International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.6, No.7, pp 3618-3627, Sept-Oct 2014

Response surface estimation and canonical quantification for the pectin degrading Fe_3O_4-SiO_2-nanobiocatalyst fabrication

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Abstract: The one-factor-at a-time (OFAT) approach followed by response surface methodology (RSM) and canonical analysis were employed to analyze and optimize the immobilization parameters (glutaraldehyde concentration, pH, temperature and pectinase loading) for the effective fabrication of pectin degrading Fe_3O_4-SiO_2 nanobiocatalyst. Based on the results obtained from the OFAT experiments; pH, temperature and pectinase loading along with its levels were optimized using central composite design (CCD) matrix under RSM. The optimum conditions for the maximum activity 54.39 IU/mg obtained after analyzing their statistical significance for the Fe_3O_4-SiO_2 nanobiocatalyst which was fabricated at pH 4.0 with the 250 µg pectinase loading at 4˚C. The fine tuning of the results obtained from the CCD was facilitated by the canonical analysis and the optimum conditions were pH, 4.03; pectinase loading, 263.125 µg; temperature, 4.08˚C.
Keywords: Canonical analysis, Nanobiocatalyst, OFAT, Optimization, RSM.

1. Introduction

The process optimization is the ultimate goal in many studies; the OFAT approach is generally used to find the most significant factors and to obtain the optimal levels of all the factors involved. The optimization by the OFAT method is tedious, extremely time-consuming, and expensive if a large number of variables are involved [1]. The statistical analysis methods are the most suitable approach for screening and optimization of the variables.

RSM is an efficient mathematical and statistical approach which has been successfully employed in process optimization of purification processes, food processing technologies, and it has been used extensively in optimization of fermentation process [2-3]. CCD under the RSM is usually employed for process optimization and firstly, it requires an experimental design and the fitting experimental data into an empirical model to determine the optimal process parameters. CCD has been the most accepted experimental design for second-order models and hence considered as a better alternative to the full factorial three-level design as its performance is comparable at a lower cost [2]. RSM is usually achieved by simultaneous testing of numerous parameters in limited number of experiments. In statistics, the functional relationship between the dependent and the independent variable can be determined by using regression analysis which also explains the significance of the variables [4].

The canonical correlation analysis is a multivariate statistical model used for finding the optimum values of independent variables by defining a stationary point and simultaneously predicts the multiple

dependent variables from multiple independent variables [5]. According to our knowledge there is no review to emphasize the use of OFAT, RSM and canonical analysis in the optimization of process parameters to obtain a maximum activity of the pectin degrading Fe_3O_4-SiO_2 nanobiocatalyst.

Thus, the main objective of our study is to disclose the optimization strategy of our previous work [6] for the fabrication of pectin degrading Fe_3O_4-SiO_2 nanobiocatalyst. The OFAT method is to be approached in order to screen the factors for the fabrication of Fe_3O_4-SiO_2 nanobiocatalyst. The interaction effects between the studied parameters and their combination that yields the maximum activity was determined using RSM and canonical analysis.

2. Experimental Methods

2.1. Chemicals and methods used for the fabrication of Fe_3O_4-SiO_2-pectinase nanobiocatalyst

All the chemicals used for the fabrication were of analytical grade, highest purity and the methods were detailed in our previous work [6]. Briefly, the co-precipitation method was employed for the synthesis of MNPs, silica coating was done using TEOS and the amino functional group was imparted through APTES and the carboxyl functional group activation was carried out by glutaraldehyde. The activity measurement was carried out by DNS method using D-(+)-galacturonic acid monohydrate as a standard. The amount of pectinase bound on the surface of activated ASMNPs was confirmed through protein estimation by Lowry's method using bovine serum albumin (BSA) as a standard [7]. Analyzing the activity of Fe_3O_4-SiO_2-pectinase nanobiocatalyst was carried out through estimating their activity under wide range pH and temperature, kinetic parameters, reusability, storage ability studies. The characterization of synthesized, surface modified MNPs and fabricated Fe_3O_4-SiO_2-pectinase nanobiocatalyst were followed through, FT-IR for the presence of functional group and binding confirmation; size distribution analysis through TEM image for its size and morphology; XRD pattern for its phase change and stractural analysis.

2.2. Overview on fabrication of Fe_3O_4-SiO_2-pectinase nanobiocatalyst

Briefly, the hydroxyl groups present on the synthesized MNPs attacks and displaces the ethoxy group ($–OC_2H_5$) on the TEOS and forms a covalent (–Si–O–) bond forming SMNPs. The hydroxyl group on the formed silica shell attacks and displaces the ethoxy group ($–OC_2H_5$) on the silanizing agent APTES and forms a covalent (–Si–O–) bond. This silanization covers the surface of the SMNPs with the functional amino ($–NH_2$) group thus forming functionalized SMNPs (ASMNPs). The glutaraldehyde and ASMNPs undergoes dehydration ie., reaction between the aldehyde group (–CHO) in the cross-linking agent glutaraldehyde and the exposed terminal amino group ($–NH_2$) of the ASMNPs to form activated ASMNPs. The aldehyde group at the other end of glutaraldehyde forms covalent bond with amino group of pectinase, thereby, immobilizing the enzyme onto the activated ASMNPs.

2.3. Experimental design of OFAT

The factors considered, analyzed and optimized for the fabrication of Fe_3O_4-SiO_2 nanobiocatalyst were glutaraldehyde concentration (for the activation of ASMNPs), pH, temperature and pectinase loading for immobilizing pectinase onto the ASMNPs. OFAT was used to estimate the possible optimum points or levels of the factors. All the chemicals used in this study were of analytical grade and of highest purity.

2.3.1. Activation of silica coated amino functionalized magnetic nanoparticles (ASMNPs)

The activation of ASMNPs was achieved by suspending about 50 mg of ASMNPs in 30 mL of glutaraldehyde solution of varying concentration (0, 2, 4, 6, 8, 10 and 12% (v/v)) under stirring at 200 rpm for 4 h in the presence of nitrogen gas. The carboxylic group activated ASMNPs were washed with deionized water and were magnetically separated and stored in toluene.

2.3.2. Pectinase immobilization over activated ASMNPs

500 µL of varying concentration of pectinase solution (50, 100, 150, 200, 250, 300 and 350 µg) using acetate buffer under various pH (3, 4, 5, 6, 7 and 8) was added to the activated ASMNPs (5.0 mg) and sonicated

for 5 min. The sonicated mixture was stored at different temperatures (0, 4, 8, 12 and 16˚C) for 1 h and again sonicated for complete dispersion. This cycle was continued for two times and finally the content was brought to room temperature. The Fe_3O_4-SiO_2 nanobiocatalyst was then decanted using permanent magnet and washed twice in water. The supernatants collected in each wash were assayed for protein analysis.

2.4. Pectin degrading Fe_3O_4-SiO_2 nanobiocatalyst activity assay

The activity of pectin degrading Fe_3O_4-SiO_2 nanobiocatalyst was determined by measuring the reducing sugar (galacturonic acid) produced as a result of the reaction between the pectinase and the pectin. Fe_3O_4-SiO_2 nanobiocatalyst suspended in 500 µL of acetate buffer was added to 1.0 mL of pectin solution (0.1 M acetate buffer, pH 4.0) containing 2.0 mg of pectin and incubated for 1h at 50°C under shaking condition. The concentration of reducing sugar in the supernatant was measured using DNS method by employing D-(+)-galacturonic acid monohydrate as the standard [8]. One unit of pectinase activity (IU/mg) is defined as the amount of galacturonic acid produced (µmol) per mg of pectinase per min at pH 4.0 and 50°C. To promote accuracy, all the experiments were done in triplicates and the mean were calculated.

2.5. Design of experiments

The independent variables such as pH, temperature and the pectinase loading were optimized for their maximal Fe_3O_4-SiO_2 nanobiocatalyst activity. The CCD under RSM was employed in order to illustrate the nature of the response surface in the experimental region and elucidate the optimal conditions of the most significant independent variables. The regression model and the experimental design were developed by the statistical software package Design Expert 8.0.6 (Stat Ease Inc., Minneapolis, USA). The independent variables and its levels were chosen based on the importance of fabrication and that could be obtained from the results of OFAT experiments. The chosen value for the extreme level of the design was $\alpha = 1.00$, this value was selected in order to obtain an orthogonal design. The coded (dimensionless) variables for the Fe_3O_4-SiO_2 nanobiocatalyst were defined as [9];

$$x_1 = \frac{pH-5}{1} \quad (1)$$

$$x_2 = \frac{PL-250}{50} \quad (2)$$

$$x_3 = \frac{T-4}{4} \quad (3)$$

where x_1, x_2, and x_3 denotes the coded variables related to pH, pectinase loading and the temperature, respectively. The coded values of pH, pectinase loading and temperature are obtained by using the equation [10];

$$t_i^* = \frac{t_i-(\max t_i+\min t_i)/2}{(\max t_i-\min t_i)/2} \quad (4)$$

where t_i denotes the uncoded observation and t_i* denotes the coded observation [5]. The coded observations are usually employed to facilitate the calculations in RSM applications. The factors and their levels for the Fe_3O_4-SiO_2 nanobiocatalyst based on the levels obtained from OFAT loading were pectinase loading, (200-300 µg); pH, (4.0-6.0) and temperature (0-8˚C).

2.6. Statistical analysis

For the quantification of the interactions of independent variables on the activity of the Fe_3O_4-SiO_2 nanobiocatalyst, an empirical equation was fitted to the experimental data. A second-order regression analysis of the data was carried out to get empirical model that defines the response in terms of the independent variables. The experimental runs were executed (Table 1) based on the CCD matrix and their corresponding observations were fitted to a second order polynomial model explaining the relation between the response and the variables which is given below,

$$\hat{y} = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (5)$$

where $\hat{y}$ is the dependent variable; A, B and C are the independent variable; β_0 is the regression coefficient at center point; β_1, β_2 and β_3 are the linear coefficients; β_{11}, β_{22} and β_{33} are the quadratic coefficients and β_{12}, β_{13} and β_{23} are the second order interaction coefficient.

Table 1 Central composite design and experimental results

Run	pH	Pectinase loading (µg)	Temperature (˚C)	Fe_3O_4-SiO_2 nanobiocatalyst activity (IU/mg)
1	4	300	8	56.95
2	5	250	4	54.89
3	5	250	0	53.56
4	5	250	8	52.87
5	5	250	4	54.39
6	6	200	8	48.2
7	4	200	4	56.56
8	5	250	4	54.67
9	5	250	4	54.5
10	6	300	0	49.98
11	4	300	8	57.45
12	6	250	4	51.95
13	4	200	8	55.43
14	5	250	4	54.78
15	4	250	4	59.3
16	6	300	8	48.67
17	5	300	4	54.34
18	5	200	4	53.08
19	5	250	4	54.74
20	6	200	0	49.02

The significance and adequacy was evaluated by analyzing the values of regression coefficients using analysis of variance (ANOVA), which includes Fisher's F-test and its associated probability p (F). The significance of the regression model and their variables can be evaluated from the Fisher's F-test having a probability value, p>F=0.0001 and p>0.005 respectively.

2.7. Canonical analysis

Canonical analysis is performed to determine the location and the nature of the stationary point from the second order model [9-10]. An empirical equation was fitted to the experimental data in order to compute the effects of independent variables and their relations. This second order equation can be represented by a matricial notation as;

$$\hat{y} = b_0 + x'b + x'Bx \quad (6)$$

where

$$x = \begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix} \quad (7)$$

$$b = \begin{bmatrix} b_1 \\ b_2 \\ b_3 \end{bmatrix} \tag{8}$$

$$B = \begin{bmatrix} b_4 & b_7 & b_8/2 \\ b_7/2 & b_5 & b_9/2 \\ b_8/2 & b_9/2 & b_6 \end{bmatrix} \tag{9}$$

where x denotes the independent variables, b is the factors vector of the first order terms, B is the factors matrix of the quadratic terms.

The maximum response expected, if it exists it will be a set of conditions ($x_1, x_2, \ldots, x_k$) such that the derivatives, $\delta\bar{y}/\delta x_1$, $\delta\bar{y}/\delta x_2$, $\delta\bar{y}/\delta x_3$, ……, $\delta\bar{y}/\delta x_k$ are simultaneously zero. This value is the stationary point of the fitted surface. So, the derivative of $\hat{y}$ with respect to the vector **x**, equated to zero, this value, say $x'_0 = (x_{1,0}, x_{2,0}, \ldots, x_{k,0})$ is the stationary point of the fitted surface. So, the derivative of $\bar{y}$ with respect to the vector **x**, equated to zero, is

$$\frac{\partial \bar{y}}{\partial x} = \frac{\partial}{\partial x}[b_0 + x'b + Bx] = b + 2Bx = 0 \tag{10}$$

The stationary point of $\hat{y}$ in (6) is represented as,

$$x_s = -B^{-1}\left(\frac{b}{2}\right) \tag{11}$$

Here the stationary point is a point at which the fitted surface attains a maximum or minimum, or a saddle point. The procedure to determine the nature of stationary point is to find the Eigen values of matrix B. when the signs of all Eigen values are positive then x_s is a minimum point, if they are negative then x_s is a maximum point, when these signs are varied then x_s is a saddle point [9-10].

3. Results and discussion

3.1. Effect of factors and its levels by OFAT

The optimum level of glutaraldehyde concentration was determined by varying the percentage from 0-12% and the maximum activity of Fe_3O_4-SiO_2 nanobiocatalyst (36.33 IU/mg) was observed with the addition of 10% glutaraldehyde and thereafter the saturation in the activity was obtained. The minimum activity observed in the maximum possible control level (without the addition of glutaraldehyde) is 6.7 IU/mg as shown in Fig.1 (a). Hence, the maximum percentage improved activity of the Fe_3O_4-SiO_2 nanobiocatalyst by the use of 10% glutaraldehyde is 81.56%.

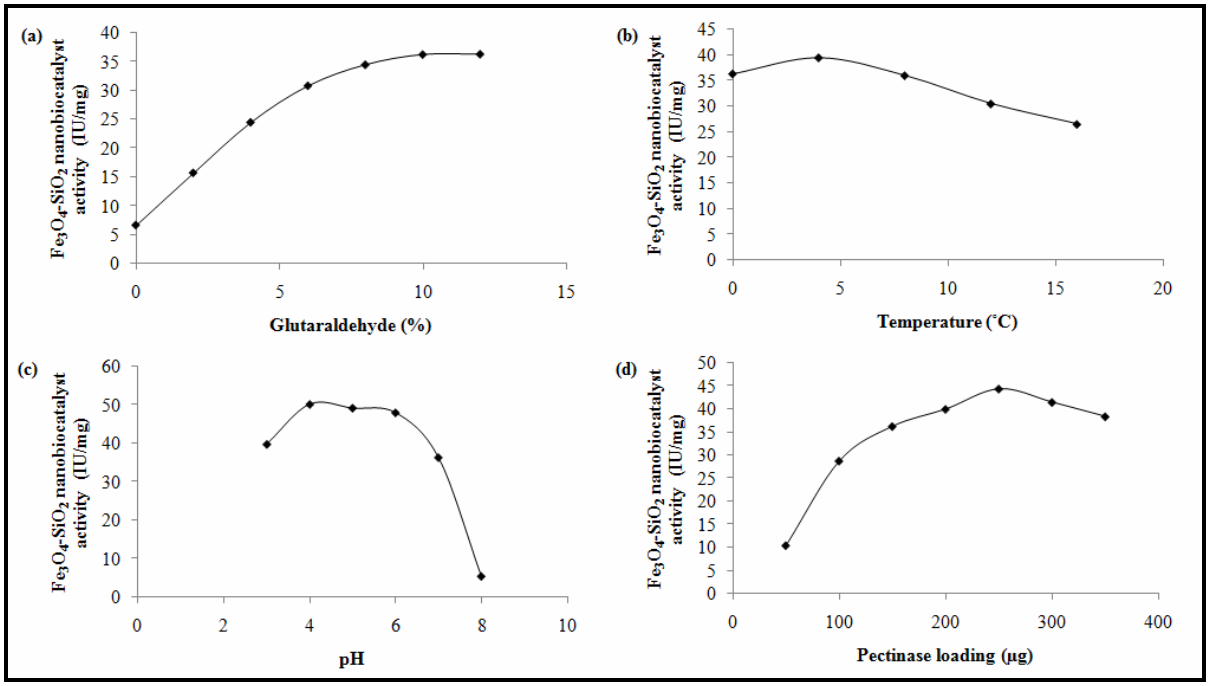

Fig.1.(a) The effect of glutaraldehyde concentration on fabrication of Fe_3O_4-SiO_2 nanobiocatalyst activity (IU/mg) (b) The effect of immobilization temperature on fabrication of Fe_3O_4-SiO_2 nanobiocatalyst activity (IU/mg) (c) the effect of pH on fabrication of Fe_3O_4-SiO_2 nanobiocatalyst activity (IU/mg) (d) the effect of pectinase loading on fabrication of Fe_3O_4-SiO_2 nanobiocatalyst activity (IU/mg). (The binding of pectinase onto ASMNPs (5.0 mg) was carried out at glutaraldehyde concentration- 12%, pH 7.0, and temperature 0˚C, pectinase loading 150 µg through OFAT approach)

The maximum activity (50.14 IU/mg) of Fe_3O_4-SiO_2 nanobiocatalyst was observed with the pH 4.0 and also the considerable relative activity was obtained at pH 5.0 and 6.0, thereof the decrease in activity was observed (Fig.1 (b)). Hence, the pH range 4.0-6.0 has been studied for further investigation. A very minimal increase in the activity of Fe_3O_4-SiO_2 nanobiocatalyst was observed at the temperature range 0-4˚C and the activity was almost maximum (39.46 IU/mg) at 4˚C. Thereof, the minimal decrease in the activity was observed up to 8˚C and then it considerably decreased (Fig.1 (c)). This implies that the optimal temperature can be defined by further investigation between 0-8˚C. An increase in the activity of Fe_3O_4-SiO_2 nanobiocatalyst was observed in the range of 50-250 µg pectinase loading and it was maximum (44.46 IU/mg) at around 250 µg. Thereafter, a linear decrease in the activity was observed (Fig.1 (d)) explaining that the pectinase loadings have to be studied further around 250 µg.

Conclusively, it was noted that the fabrication parameters except glutaraldehyde concentration needs to be further tailored based on the results obtained from OFAT experiments in order to quantify the optimal levels.

3.2. Experimental values and the fitted data

RSM employing CCD matrix was used to determine the optimal parameters for achieving the maximal activity of the Fe_3O_4-SiO_2 nanobiocatalyst. Based on the results obtained from OFAT experiments, the fabrication parameters (pH, temperature and pectinase loading) were chosen as the independent parameters for evaluation of optimized levels using CCD matrix. The Fe_3O_4-SiO_2 nanobiocatalyst activity was measured as the dependent parameter as the response and the response surface equation for the Fe_3O_4-SiO_2 nanobiocatalyst generated by the Design Expert software is given by,

$$\hat{y} = 54.7262 + 1.587\,A + 0.51\,B - 0.445\,C - 0.1225\,AB - 0.0625\,AC + 0.0175\,BC + 0.8018\,A^2 - 1.1132\,B^2 - 1.6082\,C^2 \quad (12)$$

where the response variable ($\hat{y}$) is the activity of the Fe_3O_4-SiO_2 nanobiocatalyst which is the function of pH (A), pectinase loading (B), and temperature (C).

In order to test the significance and adequacy of the model, ANOVA was performed. ANOVA shows the fitted second order response surface model as shown in Table 2. The coefficient of determination (R^2) was 0.997 with an adjusted and predicted R^2 of 0.9943 and 0.9706 respectively, which measures the fit of the model. Only 0.3% of the total variation were not explained by the Fe_3O_4-SiO_2 nanobiocatalyst activity. The value of adequate precision of 25.799 for the pectinase bound to the Fe_3O_4-SiO_2 nanobiocatalyst indicates an adequate signal (greater than 4). From the P-value presented in Table 2, it can be concluded that that A, B, C, A^2, B^2 and C^2 are significant model terms.

Table 2 ANOVA for response surface quadratic model

Source	Sum of Squares	Mean Square	F Value	p value> F
Model	168.1393	18.68215	368.0987	< 0.0001
A-pH	143.4137	143.4137	2825.713	< 0.0001
B-Pectinase loading	2.601	2.601	51.24811	< 0.0001
C-Temperature	1.98025	1.98025	39.01733	< 0.0001
AB	0.12005	0.12005	2.365373	0.1551
AC	0.03125	0.03125	0.615726	0.4508
BC	0.00245	0.00245	0.048273	0.8305
A^2	1.768009	1.768009	34.8355	0.0002
B^2	3.407728	3.407728	67.14326	< 0.0001
C^2	7.112184	7.112184	140.133	< 0.0001

The response surface (3D) and the contour (2D) plots were used to identify the type of interaction between these three variables [11-12]. The model has two factors one factor is held as the constant. Fig.2 (a), presents the maximum point of the response (pectinase activity) with respect to the pH and the pectinase loading by keeping the third variable as a constant. The predicted Fe_3O_4-SiO_2 nanobiocatalyst activity decreases gradually with the increase in the pH level. Hence, the Fe_3O_4-SiO_2 nanobiocatalyst activity was about 59.3 IU/mg when the pH, pectinase loading was about 4.0 and 180 µg respectively.

The fitted response surface plot and the corresponding contour plot for Fe_3O_4-SiO_2 nanobiocatalyst activity as a function of pH and temprature is shown in Fig.2 (b). The results concluded that the Fe_3O_4-SiO_2 nanobiocatalyst activity was significantly influenced by pH and the temperature. The activity was found decreasing beyond the pH 4.0 and temperature of 4ºC respectively.

The response plots show the variation in the Fe_3O_4-SiO_2 nanobiocatalyst activity, as a function of pectinase loading and the temperature which exhibited improved activity. Maximum nanobiocatalyst activity was recorded near the central levels of the pectinase loading and temperature, while further increase in the levels resulted in a gradual decrease in pectinase activity. Hence, the maximum fabricated Fe_3O_4-SiO_2 nanobiocatalyst activity of 54.39 IU/mg was predicted at the pectinase loading of about 250 µg and temperature was about 4˚C at pH 4.0.

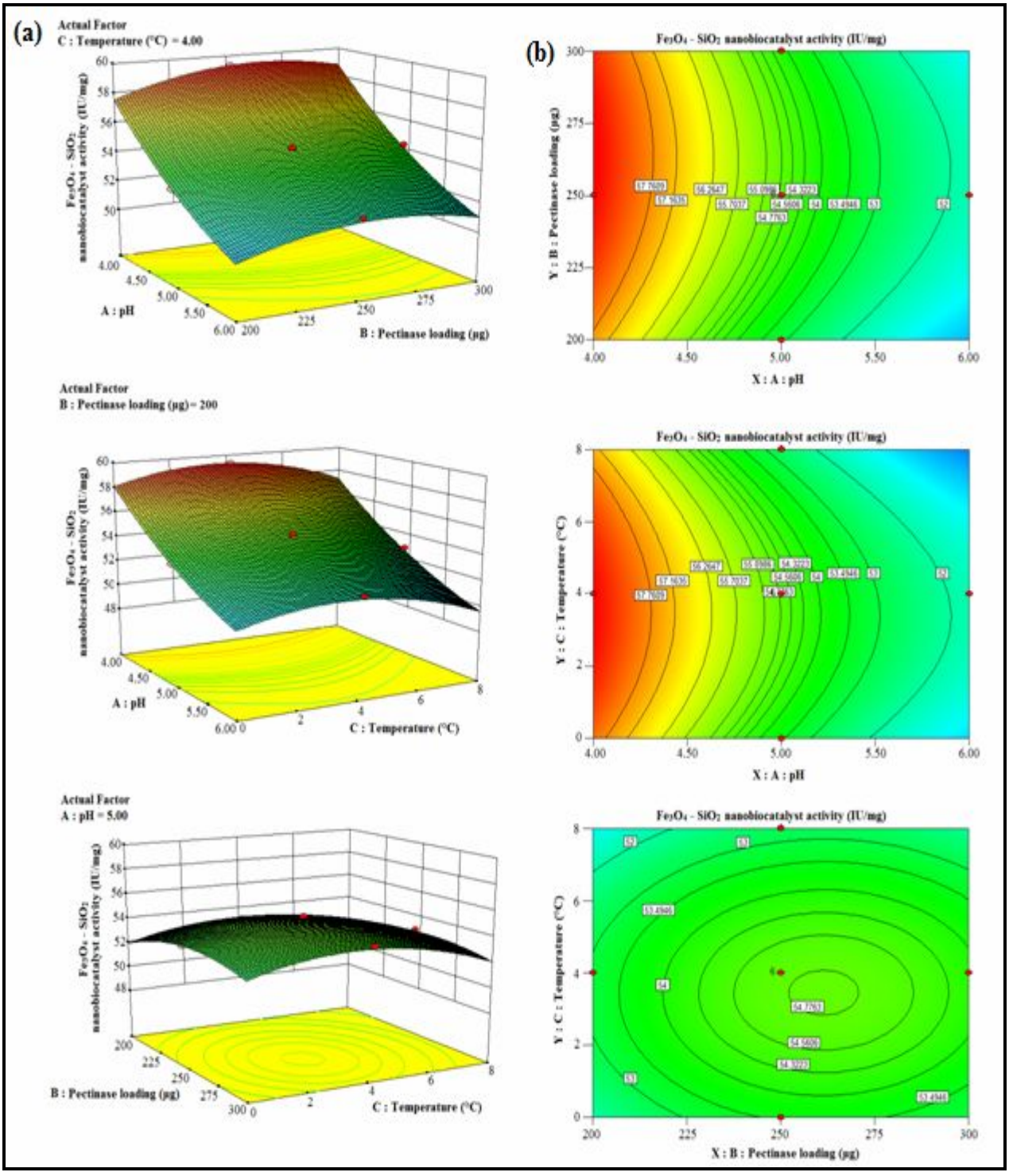

Fig.2. (a) Response surface (b) contour plots for Fe_3O_4-SiO_2 nanobiocatalyst activity (IU/mg) versus pH, temperature (°C), and pectinase loading (µg)

3.4. Canonical analysis

3.4.1. Fe_3O_4-SiO_2 nanobiocatalyst activity

Canonical analysis for optimization is the mathematical approach to determine the stationary point of the response surface and to determine whether it describes the maximum, minimum or the saddle point. The canonical form of the fitted model is given by,

$$\hat{y} = 54.7262 + x'b + x'Bx \tag{13}$$

The fitted equation (R^2 =0.9758) in matricial notation is denoted as,

$$x_0 = \begin{bmatrix} x_1 \\ x_2 \\ x_3 \end{bmatrix} \quad b = \begin{bmatrix} 1.587 \\ 0.51 \\ -0.445 \end{bmatrix} \quad B = \begin{bmatrix} 0.8018 & -0.0613 & -0.0313 \\ -0.0613 & -1.1132 & 0.0088 \\ -0.0313 & -0.0088 & -1.6082 \end{bmatrix}$$

The point of maximum response was determined from the canonical analysis of the fitted model; hence, the stationary point is,

$$x_S = \begin{bmatrix} -0.967 \\ 0.2825 \\ 0.2 \end{bmatrix} \tag{14}$$

The characteristic roots (λ_i) of matrix B are all negative: λ_1= -0.804, λ_2= -1.609 and λ_3= -1.115. Therefore, x_0 is a maximum response. From stationary point, it can be seen that the optimum coded value obtained for pH was x_1= -0.967; this value corresponds to a pH of 4.03 which is within the limit of the un-coded variables. Similarly, the optimum coded value for pectinase loading (x_2= 0.2825) and temperature (x_3= 0.2) corresponds to 263.125 µg and 4.08˚C respectively, confirming the values indicated by the RSM. As a result, the optimum conditions determined by this study are; pH 4.03, pectinase loading 263.125 µg, and temperature 4.08˚C.

4. Conclusions

In this study, the optimization strategy for the successful fabrication of pectin degrading Fe_3O_4-SiO_2 nanobiocatalyst using OFAT method followed by RSM and quantification by canonical analysis. Among the tested factors by OFAT method, glutaraldehyde concentration was fixed as 10% and other factors like pH, temperature and pectinase loading were further optimized using RSM and exhibited a maximized activity of 54.39 IU/mg when the conditions were pH, 4.0; temperature, 4˚C and pectinase loading, 250 µg. Further amelioration in the activity was approached by fine tuning the optimized factors through canonical analysis which further proved the efficient strategy for fabrication of nanobiocatalyst.

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