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Permeabilization of Yeast Cells for β-Galactosidase Activity using Mixture of Organic Solvents: A Response Surface Methodology Approach

Shweta Kumari, Parmjit S. Panesar, Manab B. Bera and Bahadur Singh
Biotechnology Research Laboratory, Department of Food Engineering and Technology, Sant Longowal Institute of Engineering and Technology, Longowal 148 106, Punjab, India

Corresponding Author: Parmjit S. Panesar, Biotechnology Research Laboratory, Department of Food Engineering and Technology, Sant Longowal Institute of Engineering and Technology, Longowal 148 106, Punjab, India Tel: +91-1672-253252 Fax: +91-1672-280057

ABSTRACT

The enzyme β-galactosidase, have been used in the dairy industry for the improvement of lactose intolerance. However, the industrial applications of enzymatic hydrolysis processes are being hampered, due to intracellular location of the yeast enzyme. The treatment of yeast cells with various chemical agents (n-butanol, n-propanol, iso-propanol, acetone, ethanol, toluene and a mixture of organic solvent) have been carried out to increase the β-galactosidase activity. Response Surfaces Methodology (RSM) using Central Composite Rotable Design (CCRD) was employed to optimize the toluene (25%, v/v): ethanol (50%, v/v) ratio, treatment time and temperature. The optimum operating conditions for permeabilization process to achieve maximum β-galactosidase activity obtained by RSM were 1:1 ratio of toluene (25%, v/v) and ethanol (50%, v/v), 23.0°C temperature and treatment time of 12 min which displayed enzyme activity of 1.68 IU mg^{-1} DW. The use of permeabilized cells can help to overcome the problems/costs associated with enzyme extraction and purification from yeast cells and in the development of a low-cost technology for β-galactosidase production.

Key words: *Kluyveromyces marxianus*, permeabilization, β-galactosidase activity, organic solvents, response surface methodology

INTRODUCTION

The enzyme β-galactosidase (EC.3.2.1.23) which hydrolyzes lactose into glucose and galactose has several applications in biotechnology, pharmaceutical and food industries. This enzyme can be obtained from a wide variety of sources, such as microorganisms, plants and animals; however, their properties differ markedly according to the enzyme source (Agrawal *et al*., 1989; El-Sawah and Ashore, 1999; Hassan *et al*., 2006; Finocchiaro *et al*., 1980; Mahoney, 1997; Zheng *et al*., 2006). β-Galactosidase is also known for trans-galactosylation reaction and synthesized lactose based derivatives including galacto-oligosaccarides, however, the industrial applications of β-galactosidase has been hampered by the difficulty and expense of releasing active enzyme in good yield from the cells and further, the cost of the purification processes especially in the case of bacteria and yeast. Thus, the use of intracellular β-galactosidase as a whole cell biocatalyst is an interesting alternative. However, a major drawback in the use of whole cells is the poor permeability of the cell membrane to lactose (Panesar *et al*., 2006).

The permeabilization methods are simple, rapid and allow the assay of enzymes under the natural environment of the cell. Thus, cell permeabilization can be used as an important tool in the biotransformation process that can be an inexpensive alternative to purified enzyme systems. In this, cell structure is altered to make it porous to allow small molecules, such as substrates or products, to cross freely and the cells are spared from the harsh treatment associated with disruption of cells (Naglak *et al*., 1990). Several chemical agents such as cetyltrimethylammonium bromide (Kaur *et al*., 2009), Chloroform (Choi *et al*., 2004), digitonin (Joshi *et al*., 1989), Ethanol (Lee *et al*., 2004) have been used for the permeabilization of yeast cells. Thus, the application of a mixture of organic solvents can also be explored to further increase permeabilization of the yeast cells.

Conventional practice of single-factor optimization by maintaining other factors at an unspecified constant level does not depict the combined effect of all the factors involved. 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 (Adinarayana *et al*., 2002) and also may result in wrong conclusions (Oh *et al*., 1995). Statistical methodologies can be applied in biotechnological processes and Response Surface Methodology (RSM) is a combination of mathematical and statistical techniques that are useful for analyzing the effects of several independent variables on the system response without the need of a predetermined relationship between the objective function and the variables (Draper and Lin, 1990). Keeping the above in view, the present study was performed to optimize the process conditions for permeabilization of yeast cells by using a mixture of organic solvents for maximum β-galactosidase activity through RSM designed with composite central design.

MATERIALS AND METHODS

This study was conducted during the period of May, 2010 to December, 2010.

Procurement of microorganism: *Kluyveromyces marxianus* NCIM 3465 was procured from the National collection of Industrial Microbiology, National Chemical Laboratory, Pune, India.

Maintenance and cultivation of the culture: The maintenance and cultivation of the culture was carried out by the method of Panesar (2008).

Permeabilization of yeast cells: The permeabilization of yeast cells was carried out by following the method of Joshi *et al*. (1987) with slight modification. The cells were harvested from 5 mL of fermentation media by centrifugation (5000 rpm for 5 min at 4°C) and washed with potassium phosphate buffer (0.1 M, pH 7.0). Different permeabilization agents such as benzene, n-butanol, n-propanol, isopropanol, toluene, acetone, ethanol and a mixture of permeabilizing agents in 1:1 ratio of 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 added to the yeast biomass and the final volume was made 5 mL using the same buffer. The contents were mixed on a vortex mixture and incubated for 10 min, under shaking conditions. After this, the cells was recentrifuged and washed twice with the same buffer and analyzed for the enzyme activity.

Table 1: Level of different process variables for β-galactosidases

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	23.41
X_3 Temperature (°C)	16.59	20	25	30	33.40

Table 2: Experimental designs of process variables and values of experimental data for optimization of enzyme activity

Coded variable			Un-coded variable			Response
x_1	x_2	x_3	Toluene: Ethanol ratio (%, v/v)	Treatment time (min)	Temperature (°C)	Enzyme activity (IU mg^{-1} Dw[a])
1.000	-1.000	-1.000	30:70	10	20	1.08 ± 0.015
1.000	-1.000	-1.000	50:50	10	20	1.48±0.026
1.000	1.000	-1.000	30:70	20	20	1.53±0.026
1.000	1.000	-1.000	50:50	20	20	1.30±0.030
-1.000	-1.000	1.000	30:70	10	30	1.12±0.038
1.000	-1.000	1.000	50:50	10	30	1.42±0.040
-1.000	1.000	1.000	30:70	20	30	0.88±0.037
1.000	1.000	1.000	50:50	20	30	0.67±0.045
-1.682	0.000	0.000	23.2:76.8	15	25	1.35±0.032
1.682	0.000	0.000	56.8:43.2	15	25	1.51±0.035
0.000	-1.682	0.000	40:60	6.59	25	1.27±0.015
0.000	1.682	0.000	40:60	23.41	25	0.99±0.030
0.000	0.000	-1.682	40:60	15	16.59	1.36±0.030
0.000	0.000	1.682	40:60	15	33.41	0.78±0.040
0.000	0.000	0.000	40:60	15	25	1.63
0.000	0.000	0.000	40:60	15	25	1.64
0.000	0.000	0.000	40:60	15	25	1.63
0.000	0.000	0.000	40:60	15	25	1.62
0.000	0.000	0.000	40:60	15	25	1.60
0.000	0.000	0.000	40:60	15	25	1.66

[a]Dry weight

Measurement of enzyme activity: β-galactosidase activity assay was carried out by the method of Miller (1972). One unit of enzyme activity is defined as one micromole of o-nitrophenol liberated per min under standard assay conditions.

Experimental design and statistical analysis: Experiments were conducted according to Central Composite Rotatable Design (CCRD) with three variables at five levels each. The design was generated by Design Expert, Trial version 6.0, Stat-Ease INC., Minneapolis, MN, USA statistical software. The variables were toluene: ethanol ratio, treatment time and temperature for the enzyme activity. The low level and high level in actual (un-coded) form were taken as 30:70-50:50 of toluene and ethanol ratio, 10-20 min (treatment time) and 20-30°C (temperature). The relationships between coded to uncoded independent process variables are given in Table 1. The experimental plan in coded and un-coded form of process variables along with result is as given in Table 2. The experiments were conducted randomly.

Statistical analysis and optimization: The second order polynomial equation was fitted to the experimental data of each dependent variable as given:

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

where, Y_i is Response {Y_1 is Enzyme activity (IU mg^{-1} DW), x_i is Independent variables, (x_1 is Toluene (25%, v/v): Ethanol (50%, v/v), x_2 is Temperature (°C), x_3 is Treatment time (min), 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.

RESULTS AND DISCUSSION

The permeabilization of *Kluyveromyces marxianus* NCIM 3465 cells was carried out by using various chemical agents (n-butanol, n-propanol, iso-propanol, acetone, ethanol and toluene) for β-galactosidase activity. From the experimentation, it has been observed that permeabilization increase with the chemical concentration up to a critical value, where a maximum enzyme activity can be observed (Fig. 1). At higher concentration of the permeabilizing agent, the enzyme activity decreases which may be due to the leakage of the enzyme from the cells or cell lysis. However, at low concentration, enzyme activity was further decreased due to the insufficient amount of the agent for the permeabilization.

The maximum β-galactsidase activity of 1.378, 1.471, 1.193, 1.432, 1.475 and 1.535 IU mg^{-1} DW have been observed with 10% (v/v) of n-butanol, 20% (v/v) n-propanol, 40% (v/v) iso-propanol, 20% (v/v) toluene, 30% acetone and 50% (v/v) ethanol, respectively (Fig. 1). Similarly, Decleire *et al.* (1987) have observed that the minimum solvent concentrations of 10% n-butanol; 20% propanol; 30% isopropanol, tert-butanol; 40% ethanol and acetone and 70% dimethylsulphoxide were required for maximum enzyme activity. Flores *et al.* (1994) reported that 40% (v/v) of ethanol was an effective permeabilizing agent for maximum β-galactosidase activity.

The results also revealed that permeabilization of yeast cells further increases by using a mixture of permeabilizing agents (ethanol and acetone, ethanol and toluene, ethanol and n-propanol, ethanol and iso-propanol, ethanol and n-butanol, ethanol and benzene). Among a mixture of different permeabilizing agents (Fig. 2), maximum enzyme activity 1.61 IU mg^{-1} DW was found with 1:1 ratio toluene (25%, v/v): ethanol (50%, v/v). Various permeabilizing agents such as iso-propanol, tert-butanol, ethanol: toluene, chloroform, CTAB have been used for the

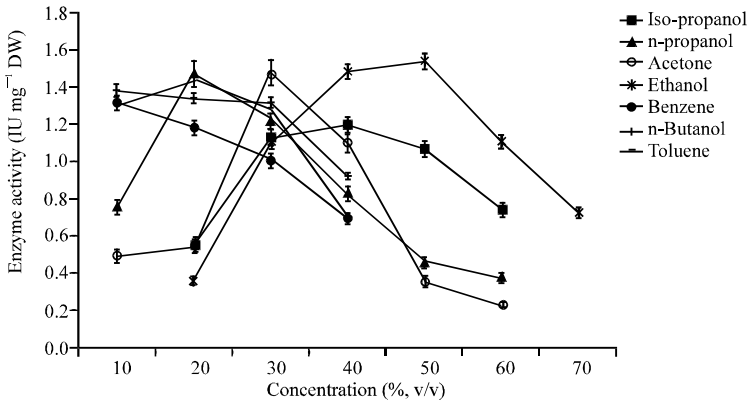

Fig. 1: Effect of concentration of iso-propanol, n-propanol, acetone, ethanol, n-butanol, toluene on the permeabilization of yeast cells

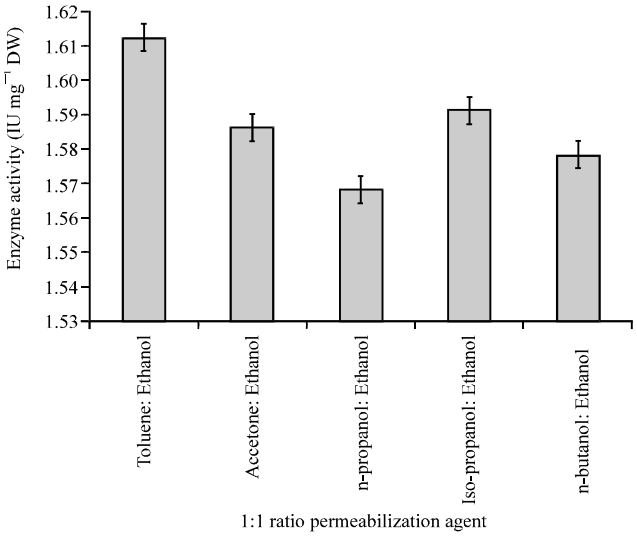

Fig. 2: Effect of a mixture of different permeabilizing agents (1:1 ratio) on the permeabilization of yeast cells

permeabilization of *K. lactis* NRRL Y-1140 and observed that a small quantity of toluene in ethanol or 70% ethanol was most effective permeabilizing agent for β-galactosidase activity (Siso *et al*., 1992).

Optimization of permeabilization of yeast cells: From the preliminary experiments to optimize of permeabilization process by using mixture of organic solvents, it was observed that the range of toluene: ethanol ratio, incubation time and temperature for maximum β-galactosidase activity were 30:70-50:50, 10-20 min and 20-40°C, respectively.

Diagnostic checking of fitted model and surface plots: The ANOVA result in Table 3 indicates that the quadratic regression to produce the second order model was significant ($p<0.0001$). The lack of fit was non significant ($p = 1.61$) and only 0.29% of the total variation were not explained by model ($R^2 = 99.71\%$). The model F-value of 383.14 also implies that the model is significant. The value of adjusted determination coefficient (adjusted $R^2 = 99.45\%$) was high to advocate a high significance of model. The magnitude of p-value in Table 3 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 3 revealed that the linear term of toluene (25%, v/v): ethanol (50%, v/v) had the positive effect ($\beta = +0.039$) on enzyme activity. The temperature have negative effect ($\beta = -0.17$) followed by treatment time ($\beta = -0.086$) on enzyme activity. This indicates that with increase the toluene (25%, v/v): ethanol (25%, v/v) ratio, there is an increase in enzyme activity which indicates that low amount of permeabilizing agent is insufficient for effective permeabilization. However, with an increase in temperature and incubation time enzyme activity decreases which may be due to enzyme inactivation or cell lysis (Joshi *et al*., 1989).

At lower levels of incubation time, the enzyme activity of yeast cells increased with the increase in toluene: ethanol ratio; but at high levels of incubation time a slight decrease in enzyme activity

Table 3: Regression summery and ANOVA table for enzyme activity fir coded value of process variable

Sources	Df	β	Sum of squares	F-value	p-value
Model	9	1.630	1.81	383.14	<0.0001
x_1	1	0.039	0.02	39.70	<0.0001
x_2	1	-0.086	0.10	192.60	<0.0001
x_3	1	-0.170	0.38	724.71	<0.0001
x_1^2	1	-0.071	0.07	138.56	<0.0001
x_2^2	1	-0.180	0.45	853.20	<0.0001
x_3^2	1	-0.200	0.57	1082.16	<0.0001
x_1x_2	1	-0.140	0.17	316.98	<0.0001
x_1x_3	1	-0.011	9.46×10^{-4}	1.80	0.2096*
x_2x_3	1	0.016	0.20	1.86	<0.0001
Residual	10	-	5.26×10^{-3}	-	-
Lack of fit	5	-	3.25×10^{-3}	1.63	0.3034*
Pure error	5	-	2.00×10^{-3}	-	
Cor total	19	-	1.82	-	
R-Squared = 0.9971					
Adj R-Squared = 0.9945					

*Non-significant at 5% level

has been observed with increase in toluene: ethanol ratio (Fig. 3). The decrease in enzyme activity with increase of toluene: ethanol ratio at higher incubation time may be due to the leakage of the enzyme from the cells or cell lysis. The maximum value of enzyme activity has been observed in the range of 45:55 to 47:53 of toluene: ethanol and 12 to 14 min of incubation time. The optimal treatment time of 14 min for the permeabilization of *Kluyveromyces marxianus* var. *lactis* NCIM 3566 using cetyltrimethylammonium bromide (0.06%, w/v), whereas an optimal time 15 min for the permeabilization of *K. lactis* NRRL Y-8279 cells using Isoamyl alcohol (20%, v/v) has been observed by previous researchers (Kaur *et al*., 2009; Dagbagli and Goksungur, 2008).

The variation of enzyme activity as function for interaction of concentration and temperature indicates that there is an increase in enzyme activity with increase of toluene: ethanol ratio (Fig. 4). The increase in temperature decreases the enzyme activity might be because of the inactivation of enzyme or cell lysis at high temperature. The maximum enzyme activity was observed at the temperature range of 20-23°C. An optimal temperature of 25 and 26°C for the permeabilization of *Kluyveromyces bulgaricus* IRC101 and *K. fragilis* NRRL Y-1196 cells has been reported, respectively (Decleire *et al*., 1987; Joshi *et al*., 1989).

At low levels of temperature, the enzyme activity of yeast cells increased with the increase in incubation time (Fig. 5). However at high incubation time, with increase in temperature a decrease in enzyme activity was observed might be due to the partial inactivation of enzyme or cell lysis at high temperature. The maximum enzyme activity was observed at the temperature range of 20.0-23.0°C and the incubation time of 12.5-15.0 min. Whereas, the optimal treatment time of 18 min and temperature of 23°C using ethanol for the optimal permeabilization of *K. marxianus* has been observed previously (Panesar, 2008).

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

$$\text{Enzyme activity (IU mg}^{-1}\text{ DW)} = -9.42366+0.10956\times X_1 + 0.46642X_2 \times +0.46662X_3 \times -7.11206\times10^{-3}\times X_1^2 - 7.05918\text{x}10^{-3}\times X_2^2 - 7.95014\text{x}10^{-3}\times X_3^2 - 2.88750\text{x}10^{-3}\times X_1\times X_2 - X_3 6.25500\text{x}10^{-3}\times X_2\times X_3 \quad (2)$$

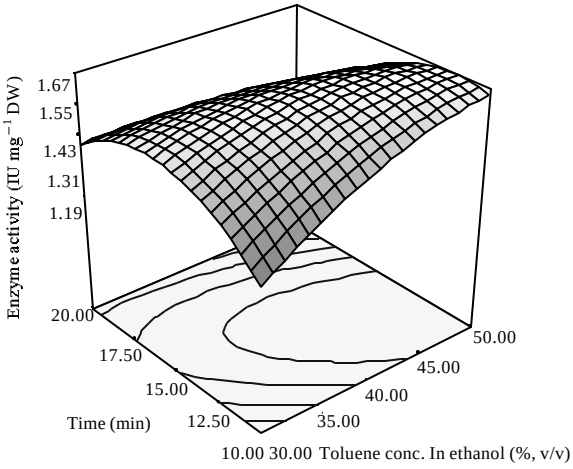

Fig. 3: Effect of treatment time and toluene (25%, v/v): ethanol (50%, v/v) on β-galactosidase activity

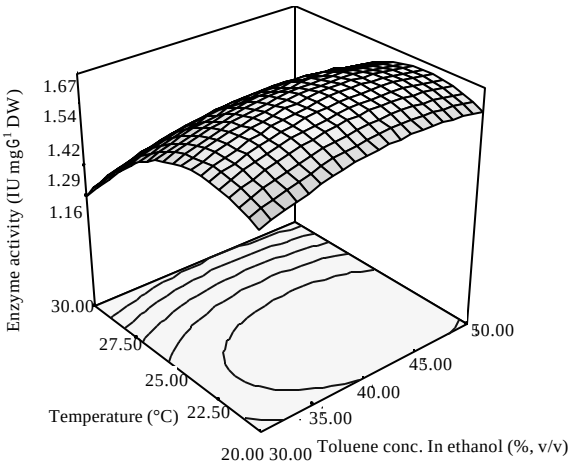

Fig. 4: Effect of temperature and toluene (25%, v/v): ethanol (50%, v/v) on β-galactosidase activity

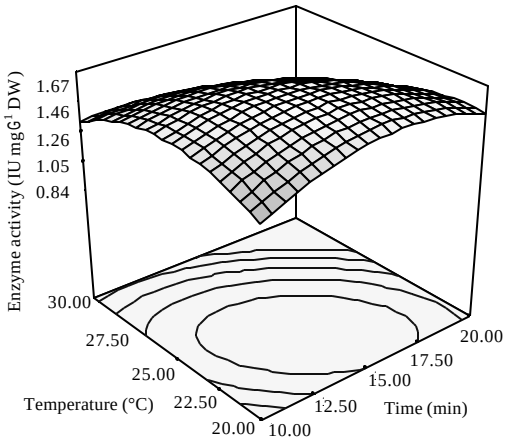

Fig. 5: Effect of increase in temperature and treatment time on β-galactosidase activity

Optimization of permeabilization process: In order to optimize the process conditions for permeabilization by numerical optimization technique, equal importance of '3' was given to all the three process parameters (viz. concentration temperature and time) and response (enzyme activity). The main criterion for constraints optimization was maximum possible enzyme activity. The optimum operating conditions for permeabilization process to achieve maximum enzyme activity were 50:50 toluene (25%, v/v): ethanol (50%, v/v) ratio, 23°C temperature and process duration of 12 min. At these conditions of process variables the predicted value of enzyme activity was found to be 1.68 IU mg^{-1} DW. The results of optimization were confirmed by conducting the experiments in triplicate at the above optimized values with±0.2% deviation in the enzyme activity values. During the experimentation, it was observed that the time and temperature are two critical factors for the effective permeabilization of yeast cells.

CONCLUSION

Response surface methodology was effective in optimizing process parameters for the β-galactosidase activity for different conditions in the range of toluene (25%, v/v): ethanol (50%, v/v) 30:70 to 50:50, treatment time 10 to 20 min and temperature 25 to 30°C. The regression equations obtained in this study can be used for optimum conditions for desired response within the range of conditions applied in study. The recommended process variables were 50:50 (toluene, 25% v/v: ethanol, 50% v/v), treatment time 12 min and 23.0°C to get maximum β-galactosidase activity. The use of permeabilized cells can help to overcome the problems/costs associated with enzyme extraction and purification from yeast cells and in the development of a low-cost technology for β-galactosidase production. Beside this, the application of the permeabilized yeast cells can also be explored for hydrolysis of lactose and the synthesis of lactulose and galacto-oligosaccharides which can be used as potential ingredients in functional foods.

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