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OPTIMIZATION OF MEDIUM COMPOSITION FOR IMPROVING NARINGINASE ACTIVITY USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Response Surface Methodology was used to optimize the fermentation medium for enhancing naringinase activity by *Aspergillus flavus.* In the first step of optimization, with plackett-Burman design, starch, peptone and naringenin were found to be the important factors affecting the naringinase activity significantly. In the second step, a 2^3 full factorial central composite design and RSM were applied to determine the optimal concentration of each significant variable. The optimum values for the critical components were obtained as follows: starch 1.5 (15 g/L), peptone 0.5 (5 g /L) and 0.005 (0.055 g/L) naringenin. Under the optimal conditions, the practical naringinase enzyme activity was increased up to 3606.6U/lit an approximate 6.4 fold improvement over the previous enzyme activity (560U/lit) with un-optimized medium. The determination coefficient (R^2) was 0.8949, which ensures adequate credibility of the model.

KEYWORDS: Naringinase; Plackett-Burman design: Response Surface Methodology; *Aspergillus flavus*; Medium Optimization

INTRODUCTION

Naringin, a bitter flavonone glycoside which is responsible for the bitterness in citrus fruits (Akira konno et al. 1982).Naringinase (E.C.3.2.1.40) is an enzyme which can hydrolyze the naringin into prunin and then into naringenin, which is non-bitter and tasteless (Habelt et al. 1983). Naringinase has significant application in fruit juice industry to de-bitter the citrus fruit juices during its processing, and helps to improve the properties and stability of the juices (Munish Puri et al. 2000). The debittering process could be more cost effective and economically viable if naringinase production is achieved industrially using microorganisms. With this background, the study was designed to optimize the medium composition for naringinase production by *Aspergillus flavus* using Response surface methodology in order to predict the best performance conditions with the minimum number of experiments and thereby increasing the activity of enzyme. This is a non-conventional method that has been successfully used for the optimization of enzymatic reactions conditions (Ribeiro et al. 2003), medium composition (Ribeiro et al. 2006, Kapat et al. 1996, Montserrat et al. 1993) and food preservation parameters (King, 1993). It can give information about the interaction between the variables, provide information necessary for design and process optimization, and give multiple responses at the same time. In the first Optimization step, a Plackett-Burman design was used to determine the likely effects of medium components on naringinase activity. In the second step, the factors that had significant effects were optimized using a central composite design (CCD) and response surface analysis.

MATERIALS AND METHODS

Chemicals

Naringin and Narigenin were obtained from sigma, St. Louis, USA. The other culture media (Czapeck-dox Broth and Agar) and different carbon and nitrogen sources were obtained from Hi-Media, Laboratories, Mumbai, India. All other reagents used were of analytical grade.

Microorganism and its Cultivation Condition

Aspergillus flavus was obtained from Microbial Type Culture Collection, Institute of Microbial Technology, and Chandigarh and maintained on sterilized potato dextrose agar medium, 4.5. The slants of Aspergillus *flavus* medium were stored at 5°C in the refrigerator and sub cultured every month. All the culture media, unless otherwise stated, were sterilized at 15 lbs/inch^2 pressure (121ºC) for 15 minutes. The vegetative spores, taken from slants and suspended in 0.85% sterile sodium chloride, were inoculated in the potato dextrose medium, pH 4.5. Flasks were incubated (28°C, 200rpm) in a rotary shaker for 8-10 days. To study the effect on enzyme production, starch (carbon, 1%, w/v), peptone (nitrogen, 1%, w/v)) and Mg^{2+} (metal ions) and trace naringenin were added to the culture medium from preliminary studies. Samples were withdrawn aseptically at regular time intervals and analyzed for cell mass and naringinase activity. Maximum activity was found at the seventh day old culture after inoculation.

Assay for Naringinase Activity

Naringinase activity was estimated using Davis method (Davis, 1947). A typical assay mixture comprised of 1ml 0.1% naringin dissolved in 300 µl 0.1M sodium acetate buffer (pH 4) and 200 µl culture filtrate. The assay mixture was incubated at 50 ◦C for 60 min after which 100 µl aliquot was added to 5ml 90% diethylene glycol followed by the addition of 100 µl 4N NaOH. Samples were kept at room temperature (28 ◦C) for 10 min. The intensity of the resultant yellow color was determined at 420 nm. One unit of naringinase activity was defined as 1µmol of naringin hydrolyzed under the above assay condition. The dry weight of the *A. flavus* mycelium was determined after filtering through Whatman (No. 1) filter paper, washing thoroughly (three times) with distilled water and drying overnight at 80 ◦C. Extracellular protein was measured by the method of Lowry et al. (Lowry OH, 1951). Total sugars were determined with anthrone (Updegraff DM, 1969) method.

MEDIUM OPTIMIZATION WITH STATISTICALLY-BASED EXPERIMENTAL DESIGNS

Plackett-Burman Design

A Plackett-Burman design was used to determine the important factors influencing naringinase activity describes no interaction among factors and remove the dispensable ones to conclude a smaller and more manageable set of factors. The different factors were prepared in two levels, -1 for low level and +1 for high level based on placket-Burman design (Table 1 and 2). The inoculums size (10%, v/v) was taken as a dummy variable. The design matrix (Table 2) was developed using Minitab software (Version 15, Minitab Co., PA, USA)

Central Composite Design

The maximum activity of naringinase was investigated using CCD with three variables. Each factor in the design was studied at five different levels ($-\alpha$, -1, 0, $+1$, $+\alpha$), which is shown in Table 3. Based on the Plackett-Burman design, the processing variables (factors) including the concentrations of starch (X_1), peptone (X_2) and naringenin (X_3) were chosen for the CCD.

As shown in Table 4, a set of 20 experiments was carried out. All variables were taken at a central coded value considered as zero, which was determined by the “one-variable-at-a-time” approach. The minimum and maximum ranges of variables were investigated and the full experimental plan with respect to their values in actual and coded forms was also listed in Table 3. Upon completion of experiments, the activity of nariniginase was taken as the response (Y). A second order polynomial equation was then fitted to the data by a multiple regression procedure. The equation resulted in an empirical model that relates the measured response to the independent variables of the experiment. When several factors are involved, the model is expressed as follows:

$$Y = \beta_o + \beta_i \sum x_i + \beta_{ij} \sum x_i x_j + \beta_{ii} \sum x_{ii}^2 \quad (1)$$

where Y is the measured response; β_0, β_i, β_{ij}, and β_{ii} are the intercept term, linear coefficient, interactive coefficient, and quadratic coefficient, respectively; and x_i is the coded independent variable (i=1, 2, 3). Low and high factor settings are coded −1 and +1, and the midpoint is coded 0. The factor setting of trials that ran along axes drawn from the middle of the cube through the center of each face of the tube is coded +1.682 or −1.682. Minitab software (Version 15, Minitab Co., PA, USA) was used to analyze the results. *F*-test was employed to evaluate the statistical significance of the quadratic polynomial. The multiple coefficients of correlation R and the determination coefficient of correlation R^2 were calculated to evaluate the performance of the regression equation. The optimum levels of the selected variables were obtained by analyzing the response-surface plots (Khuri and Cornell, 1987). The fit of the models was evaluated by the determination coefficients (R^2) and adjusted R^2 (R^2 adj).

Statistical Analysis of Data

The data of the nariginase activity were subjected to analysis of variance (ANOVA) using Minitab software to estimate *t*-value, *P*-value and confidence levels. Optimal values of Naringinase activity were estimated using the 3D graph generated by Minitab software (Version 15, Minitab Co., PA, USA).

RESULTS AND DISCUSSIONS

The first optimization step was using a 12-run PB design to identify the significant factors for nariniginase activity by *Aspergillus flavus*. According to the resulting effects of these six variables on naringinase activity and the associated significant levels presented in Table 5, it can be seen that with the increase in the concentration of starch, peptone, Mg^{2+} and naringenin, all have positive effects on Naringinase activity. pH increase has negative effects on naringinase activity. With the help of relative ranking, starch, peptone and naringenin within the tested limits were selected for further optimization, which had the most significant effects on naringinase activity.

The PB design was proved to be a powerful tool to rapidly determine the effects of medium constituents on naringinase activity of *Aspergillus flavus*. However the optimal concentrations of medium components that significantly affect naringinase activity could not be obtained. Further work need to be done to find out this information.

Central Composite Design

This is a very useful tool to determine the optimal level of medium constituents and their interaction. Based on the PB design, where starch, Peptone and Naringenin were selected for their significant effects on the naringinase activity, a CCD was used for further optimization. Table 3 gave the variation levels at which these components were supplemented to naringinase activity. Other nutrients concentrations were set at their centre point tested in the PB design. Table 4 gives the design and results of experiments carried out by the CCD design. The results obtained were submitted to analysis of variance on of Minitab software, with the regression model given as

$$Y=3481.53+28.9X_1-196.86X_2-33.78X_3-389.92X_1^2-357.64X_2^2+59.06X_3^2+158.35X_1X_2+4.80X_1X_3-42.25X_2X_3, \quad (2)$$

where Y is the response value, that is, the naringinase activity and $X1$, $X2$ and $X3$ are the coded levels of starch, peptone and naringenin , respectively.

ANOVA

ANOVA analysis was performed to check the adequacy of the suggested models and identify the significant factors. The analysis of variance of the quadratic regression model demonstrated that Eq. (2) was an appreciably significant model. The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of the

determination coefficient (R^2=0.8949) indicated the measure of the goodness of fit of the model. The value of the adjusted determination coefficient [Adj (R^2)=0.0.8002] was also good in supporting the significance of the model. Among the model terms, X_1, X_2 and X_3 were significant with a probability of 95% (Table 6). The interaction between X_1, X_2 and X_3, however, had no significant influence on naringinase activity. Also, Table 6 shows a value of 4008110 for the predicted residual sum of squares, a measure of how a particular model fits each point in the design.

The fitted response for the above regression model was plotted in Figure 1. 3D graphs were generated for the pair-wise combination of the three factors while keeping the other one at its optimum levels for naringinase activity (Figure 1&2). The Optimum components (per liter) consisted of 15 g starch, 5 g Peptone and 0.055 g naringenin. Under the optimal condition, the naringinase activity of 3606.6U/lit could be achieved. This result corroborated the validity and the effectiveness of this model.

Verification of the Predicted Concentration in the Optimal Medium

The formulated optimal medium from RSM experiments was verified experimentally and compared with the predicted data from the model. The average activity of naringinase in the broth was 3557.9 U/lit from triple-duplicated experiments, which suggests the accuracy of the model is over 95%.

CONCLUSIONS

The fermentation medium was optimized systematically with regard to the production of naringinase with *A.flavus*. The experiments of Plackett-Burman design indicated that starch, peptone and naringenin were beneficial for increasing naringinase activity. Furthermore, the CCD formulated an optimal fermentation medium, and a maximum catalytic activity of naringinase (3606.5 U/L) at pH 4.5. Compared with the enzymatic activity of nariniginase in unoptimized medium, the systematically optimized medium produced naringinase with increasing activity (3606.5 U/L).This is an innovative work conjugating the effect of media components on increasing the naringinase activity thereby supporting to reduce the bitterness of citrus fruit juices. This may support a further process development to produce naringinase on large scale.

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REFERENCES

1. Akira Konno., Masaru Misaki., Jun Toda., Takeo Wada., & Katsuharu Yasumatsu. (1982). Bitterness reduction of naringin and limonin by β-cyclodextrin. Agricultural biology and chemistry, 46(9), 2203-2208.
2. Habelt, K., & Pittner, F. (1983). A rapid method for the determination of naringin, prunin and naringenin applied to the assay of naringinase. Anal Biochem, 134, 393-7.
3. Munish Puri., & Sukirti Kalra. (2000). Purification and characterization of naringinase from a newly isolated strain of *Aspergillus niger* 1344 for the transformation of flavonoids. World journal of microbiology and biotechnology, 21, 753-758.
4. Ribeiro, M.H.L., Silveira, D., & Ferreira-Dias, S. (2003). Response surface modelling of the consumption of bitter compounds from orange juice by *Acinetobacter calcoaceticus*. Journal of Molecular Catalysis B-Enzymatic, 21, 81–88.

5. Ribeiro, M.H.L., Manha, S., & Brito, L. (2006). The effects of salt and pH stress on the growth rates of persistent strains of *Listeria monocytogenes* collected from specific ecological niches. Food Research International, 39, 816–822.

6. Kapat, A., Rakshit, S.K., & Panda, T. (1996). Optimization of carbon and nitrogen sources in the medium and environmental factors for enhanced production of chitinase by *Trichoderma harzianum.* Bioprocess Engineering, 15, 13–20.

7. Montserrat, A., Inaki, R., Franc ois, O., Francesco, G., & Carles, C. (1993). Application of factorial design to the optimization of medium composition in the batch cultures of *Streptomyces lividans* TK21 producing a hybrid antibiotic. Biotechnology Letters, 15, 559–564.

8. King, V.A. (1993). Studies on the control of the growth of *Saccharomyces cerevisiae* by using response surface methodology to achieve effective preservation at high water activities. International Journal of Food Science and Technology, 28, 519–529.

9. Davis, W.D. (1947). Determination of flavonones in citrus juice. Anal chemistry, 19, 476.

10. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951). Protein measurement with the folin phenol reagent. J Biol Chem,193, 265–75.

11. Updegraff, D.M. (1969). Semimicro determination of cellulose in biological materials. Anal Chem, 32, 420–4.

12. Khuri, A.I., & Cornell, J.A. (1987). Response Surfaces: Design and Analyses. Dekker, New York

Table 1: Factors and levels in Plackett-Burman Design

S.No	Factors	High level (+1)	Low level (-1)
1	Glucose (g/L)	20	10
2	Peptone (g/L)	7.5	2.5
3	Mg^{2+} (g/L)	0.8	0.3
4	Naringenin (g/L)	0.08	0.03
5	pH	5.0	4.0

Table 2: The Experimental Design Using the PB Method for Screening of Medium Components

	Variables						
Run	Starch	Peptone	Mg2+	Naringenin	pH	Inoculum size	Activity U/lit
1	1	1	-1	1	1	-1	1158.33
2	1	1	-1	1	-1	-1	1268
3	-1	-1	-1	-1	-1	-1	133
4	1	-1	1	-1	-1	-1	173
5	-1	-1	-1	1	1	1	180
6	-1	1	1	1	-1	1	278.3
7	-1	1	-1	-1	-1	1	1.68
8	-1	-1	1	1	1	-1	1.3
9	1	-1	1	1	-1	1	2.16
10	1	-1	-1	-1	1	1	0.3
11	-1	1	1	-1	1	-1	8.16
12	1	1	1	-1	1	1	4.6

Table 3: Factors and levels in CCD

Variables	Ranges and levels				
	−1.682	−1	0	1	1.682
Starch (g/L)	6.59	10.00	15.00	20.00	23.40
Peptone (g/L)	0.79	2.5	5	7.5	9.2
Naringenin (g/L)	0.02	0.03	0.05	0.08	0.1

Table 4:Experimental design and results of CCD

Run	Variables			Naringinase Activity,Y(U/L)
	Starch(X_1)	Peptone (X_2)	Naringenin (X_3)	
1	0	0	-1.682	3535
2	0	0	1.682	3553.2
3	-1	1	1	2207.8
4	0	0	0	3532.8
5	-1.682	0	0	2224.4
6	0	0	0	3487.2
7	1	1	-1	2788.8
8	1.682	0	0	2324
9	1	-1	1	3037.8
10	-1	-1	-1	3336.2
11	0	1.682	0	2373.8
12	0	-1.682	0	2357.2
13	-1	1	-1	2473.4
14	1	1	1	2639.4
15	-1	-1	1	3336.6
16	0	0	0	3470.2
17	0	0	0	3606.6
18	0	0	0	3424
19	0	0	0	3404.2
20	1	-1	-1	3115.2

Table 5: Coefficients, *T* Values and Significance Levels Calculated from the Naringinase Activity Obtained in the Screening Experiments (PB Design)

Term	Effect	Coefficient	t value	P
Starch	334	167	4.97	0.004**
Peptone	371.6	185.8	5.53	0.003**
Mg^{2+}	-379	-189.5	-5.64	0.002**
Naringenin	427.9	213.9	6.36	0.001**
pH	-83.9	-42	-1.25	0.267
Inoculum	-379.1	-189.6	-5.64	0.002

**Statistically significant at 99% confident level.

Table 6: Analysis of Variance (ANOVA) for Enzyme Activity

Source	DF	Seq SS	Adj MS	F	P
Regression	9	4677866	519763	9.46	0.001
Linear	3	556249	185416	3.37	0.063*
Square	3	3906554	1302185	23.69	<0.0001
Interaction	3	215063	71688	1.3	0.326
Residual Error	10	549646	54965		
Lack-of-Fit	5	522138	104428	18.98	0.003
Pure Error	5	27507	5501		
Total	19	5227511			
PRESS		4008110			

PRESS (predicted residual sum of squares), *statistically significant at 95% of probability level

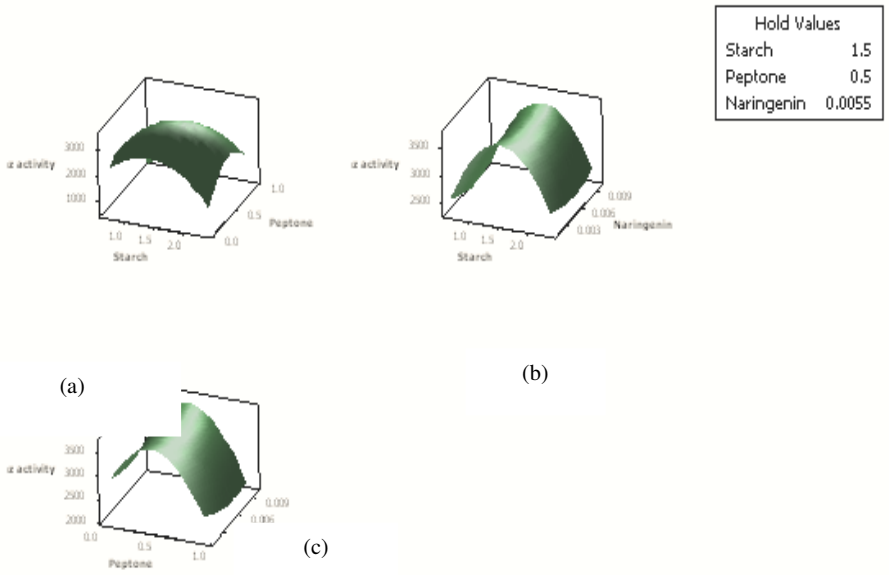

Figure 1 :Response Surface plot for the combinatory effects (a) Starch (X_1) and Peptone (X_2); (b) Starch (X_1) and Naringenin (X_3); (c) Peptone (X_2)and Naringenin (X_3).

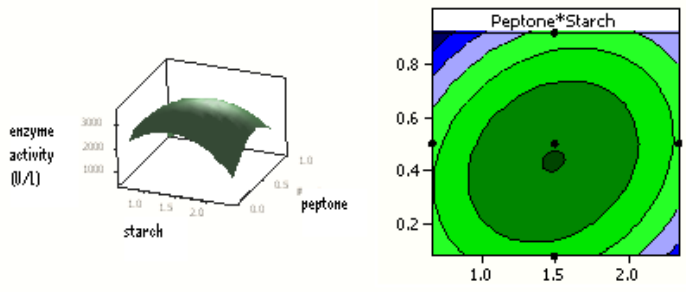

Figure 2: Three-Dimensional (3D) Response Surface and Contour Plot for Starch (X_1) and Peptone (X_2). Fixed Level: Naringenin (X_3) =0 (0.05g/L).