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RESEARCH ARTICLE

Media optimization for extracellular amylase production by *Pseudomonas balearica* vitps19 using response surface methodology

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BACKGROUND: In this study, we optimized the process for enhancing amylase production from *Pseudomonas balearica* VITPS19 isolated from agricultural lands in Kolathur, India.
METHODS: Process optimization for enhancing amylase production from the isolate was carried out by Response Surface Methodology (RSM) with optimized chemical and physical sources using Design expert v.7.0. A central composite design was used to evaluate the interaction between parameters. Interaction between four factors – maltose (C-source), malt extract (N-source), pH, and $CaCl_2$ was studied.
RESULTS: The factors pH and $CaCl_2$ concentration were found to affect amylase production. Validation of the experiment showed a nearly twofold increase in alpha amylase production.
CONCLUSION: Amylase production was thus optimized and increased yield was achieved.

Keywords *Pseudomonas balearica VITPS19*, alpha amylase, optimization, response surface methodology, central composite design, pH

Introduction

Alpha-amylases [EC 3.2.1.1] are enzymes that randomly cleave the 1,4-α-D-glycosidic linkages between adjacent glucose molecules in linear polysaccharides such as starch, glycogen, and oligosaccharides (Rameshkumar and Sivasudha, 2011). Alpha-amylases are mainly exploited as degrading agents in industries where starch is majorly used, including food, paper, textile, and brewing industries (Nigam and Singh, 1995). Amylases have replaced the chemical methods of starch hydrolysis and their use has dominated the enzyme market by 25% (Kumar and Mehta, 2013).

The composition and concentration of various media components greatly influences the cellular growth and extracellular amylase production (Viswanathan et al., 2014). Nutritional and physicochemical factors such as pH, temperature, and carbon and nitrogen source greatly influence amylase production which differs significantly with the nature of the microbe being used (Stergiou and Papamichael, 2014). Being metalloenzymes, amylases require Ca^{2+} for their activity, integrity, and structural stabilization (Saha et al., 2014). In this regard, media components and conditions should be optimized for the maximum enzyme production.

The classical method of media optimization involves changing of one independent variable while other factors are kept constant; this might lead to misinterpretation of results due to the effect of interaction between different factors (Gangadharan et al., 2008). The multifactor experimental design is usually time-consuming and is incapable of determining the true optima due to interactions between the factors. Fermentation processes involve interactions between operational variables, which influence the final response. It is important for the optimization method to account for the interaction between variables in order to determine a set of optimal experimental conditions. The limitations encountered with the classical method of optimization could be overcome by other techniques. Response surface Methodology (RSM) is a technique in which the effect of all factors in a fermentation process can be explained. RSM is a collection of mathematical methods, experimental strategies, and statistical inference for exploring and constructing an

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approximate functional relationship between a response variable and a set of design variables (Elibol et al., 2005 ; Gangadharan et al., 2008).

In this study, the fermentation medium for extracellular amylase production was optimized with a central composite design using RSM, by studying the effect of four factors namely, maltose (carbon source), malt extract (nitrogen source), pH, and $CaCl_2$.

Materials and methods

Strain and medium

Pseudomonas balearica VITPS19 isolated from the agricultural fields of Kolathur, Tamil Nadu was reported previously (Kizhakedathil and Chandrasekaran, 2017). The isolate was identified by 16S rRNA sequencing followed by a BLAST homology search. The nucleotide sequences have been deposited in GenBank under the accession number MF164145.1. *Pseudomonas balearica* VITPS19 was initially grown in a liquid medium containing soluble starch, 1% (w/v); yeast extract, 0.2% (w/v); malt extract, 0.5% (w/v); $MgSO_4$, 0.05% (w/v); KH_2PO_4, 0.05% (w/v); NaCl, 0.15% (w/v); and $CaCl_2$, 0.1% (w/v) (Meena et al., 2013). The pH of the medium was adjusted to 7.3 and incubated at 28°C for 7 days. After incubation, the culture broth was centrifuged at 12500 r/min for 15 min at 0°C to remove the cell components, and the supernatant was used for enzymatic studies.

Enzyme assay and protein estimation

α-amylase activity was assayed by incubating 0.5 ml enzyme with 0.5 ml soluble starch (1.0 w/v) prepared in 0.1 M phosphate buffer, pH 6.9. Enzyme activity was assayed using the DNSA method by estimating the amount of glucose released (Miller, 1959). One unit of α-amylase activity was defined as the amount of enzyme that liberates 1 μmol of glucose per minute under standard conditions. Total protein was determined by the Bradford's method using bovine serum albumin as a standard (Bradford, 1976).

Optimization of α- amylase by response surface methodology

Experimental design of rsm

Amylase production was initially optimized by the classical method. The initial optimization experiments revealed that a higher yield was observed with 1% maltose as the carbon source, 0.5% malt extract as the nitrogen source, and 0.1% $CaCl_2$; along with these conditions, pH was chosen as an additional variable.

Parameters

RSM was carried out using the CCD design to optimize and identify the interactions between the significant factors obtained from the classical method of optimization. The four variables chosen in this experiment were the carbon source-maltose, nitrogen source-malt extract, pH, and $CaCl_2$ concentration with 5 coded levels (-α, −1, 0, + 1, + α) used for their combined influence on amylase production. In total, 30 experimental trials were carried out with 16 factorial points, 8 axial points with α = 2, and 6 replications of central points. The test factors were coded according to the Equation given below while developing the regression equation,

$$X_i = (X_i - X_0) / \delta X_i \quad (1)$$

where X_i is the dimensionless coded value of the i^{th} independent variable; X_i is the natural value of the i^{th} independent variable; X_0 is the natural value of the i^{th} independent variable at the center point, and δX_i is the difference in effect. A second order polynomial equation was generated and the response data obtained from the design was fitted to the equation. The general polynomial equation is as follows:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_{12} \\ & + \beta_{22} X_{22} + \beta_{33} X_{32} + \beta_{44} X_{42} + \beta_{12} X_1 X_2 \\ & + \beta_{13} X_1 X_3 + + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 \\ & + \beta_{34} X_3 X_4 \end{aligned} \quad (2)$$

where Y is the predicted response; β_0 is the model constant; β_1, β_2, β_3, and β_4 are the linear coefficients; β_{11}, β_{22}, β_{33}, and β_{44} are the squared coefficients; and β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are the interaction coefficients (Suganthi and Mohanasrinivasan, 2014).

Data analysis

Design expert v 7.0. (Stat-Ease, Inc., Minneapolis, USA) was used to analyze the experimental data obtained. The quality of fit of the model equation was expressed by analyzing the coefficient of determination, R^2, and by analysis of variance

Table 1 Range of values for the response surface method- CCD

Factors	Independent variables	Coded			Levels	
		−α (−2)	−1	0	+ 1	+ α (+ 2)
A	Maltose %	0	0.5	1	1.5	2
B	Malt extract %	0	0.25	0.50	0.75	1
C	$CaCl_{2\%}$	0	0.05	0.10	0.15	0.2
D	pH	6	6.25	6.5	6.75	7

Table 2 Response of the experimental design by RSM

Runs number	Coded value order	A	B	C	D	Enzyme activity (U)	Predicted response (U)
8	1	1.5	0.75	0.15	6.25	0.52	0.16
5	2	0.5	0.25	0.15	6.25	0.76	0.926
28	3	1	0.5	0.1	6.5	1.90	1.90
19	4	1	0	0.5	6.5	1.77	2.108
26	5	1	0.5	0.1	6.5	1.90	1.90
30	6	1	0.5	0.1	6.5	1.90	1.90
12	7	1.5	0.75	0.05	6.75	2.10	1.186
22	8	1	0.5	0.2	6.5	0.49	0.46
11	9	0.5	0.75	0.05	6.75	2.42	1.996
27	10	1	0.5	0.1	6.5	1.90	1.90
15	11	0.5	0.5	0.15	6.75	2.48	1.958
10	12	1.5	0.25	0.05	6.75	1.92	1.986
13	13	0.5	0.25	0.15	6.75	1.94	1.79
17	14	0	0.5	0.1	6.5	1.92	2.416
23	15	1	0.5	0.1	6	0.30	0.12
24	16	1	0.5	0.1	7	0.59	1.44
4	17	1.5	0.75	0.05	6.25	0.55	0.73
7	18	0.5	0.75	0.15	6.25	0.80	0.766
9	19	0.5	0.25	0.05	6.75	1.96	1.056
1	20	0.5	0.25	0.05	6.25	1.89	1.292
29	21	1	0.5	0.1	6.5	1.90	1.90
21	22	1	0.5	0	6.5	0.42	1.1
16	23	1.5	0.75	0.15	6.75	0.28	0.912
25	24	1	0.5	0.1	6.5	1.90	1.90
3	25	0.5	0.75	0.05	6.25	1.08	1.244
14	26	1.5	0.25	0.15	6.75	2.73	1.824
18	27	2	0.5	0.1	6.5	1.73	1.936
2	28	1.5	0.25	0.05	6.25	2.05	1.856
6	29	1.5	0.25	0.15	6.25	0.80	1.256
20	30	1	1	0.1	6.5	0.78	1.148

Table 3 Analysis of variance (ANOVA) for the quadratic model of amylase production obtained from the experimental results

Source	Sum of squares	*df*	Mean square	*F* value	*p*-value	Prob > *F*
Model	11.01	14	0.79	2.40	0.0479	Significant
A-A	*0.32*	*1*	*0.32*	*0.99*	*0.3345*	
B-B	*1.39*	*1*	*1.39*	*4.23*	*0.0563*	
C-C	*0.58*	*1*	*0.58*	*1.77*	*0.2021*	
D-D	*2.62*	*1*	*2.62*	*8.00*	*0.0121*	Significant
AB	*1.13*	*1*	*1.13*	*3.45*	*0.0819*	
AC	*0.056*	*1*	*0.056*	*0.17*	*0.6837*	
AD	*0.089*	*1*	*0.089*	*0.27*	*0.6103*	
BC	*0.013*	*1*	*0.013*	*0.039*	*0.8467*	
BD	*0.11*	*1*	*0.11*	*0.33*	*0.5751*	
CD	*0.18*	*1*	*0.18*	*0.54*	*0.4711*	
A^2	*0.14*	*1*	*0.14*	*0.42*	*0.5279*	
B^2	*0.13*	*1*	*0.13*	*0.41*	*0.5316*	
C^2	*2.32*	*1*	*2.32*	*7.07*	*0.0171*	Significant
D^2	*2.18*	*1*	*2.18*	*6.65*	*0.0202*	Significant
Residual	5.24	16	0.33			
Lack of Fit	*5.24*	*10*	*0.52*			
Pure Error	*0.000*	*6*	*0.000*			
Cor total	*16.25*	30				

Table 4 *R*-Squared, Adj *R*-Squared, Pred *R*-Squared, and Adeq Precision value of the model

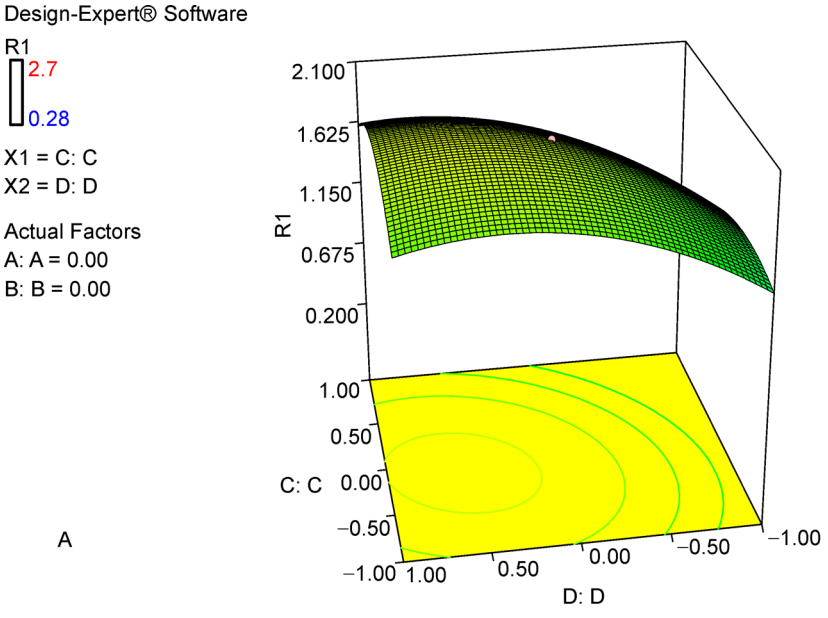
Std. Dev.	0.57	*R*-Squared	0.6774
Mean	1.47	Adj *R*-Squared	0.3951
C.V. %	39.03	Pred *R*-Squared	-0.8581
PRESS	30.19	deq Precision	5.972

(ANOVA). The significance of the model was assessed by the determination coefficient and correlation coefficient. The model was statistically analyzed using Fisher's test (Myers and Montgomery, 2002).

Results and discussion

The optimum levels of significant factors and the effect of their interactions on amylase production were determined by CCD experiments. Maltose, malt extract, pH, and $CaCl_2$ were selected as variables based on the results of the classical method. The experimental design was carried out to determine the parameter ranges. The coded and actual values of the four independent variables for amylase production are presented in Table 1.

The results of 30 runs from CCD experiments for studying the effects of 4 independent variables on amylase production are represented in Table 2.



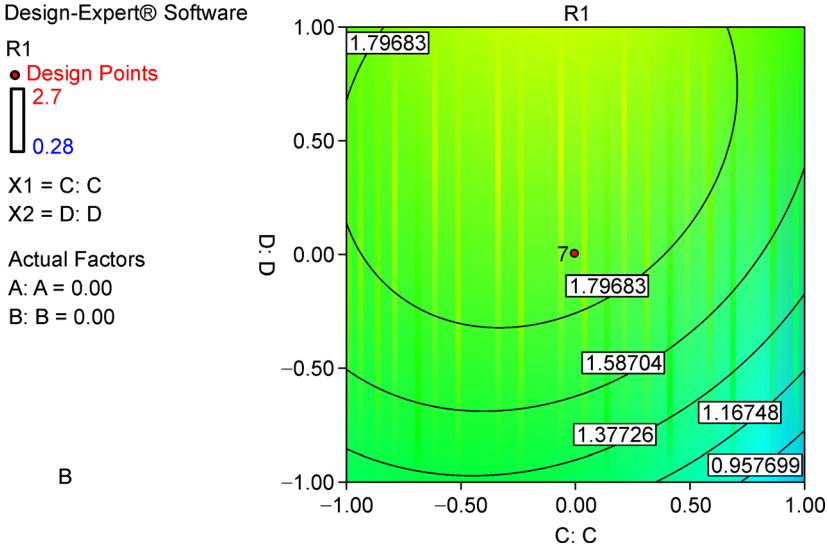


Figure 1 (A) Three dimensional curves showing the effects of factors C ($CaCl_2$) and D (pH). (B) Contour plots showing the effects of factors C ($CaCl_2$) and D (pH).

From the RSM results, the maximum experimental value for amylase production was 2.732 U/mL. The regression analysis data was fitted to a quadratic model. The second order regression equation obtained was as follows:

$$\begin{aligned} Y = {} & +1.90 - 0.12^{*}A - 0.24^{*}B - 0.16^{*}C \\ & + 0.33^{*}D - 0.27^{*}A^{*}B - 0.059^{*}A^{*}C - 0.074^{*}A^{*}D - 0.028^{*}B^{*}C \\ & + 0.082^{*}B^{*}D + 0.11^{*}C^{*}D \\ & + 0.069^{*}A^{2} - 0.068^{*}A^{2} - 0.28^{*}C^{2} - 0.28^{*}D^{2} \end{aligned} \quad (3)$$

where Y is amylase activity U/mL, A is maltose (g/L), B is malt extract (g/L), C is $CaCl_2$ (g/L), and D is pH.

The statistical significance was determined by the F-test and the analysis of variance (ANOVA) for the response surface quadratic model is presented in Tables 3 and 4.

The *F* value of 2.40 from ANOVA for amylase production implies that the model is significant. This is also evident from the model *F* value and the probability value at $P > F$ value, which was about 0.0479 (less than 0.05). The goodness of the model can be determined from the determination coefficient (R^2) and the correlation coefficient (*R*) (Sunitha et al., 1999; Osorio, 2001). The R^2 value of 0.6674 suggests 66.7% variability in amylase production. The closer the value of *R* (*R* = multiple correlation coefficient) to 1, better is the correlation between the experimental and predicted values (Pujari and Chandra, 2000; Liu et al., 2003). The ratio of signal to noise is measured by adequate precision and is believed to be desirable if the value is greater than 4. The value obtained was 5.972, showing that the polynomial quadratic model is of an adequate signal and can be used to navigate the design space.

The coefficient estimates of Eq. (3), along with the corresponding *P* values are given in Table 3. The *P* values correlate with the significance of each coefficient. It is important to indicate the pattern of mutual interaction between the coefficients. The smaller the *P* value, more significant is the corresponding coefficient (Rao et al., 2000). The linear coefficients C and D, all quadratic coefficients, and two interaction coefficients i.e. D, C^2, D^2 were observed to be significant. Since it is a hierarchical model, insignificant coefficients were not omitted (Fig. 1A and B).

RSM plots provide the relation between the response and experimental levels of each variable. These plots are useful in understanding the kind of interaction among test variables in order to deduce the optimum conditions (Dey et al., 2001; Vohra and Satyanarayana, 2002). The results of the classical approach evidenced that an increase in $CaCl_2$ concentration (0.15%), interaction with pH at a maximum level of 6.25–6.75, Maltose at 1.5%, and Malt extract at 0.25% increased the amylase activity. It was also observed that an increase in malt extract concentration or a decrease in pH and $CaCl_2$ concentration decreases the amylase activity.

The results suggest that $CaCl_2$ and pH are the important components that interact with maltose and malt extract for enhanced amylase activity. The effect of variations in the levels of all 4 independent variables on amylase production is shown in the perturbation graph (Fig. 2). From the graph, it can be concluded that $CaCl_2$ plays an important role in enhanced amylase activity and production, followed by pH with maltose and malt extract. Normal probability versus residuals was plotted on a graph showing that the data were

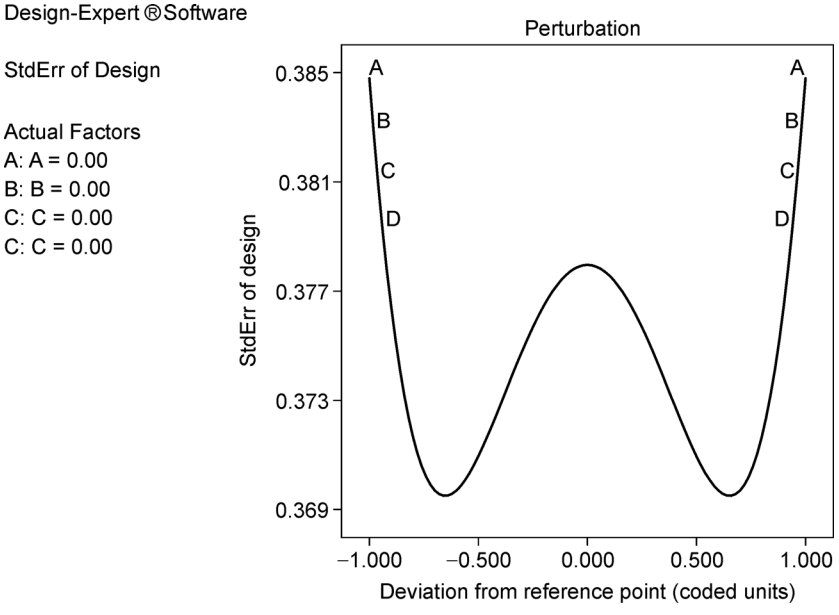


Figure 2 Perturbation graph showing the effect of all independent variables on amylase production.

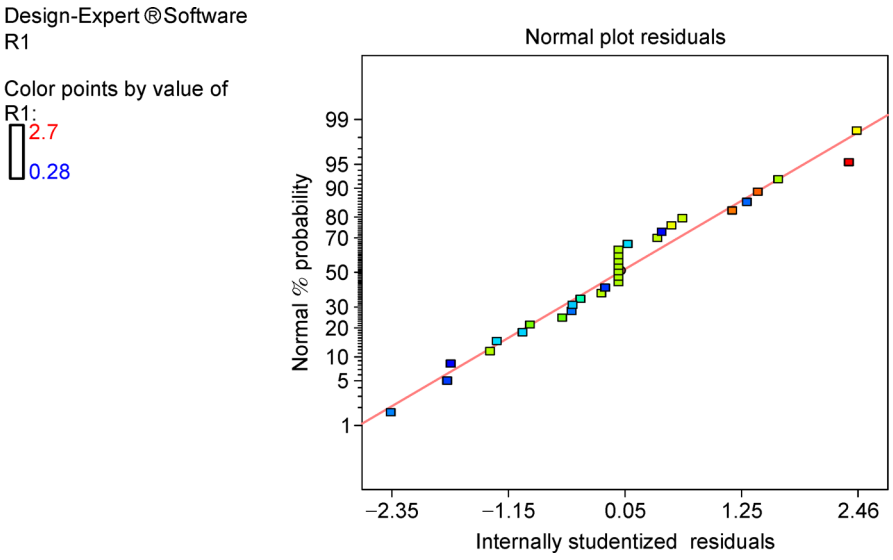


Figure 3 Plot between expected normal values versus residuals.

very close to the straight line and are placed on both sides of the line, thus indicating that the model is fairly good (Fig. 3).

Amylase production by the classical method yielded 1.57 U/mL activity. The media was modified based on the results obtained from the RSM, and contained maltose (1.5%), malt extract (0.2%), and $CaCl_2$ (0.15%), at pH 6.8. This yielded around 3.02 U/mL of enzyme activity which was an approximately twofold increase in alpha amylase when compared to the initial media.

Conclusion

The use of an experimental design helped in understanding the influence of different concentrations of various factors on amylase production. This enabled screening of large experimental data in search of the best culture conditions for optimizing amylase production in a short period. The statistical approach showed significant results in optimizing process parameters for maximum amylase production by *Pseudomonas balearica VITPS19*. The present study identified the effect of various parameters on the enzyme production and yield. Based on the study, pH and $CaCl_2$ concentration were found to influence the enzyme production significantly.

Conflicts of interest

The authors declare that they have no competing interests.

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