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Invertase production by *S. cerevisiae* MTCC 170 using statistical tool Central Composite Design

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ABSTRACT: Invertase producing yeast *S. cerevisiae* was obtained from MTCC. 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 (orange peel - 4%; yeast extract - 0.5% and methionine - 0.5%). The nearness of the coefficient of determination (R^2= 0.9948) to 1 ensures the satisfactory adjustment of the quadratic model to the experimental data. The maximum invertase production was calculated as 0.37 IU/ml.

***Keywords*:** Invertase, CCD, RSM, *Saccharomyces cerevisiae* MTCC 170, orange peel, yeast extract and methionine.

INTRODUCTION

Invertase catalyses α-1,4 glycosidic linkage between α-D-glucose and β-D-fructose molecules of sucrose by hydrolysis releasing monosaccharides such as glucose and fructose. This mixture is called invert syrup. It also hydrolyses β-fructans such as raffinose into simple sugars (Shafiq, 2004). *Saccharomyces cerevisiae* is particularly interesting microorganism, it synthesizes two invertases: a glycosilated periplasmic protein and a cytosolic non glycosilated protein. The enzymatic activity of invertase has been characterized in plants, filamentous fungi, yeast, bacteria, and etc (Mona and Mohamed, 2009). *Saccharomyces cerevisiae* have many advantages as a host for the production of heterologous proteins (De-Nobel, 1989).

Invertase [β-fructofuranosidases (EC.3.2.1.26)] is member of glycoside hydrolases, which include more than 370 enzymes of plant and microbial origin. Invertase or β-fructonosidase is the yeast derived enzyme. Invertase from *Saccharomyces cerevisiae* is the high cost enzyme. Invertases are intracellular as well as extracellular enzymes (Ul-Haq and Ali, 2007). Invertase acts on non-reducing fructofuranoside terminal residues of β-fructofuranosides (Veana, 2011). Invertase is an enzyme mainly used for the production of invert sugar in the food industry. They include acid invertase and neutral invertase, which have widely optimum pH range for their activity (Thang, 2010).

Many organisms produce invertase such as *Neurospora crassa, Fusarium oxysporium, Phytophthora meganosperma, Bacillus macerans, Aspergillus niger, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Cladosporium cladosporioides, Kluyveromyces marxianus, Saccharomyces fragilis, Aspergillus caespitosus, Candida guilliermondii, Penicillium chrisogenum, Candida utilis, Pichia pastoris* (Ul-Haq and Ali, 2005).

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 (Ferreira, 2009). 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. RSM is now considered as a standard statistical approach for designing experiments, building models, evaluating the effects of many factors and finding the optimal conditions for desirable responses and reducing the number of required experiments (Coninck, 2000). In

biological processes, especially in the production of invertase, RSM has been adopted to optimize the growth of microorganisms and the production of invertase (Shankar and Isaiarasu, 2012).

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 (Balusu, 2005). 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 (Sivakumar, 2012a). Therefore, this study was mainly focused on statistical optimization of invertase production using central composite design for high yield with low cost. In this work, RSM was adopted to determine the optimal conditions for the production of invertase from *Saccharomyces cerevisiae* MTCC 170 and the interactions among the factors that influence the response of the invertase production.

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 2^3 factorial design with six axial points ($\alpha = 1.68$) and six replication of the centre 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.

$$Y= \beta_o + \Sigma \beta i \chi i + \Sigma \beta ii \chi i^2 + \Sigma \beta ij \chi i \chi j$$

Where, Y is the predicted response, β0 the intercept term, βi the linear coefficients, βii the quadratic coefficients, βij the interactive coefficients, and xi and xj the coded independent variables (Song, 2007).

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, 30^0C] 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 (Sivakumar, 2012b).

Invertase activity was assayed as per the method of Sumner and Howells (1935) 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 then the 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 1µ mol of glucose/ml/minute under assay condition. Enzyme activity was expressed in units.

Invertase activity was calculated using this formula:

IU/ml = concentration of glucose / 0.5×30×0.180

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).

RESULTS AND DISCUSSION

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 0.37 IU/ml. By applying multiple regression analysis to the experimental data (Table 4), the following second order polynomial equation was established:

Final Equation in Terms of Coded Factors

INVERTASE=+0.37+7.089E-003*A-0.011*B+0.016*C-7.500E-003*A*B+0.010*A*C-0.020*B*C-0.059*A2-0.071*B2-0.073*C2

Final Equationin Terms of Actual Factors

INVERTASE=-4.07696+1.88527*ORANGE PEEL+1.49540 *YEAST EXTRACT+2.68114*METHIONINE-0.060000*ORANGE PEEL*YEAST EXTRACT+0.20000*ORANGE PEEL*METHIONINE-0.80000*YEASTEXTRACT*METHIONINE-0.23514*ORANGE PEEL2-1.13854*YEAST EXTRACT2-7.29263*METHIONINE2

Where, Y1 was the invertase production, X1 the orange peel, X2 the yeast extract and X3 the methionine. The Model F-value of 211.25 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, C, AC, BC, A2, B2, C2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Pred R-Squared" of 0.9600 is in reasonable agreement with the "Adj R-Squared" of 0.9901. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 34.254 indicates an adequate signal. This model can be used to navigate the design space.

Table 1. Independent variables and their coded levels for the central composite design used for invertase production by Saccharomyces cerevisiae MTCC 170

Variables	-α	Low value	Coded variable	High value	+α
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 MTCC 170

Std	Run	Factor 1 Orange peel%	Factor 2 Yeast extract%	Factor 3 Methionine%	Invertase IU/ml
16	1	4.00	0.50	0.20	0.37
4	2	4.50	0.75	0.10	0.16
8	3	4.50	0.75	0.30	0.17
14	4	4.00	0.50	0.37	0.18
9	5	3.16	0.50	0.20	0.19
19	6	4.00	0.50	0.20	0.37
12	7	4.00	0.92	0.20	0.14
5	8	3.50	0.25	0.30	0.19
1	9	3.50	0.25	0.10	0.14
11	10	4.00	0.08	0.20	0.18
10	11	4.84	0.50	0.20	0.20
17	12	4.00	0.50	0.20	0.37
3	13	3.50	0.75	0.10	0.17
13	14	4.00	0.50	0.03	0.13
20	15	4.00	0.50	0.20	0.37
6	16	4.50	0.25	0.30	0.25
7	17	3.50	0.75	0.30	0.15
2	18	4.5	0.25	0.10	0.15
18	19	4.00	0.50	0.20	0.37
15	20	4.00	0.50	0.20	0.37

Table 3. The matrix of the CCD experiment and the corresponding experimental data by Saccharomyces cerevisiae MTCC 170

Std	Run	Factor 1 Orange peel%	Factor 2 Yeast extract%	Factor 3 Methionine%	Actual value	Predicted value
16	1	4.00	0.50	0.20	0.37	0.37
4	2	4.50	0.75	0.10	0.16	0.14
8	3	4.50	0.75	0.30	0.17	0.16
14	4	4.00	0.50	0.37	0.18	0.19
9	5	3.16	0.50	0.20	0.19	0.19
19	6	4.00	0.50	0.20	0.37	0.37
12	7	4.00	0.92	0.20	0.14	0.15
5	8	3.50	0.25	0.30	0.19	0.18
1	9	3.50	0.25	0.10	0.14	0.13
11	10	4.00	0.08	0.20	0.18	0.18
10	11	4.84	0.50	0.20	0.20	0.21
17	12	4.00	0.50	0.20	0.37	0.37
3	13	3.50	0.75	0.10	0.17	0.17
13	14	4.00	0.50	0.03	0.13	0.13
20	15	4.00	0.50	0.20	0.37	0.37
6	16	4.50	0.25	0.30	0.25	0.23
7	17	3.50	0.75	0.30	0.15	0.12
2	18	4.5	0.25	0.10	0.15	0.14
18	19	4.00	0.50	0.20	0.37	0.37
15	20	4.00	0.50	0.20	0.37	0.37

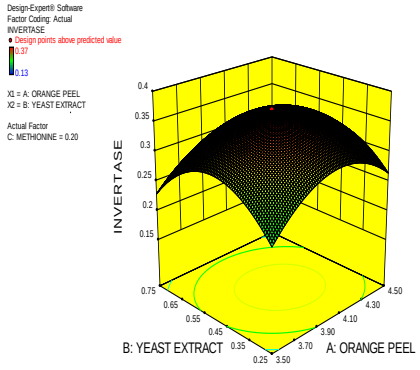

Figure 1a. Interaction between orange peel and yeast extract

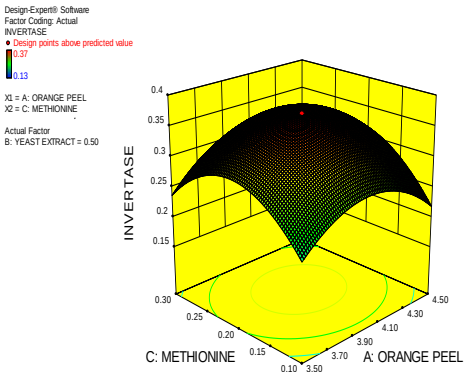

Figure 1b. Interaction between orange peel and methionine

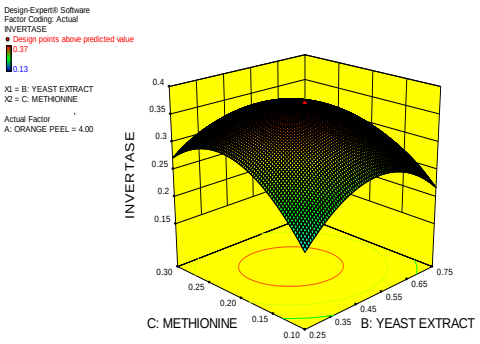

Figure 1c. Interaction between yeast extract and methionine

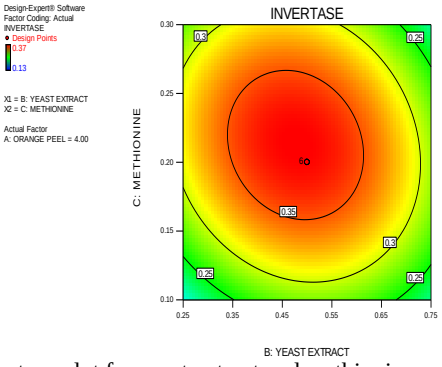

Figure 2a. Contour plot for yeast extract and methionine

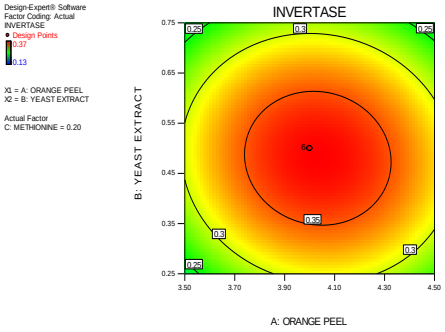

Figure 2b. Contour plot for orange peel and yeast extract

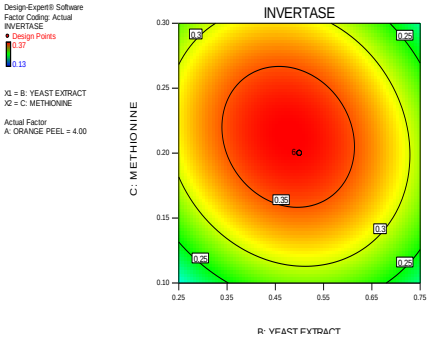

Figure 2c. Contour plot for orange peel and methionine

Table 4. Variance analysis of response surface quadratic model for invertase production by S. cerevisiae MTCC 170

Source	Sum of squares	df	Mean square	F-value	p-value Prob > F
Model	0.18	9	0.020	211.25	< 0.0001
A-orange peel	6.864E-004	1	6.864E-004	7.36	0.0218[C]
B-yeast extract	1.588E-003	1	1.588E-003	17.04	0.0021[C]
C-methionine	3.677E-003	1	3.677E-003	39.44	< 0.0001[C]
AB	4.500E-004	1	4.500E-004	4.83	0.0527
AC	8.000E-004	1	8.000E-004	8.58	0.0151[C]
BC	3.200E-003	1	3.200E-003	34.33	0.0002[C]
A2	0.050	1	0.050	534.18	< 0.0001[C]
B2	0.073	1	0.073	782.74	< 0.0001[C]
C2	0.077	1	0.077	822.12	< 0.0001[C]
Residual	9.323E-004	10	9.323E-005		
Lack of fit	9.323E-004	5	1.865E-004		
Pure error	0.000	5	0.000		
Cor total	0.18	19			

R^2= 0.9948; Adj R^2= 0.9901; CV%= 4.18; [c]Model terms are significant

DISCUSSION

The yeast invertase recovered from optimized medium was 0.37 IU/ml. In this case of Saccharomyces cerevisiae MTCC 170 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 0.37 IU/ml. The three-dimensional response surfaces Figure 1a, 1b, 1c and contour plots are shown in Figure 2a, 2b, 2c (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 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 (Shankar and Isaiarasu, 2012).

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 0.37 IU/ml of invertase at 48 hours. The nearness of the coefficient of determination (R^2=0.9948) 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.

CONCULSION

In the present work the optimum culture conditions for invertase production by *Saccharomyces cerevisiae* MTCC 170 was studied by RSM using central composite design with three variables orange peel, yeast extract and methionine for maximizing the production of invertase.

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