiae* MK was isolated from toddy sample. The optimal level of the key variables (orange peel, yeast extract and methionine) used to determine the effect of their interactions on invertase production using the statistical tool (CCD of RSM). The second-order quadratic model with the optimum conditions was orange peel - 4%; yeast extract - 0.5% and methionine - 0.5%. The nearness of the coefficient of determination ( $R^2=0.9994$ ) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The maximum invertase production was calculated as 0.50 IU/ml.

**Key words:** Invertase, CCD, RSM, *Saccharomyces cerevisiae* MK, orange peel, yeast extract and methionine

### INTRODUCTION

Invertase ( $\beta$ -D-fructofuranoside-fructohydrolase, E.C.3.2.1.26) catalyzes the hydrolysis of sucrose to glucose and fructose. Invertase is one of the most widely used enzymes in food industry, especially in the preparation of jams and candies [1]. Invertase is also referred as  $\beta$ -fructofuranosidase it catalyses hydrolysis of the terminal non-reducing residue of  $\beta$ -fructofuranoside [2]. The enzyme is a glycoprotein, with some residues of mannose being the major component of the carbohydrate moiety. Invertase is mainly used in the food industry, where fructose is preferred over sucrose because it is sweeter and does not crystallize easily [3].

Invertases play a key role in the sugar metabolism of higher plants and they are classified as acid or neutral (alkaline) invertases on the basis of their pH optima for acid invertase and neutral invertase [4]. Invertase is classified in the GH32 family of glycoside hydrolases, that includes over 370 members and has been reported in plant, bacteria, yeast and filamentous fungi, as *Aspergillus ochraceus*, *Aspergillus niger*, *Aspergillus japonicus* and *Thermomyces lanuginosus* [5]. Invertase is biosynthesized by yeast strains, *Aureobasidium spp*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, *Saccharomyces carlsbergensis* [6].

Growth conditions have a great influence on invertase production capacity of *Saccharomyces cerevisiae*. The production of the extracellular invertase

shows a cyclic behaviour that coincides with the budding cycle. The invertase activity increases during bud development and ceases at bud maturation and cell scission [7].

Agricultural and industrial wastes have motivated curiosity in converting waste materials into commercially valuable products. The agro-food industry produces large volumes of wastes, both solids and liquids resulting from the production, preparation and consumption of food. Besides their pollution and hazardous aspects, in many cases, food processing wastes might have potential for recycling raw materials or for conversion into useful product of higher value [8].

Statistical methods are increasingly preferred for fermentation optimization because they reduce the total number of experiments needed and provide a better understanding of the interactions among factors on the outcome of the fermentation. Statistical techniques such as the Taguchi method have gained broad acceptance in fermentation optimization. Taguchi's method specifies orthogonal arrays for combining the various variables and levels in a minimum acceptable number of experimental trials [9].

Response Surface Methodology (RSM) is a statistical technique for the modelling and optimization of multiple variables, which determine optimum process conditions by combining experimental designs with interpolation by first-or second-polynomial equations in a sequential testing procedure [10]. RSM has already been

successfully applied for the optimization of enzymatic hydrolysis of other bioprocesses. Response surface methodology (RSM) is a useful tool which integrates mathematical and statistical approaches to analyze the effects of defined independent variables on the response without the need for prior knowledge of a predetermined relationship between the response function and the variables [11].

Optimization of the fermentation process parameters through a statistical approach, such as central composite design and response surface methodology (RSM), has been well appreciated for a significant improvement in yield as well as a decrease in the production cost of the enzyme [12]. Therefore, this study was mainly focused on statistical optimization of invertase production by *Saccharomyces cerevisiae* MK using central composite design for high yield with low cost. Optimization of the fermentation process parameters through a statistical approach, such as CCD and response surface methodology (RSM), has been well appreciated for a significant improvement in yield as well as a decrease in the production cost of the enzyme.

## MATERIALS AND METHODS

**Optimization of Significant Variables for Invertase Production Using (CCD):** To find the optimal cultivation conditions for invertase production, CCD with five coded levels was used for locating the true optimum conditions of orange peel (carbon source), yeast extract (nitrogen source) and methionine (amino acid). For the three factors, this trial was essentially a full 2<sup>3</sup> factorial design with six axial points ( $\alpha = 1.68$ ) and six replication of the center points, resulting in a total number of 20 experiments. The levels of the variables and the experimental design are shown in Table 1. The results of CCD were expressed as the following second-order polynomial Eq. 2 using a multiple regression technique.

$$Y = \beta_0 + \sum \beta_i \chi_i + \sum \beta_{ii} \chi_i^2 + \sum \beta_{ij} \chi_i \chi_j$$

where, Y is the predicted response,  $\beta_0$  the intercept term,  $\beta_i$  the linear coefficients,  $\beta_{ii}$  the quadratic coefficients,  $\beta_{ij}$  the interactive coefficients and  $\chi_i$  and  $\chi_j$  the coded independent variables [13].

**Invertase Production by Optimized Parameters:** After 48 hours of incubation on optimized medium [orange peel (carbon source) - 4.0%, yeast extract

(nitrogen source) - 0.5%, methionine (amino acid) – 0.2%, calcium chloride (metal ions) – 0.02%, inoculum concentration – 2.0%, citrate buffer - 0.1M (pH-5), poly ethylene glycol – 0.2% at pH 6.0, 30°C].

The culture medium was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was used as crude enzyme source for invertase assay. Invertase activity was assayed as per the method of Sumner and Howells (1935) [14] using 0.5ml of sucrose as the substrate in 0.03M acetate buffer (pH- 5.0) and incubated at 45°C for 30 minutes. The reaction was terminated by addition of 1ml of DNS reagent and tubes were kept at boiling water bath for 5 minutes. After cooling the tubes at room temperature, 3ml of distilled water was added in each tube. The intensity of the colour was read at 540nm in UV-Vis spectrophotometer (Systronics, 119). Standard curve was performed with glucose solution. One unit of enzyme activity was defined as the amount of enzyme required for release 1 $\mu$ mol of glucose/ml/minute under assay condition. Enzyme activity was expressed in International units.

Invertase activity was calculated using this formula:

$$\text{IU/ml} = \text{concentration of glucose} / 0.5 \times 30 \times 0.180$$

**Statistical Analysis:** Experimental designs and the polynomial coefficients were calculated and analyzed using a trial version of Design-Expert software (version 8.0.4, Stat-Ease Inc., Minneapolis, USA). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

## RESULT

**Central Composite Design (CCD) and Response Surface Methodology (RSM):** The optimal level of the key variables (orange peel, yeast extract and methionine) and the effect of their interactions on invertase production were further explored using the CCD of RSM. The design matrix and the corresponding experimental data to determine the effects of three independent variables are shown in Table 1. The mutual interactions between every two of the three variables which were significant under the optimum condition, the predicted maximum invertase production were calculated as 0.50 IU/ml. By applying multiple regression analysis to the experimental data (Table 4), the following second order polynomial equation was established:

Table 1: Independent variables and their coded levels for the central composite design used for invertase production by *Saccharomyces cerevisiae* MK

Variables	- $\alpha$	Low value	Coded variable	High value	+ $\alpha$
Orange Peel	3.1591	3.5	4	4.5	4.8409
Yeast extract	0.0795518	0.25	0.5	0.75	0.920448
Methionine	0.0318207	0.1	0.2	0.3	0.368179

Table 2: Central composite design for Invertase production by *Saccharomyces cerevisiae* MK

Std	Run	Factor 1 Orange peel%	Factor 2 Yeast extract%	Factor 3 Methionine%	Invertase IU/ml
15	1	4.00	0.50	0.20	0.50
16	2	4.00	0.50	0.20	0.50
6	3	4.50	0.25	0.30	0.30
9	4	3.16	0.50	0.20	0.26
1	5	3.50	0.25	0.10	0.24
13	6	4.00	0.50	0.03	0.32
12	7	4.00	0.92	0.20	0.23
11	8	4.00	0.08	0.20	0.22
7	9	3.50	0.075	0.30	0.21
14	10	4.00	0.50	0.37	0.32
17	11	4.00	0.50	0.20	0.50
18	12	4.00	0.50	0.20	0.50
8	13	4.50	0.75	0.30	0.32
3	14	3.50	0.75	0.10	0.24
4	15	4.50	0.75	0.10	0.33
5	16	3.50	0.25	0.30	0.24
19	17	4.00	0.50	0.20	0.50
20	18	4.00	0.50	0.20	0.50
10	19	4.84	0.50	0.20	0.37
2	20	4.50	0.25	0.10	0.28

Table 3: The matrix of the CCD experiment and the corresponding experimental data by *Saccharomyces cerevisiae* MK

Std	Run	Factor 1 Orange peel%	Factor 2 Yeast extract%	Factor 3 Methionine%	Actual value	Predicted value
15	1	4.00	0.50	0.20	0.50	0.500
16	2	4.00	0.50	0.20	0.50	0.500
6	3	4.50	0.25	0.30	0.30	0.301
9	4	3.16	0.50	0.20	0.26	0.253
1	5	3.50	0.25	0.10	0.24	0.243
13	6	4.00	0.50	0.03	0.32	0.320
12	7	4.00	0.92	0.20	0.23	0.230
11	8	4.00	0.08	0.20	0.22	0.216
7	9	3.50	0.075	0.30	0.21	0.214
14	10	4.00	0.50	0.37	0.32	0.315
17	11	4.00	0.50	0.20	0.50	0.500
18	12	4.00	0.50	0.20	0.50	0.500
8	13	4.50	0.75	0.30	0.32	0.320
3	14	3.50	0.75	0.10	0.24	0.242
4	15	4.50	0.75	0.10	0.33	0.328
5	16	3.50	0.25	0.30	0.24	0.245
19	17	4.00	0.50	0.20	0.50	0.500
20	18	4.00	0.50	0.20	0.50	0.500
10	19	4.84	0.50	0.20	0.37	0.373
2	20	4.50	0.25	0.10	0.28	0.279

Table 4: Variance analysis of response surface quadratic model for invertase production *S. cerevisiae* MK

Source	Sum of squares	Df	Mean square	F-value	p-value Prob > F
Model	0.24	9	0.027	1723.80	< 0.0001 <sup>c</sup>
A-orange peel	0.017	1	0.017	1111.88	< 0.0001 <sup>c</sup>
B-yeast extract	2.364E-004	1	2.364E-004	15.26	0.0029 <sup>c</sup>
C-methionine	2.929E-005	1	2.929E-005	1.89	0.1991
AB	1.250E-003	1	1.250E-003	80.69	< 0.0001 <sup>c</sup>
AC	2.000E-004	1	2.000E-004	12.91	0.0049 <sup>c</sup>
BC	4.500E-004	1	4.500E-004	29.05	0.0003 <sup>c</sup>
A <sup>2</sup>	0.063	1	0.063	4076.29	< 0.0001 <sup>c</sup>
B <sup>2</sup>	0.14	1	0.14	8937.25	< 0.0001 <sup>c</sup>
C <sup>2</sup>	0.060	1	0.060	3861.48	< 0.0001 <sup>c</sup>
Residual	1.549E-004	10	1.549E-005		
Lack of fit	1.549E-004	5	3.098E-005		
Pure error	0.000	5	0.000		
Cor total	0.24	19			

R<sup>2</sup>= 0.9994; Adj R<sup>2</sup>=0.9988; CV%= 1.14; <sup>c</sup>Model terms are significant

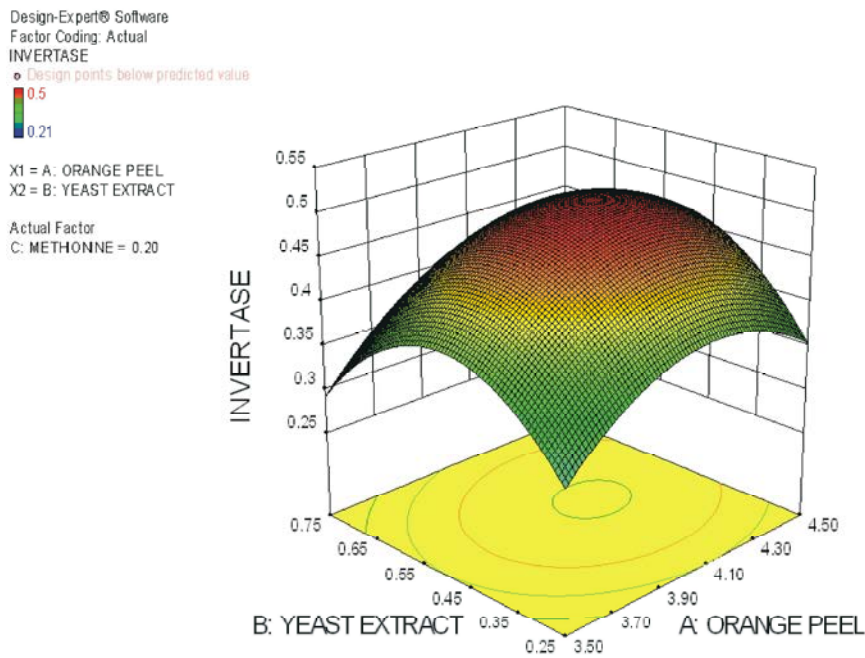


Fig. 1a: Interaction between orange peel and yeast extract

**Final Equation in Terms of Coded Factors:**

$$\text{INVERTASE} = +0.50 + 0.036A + 4.160E-003 B - 1.464E-003 C + 0.013A*B + 5.000E003A*C - 7.500E-003 B*C - 0.066A^2 - 0.098B^2 - 0.064C^2$$

**Final Equation in Terms of Actual Factors:**

$$\begin{aligned} \text{INVERTASE} = & 4.42552 + 2.11922 * \text{ORANGEPEEL} + 1.24486 * \\ & \text{YEASTEXTRACT} + 2.31239 * \\ & \text{METHIONINE} + 0.10000 * \text{ORANGE PEEL} * \text{YEAST} \\ & \text{EXTRACT} + 0.10000 * \text{ORANGE PEEL} * \text{METHIONINE} - \\ & 0.30000 * \text{YEAST EXTRACT} * \text{METHIONINE} - 0.26477 \end{aligned}$$

$$\begin{aligned} & * \text{ORANGE PEEL}^2 - 1.56821 * \text{YEAST EXTRACT} - 6.44259 \\ & * \text{METHIONINE}^2 \end{aligned}$$

where, Y1 was the invertase production, X1 the orange peel, X2 the yeast extract and X3 the methionine. The Model F-value of 1723.80 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, AB, AC, BC, A<sup>2</sup>, B<sup>2</sup>, C<sup>2</sup> are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Pred R-Squared" of 0.9951 is in reasonable agreement with the "Adj R-Squared" of 0.9988.

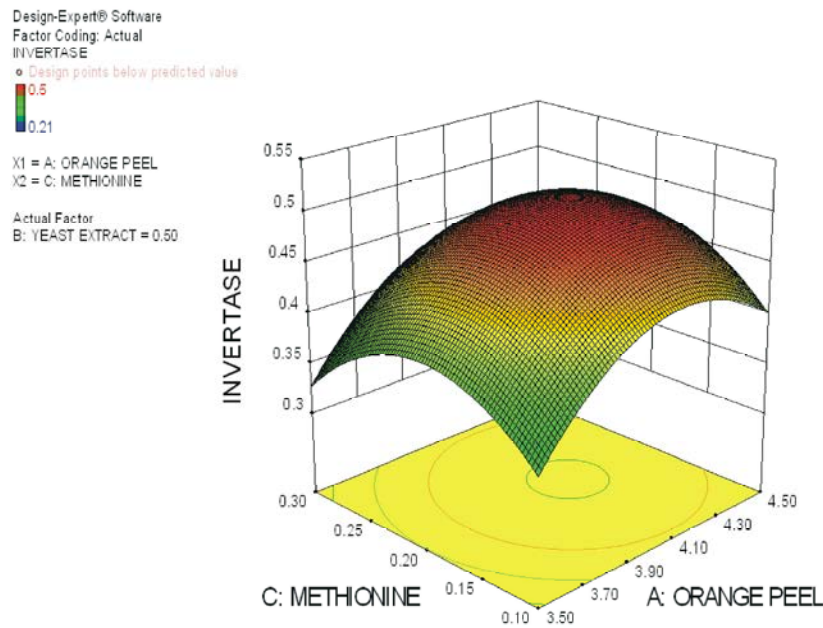


Fig. 1b: Interaction between orange peel and methionine

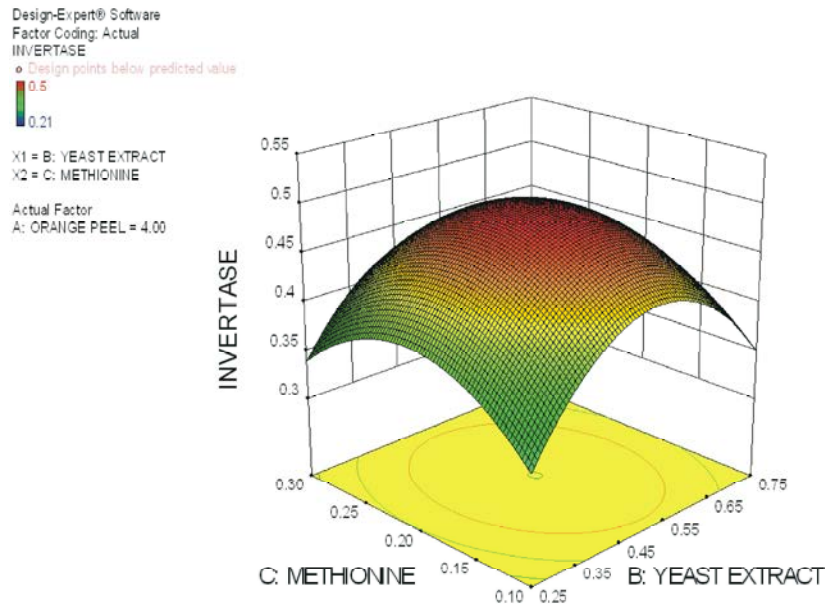


Fig. 1c: Interaction between yeast extract and methionine

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. This ratio of 102.927 indicates an adequate signal. This model can be used to navigate the design space.

### DISCUSSION

The yeast invertase recovered from optimized medium was 0.50 IU/ml. In this case of *Saccharomyces cerevisiae* MK showed the mutual interactions between

every two of the three variables which were significant under the optimum condition, the predicted maximum invertase production was calculated as 0.50 IU/ml. The three-dimensional response surfaces and contour plots are shown in Figure 2 (invertase production) which depicts the interactions between the two variables by keeping the other variables at their zero levels. The shapes of the contour plots, circular or elliptical, indicate whether the mutual interactions between the variables are significant or not. A circular contour plot of response

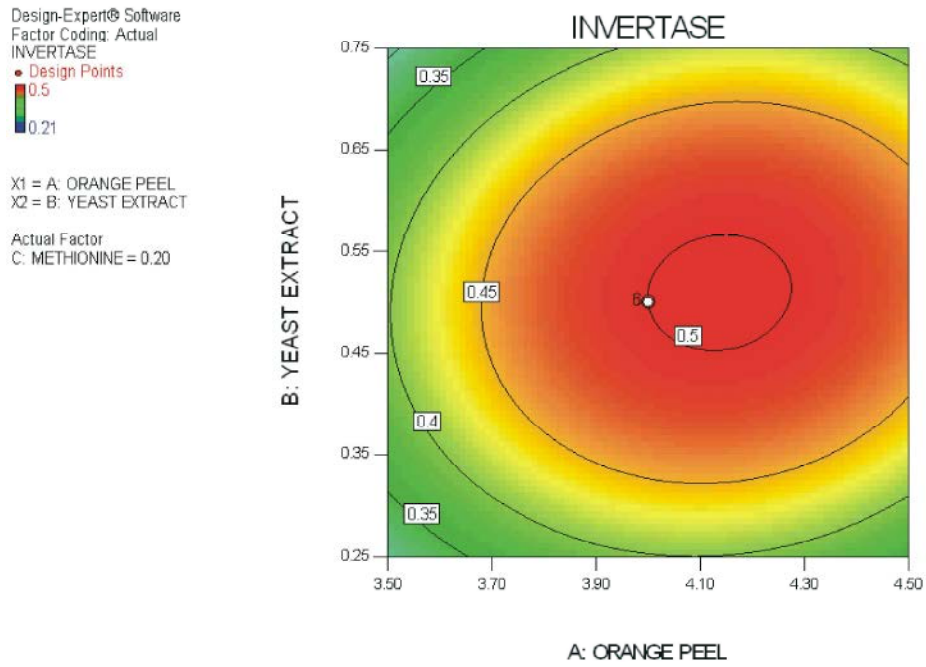


Fig. 2a: Contour plot for orange peel and yeast extract

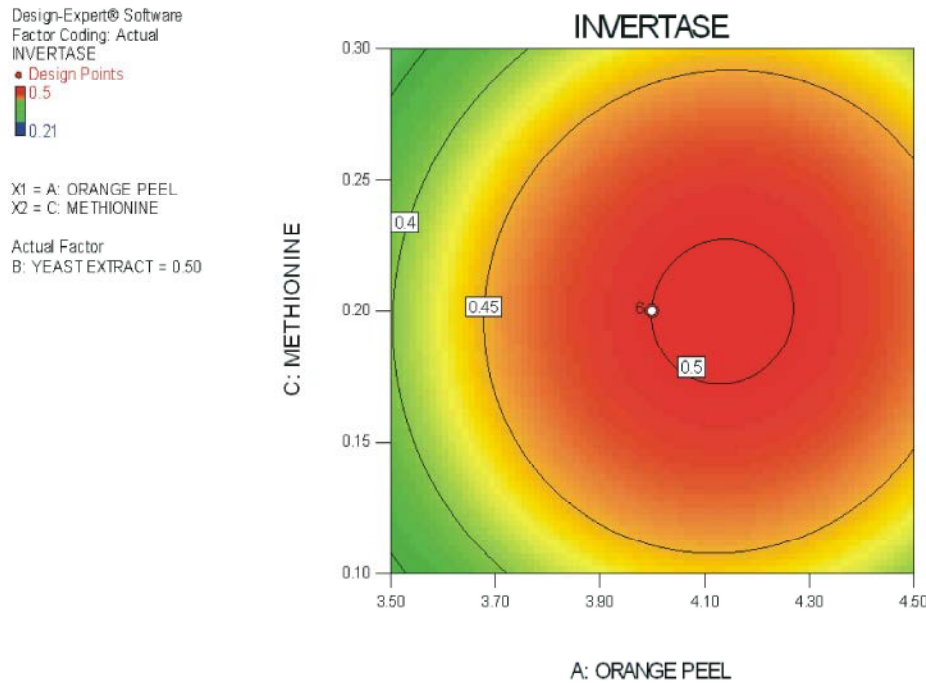


Fig. 2b: Contour plot for orange peel and methionine

surfaces indicates that the interaction between the corresponding variables can be ignored, while an elliptical or saddle nature of the contour plot suggests that the interaction between the corresponding variables is significant [12].

The second-order quadratic model with the optimum conditions (orange peel - 4%; yeast extract - 0.5% and

methionine - 0.5%) resulted in a maximum titre of 0.5 IU/ml of invertase at 48 hours. The nearness of the coefficient of determination ( $R^2=0.9994$ ) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. Likewise model of RSM was employed in the optimization of major invertase producing conditions such as orange peel, yeast extract and methionine.

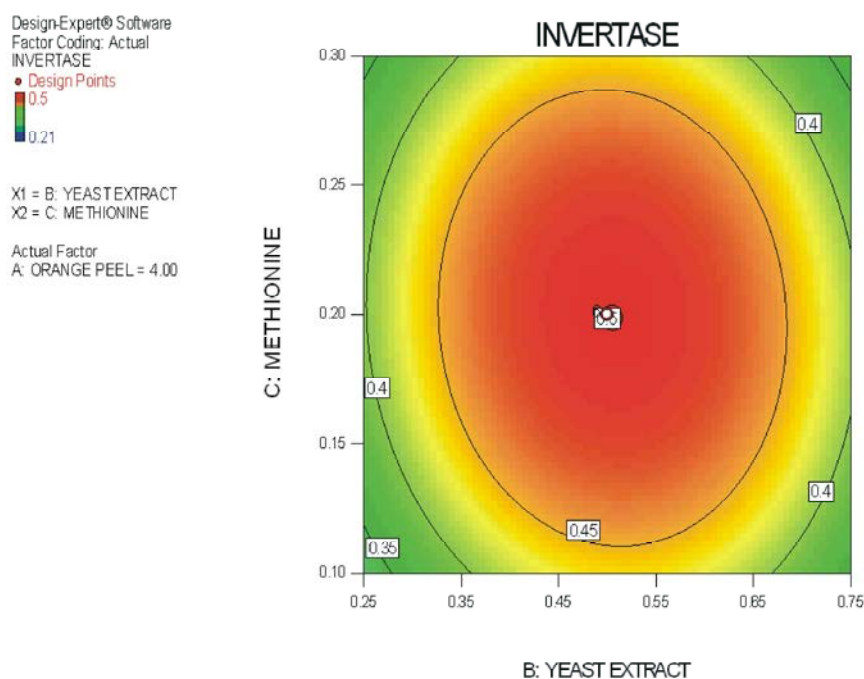


Fig. 2c: Contour plot for yeast extract and methionine

### CONCLUSION

In the present work the optimum culture conditions for invertase production by *Saccharomyces cerevisiae* MK was studied by RSM using central composite design with three variables orange peel, yeast extract and methionine for maximizing the production of invertase which resulted in a maximum titre of 0.50 IU/ml of invertase at 48 hours.

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