Appl Biochem Biotechnol (2010) 162:181–191
DOI 10.1007/s12010-009-8765-y

Response Surface Optimization of Medium Components for Naringinase Production from *Staphylococcus xylosus MAK2*

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Received: 13 May 2009 / Accepted: 24 August 2009 /
Published online: 8 September 2009

Abstract Response surface methodology was used to optimize the fermentation medium for enhancing naringinase production by *Staphylococcus xylosus.* The first step of this process involved the individual adjustment and optimization of various medium components at shake flask level. Sources of carbon (sucrose) and nitrogen (sodium nitrate), as well as an inducer (naringin) and pH levels were all found to be the important factors significantly affecting naringinase production. In the second step, a 22 full factorial central composite design was applied to determine the optimal levels of each of the significant variables. A second-order polynomial was derived by multiple regression analysis on the experimental data. Using this methodology, the optimum values for the critical components were obtained as follows: sucrose, 10.0%; sodium nitrate, 10.0%; pH 5.6; biomass concentration, 1.58%; and naringin, 0.50% (*w*/*v*), respectively. Under optimal conditions, the experimental naringinase production was 8.45 U/mL. The determination coefficients (R^2) were 0.9908 and 0.9950 for naringinase activity and biomass production, respectively, indicating an adequate degree of reliability in the model.

Keywords Naringinase · Response surface methodology · Central composite rotatable design · *Staphylococcus xylosus* · Naringin

Introduction

Naringinase, an enzyme detected in different species of microorganisms, is widely used to remove rhamnose and glucose from naringin and other glycosides to obtain aglycon.

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Naringin contains the glycon 4′,5,7-trihydroxy flavonone 7-rhamnoglucoside, which on hydrolysis with naringinase yields rhamnose as well as naringenin, a nonbitter derivative which cannot be converted to naringin [1]. Naringinase has been reported in plants, fungi, and bacteria. The characteristics of naringinase from fungi and plants have been thoroughly documented, although the production of this enzyme in bacteria has to date received little attention. Some reports on rhamnosidase activity from bacteria have however recently appeared. These reports have documented bacterial production of naringinase in *Clostridium stercorarium* [2], *Bacillus* sp. GL1 [3], *Thermomicrobium roseum* [4], and *Sphingomonas paucimobilis* [5].

Naringinase has several important industrial applications, as reviewed in one of our earlier studies [6]. This enzyme is of particular interest to the citrus fruit processing industry, where bitterness can be removed by α-L-rhamnosidase with the hydrolysis of naringin [7]. It is also used for the improvement of aroma in wines, and for the preparation of hydrolysis products of natural glycosides [8]. Through the deglycosylation of novel glycopeptide antibiotics, chloropolysporin, was produced successfully with rhamnosidase activity of naringinase [9]. Some α-L-rhamnosidases have been utilized for structural analysis of the polysaccharides, glucosides, and glycolipids [10, 11].

Recent research efforts have been focused on further process development (optimization) and scale-up of naringinase production. It is well known that medium compositions play an important role in enhancing the enzyme's accumulation. For this reason, optimization of constituents of the media and their concentration are required. In general, medium optimization by the traditional technique of varying one variable at a time to achieve optimal output is used [12]. The classical method of medium optimization involves the change of one variable at a time, which is tedious, extremely time consuming, and expensive if a large number of variables are involved. This method does not bring about interaction between various parameters and often leads to an incomplete understanding of system behavior, resulting in unclear results and a lack of accuracy [13]. To overcome these difficulties, response surface methodology (RSM) was employed to optimize the medium components [14, 15].

RSM is a powerful and efficient mathematical approach widely applied in the optimization of fermentation processes, including media components for enzyme [16–18], other metabolites [19], spore [20], and biomass production [21, 22]. It can give information about the interaction between variables, provide the information necessary for design, and process optimization, simultaneously. In the first step, critical medium components were optimized at shake flask level to determine their effects on naringinase production. In the second step, the selected variables demonstrating a significant degree of influence were optimized using a central composite design (CCD) and response surface analysis.

Central composite rotatable design (CCRD) is a widely used statistical technique for determining key factors from a large number of medium components with a small number of experiments. Experimental designs for optimization have been commonly used for the optimization of multiple variables with a minimum number of required experiments [21–23]. RSM has been used extensively in recent times and in many areas of biotechnology for the optimal production of pullulan [24], biosurfactants [25], biodiesel [26], nicotinamide [27], and dextran sucrose [28].

In this study, optimization of medium production by *Staphylococcus xylosus MAK2* was attempted using central composite rotatable design, where the simultaneous effect of four variables (sucrose, sodium nitrate, inducer, and pH levels) were investigated for optimum naringinase production. The literature survey reveals that it is a new report for the production of naringinase using central rotatable design at shake flask level.

Materials and Methods

Micro Organism

Staphylococcus xylosus MAK2, an isolate of our laboratory, was used for the production of naringinase in this study. The culture was isolated from soils of Uttranchal (India), identified, deposited in Microbial Type Culture Collection (MTCC), Chandigarh, India, and assigned accession # MTCC 7443. It was maintained on agar plates containing (g L^{-1}): glucose, 30; peptone, 10; agar, 20; KH_2PO_4, 1.0; and $MgSO_4H_2O$, 0.5.

Medium and Culture Conditions

The starter and fermentation mediums both consisted (g L^{-1}) peptone, 10.0; beef extract, 5.0; sodium chloride, 5.0; with naringin, 0.5. The components in the fermentation medium were added in accordance with the design of each experiment. The initial pH of all media was adjusted to 5.6. A loopful of cells from the slant was transferred into a 250 mL conical flask containing 50 mL starter medium and incubated in a refrigerated rotary shaker (Innova 4335, New Brunswick Scientifc, USA) under agitation (200 rpm) at 30°C for 16 h. Inoculum (5%, *v*/*v*) was transferred to the production medium (50 mL). After inoculation, Erlenmeyer flasks were incubated on a rotary shaker operating at 200 rpm and 30°C for 36 h.

Experiment Design and Statistical Analysis

A Central Composite Experimental Design [13] with four variables was used to study the response patterns and to determine the optimum combination of the variables. These critical components of the medium were selected on the basis of the optimized medium conditions, which were concluded from the classical variable method [29]. The variables chosen were concentration level of sucrose (5–10%,*w*/*v*), sodium nitrate (5–10%,*w*/*v*), naringin (0.3–0.5%, *w*/*v*), and pH (5.1–5.6) each at different levels −1, 0, and +1 (Tables 1 and 2). The complete design considered 22 experiments which included three replications at the center point (0). Each trial was performed twice. The statistical analysis of the results was performed using the Design Expert 7.0.3 statistical software (Stat-Ease Inc., MN, USA). The naringinase production, biomass production, sugar utilization, and pH optimum were analyzed using the analysis of variance method (ANOVA) combined with the Fisher's test

Table 1 Experimental factors and coded levels in the four factor three-level response surface design used for optimizing the production of biomass and enzyme activity.

Coded unit	Experimental factors			
	pH concentration	Carbon concentration (%, *w*/*v*)	Nitrogen concentration (%, *w*/*v*)	Inducer (%,*w*/*v*)
Symbols	A	B	C	D
−1.00	5.1	5	5	0.30
0.00	5.6	10	10	0.50
+1.00	6.1	5	15	0.70

Table 2 Predicted values vs. experimental values for maximum naringinase activity and biomass production.

Variables	Culture conditions	Naringinase activity (IU/mL)		Biomass (%, *w*/*v*)	
		Predicted	Experimental	Predicted	Experimental
Sucrose concentration	10 g/L				
Sodium nitrate concentration	10 g/L	7.92	8.45	1.51	1.58
Naringin concentration	0.5 g/L				
pH	5.6				

to evaluate if a given term possess a significant effect. The optimum levels of the variables were obtained by graphical and numerical analysis using Design Expert Program.

Analytical Techniques

Determination of Biomass

A sample of culture broth (10 mL) was centrifuged at 3,000×*g* for 10 min. The sediment cells were then washed twice and resuspended in deionized water. Biomass (percent) was estimated as grams of biomass (dry weight) produced per 100 mL of fermented broth.

Naringinase Activity Assay

Naringin hydrolysis was estimated using a minor variation of the Davis method [1]. After fermentation, the biomass was harvested by centrifugation (3,000×*g* for 10 min at 4°C), and the supernatant was analyzed for enzyme activity. A typical assay mixture comprising 900 µl of naringin (0.05%, *w*/*v*) dissolved in sodium acetate buffer (0.1 M, pH 4.5) and 100 µl of supernatant were incubated at 50°C for 1 h. Aliquot (100 µl) of reaction mixture was added to 5 mL of diethylene glycol (90%, *v*/*v*) followed by the addition of 100 µl NaOH (4 N). The reaction was maintained at ambient temperature (27°C) for 10 min, and the intensity of the resulting yellow color was measured at 420 nm in a UV-visible spectrophotometer (Shimadzu UV-1601). Naringinase activity was calculated in terms of percent hydrolysis of naringin and expressed as 1 µmol of naringin hydrolyzed per minute under the standard assay conditions.

Results and Discussion

Response Surface Method Optimization for Naringinase Production

The central composite design was used to find suitable concentrations of variables on naringinase production by *S. xylosus*. Using CCRD, a total of 22 experiments with appropriate combinations of sucrose, sodium nitrate, naringin, and pH were conducted. The range of variable is given in Table 1. The experimental design and the results obtained for naringinase activity and biomass production was varied from 4.5 to 8.55 IU/mL and 1.03 to 1.58 g/L, respectively (Table 3). The experimental results of the CCD were fitted with a second order

Table 3 Central composite design matrix for the experimental design and predicted results for naringinase activity and biomass production.

Standard order	FACTORS	(%,*w*/*v*)			Predicted results		Experimental results	
	pH	Sucrose	Sodium nitrate	Naringin	Biomass (%,*w*/*v*)	Enzyme activity (IU/mL)	Biomass (%,*w*/*v*)	Enzyme Activity (IU/mL)
	A	B	C	D				
1	6.10	15.00	15.00	0.30	1.10	6.50	1.20	6.43
2	6.10	15.00	5.00	0.30	1.15	6.86	1.32	6.84
3	6.10	5.00	15.00	0.70	1.18	7.07	1.37	6.97
4	5.10	15.00	5.00	0.70	1.15	6.50	1.33	6.49
5	6.10	5.00	5.00	0.70	1.01	4.49	1.03	4.50
6	5.10	5.00	15.00	0.30	1.12	6.00	1.24	5.98
7	5.10	15.00	15.00	0.70	1.17	6.50	1.35	6.51
8	5.10	5.00	5.00	0.30	1.11	6.10	1.23	6.11
9	5.60	10.00	10.00	0.50	1.24	8.17	1.52	8.55
10	5.60	10.00	10.00	0.50	1.24	8.17	1.53	8.30
11	5.60	10.00	10.00	0.50	1.24	8.18	1.57	8.31
12	5.60	10.00	10.00	0.50	1.24	7.89	1.58	7.98
13	4.76	10.00	10.00	0.50	1.25	8.23	1.49	7.48
14	6.44	10.00	10.00	0.50	1.20	7.18	1.36	6.50
15	5.60	1.59	10.00	0.50	1.07	5.15	1.16	5.23
16	5.60	18.41	10.00	0.50	1.09	5.47	1.19	5.55
17	5.60	10.00	1.59	0.50	1.08	5.56	1..31	5.54
18	5.60	10.00	18.41	0.50	1.13	6.55	1.30	6.75
19	5.60	10.00	10.00	0.16	1.14	5.85	1.18	5.93
20	5.60	10.00	10.00	0.84	1.19	7.12	1.51	7.22
21	5.60	10.00	10.00	0.50	1.22	7.67	1.53	8.41
22	5.60	10.00	10.00	0.50	1.22	7.67	1.48	7.89

polynomial equation. The values of regression coefficient were calculated, and the fitted equations (in terms of coded value) for predicting biomass production (X) and naringinase activity (Y) were given succeedingly regardless of the significance of the coefficients:

$$X = +1.23 - 0.016 \times A + 4.114E003 \times B + 0.017 \times C + 0.017 \times D + 0.012 \times A \times B + 0.011 \times A \times C - 0.015 \times A \times D - 0.026 \times B \times C - 2.687E - 003 \times B \times D + 0.027 \times C \times D - 0.051 \times B^2 - 0.043 \times C^2 - 0.021 \times D^2 \quad (1)$$

$$Y = +2.81 - 0.055 \times A + 0.20 \times B + 0.061 \times C + 0.074 \times D + 0.100 \times A \times B + 0.058 \times A \times C - 0.051 \times A \times D - 0.071 \times B \times C - 0.042 \times B \times D + 0.079 \times C \times D - 0.16 \times B^2 - 0.11 \times C^2 - 0.081 \times D^2 \quad (2)$$

Where A, B, C, and D are pH value, sucrose, sodium nitrate, and naringin concentrations, respectively. The statistical significance of Eqs. 1 and 2 was checked by ANOVA for

response surface quadratic model and is summarized in Table 4. The quadratic model in Eqs. 1 and 2 with 13 terms contain four linear, four quadratic, and three two-factorial interactions. Out of these, insignificant terms (on the basis of p values, which are more than 0.1000 for each response) are neglected (Table 4). The analysis of variance indicated that the model F value for the overall regression model (12.29) is significant at 5% and the lack of fit is insignificant, indicating that the first-order model with interaction is adequate for approximating the response surface of the experimental design. The regression analysis of the experimental design for biomass production and enzyme activity is based on the p value. The smaller p value (<0.05) indicates significant model, whereas values (0.10) indicates that the model is insignificant [30].

The minimum naringinase activity of 4.50 IU/mL was obtained with the minimum biomass value of 1.03%; however, maximum naringinase activity of 8.55 IU/mL was obtained with a biomass value of 1.52%. The maximum biomass production of 1.58 was obtained in naringinase activity of 7.98 IU/mL (Table 3). Figure 1a represents the relationship between the actual and predicted naringinase activity. The cluster of measurements near the diagonal line in the parity plot indicates a good fit of the model and demonstrates a satisfactory correlation between the actual and predicted values.

The minimum naringinase activity was obtained with a sucrose concentration (5%), sodium nitrate (5%), naringin concentration (0.7%), and a pH level of 6.10. However, the maximum naringinase activity occurs with sucrose (10%), sodium nitrate (10%), naringin (0.5%), and a pH level of 5.6 (Table 3).

The naringinase activity and biomass production data were then analyzed employing a multiple regression technique. The regression equation obtained from ANOVA indicated

Table 4 Model coefficients estimated by multiples linear regression for naringinase activity and biomass production.

Factors	Naringinase activity			Biomass		
	Coefficient estimate	Standard error	F value	Coefficient	Standard error	F value
Intercept	2.81	8.000E-003	7.94	1.23	0.034	2.90
Block 1	0.042	9.516E-003	1.91	7.268E-003	0.040	0.19
Block 2	−0.402	9.516E-003	0.26	−7.268E-003	0.040	8.00
A	−0.055	6.125E-003	5.57	−0.016	0.026	3.02
B	0.020	9.516E-003	3.42	4.114E-003	0.040	0.99
C	0.061	0.012	3.66	0.017	0.052	1.93
D	0.074	8.002E-003	2.96	1	0.034	1.42
AB	0.100	0.012	0.95	1	0.052	10.25
AC	0.058	8.002E-003	4.48	1	0.034	0.047
AD	−0.051	0.012	0.65	1	0.024	11.09
BC	−0.071	8.002E-003	5.49	1	0.024	0.0001
BD	−0.042	5.762E-003	46.50	1	0.024	0.0002
CD	0.079	5.762E-003	20.77	1		0.0074
B^2	−0.16	5.762E-003		1		
C^2	−0.11			1		
D^2	−0.081			1		

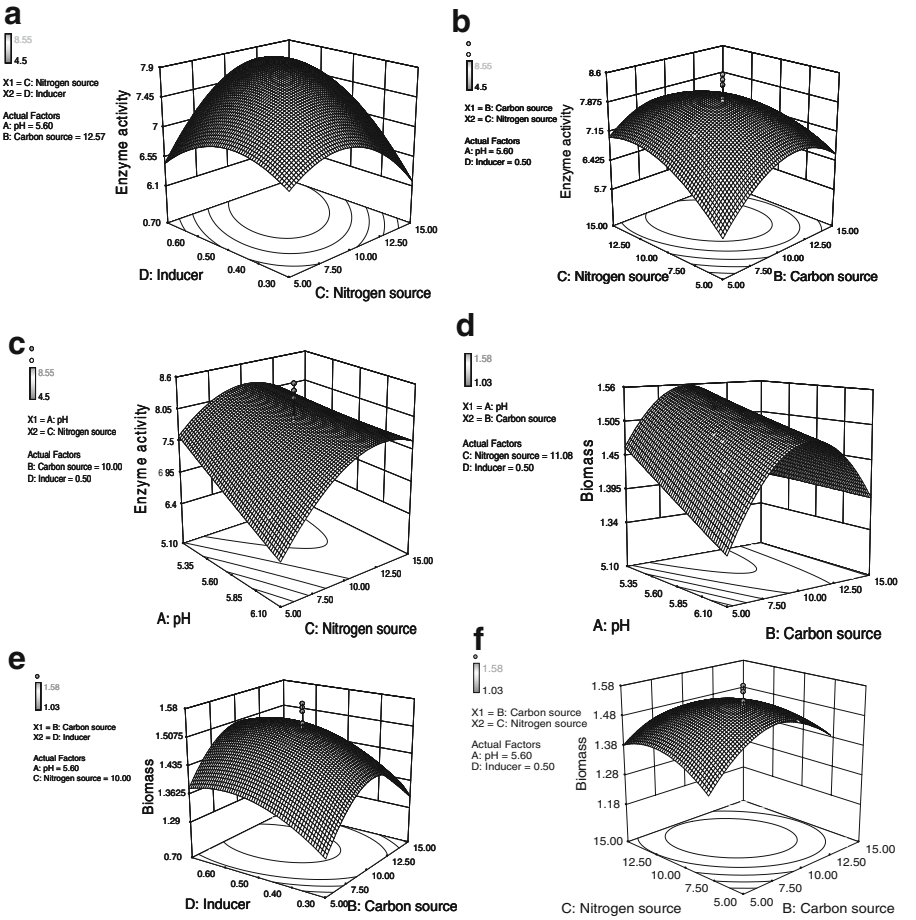

Fig. 1 **a**–**f** Response surfaces showing the effect of sucrose, sodium nitrate, naringin, and pH on naringinase and biomass production

the response surface experiments (Tables 4, 5, and 6); the *F*-values with low probability value ($p_{\text{model}}<0.05$) demonstrate a very high significance for the regression model. The associated *p* value is used to estimate whether *F* is large enough to indicate statistical significance. *p* values lower than 0.0500 indicate that the model is statistically significant [31].

Table 5 Analysis of variance for the regression model for naringinase production and biomass obtained from response surface methodology.

Source of variation	Sum of square	Degree of freedom	Mean square	*F* value	Prob (p)≤0.0500
Residual total	0.063	7	9.005E-003	7.89	0.0372
	1.01	21			
Residual total	3.586E-003	7	5.123E-004	8.38	0.0337
	0.098	21			

Table 6 Regression coefficients and their significance in the quadratic model of biomass production and enzyme activity.

Source	Biomass				Enzyme activity			
	Sum of square	*(df)*	Mean square	*p* value	Sum of square	*(df)*	Mean square	*p* value
Block	4.621E005	1	4.621E-005		0.013	1	0.013	
Model	0.095	13	7.285E-003	0.009	0.93	13	0.071	0.0053
A	1.483E-003	1	1.483E-003	0.1326	0.017	1	0.017	0.2095
B	9.575E-005	1	9.575E-005	0.6785	2.375E-003	1	2.375E-003	0.6233
C	4.100E-003	1	4.100E-003	0.0254	0.050	1	0.050	0.0503
D	1.548E-003	1	1.548E-003	0.1257	0.031	1	0.031	0.1069
AB	5.056E-004	1	5.056E-004	0.3536	0.033	1	0.033	0.0972
AC	9.896E-004	1	9.896E-004	0.2072	0.027	1	0.027	0.1291
AD	7.287E-004	1	7.287E-004	0.2719	8.599E-003	1	8.599E-003	0.3610
BC	5.248E-003	1	5.248E-003	0.0150	0.040	1	0.040	0.0721
BD	2.393E-005	1	2.393E-005	0.8350	5.890E-003	1	5.890E-003	0.4452
CD	5.681E-003	1	5.681E-003	0.0126	0.049	1	0.049	0.0517
B2	0.040	1	0.040	0.0001	0.42	1	0.42	0.0002
C2	0.028	1	0.028	0.0002	0.19	1	0.19	0.0026
D2	7.100E-003	1	7.100E-003	0.0074	0.10	1	1.10	0.0125
Residual	3.586E-003	7	5.123E-004		0.063	7	9.005E-003	
Lack of fit	3.094E-003	3	1.031E-003	0.0337	0.054	3	0.018	0.0372
Pure error	4.922E-004	4	1.231E-004		9.109E-003	4	2.277E-003	
Core total	0.098	21			1.01	24		

The squared regression statistic R^2 value provided a measure of the variability in the actual response values that could be explained by the experimental factors and their interactions. A value of 1 represents the ideal case at which 100% of the variation in the observed value can be explained by the model. The matching quality of the data obtained by the model was evaluated with respect to the correlation coefficient, R^2, in the experimental and modeled data. The mathematical adjustment of those values generated R^2=0.9908 (99.08%) for naringinase activity and R^2=0.9950 (99.05%) for biomass production, revealing that the model was unable to explain only 0.92% and 0.95% of the overall effects, demonstrating it to be a robust statistical model.

The three dimensional (3D) response surface graphs based on the final model are depicted in Fig. 1a–f, and are obtained by holding two variables at their optimum level while varying the other two within their experimental range. The coded model was used to generate response surfaces and contour curves for the analysis of the variable effects on naringinase production. The statistically optimal values of variables are obtained when moving along the major and minor axis of the contour. The response at the central point corresponds to a maximum degree of achievable naringinase production for that set of variables.

Figure 1a was obtained by fixing the concentration of the carbon source and pH level at 12.57 g/L and 5.6, respectively. Further increase in either naringin or sodium nitrate concentration decreases naringinase activity. It is clear from the three dimensional plots that the optimum response of naringinase activity (7.9 IU/mL) occurred when inducer and sodium nitrate concentrations were at moderate levels and sucrose at its highest.

The pH of the medium also played a significant role in naringinase as well as biomass production (Fig. 1c, d). Changes in acidity of the fermentation medium by the addition of inducer at higher concentrations may contribute to a decrease in the pH of culture broth, which could in turn result in death of cells and decreases in enzyme production.

Figure 1b, c were generated by fixing the concentration of sucrose, inducer and pH at values of 10.00 (g/L), 0.50 (g/L), and 5.6 respectively. In these conditions, a decrease in naringinase production was observed, suggesting that there is a considerable interaction between sodium nitrate and sucrose levels. The response was varied at different concentrations of sodium nitrate, but the ideal concentration was found to be 10.00 g/L for optimum naringinase production. Further increases in either nitrogen source or pH decreased enzyme activity. This indicates that sodium nitrate concentrations in the medium have a significant effect on the naringinase production response.

The 3D plots shown in Fig. 1d, e, and f were provided by the regression equation for biomass production. At fixed values of sucrose (10 g L^{-1}) and pH (5.6), as the concentration of sucrose was increased in the medium, a decrease in cell mass was observed, indicating that sucrose concentration in the medium formulation for biomass production has a significant effect on the responses (Fig. 1d).

A combined effect of changes in inducer and sucrose concentration was found to increase the response of biomass considerably, but sodium nitrate appears mainly to contribute to the cell growth when the pH level of the culture broth was 5.6. At fixed values of sucrose 10.00 (g/L) and pH (5.6), an optimum biomass concentration was obtained. Further increases in the other two variables, naringin and sodium nitrate, decrease the production of biomass. This may be due to the inhibition of higher concentrations of inducer in the medium (Fig. 1e).

Figure 1f represents the contribution of sodium nitrate and sucrose at moderate levels for an optimum production of biomass in the medium, when inducer and pH values were fixed. It is clear from this figure that higher concentrations of sucrose resulted in a significant decrease of biomass. The model predicted the maximum biomass and enzyme production (in terms of activity) levels at 1.51721 and 7.92289 U/mL. This occurred at sucrose concentration, sodium nitrate concentration, inducer concentration, and pH at 10 g/L, 10 g/L, 0.50 g/L, and 5.6, respectively. Verification of this result was achieved by carrying out the experiment under these predicted optimal conditions.

Conclusions

RSM was performed to optimize the medium components for naringinase production of *S. xylosus*. A highly significant quadratic polynomial obtained by the CCD for the present study was very useful in determining the optimal concentration of constituents that have significant effects on naringinase production. Almost all interactions in the designed experiments yielded a “spherical” or “nearly spherical” variance function. This indicated that the effects of variables are both individual as well as interrelated, allowing for the prediction of optimum concentration levels for maximized naringinase productions. Such a design ensures the estimated response has a constant variance at all points equidistant from the centre of design. Most of the elliptical contour curves generated in this study represent perfect interaction between the independent variables. This statistical method reduced the number of experiments without affecting the accuracy of the resulting predictions. Results of the study illustrated the importance of various factors for enzyme production. A value of R^2 approaching unity indicated its ability to produce a strong response. Statistical analysis

applied in this work proved to be efficient for optimizing enzyme production by fermentation providing suitable designs and models for the experiments. The experimental results clearly showed that naringinase production by *S. xylosus* heavily depends on sucrose, sodium nitrate and pH. Thus, this bacterial strain has the potential to overcome the limitations of producing naringinase enzyme present in its available fungal counterparts. The optimum conditions developed in this experimental setup achieved a production rate of 8.55 IU/mL of the naringinase enzyme. From this it is clear that further investigations on naringinase production by this bacterial strain at a pilot scale could be highly beneficial.

Acknowledgement The authors would like to thank the Council of Scientific and Industrial Research (CSIR), New Delhi, India for providing financial assistance to carry out this study from a project grant CSIR 38(1133)/07/ EMR-II. Aneet Kaur gratefully acknowledges the award of SRF (Senior Research Fellowship).

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