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Statistical Modeling for Permeabilization of a Novel Yeast Isolate for β-Galactosidase Activity Using Organic Solvents

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Abstract**—**The hydrolysis of lactose using β-galactosidase is one of the most promising biotechnological applications, which has wide range of potential applications in food processing industries. However, due to intracellular location of the yeast enzyme, and expensive extraction methods, the industrial applications of enzymatic hydrolysis processes are being hampered. The use of permeabilization technique can help to overcome the problems associated with enzyme extraction and purification of yeast cells and to develop the economically viable process for the utilization of whole cell biocatalysts in food industries. In the present investigation, standardization of permeabilization process of novel yeast isolate was carried out using a statistical model approach known as Response Surface Methodology (RSM) to achieve maximal β-galactosidase activity. The optimum operating conditions for permeabilization process for optimal β-galactosidase activity obtained by RSM were 1:1 ratio of toluene (25%, v/v) and ethanol (50%, v/v), 25.0 ^{o}C temperature and treatment time of 12 min, which displayed enzyme activity of 1.71 IU /mg DW.

Keywords**—**β-galactosidase, optimization, permeabilization, response surface methodology, yeast.

I. Introduction

A wide range of microbial products have been made possible using micro-organisms through biotechnological approach. Although many microbial enzymes are produced by extracellular means, large proportion of the potentially useful microbial products is retained within the cells (intracellular). The cell disruption processes applied to extract the intracellular products involves the breaking of the cell envelope and release of all the intracellular components into surrounding medium [1]. Further, the high cost of the enzyme purification becomes the main problem of this process. However, these types of problems can be overcome using permeabilization technique.

The enzyme β-galactosidase can be obtained either in extracellular or intracellular form from wide variety of micro-organisms such as bacteria, fungi and yeast, which has wide range of applications in food processing industries [2]. Due to its hydrolyzing property, it has significant role in the hydrolysis of lactose in milk and in fermented milk products to overcome lactose intolerance. The use of β-galactosidase to avoid lactose crystallization in condensed and frozen dairy products such as ice cream and condensed milk raises its industrial importance [3]. Recently, β-galactosidase has also been employed in the production of different types of prebiotics such as lactulose, galacto-oligosaccharide and lactosucrose [4]-[6]. Therefore, it is important to search new strains, which can display higher enzyme activity for its wider utilization and industrial prospective.

The optimization of parameters by the conventional method involves changing one independent variable while unchanging all others at a fixed level. This is extremely time-consuming and expensive for a large number of variables [7]. Response surface methodology (RSM) is a collection of statistical and mathematical techniques that are useful for developing, improving, and optimizing processes [8]. In addition to analyzing the effects of the independent variables, this RSM generates a mathematical model that accurately describes the overall process [9]. Keeping the above in view, Response surface modeling technique was applied in present investigation to optimize the permeabilization conditions of yeast cells using organic solvents for enhanced β-galactosidase activity.

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II. Materials and Methods

A. Microorganism

The yeast strain isolated in our laboratory was used for the permeabilization for β-galactosidase activity. The identification of a novel yeast isolate was carried out with the help of partial 18S rRNA, ITS1, 5.8S rRNA, ITS2 and partial 28S rRNA gene analysis from Merck Specialities Private Limited GeNeiTM, Bangalore (India) and was identified as *Kluyveromyces marxianus.*

B. Inoculum Preparation

Loopful yeast from slant was added to sterile media containing (%, w/v) glucose (1.0), malt extract (0.3), yeast extract (0.3) and peptone (0.5) at temperature 30^{o}C for the incubation period of 20h and used as inoculum.

C. Submerged Fermentation of Yeast Isolate for β-Galactosidase Production

The isolated yeast cells were inoculated into the media containing (%, w/v) lactose (5.0), yeast extract (0.36), magnesium sulphate (0.05), urea (0.11) and incubated at temperature 30^{o}C, pH 5.25, for 28h.

D. Measurement of Enzyme Activity

β-Galactosidase activity assay was carried out using the method of Miller [10]. One unit of enzyme activity is equivalent to one micromole of o-nitrophenol liberated per min under standard assay conditions.

E. Permeabilization of Yeast Cells

The permeabilization of yeast cells was carried out by following the method of Panesar [11] with slight modifications. Different mixture of permeabilizing agents (1:1 ratio) such as ethanol (50%, v/v) and acetone (30%, v/v), ethanol (50%, v/v) and toluene (25%, v/v), ethanol (50%, v/v) and n-propanol (20 %, v/v), ethanol (50%, v/v) and iso-propanol (40%, v/v), ethanol (50%, v/v) and n-butanol (10%, v/v) were used for permeabilization of yeast cells.

F. Experimental Design for the Process Optimization

For the optimization of permeabilization process, the experiments were conducted according to Central Composite Rotatable Design [12], with three variables (toluene: ethanol, temperature and treatment time) at five level each. The experimental design was generated by using Design Expert statistical software (Trial version 6.0, Stat-Ease Inc., Minneapolis, MN, USA Statistical software).

Three factors and Central Composite Rotatable design (CCCD) with 20 design points having 14 combinations with 6 replications of the central point were adapted in this study. The independent variables and their levels are presented in Table I.

The highest and lowest levels of the interested range for each variable were coded as plus and minus one, respectively, and the center point of the range was coded to be zero.

Five different levels for each experiment in coded form are

$$-\alpha, -1, 0, +1, +\alpha,$$

where $\alpha = [2]^{(No.of\ variable/4)} = [2]^{3/4} = 1.682$

The relationship between the coded and uncoded form of the variables is:

$$\text{Coded value} = x_i = \frac{2\,(X_i - \bar{X}_i)}{R_i} \quad (1)$$

where *Xi* is the actual setting in the uncoded units of the *i*th factor, $\bar{X}_i$ is the average of the low and high settings for the *i*th factor, and *Ri* is the range between the low and high settings.

TABLE I
LEVEL OF DIFFERENT PROCESS VARIABLES FOR THE PERMEABILIZATION OF YEAST CELLS FOR *β*-GALACTOSIDASE ACTIVITY

Factor	Process parameter	Level				
		-1.682	**-1**	**0**	**+1**	**+1.682**
X_1	Toluene and ethanol ratio (%, v/v)	23.18:76.82	30:70	40:60	50:50	56.82:43.18
X_2	Treatment time (min)	6.59	10	15	20	23.40
X_3	Temperature (°C)	16.59	20	25	30	33.41

Based on our preliminary investigation, the low and high levels chosen for three independent variables for toluene: ethanol ratio, temperature and treatment time were 30:70-50:50, 20-30°C and 10-20min respectively. The value of enzyme activity was taken as response for the process optimization. The results of the enzymatic activity in the CCRD for the three process variables: solvent concentration, incubation time and temperature and the values predicted by the model are presented in Table II. The experiments were conducted randomly.

The second order polynomial equation was fitted to the experimental data of each dependent variable as given below:

$$Y_i = \beta_0 + \sum_{i=1}^{n} \beta_i x_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} x_i x_j + \sum_{i=1}^{n} \beta_{ii} x_i^2 \quad (2)$$

where Y_i = Response {Y_1 = Enzyme activity (IU/ mg DW) x_i = Independent variables (x_1 = Toluene (25%, v/v): ethanol (50%, v/v), x_2 = Treatment time (min), x_3 = Temperature (°C), β_o is the value of coefficient of fitted response at the central point of design, β_i, β_{ij}, β_{ii} are the linear, quadratic and cross product regression coefficients, respectively.

III. RESULTS AND DISCUSSION

The effect of the process parameters was monitored to optimize the permeabilization process during the course of the present investigation.

A. Permeabilization of Yeast Isolate

Different organic solvents (ethanol and acetone, ethanol and toluene, ethanol and propanol, ethanol and iso-propanol, ethanol and n-butanol) were applied for the permeabilization of yeast cells for *β*-galactosidase activity (Fig. 1). Among these permeabilizing agents, the maximum enzyme activity 1.631 IU/ mgDW was observed with toluene: ethanol mixture. However, minimum enzyme activity was observed with ethanol and n-butanol mixture. Therefore, toluene: ethanol mixture was selected for further experimentation.

B. Selection of Factor Levels

From the preliminary experiments, the low and high levels chosen for three independent variables for toluene: ethanol ratio, incubation time and temperature were 30:70-50:50, 10-20min, and 20-40ºC respectively, for the permeabilization of yeast cells to get maximum *β*-galactosidase activity.

C. Diagnostic Checking of Fitted Model and Surface Plots

The result of second order response model in the form of analysis of variance (ANOVA) is given in Table III. The ANOVA result indicated the quadratic regression to produce the second order model was significant (P< 0.0001). The lack of fit was non-significant (P=4.54), and only 0.01 % of the total variation were not explained by model (R^2=99.61%). The model F-value of 297.55 also implies that the model is significant. The value of adjusted determination coefficient (adjusted R^2 = 99.26%) was high to promote a high significance of model.

The magnitude of P-value in Table III indicates that the linear and quadratic terms of all the process variables have significant effects on β-galactosidase activity at 5% level of significance (P< 0.05). The magnitude of β-coefficient as given in Table III revealed that the linear term of toluene (25%, v/v): ethanol (50 %, v/v) had the positive effect (β= +0.033) on enzyme activity. The temperature have negative effect (β= -0.15) followed by treatment time (β=-0.088) on enzyme activity. This indicates that increase in enzyme activity with an increase of toluene (25%, v/v): ethanol (25%, v/v) ratio is due to the fact that low amount of permeabilizing agent is insufficient for effective permeabilization. To analyze the results, the response surface and contour plots were generated for different interaction of any two independent variables, while holding the value of third variable as constant at the central level. Such three-dimensional surfaces could give accurate geometrical representation and provide useful information about the behaviour of the system within the experimental design. Thus, effects of the independent variables on the enzyme activity were represented via response surface plots.

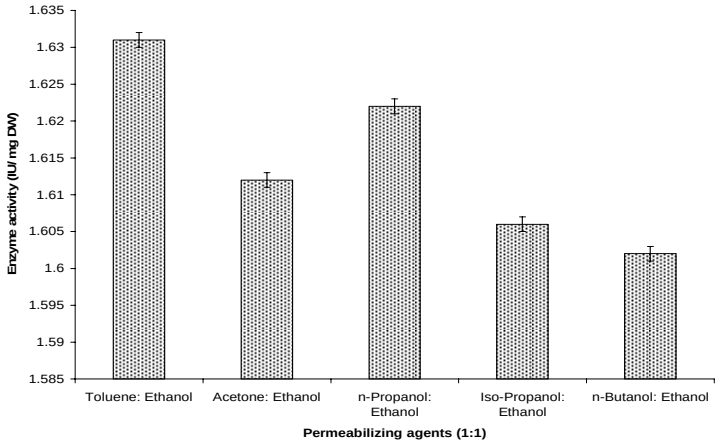

Fig. 1 Effect of a mixture of permeabilizing agents on the permeabilization of yeast cells for β-galactosidase activity

TABLE II
EXPERIMENTAL DESIGN OF PROCESS VARIABLES AND VALUES OF EXPERIMENTAL DATA FOR THE OPTIMIZATION OF PERMEABILIZATION PROCESS FOR β-GALACTOSIDASE ACTIVITY

	Coded variable			Actual response
Std	**x_1**	**x_2**	**x_3**	**Enzyme activity (IU/ mg DW)**
1	-1.000	-1.000	-1.000	1.096±0.021
2	-1.000	-1.000	-1.000	1.484±0.024
3	-1.000	1.000	-1.000	1.545±0.025
4	-1.000	1.000	-1.000	1.320±0.024
5	-1.000	-1.000	1.000	1.178±0.022
6	-1.000	-1.000	1.000	1.487±0.024
7	-1.000	1.000	1.000	0.920±0.018
8	-1.000	1.000	1.000	0.736±0.015
9	-1.682	0.000	0.000	1.438±0.023
10	1.682	0.000	0.000	1.535±0.025
11	0.000	-1.682	0.000	1.286±0.021
12	0.000	1.682	0.000	0.998±0.020
13	0.000	0.000	-1.682	1.327±0.024
14	0.000	0.000	1.682	0.768±0.016
15	0.000	0.000	0.000	1.655
16	0.000	0.000	0.000	1.634
17	0.000	0.000	0.000	1.646
18	0.000	0.000	0.000	1.656
19	0.000	0.000	0.000	1.652
20	0.000	0.000	0.000	1.682

TABLE III
REGRESSION SUMMARY AND ANOVA TABLE FOR ENZYME ACTIVITY FOR CODED VALUE OF PROCESS VARIABLE

Sources	*Df*	*β*	Sum of squares	F-value	p-value
Model	9	1.65	1.79	285.80	< 0.0001
x_1	1	0.033	0.015	21.41	< 0.0001
x_2	1	-0.088	0.107	153.62	< 0.0001
x_3	1	-0.151	0.312	448.27	< 0.0001
x_1^2	1	-0.055	0.043	62.087	< 0.0001
x_2^2	1	-0.176	0.449	645.49	< 0.0001
x_3^2	1	-0.209	0.635	912.90	< 0.0001
x_1x_2	1	-0.138	0.153	219.70	< 0.0001
x_1x_3	1	-0.005	1.805×10^{-4}	0.26	0.6216*
x_2x_3	1	-0.162	0.21	300.74	< 0.0001
Residual	10	-	6.96×10^{-3}	-	-
Lack of Fit	5	-	5.703×10^{-3}	4.54	0.3234*
Pure Error	5	-	1.257×10^{-3}	-	
Cor Total	19	-	-	-	
R-Squared	0.9961		-	-	
Adj R-Squared	0.9926		-	-	

* Non-significant at 5% level

The effect of solvent concentration and treatment time on enzyme activity has been presented in Fig. 2. At lower value of treatment time, the enzyme activity of yeast cells increased with the increase in toluene: ethanol ratio, but at higher value of incubation time a slight decrease in enzyme activity has been observed with increase in toluene: ethanol ratio, it may be due to might be due to the partial inactivation of enzyme at high temperature. The maximum enzyme activity has been found in the range of 45: 55 to 47: 53 (%, v/v) of toluene: ethanol and 11 to 13 min of incubation time. However, the optimal treatment time of 14 min has been reported in case of permeabilization of *Kluyveromyces marxianus* var. *lactis* NCIM 3566 with cetyltrimethylammonium bromide [13], whereas an optimal time for the permeabilization of *K. fragilis* NRRL Y-1196 cells with digitonin was 30min [14].

The effect of solvent concentration and incubation temperature on the enzyme activity was shown in Fig. 3. At lower values of solvent concentration and at higher values of temperature, a decrease in enzyme activity was observed. However at high temperature, a decrease in enzyme activity was observed with increase in incubation time. On the other hand, at low temperature, the enzyme activity of yeast cells increased with the increase in treatment time (Fig. 4), it may be due to the partial inactivation of enzyme at high temperature. The maximum enzyme activity was found at the temperature range of 23.5-25.0ºC and the incubation time of 10.5-12.0min. The higher enzyme activity was observed at the temperature range of 23.5-25.0ºC. An optimal temperature of 26ºC for the permeabilization of *K. fragilis* NRRL Y-1196 cells has been reported previously [15]. However, the optimal treatment time of 18 min and temperature of 23ºC using ethanol for the permeabilization of *K. marxianus* has been reported previously [16].

The results of second order responses model in the form of Analysis of variance (ANOVA) are given in Table III. The high value of R^2 indicate that only 0.39% of the total variation was not explained by the model (R^2 = 99.61%). Fischers's F Test and p values demonstrate significance for the regression model.

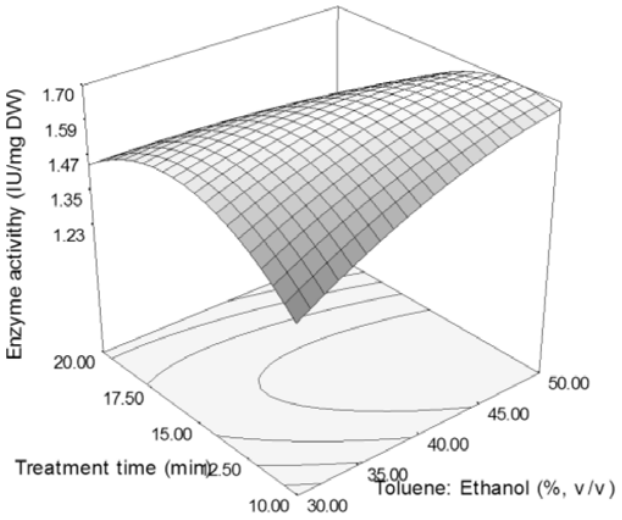

Fig. 2 Effect of treatment time and toluene: ethanol mixture on the permeabilization of yeast cells for *β*-galactosidase activity

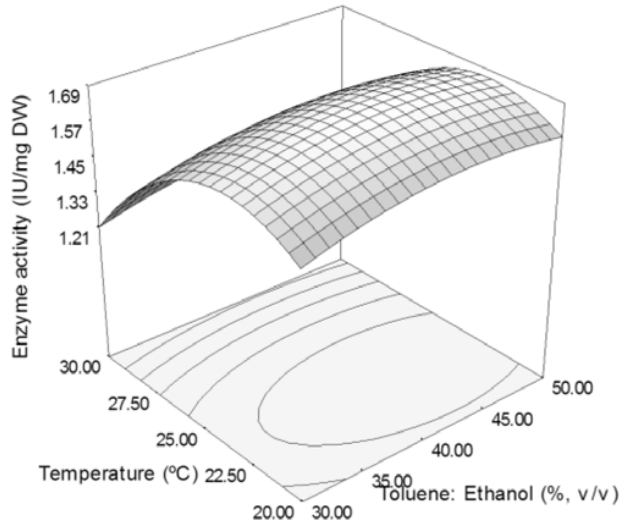

Fig. 3 Effect of temperature and toluene: ethanol mixture on the permeabilization of yeast cells for *β*-galactosidase activity

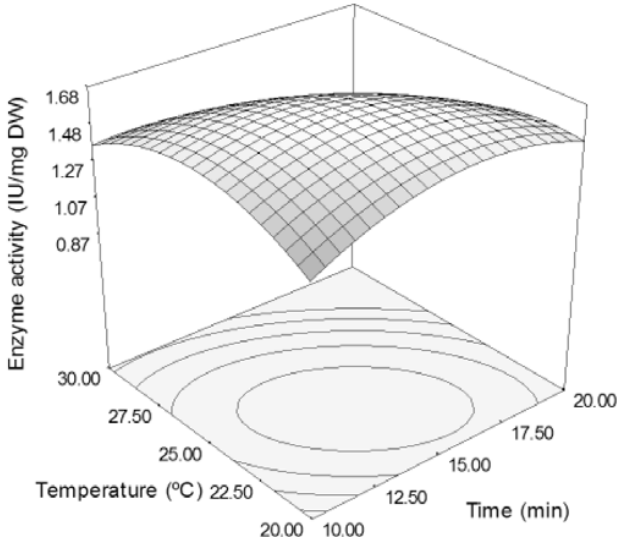

Fig. 4 Effect of temperature and treatment time on the permeabilization of yeast cells for *β*-galactosidase activity

The second-order polynomial equation of fitted model after neglecting the effect of non-significant term in term of uncoded (actual) terms of process variable is:

$$\text{Enzyme activity (IU/mgDW)} = -9.35205 + 0.090959\,X_1 + 0.46652\,X_2 + 0.49056\,X_3 - 5.47569\times10^{-4}\,{X_1}^2 - 7.06224\times10^{-3}\,{X_2}^2 - 8.39867\times10^{-3}{X_3}^2 - 2.76500\times10^{-3}\,X_1X_2 - 6.47000\times10^{-3}\,X_2X_3 \quad (3)$$

D. Optimization and Validation of Predictive Model

The numerical optimization technique was used for the optimization of process conditions for permeabilization. The main aim was to get maximum *β*-galactosidase activity. The optimum values of the selected variables were obtained by solving the regression equation. The optimized operating conditions for permeabilization were 50: 50 toluene (25%, v/v): ethanol (50%, v/v) ratio, 25ºC temperature and process duration of 12min. The experimental rechecking was performed to ensure the predicted result was not biased towards the practical value. The set of optimal conditions determined by the RSM optimization approach was also used to validate experimentally and predict the values of the responses using the model equation. A mean value of 1.71±0.022 IU/ mgDW for *β*-galactosidase activity obtained from real experiments demonstrated the validation of the RSM model indicating that the model was adequate for the permeabilization process.

IV. Conclusions

It is evident from the above, that the use of this statistical method not only helped in searching the optimum levels of the most significant factors but also proved to be useful and satisfactory in this process-optimization. By fitting the experimental data to a second order polynomial equation, the recommended process variables were 50:50 (toluene, 25% v/v: ethanol, 50% v/v), treatment time 12min and 25.0°C to get maximum *β*-galactosidase activity. Further, these optimal conditions were validated using validation experiments. Using the optimum levels of fermentation parameters, a maximum enzyme activity of 1.71 IU mg/ DW was obtained. The kinetic model described here can provide effective guidelines for scale-up studies on the permeabilization of yeast cells for *β*-galactosidase production. The developed permeabilized cell technology can be helpful to overcome the problems associated with enzyme extraction and purification of yeast cells, which can be applied in lactose hydrolysis in dairy industry and synthesis of lactose-derived functional food ingredients.

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