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Immobilization of *Aspergillus nidulans* SU04 cellulase on modified activated carbon

Sorption and kinetic studies

S. Anuradha Jabasingh · C. Valli Nachiyar

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Abstract The present study deals with the immobilization of *Aspergillus nidulans* SU04 cellulase onto modified activated carbon (MAC). The effect of contact time, cellulase concentration, MAC dosage, and temperature for maximum immobilization percentage and immobilization capacity is investigated. The equilibrium nature of immobilization is described by Langmuir and Freundlich isotherms. The kinetic data were tested using the pseudo first order. The activation energy of immobilization was evaluated to be 11.78 J mol^{-1}. Results of the thermodynamic investigation indicate the spontaneity ($\Delta G < 0$), slightly endothermic ($\Delta H > 0$), and irreversible ($\Delta S > 0$) nature of the sorption process. Entropy and enthalpy were found to be 41.32 J mol^{-1} mg^{-1} and 10.99 kJ mol^{-1}, respectively. The Gibbs free energy was found to be -22.79 kJ mol^{-1}. At 80 rpm, 323 K, 2 h, 5 mg of MAC, immobilization capacity was 4.935 mg cellulase per mg of MAC from an initial cellulase concentration of 16 mg ml^{-1} with retention of 70% of native cellulase activity up to 10 cycles of batch hydrolysis experiments. The diffusion studies that were carried out revealed the reaction rate as μmol min^{-1}. At optimized conditions, immobilized cellulase had a higher Michaelis–Menten constant, K_m of 1.52 mmol and a lower reaction rate, V_{max} of 42.2 μmol min^{-1}, compared with the free cellulase, the K_m and V_{max} values of which were 0.52 mmol and 18.9 μmol min^{-1}, respectively, indicating the affinity of cellulase for MAC matrix.

Keywords Immobilization · Cellulase · Modified activated carbon · Isotherms · Kinetic models · Reaction rate · Hydrolysis

S. Anuradha Jabasingh (✉)
Department of Chemical Engineering, Sathyabama University, Jeppiaar Nagar, Old Mamallapuram Road, Chennai 600119, Tamilnadu, India
e-mail: anu3480@yahoo.com

C. Valli Nachiyar
Department of Biotechnology, Sathyabama University, Chennai 600119, Tamilnadu, India

Introduction

In recent years, the enzyme, cellulase finds wide application to a variety of fields such as textile, paper and pulp, food and animal feed, and fuel and chemical industry. In addition, they can be used in waste management, pharmaceutical industry, protoplast production, genetic engineering, and pollution treatment [1]. High specificity, catalytic activity, efficiency, non-toxicity, water solubility, biodegradability, and mild operating conditions including pH, temperature, and pressure favor its application in these industrial sectors. But, the relative instability and high cost incurred in enzyme isolation and recovery, during the downstream processes, after the catalysis reaction, limits its application. To overcome these problems, cellulase immobilization is established. The process of immobilization eliminates enzyme recovery and purification. Further, it aids in the enzyme utilization and better environment for biocatalytic reactions. The technique also improves the product purity and minimizes the effluent handling problems during product development.

Several techniques employed for immobilization, including, entrapment and surface immobilization are commonly based on physical and chemical mechanism [2–4]. Entrapment methods include, matrix entrapment employing matrices made of polymeric materials, Ca-alginate, agar, K-Carrageenin, polyacrylamide, and

collagen and membrane entrapment employing membrane made of nylon, cellulose, polysulfone, and polyacrylate. Enzyme entrapment has its inherent problems including enzyme leakage, diffusional limitation, reduced activity, stability, and lack of control over the micro-environmental conditions [5–7]. Surface immobilization methods include, adsorption on inorganic materials such as alumina, silica, porous glass, ceramics, diatomaceous earth, clay, and bentonite or on organic materials such as cellulose, starch, activated carbon [8], and covalent binding where the enzyme molecules bound to surface of support material via amino, carboxyl, hydroxyl sulfhydryl groups employing chemical reagents cyanogen bromide, glutaraldehyde, and carbodiimide [9].

Several factors including the overall biocatalytic activity, biocatalyst effectiveness, deactivation, regeneration, and cost has chosen us to employ the adsorption technique for the immobilization of *A. nidulans* SU04 cellulase onto activated carbon. Physical sorption has advantages as well as several disadvantages; these are overcome by increasing the adherence of the cellulase on to the matrix throughout the condition range of enzyme exposure, when the same is in service. Conditions for the immobilization must be consistent for this means. The initial cellulase concentration, modified activated carbon (MAC) dosage, temperature, contact time and agitation rate on immobilization needs to be assessed carefully [10, 11].

Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials [12]. Cellulase production in fungi is found to be extra cellular and has three components, endoglucanase(endo-1,4-β-D-glucanase, EC 3.2.1.4), exoglucanase (exo-1,4-β-D-glucanase, EC 3.2.1.91),and β-glucosidase(1,4-β-D-glucosidase, EC 3.2.1.21). This enzyme has been immobilized on a number of soluble and insoluble carriers, calcium alginate [13], liposome [14], PVA membranes [15], silicate clay [16], loofa sponge [17], pretreated lignocelluloses [18], and activated carbon [19]. Most of the studies have concluded that enzymes with inorganic carriers were more stable than those attached onto organic polymers. Also, porous materials have favoring features compared with non porous materials [20]. Activated carbon is one of the most abundant organic materials, and it has been processed to make it extremely porous and thus to have a very large surface area available for adsorption or chemical reactions [21]. Our matrix, MAC was developed by aqueous oxidation using hydrogen peroxide followed by drying at 393 K. Oxidation generated cyclic acid anhydride groups, CO groups associated with polynuclear aromatic systems, and several types of C–O species. The aim of our present study is to bring out the feasibility of employing MAC in a batch sorption process for the immobilization of cellulase produced by *A. nidulans*.

Experimental

Immobilization matrix

A commercial activated carbon powder (purity ≥60%) was purchased from Merck, Germany. Activated carbon was modified by dissolving 5 g of activated carbon in 200 ml of 37% H_2O_2 below 5 °C with rapid stirring. The temperature was gradually raised and at 40 °C, the viscosity of the solution showed an abrupt increase and a rapid decrease. At this point, the solution was filtered using glass wool into a beaker containing 3 L of deionised water, below 5 °C with moderate stirring. The suspension was stirred for 30 min, and placed overnight in a cold room below 5 °C followed by decantation. Activated carbon was filtered out using Whatman no. 40 filter paper and subjected to overnight dialysis using bio-design dialysis tubing 8000 MWCO. This was carried out in a pipette washer with a diameter of 20 cm and a water flow rate of 1 mL s^{-1}. The sample was dried overnight at 120 °C in a vacuum oven at 400 mmHg vacuum [22]. The BET surface area and total pore volume were determined using nitrogen gas adsorption analyzer at 77 K with an ASAP2010 instrument. Specific surface area and total pore volume of MAC was 63.33 m^2 g^{-1} and 0.085 cm^3 g^{-1}. This MAC, large, crystalline, and greyish black in color was selected for the immobilization of cellulase.

Enzyme and reagents

Enzyme cellulase was procured from *A. nidulans* SU04 by a previous procedure adopted [23]. It was seen to possess an activity of 51.98 U ml^{-1}. Na-Carboxy methyl cellulose powder (Na-CMC) purchased from Sigma-Mumbai, India was used as substrate. Diluted solution of Na-CMC was prepared using citrate buffer. Diluted solutions of D-glucose anhydrous (purity ≥99.0%), obtained from Merck-Mumbai, India were used as standards. All other reagents were of analytic grade.

Immobilization experiments

The immobilization of cellulase from aqueous solution onto MAC was performed using batch technique [24]. The dependency of contact time, initial cellulase concentration, MAC dosage, and temperature on immobilization were inferred. These four variables were chosen to determine optimal values of these factors. Immobilization experiments were carried out at temperatures (293–333 K) on an incubator shaker at agitation speed of 80 rpm. Immobilization studies involving cellulase by MAC were conducted by taking different amounts 1, 2, 3, 4, 5, and 6 mg of MAC in glass tubes containing 4, 8, 12, and 16 mg mL^{-1} of

cellulase solution. Experiments were carried out at contact times 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 h. The initial pH of the solution was adjusted to 4.8 using 1% citrate buffer. Upon equilibration, the solution was centrifuged, and the equilibrium concentration of cellulase in the solution was determined using UV–Vis spectrophotometer UV 2700 model (Thermo Fisher, Nashik, India) at $\lambda_{max} = 540$ nm. The equilibrium immobilization capacity, I_e (mg cellulase mg^{-1} MAC) was calculated using the following equation:

$$I_e = \frac{E_0 - E_e}{M} \cdot V \quad (1)$$

where E_0 is the initial concentration of cellulase solution (mg mL^{-1}), E_e is the equilibrium concentration of cellulase solution (mg mL^{-1}), V is volume of cellulase solution (mL), and M is the mass of MAC (mg). The immobilization percentage of cellulase is calculated using the following equation:

$$\text{Immobilization}\% = \frac{E_0 - E_f}{E_0} \times 100 \quad (2)$$

E_f is the final concentration of cellulase in the solution (mg mL^{-1}).

Experimental validation

The experimental characteristics were evaluated to determine the efficacy of the performed experiments in predicting the described situation [25–28]. The goal fixed for the maximum immobilization percentage was to employ a maximum cellulase concentration 16 mg mL^{-1} at pH 5 and 323 K, minimal activated carbon dosage of 5 mg, equilibrium contact time of 2 h, and agitation rate fixed at 80 rpm [29].

Hydrolysis experiments and analytical methods

Determination of cellulase activity in immobilized MAC matrix was performed using 1% Na-CMC. Substrate solutions were treated with free *A. nidulans* SU04 cellulase (51.98 U mL^{-1}) and 50 mg immobilized cellulase (49.82 U mL^{-1}) in separate test tubes at 323 K, pH 5.0 for 20 h to access the extent of hydrolysis [23]. Assay for cellulase activity (CMCase) were carried out by measuring the release of reducing sugars in the reaction mixture of 1 mL crude enzyme and 1 mL of 1% (w/v) CMC solution in 0.05 M sodium citrate buffer (pH 4.8) incubated at 50 °C for 1 h, according to IUPAC recommendation [30]. At 2 min interval, 1 mL of solution was withdrawn, and the residual glucose concentration is measured by Dinitrosalicylic acid method using UV–Vis spectrophotometer UV 2700 model (Thermo Fisher, Nashik, India) at $\lambda_{max} =$ 540 nm [31]. One unit of CMC activity is defined as the amount of enzyme needed to liberate 1 μmol of glucose per min from 1 mL of culture broth under assay conditions. The surface morphology of activated carbon, before modification, after modification, after cellulase immobilization, and after ten times of repeated usage was observed by subjecting the samples to scanning electron microscopy (SEM) using Philips XL30 scanning electron microscope with electron acceleration voltage of 20 kV and probe current of 5×10^{-11} A after subjecting them to gold-sputtering in a denton vacuum desk I for 1.5 min under a 200 m torr Argon atmosphere and a current of 30 mA.

Immobilization kinetics

The kinetic data of immobilization were treated with the first order model [32].

$$\frac{dI}{dt} = k_1(I_e - I) \quad (3)$$

Integrating the above equation between the limits 0 to t from $q = 0$ to $q = q_t$, the above kinetic expression becomes

$$\log(I_e - I_t) = \log I_e - \left[\frac{k_1}{2.303}\right] t \quad (4)$$

where, I_t is the Immobilization capacity of MAC (mg cellulase mg^{-1} MAC) at any time t and k_1 is the first order rate constant (min^{-1}). The initial immobilization rates were given by

$$h = k_1 I_e^2 \quad (5)$$

Thermodynamic analysis

The activation energy E_a for the immobilization of cellulase on MAC was determined using Arrhenius equation.

$$k = Ae^{\frac{-E_a}{RT}} \quad (6)$$

The Gibb's free energy, enthalpy, and entropy for the process of immobilization are investigated using the following equation:

$$K_D = \frac{I_e}{C_e} \quad (7)$$

$$\Delta G^{\circ} = -RT \ln K_D \quad (8)$$

$$\ln K_D = \frac{-\Delta G}{RT} = \frac{-\Delta H}{RT} + \frac{\Delta S}{R} \quad (9)$$

where K_D is the distribution coefficient in mL mg^{-1}. The values of K_D, indicate retentability of MAC and the mobility of cellulase in MAC as well as in the solution phase [33, 34].

Immobilization isotherms

The distribution of cellulase between MAC and the cellulase solution, when the system is at equilibrium is used to determine the maximum immobilization capacity of MAC. Langmuir equation, used for fitting equilibrium data is given by

$$I_{eL} = \frac{K_L E_e}{1 + a_L E_e} \tag{10}$$

The amount cellulase immobilized at equilibrium per unit mass of the MAC is given by the above equation, where

$$a_L = \frac{K_L}{X_m} \tag{11}$$

The linear form of Langmuir isotherm is given by

$$\frac{E_e}{I_{eL}} = \frac{1}{K_L} + \frac{a_L}{K_L} E_e \tag{12}$$

where K_L (mL mg^{-1}) and a_L (mL mg^{-1}) represent Langmuir constants. The maximum immobilization capacity in the Langmuir model is given by X_m (mg cellulase mg^{-1} MAC). The essential features of Langmuir isotherm can be expressed in terms of a dimensionless equilibrium parameter

$$R_L = \frac{1}{1 + a_L E_O} \tag{13}$$

Values of R_L between 0 and 1 indicate favorable immobilization. The Freundlich isotherm gives the relationship between the equilibrium liquid and immobilization capacity based on multilayer immobilization. This isotherm is widely used in studies at low concentrations of solute in aqueous medium. The expression for Freundlich equation is given by

$$I_{eF} = K_F E_e^{1/n} \tag{14}$$

The linear form of Freundlich equation is given by

$$\ln I_{eF} = \ln K_F + \frac{1}{n} \ln E_e \tag{15}$$

where K_f and n are multilayer immobilization capacity (mg g^{-1}) and immobilization intensity respectively. Values of n between 1 and 10 represent beneficial immobilization [35].

Reuse and shelf life of immobilized cellulase

After the hydrolysis experiments, the solution was centrifuged and cellulase immobilized on MAC was separated and washed exhaustively with citrate buffer to enhance stability [36]. Reusability was monitored by following the change in the activity of immobilized cellulase with its repeated usage in the Na-CMC hydrolysis. The cellulase activity was measured after each usage in succession for repeated hydrolysis experiments.

Results and discussion

The results of immobilization experiments with different combinations of factors are presented in Figs. 1 and 2. The effect of contact time on the immobilization of cellulase on MAC was studied at 50 °C by varying the contact time from 0 to 3 h for cellulase solution with initial concentration from 4 to 16 mg ml^{-1}. The study was made for MAC dosage of 5 mg with initial concentration at pH of 5.0. The result is shown in Fig. 1. The amount of cellulase immobilized was found to increase with an increase in the contact time and attained equilibrium at 2 h for all the cellulase concentrations studied. This behavior is attributed to the relatively less available immobilization sites on the surface of the MAC as contact time increases. At equilibrium, the maximum sorption percentages were found to be 79.5, 74.88, 65.67, and 52.56 for initial cellulase concentration of 4, 8, 12, and 16 mg mL^{-1}, respectively. Immobilization studies were carried out using MAC dosages ranging from 1 mg to 6 mg in cellulase solution for the equilibrium time of 2 h at optimum pH 5.0 and at 50 °C. The effect of MAC dosage on immobilization is shown in Fig. 2. It was found that the surface area available for the sorption is limited for a specific dosage of MAC. The reduced immobilization of cellulase at low concentration was due to the lack of enough cellulase molecules near to the sorption sites. It is clear from the Fig. 2 that the immobilization capacity increased from 0.692 to 1.65 mg cellulase mg^{-1} MAC and 2.328 to 4.935 mg cellulase mg^{-1} MAC for cellulase concentrations of 4 and 16 mg mL^{-1}, respectively, for the variation in MAC

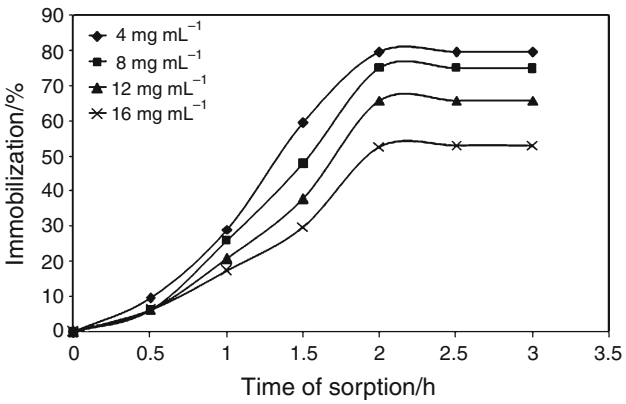

Fig. 1 The effect of contact time on cellulase immobilization on MAC

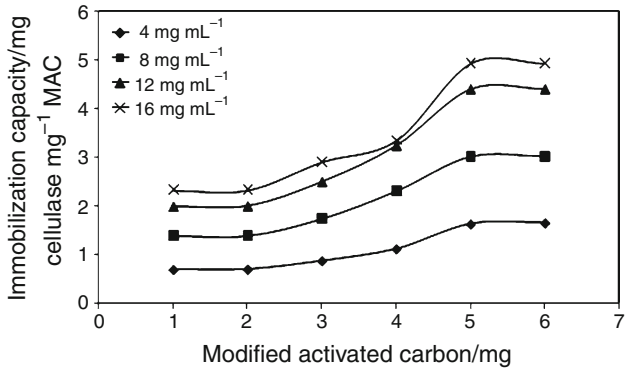

Fig. 2 The effect of MAC dosage on immobilization capacity

dosage from 1 to 6 mg. This is owed to the availability of more binding sites for the complexation of cellulase [37].

However, increasing the MAC dosage above 5 mg had very little influence on the immobilization which was just about 0.0001 at equilibrium for 5 mg of MAC. This effect may be due to the decline in the cellulase molecules in the solution with the increase in the MAC dosage. Hence, further addition of MAC above 5 mg was considered to be economically unsuitable for the immobilization of cellulase molecules.

The results of kinetic analysis are shown in the Fig. 3. From the slope of log (I_e–I_t) versus t plot, the first order rate constants k_1 are found to be 2.111, 3.022, 2.819, and 2.059 h^{-1} for initial cellulase concentration of 4, 8, 12, and 16 mg mL^{-1} at 323 K, respectively. The regression coefficients values shown in Table 1 confirm the applicability of the model.

The pseudo first order kinetic model fitted the data well and represented the rapid stages of immobilization. Hence, the use of Lagergren kinetic model for cellulase immobilization on MAC for the entire immobilization period was found to be appropriate and substantiates the immobilization of cellulase onto MAC. The prediction of rate limiting

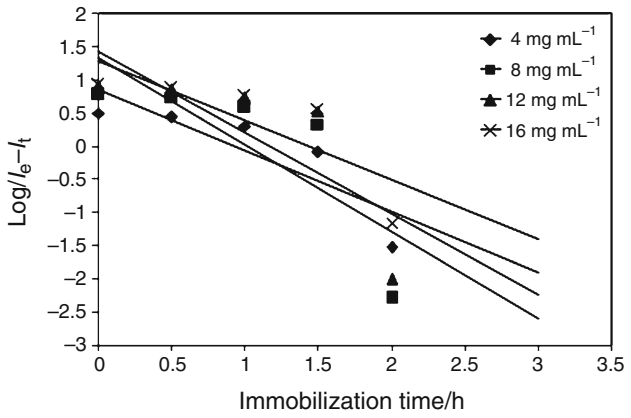

Fig. 3 Pseudo first order kinetic plot

step is an important factor to be considered in the process of immobilization [32]. The solute transfer is usually characterized by the external mass transfer (boundary layer diffusion) or by intraparticle diffusion or both for a solid–liquid immobilization. Mechanism of immobilization involves enzyme transport from the bulk solution through liquid film to the MAC exterior surface. After which, the enzyme may be transported within the pores of MAC or immobilized on the exterior surface. The equilibrium reaction is the last and the most rapid step. The slowest step determined the rate controlling parameter in the immobilization process.

The rate controlling parameter might be distributed between intraparticle and film diffusion mechanisms [38]. Immobilization is controlled due to the film diffusion at earlier stages and later by particle diffusion. By fitting the data in the intraparticle diffusion plot, the mechanism involved in the immobilization process can be identified. The intraparticle immobilization coefficient I_{id} is given by the equation

$$I_t = I_{id} t^{0.5} \tag{16}$$

In the immobilization diffusion plot of Fig. 4, the initial curved portion relates to the boundary layer diffusion and the latter linear portion represents intraparticle diffusion. These two regions suggested the ensuing of immobilization process by both surface immobilization and pore diffusion. From the slope of the second linear portion of the plot, the intraparticle immobilization parameter I_{id} was found to be 2.188, 4.131, 5.403, and 5.763 mg cellulase mg^{-1} MAC $min^{-0.5}$. The boundary layer effect was depicted by the surface immobilization factor "I_f" mg cellulase mg^{-1} MAC characterized by the intercept of diffusion plot. Large values of intercept, emphasis more contribution of surface phenomena to the immobilization process. In this case, I_f factor was found to increase for the initial cellulase concentrations. Surface immobilization becomes more predominant as the rate controlling step at 16 mg mL^{-1} after which the immobilization process is governed by pore diffusion. The values for the intraparticle immobilization coefficient and surface immobilization factor are given in the Table 1 for all the cellulase concentrations studied. Langmuir and Freundlich isotherms for the immobilization of Cellulase onto MAC are depicted in Figs. 5 and 6.

Variation in temperature leads to smaller changes in the immobilization capacity of cellulase on MAC. Large molecular weight of cellulase leads to minor changes in the immobilization capacity, since at specific temperatures molecules with large molecular weight possess smaller velocity. Hence, the equilibrium immobilization capacity of cellulase on MAC had marked increase with lowering temperature, testifying the hypothesis put forth by the kinetic theory. The amount of cellulase immobilized on

Table 1 Kinetic parameters for the immobilization of *A. nidulans* SU04 cellulase on MAC

Initial cellulase concentration C_o/mg mL^{-1}	Pseudo first order kinetics				
	Pseudo first order rate constant (k_1/h^{-1})	Correlation coefficient R^2	Initial sorption rate h/mg cellulase mg^{-1} MAC min^{-1}	Intraparticle diffusion coefficient I_{id}/mg cellulase mg^{-1} MAC min$^{-0.5}$	Surface sorption factor I_f/mg cellulase/mg^{-1} MAC
4	2.111	0.739	21.35	2.188	0.477
8	3.022	0.623	107.99	4.131	1.046
12	2.819	0.610	175.04	5.403	1.426
16	2.059	0.649	148.06	5.763	1.467

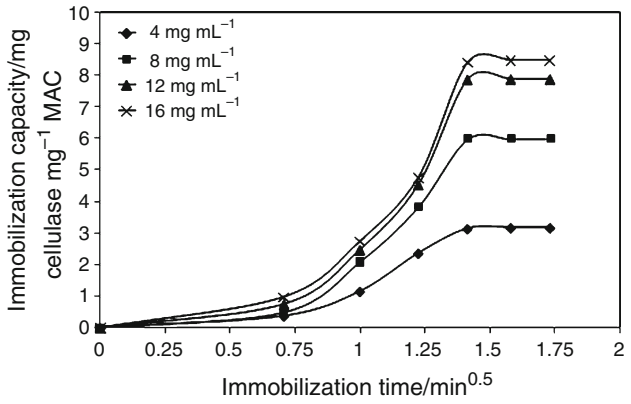

Fig. 4 Immobilization diffusion plot

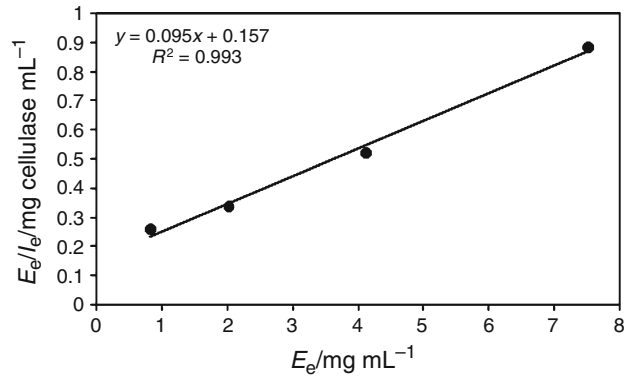

Fig. 5 Langmuir isotherm for the cellulase immobilization on MAC

MAC at equilibrium per unit mass of the MAC (a_L) was found to be 0.605 mL mg^{-1} and K_L was found to be 6.37 mL mg^{-1} representing Langmuir constants. From the linear form of Langmuir isotherm, the maximum immobilization capacity X_m in Langmuir model was found to be 10.53 mg cellulase mg^{-1} MAC. The linear plot of E_e/I_e versus E_e confirms the applicability of Langmuir model. The effect of isotherm shape had been considered with a view to predict whether the immobilization is favorable or unfavorable.

Values of R_L between 0 and 1 indicated favorable immobilization. The Freundlich isotherm gives the

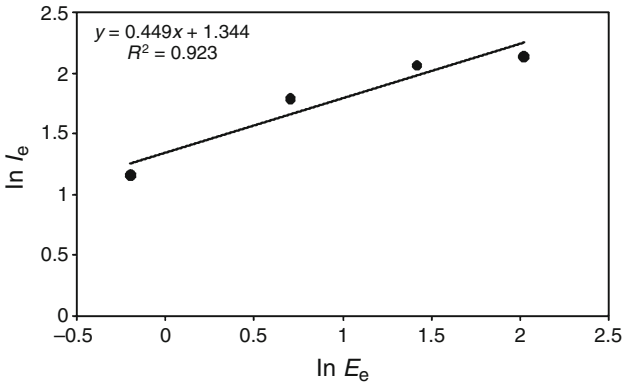

Fig. 6 Freundlich isotherm for the cellulase immobilization on MAC

relationship between equilibrium liquid and immobilization capacity based on multilayer immobilization. This isotherm is widely used in studies at low concentrations of solute in aqueous medium [39]. Multilayer immobilization capacity, K_f and immobilization intensity, n were found to be 3.834 mg mg^{-1} and 2.23, respectively. Values of n between 1 and 10 represented beneficial immobilizaton. Langmuir and Freundlich parameters for the immobilization of cellulase onto MAC are shown in Table 2.

The immobilization capacity increased from 0.078 to 1.52 mg per mg of MAC as the temperature increased from 293 to 323 K after which the sorption capacity decreased to 0.089 mg mg^{-1} of MAC as the temperature was further raised to 333 K. This indicates the endothermic nature of the process.

The increase in temperature increased the mobility of the cation. The augmentation of sorption capacity at higher temperatures indicated the involvement of physisorption, chemisorption, and an increase in the number of active sites due to the bond rupture [32]. Further above 323 K, there occurs deactivation of cellulase. Plot of ln k versus T^{-1} gives activation energy E_a as 11.781 kJ mol^{-1} and Arrhenius constant as 267.74 h^{-1} (Fig. 7). R and k represent the Universal gas constant and the pseudo first order rate constant, respectively. In the process of immobilization, Gibbs free energy, enthalpy, and entropy play a vital role in determining the spontaneity of the process. The plot

Table 2 Langmuir and Freundlich parameters for the immobilization of *A. nidulans* SU04 cellulase on MAC

Initial cellulase concentration E_o/mg mL^{-1}	Equilibrium cellulase concentration E_e/mg mL^{-1}	Equilibrium immobilization capacity, I_e/mg cellulase mg^{-1} MAC	Langmuir parameters			Freundlich parameters	
			Langmuir equilibrium parameter R_L	Langmuir equilibrium immobilization capacity I_{eL}/mg cellulase mg^{-1} MAC	Correlation coefficient R^2	Equilibrium immobilization capacity I_{eF}/mg cellulase mg^{-1} MAC	Correlation coefficient R^2
4	0.82	3.18	0.292	3.49	0.993	3.51	0.923
8	2.02	5.98	0.171	5.79		5.26	
12	4.12	7.88	0.121	7.51		7.24	
16	7.52	8.48	0.093	8.63		9.49	

of ln K_D versus T^{-1} results in a straight line, the slope and intercept of which gives $\Delta H°$ and $\Delta S°$, respectively (Fig. 8).

The values of K_D were seen to increase with an increase in temperature. The values of $\Delta G°$, $\Delta H°$, and $\Delta S°$ are given in Table 3. Temperature was seen to adversely affect the process of immobilization. As the temperature was increased, the immobilization was seen to increase till 323 K. This may be due to the effect of a more negative value of Gibb's free energy which makes the reaction spontaneous [32, 40]. The negative $\Delta G°$ values indicate the thermodynamic favorability of the reaction toward the immobilization of cellulase on MAC. Positive $\Delta S°$ and $\Delta H°$ values indicate the spontaneity of immobilization at high temperatures. Affinity factor plays a major role in determining the immobilization capacity of cellulase. The increase in temperature increases the mobility of solute through the solution. It also increases the pore diameter of the immobilizing agent. The cellulase molecules are dehydrated and get accessed to the micropores of MAC at 323 K. The entire surface was seen to be covered by a monolayer of cellulase molecules. The morphology of activated carbon before modification, MAC after modification, MAC after immobilization, MAC after 10 repeated cycles of usage during hydrolysis is shown in SEM images (Fig. 9).

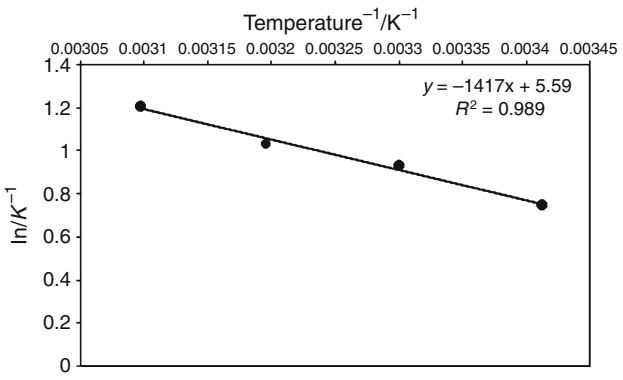

Fig. 7 Arrhenius plot for activation energy calculation

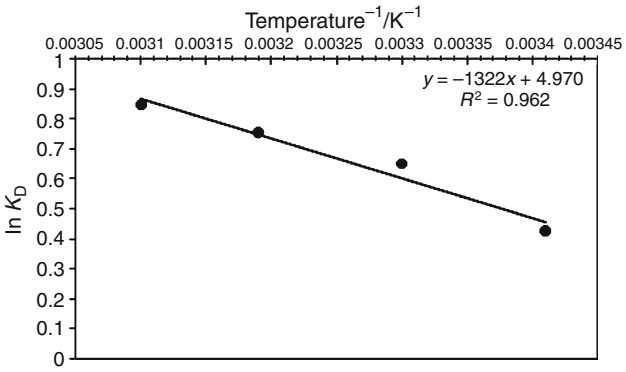

Fig. 8 Gibb's free energy, enthalpy, and entropy from the plot of ln K_D versus T^{-1}

Figure 9a shows the smooth surface of activated carbon without any eruptions on the texture. On subjecting activated carbon to modification using H_2O_2, a large number of pores were formed, possibly due to the penetration of H_2O_2 into the matrix and the subsequent erosion of the matrix due to infiltration (Fig. 9b) [41]. During immobilization, these pores are accumulated with cellulase (Fig. 9c). After 10 times of repeated usage, cellulase seems to get dilapidated out of the surface of MAC, as shown in Fig. 9d. The SEM images clearly picturizes the entire scenario of cellulase immobilization on the sorbent.

Hydrolysis and reusability of immobilized cellulase

The entire scenario of CMC hydrolysis and glucose liberation is shown in Fig. 10. CMC solutions 0.125, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, and 2.00% (w/v) were treated with free and immobilized cellulase to access the extent of hydrolysis at 323 K. The immobilized cellulase had a higher K_m (1.52 mmol) and lower V_{max} (42.2 µmol min^{-1}). The free cellulase had lower K_m (0.52 mmol) and higher V_{max} (18.9 µmol min^{-1}).

Table 3 Thermodynamic parameters for *A. nidulans* SU04 cellulase immobilization on MAC

Sorption temperature T/K	Distribution coefficient K_D/mL mg^{-1}	Gibbs free energy ΔG/kJ mol^{-1}	Enthalpy ΔH/kJ mol^{-1}	Entropy ΔS/J mol^{-1} K^{-1}
293	1.534	−1.042	10.99	41.32
303	1.919	−1.642		
313	2.132	−1.969		
323	2.337	−2.279		

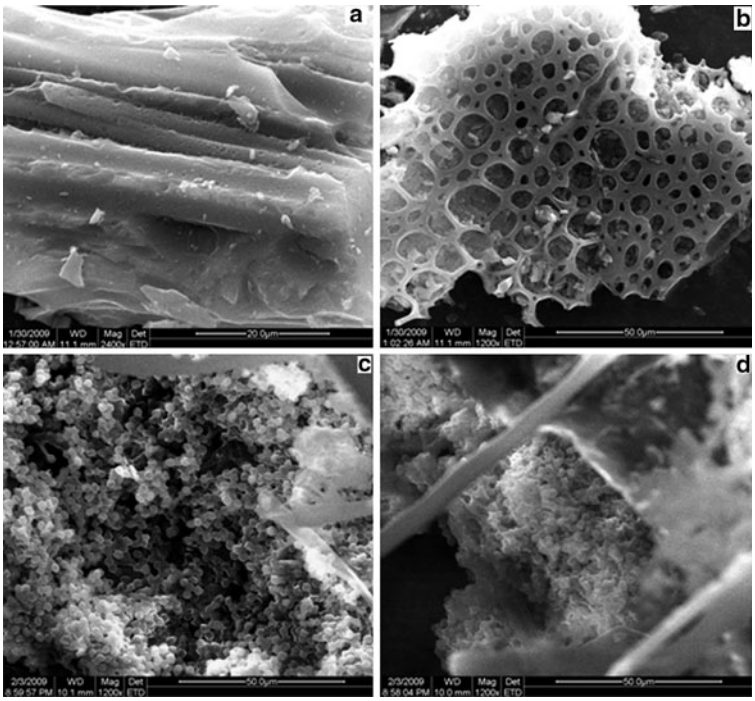

Fig. 9 **a** Activated carbon morphology before modification, **b** after modification, MAC, **c** after cellulase immobilization on MAC, **d** after 10 times of repeated usage in hydrolysis

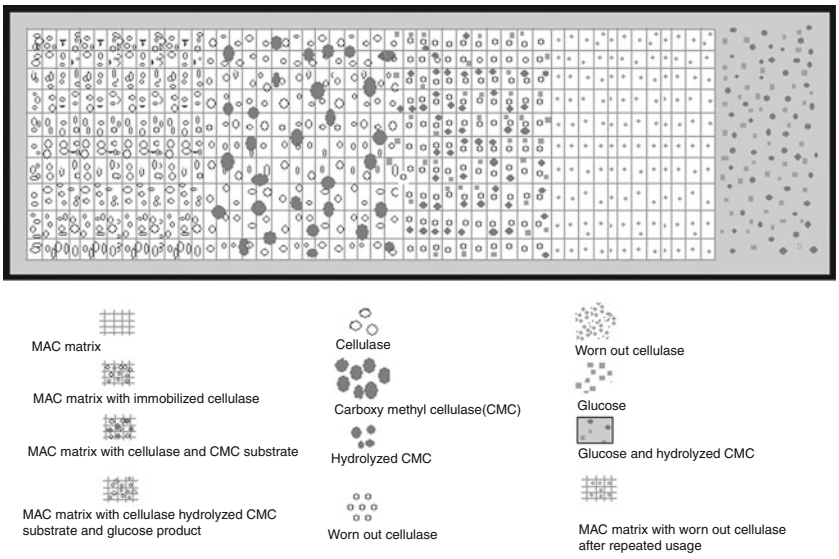

Fig. 10 Schematic representation of CMC hydrolysis

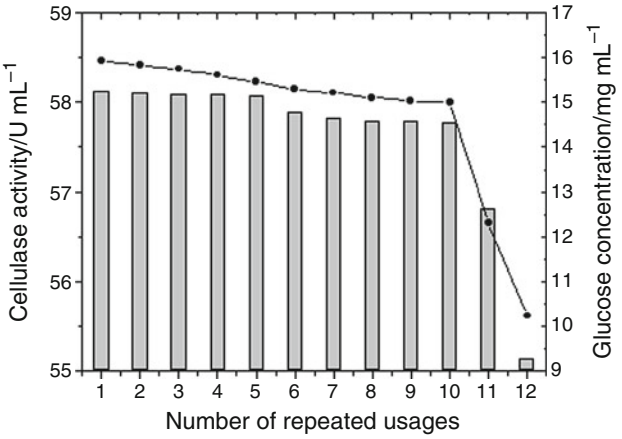

Fig. 11 Reusability of MAC on cellulase activity and glucose yield

The glucose units formed as a result of hydrolysis were measured. There was not any remarkable extent of hydrolysis due to the same activity possessed by both free and immobilized cellulase. Maximum amount of glucose released was found to be at 13 min in both cases. This indicates the negligible effect of immobilization on the activity of cellulase [19, 37]. The reusability of the immobilized cellulase was monitored by following the change in the enzymatic activity with its repeated usage in CMC hydrolysis (Fig. 11).

The glucose yield was seen to decrease at each repeated usage whereas a slight decrease in cellulase activity was observed after ten times of usage. The immobilized cellulase activity was less by 0.44% on an average, but during the course of CMC hydrolysis, the immobilized cellulase was found to retain its average activity by 57.12 ± 0.05 U mL^{-1} after 10 times of repeated usage. A maximum of 15.92 mg ml^{-1} of glucose was synthesized using immobilized cellulase.

Conclusions

The present study on the optimization of *A. nidulans* SU04 cellulase immobilization on MAC located the optimum levels of the most significant factors playing a key role in maximizing the immobilization capacity. The simplicity, accuracy, efficiency, and robustness of the technique adopted, encourages its application in similar immobilization studies. The equilibrium time of immobilization was found to be 2 h for the cellulase concentration 16 mg L^{-1} at 323 K using 5 mg of MAC. The kinetics of the process was predicted by the pseudo first order model. Cellulase immobilization as indicated by the thermodynamic parameters was found to be a spontaneous, feasible, and endothermic process. In conclusion, MAC is a good supporting material for the immobilization of cellulase and cheaper when compared to other carriers. This immobilized system produced 15.92 mg mL^{-1} of glucose at 50 °C and pH 5 in 13 min, hence can be employed for the continuous production of glucose from cellulose derivatives in industries. Also, it can be recycled and recharged after prolonged use. Our study will be continued with hydrolysis experiments on column, packed with MAC immobilized cellulase, aiming at higher immobilization capacities, and more glucose yield. The data obtained in this and future studies will be employed to design a bioreactor for the enhanced production of glucose using immobilized cellulase as biocatalyst.

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