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Optimization of β-galactosidase production by response surface methodology

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Abstract

β-galactosidase production is carried out using lactose or lactose rich substrates like whey. Tamarind seed powder has not been reported as a substrate for production of this enzyme before. In this study, statistical optimization of medium components for production of β-galactosidase, using tamarind seed powder, by *Aspergillus terreus* was attempted. Screening for the effects of eleven medium components on enzyme activity was carried out by Plackett-Burman design which showed that $NH_4(SO_4)_2$, lactose and $MgSO_4$ has significant ($p<0.001$) positive influence and pH, yeast extract, maltose and $NaNO_3$ has significant negative influence. Optimal levels of positively influencing parameters were determined by ridge analysis and was found to be 2.97, 2.88 and 2.67 g/L of $NH_4(SO_4)_2$, lactose and $MgSO_4$ respectively. In the optimized medium, enzyme activity increased 2.8 folds in comparison with basal medium. Improved activity, being achieved by the use of a cheaper substrate, could reduce the cost of production of the enzyme.

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Introduction

The enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC.3.2.1.23), most commonly known as lactase, which hydrolyses lactose into its monomers that is glucose and galactose, has a lot of potential in research as also applications in food processing, bioremediation, biosensor, diagnosis and treatment of disorders [Panesar *et al.*, 2010]. The enzyme β-galactosidase have been employed for the production of ethanol, exopolysaccharide and single cell protein from whey, used in transglycosylation of lactose for production of glucose, galactose, heteropolysaccharides (Asraf & Gunasekaran, 2010) and lately, for the production of probiotic galactooligosaccharides (Hsu *et al.*, 2007; Neri *et al.*, 2009). The transgalactosylation property has other prominent medical applications such as treatment of disorders, development of digestive supplements, cleavage of blood group A and B glycotopes, biosensor for specific lactose determination in milk, disease diagnosis, and treatment of lactose malsorption and production of lactose hydrolysed milk (Asraf & Gunasekaran, 2010).

The enzyme β-galactosidase can be obtained from a wide variety of sources such as microorganisms, plants and animals. Enzymes of plants and animal origin have little commercial value but several microbial sources of β-galactosidase are of great technological interest. Microorganisms offer various advantages over other available sources such as easy handling, higher multiplication rate, and high production yield. As a result of commercial interest in β-galactosidase, a large number of microorganisms have been researched for their production (Panesar *et al.*, 2010). Among these, fungi are the preferred candidates for industrial production of enzymes (Mathew *et al.*, 2008).

The production of microbial enzymes is influenced primarily by the physical parameters of fermentation (temperature, pH, aeration, agitation, incubation time) and the composition of the growth medium (particularly carbon and nitrogen sources) (Jurado *et al.* 2004; Tari *et al.* 2007). The most recent approach to identify the best conditions for fermentation is the use of statistical design of experiments. Response surface methodology (RSM) is a mathematical and statistical technique widely used to determine the effects of several variables and to optimize different biotechnological processes (He and Tan, 2006). RSM has been extensively applied to optimize production of cellulases (Pothiraj *et al.*, 2006; Hao *et al.*, 2006; Alam *et al.* 2008), endoglucanase (Youssef & Berekaa, 2009), α-amylase (Rao and Satyanarayana, 2007), chitinase (Akhir *et al.*, 2009) and other enzymes.

Production of β-galactosidase is primarily carried out using lactose as the substrate. Most reports of optimization of production of this enzyme have used the same substrate [Manera *et al.*, 2008; Anumukonda and Tadimalla, 2010]. This study attempts optimization of β-galactosidase production using a cheap and locally available substrate, tamarind kernel powder (TKP) as substrate by *Aspergillus terreus*. The aim of the study was to evaluate the possibility of enhancing β-galactosidase production using TKP as the main substrate.

Materials and methods

Enzyme production

A locally isolated strain of fungi, *Aspergillus terreus* NFCCI 1840, identified at Agharkar Research Institute, was used in the present study. A slurry of commercially available tamarind kernel powder (TKP) was suspended (1.0 g) in 100 ml of distilled water, boiled for 10 min, centrifuged at 10,000×g for 10 min and the supernatant was used as substrate for β-galactosidase production (Rao & Srivastava, 1973). The basal medium used for fermentation was of the following composition (g/L): $NaNO_3$-2.0; K_2HPO_4-1.0; $MgSO_4.7H_2O$-0.5; KCl-0.5; $FeSO_4.7H_2O$-0.05; Yeast Extract-0.2 & TSP-10.0 (pH-4.5) [Atlas, 2004]. β-galactosidase production was carried out by submerged fermentation by taking 100 ml of the basal

medium in 250 ml Erlenmeyer flasks. It was inoculated with approximately 10^5 spores/ml and incubated at 28±2°C, 150 rpm for 8 days. After completion of fermentation, the broth was centrifuged at 4000g for 15 min and the separated culture filtrate (CF) was used for assays. All experiments were performed in triplicate.

Enzyme assay

β-galactosidase activity was assayed by the method of Pressey (1983) using PNP-gal as substrate. One unit of activity was defined as the micromoles of PNP liberated per ml of the enzyme per minute.

Fractional factorial design

Preliminary studies using the basal medium (data not shown) indicated that the culture filtrate had β-galactosidase activity. Hence optimization was attempted using the following methods.

Table 1. The Plackett-Burman experimental design matrix for screening of medium components for β-galactosidase production.

Factors are listed as F1-F11and their – and + levels are indicated in parenthesis as g/L(except F2): F1- TSP (5.0, 25.0); F2- pH (5.0, 8.5); F3-$NaNO_3$ (2.0, 5.0); F4-$(NH_4)_2SO_4$ (1.0, 5.0); F5- Yeast extract (2.0, 10.0); F6-Urea (1.0, 10.0); F7- Lactose (1.0, 5.0); F8- Maltose (1.0, 5.0); F9- $MgSO_4$ (1.0, 5.0); F10- KH_2PO_4 (1.0, 6.0); F11- Cellobiose (0.5, 1.0).

Run ..	Variables										
	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11
1	+	+	-	+	+	+	-	-	-	+	-
2	+	-	+	+	+	-	-	-	+	-	+
3	-	+	+	+	-	-	-	+	-	+	+
4	+	+	+	-	-	-	+	-	+	+	-
5	+	+	-	-	-	+	-	+	+	-	+
6	+	-	-	-	+	-	+	+	-	+	+
7	-	-	-	+	-	+	+	-	+	+	+
8	-	-	+	-	+	+	-	+	+	+	-
9	-	+	-	+	+	-	+	+	+	-	-
10	+	-	+	+	-	+	+	+	-	-	-
11	-	+	+	-	+	+	+	-	-	-	+
12	-	-	-	-	-	-	-	-	-	-	-

Eleven variables were screened using a fractional factorial design to identify the parameters that significantly influenced enzyme production. A Plackett and Burman design [Plackett & Burman, 1946] was employed to determine the effect of individual parameters affecting β-galactosidase production by the fungus under submerged fermentation. Plackett-Burman (P-B) matrix design is an orthogonal matrix, a fraction of two level factorial designs and allows the screening of n-1 variables in just n-experiments. Generally 4n experiments are needed to screen 4n-1 factors. Each factor is taken at two levels (the lower '-'and higher '+' levels). Table 1 represents the total design with the rows representing the different experiments and each column representing a different variable.

Fermentation experiments were carried out by addition of the medium components to the basal medium, according to the design matrix. A factorial model was fitted for the main effects using SAS 9.0 software, USA. The effects of individual parameters on β-galactosidase production was calculated by the following equation

$$Y=\beta_o+\Sigma\,\beta_i x_i \qquad (1)$$

where, Y is the predicted response (activity of β-galactosidase in IU/ml), β_o, β_i are constant coefficients and x_i is the coded independent variables estimates or factors. Regression analysis was performed on the data to test the significance of the effect of individual parameters and the most significant parameters affecting β-galactosidase production were identified.

Respose surface methodology

Response surface methodology (RSM) was used to identify the optimum levels of the parameters identified by P-B experiments. RSM is an empirical statistical modeling technique employed for multiple regression analysis using quantitative data obtained from factorial design to solve multivariable equations simultaneously (Montgomery, 2001). The selected

variables: ammonium sulphate, lactose and magnesium sulphate were coded as X1, X2 and X3 respectively. The behavior of the system was explained by a second order polynomial equation given by

$$Y = \beta_o + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad (2)$$

where Y is the predicted response, β_o the intercept term, β_i the linear effect, β_{ii} the squared effect and β_{ij} is the interaction effect. SAS package, version 9.0, was used for multiple regression analysis of the experimental data obtained. *F*-test was employed to evaluate the statistical significance of the quadratic polynomial. Optimal concentrations of the critical medium components were obtained by ridge analysis and also by analyzing the response surface plots.

Table 2. Regression Results of P-B experiment.

Factors	Parameter co-efficients	Significance
1. Tamarind Kernel Powder	+0.004	<0.001**
2. pH	-0.224	<0.001**
3. Sodium nitrate	-0.061	<0.001**
4. Ammonium sulphate	+0.039	<0.001**
5. Yeast extract	-0.133	<0.001**
6. Urea	-0.014	0.152
7. Lactose	+0.034	<0.001**
8. Maltose	-0.106	0.001**
9. Magnesium sulphate	+0.019	<0.001**
10. Potassium dihydrogen phosphate	-0.046	0.325
11. Cellobiose	-0.023	<0.001**

Parameter co-efficients with + sign indicate positive influence and those with – sign indicate negative influence on the enzyme activity; greater the numerical value, greater their influence.

Results

Fractional factorial design

Plackett-Burman design calculates the main effect of each constituent on the β-galactosidase production, as the difference between the average measurement calculated at the higher (+) and lower (-) levels of the constituent. The parameters with statistically significant effects ($p<0.001$) were identified using regression analysis. F1, F4, F7 and F9 had positive effect on β-galactosidase production (Fig. 1). This indicates that TKP supports production of this enzyme. It has not been reported so far as substrate for β-galactosidase production. Three factors with higher positive co-efficients i.e., ammonium sulphate, lactose and magnesium sulphate were chosen. It is evident that β-galactosidase production increases with increasing concentration of all three factors. Lactose is a known inducer of the enzyme and ammonium sulphate has greater influence than lactose as evident from the regression co-efficients.

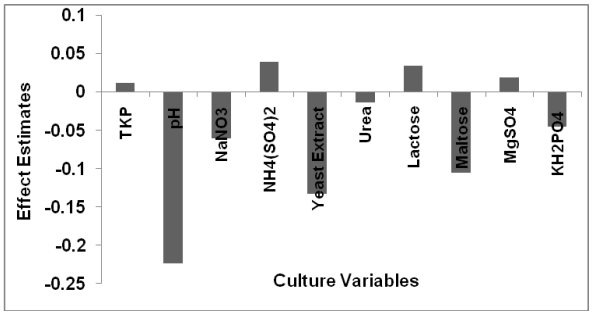

Fig. 1. Pareto chart to rank the effect and magnitude of different variables on the measured response.

The regression coefficients for the linear regression model of β-galactosidase production are presented in Table 2. The determination coefficient (R^2) was 0.997. The model was highly significant ($p<0.001$) which suggests that P-B design was an efficient tool to determine the effects of medium constituents on β-galactosidase by *Aspergillus terreus* NFCCI 1840.

Response surface methodology

RSM was employed to further optimize the levels of these three parameters. The data was subjected to multiple regression analysis and a second order polynomial equation (Eq. 4) was derived to represent

the β-galactosidase production as a function of the independent variables tested.

Y=0.177 + 0.013X1 - 0.004X2 - 0.021X3 + 0.077X1X2 + 0.063X1X3 + 0.020X2X3 (3)

Where Y = predicted response (β-galactosidase activity), X1, X2 and X3 are coded values of ammonium sulphate, lactose and magnesium sulphate respectively. Multiple regression analysis suggests that though the total model was highly significant (P<0.001), the interaction effects are negated (quadratic co-efficients = 0.0). Also the response obtained was identified to be a saddle point, so the estimated surface does not have a unique optimum i.e., β-galactosidase activity is neither optimally minimal nor optimally maximal. This implies that further analysis is warranted for and hence, ridge analysis was carried out.

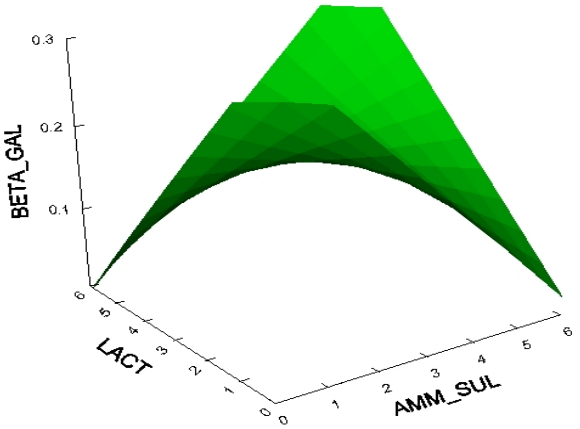

Fig. 2. Response and Contour graphs for lactose versus ammonium sulphate.

Ridge analysis

Ridge analysis is used when multiple regression cannot determine the region in which the optimum lies, i.e., when the estimated surface does not have a unique optimum. Ridge regression is generally used to investigate second-order mixture surfaces involving many factors [Draper & Pukelsheim, 2002]. Ridge regression analysis was carried out between the coded radii of 0 to 0.5 in order to identify the amounts of ammonium sulphate, lactose and magnesium sulphate that can maximize the yield. The estimated response and the optimized values are given in Table 3.

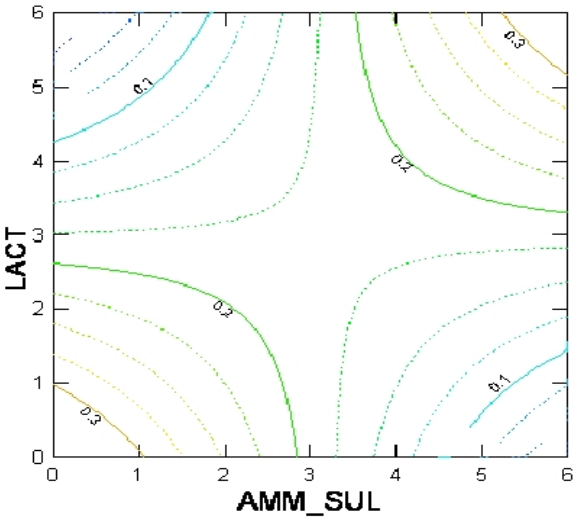

Fig. 3. Response and Contour graphs for magnesium sulphate versus ammonium sulphate.

Table 3. Ridge regression analysis.

Coded Radius	Estimated Response (β-galactosidase activity)	Values of Parameters		
		$(NH_4)_2SO_4$	Lactose	$MgSO_4$
0.000	0.177	3.000	3.000	3.000
0.100	0.219	3.052	2.961	2.811
0.200	0.281	2.967	2.875	2.671
0.300	0.281	2.967	2.875	2.671
0.400	0.281	2.967	2.875	2.671
0.500	0.281	2.967	2.875	2.671

Ridge analysis indicated that maximum yield results at higher levels of all the three variables and that the quadratic terms are unnecessary. Also, at radii greater than 0.2, the response is no longer enhanced. Surface and contour graphs of the predicted response surface, generated pair wise with the other factor set at mid-level are shown in Fig. 2-4. Fig. 2 shows the effect of lactose and $(NH_4)_2SO_4$ on enzyme activity. When lactose and $(NH_4)_2SO_4$ concentration increased, the production of the enzyme remained between 0.24-0.28 IU/ml. Further increase in amounts of the factors did not increase the activity. The same is seen in values obtained by ridge analysis. A similar pattern of

interaction was observed between $MgSO_4$ and $(NH_4)_2SO_4$ as well as between $MgSO_4$ and lactose. The maximum predicted yield is indicated in the contour graphs by the surface confined at the centre of the ellipses and is seen to be 0.28 IU/ml.

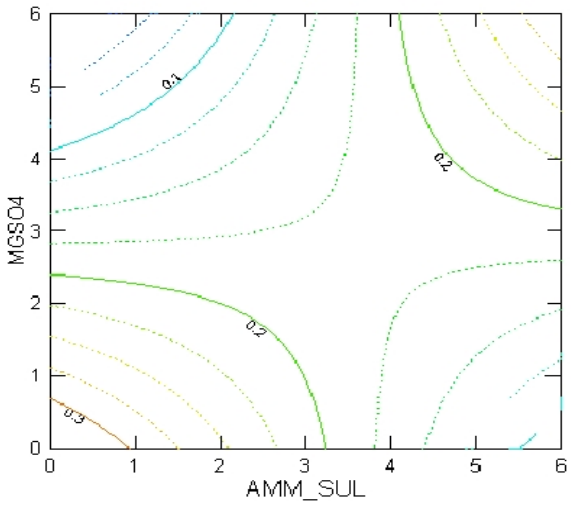

Fig. 4. Response and contour graphs for magnesium sulphate versus lactose.

The predicted optimum levels of ammonium sulphate, lactose and magnesium sulphate obtained by ridge regression and surface plot analyses were 2.97, 2.88 and 2.67 g/L respectively, with the corresponding Y = 0.28 IU/ml. The predicted values were verified by carrying out fermentation using optimized medium. Sodium nitrate and yeast extract which were components of the basal medium, were eliminated since they had significant negative effect. The pH was maintained at the lower level (pH 4.5) and magnesium sulphate, which is also a component of the basal medium, was used at the optimized level along with optimized amounts of lactose and ammonium sulphate. The practical corresponding response in the optimized medium, after fermentation, was 0.31 IU/ml. This result corroborated the validity of the model.

Discussion

RSM has been previously utilized for the optimization of β-galactosidase (Manera *et al.*, 2008; Anumukonda and Tadimalla, 2010; Dagbagli and Goksungur 2008), but this is the first report on the production of this enzyme from TKP. The identification of important process variables by Plackett and Burman experiments and the optimization of their levels by ridge analysis caused 2.8 fold increases in β-galactosidase activity. Anumukonda and Tadimalla (2010) have achieved 1.95 fold and Manera *et al.*, (2008) fourfold increase in activity after using central composite design.

Lactose is the best reported substrate for β-galactosidase production and has been widely used. Most studies report much greater amounts of lactose (28.2 g/L by Manera *et al.*, 2008, 1.3% by Anumukonda and Tadimalla, 2010, 80.6 g/L by Chen *et al.*, 1992) in the optimized medium. Chen et al. (1992) have optimized a medium by RSM where, in addition to lactose, glucose and glycerol have also been used. Pavani *et al.* (2011) have used one-factor-at-a-time approach to optimize the medium components and report best β-galactosidase activity in the presence of lactose, galactose and soya peptone. The high cost of these substances has led to a search for alternates and brought to light substrates like whey, wheat bran, rice husk etc. Identification of TKP adds another substrate to the above list. Studies on wheat bran and rice husk have been more effective with solid substrate fermentation (Nizamuddin *et al.*, 2008). TKP can be used in submerged fermentation, which is advantageous as it is the preferred method of fermentation for industrial scale production.

Among the nitrogen sources studied, positive influence of ammonium salts during optimization of β-galactosidase has been reported (8.8 g/L by Manera *et al.*, 2008 and 0.7% by Anumukonda and Tadimalla, 2010) as determined by central composite design. Concentration of both components is much higher than what is reported in this study. In contrast to our study, Manera *et al.* (2008) have reported the positive influence of yeast extract. Braga *et al.* (2011) report an optimized medium having yeast extract and peptone. Dagbagli and Goksungur (2008) and Manera *et al.*

(2008) report that increase in pH improves enzyme activity. Our study indicates that greater pH is unfavourable for this organism and optimization of pH to 4.65 by Matheus and Rivas (2003) and 4.5 by Ruchi Gaur (2006) supports the findings of this study.

Use of TKP as the main substrate, use of lactose in small amounts as an inducer and absence of any organic nitrogen source (yeast extract, peptone, soya peptone etc.) in the optimized medium of this study makes the medium relatively cheaper and can economize the production costs. The results obtained here have been derived by ridge regression analysis which was applied to the data of screening experiments and has proved efficient in enhancing β-galactosidase activity. It would be worthwhile to further optimize the medium by applying other suitable designs. Other organisms can be researched in order to improve upon the basal activity.

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