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Immobilization of fungal cellulase on chitosan beads and its optimization implementing response surface methodology

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The present study deals with the immobilization of cellulase produced from our isolated fungus *Aspergillus niger* ISSFR-019 on different matrices of which chitosan beads has been found to be the most suitable one. The optimization of the amount of cellulase, amount of beads, temperature and amount of glutaraldehyde used as a cross-linking agent for the procurement of maximum immobilization efficiency has been done on the basis of statistical approach BBD (Box-Behnken Design). Cellulase is found to be successfully immobilized on the chitosan bead surface, with 94.2% efficiency and immobilization yield is found to be 54.1% after statistical optimization. Significant enzymatic activity is recorded after 12 cycles of reuse of the beads. The kinetic study revealed that the Michael's Menten constant (K_m) is found to be 1.3 mM and 2 mM for free enzyme and immobilized enzyme respectively, the higher Km value for immobilized enzyme indicated conformational change in the enzyme that altered the accessibility of substrate towards the active site of the enzyme. The V_{max} is found to be 2.7 and 4.4 U/mg-1 respectively for the immobilized and free enzyme. Fourier Transform Infrared Spectroscopy studies revealed the change in absorbance of functional groups when the enzyme is bound to chitosan beads.

Keywords: BBD, *Aspergillusniger*, Cellulase, Immobilization, FTIR.

Cellulase is one of the most industrially significant enzyme[1]. Unfortunately difficulty in separation and recovery process of free cellulase limits the reusability of the enzyme[2]. The implementation of immobilized carriers and the immobilization methods are two important aspects that significantly influence the properties of biocatalysts that have industrial application and in a way solves the problem of separation and recovery making the enzyme reusable[3]. The widely accepted methods of immobilization are physical adsorption, covalent binding, cross-linking and entrapment or encapsulation of enzyme in polymers of which the former is the earliest immobilization methods reported. Physical methods are characterized by weaker interactions such as hydrogen bonds, hydrophobic interactions, van der Waals forces, affinity binding and ionic binding of the enzyme with the chosen matrix[4]. In the chemical method, formation of covalent bonds achieved through ether, thio-ether, amide or carbamate bonds between the enzyme and support material chosen are usually involved. The method of covalent bonding is advantageous due to its high reusability which results due to the strong bonding between the enzyme and the matrix used for immobilization[3]. This paper deals with (i) selection of appropriate matrix for immobilization of cellulase obtained from isolated *Aspergillus niger* ISSFR-019 (ii) Statistical optimization of parameters involved in immobilization by Box-Behnken Design to evaluate the combined effect of theinfluentialparameters on immobilization yield and immobilization efficiency (iii) Determination of the kinetic parameters of the immobilized and free enzyme (iv) FTIR analysis to figure out the change in the absorbance of the functional groups of chosen matrix before and after immobilization of the enzyme.

Experimental Section

Screening of carriers for immobilization

Different supports were screened for immobilization of cellulase viz. coconut fiber, alginate beads, ceramic and chitosan beads. The characteristics that are preferred while selecting a carrier for immobilization includes chemical stability, physical strength, and cost effectiveness[5].

(i) *Preparation of immobilized enzyme on coconut fiber*

The coconut fibers of approximately 1cm length were separated and boiled in water containing 0.01% SDS for 1 h followed by drying at room

temperature. 1 mL of cellulase (0.5%) and 0.1 g of coconut fiber was placed in shaking water bath at 30°C for 2 h[6]. After adsorption, the enzyme solution was decanted and stored for further assay. The unbound enzyme was washed away by means of distilled water until no cellulase activity was detected. Enzyme assay was done by incubating the reaction concoction containing the coconut fiber bound enzyme, CMC as substrate at 50°C for 1 h. The reaction was terminated by DNS acid followed by measuring the absorbance at 540 nm in a spectrophotometer (HITACHI-U2000).

(ii) *Preparation of immobilized enzyme on ceramic*

Ceramics was obtained from local suppliers. A buffered enzyme solution containing 2 mL cellulase (0.5%) in 0.1M citric acid was stirred with 1 g of ceramic support for 1.5 h. The concoction was then filtered and washed with 3.5 mL distilled water and 3.5 mL acetone. It was later dried at 4°C overnight and assay of CMCase was done to calculate the yield of the enzyme[7]. Immobilization yield and specific activity (activity/ protein) was calculated for the matrix.

(iii) *Preparation of pre-treated chitosan beads*

The chitosan beads were prepared based on a reported method of Cetinus and Oztop and Hung *et al.*[8,9] with minor modification. 2 g of chitosan flakes were dissolved in 100 mL of acetic acid solution (5%, *v*/*v*) by needles (0.5 mm and 0.7 mm diameter). The resulting solution was added into 200 mL of 2M NaOH solution containing 40 mL of ethanol at room temperature eventually leading to the formation of chitosan gelled spheres. After hardening, the pre-treated chitosan beads were separated and washed with distilled water until the filtrate became neutral (*p*H=7). The diameter of the pre-treated beads was approximately 1.1 to 1.5 mm. Then the pre-treated chitosan beads (0.1 g) were added into 10 mL of glutaraldehyde solution (3%) and the mixtures were stirred at 28°C and 150 rpm for 2 h. The crosslinking chitosan beads were separated on a filter and washed with 100 mM phosphate-citric acid buffer (*p*H=5)[10]. 0.3 g pre-treated chitosan beads were added into 1 mL CMCase solution (0.1 mg/mL) and the solution was stirred at 28°C, for 5 h. The chitosan beads with adsorbed enzyme were separated and washed with 100 mM phosphate-citric acid buffer (*p*H=5.0) to remove unbound enzymes. Immobilization yield and specific activity (activity/ protein) was calculated for the matrix.

(iv) *Preparation of alginate beads*

Cellulase and sodium alginate (2%) was mixed followed by addition of $CaCl_2$ (0.1M) which eventually lead to formation of calcium alginate bead[11]. The beads were washed with citrate buffer *p*H=5 and was kept for CMCase assay to determine the immobilization yield.

Analytical methods

The immobilization yield (%) is used to describe the percentage of total cellulase activity from the free cellulase solution that is immobilized onto the chitosan beads. The immobilized activity was calculated by determining the total activity from the starting enzyme solution subtracting the total residual enzyme activity that remains in the enzyme solution after immobilization[12.]

Yield % = 100 (Immobilized activity/Starting activity)

The immobilization efficiency describes the percentage of bound enzyme activity that is observed in the immobilizate[12].

Efficiency % = 100 (Observed activity/Immobilized activity)

The process of CMCase assay was carried out following the method of Ghose[13].

Statistical optimization of immobilization of CMCase on chitosan beads

RSM was implemented to optimize the immobilization process of cellulase (CMCase)[14]. Box-Behnken design (BBD) was used to analyze the effects of the four variables (percentage of glutaraldehyde, temperature, enzyme dosage and amount of chitosan bead) on the responses, immobilization yield (%) [IY] and immobilization efficiency (%) [IE] which were considered as the dependent variables. For each independent variable three levels, i.e. -1, 0, and +1 were considered (Table 1). The experiments were designed using Design Expert software version 7 (Statease, Inc.,

Table 1 — Coded levels of independent variables selected

Variable	Code	Unit	Range and Level		
			-1	0	+1
Amt of glutaraldehyde	A / X_1	%	1	2	3
Temperature	B / X_2	degree C	30	50	70
Amount of enzyme	C / X_3	%	0.3	0.5	0.7
Amount of bead	D / X_4	gram	0.2	0.4	0.6

Minneapolis, MN, USA). The designed levels of the parameters are shown in Table 1. A total of 29 experimental trials were carried out according to the list provided by the software. Effects of the variables on immobilization yield and efficiency were simulated based on the obtained numerical data and then the optimal immobilization condition was predicted. The RSM model for analysing and predicting is as follows,

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j \quad \ldots (1)$$

where Y is the response (immobilization yield and immobilization efficiency), X is thevariable, $ß_0$,$ß_i$, $ß_j$ and $ß_{ij}$ are respectively the coefficients of the constant, liner, quadratic and interaction terms of the regression model.

Kinetics of immobilized enzyme

The rate of enzyme catalyzed reaction follows the Michaelis-Menten equation[15]

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

Where, V is the velocity of the reaction, V_{max} is the maximum velocity and K_m is the Michaelis-Menten constant and [S] is the concentration of substrate that participates in the reaction with the enzyme. The kinetic parameters K_m and V_{max} of free and immobilized cellulase were determined accordingly. Here, CMC was used as the substrate over a varying range of concentration (1-6 Mm).

Reusability

For the assessment of reusability, the stored immobilized CMCase was reused 12 times and the residual activity was measured to calculate the immobilized activity[3].

FTIR analysis

The Fourier transform infrared spectroscopy has been used to identify the new functionalities on the enzyme bound chitosan beads and chitosan beads without enzyme. IR transmission spectra were obtained using an FTIR spectrophotometer (FTIR-8300, Shimadzu, Japan). The test is aimed to prove the presence of the new functional group or changes in vibration of the bonds after the enzyme is bound with the matix[3, 16].

Results and Discussion

Selection of matrix for immobilization of CMCase

Selection of immobilized carriers plays an imperative role in enzyme immobilization. Among the different immobilization supports that were screened for immobilizing cellulase (Fig. 1), chitosan beads cross-linked with glutaraldehyde showed maximum immobilization yield at the level of 43.4% followed by coconut fiber (31.3%), alginate (30.8%) and ceramic (29.5%). The effective support chitosan is also reported to be advantageous as it is biocompatible, cheap, it has good hydrophobicity and porosity besides that its structure ensures minimal steric hindrance during immobilization[17].

Statistical optimization of parameters involved in immobilization of enzymeon chitosan beads

To optimize and comprehend the relationship between the chosen independent variables (percentage of glutaraldehyde and enzyme, the amount of chitosan beads (g) and temperature degree C) the obtained experimental data or the responses were analysed by second-order polynomial equations obtained from the software of the RSM. The analysis of variance (Tables 2 and 3) of the polynomial quadratic model showed lower p-values and higher determination coefficients (R^2). The low p-value obtained indicated that the model accurately represented the relationship between dependent variables (Immobilization yield and efficiency) and the independent variables selected. The p-values obtained from the regression analysis showed that four interaction terms AB (amount of glutaraldehyde and temperature), AD (amount of glutaraldehyde and bead),

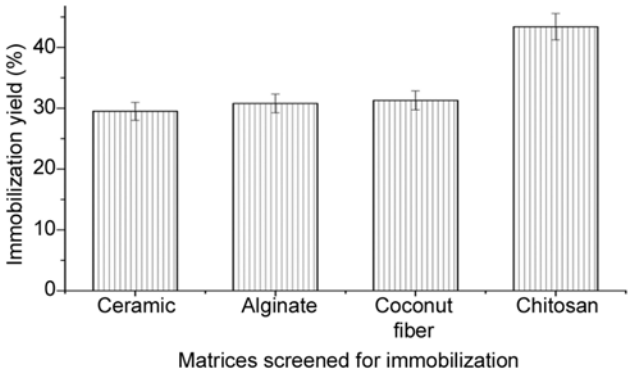

Fig. 1 — Selection of immobilization matrices for efficient cellulase immobilization

Table 2 — ANOVA for CMCase immobilization yield

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P –valueProb>F	
Model	862.75	14	61.63	168.22	<0.0001	Significant
A-Amt of glutaraldehyde	0.96	1	0.96	2.63	0.1274	
B-Temperature	7.84	1	7.84	21.40	0.0004	
C-Amt of Enzyme	0.47	1	0.47	1.28	0.2773	
D-Amt of bead	1 0.32	1	1 0.32	28.18	0.0001	
AB	3.24	1	3.24	8.84	0.0101	
AC	0.16	1	0.16	0.44	0.5194	
AD	5.29	1	5.29	14.44	0.0020	
BC	2.25	1	2.25	6.14	0.0226	
BD	2.40	1	2.40	6.56	0.0226	
CD	0.38	1	0.38	1.03	0.3268	
A^2	2.70	1	2.70	7.36	0.0168	
B^2	693.90	1	693.90	1894.12	<0.0001	
C^2	22.76	1	22.76	62.14	<0.0001	
D^2	0.048	1	0.048	0.13	0.7234	
Residual	5.13	14	0.37			
Lack of fit	4.33	10	0.43	2.16	0.2376	Not significant
Pure Error	0.80	4	0.20			
Cor total	867.88	28				

*P ≤ 0.0001 indicates highly significant values, P ≤ 0.05 indicates significant values, P> 0.05 indicates values that are not significant.

Table 3 — ANOVA table for CMCase immobilization efficiency

Source	Sum of squares	Degrees of freedom	Mean square	F-value	P –valueProb>F	
Model	203.76	14	14.55	291.78	<0.0001	Significant
A-Amt of glutaraldehyde	0.24	1	0.24	4.83	0.0453	
B-Temperature	5.74	1	5.74	115.09	<0.0001	
C-Amt of Enzyme	0.12	1	0.12	2.41	0.1432	
D-Amt of bead	3.333E-003	1	3.333E-003	0.067	0.7998	
AB	3.24	1	3.24	64.95	<0.0001	
AC	0.12	1	0.12	2.46	0.1394	
AD	1.93	1	1.93	39.29	<0.0001	
BC	2.25	1	2.25	45.11	<0.0001	
BD	1.56	1	1.56	31.32	<0.0001	
CD	0.063	1	0.063	1.25	0.2818	
A^2	0.095	1	0.095	7.36	0.1881	
B^2	175.15	1	175.15	3511.21	0.3553	
C^2	0.046	1	0.046	0.91	0.6460	
D^2	0.011	1	0.011	0.22	0.7234	
Residual	0.70	14	0.050			
Lack of fit	0.63	10	0.063	3.74	0.1078	Not significant
Pure Error	0.068	4	0.017			
Cor total	204.46	28				

*P ≤ 0.0001 indicates highly significant values, P ≤ 0.05 indicates significant values, P> 0.05 indicates values that are not significant.

BD (temperature and amount of bead) and BC (temperature and amount of enzyme) were significant and had a substantial effect on cellulase immobilization yield (%) and immobilization efficiency. The “Model F-value” of 168.22 and 291.78 for immobilization yield and immobilization efficiency implies that the model is significant and 0.01% chance of obtaining this value due to noise exists. Values of "Prob> F" less than 0.05 indicates the model terms are significant. A lower value for coefficient of variation (C.V) designates higher relability of the experiment, in this case the C.V was found to be 1.30 and 0.25 % respectively for our studied responses (IY and IE). In the case of immobilization yield (response 1) temperature (B) and the amount of bead (D) were the significant model terms on the other hand glutaraldehyde (A) and temperature (B) were the significant model terms for

response 2 i.e immobilization efficiency. The Adjusted R^2 value of the model was found to be 0.98 and 0.99 respectively for response 1 and 2 which is close to unity. The results of the RSM experiments were analyzed by Design Expert 7. The contour plots of only the significant terms are given in Figs. 2 and 3.

Final equation in terms of coded factors

$$Im\,mobilization\ yield\ (R_1) = +49.70 + 0.28A - 0.81B - 0.20C - 0.93D - 0.90AB + 0.20AC + 1.15AD - 0.75BC + 0.77BD - 0.31CD + 0.64A^2 - 10.34B^2 + 1.87C^2 + 0.086D^2 \quad \ldots (2)$$

$$Efficiency(R_2) = +93.08 - 0.14A - 0.69B + 0.10C - 0.017D + 0.90AB - 0.18AC + 0.70AD + 0.75BC + 0.63BD - 0.12CD - 0.12A^2 - 5.20B^2 - 0.084C^2 + 0.041D^2 \quad \ldots (3)$$

Authentication of the statistical model

Immobilization of cellulase on chitosan beads was performed according to the conditions suggested by the response surface model (Table 4). In reality the given conditions are not possible to maintain so the process was carried out with 1% glutaraldehyde, 0.7% CMCase, 0.2 g of bead at 50°C. It was predicted that the immobilization yield and efficiency obtained after carrying out the process stringently under the

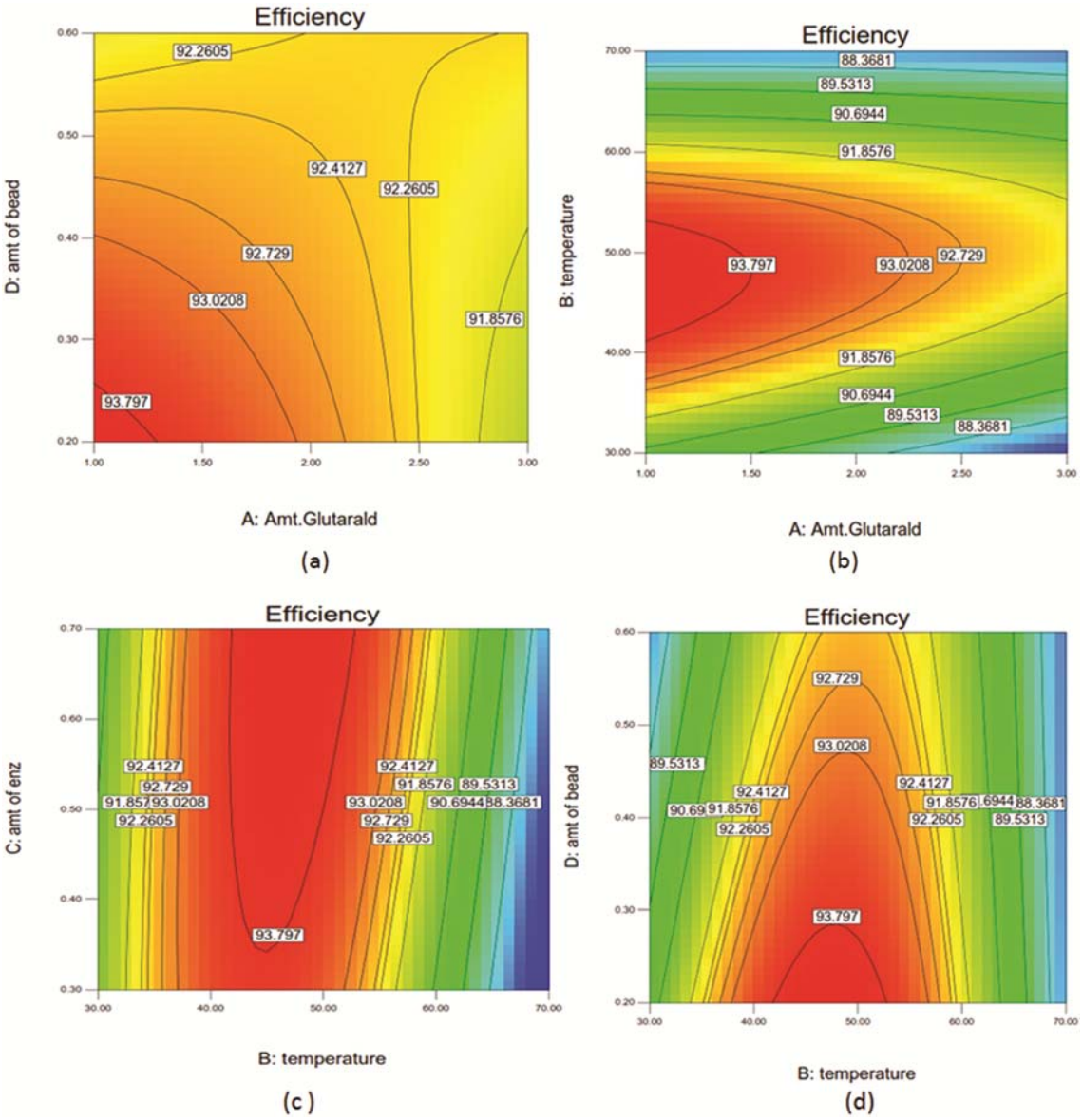

Fig. 2 — Contour plots of the significant factors (BD,AD,BC,AB) affecting the cellulase immobilization yield (%)

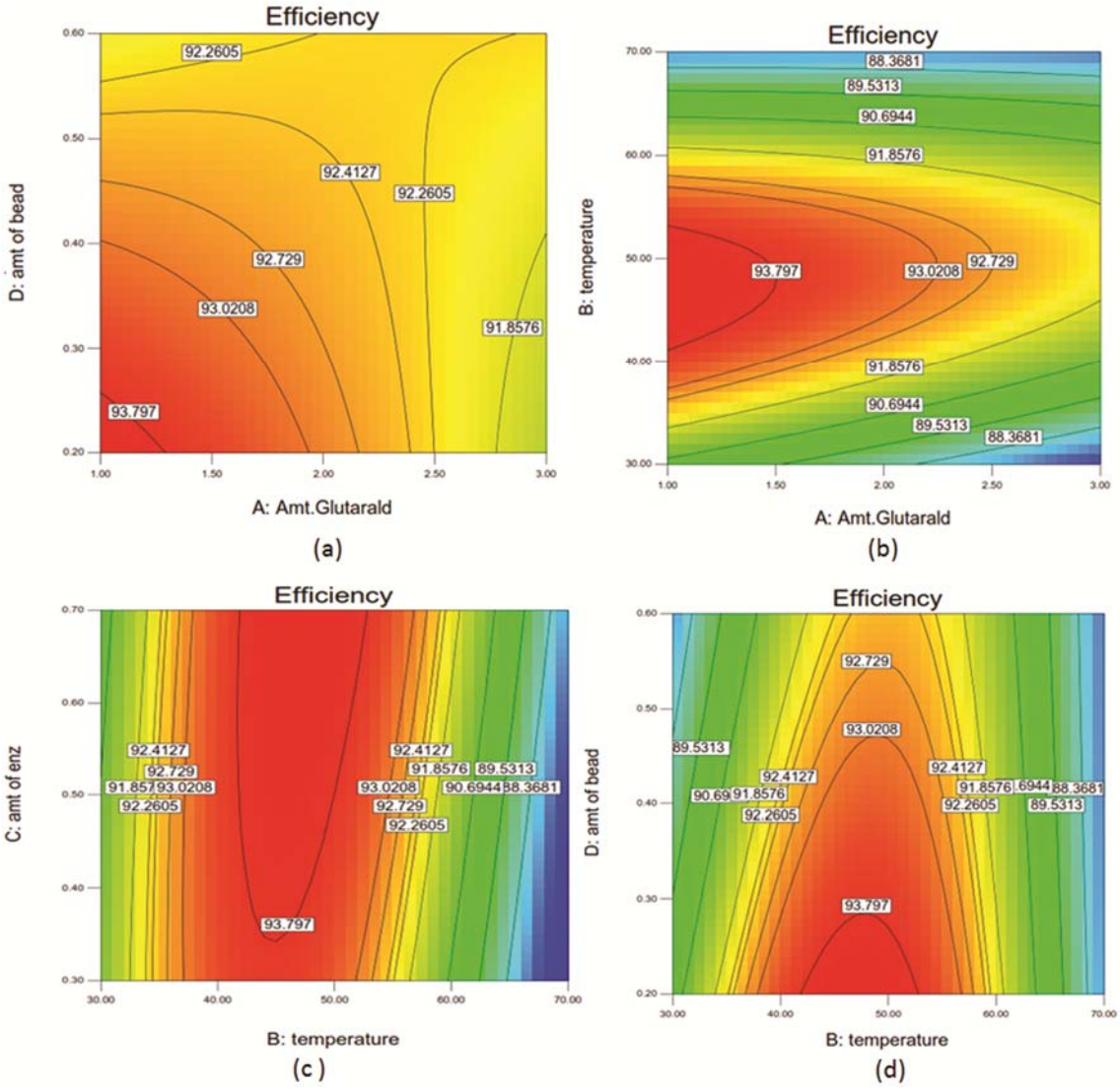

Fig. 3 — Contour plots of the significant factors (AD, AB, BC,BD) affecting the cellulase immobilization efficiency(%)

Table 4 — Validation of the model

Factors	Name	Optimized level	Immobilization yield (%)		Immobilization efficiency (%)	
			Predicted	Observed	Predicted	Observed
A	Amt of Glutaraldehyde	1.03% (v/v)	53.54	54.1	94.05	94.5
B	Temperature	50.7°C				
C	Amount of enzyme	0.68% (w/v)				
D	Amount of bead	0.2 g				

given conditions were 53.4 and 94.05% respectively. The observed values (54.1% and 94.5% respectively for immobilization yield and efficiency) were not significantly different from the predicted ones. Moreover, the optimum values of the independent variables were different from the central values selected for the experiment. Therefore, the statistical model has good correspondence between the observed and predicted values and this model can be successfully applied to increase the immobilization efficiency of cellulase on chitosan beads.

Characterization of immobilized and free cellulase

Kinetics of immobilized and free enzyme

The kinetic constant (K_m) and the maximum velocity of enzyme-catalyzed reaction (V_{max}) were

obtained from Michaelis-Menten plots. The kinetic behaviour of cellulase was altered by immobilization. Km, which is a measure of the substrate's affinity for the enzyme, was found to be 2 mM for the immobilized enzyme which was greater than the free enzyme (Fig. 4). This increase in K_m value might be due to the conformational change in the enzyme that alters the accessibility of substrate to the active site of the enzyme[18,19]. Values for V_{max} decreased for immobilized enzymes compared to the free enzyme (Table 5). Similar results for K_m was obtained by Romo-Sanchez *et al.* while no change in Vmax in the case of the free and immobilized enzyme was seen in their case[20].

Reusability

Reusability is a vital issue for immobilized cellulase in industrial application[21]. The reusability of immobilized cellulase on chitosan beads shown in Fig. 5, depicts that the relative activity of immobilized cellulase diminished with reuse after 12 cycles. The relative activity (maximum activity taken as 100%) of the immobilized cellulase retained 100–90.9 % activity from 1st to 4th cycle and dropped from 90.9–44.31 % following 4th cycle to 12th cycle. This decrease in the activity can be due to the frequent interaction of the active site and substrate leading to distortion of the immobilized enzyme [10]. Reusability up to 6th cycle was achieved by Ikeda *et al.*[22] Zhou *et al* reported that 89% of enzyme activity was retained after 10 cycles of reuse[10]. Yin *et al.* observed 88% of initial enzyme activity after 11 cycles of reuse[23].

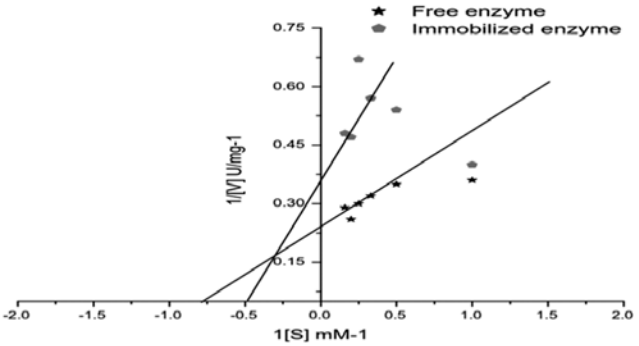

Fig. 4 — Kinetic parameters of free and immobilized cellulase

Table 5 — Kinetic sparameters of free and immobilized enzyme

Kinetic parameters	Immobilized enzyme	Free enzyme
Km (mM)	2	1.3
Vmax (U/mg-1)	2.7	4.4

FTIR analysis

The FTIR spectroscopic analysis of chitosan beads and immobilized enzyme were carried out from 400 to 4000 cm^{-1}, as shown in Fig. 6 (% Transmittance *vs* Wave number [cm^{-1}]). The FTIR bands of the chitosan bead (blue line) showed that the characteristic functional groups (–COO-stretching) were present, with a broad asymmetrical band at 1610 cm^{-1}. The sharp peak near 1622 cm^{-1} in chitosan bead without enzyme was probably due to C=O in amide group (amide I)[24], a peak near 1394 cm^{-1} may be due to CH_3 in amide group[24]. C-O-C in glycosidic linkage was represented near 1155 cm^{-1} range. The C-O stretching vibration in the spectrum of chitosan was represented in 1073 cm^{-1} range[25]. No remarkable change in peak was observed after the enzyme was bound to the bead. Intense peak observed around 1567 cm^{-1} range denoted C-O or C-N stretching. The characteristic peak near 1411cm^{-1} is associated with C-N and COO stretching[26].

Conclusion

Theabovestudy deals with the immobilization of cellulase produced from *Aspergillusniger*onto different matrices among which chitosan beads was found to be the most suitableone. The optimization of the immobilization parameters to determine the maximum immobilization efficiency was done on the basis of statistical approach BBD (Box-Behnken Design). Cellulase was found to be successfully immobilized on the chitosan bead surface, with 94.2% efficiency and immobilization yield was found to be 54.1% after statistical optimization. Significant activity after 12 cycles of reuse was observed (44% relative activity). Thus our immobilization process using chitosan beads promotes enzyme recycling which is extremely promising from the industrial perspective. The Michael's Menten constant K_m was found to be 1.3 mM and 2 mM for free enzyme and immobilized enzyme respectively. The V_{max} was found to be 2.7 and 4.4 U/mg-1 respectively for the immobilized and free enzyme. The immobilized enzyme displayed higher Km but lower Vmax indicating decrease in catalytic efficiency after immobilization. FTIR studies revealed the change in absorbance of functional groups when the enzyme was bound to chitosan beads.

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