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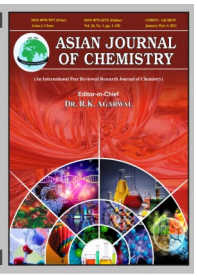

Study on Alkaline Protease Immobilized on Mesoporous Materials

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In the present study, MCM-41 and SBA-15 were used to immobilize alkaline protease. SBA-15 was selected as the better material to immobilize alkaline protease because of its higher enzyme loading and energy recovery of immobilized alkaline protease. Factors affecting the catalytic activity of the immobilized enzyme, such as immobilization's time, pH and enzyme concentration were studied. The properties of immobilized enzyme were also investigated. Under the optimal condition (1 h, pH 8, enzyme concentration 10 mg/mL), the immobilized enzyme displayed maximum activity (enzyme loading 589.43 μg/mg, energy recovery 70.86 %). Obtained immobilized enzyme had a good pH and temperature stability.

Keywords: Mesoporous materials, Immobilization, Alkaline protease, Stability.

INTRODUCTION

Enzymes as specific biocatalysts play an important role in biochemical reactions owing to its properties (high activity, selectivity and specificity)[1]. However, the application of enzyme for a given reaction is often restricted by major limitations such as high cost, short catalytic lifetime and the difficulty in recovery and recycling. These disadvantages can be overcome by immobilizing enzyme onto suitable supports, as the immobilized enzyme has better thermal and pH stability, reusability, easy separation and greater efficiency for practical applications[2].

Microbial proteases account for approximately 40 % of the total worldwide enzyme sales[3] and among them alkaline proteases have ample biotechnological potential for industrial sectors like laundry detergents, leather processing, brewing, food and pharmaceutical industries. Alkaline protease, produced from alkalophilic *Bacillus*, which can withstand high temperature, pH, chemical denaturing agents and non-aqueous environments have attracted a great deal of attention due to their multitude of industrial applications. The industrial applications have been limited by several factors such as the high cost of the enzymes, their instability at high pH and temperature and their availability in small amounts[4]. So how to improve the activity of alkaline protease is one of the challenges we meet. Like many other enzymes, enzyme immobilization is one of the effective ways to solve the problem.

Nelson and Griffin[5] found that the activity of sucrose was little changed after sucrose was adsorbed on activated carbon and colloid (saponins, serum and ovalbumin). Immobilization of enzyme has been an important process in the biotechnological applications such as separation, catalysis and sensors, which typically depend in large part on the successful immobilization of the biomolecules onto or within a suitable carrier[6]. Researchers have been paying great attention to exploitation and application of immobilized enzymes. However, finding an economical and practical carrier with high loading capacity and high stability has always been one of the focuses of enzyme immobilization and different methods were conducted to obtain a more excellent one[7-9]. In recent years, the application of enzyme immobilization techniques in mesoporous silica have been attractive, such as physical adsorption[10-14], chemical adsorption[15-17], enzyme cross-linking[18] and enzyme encapsulation[19,20]. Besides, a number of processes for enzyme immobilization in silica nanotubes[21], on phospholipid-sepiolite biomimetic interfaces[22], on self-assembled monolayers[23], in Langmuir Blodgett films[24], within a polymer matrix[25], mesoporous materials[26], A heterofunctional support[27]on polystyrene latex particles[28], gold nanoparticles assembled on polymer and zeolite[29] and in thermally evaporated fatty lipid films[30] have been developed, each with its characteristic pros and cons[31].

Among various carriers, mesoporous silica materials showed promising application in enzyme immobilization due to its ordered nano-pore structure, narrow pore diameter distribution, high surface-to-volume ratio, large pore volume and great stability and other properties[31]. International Union of Pure and Applied Chemistry (IUPAC) defined the mesoporous material as a porous material with pore diameter of 2-50 nm. Mesoporous materials can be easily modified because of its active hydroxyl on the surface. Since Díaz and Balkus[32]

prepared immobilized enzyme on MCM-41 in 1996 for the first time, SBA-15, MCM-48, MCF, SBA-16 and other different pore diameter of mesoporous materials have been prepared and used to immobilize enzyme successfully and at the same time, enzyme immobilized on mesoporous materials is becoming a very exciting area in research on enzyme immobilization[33].

As XRD, N_2 adsorption desorption isotherms, Fourier transformed infrared (FT-IR) and other characterization methods emerge, the microstructure and special parameters of mesoporous materials can be known accurately[34,35], which is helpful to select the most suitable carrier with respect to an enzyme of unique diameter. In the present study, the effects of two different mesoporous materials on alkaline protease immobilization were discussed. The more appropriate one for the alkaline protease immobilization was picked out at the hand of new characterization methods mentioned above. In addition, the optimum catalysis condition with high enzyme loading and energy recovery for alkaline protease immobilization was obtained. And the properties of immobilized enzyme have also been evaluated.

EXPERIMENTAL

Alkaline protease was purchased from Pangbo biological engineering Co., Ltd (Guangxi, China). SBA-15 and MCM-41 were prepared by State Key Laboratory of Inorganic Synthesis and Preparative Chemistry. Bovine serum albumin (BSA) was obtained from Amresco (America). KBr (FT-IR grade) was purchased from Sigma. All of other chemicals were analytical grade and were used as received without further purification. All solutions were prepared with deionized water as a solvent.

Mesoporous characterization: Mesoporous structures of the two hosts were obtained by X-ray diffraction methods. Powder XRD of the immobilized enzyme systems and the support were taken on a Phillips diffractometer with CuK_α radiation (40 kV, 30 mA) over a 2θ range from 5 to 45°. The equipment was connected to a DACO-MP microprocessor using a Diffract-AT software. The pore size, pore volume and surface-to-volume were obtained by a series of analysis and calculations on N_2 adsorption desorption isotherms. And low-temperature N_2 adsorption desorption experiments were performed on a Gemini V 2380 system. FT-IR spectra were collected on a Bruker Tensor 27 spectrometer. Samples were prepared using the standard KBr disk method and were measured at 500-4000 cm^{-1}. The differences between free and immobilized enzyme can be recognized by FT-IR, through which, whether alkaline protease was immobilized or not can be known clearly.

Enzyme immobilization: 10 mg of mesoporous materials was dissolved in 1 mL 5 mg/mL of alkaline protease solution (with 0.1 M pH 7.5 phosphate buffer). And the mixture was incubated in constant temperature oscillation incubator (HZQ-X100) at 50 °C under stirring of 150 rpm for 2 h. After incubation, the mixture was transformed in 1.5 mL eppendorf tubes and centrifuged (1-15 K, sigma) at 10000 rpm for 10 min. The supernatant was collected and the suspension was washed twice with 1 mL buffer mentioned above and immobilized enzyme was obtained. All the supernatant was mixed together to determine its protein assay. Each adsorption experiment was done in triplicate. Finally, the amount of immobilized enzyme was calculated according to mass balance of alkaline protein concentration before and after the absorption. And then enzyme loading was determined by the amount of enzyme immobilized on each unit of mesoporous material.

Enzyme loading: Protein assay was determined using the Bradford method[36] and using BSA as the standard for protein. According to the latter method, 1 mL of enzyme solution is mixed with 1 mL of Bradford reagent. Followed by 10 min of incubation, the concentration of the protein is determined at 595 nm using UV spectrophotometer (UV-2550, Shimadzu Corporation). The amount of protein immobilized on the support was calculated by substracting the protein recovered in the combined washing of the mesoporous material complex from the added protein[37].

Enzyme loading can be calculated according to the formula below:

$$\text{Enzyme loading } (\mu g/mg) = (C_1 \cdot V_1 - C_2 \cdot V_2) \cdot m_1 / (m_2 \cdot C_1 \cdot V_1) \quad (1)$$

where C_1 represents the enzyme concentration in the initial solution and V_1 is the volume of the initial enzyme solution, C_2 the enzyme concentration of supernatant and V_2 correspondingly the volume of supernatant. Besides, m_1 refers to the mass of enzyme in the initial solution and m_2 the mass of mesoporous carrier added in the initial solution.

Measurement of enzyme activity: The activity of free alkaline protease was measured using mixture of 1 mL of pH 8 phosphate buffer solution containing 10 mg alkaline enzyme and 1 mL of 0.5 % of casein solution. The mixture was incubated at 37 °C for exact 5 min and the reaction was stopped by adding 4 mL of 10 % trichloroacetic acid. The final mixture was centrifuged at 4000 rpm for 10 min. The supernatant was obtained for calculating the absorbance at 275 nm. One unit of activity (1 U/g) is defined as the amount of enzyme required to release the trichloroacetic acid hydrolysis of casein whose absorbance is equivalent of the absorbance of 1 μg of tyrosine.

The activity of immobilized enzyme was similar to the free enzyme with a little adjustment. That is 10 mg of immobilized enzyme instead of 10 mg of free enzyme. Other steps are the same with the measurement of free enzyme activity. Activity retention for the immobilized enzyme was determined by the ratio between the activity of the immobilized enzyme and the activity of the same amount of the free enzyme.

Stability of immobilized enzyme

Effect of pH on free and immobilized enzyme activity: Effect of pH on stability of immobilized enzyme was investigated by dispersing 10 mg of free and immobilized enzyme into 1 mL buffer of broad range of pH (5, 6, 7, 8, 9). The mixture was incubated at 37 °C for 2 h. The measurement of activity followed the steps of the measurement of enzyme activity.

Effect of temperature on free and immobilized enzyme activity: Effect of temperature on stability of immobilized enzyme was investigated by dispersing 10 mg of free and immobilized enzyme into pH 8.0 of phosphate buffer solution at different temperatures, 30, 40, 50 and 60 °C, respectively and incubating for 2 h. The measurement of activity followed the steps of the measurement of enzyme activity.

Enzyme kinetics: The initial reaction velocity of casein hydrolyzation catalyzed by alkaline protease was measured with various concentrations of casein from 0.5 to 3 % at 37 °C, pH 8.0. The Michaelis-Menten constant (Km) and apparent maximum velocity (v_{max}) were obtained from Lineweaver-Burk plot.

Calculation of enzymatic activity recovery: The activity recovery of alkaline protease was determined by the formula below.

$$\text{Enzymatic activity recovery (\%)} = \frac{v_2}{v_1} \times 100 \qquad (2)$$

where v_2 is the activity of final immobilized enzyme and v_1 is the activity of initial immobilized enzyme.

RESULTS AND DISCUSSION

XRD results of mesoporous materials structure: Fig. 1 shows the powder XRD patterns of SBA-15 and MCM-41. Three well-resolve XRD peaks corresponding to (100), (110) and (200) reflections were observed, which can be indexed to a two-dimensional hexagonal lattice (p6mm)[38] of the mesoporous material SBA-15. The powder X-ray diffraction pattern of the sample exhibits four diffraction peaks. By assuming a hexagonal symmetry for the MCM-41 structure[39], these peaks corresponding to d (100) of 3.908 nm, d (110) of 2.28 nm, d (200) of 1.97 nm and weak d (210), indicative of a typical MCM-41 XRD reflection pattern. The figures reveal the well-ordered mesoporous structure of SBA-15 and MCM-41.

Measurement of pore size of mesoporous materials: The pore size of mesoporous materials were determined by N_2 adsorption instrument and pore structure parameters of materials were given in Table-1. The results show pore volume and pore diameter of SBA-15 are larger than MCM-41, while the latter is better than the former as for the surface area.

TABLE-1
PORE STRUCTURE PARAMETERS OF MATERIALS

Samples	Surface area (m^2/g)	Pore volume (cm^3/g)	Pore diameter (nm)
SBA-15	662.2186	1.181367	6.5087
MCM-41	806.103	1.030188	3.793

Condition: Cu K_α radiation λ = 1.54060Å at 40 kV and 30 mA

Nitrogen adsorption desorption isotherms of samples were shown in Fig. 2. Both samples exhibited typical type IV isotherms with clear hysteresis loops of H1 type associated with capillary condensation at high relative pressure, which is typical of hexagonal cylindrical channel mesoporous materials. Furthermore, the relatively sharp increase of SBA-15 in

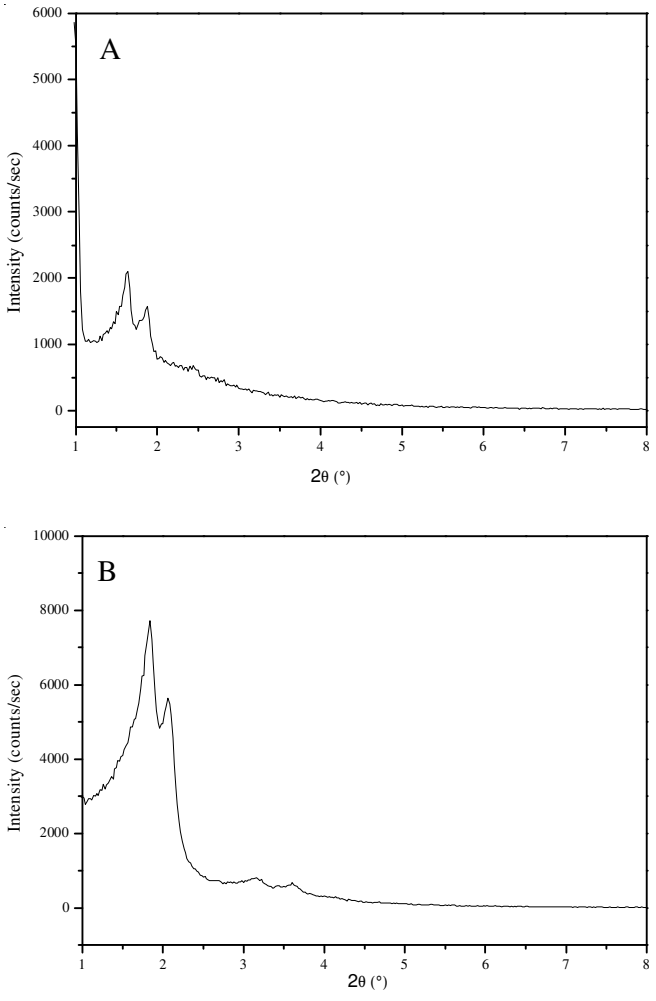

Fig. 1. Powder X-ray diffraction patterns of mesoporous materials, A SBA-15, B MCM-41

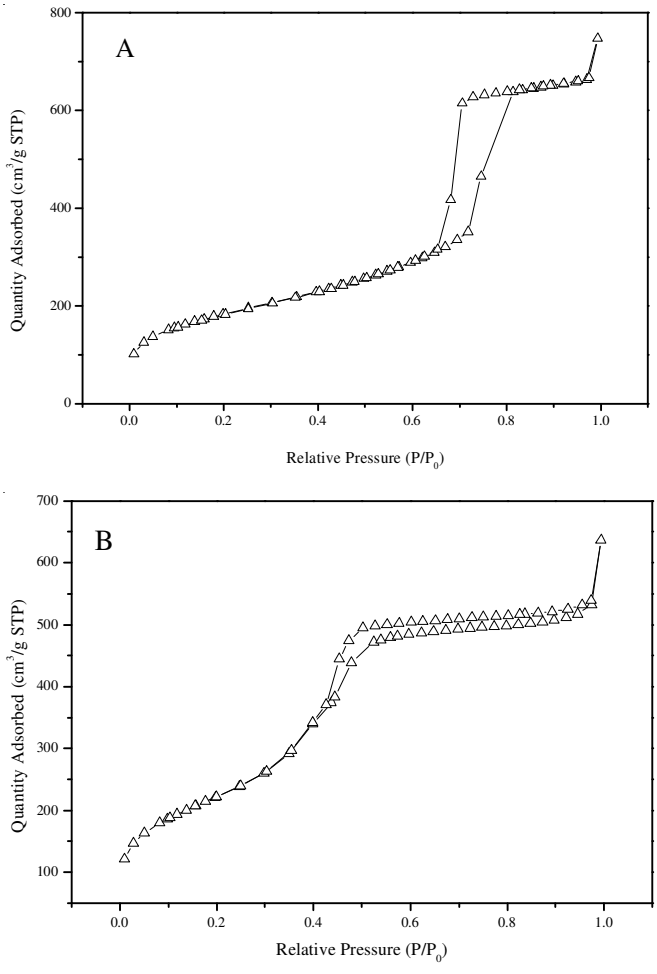

Fig. 2. N_2 adsorption isotherms of SBA-15 and MCM-41, A SBA-15, B MCM-41

adsorbed volume between $P/P_0 = 0.65$-0.80 suggests a uniform size pore system. Hysteresis loop shape of MCM-41 may be caused by the use of CTMB (hexadecyl trimethyl ammonium bromide) as surfacant leading to a smaller pore size.

Screening of mesoporous materials for alkaline protease immobilization: Parameters of immobilized enzyme and free enzyme were given in Table-2. As seen in the table, enzyme loading and enzymatic activity recovery of alkaline protease immobilized on SBA-15 were better than that of enzyme immobilized on MCM-41, revealing eminent application potential of SBA-15 in alkaline protease immobilization.

Comparison of structure of carrier and immobilized enzyme: The differences between carrier and immobilized enzyme were shown by FT-IR. The FT-IR spectrums between carrier and immobilized enzyme were quite different except several peaks of SBA-15 at 810 and 1080 cm^{-1} (Fig. 3). The two peaks correspond to the stretching of nonbridging oxygen atoms of Si-O-H bonds and symmetric stretching (Si-O-Si) of the inter tetrahedral oxygen atoms in calcined SBA-15, respectively[40]. The brand peak area from 2000 to 1600 cm^{-1} originates from amide band I and amide band II of alkaline protease, which verifies the linkage between carrier and alkaline protease[41], verifying that alkaline protease has been immobilized successfully.

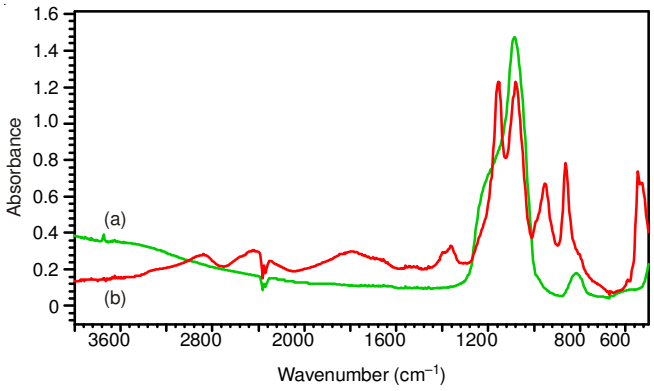

Fig. 3. Fourier transform infrared spectrum of carrier (SBA-15) and immobilized enzyme, (a) carrier, (b) immobilized enzyme. Condition: 1 mg of sample baked for 5 h at 130 °C mixed with 200 mg of KBr in disk method.

Optimum immobilization condition of immobilized enzyme

Effect of immobilization's time on immobilization efficiency: The effects of immobilization's time on enzyme loading and enzymatic activity recovery were shown in Fig. 4A. Enzyme loading increased as immobilization's time increased up to 4 h, but this increase leveled off after 4 h because of the resistance from the increasing amount of enzyme adsorbed in

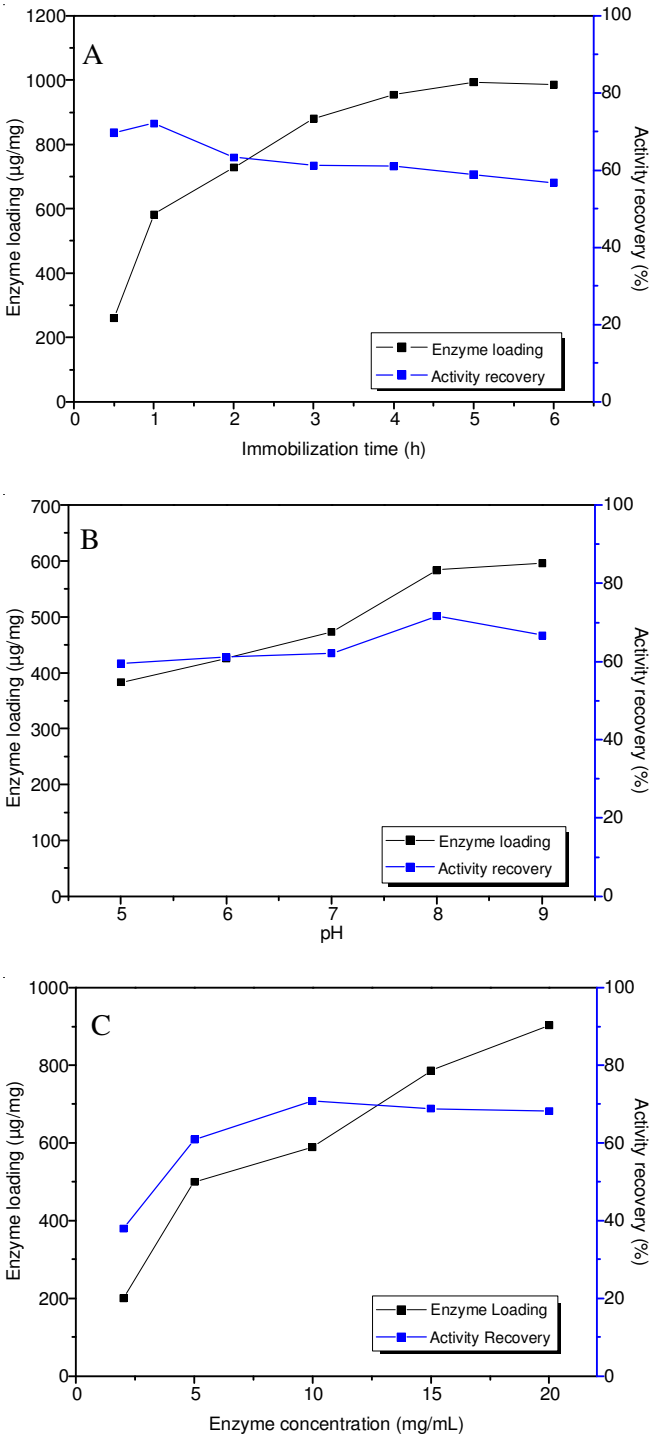

Fig. 4. Effects on alkaline protease immobilization efficiency, A immobilization's time, B pH, C enzyme concentration. Immobilization condition: 0.5 g/mL of enzyme solution per gram of support. The optimum conditions were determined weighing the enzyme loading and activity recovery

TABLE-2
PARAMETERS OF IMMOBILIZED ENZYME AND FREE ENZYME

Sample	Enzyme loading (μg/mg)	Activity recovery (%)	K_m (g/mL)	v_{max} g/(mL min)
SBA-15	487.00	71	0.558	0.4205
MCM-41	211.17	41	0.623	0.3742
Free enzyme	-	-	0.509	1.9238

Conditions: Enzyme immobilization condition,10 mg mesoporous materias dispersed in 1 mL 5 mg/mL of alkaline protease solution (with 0.1 M pH 7.5 phosphate buffer). Protein essay was determined using the Bradford method and BSA (bovine serum albumin) as the standard protein. Enzyme kinetics was determined with casein of different concentration ranging from 0.5 to 3 % at 37 °C, pH 8.0.

mesoporous materials pores. Thus, the maximum of enzyme loading of 1000 μg/mg was observed. However, enzymatic activity recovery reached its maximum and decreased suddenly at time range from 1 to 2 h of the process. A slow decrease was observed with the prolongation of immobilization's time after 2 h. So the optimum coupling time for alkaline protease immobilized on SBA-15 was 1 h.

Effect of pH on immobilization efficiency: The pH activity profile of alkaline protease was shown in Fig. 4B. Optimum pH for alkaline protease immobilization was determined by enzyme loading and enzymatic activity recovery in different buffers within the pH range of 5-9. Enzyme loading and enzymatic activity recovery increased in the pH range of 5 to 8, which may be attributed to the interactions of charges caused by the difference of isoelectric point between SBA-15 and alkaline protease. Further increase of pH had little effect on the enzyme loading. While high pH value led to the decrease of enzymatic activity recovery from pH 8 to 9. Considering the both factors, 8 was the optimum pH for alkaline protease immobilized on SBA-15.

Effect of enzyme concentration on immobilization efficiency: The effect of various enzyme concentration on the immobilization efficiency was presented in Fig. 4C. The immobilization pH was kept at 8.0 for 1 h. Enzyme loading increased along with enzyme concentration from 2.5 to 20 mg/mL. And the maximum enzyme loading reached 900 μg/mg when the enzyme concentration was 20.0 mg/mL. Enzymatic activity recovery kept a similar increase tendency to enzyme loading as enzyme concentration increased from 2.5 to 10 mg/mL. The curve reached its highest point where the enzymatic activity recovery was 70 % at the enzyme concentration of 10 mg/mL. After which, the further increase of enzyme concentration had an opposite effect on the enzymatic activity recovery. Considering the actual cost, 10 mg/mL was the optimum enzyme concentration of alkaline protease immobilized on SBA-15.

Operational stability of enzyme immobilization

pH stability study of free and immobilized enzyme: Free and immobilized enzyme were incubated at different solution with pH range from 5 to 9 at 37 °C for 1 h. Enzymatic activity recovery of free and immobilized enzyme were shown in Fig. 5A. Enzymatic activity recovery of immobilized enzyme was higher than that of free enzyme at various pH and a large increase was observed in pH range from 6 to 9. The optimum pH was 9 for both free and immobilized enzyme, while immobilized alkaline protease was more stable than free enzyme in pH range from 5 to 9.

Temperature stability of free and immobilized enzyme: Free and immobilized enzyme (pH 9.0) were incubated at different temperature for 1 h. The effect of temperature profile on free and immobilized enzyme was studied in temperature range of 30-60 °C (Fig. 5B). Enzymatic activity recovery of immobilized enzyme was higher than that of free enzyme at various temperature, which revealed that temperature stability of enzyme was improved after being immobilized. Whereas, enzymatic activity recovery of both free and immobilized enzyme decreased along with the further increase of temperature and a significant decrease appeared at a relatively high temperature of 50 °C, explaining that both free and immobilized enzyme cannot stand the high temperature of 50 °C or even higher.

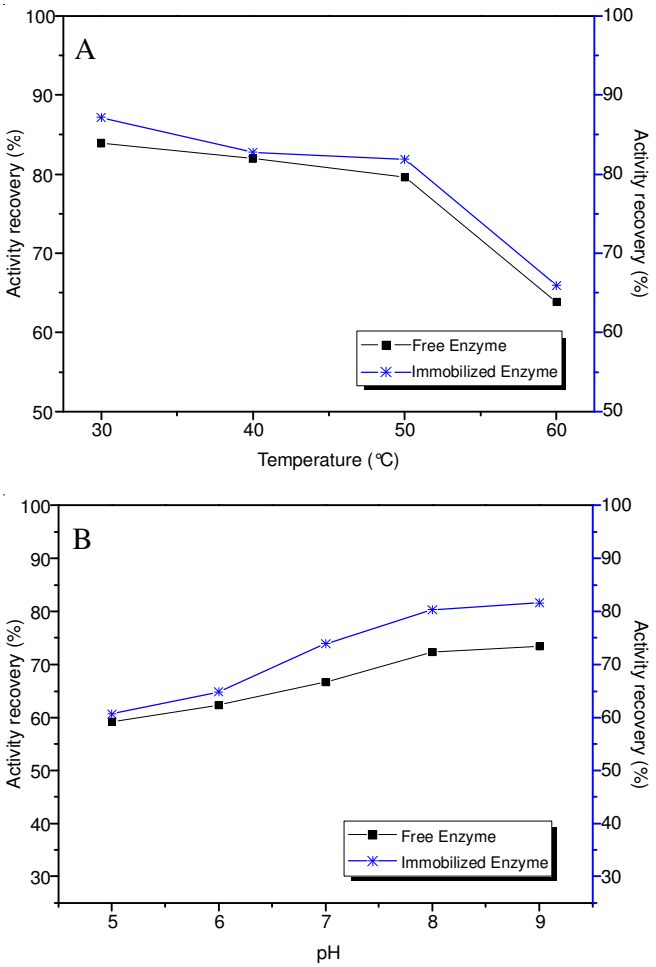

Fig. 5. Stability of immobilized enzyme, A temperature, B pH. Activity recovery is the ratio of enzyme activity after and before processing

Kinetics of free and immobilized alkaline protease: Change in kinetic parameters upon immobilization is also a critical point for evaluating the success of an immobilization process. The effects of substrate concentration on the initial velocity of free and immobilized alkaline protease were shown in Fig. 6. The apparent Km and ν_{max} values for the free and immobilized alkaline protease were calculated from Lineweaver-Burk plots by using the initial rate of the enzymatic reaction. Apparent K_m and ν_{max} values were calculated from the interception x- and y-axis, respectively[42]. The reaction rate increased linearly as the concentration of casein went up before reaching the plateau values. Substrate inhibition occurred at higher than 2 % g/mL. It is evident that the apparent K_m value for the immobilized enzyme is (0.558 g/mL) a little higher than that of the free one (0.509 g/mL), suggesting that enzyme immobilization by this method caused a little decrease in the enzyme-substrate affinity. This higher K_m value can be attributed to the parting or mass diffusional resistance of the carrier against substrate and/or product and less porous structure of SBA-15. The apparent Km value of immobilized alkaline protease was the same order of magnitude whereas the ν_{max} of the immobilized form was 4.6 times lower than

that of free form. Thus, the hydrophilic modification of SBA-15 may be the major factor leading to a lower value of apparent K_m and obtaining a better affinity for immobilized alkaline protease.

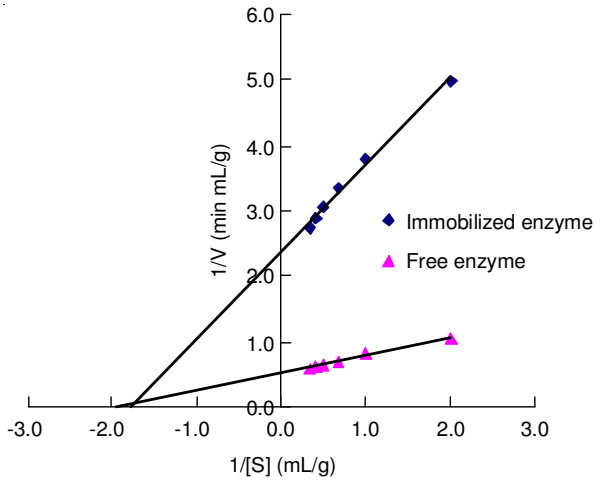

Fig. 6. Lineweaver-Burk plot for the free and immobilized enzyme. Using casein as the enzyme substrate, casein was dissolved in water containing 0.01 M NaOH

Conclusion

Two kinds of mesoporous materials of SBA-15 and MCM-41 were compared in the study. SBA-15 was the more suitable carrier for alkaline protease with two higher indexes than MCM-41. Optimum immobilization conditions (1 h, pH 8, enzyme concentration 10 mg/mL) were obtained by studying the effects of immobilization temperature, pH and enzyme concentration on immobilization efficiency. And the enzyme loading reached 589.43 μg/mg, energy recovery 70.86 % at the optimum condition. Stability of temperature and pH on immobilized enzyme were studied and the results showed that immobilized enzyme prepared in the method had a high stability of temperature and pH and was more convenient to be applied in industrial process.

ACKNOWLEDGEMENTS

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