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Research Article

Optimization of process variables using Response Surface Methodology (RSM) in the solid-state fermentative production of pectinase by *Aspergillus awamori*

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Abstract

Pectinase is an important hydrolytic enzyme playing a vital role in the hydrolysis of pectin molecules and finds variety of applications in food processing industries. The aim of this present research work is to evaluate the potential use of natural substrates (rice bran) in the solid-state fermentative production of pectinase by using Response Surface Methodology (RSM). The 2^4 five level Central Composite Rotatable Design (CCD) was used to develop a statistical model for the optimization of process variables such as substrate concentration (5 – 25 % w/w) X_1, initial pH (3.0 – 7.0) X_2, fermentation temperature (25 – 37°C) X_3 and inducer concentration (2 – 10%) X_4 by *Aspergillus awamori* MTCC 0548. The design contains a total of 31 experimental runs involving replications of the central points and organized in a randomized factorial design. Data obtained from RSM on pectinase production were subjected to the analysis of variance (ANOVA) and were analyzed using a second order polynomial equation. Maximum pectinase production of 6.56 g/Kg substrate was obtained from rice bran (18.5% w/w) at the optimized process conditions (temperature 31°C, pH 5.4 and inducer concentration 5%) in 144 h aerobic batch fermentation.

Keywords: enzyme, food processing, CCD, rice bran, yeast, India

Introduction

Pectinases have great commercial importance for various industrial food applications like increasing the juice yield and its clarity, preventing haziness of wine in brewing industries, extraction of oil, fermentation of coffee and tea and in the preparation of animal feed [1]. As a result the importance of the pectinase enzyme has gained momentum and lot of research is ongoing for efficient and economical production of pectinase enzymes using cheaper substrates of agro-residue origin. Primarily, these enzymes are responsible for the degradation of the long and complex molecules know as pectin that occur as structural polysaccharides in the middle lamella and the primary cell walls of young plant cells [2]. The enzymes that hydrolyze pectic substances are known as pectic enzymes, pectinases or pectinolytic enzymes. In pectic substances, D-galacturonic acid units are linked together by α-1,4-glycosidic linkages. The three major types of pectinases are Pectinesterases (PE), Depolymerizing enzymes (Polymethylgalacturonases, Polygalacturonases and Polygalacturonate lyases) and Protopectinase [3].

Pectinases are produced during the natural ripening process of some fruit and they help to soften the cell walls in combination with cellulase. A large number of microbial strains have been studied for the production of pectinase [4]. The main sources for the pectinolytic complex enzymes are yeast, bacteria and a large number of filamentous fungi of which the most relevant ones are *Aspergillus* sp. The pectinase production in yeast has received less attention due to less yield obtained [5]. In this sense the biochemical characterization of polygalacturonases (PG) using yeast has been reported and heterologous genes have been successfully expressed in *Saccharomyces cerevisiae* [6]. Endo-polygalacturonase production by yeasts was first reported in the year 1951 using *Saccharomyces fragilis*. The ability of certain yeasts to attack cell wall pectin indicating that they contain true PG was investigated. These yeasts belong to the genera *Candida, Pichia, Saccharomyces* and *Zygosaccharomyces*. Yeast pectinase are usually exocellular enzymes of a varying molecular means and are of glycoproteinaceous nature [7]. Yeast PG's may have some advantages over fungal ones and could offer a good alternative to fungal enzyme production. Probably the main problem in yeast pectinolytic enzymes in industrial processes lies in the low fermentation yield [6].

Solid State Fermentation (SSF) is generally preferred because highly concentrated crude enzymes are obtained at low costs [4, 8]. Since the culture conditions in SSF are much more similar to the natural habitat of filamentous fungi, these are able to grow well and excrete large quantities of enzymes. Additionally these processes are of special interest for countries like India with abundance of agro-industrial wastes which can be used as cheap raw materials and also it allows the utilization of wastes to produce useful products [9]. Optimization of process conditions is one of the most critical stages in the development of an efficient and economic bioprocess. The classical method of studying one variable at a time can be effective in some cases but it is useful to consider the combined effects of all the factors involved. Response Surface Methodology (RSM) is a powerful mathematical model with a collection of statistical techniques wherein interactions between multiple process variables can be identified with fewer experimental trials [10]. There are various advantages in using statistical methodologies in terms of rapid and reliable short listing of process conditions. Thus, RSM experimental design is an efficient approach to deal with a large

number of variables and there are several reports on application of RSM for the production of primary and secondary metabolites through microbial fermentation [11, 12].

In the present work rice bran, which is largely available in the southern part of India as an agricultural waste was selected as the substrate for the fermentative production of pectinase enzyme using *Aspergillus awamori* by solid state fermentation. This fermentation was carried out and the optimization of the important process variables such as substrate composition, fermentation temperature, initial pH and inducer concentration were standardized using Response Surface Methodology (RSM).

Materials and Methods

Materials

Rice bran samples were obtained from the agricultural field, Salem District, Tamilnadu. The sample was made into 100 mesh (0.15 mm) fine powder by the use of laboratory blender at 3000 rpm and was preserved in a sealed plastic bag at 4°C to prevent any possible degradation or spoilage.

Microorganism and culture conditions

Fungal strain *Aspergillus awamori* MTCC 548 was obtained from Microbial Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Culture was maintained on Czapek's Agar medium. After three days incubation at 30°C the agar slants were stored at 4°C. The liquid medium for the growth of inoculum for yeast was yeast extract – malt extract – peptone – glucose medium (YMP) composed of 3 g/l of yeast extract, 3 g/l of malt extract, 5 g/l of peptone and 10 g/l of glucose.

Inocula were grown aerobically in 250 ml Erlenmeyer flasks containing the above mentioned medium at 30°C in an Environmental Shaker (Remi Scientific) at 200 rpm for 24 h. Active cells were centrifuged in a clinical centrifuge (1200 rpm), washed with sterile water and were used as inoculum. Fermentations for pectinase production were conducted on a shaker at 200 rpm in 250 ml flasks and the samples were withdrawn periodically for the analysis of enzyme concentration.

Enzyme extraction

The crude pectinase was extracted by mixing 10g of fermented materials with distilled water, stirred for 20 minutes in the shaker, filtered and then centrifuged for 20 minutes. The supernatant was used as the crude enzyme and then studied for enzymatic measurements by DNS method [13].

Total pectinase assay

A suitably diluted sample of 0.5ml was added to a solution containing 2 ml of 1% citrus pectin in acetate buffer (pH 4.8) in a test tube. Samples are kept at 45°C for 30 minutes in a water bath, cooled, added with 2.5 ml of DNS reagent, seethed for 5 min. Finally the contents were cooled and added with distilled water and were measured at 540 nm using

UV/Vis Biospectrophotometer (ELICO BL 198). The concentration of β-galacturonic acid was determined from the standard β-galacturonic calibration curve.

Solid State Fermentation (SSF)

The powdered rice bran samples of different compositions were weighed (10 g/flask) and distributed into 250 ml Erlenmeyer flasks with the addition of Czapek's nutrient medium (without carbon source) to a desired solid-liquid ratio (up to 20% solid) and 0.1 M Potassium phosphate buffer (pH = 6.0), followed by sterilization for 15 min at 15 psi (121°C) in an autoc1ave. To the production medium 10^8 spores of *A. awamori* were inoculated aseptically and the flasks were then covered with cotton to allow CO_2 produced during fermentation to escape. The flasks were incubated in a rotary shaker (200 rpm) at 30°C for 144 h. Samples were withdrawn periodically (12h interval) and were analyzed for total pectinase enzyme activity.

Experimental design and statistical analysis

The RSM used in the present study is a central composite design (CCD) involving four different factors. Experiments were conducted in a randomized fashion. The CCD contains a total of 31 experimental trials involving the replications of the central points (Table 2). The dependent variables selected for this study were pectinase yield (g/kg). The independent variables chosen were substrate composition, X_1 (%); incubation temperature, X_2 (°C); initial pH, X_3; inducer concentration, X_4 (%). Once the experiments were performed, a second order polynomial equation (1) shown below was used to describe the effect of variables in terms of linear, quadratic and cross product terms [14].

$$Y = b_0 + \sum_{i=1}^{k} b_i Xi + \sum_{i=1}^{k} b_{ij} X_i^2 + \sum_{i_i<j}^{k} \sum_{j}^{k} b_{ij} X_i X_j + e \qquad \text{----------------(1)}$$

Where, *i*, *j* are linear, quadratic coefficients, respectively, while '*b*' is regression coefficient, Y is the pectinase yield, *k* the number of factors studied and optimized in the experiment and 'e' is random error. When developing the regression equation, the test factors were coded according to the following equation:

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \qquad i = 1,2,3,\ldots..,k, \qquad \text{----------------(2)}$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of the independent variable at the center point, and ΔX_i is the step change value.

The quality of fit of the second order equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by *F*-test. The significance of each coefficient was determined using Student's *t*-test. The coefficients of the equation were determined by employing MINITAB software version 15. Analysis of variance (ANOVA) for the final predictive equation was done using the same software package. The response surface equation was optimized for maximum yield in the range of process variables using MATLAB software version 7.0.1. Three dimensional plots and their

respective contour plots were obtained based on the effect of the levels of two parameters (at five different levels each) and their interactions on the yield of pectinase by keeping the other two parameters at their optimal concentrations. From these contour plots, the interaction of one parameter with another parameter was studied and also the model is developed to explain the quadratic interaction effects by conducting the pairwise regression analysis of experimental data which is not fully described in the multiple regression analysis due to limited degrees of freedom. The optimum concentration of each parameter was identified based on the hump in the three-dimensional plots.

Results and Discussion

Optimization of process variables in pectinase production using RSM

Table 1 shows the four independent variables (substrate composition, pH, temperature, inducer concentration) and their concentrations at different coded and actual levels of the variables employed in the design matrix. Five level central composite design matrix and the experimental responses of the dependent variable (pectinase yield) are listed in Table 2. Using the designed experimental data presented in Table 2, the polynomial proposed model for pectinase yield was regressed by only considering the significant terms. The expanded equation (3) is shown below.

$$Y = 6.22 - 0.915 X_3 - 0.523 X_4 + 0.711 X_1^2 - 1.283 X_2^2 - 0.956 X_3^2 - 0.516 X_4^2 + 0.448 X_1 X_3 - 0.472 X_1 X_4 + 0.328 X_2 X_4 - 0.187 X_3 X_4 \quad (3)$$

Based on the experimental response, the quantity of pectinase enzyme produced by *A. awamori* ranged from 0.48 to 6.22 g/kg of substrate. The ANOVA result of quadratic regression model for pectinase yield is described in Table 3. ANOVA of the regression model for pectinase yield demonstrated that the model was significant due to an *F*-value of 61.66 and a very low probability value (P model >*F* = 0.001). ANOVA (*F*-test) for the model explained the response of the dependent variable Y.

Table 1. Coded and actual levels of the independent variables for the design of experiment.

Independent variables	Symbols	Coded levels				
		– 2	–1	0	1	2
Substrate conc. (% w/w)	X_1	5	10	15	20	25
pH	X_2	3	4	5	6	7
Temperature (°C)	X_3	25	28	31	34	37
Inducer concentration (% w/v)	X_4	2	4	6	8	10

Table 3 also shows that the experimental yields fitted the second order polynomial equation well as indicated by high R^2 values (0.984). R^2 value being the measure of the goodness of fit of the model, indicated that 98.4% of the total variation was explained by the model.

Tabulated *F*-value shows that the model predicts the experimental results well and the estimated factors effects were real.

The regression coefficients, along with the corresponding *P*-values, for the model of pectinase production by *A.awamori*, are described in Table 4. It shows that the regression coefficients of all the linear terms and all quadratic coefficients of X_3, X_4 and X_1X_1, X_2X_2, X_3X_3 and X_4,X_4 were significant at < 1% level and interaction coefficients of *X1X3, X1X4, X2X3,* and *X3X4* were significant at < 5% level. ANOVA suggests the model to be significant at $P<0.01$. The *P*-values used as a tool to check the significance of each of the coefficients, in turn indicate the pattern of interactions between the variables. Smaller value of *P* was more significant to the corresponding coefficient. The contour plots based on independent variables were obtained using the same software package (Figs.1 to 3), indicating that a local optimum exists in the area experimentally investigated. The orientation of the principal axes of the contour plots between the variables substrate concentration and inducer concentration, pH and inducer concentration, temperature and inducer concentration indicated that the mutual interactions between these set of variables had a significant effect on the pectinase yield (6.22 g/kg of substrate).

Table 2. Five level Central Composite Rotatable Design (CCRD) and the experimental responses of dependent variable Y (Pectinase, g/kg of substrate).

Run No.	Coded levels				Real values				Pectinase yield (g/kg)	
	x_1	x_2	x_3	x_4	$^aX_1$	$^bX_2$	$^cX_3$	$^dX_4$	Experimental	Predicted
1	1	1	1	-1	20	6	34	2	5.05	4.97
2	-1	-1	-1	-1	10	4	28	2	2.45	2.47
3	-1	-1	1	-1	10	4	34	2	3.24	3.78
4	1	-1	1	-1	20	4	34	2	5.33	5.62
5	1	-1	1	1	20	4	34	8	2.31	2.60
6	0	0	0	0	15	5	31	6	6.22	6.22
7	0	0	0	-2	15	5	31	2	5.20	5.20
8	-2	0	0	0	5	5	31	6	3.58	3.37
9	0	0	0	0	15	5	31	6	6.22	6.22
10	0	0	0	0	15	5	31	6	6.22	6.22
11	-1	1	-1	1	10	6	28	8	2.98	2.75
12	0	0	0	0	15	5	31	6	6.22	6.22
13	-1	1	1	1	10	6	34	8	4.05	3.31
14	0	0	0	0	15	5	31	6	6.22	6.22
15	-1	1	-1	-1	10	6	28	2	2.05	1.82
16	0	2	0	0	15	7	31	6	1.40	1.08
17	0	0	0	0	15	5	31	6	6.22	6.22
18	1	-1	-1	1	20	4	28	8	0.47	0.25
19	1	1	1	1	20	6	34	8	3.35	3.26
20	-1	-1	-1	1	10	4	28	8	2.06	2.09
21	-1	1	1	-1	10	6	34	2	3.87	3.13
22	0	0	-2	0	15	5	25	6	0.56	0.56
23	0	-2	0	0	15	3	31	6	0.76	1.08
24	2	0	0	0	25	5	31	6	3.17	3.37
25	1	1	-1	-1	20	6	28	2	1.44	1.87
26	0	0	0	2	15	5	31	10	3.11	3.11
27	0	0	0	0	15	5	31	6	6.22	6.22
28	1	-1	-1	-1	20	4	28	2	2.74	2.52
29	1	1	-1	1	20	6	28	8	0.48	0.91
30	-1	-1	1	1	10	4	34	8	2.11	2.65
31	0	0	2	0	15	5	37	6	4.22	4.22

$^aX_1$ (Substrate concentration,%) is calculated as: $X_1 = 15 + x_1\,(5)$
$^bX_2$ (initial pH) is calculated as: $X_2 = 5.0 + x_2\,(1.0)$
$^cX_3$ (fermentation temperature, °C) is calculated as: $X_3 = 31 + x_3\,(3)$
$^dX_4$ (inducer concentration, % w/v) is calculated as: $X_3 = 6 + x_3\,(2)$

Table 3. Regression analysis and corresponding *t* and p- value of second order polynomial model for the optimization of pectinase production.

Term constant	Regression coefficient	Std deviation	*t*- statistic	P-value
Intercept	6.22	0.12924	48.127	< 0.001
X_1	-0.103	0.06980	-1.474	0.160
X_2	0.16	0.06980	2.298	0.035
X_3	0.915	0.06980	13.115	< 0.001
X_4	-0.523	0.06980	-7.492	< 0.001
X_1X_1	-0.711	0.06394	-11.115	< 0.001
X_2X_2	-1.283	0.06394	-20.068	< 0.001
X_3X_3	-0.956	0.06394	-14.946	< 0.001
X_4X_4	-0.516	0.06394	-8.065	< 0.001
X_1X_2	-0.227	0.08549	-2.654	0.017
X_1X_3	0.448	0.08549	5.242	< 0.001
X_1X_4	-0.472	0.08549	-5.520	< 0.001
X_2X_3	0.258	0.08549	3.020	0.008
X_2X_4	0.328	0.08549	3.838	0.001
X_3X_4	-0.187	0.12924	48.127	< 0.001
	R^2 = 0.984			

Table 4. Analysis of variance (ANOVA) for the quadratic polynomial model for pectinase production.

Source	Sum of squares	Degrees of freedom (DF)	Mean square (MS)	*F*-value	P-value
Regression	114.100	14	8.1500	69.70	<0.001
Linear	27.546	4	6.8860	58.90	<0.001
Square	75.607	4	18.901	61.66	<0.001
Interaction	10.947	6	1.8245	15.60	<0.001
Residual Error	1.871	16	0.1169	-	-
Lack-of-Fit	1.871	10	0.1871	-	-
Pure Error	0	6	0	-	-
Total	115.971	30	-	-	-

The isoresponse contour of RSM as a function of two factors at a time, holding all other factors at fixed level was helpful for understanding both the main and the interaction effects of these two factors. The response values for the variables can be predicted from these plots. Figures 1 – 3 represent the isoresponse contour plots for the pectinase production during batch solid state fermentation. The effect of varying inducer concentration and substrate concentration on pectinase production, while other two variables (pH and temperature) were fixed at central concentrations (5 and 31°C) respectively), is shown in Figure 1. It was evident that as the substrate concentration increased, the pectinase production decreased at low inducer concentration, but drastically increased at higher inducer concentrations. Figure 3 shows a similar plot of the above was obtained at various values of temperature and inducer concentration and at fixed substrate concentration (15%) and pH (5). From Figure 2, as the temperature was increased, the pectinase production decreased at low inducer concentration, but drastically increased at higher inducer concentration. The interaction effect of temperature and inducer concentration was responsible for this behavior. Figure 3 depicts contour plot showing the effects of pH and inducer concentration on pectinase production at fixed substrate concentration (15%) and temperature (31°C). The drastic interactions between pH and inducer concentration were apparent from the elliptical contour plot. The shape of the contour plots indicates whether the mutual interactions between the independent variables are significant or not. A circular contour plot indicates that the interactions between the corresponding variables are negligible, while an elliptical contour plot indicates that the interactions between them are significant. Interactions of variables can be better determined by the orientation of the principal axes of the contour plots. As can be seen from Fig. 3, an increase in pH markedly decreased the pectinase production within the tested inducer concentration. Increasing the pH resulted in a marked decrease in pectinase production at higher inducer concentration.

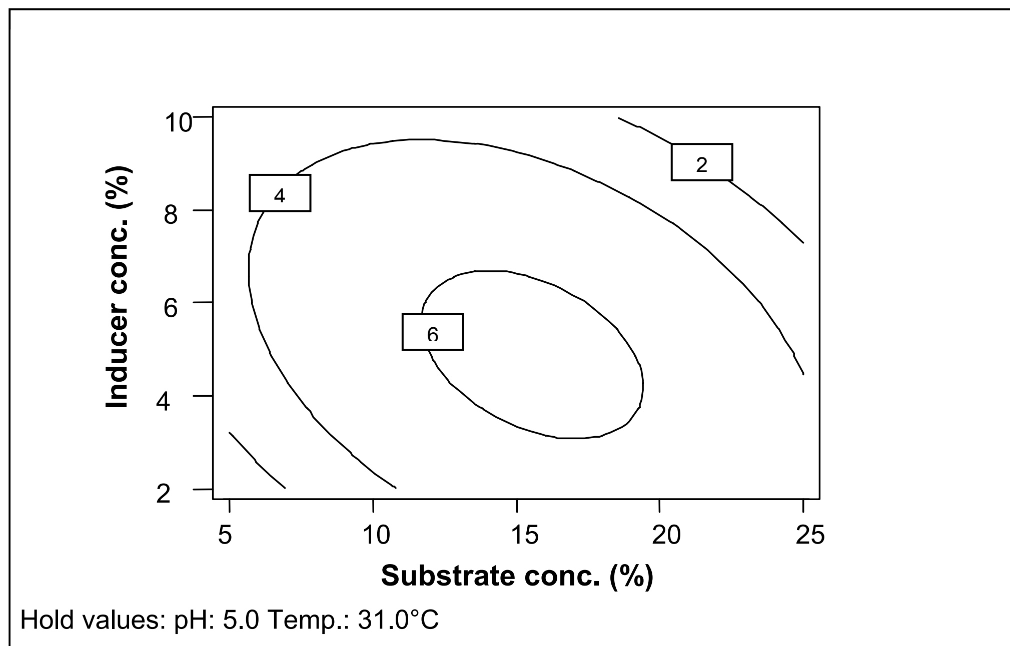

Figure 1. Isoresponse contour plot of substrate concentration versus inducer concentration on pectinase production.

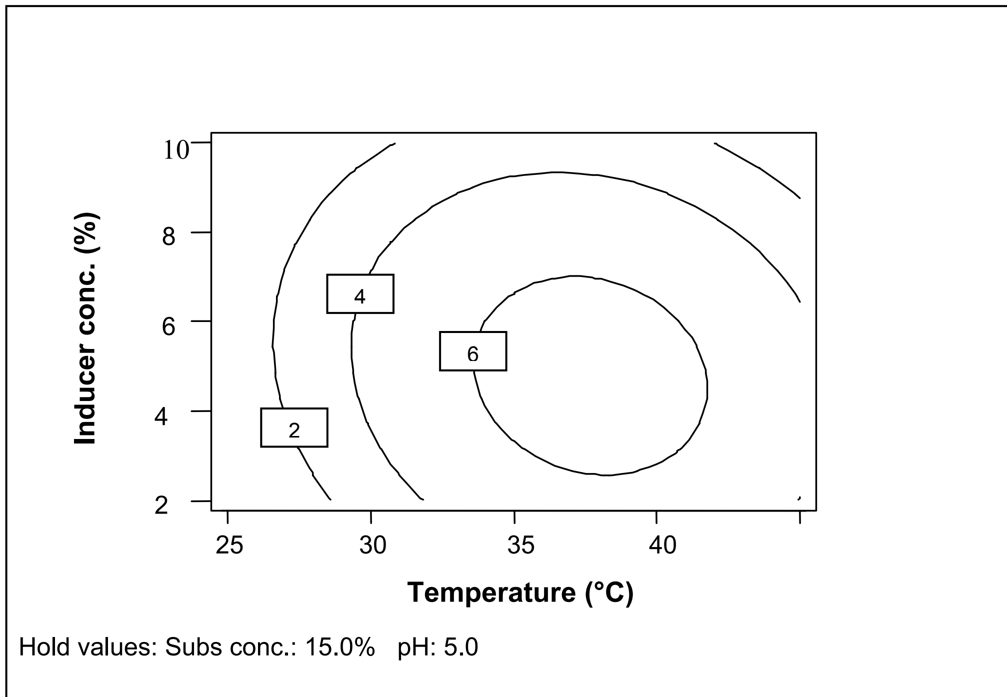

Figure 2. Isoresponse contour plot of fermentation temperature versus inducer concentration on pectinase production.

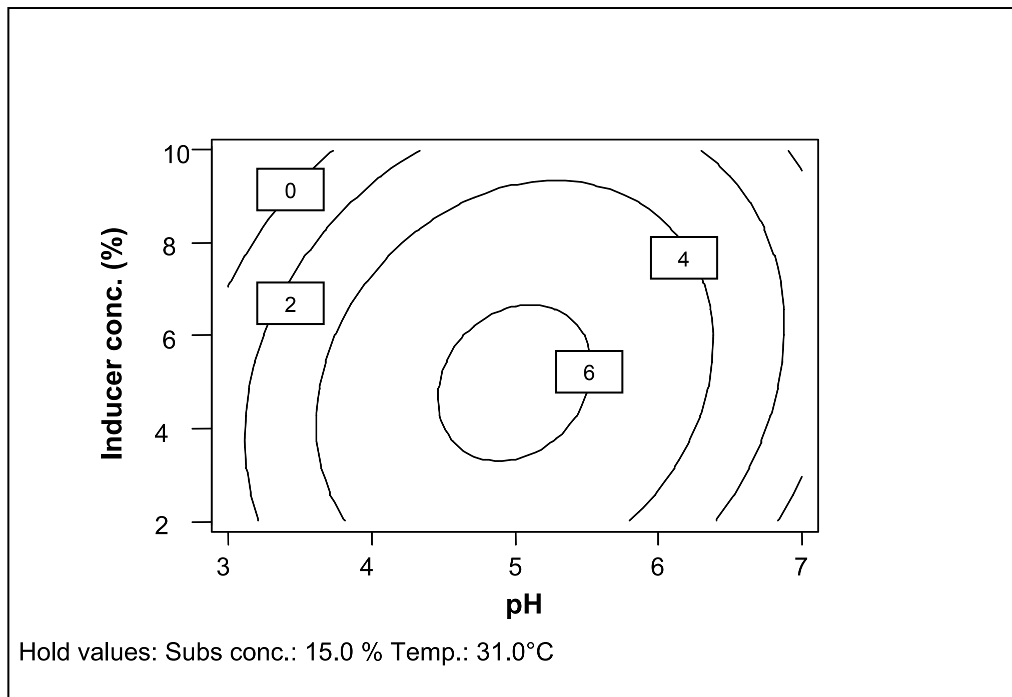

Figure 3. Isoresponse contour plot of initial pH versus inducer concentration on pectinase production.

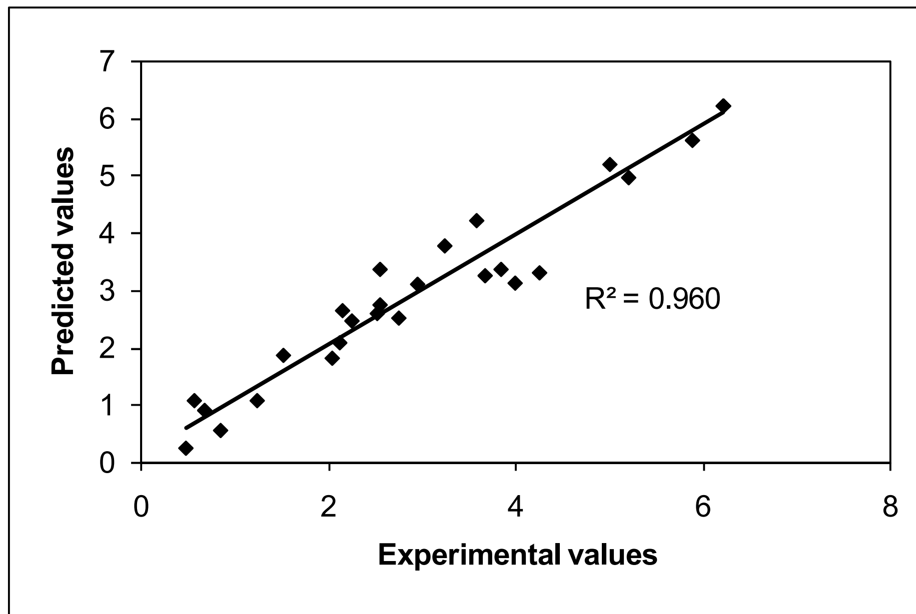

Figure 4. Parity plot showing the distribution of experimental versus predicted values by the mathematical model of the Y (pectinase yield, g/kg of substrate) values.

These results show that substrate concentration, pH; fermentation temperature and inducer concentration significantly affect pectinase enzyme production. From the contour plots, the optimal values of the independent variables could be observed and the interaction between each independent variable pair can de described. The optimal levels in coded values for pH lie between 0 and + 1, substrate concentration between 0 and +1, inducer concentration between 0 and -1 and temperature between 0 and +1. From equations derived by differentiating equation (2), the optimum values for the independent variables investigated were substrate concentration 18.45% of rice bran, initial pH of fermentation media 5.43, fermentation temperature 30.98°C and inducer concentration 5.03% (w/w) with the corresponding pectinase yield 6.56 g/kg of substrate. To confirm the results, solid state fermentation was carried out by *A. awamori* under these optimum conditions and a variability of 6.56±1.23 % (No. of runs = 3) was obtained. The good correlation between the experimental and predicted results verified the goodness of fit of the model (R^2 = 0. 960) shown in Figure 4.

Conclusions

The conventional method (i.e., change-one-factor-at-a-time) traditionally used for optimization of multifactor experimental design had limitations because (i) it generates large quantities of data which are often difficult to interpret (ii) it is time consuming and expensive (iii) ignores the effect of interactions among factors which have a great bearing on the response. To overcome these problems, a central composite design (CCD) and RSM were applied to determine the optimal levels of process variables on pectinase enzyme production. Only 31 experiments were necessary and the obtained model was adequate ($P < 0.001$). By

solving the regression equation, the optimum process conditions were determined; substrate concentration 18.5% (w/w) of rice bran, initial pH of fermentation media 5.4, fermentation temperature 31°C and inducer concentration 5% (w/w). A maximum pectinase yield of 6.56g/kg of substrate was obtained at the optimized process conditions. The research results indicated that RSM not only helps us locate the optimum conditions of the process variables in order to enhance the maximum pectinase enzyme production, but also proves to be well-suited to evaluating the main and interaction effects of the process variables on pectinase production from waste agricultural residues.

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