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Enhanced Production of Tannase through RSM by *Bacillus haynesii* SSRY4 MN031245 under Submerged Fermentation

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Present investigation reports enhanced production of tannase by *Bacillus haynesii* SSRY4 MN031245 through application of Response Surface Methodology (RSM). Central Composite Design (CCD) of RSM was employed to determine the most important factors contributing to enzyme production and their interactions were analysed through graph models. Optimum production of enzyme (11.19 U/mL) was achieved at pH 5.5, temperature 37°C, incubation period 72 hours and agitation speed of 150.0 rpm. The statistically optimized values of variables enhanced the tannase production by 2.49 folds.

Keywords: Design matrix, Enzyme synthesis, Interaction, Response, Statistical optimization

Introduction

Enzyme production and optimization is one of the integral parts of the study which can be implemented for commercial manufacturing. Industries spend billions of rupees to achieve maximum enzyme production through various strategies including genetic engineering. Tannase is one among most versatile biocatalysts that play a key role in vast-ranging biotransformation reactions and industrial applications.[1–3] However, on account of high production costs of this enzyme, its industrial production is markedly restricted. Optimization of key process parameters and fermentation strategies play a major role in reducing the production cost.[4] Temperature, medium pH, incubation period and agitation speed are very important process parameters for optimum enzyme production. Thus, determining the most suitable conditions of these parameters is of utmost importance for optimum enzyme production. Looking at the tremendous biocatalytic potential of tannase and its utility in numerous applications; it's a worthwhile endeavour to optimize its production.

Tannase production from fungi and its optimization has been reported in several research studies. However, the reports on bacterial tannase production and its subsequent optimization are scanty in literature. Thus, researchers are continuously trying to explore a potent bacterial strain which can be marketed globally and improve the economy by obtaining maximum revenue. Optimization of process parameters is an important aspect in developing an economically feasible bioprocess. Traditionally, the factors which are directly responsible for culture conditions and composition of media are carefully examined through One Factor At a Time (OFAT) approach for enhancing either growth of microorganisms or enzyme production. This approach of optimization has certain drawbacks; it does not consider interactions between variables, becomes costlier, laborious, and time-consuming and may also lead to unreliable and less accurate results. Thus the classical OFAT strategy of optimization is costlier, arduous and may also lead to unreliable and less accurate results.[5]

Response Surface Methodology (RSM) can be applied to overcome this problem.[6–8] Determining the most suitable conditions of process parameters is of utmost importance for optimum enzyme production. Statistical optimization permits the interactions among possible influencing parameters within minimum experiments thereby reducing the overall cost of elaborative and laborious analysis and minimizing the errors.[9] Response surface methodology is a flexible procedure that reduces the number of experiments required for optimization.[10] Response surface methodology is widely used in biotechnological and enzymology experiments such as optimization of media, process parameters, etc.[11] In our study, production of tannase by *Bacillus haynesii* SSRY4

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MN031245 has been optimized under submerged conditions using Central Composite Design (CCD) in a batch process.

Materials and Methods

Microorganism and Enzyme Production

Bacillus haynesii SSRY4 MN031245 previously isolated from rhizospheric soil of *Casia* species was used to produce tannase. An inoculum level of 5% (v/v) of overnight grown culture was used for tannase production. Fermentation was accomplished in 100 ml minimal media containing KH_2PO_4 (0.05 g), K_2HPO_4 (0.05 g), Glucose (0.5 g), $NaNO_3$ (0.6 g), KCl (0.5 g), $MgSO_4$ (0.05 g). The solution of tannic acid (2.5% (w/v)) was sterilized separately by passing through membrane filter (0.22 µm) before adding to the medium. Enzyme activity was quantified by adopting rhodanine assay method.[12]

RSM for Optimum Enzyme Production

Response surface methodology was adopted to determine the most favourable conditions of four physical process parameters for maximizing the tannase production. In our study, CCD involving four factors and five levels (−2, −1, 0, +1, +2) comprising of total 30 experiments were used to maximize tannase production. Thirty set of trials involved replications of central points. The effects of independent variable or response were calculated in the form of yield (Y), expressed as U/mL.

Statistical Analysis

Design Expert (12.0.7.0) was used for RSM design, for regression analysis of the experimental data and to plot the three-dimensional graphs for understanding the effects of selected variables discretely as well as in conjunction on enzyme production. Analysis of variance was applied to establish the most significant terms for each response. An equation based on set of algorithms and second-order polynomial was used to describe the test variables and their combined effects in the response.

Results and Discussion

RSM for Optimum Enzyme Production

In the present study, optimization of four physical process parameters for optimum enzyme synthesis was accomplished by employing RSM using CCD (Table 1). The experiments were performed in randomized fashion with replications (Table 2).

The second order polynomial model describing the effect of independent variables on tannase activity is measured by following formula:

$$Y = 11.03 + 0.1515X_1 - 1.10X_2 + 0.5232X_3 - 0.1001X_4 + 0.1410X_1X_2 - 0.8393X_1X_3 + 0.2947X_1X_4 - 1.03X_2X_3 - 1.07X_2X_4 - 0.1248X_3X_4 - 2.45X_1^2 - 2.27X_2^2 - 2.49X_3^2 - 0.2649X_4^2$$

where Y is tannase activity, X_1 is pH, X_2 is incubation temperature (°C), X_3 is incubation time period (h), and X_4 is agitation rate (rpm). Tannase activity of *Bacillus haynesii* SSRY4 MN031245 ranged from 0.0029 U/mL to 11.19 U/mL.

Statistical Analysis

The experimental data and response were evaluated and analysed by Design Expert software. It is revealed from ANOVA that the model was significant with p-value < 0.001 with Model F-value of 1274.36. Values of "Prob>F" less than 0.05 signifies that the model terms were significant. Analysis of variance expressed in terms of degree of freedom, standard error, p values of each variable and interactions between them is compiled in Table 3. The coefficient of determination (R^2) for the tannase activity was calculated as 0.9992, which was close to 1 and can be considered up to as 99.92% of variability of the response. The "adequate precision value" in the present study was 98.8881. The lower value of coefficient of variation (3.19) indicates that experiments were done precisely and are authentic.

Interaction effects of variables

All the variables studied in present study were found to affect tannase activity significantly. To study the effects of interaction of variables on the enzyme synthesis, 3-D response surface plots were used (Figs 1 & 2). In the present study the strain *Bacillus haynesii* SSRY4 MN031245 is able to produce tannase in the pH range of 4.0 to 7.0 and in the temperature range of 25°C to 49°C. Optimum tannase production was observed in the acidic pH range. However, tannase production showed a downfall in the alkaline range. Similar pattern of decline in tannase activity was observed in previous reports.[9,13] In the similar fashion, reduced tannase production was observed at lower and

Table 1 — Experimental range and levels of process parameters

Variable	Symbol	−2	−1	0	1	2
pH	X_1	2.5	4.0	5.5	7.0	8.5
Temperature (°C)	X_2	13	25	37	49	61
Incubation time (h)	X_3	24	48	72	96	120
Agitation speed (rpm)	X_4	50	100	150	200	250

Table 2 — Design matrix and responses

Run order	X_1: pH	X_2: Temperature (°C)	X_3: Incubation time (h)	X_4: Agitation speed (rpm)	Experimental value (U/mL)	Predicted value (U/mL)
1	−1	−1	1	1	7.65	7.60
2	0	0	−2	0	0.213	0.0136
3	−1	−1	1	−1	6.63	6.50
4	1	1	−1	−1	4.79	4.82
5	0	0	0	0	10.92	11.03
6	1	1	1	1	0.4970	0.4238
7	0	0	2	0	1.94	2.06
8	0	2	0	0	0.0029	−0.2330
9	1	−1	−1	1	5.48	5.32
10	2	0	0	0	1.46	1.57
11	−1	1	−1	−1	3.09	3.15
12	0	−2	0	0	3.96	4.18
13	0	0	0	0	11.02	11.03
14	−1	1	1	1	0.8840	0.9284
15	1	−1	1	1	6.56	6.53
16	−2	0	0	0	1.07	0.9624
17	1	−1	1	−1	4.37	4.25
18	0	0	0	−2	10.19	10.19
19	1	1	1	−1	2.42	2.42
20	−1	1	−1	1	0.3777	0.4710
21	0	0	0	0	10.96	11.03
22	1	−1	−1	−1	2.57	2.55
23	−1	−1	−1	−1	1.39	1.44
24	1	1	−1	1	3.17	3.32
25	−1	−1	−1	1	3.01	3.03
26	0	0	0	0	11.19	11.03
27	0	0	0	2	9.79	9.79
28	−1	1	1	−1	3.97	4.10
29	0	0	0	0	10.98	11.03
30	0	0	0	0	11.13	11.03

higher levels of temperature. Low temperature led to decrease in tannase production possibly due to reduced transport of substrate across the cell. At optimum temperature, the tannase yield increased probably due to surge in kinetic energy of reactants. Highest tannase production was observed at a temperature of 37°C. Tannase production got restrained at higher temperatures probably due to thermal denaturation of metabolic pathways. These findings are in concordance with other reported work.[14] It was also observed that increasing the fermentation period up to 72 h resulted in enhanced enzyme production. Drop in enzyme synthesis beyond 72 h may be ascribed to decreased availability of nutrients in the production medium. Reducing the agitation speed from 250 rpm to 150 rpm enhanced the enzyme activity to 11.19 U/mL which is also supported by previous study.[13] Increasing the agitation speed beyond the optimum level (150 rpm) observed decline in tannase production possibly due to shear stress generation at high speeds. In our study, Tannase production was maximum at pH 5.5, temperature 37°C, 72 h incubation period and agitation speed 150 rpm. Response surface plots revealed that all the parameters showed significant interactive effects. The optimum conditions obtained in our study are in agreement with the previous studies.[5,13–20]

Model Validation

For confirming the validation of experimental model triplicate experiments were conducted under optimized conditions of process parameters of the study and enzyme activity of each experiment was determined. The average maximum activity was 11.19 ± 0.11 U/mL which is comparable to predicted maximum activity of 11.03 U/mL. Thus, the proposed model is validated, and found accurate and reliable.

Concluding Remarks

Thus, present study was aimed at optimizing the selected process parameters for enhancing Tannase production by *Bacillus haynesii* SSRY4 MN031245

Table 3 — Experimental CCD data analysis using ANOVA

Source	Coefficient factor	Sum of Squares	df	Mean Square	F-value	p-value	—
Model	11.03	463.15	14	33.08	1274.36	< 0.0001	significant
X_1-pH	0.1515	0.5506	1	0.5506	21.21	0.0003	—
X_2-Temperature	−1.10	28.99	1	28.99	1116.58	< 0.0001	—
X_3-Incubation period	0.5232	6.57	1	6.57	253.09	< 0.0001	—
X_4-Agitation speed	−0.1001	0.2403	1	0.2403	9.26	0.0082	—
X_1X_2	0.1410	0.3179	1	0.3179	12.25	0.0032	—
X_1X_3	−0.8393	11.27	1	11.27	434.20	< 0.0001	—
X_1X_4	0.2947	1.39	1	1.39	53.53	< 0.0001	—
X_2X_3	−1.03	16.84	1	16.84	648.86	< 0.0001	—
X_2X_4	−1.07	18.24	1	18.24	702.46	< 0.0001	—
X3X4	−0.1248	0.2492	1	0.2492	9.60	0.0073	—
X_1^2	−2.45	164.13	1	164.13	6322.38	< 0.0001	—
X_2^2	−2.27	140.97	1	140.97	5430.41	< 0.0001	—
X_3^2	−2.49	170.51	1	170.51	6568.33	< 0.0001	—
X_4^2	−0.2649	1.93	1	1.93	74.16	< 0.0001	—
Residual	—	0.3894	15	0.0260	—	—	—
Lack of Fit	—	0.3343	10	0.0334	3.03	0.1163	not significant
Pure Error	—	0.0551	5	0.0110	—	—	—
Cor Total	—	463.54	29	—	—	—	—

X_1: pH, X_2: incubation temperature (°C), X_3: incubation time period (h), X_4: agitation rate (rpm)

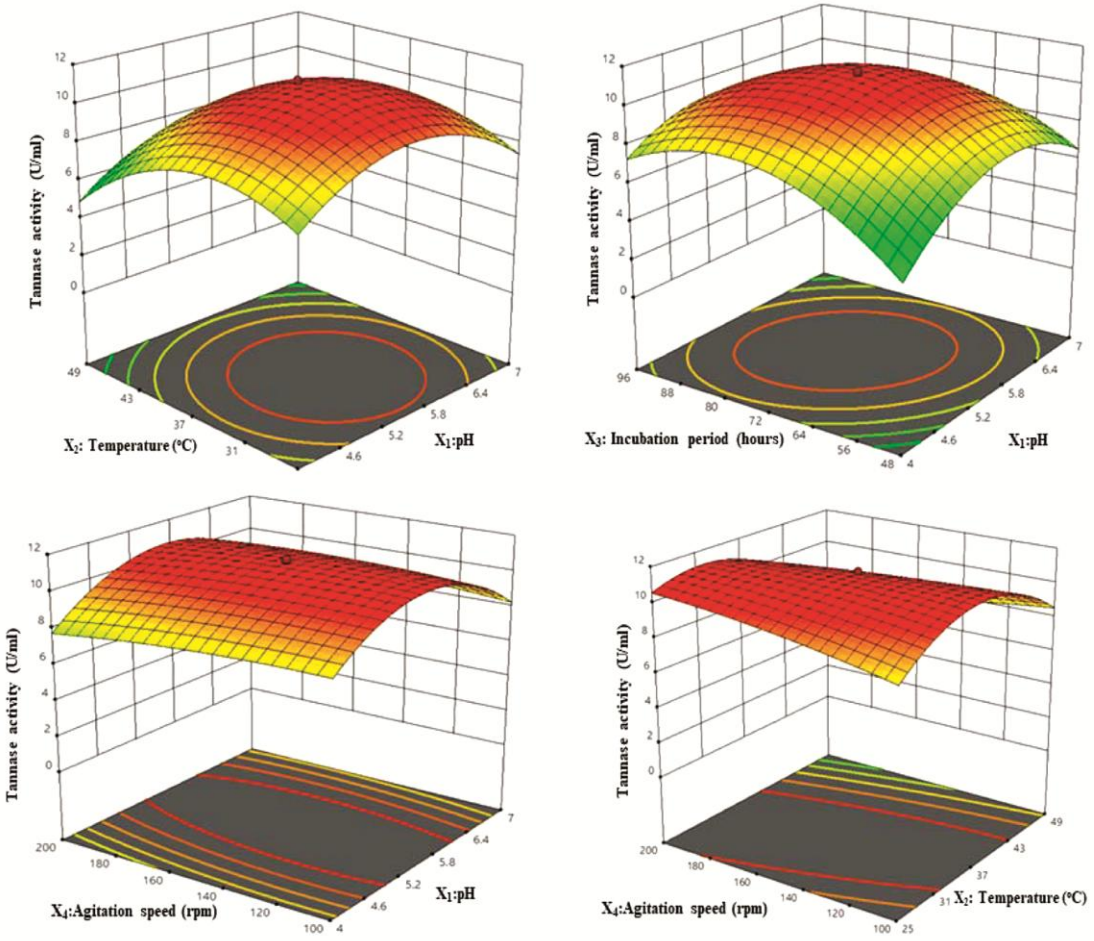

Fig. 1 — Response surface plots representing the response of interaction between temperature-pH, incubation period-pH, agitation speed-pH, agitation speed-temperature

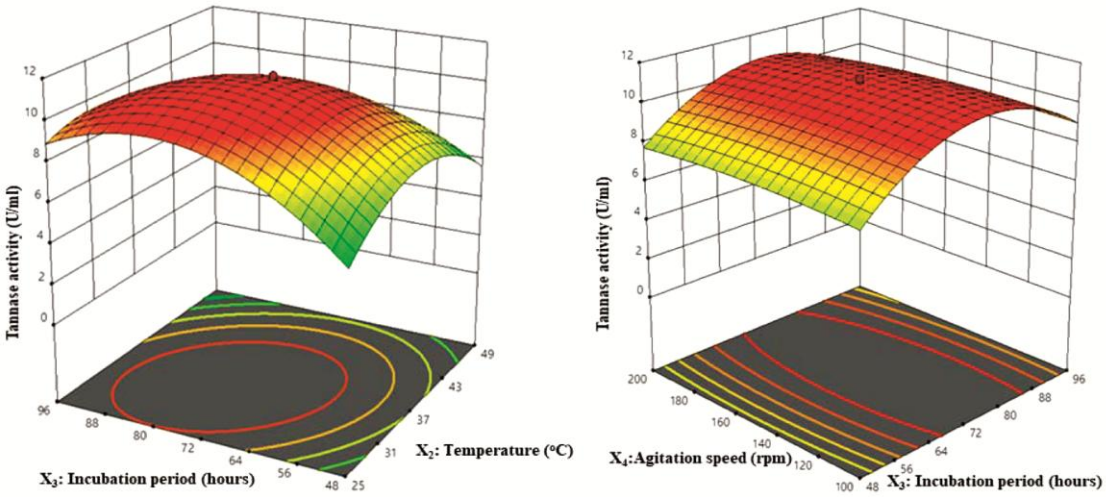

Fig. 2 — Response surface plots representing the response of interaction between incubation period-temperature, agitation speed-incubation period

through RSM using CCD. In our study, agglomerating all the important observations it can be concluded that tannase activity was enhanced from 4.49 U/ml to 11.19 U/mL after employing statistical design models, which was almost 2.49-fold higher than the initial enzyme activity. The statistical design of experiments used in present study could efficiently optimize the process parameters for optimum enzyme production by *Bacillus haynesii* SSRY4 MN031245. This work provides an attractive approach to produce a higher titer of tannase under optimized culture conditions, however still there exists a scope of improvement in terms of production and industrial applications. For the cost-effective enzyme production there is a strong need to develop more efficient fermentation protocols with continuous production process for higher yield and turnover. There is also a need to carry out extensive research studies to assure improved process controls for enhanced tannase production in future.

Conflicts of interest

None

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