Short Communication

Response Surface Optimization of the Critical Medium Components for the Production of α-Galactosidase from *Aspergillus parasiticus* MTCC-2796

(α-galactosidase / culture condition / *Aspergillus parasiticus* / submerged fermentation / response surface methodology)

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Abstract. Response surface methodology was used to evaluate the effect of main variables such as concentration of galactose, yeast extract and wheat bran on α-galactosidase production from *Aspergillus parasiticus* MTCC-2796 under submerged fermentation conditions. A full factorial Central Composite Design was applied to study these main factors that affected α-galactosidase production. The experimental results showed that the optimum concentration of galactose, yeast extract and wheat bran were 1.5 %, 0.06 % and 1.5 %, respectively. This method was efficient as only 20 experiments were necessary to asses these conditions, and model adequacy was very satisfactory as the coefficient of determination was 0.9921.

Introduction

α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) is used for hydrolysing the α-galactosyl linkages present in simple raffinose family oligosaccharides as well as more complex polysaccharides (Manzanares et al., 1998). The wide specificity of hydrolytic action of α-galactosidase finds its potential application in biotechnology: in beet sugar industry, this enzyme is used to remove raffinose and to increase the yield of sucrose (Shibuya et al., 1995); α-galactosidase is also used to improve the gelling properties of galactomannans to be used as food thickeners and to degrade the raffinose family sugars in food and feed materials (Guimarães et al., 2001).

In commercial practice, optimization of the medium composition is done to maintain a balance between the various medium components in order to get maximum enzyme production at the end of fermentation. Research efforts have been directed to evaluate the effect of various medium components such as carbon sources, nitrogen sources and inorganic salts on the yield of enzymes. In addition, environmental factors and growth conditions such as pH, temperature and agitation also affect enzyme production through their effects on cellular growth or activity.

The conventional method for optimization involves varying one parameter at a time and keeping the others constant. Being single-dimensional, this laborious and time-consuming method often does not guarantee determination of optimal conditions. On the other hand, designing experiments with every possible factorial combination of the test variables is impractical because of the large number of experiments required (Sen, 1997). Using the response surface methodology (RSM), a large number of experimental variables can be investigated without having to increase the number of experiments to the extreme (Montgomery, 1997). In the first screening, it is recommended to evaluate the result and estimate the main effects according to a linear model. After this evaluation, the critical variables that influence the result to a large extent are selected for the optimization of the culture media applying RSM. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of variables and searching for the optimum conditions. At present, RSM is widely used in bioprocess technology for optimization of different types of fermentation media.

At present, only a few reports are available related to the optimization of culture media for the production of α-galactosidase using RSM (Liu et al., 2007; Anisha et al., 2008a, b). However, as the different organisms or strains have their own special conditions for maximum production of the enzyme, the reported optimized cul-

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Abbreviations: ANOVA – analysis of variance, CCD – central composite design, oNPG – o-nitrophenyl-α-D-galactopyranoside, RSM – response surface methodology, SmF – submerged fermentation.

ture conditions may not be applicable to α-galactosidase production from *Aspergillus parasiticus* MTCC-2796. In our preliminary studies, for the development of the production medium, galactose, yeast extract and wheat bran were found to be crucial factors in enhancing α-galactosidase formation in submerged fermentation conditions (SmF) (unpublished data). The present investigation is aimed at optimization of these medium components by applying RSM, which is an innovative step towards evaluating the industrial relevance of *Aspergillus parasiticus* MTCC-2796 in α-galactosidase production.

Material and Methods

Materials

o-Nitrophenyl-α-D-galactopyranoside was obtained from Sigma Chemical Co. (St. Louis, MO). Galactose, yeast extract, and all other chemicals used in these investigations were of analytical grade purchased from SRL Chemicals (Mumbai, India).

Microorganism

Various strains of *Aspergillus* sp. used in the present study were obtained from the Institute of Microbial Technology (Chandigarh, India). *A. parasiticus* MTCC 2796, *A. japonicus* MTCC 2733, *A. foetidus* MTCC 508, *A. terreus* MTCC 2803, three strains of *A. niger* (MTCC 281, 872 and 1781), three strains of *A. flavus* (MTCC 873, 1973 and 2786) and two strains each of *A. fumigatus* (MTCC 870 and 1811), *A. nidulans* (MTCC 344 and 1857) and *A. oryzae* (MTCC 1846 and 3567) were tested for α-galactosidase production. The fungal cultures were maintained on Potato-dextrose-agar slants at 4 °C.

α-Galactosidase production

In order to select the best α-galactosidase-producing microorganism, submerged fermentations were carried out in basal liquid culture medium, composed of gl^{-1} KH_2PO_4 7.0, K_2HPO_4 2.0, $MgSO_4.7H_2O$ 0.1, $(NH_4)_2SO_4$ 1.0, yeast extract 0.6, and 1 % (w/v) of galactose, pH 6.5. Further, 1 % (w/v) wheat bran was supplemented into the basal medium to study its effect on the production of α-galactosidase by the best enzyme-producing fungal strain. The fermentations were carried out in 100 ml Erlenmeyer glass flasks (Sigma-Aldrich, St. Louis, MO) containing 25 ml of culture medium inoculated with fungal spores (10^8 ml^{-1}) and incubated statically at 30 °C for 24 h in an automatic incubator. After incubation, the culture broths from triplicate flasks were centrifuged at 8,700 *g* for 10 min at 4 °C in a refrigerated centrifuge. The clear supernatant was used for α-galactosidase assay.

Enzyme assay

α-Galactosidase assay was carried out by the modified version of the method of Garro et al. (2004). The reaction mixture contained: 20 mM o-nitrophenyl-α-D--galactopyranoside (oNPG) 50 µl, McIlvaine buffer (0.2 M-Na_2HPO_4/0.1 M citric acid, pH 5.0) 50 µl, cell-free extract 100 µl; final volume: 200 µl. The mixture was incubated at 50 °C for 10 min, and the reaction was stopped by adding 3 ml of sodium carbonate (0.25 mM). One enzyme unit (U) was defined as the amount of enzyme that released 1.0 µmol of o-nitrophenol from its substrate oNPG per min under the given assay conditions. The results are expressed as Uml^{-1} of cell-free extract.

Optimization of α-galactosidase production by applying response surface methodology

Characterization of the different factors for α-galactosidase production was optimized by applying RSM. The statistical model was obtained using Central Composite Design (CCD) with three independent variables [galactose concentration (A), yeast extract concentration (B) and wheat bran concentration (C)]. CCD maximizes the amount of information that can be obtained while limiting the number of individual experiments (Kunamneni and Singh, 2005). Each factor in this design was studied at five different levels (Table 1). A set of 20 experiments were performed. All variables were taken at a central coded value considered as zero. The full experimental plan with respect to their coded values is listed in Table 2. Upon completion of experiments, the average of α-galactosidase production was taken as the dependent variable or response.

Statistical analysis and modelling

The data obtained from RSM on α-galactosidase production were subjected to the analysis of variance (ANOVA). The results of RSM were used to fit a second-order polynomial equation (1) as it represents the behaviour of such a system more appropriately:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_1 \beta_1 A^2 + \beta_2 \beta_2 B^2 + \beta_3 \beta_3 C^2 + \\ + \beta_1 \beta_2 AB + \beta_1 \beta_3 AC + \beta_2 \beta_3 BC \quad (1)$$

where Y is response variable, β_0 is intercept, β_1, β_2, and β_3 are linear coefficients, $\beta_{1,1}$, $\beta_{2,2}$, and $\beta_{3,3}$ are squared coefficients, $\beta_{1,2}$, $\beta_{1,3}$ and $\beta_{2,3}$ are interaction coefficients and A, B, C, A^2, B^2, C^2, AB, AC, and BC are levels of independent variables. Statistical significance of the

Table 1. Range of the values for the response surface methodology

Factors	Coded factor levels				
	-α (%)	-1 (%)	0 (%)	+1 (%)	+α (%)
Galactose	0.6591040	1.00	1.50	2.00	2.3409000
Yeast extract	0.0263641	0.04	0.06	0.08	0.0936359
Wheat bran	0.6591040	1.00	1.50	2.00	2.3409000

Table 2. Experimental design and result of CCD of response surface methodology

Run No.	Factor A: Galactose	Factor B: Yeast Extract	Factor C: Wheat Bran	Enzyme production (Units) Actual	Predicted
1	–1	–1	–1	45.80	48.26
2	1	–1	–1	43.12	44.02
3	–1	1	–1	50.00	47.67
4	1	1	–1	67.33	67.67
5	–1	–1	1	70.00	67.53
6	1	–1	1	44.00	44.20
7	–1	1	1	33.13	30.11
8	1	1	1	35.60	31.01
9	–α	0	0	66.81	68.97
10	+α	0	0	65.32	66.17
11	0	–α	0	43.30	41.63
12	0	+α	0	25.36	30.04
13	0	0	–α	58.30	56.46
14	0	0	+α	37.00	41.85
15	0	0	0	107.50	105.18
16	0	0	0	107.50	105.18
17	0	0	0	104.10	105.18
18	0	0	0	102.40	105.18
19	0	0	0	105.60	105.18
20	0	0	0	104.50	105.18

model equation was determined by Fisher's test value and the production of variance explained by the model was given by the multiple coefficient of determination, R squared value. Design Expert (version 7.1.5) was used in this investigation.

Results and Discussion

Screening of the microorganism for α-galactosidase production

Only five strains (out of 16 analysed) viz. *A. niger* MTCC-872, *A. flavus* MTCC-873, *A. flavus* MTCC-1973, *A. flavus* MTCC-2786 and *A. parasiticus* MTCC-2796 were found to produce the enzyme at different levels (Table 3). Further, among the five strains of *Aspergillus* capable of producing extracellular α-galactosidase, *A. parasiticus* MTCC-2796 (Table 3) was found to be the best producer of the enzyme; therefore, it was selected for the optimization trials of the culture medium by applying RSM.

Optimization of α-galactosidase production by applying response surface methodology

The results of CCD experiments for studying the effect of three independent fermentation variables (concentration of galactose, yeast extract and wheat bran) for α-galactosidase production from *Aspergillus parasiticus* MTCC-2796 are presented along with the mean predicted and observed responses in Table 2.

Table 3. Enzymatic activity of the microorganism tested for α-galactosidase production

Strains	Enzyme production (Units)
Aspergillus niger MTCC-872	19.3
Aspergillus flavus MTCC-873	34.5
Aspergillus flavus MTCC-1973	22.7
Aspergillus flavus MTCC-2786	20.0
Aspergillus parasiticus MTCC-2796	50.0

The regression equations obtained after the ANOVA gave the level of α-galactosidase production as a function of the initial values of the concentration of galactose, yeast extract and wheat bran. The final response equation that represented a suitable model for α-galactosidase production is given below,

$$Y = 105.18 - 0.83\,A - 3.44\,B - 4.35\,C - 13.30\,A^2 - 24.52\,B^2 - 19.81\,C^2 + 6.06\,AB - 4.77\,AC - 9.21\,BC$$

where Y is enzyme production, 'A' is concentration of galactose (%), 'B' is concentration of yeast extract (%) and 'C' is concentration of wheat bran (%). The coefficient of determination (R^2) was calculated as 0.9921 for α-galactosidase production, indicating that the statistical model can explain 99.21 % of variability in the response. The R^2 value is always between 0 and 1. The closer the value of R^2 is to 1.0, the stronger the model and the better it predicts the response (Rao and Satyanarayana, 2003). An adequate precision of 29.935 of α-galactosidase production was recorded. The predicted R^2 of 0.9473 is in reasonable agreement with the adjusted R^2 of 0.9851. This indicated a good agreement between the experimental and predicted values for α-galactosidase production.

The model F-value of 140.27 and values of prob > F (< 0.05) indicated that the model terms are significant. For α-galactosidase production B, C, AB, AC, BC, A^2, B^2, and C^2 are significant model terms. The 'lack of fit F-value' of 5.22 implied that the lack of fit is significant.

Response surface was generated by plotting the response (α-galactosidase production) on the z-axis against any two independent variables while keeping the other independent variable at zero level. Therefore, three response surfaces were obtained by considering all the

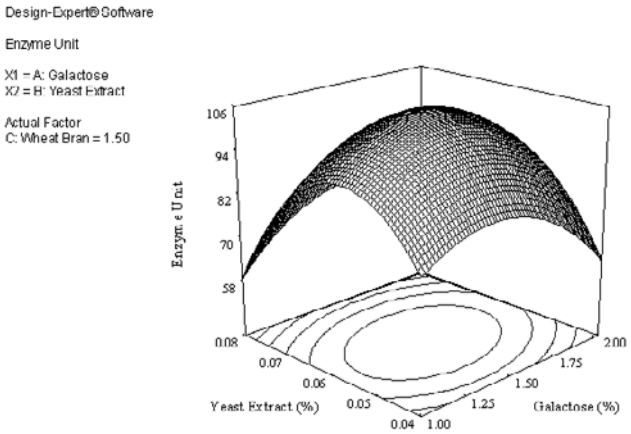

Fig. 1. Response surface plot showing a mutual effect of yeast extract and galactose concentration on the enzyme production

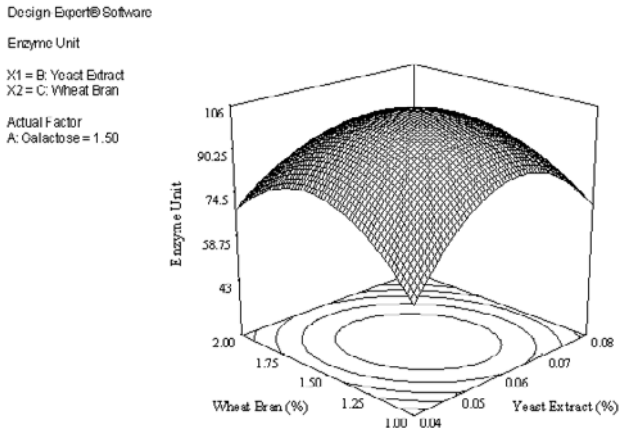

Fig. 3. Response surface plot showing a mutual effect of wheat bran and yeast extract concentration on the enzyme production

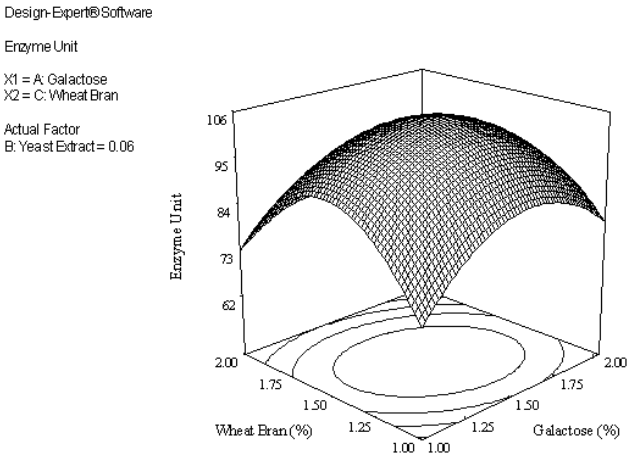

Fig. 2. Response surface plot showing a mutual effect of wheat bran and galactose concentration on the enzyme production

possible combinations. Fig. 1 represents a 3-D diagram and a contour plot of calculated response surface from the interaction between the concentration of yeast extract and galactose, while keeping the other variable (concentration of wheat bran) at '0' level. A linear increase in the enzyme production was observed when galactose concentration was increased up to 1.5 % and thereafter it declined. When the level of yeast extract concentration was increased from 0.04 % to 0.06 %, a linear increase in α-galactosidase production was recorded. At the '0' level of yeast extract concentration, the response between wheat bran concentration and galactose concentration indicated that wheat bran concentration of 1.5 % was optimum (Fig. 2) with 1.5 % of galactose concentration for α-galactosidase production. The interaction between the remaining two parameters (concentration of wheat bran and concentration of yeast extract) (Fig. 3) depicts only a marginal difference with the earlier responses. Thus, concentration of galactose (1.5 %), concentration of yeast extract (0.06 %) and concentration of wheat bran (1.5 %) were adequate parameters to attain optimum enzyme production. Anisha et al. (2008a) have optimized the culture conditions consisting in 1.5% galactose as one of the factors for maximum α-galactosidase yield in solid-state fermentation from *Streptomyces griseoloalbus*, a filamentous bacterium. Further, in the same year they also reported the screening of various factors to find their relative effect on α-galactosidase production from the same strain in shake flask culture using RSM, and 194% increase in enzyme production was achieved as compared to the unoptimized conditions (Anisha et al., 2008b). Liu et al. (2007) reported optimal fermentation medium consisting of 3.2% soybean meal (w/v) and 2% wheat bran (w/v) for α-galactosidase production by *Aspergillus foetidus* ZU-G1, in addition to other factors such as 0.1% KH_2PO_3 (w/v), and 0.05% $FeSO_4 \cdot 7H_2O$ (w/v) with initial medium pH 6.31. There is also a report for rapid screening of various nutritional components for the enhanced production of α-galactosidase up to 73 % by *Aspergillus niger* MRSS 234 in a solid-state fermentation system using Plackett-Burman design by Srinivas et al. (1994).

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