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Immobilization of alkaline serine endopeptidase from *Bacillus licheniformis* on SBA-15 and MCF by surface covalent binding

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ABSTRACT

An industrial enzyme, alkaline serine endopeptidase, was immobilized on surface modified SBA-15 and MCF materials by amide bond formation using carbodiimide as a coupling agent. The specific activities of free enzyme and enzyme immobilized on SBA-15 and MCF were studied using casein (soluble milk protein) as a substrate. The highest activity of free enzyme was obtained at pH 9.5 while this value shifted to pH 10 for SBA-15 and MCF immobilized enzyme. The highest activity of immobilized enzymes was obtained at higher temperature (60 °C) than that of the free enzyme (55 °C). Kinetic parameters, Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}), were calculated as K_m = 13.375, 11.956, and 8.698×10^{-4} mg/ml and V_{max} = 0.156, 0.163 and 0.17×10^{-3} U/mg for the free enzyme and enzyme immobilized on SBA-15 and MCF, respectively. The reusability of immobilized enzyme showed 80% of the activity retained even after 15 cycles. Large pore sized MCF immobilized enzyme was found to be more promising than the SBA-15 immobilized enzyme due to the availability of larger pores of MCF, which offer facile diffusion of substrate and product molecules.

1. Introduction

Alkaline proteases are important enzymes having diverse applications in a wide variety of industries such as food, pharmaceutical, detergent, leather, silk, diagnostics, and for the recovery of silver from used X-ray films [1–4]. Alkaline serine endopeptidase from selected strain of *Bacillus licheniformis*, which hydrolyses casein, plays a vital role in dairy industries. Casein is a slowly digesting protein which is essential in building muscles in human body and is a major component of milk, whey and soy [5]. The industrial application of this enzyme requires specificity, stability at higher pH, temperature and reusability. Numerous techniques have been used for immobilization of free enzymes on solid support to obtain efficient biocatalysts [6]. Mesoporous silicas (MPSs) obtained by different templating methods demonstrate high potential as solid supports for enzyme immobilization [7–22]. These supports are environmentally acceptable, structurally more stable, and resistant to microbial attack. MPSs have a large specific surface area (~1000 m^2/g) and pore diameter, in the range of 2–50 nm, which can be tuned to host the enzymes of varied size. As such, the enzymes have considerable affinity towards the MPSs surfaces [7–14]. Besides, the MPSs surface can be modified with various anchor groups to covalently bind the enzyme molecules, which could reduce the enzyme leaching from the support during the recycling of the catalyst [14–22]. The uniform distribution of pores in MPSs favors the uniform loading of enzyme as well as facile diffusion of the substrate and product molecules inside the channels. The loading of an enzyme and its activity depend upon the surface area and pore size of the MPSs [17–22]. Among the MPSs, SBA-15 and MCF have been shown to be efficient supports for covalent immobilization of α-amylase, trypsin, chloroperoxidase, penicillin G acylase, organophosphorus hydrolase, glucose oxidase, glucoamylase, and invertase [8–11,14,19–22]. SBA-15 and MCF have pore sizes in the range of 9–25 nm and they possess 600–800 m^2/g surface area, which make them suitable to host the alkaline endopeptidase comfortably and also allow substrate and product molecules facile diffusion towards and from the active site of the enzyme [18,21]. The average size of the alkaline endopeptidase is 4.7 nm as shown in Fig. 1, produced using Chem3D Pro 10.0 from RCSB enzyme database [23].

Alkaline endopeptidase immobilized on polymeric support has been evaluated [24–26] for stability, activity and reusability with reference to free enzyme. In the present study, covalent immobilization of alkaline serine endopeptidase has been done through amide bond formation on modified SBA-15 and MCF. Surface

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modification of MPSs was done with 3-aminopropyltriethoxy silane followed by succinic anhydride. The specific activity of the immobilized enzyme was studied using casein as a substrate.

2. Materials and methods

2.1. Materials

Tetraethoxysilane (98%, TEOS), Triblock poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) copolymer (Pluronic P123, M.W. = 5800), Bradford reagent (B6916), Na_2HPO_4 (99%, A.R.) were from Aldrich (USA). 3-Aminopropyl triethoxy silane (98%, APTES), 1-[3-(dimethylamino)propyl]-3-ethyl carbodiimide hydrochloride (EDAC) were purchased from Fluka (USA). Bacterial alkaline protease (E.C.3.4.21.62) from *B. licheniformis* was donated by Genencor International, Netherlands. Trichloroacetic acid (98%, A.R.) was procured from Merck, Germany. 4-*N*,*N*-dimethylaminopyridine (98%, A.R., DMAP), 1,3,5-trimethylbenzene (98%, A.R., TMB), Soluble casein A.R., $NaH_2PO_4{\cdot}2H_2O$ (99% A.R.), Na_2CO_3 (98%, A.R.) $NaHCO_3$ (98%, A.R.) were purchased from S.d. Fine Chemical, India. Glycine (98%, A.R.), NaOH (98%, A.R.) were from Qualigens Fine Chemicals, India.

2.2. Synthesis of SBA-15

Highly ordered mesoporous SBA-15 was synthesized [27] using Pluronic P123 triblock copolymer (EO20–PO70–EO20, BASF) as a template and TEOS as a silica source in acidic conditions. In a typical synthesis, 12 g of triblock P123 was dissolved in 90 g of de-ionized water and 360 g of 2 M HCl was added under stirring at ambient temperature (25–30 °C) for 90 min. TEOS, 27 g was added to the homogeneous surfactant solution and the mixture was stirred at 40 °C for 24 h. Then, it was allowed to stand for crystallization under static hydrothermal conditions at 110 °C for 48 h in a Teflon lined autoclave reactor. The crystallized product was filtered, washed with de-ionized water and dried. Finally, it was calcined at 550 °C in air for 6 h to remove the template.

2.3. Synthesis of MCF

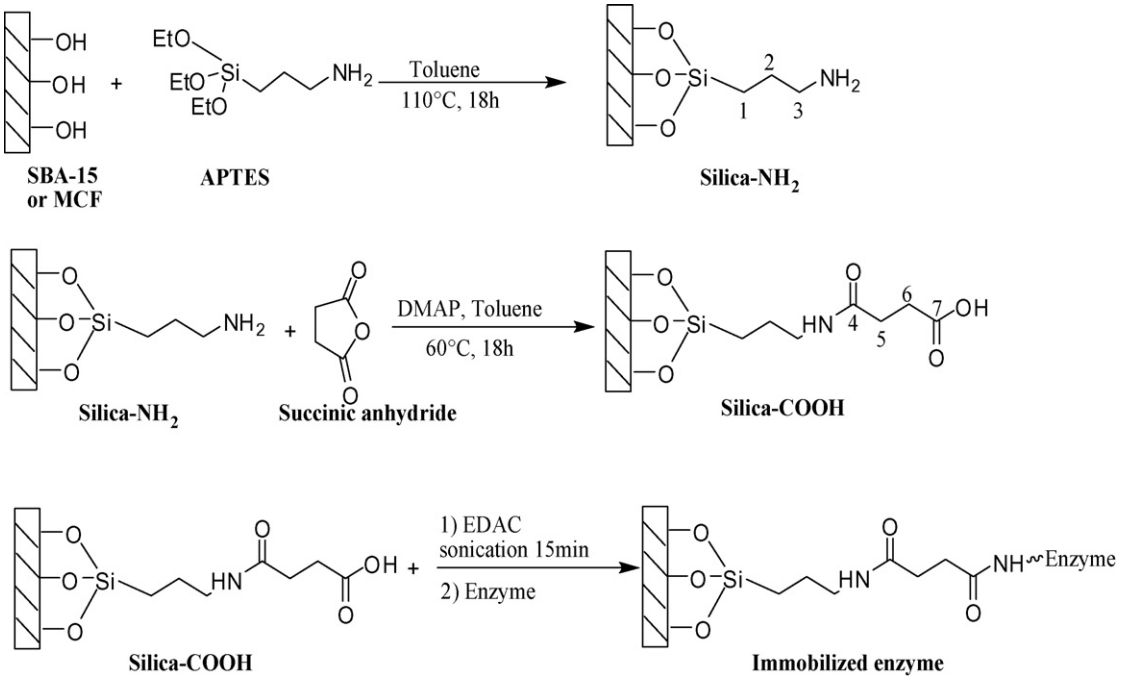
Siliceous MCF syntheses are reported in the literature [28–33]. However, in the present work, MCF was synthesized using the procedure reported by Lettow et al. [29]. In a typical synthesis, 4 g of BASF Pluronic P123 was dissolved in 150 ml of 1.6 M HCl followed by addition of 2 g of TMB to the polymer solution. The mixture was stirred for at least 90 min at 35–40 °C. Amount of 8.5 g TEOS was added as the silica source to this homogeneous emulsion. This mixture was stirred for 24 h at 35–40 °C and aged for 24 h at 110 °C, the product was filtered, washed with de-ionized water and dried. Finally, it was calcined at 500 °C in air for 6 h to remove the template.

2.4. Surface modification of SBA-15 and MCF

The surfaces of MPSs were amino functionalized by refluxing 1 g of SBA-15 or MCF with 3 ml of APTES in 100 ml of dry toluene at 110 °C for 16 h with stirring under N_2 atmosphere. Product was separated by filtration, washed with toluene, dichloromethane and methanol. It was further Soxhlet-extracted using dichloromethane to remove excess APTES. APTES modified samples were labeled as SBA-A and MCF-A. The amino functionalized SBA-15 or MCF was succinylated typically, by adding 1 g into 0.4 g of succinic anhydride and a catalytic amount of DMAP in 100 ml of dry toluene at 60 °C for 16 h shown in Scheme 1. The product was filtered, washed with toluene and dichloromethane. The samples obtained after succinylation were designated as SBA-S and MCF-S.

2.5. Characterization

Powder X-ray diffraction patterns of calcined samples were recorded with a Philips X'Pert MPD system using Cu Kα X-ray radiations (λ = 1.54056 Å) in (2θ = 0.5–10°) range. The textural parameters (surface area, S_{BET}; pore volume, V_p and pore diameter d_p) of calcined and surface modified MPSs were obtained from N_2 adsorption data measured at 77.4 K using a volumetric adsorption set-up (Micromeritics ASAP 2010, USA). All the samples were degassed at 50 °C for 3 h prior to N_2 adsorption. The



Scheme 1.

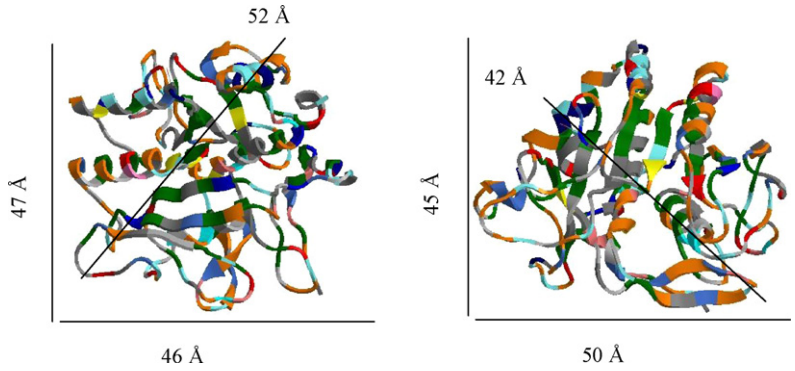


Fig. 1. Alkaline serine endopeptidase structure from RCSB protein database.

specific surface area of the sample was calculated by using the multiple-point Brunauer–Emmett–Teller (BET) method in the relative pressure range (P/P_0) of 0.05–0.3. The pore size distribution was determined using the Barrett–Joyner–Halenda (BJH) method, and pore sizes were obtained from the peak positions of the distribution curves. Thermogravimetric analysis of calcined, aminated and succinylated MPSs were carried out with a thermal analyzer (Mettler–Toledo TGA/SDTA 851e) up to 850 °C with a heating rate of 10 °C/min under N_2 flow (100 ml/min). Fourier transform infrared (FTIR) spectra were collected on a PerkinElmer Spectrum GX FT-IR instrument in the range of 400–4000 cm^{-1}. CHN elemental analyses were performed on a PerkinElmer (CHNS/O, 2400) analyzer. ^{13}C solid-state magic-angle spinning (MAS) nuclear magnetic resonance (NMR) spectra were obtained on a Bruker DRX500 MHz with MAS speed of 8 kHZ.

2.6. Immobilization of enzyme

In a typical procedure, functionalized SBA-15 or MCF (100 mg) was dried at 100 °C in a vacuum oven for 30 min. To these dried samples, 25 mg of EDAC was added and the mixture ultrasonicated for 15 min. Alkaline serine endopeptidase (250 μl) diluted with an equal amount of buffer solution was added drop by drop into carboxyl activated SBA-15 or MCF and allowed to incubate for 30 min. The excess protein was removed by washing with excess of buffer and unbound enzyme was estimated by Bradford method [34].

2.7. Activity assay of the enzyme

Activity of both the free and immobilized enzyme was measured by incubating free (70 μl) or immobilized enzyme with 1 ml of casein (0.5%) solution in 0.1 M bicarbonate buffer at pH 9.5, for exactly 20 min at 45 °C with a stirring speed of 150 rpm. The reaction was stopped by the addition of 3 ml of 10% (w/v) of trichloroacetic acid and the precipitate was removed by centrifugation. The absorbance due to the amino acid produced was measured at 280 nm using a UV–vis–NIR scanning spectrophotometer (CARY 500 SCAN), with L-tyrosine as a standard. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyse casein to increase one absorbance unit at 280 nm due to 1 μmol of tyrosine produced per min at 45 °C. The kinetic parameters of free and immobilized enzyme were evaluated by incubating with casein concentration of 0.1–0.65% and following the above described procedure.

2.8. Effect of pH and temperature on enzyme activity

The enzymes were incubated with casein in 0.1 M bicarbonate buffer solution having a pH of 9.5 at temperatures ranging from 30 to 80 °C, and alternatively incubated at a fixed temperature of 45 °C with pH ranging from 7 to 12 (phosphate buffer, 7–8.5; bicarbonate 9–11; glycine–NaOH 11.5–12). The activity of alkaline serine endopeptidases at different pH and temperature was then measured according to the previously described (Section 2.7) method.

2.9. Reusability and deactivation stability of the immobilized enzyme

The initial activity of the immobilized enzyme was measured and then compared with the activity of the used enzyme obtained after its repeated use for 15 cycles with 3 cycles per day. After each cycle, the immobilized enzyme was immediately filtered, washed with buffer solution and stored at 5 °C. The deactivation stability of free and immobilized enzyme was studied by deactivating at pH 11, holding for different time intervals at a temperature of 60 °C and comparing the activities with the activity of fresh enzymes.

3. Results and discussion

3.1. Characterization of synthesized and surface modified silicas

X-ray diffraction patterns of the calcined SBA-15 and MCF samples given in Fig. 2 show the presence of the three reflection peaks corresponding to 100, 110 and 200 confirming the presence of the ordered hexagonal mesoporous structure of SBA-15. There was no significant peak observed for MCF. As shown in Table 1(a) the synthesized and modified MPSs with organosilane

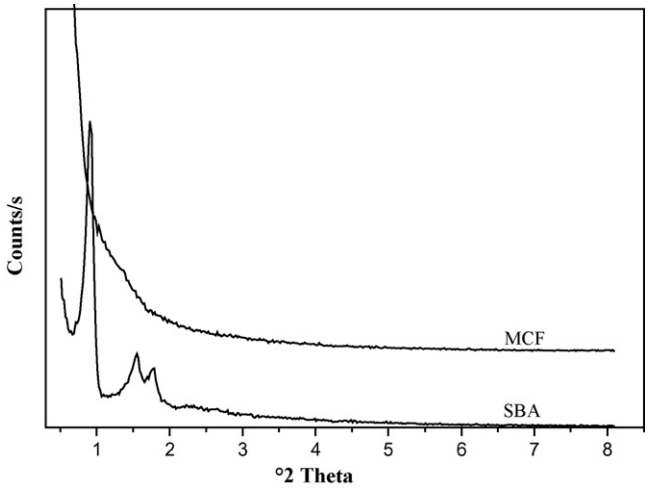


Fig. 2. Powder XRD pattern of calcined SBA-15 and MCF.

Table 1
Physico-chemical characterization data of calcined (SBA, MCF), aminated (SBA-A, MCF-A) and succinylated (SBA-S, MCF-S)

Sample	N_2 adsorption (a)			Elemental (CHN) analysis (b)		
	Surface area S_{BET} (m^2/g)	Total pore volume V_p N_2 (cm^3/g)	Pore diameter d_m (Å)	C (%)	H (%)	N (%)
SBA	638	0.91	94	–	–	–
SBA-A	352	0.23	80	09.41	4.82	3.36
SBA-S	116	0.20	63	19.73	3.21	2.85
MCF	682	0.82	218	–	–	–
MCF-A	336	0.42	187	11.26	3.00	3.91
MCF-S	116	0.33	178	20.00	3.35	2.89

followed by succinylation resulted in a decrease of all textural parameters like surface area, pore volume and pore diameter. The calcined samples have higher BET surface area, pore volume and pore diameter. On amination of SBA-15 and MCF, all the above parameters were observed to decrease sharply. The sharp decrease in adsorbed volume at relative pressure (P/P_o) in the range of 0.2–0.9 is an indication of uniform functionalization on the surface (Fig. 3(a) and (b)). However, succinylated MPSs were observed to have slightly lower structural parameter values compared to aminated MPSs. This shows that amination and then succinylation have occurred inside the pores as well as on the surfaces of the MPSs. The pore diameter values of succinylated SBA (63 Å) and MCF (178 Å) are large enough to host the alkaline serine endopeptidase inside their channels. However, the possibility of some enzyme molecules to be present on the external surface cannot be ruled out.

The amino functionalization followed by succinylation of SBA-15 and MCF was analyzed by FTIR spectroscopy. Fig. 4(a) shows the FT-IR spectra for calcined SBA, SBA-A and SBA-S. FT-IR spectra for calcined MCF, MCF-A and MCF-S are given in Fig. 4(b). The broad band at 3600–3000 cm^{-1} for hydrogen bonded silanol [35,36] was appreciably reduced in the modified samples. The organosilane presence was identified by the absorbance of the band 2950–2850 cm^{-1} for the propyl chain [9] and the deformation bands at 1455–1410 cm^{-1} [36]. The N—H absorption band overlapped with O—H bands at 3300–3500 cm^{-1} [9]. The presence of bands at 1710 cm^{-1} (—C=O, acid), 1695–1650 cm^{-1} (—C=O amide I band), 1566–1561 (—NH amide II band) and 1415–1419 cm^{-1} (—C—N amide) confirmed that succinylation had taken place [37].

The calcined silicas have no C, H and N. However, upon functionalization with APTES followed by succinylation, C, H and N were observed in modified MPSs (Table 1(b)). On succinylation of aminated MPSs the overall N% is expected to decrease and the data indeed show this trend.

The data shown in Fig. 5(a) and (b) are TGA of calcined, aminated and succinylated SBA-15 and MCF samples, respectively. The TGA curves of pristine calcined samples showed no appreciable weight losses, aminated samples showed one sharp weight loss between 368 and 617 °C with 9% weight loss due to aminopropyl group decomposition. Succinylated samples gave two sharp weight losses, 123–226 °C with 8% weight loss due to succinyl and 436–587 °C with 3% weight loss due to aminopropyl decompositions on MPSs.

The amination of MPSs [34] and succinylation were further confirmed by solid state ^{13}C MAS–NMR spectroscopy as shown in Fig. 6.

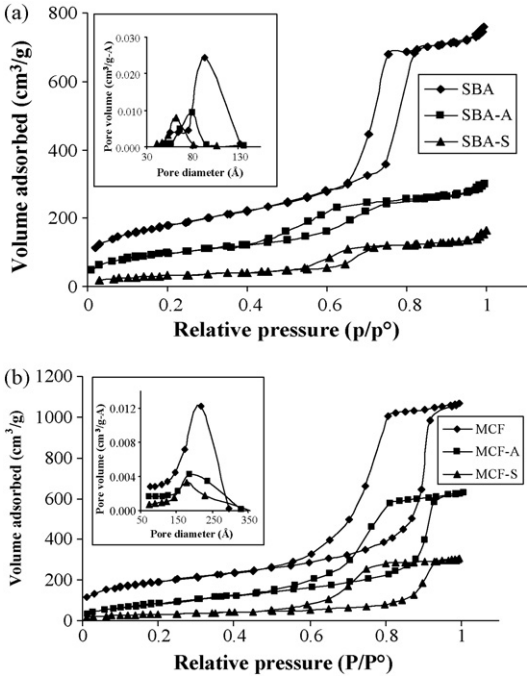


Fig. 3. (a) N_2 adsorption–desorption isotherm and pore size distribution of SBA, SBA-A and SBA-S. (b) N_2 adsorption–desorption isotherm and pore size distribution of MCF, MCF-A and MCF-S.

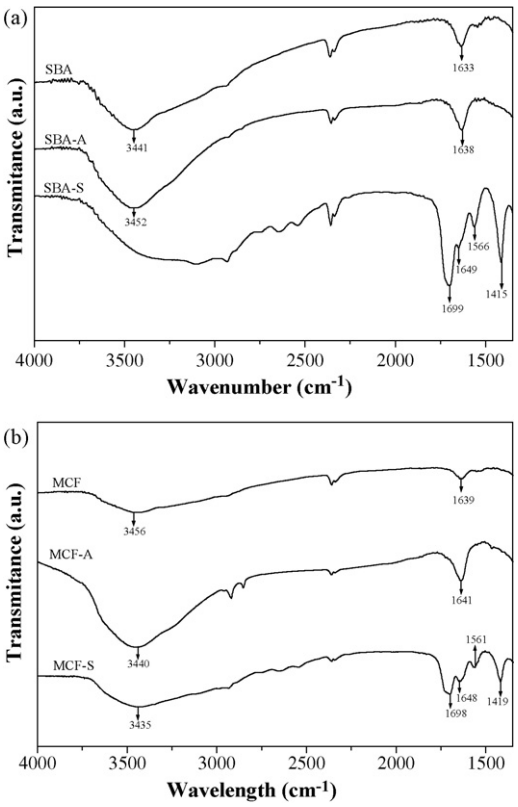


Fig. 4. (a) IR spectra of calcined (SBA), aminated (SBA-A) and succinylated (SBA-S). (b) IR spectra of calcined (MCF), aminated (MCF-A) and succinylated (MCF-S).

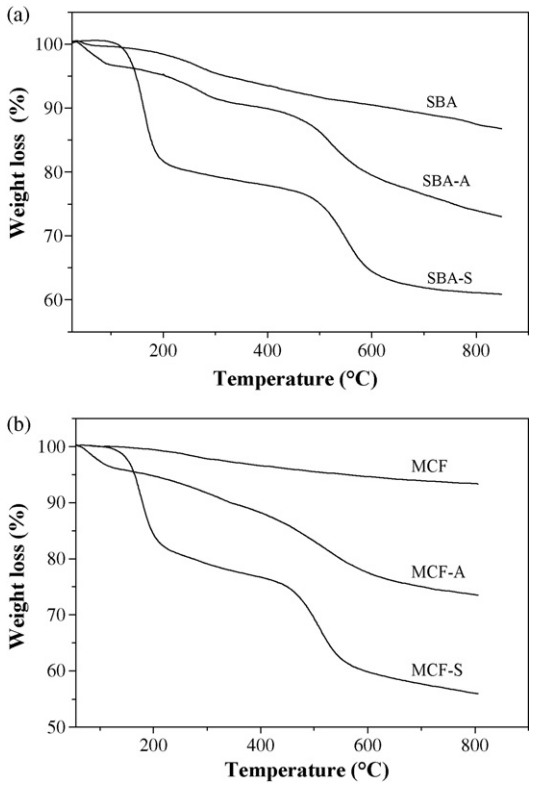


Fig. 5. (a) TGA curve of calcined (SBA), aminated (SBA-A) and succinylated (SBA-S). (b) IR spectra of calcined (MCF), aminated (MCF-A) and succinylated (MCF-S).

The SBA-A sample gave peak values at 7.51 ($-C_1-$), 19.13 ($-C_2-$) and 39.81 ($-C_3-$) ppm and the MCF-A sample at 39.87 ($-C_1-$), 19.04 ($-C_2-$) and 7.27 ($-C_3-$) ppm. The chemical shift values for the SBA-S methylene carbons were 7.08 ($-C_1-$), 19.85 ($-C_2-$), 39.76 ($-C_3-$), 25.53 ($-C_5-$) and 28.27 ($-C_6-$) and for the carbonyl carbons 174.71 ($-C_4-$, amide) and 177.31 ($-C_7-$, acid) ppm. In case of MCF–S methylene carbons were at 7.37 ($-C_1-$), 18.66 ($-C_2-$), 39.49 ($-C_3-$), 25.39 ($-C_5-$), 27.21 ($-C_6-$) and carbonyl carbons were at 174.40 ($-C_4-$, amide) and 177.24 ($-C_7-$, acid) ppm.

3.2. Activity of the enzyme

The kinetic parameters of the Michaelis–Menten equation were determined for free and immobilized enzymes. Experiments were carried out by using various substrate concentrations 0.1–0.65% as shown in Fig. 7(a). The MCF immobilized enzyme shows higher specific activity than the SBA immobilized and the free enzyme. The Michaelis–Menten constant (K_m) and the maximum reaction velocity (V_{max}) values were calculated from Lineweaver–Burk plots (Fig. 7(b)) by the linear regression method with R^2 value of 0.95. K_m values were found to be 13.375, 11.956 and 8.698×10^{-4} mg/ml for free, SBA-15 immobilized, and MCF immobilized enzymes, respectively. V_{max} values were calculated as 0.156, 0.163 and 0.17×10^{-3} U/mg for free, SBA-15 immobilized, and MCF immobilized enzymes, respectively. For immobilized enzymes, the calculated V_{max} value was found to be higher than that of the free enzyme. The calculated V_{max} value for MCF immobilized enzyme was higher than that of the SBA-15 immobilized enzyme though the loading of the enzyme was the same. The difference observed in the kinetic parameters could be due to the difference in diffusion of substrate and product molecules in SBA-15 and MCF. There were no significant structural changes of the enzyme upon immobilization on SBA-15 and MCF. The specific activity of MCF (218 Å) immobilized enzyme was higher than that of SBA (94 Å) immobilized enzyme, which implies that larger pore diameter facilitates the diffusion of the casein (dimension ~50 Å, M.W. = ~30 kDa) towards the active sites of the enzyme where it is gets hydrolyzed [8]. This increased activity could also be due to the easy accessibility of substrate molecules to active sites of covalently attached enzyme particularly with the 8-atom long chain carrier inside the pore as well as on the external surface of the MPSs.

Fig. 7(c) shows the relative specific activity of the free enzyme and the immobilized enzymes at pH values ranging from 7 to 12. In the free enzyme, the specific activity increased with increasing pH upto a pH of 9.5, after that the activity started to decrease. In case of SBA and MCF immobilized enzymes, the optimum pH was found to be 10 beyond which a decrease in specific activity was seen.

Fig. 7(d) shows the relative specific activity of the enzyme at different temperatures. In the free enzyme, the specific activity increased with increasing temperature, but beyond 55 °C the activity showed a sharp decrease. But in the case of SBA and MCF immobilized enzymes, the optimum temperature was found to be

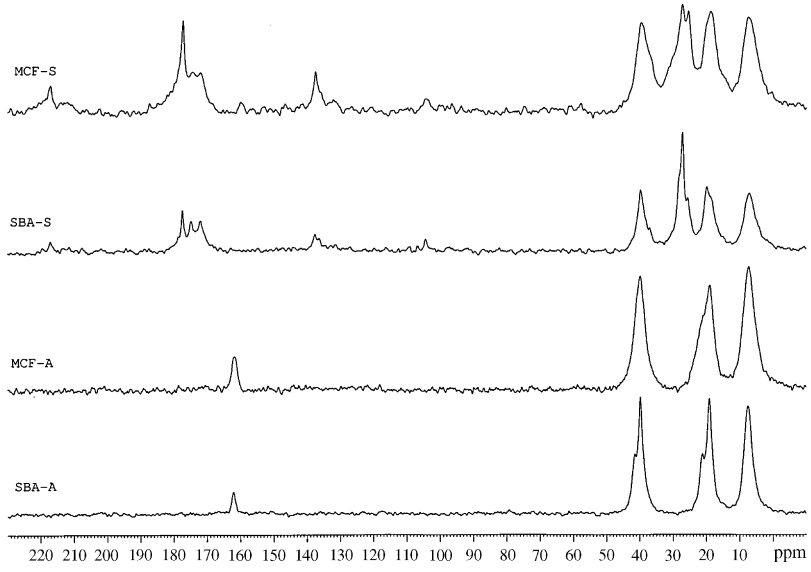


Fig. 6. Solid ^{13}C MAS–NMR spectra of aminated (SBA-A, MCF-A) and succinylated (SBA-S, MCF-S).

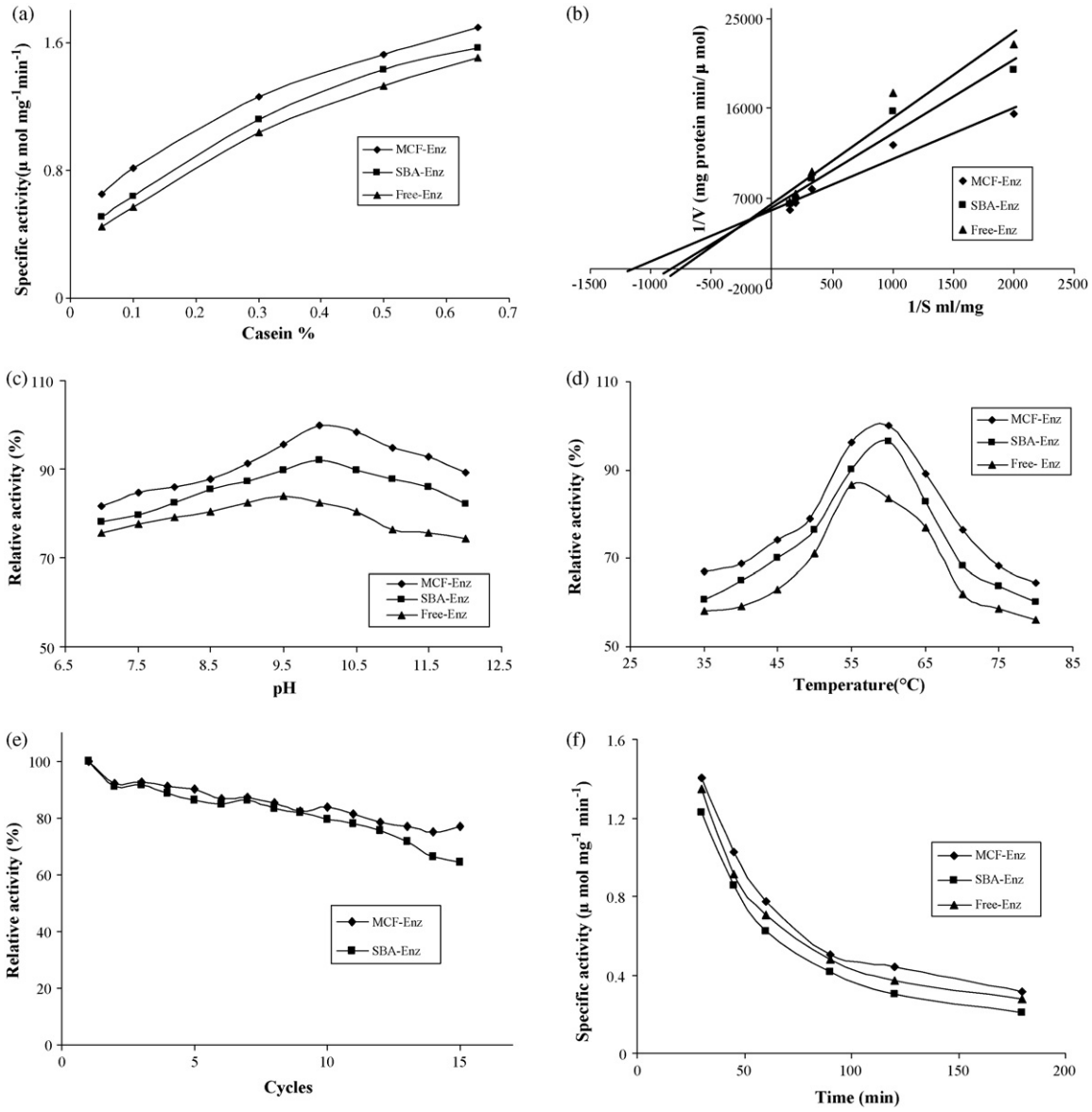


Fig. 7. (a) Comparison of activity at different substrate (casein) concentrations. (b) L–B plot for kinetic parameters of free and immobilized enzymes. (c). Effect of pH on free and immobilized enzymes activities. (d). Effect of temperature on free and immobilized enzymes activities. (e). Effect of reuse number on the enzyme's activities. (f). Effect of deactivation of free and immobilized enzyme's activities.

60 °C and the specific activity decreased gradually at temperatures higher than that.

The reusability data of SBA and MCF immobilized enzymes are shown in Fig. 7(e). The relative activity of MCF immobilized enzyme was higher than that of the SBA immobilized enzyme. Initially, there was a rapid decrease which could be due to the leaching of ionic immobilized and encapsulated enzyme. The activity was found to decrease after every cycle because of loss of small amount of enzyme immobilized MPSs in each cycle. The relative specific activity of deactivation studies at different time intervals is shown in Fig. 7(f). The specific activities of free and immobilized enzymes decrease with increasing time. However, the SBA and MCF immobilized enzymes showed higher specific activity than the free enzyme.

4. Conclusions

An efficient biocatalyst was prepared by covalent immobilization of the enzyme alkaline serine endopeptidase on SBA-15 and MCF. Mesoporous SBA-15 and MCF immobilized enzyme have higher specific activity, pH and temperature stability and better reusability compared to free enzyme. However, due to its large pore size MCF was found to be a more suitable support for hosting the enzyme comfortably in its pores and offer access to enzyme active sites for casein substrates.

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