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# Isolation and immobilization of alkaline protease on mesoporous silica and mesoporous ZSM-5 zeolite materials for improved catalytic properties

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ABSTRACT

Alkaline protease from brinjal leaf (*Solanum melongena*) having milk clotting activity has been purified to 9.44 fold to a final specific activity of 45.71 U/mg. SDS-PAGE of the final preparation revealed a single protein band of approx 14 kDa. Purified enzyme was characterized and was successfully immobilized into the amorphous mesoporous silica (SBA-15) and crystalline mesoporous zeolite (Nano-ZSM-5) using entrapment method. Maximum immobilization of 63.5% and 79.77% was obtained with SBA-15 and Nano-ZSM-5, respectively. This protocol serves as a novel approach for bioprocesses, mainly as milk coagulant for local dairy products and particularly, cheese making, and opens the new dimension of further research and other innovation.



## 1. Introduction

Proteases are among the most important hydrolytic enzymes that have been extensively studied since the advent of enzymology. Plants proteases are gaining importance due to their compatibility for biotechnological applications, their broad substrate specificity and catalytic performance [1]. Proteases accounts for approximately 60% of global market of industrial enzymes. *In vivo,* they play very important role in metabolic and other regulatory functions from digestion to apoptosis. Applications of proteases ranges from tannery, brewing, hydrolyzed protein products to meat processing and in dairy industry [2]. Dairy use of protease is determined by the production of cheese/curd from milk. Most cheese is made by coagulating milk to produce curds (solids) and whey (liquid) as by-product. Milk coagulation is caused by the action of proteases on milk proteins. Though, worldwide most of the cheese production processes use chymosin protease from the animal resources, but demand of vegetarian resources high protease driven coagulating efficiency and lower general proteolytic activity is still highly desired.

Stability of enzymes is key importance in a variety of commercial and industrial applications due to harsh conditions than in the laboratory assays. For economical and environmental considerations, it is also very important that catalyst should be reusable through a number of duty cycles. Nowadays mesoporous silica materials have attracted significant attention for enzyme immobilization because of their better biocompatibility, stable mesoporous structures, larger surface areas, and tunable pore sizes and volumes [3–11]. As a protein/enzyme immobilizing matrix, mesoporous materials can incorporate proteins through physical or chemical action with good adsorption due to its large specific surface area. The available size range, approximately 3–10 nm, is comparable with the hydrodynamic radius of most enzymes of practical interest in biotechnology. Zeolites are microporous materials and have pore dimensions in the range of 0.4–1.5 nm [12,13]. These pore dimensions of microporous zeolites are too small for the purpose of biomolecule encapsulation, thereby limiting their use in biocatalysis. Recently, there is a surge of efforts made to prepare mesoporous zeolites that have inter-/intra-crystalline mesoporoes in the range of 2–10 nm. [14–16].

Therefore, objective of the present study was to isolate a protease from brinjal leaf (*Solanum melongena*) and immobilized on the matrix of amorphous mesoporous silica SBA-15 and mesoporous zeolite Nano-ZSM-5. Stability and catalytic activity of the immobilized enzyme were investigated under various storage and operational conditions. Further, potential use of the isolated and immobilized proteases was investigated in the cheese production.

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## 2. Material and methods

### 2.1. Plant material and chemicals

*S. melongena* was grown at the experimental farm of Centre of Innovative and Applied Bioprocessing (CIAB), Mohali, India. The leaves were sampled and surface sterilized with 1% (v/v) sodium hypochlorite for 10 min and then rinsed thoroughly with Milli Q water followed by surface demoistened with a tissue paper. Protease was isolated from the dried leaves. All the chemicals were of analytical or electrophoresis grade. Unless stated otherwise, all chemicals were purchased from Sigma Aldrich, USA. Deionized water from Millipore Milli-Q system (resistivity 18.2 MΩ cm) was used throughout the experiments.

### 2.2. Enzyme extraction and purification

All purification steps were carried out at 4 °C and centrifugations were performed at 14,938*g* rcf using eppendorf centrifuge 5804 R for 30 min, unless stated otherwise. In a typical batch of purification, crude extract was prepared by homogenization of the tissue in 10 mM Tris–HCl buffer (pH 9.0). The extract was squeezed through two layers of muslin cloth and the supernatant was obtained from the homogenate by centrifugation for uses as crude enzyme preparation.

Differential ammonium sulfate fractionation was carried out and the protein precipitating between 35–70% saturation was retained for further purification of the enzymes. The precipitate was dissolved in minimum volume of Tris–HCl buffer (pH 9.0), and dialyzed extensively against the same buffer. Activity and protein concentration in dialyzed samples were determined by following the methods as described ahead.

The dialyzed ammonium sulfate fraction was loaded onto a DEAE Sepharose column (1.6 cm × 20 cm) pre-equilibrated with 10 mM Tris–HCl buffer (pH 9.0) and eluted with same buffer with a salt concentration gradient of 0–1 M NaCl. The active and catalytically enriched fractions were pooled, concentrated by Amicon Ultracentricon (Millipore, Bedford, MA, USA cutoff MW 10 kDa), assayed for protein content and analyzed for its catalytic traits.

### 2.3. Enzyme assay

Protease activity was assayed by following the modified method of Anson (1938) for soluble and immobilized enzyme using casein (0.65%) as substrate [17]. In a typical experiment, 1 mL of suitably diluted enzyme was incubated with 1 mL of 0.65% casein solution in 10 mM Tris–HCl buffer (pH 9.0) for 10 min at 37 °C. Reaction was terminated by the addition of an equal volume of 10% (w/v) trichloroacetic acid. In the case of immobilized enzyme, the reaction mixture was transferred to fresh test tube after incubation. The precipitate was removed by filtration and centrifugation. The filtrates were mixed with 1 mL of 500 mM $Na_2CO_3$ solution and 0.5 mL of two-fold diluted Folin's reagent. After vigorous mixing, the color was allowed to develop for 30 min at 37 °C and was monitored using UV–visible spectrophotometer at 660 nm. A control/blank assays (containing no enzyme) was run in parallel in each assay.

One unit (IU) protease was defined as the amount of enzyme that hydrolyzed casein to releases 1.0 μmol (181 μg) of tyrosine per minute at 37 °C and pH 9.0. The specific activity was expressed in the units of enzyme activity per milligram of protein (U/mg). The enzyme activity computations in IU/ml were made using the following equation:

$$IU=\frac{\mu M \text{ tyrosine released X Volume of the reaction mixture (ml) X Dilution factor}}{\text{Reaction Time (min) X Volume of the enzyme used (ml) X Volume used for calorimetric estimation}}$$

Protein concentration was determined by following the Bradford method using DL-tyrosine as standard [18].

### 2.4. Electrophoresis

Purity of the enzyme preparation was assessed through with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system (Mini-PROTEAN II gel system (Bio-Rad Laboratories), using slab gels (0.5 mm thick, 10% polyacrylamide)) by following the Laemmli method [19]. Apparent subunit molecular mass of protease was calculated by linear regression analysis from the data obtained by SDS-PAGE using protein markers with molecular mass ranging from 10 to 250 kDa. SDS-PAGE gels were stained by Coomassie Brilliant Blue R.

### 2.5. Zymography

For Casein zymography, 10% polyacrylamide gels were copolymerized with 0.12% w/v casein by following the reported procedure of Choi et al. [20]. After electrophoresis, the gel was incubated in 10 mM Tris–HCl buffer (pH 9.0) at room temperature for 1 h containing 2.5% Triton X-100 with gentle agitation, to remove the excess of SDS. The gel was then incubated in a zymogram development buffer (0.1 M Tris–HCl buffer (pH 9.0) containing 0.2 M NaCl and 5 mM $CaCl_2$ ) at 40 °C for 2 h. The gel was stained with Coomassie brilliant blue R-250 (0.5% w/v) for 30 min. The activity band was observed as a clear colorless area depleted of casein in the gel against the blue background after de-staining.

### 2.6. Synthesis of Nano-ZSM-5 and SBA-15

In a typical synthesis of Nano-ZSM-5, 1.2 g of sodium aluminate (53 wt% $Al_2O_3$, 43 wt% $Na_2O$, Riedel-deHaën) was dissolved in 25 mL of distilled water (Solution A). 2.06 g of propyl triethoxysilane (PrTES, 97% Aldrich) was mixed with 25 mL of tetrapropylammonium hydroxide (TPAOH, 1 M aqueous solution) (Solution B). Solution A and solution B were mixed and the resultant solution was stirred for 15 min at ambient condition until it became a clear solution. 18.7 g of tetraethoxy orthosilicate (TEOS, 98% Aldrich) was added into the resultant solution and stirring was continued for 6 h. The molar composition of the gel was 90 TEOS/10 PrTES/2.5 $Al_2O_3$/3.3 $Na_2O$/25 TPAOH/2500 $H_2O$. This mixture was transferred to a Teflon-lined autoclave, and hydrothermally treated at 170 °C for 3 days under static conditions. The final product was filtered, washed with distilled water, and dried at 100 °C. Material was calcined at 550 °C for 6 h under flowing air.

SBA-15 was synthesized using molar gel composition: TEOS/0.016 P123/0.46 HCl/127 $H_2O$. In a typical synthesis, 1.78 g of pluronic block copolymer P123 (MW 5800, Aldrich) was added to 24 mL of distilled water. After stirring for 4 h, a clear solution was obtained. Then, dilute aqueous HCl solution (0.92 g HCl+20 mL water) was added and the solution was stirred for another 2 h. This was followed by addition of 4 g of TEOS and stirring for another 24 h at 40 313 °C. The reaction mixture was transferred to an autoclave and aged under static condition for 48 h at 100 °C to get the product. The resultant solid was filtered, washed, dried at 100 °C, and calcined at 550 °C for 6 h.

### 2.7. Immobilization of brinjal protease on porous matrix

Immobilization of protease in SBA-15 and Nano-ZSM-5 was performed as follow: 1 g of the SBA-15/ Nano-ZSM-5 was suspended in enzyme solution having specific activity 47.95 U/mg in 0.1 M Tris–HCl buffer (pH 9.0) at 37 °C under stirring condition for 24 h in 2 mL eppendorf tube. The suspension was centrifuged at rcf 6708g in a minispin centrifuge (from eppendorf) to settle down the bounded enzymes and further it was re-suspended in the same buffer by gentle tapping the eppendorf tube. Process was repeated thrice in order to remove the unbound enzyme. For the comparative stability, the biological activity of both the free as well as immobilized enzymes was expressed in terms of a "specific activity (U/mg)".

### 2.8. Steady state kinetics

#### 2.8.1. Influence of reaction parameters

The effect of pH on the activity of soluble and immobilized protease was investigated by assaying the catalytic activity at different pH and appropriate buffers, such as 10 mM sodium acetate buffer (pH range 3.0–5.6), 10 mM phosphate buffer (pH range 5.7–8.0), and 10 mM Tris–HCl buffer (pH range 8.0–10.0). The substrate solution (0.65% casein) was prepared in the respective pH buffers and the enzyme preparation (soluble and immobilized) were incubated for 10 min at 37 °C. The % of maximum enzyme activity was computed and the values were plotted against different pH. Further, the activity of immobilized enzyme was compared with the soluble counterpart.

The optimum temperatures of catalytic activity for soluble and immobilized enzyme were determined by assaying the enzyme activity at different temperature in the range of 25–100 °C using 10 mM Tris–HCl buffer (pH, 9.0) in a water bath. The relative activity level as of % of maximum activity were calculated by the method described above and were plotted against the respective temperature.

#### 2.8.2. Determination of $K_m$ and $V_{max}$

Effect of substrate concentration on protease activity was investigated at 37 °C by varying the casein concentration from 0 to 10 mg/mL in 10 mM Tris–HCl buffer at their optimum pH for soluble and immobilized enzymes. The activity assays were performed as stated above. Michaelis–Menton constant $K_m$ was determined using a double reciprocal (Lineweaver–Burk) plot. All calculated parameters were the mean of triplicate determinations from three independent preparations.

#### 2.8.3. Storage stability and reusability

For storage stability studies, soluble and immobilized proteases were incubated in 10 mM Tris–HCl buffer at their optimal pH. Both the enzymes preparations were stored at 4 °C under identical conditions. The activity of immobilized enzyme was determined using the assay procedure mentioned in the previous section. Immobilized protease was tested for % initial activity at regular intervals. Similarly the activity of fresh soluble protease was also checked at regular intervals. After each assay, the immobilized enzyme was washed with 10 mM Tris–HCl buffer and stored at 4 °C as stated above and % of initial activity was plotted as a function of time.

The initial activity of the immobilized enzyme was measured and the conjugate was reused 12 times over a period of 10 days, and measuring % of initial activity of first cycle was measured. For each cycle assay, the immobilized protease were washed with 10 mM Tris–HCl buffer (at their optimal pH), dried and stored at 4 °C.

#### 2.8.4. Milk coagulation/cheese production

For milk coagulation, 1 mL (47.95 U/mg) of the extract was mixed with 25 mL of low fat (12 wt% milk powder in water) pasteurized milk (containing 0.02 g of $CaCl_2$ in 100 mL of water) and incubated at 37 ° C. The clotting time for the period elapsed from the time when the coagulant was added and when milk clotting occurred was noted.

### 2.9. Material characterizations

X-ray diffraction (XRD) patterns were recorded in the 2θ range of 5-50° with a scan speed of 2°/min on a PANalytical X'PERT PRO diffractometer using Cu Kα radiation ($\lambda = 0.1542$ nm, 40 kV, 20 mA). Transmission electron microscopy (TEM) measurements were carried out on a JEOL (model 1200 EX) microscope operating at 100 kV. Nitrogen adsorption measurement at 77 K was performed by Autosorb-IQ Quantachrome Instruments volumetric adsorption analyzer. Sample was out-gassed at 423 K for 4 h in the degas port. The specific surface area was determined by Brunauer–Emmett–Teller (BET) method using the data points of $P/P_0$ in the range of about 0.05–0.3.

## 3. Result and discussion

### 3.1. Purification, biochemical properties, and immobilization of brinjal protease in porous matrix

Alkaline protease was extracted from brinjal leaf and was purified 9.44 fold to a final specific activity of 45.71 U $mg^{-1}$ (after purification). The results of the protease purification process procedure are summarized in Table 1. The purified enzyme showed a single band corresponding to a molecular mass of- 14 kDa on SDS-PAGE (pH 9.0), under reducing conditions (Fig. 1A). The molecular mass of the purified brinjal protease obtained in this study was similar to the molecular mass of protease isolated from other plant sources [21]. Zymogram activity staining revealed clear zone of proteolytic activity against the blue background for purified sample at corresponding positions in SDS-PAGE using a simple renaturing and staining protocol described by Laber and Balkwill [22] (Fig. 1, lane D). The amount of protease immobilized on SBA-15/Nano-ZSM-5 was estimated by subtracting the specific activity protease determined in supernatant after immobilization from the specific activity of protease used for immobilization Table 2. Maximum immobilization of 63.5% and 79.77% was obtained with SBA-15 and Nano-ZSM-5, respectively Table 2.

### 3.2. Synthesis and characterization of silicate materials

In this study, amorphous mesoporous silica SBA-15 (known as Santa Barbara Amorphous-15) and crystalline mesoporous Nano-ZSM-5 were prepared and used for the protease immobilization. SBA-15 exhibited low angle XRD pattern corresponding to hexagonal mesoporous silica materials (JCPDS card number 00-049-1711). SBA-15 showed only broad XRD patterns in the range of 23-27 degree, which confirmed the amorphous nature of the material. Nano-ZSM-5 exhibited only wide angle XRD pattern, which corresponds to MFI-framework structure (JCPDS card number 00-044-0002) (Fig. 1B). MFI is a three letter code suggested by the International Zeolite Association for ZSM-5 (also known as Zeolite Socony Mobil-5) framework topology. $N_2$-adsorption isotherms showed that SBA-15 and Nano-ZSM-5 both exhibited type IV isotherm (Fig. 1C). However, the hysteresis loop obtained in the $N_2$-adsorption studies for these materials was found to be different. SBA-15 shows H1 hysteresis, which is characteristic of mesoporous materials with one-dimensional cylindrical pores. A sharp increase

**Table 1**
Purification of alkaline protease from 100 g brinjal leaf.

| Steps | Total activity (units) | Total protein (mg) | Specific activity (units/mg) | Purification (fold)[a] | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 1468 | 303 | 4.84 | – | 100 |
| (NH4)2SO4 (35–70%) | 1206 | 115 | 10.47 | 2.16 | 82.15 |
| DEAE Ion-Exchange chromatography | 960 | 21 | 45.71 | 9.44 | 65.39 |

[a] Fold purification calculated with respect to the specific activity of the crude extract.

**Table 2**
Biological activity and protein concentration used for enzyme immobilization.

| Material | Bound enzyme specific activity (U/mg) | Unbound enzyme specific activity (U/mg) | % efficiency |
|---|---|---|---|
| Protease immobilized on SBA-15 | 30.45 | 17.5 | 63.5 |
| Protease immobilized on Nano-ZSM-5 | 38.25 | 9.7 | 79.77 |
| Soluble/free protease | 47.95 | – | – |

in the volume of $N_2$ adsorbed above a $P/P_0$ of 0.6 is characteristic of the capillary condensation within the mesopores. Whereas, Nano-ZSM-5 exhibited H3 hysteresis loop, which confirmed non-uniform slit shape pores (Fig. 1C Inset). $N_2$ adsorption in the region $0.4 < P/Po < 0.9$ in the case of Nano-ZSM-5 is interpreted as condensation in intercrystalline mesoporous void spaces. Surface area and pore volume were obtained from the sorption data and summerized in Table 3. High resolution TEM (HR-TEM) micrographs of SBA-15 shows two different views observed with the electron beam, perpendicular (Fig. 2A) and parallel (Fig. 2B) to the axis of the hexagonal arranged mesopores. Fig. 2A shows uniform channels, whereas Fig. 2B shows hexagonal array of uniform channels of the SBA-15. TEM images of Nano-ZSM-5 showed that the round/egg shape particles were built with an assembly of tiny crystallites of about 20 nm in diameter or less (Fig. 2 C). No changes in the morphology and XRD pattern of the materials were observed after the immobilization of the enzyme, which confirmed that the material was stable after the enzyme immobilization (Supporting information, Fig. S1 A,B).

### 3.3. Steady state kinetics

#### 3.3.1. Optimum pH and temperature

The influence of pH on the activity of free and immobilized protease is presented in Fig. 3A. The optimum pH for the soluble protease and immobilized protease on Nano-ZSM-5 was found to be 9.0. However, optimum pH of 10.0 was found for SBA-15. It may be noted that surface and residual charges on the solid matrix and the nature of the bound enzyme may lead to the change in the immediate vicinity of the enzyme molecule, which in turn changes the nature of the active enzyme protein and finally cause a shift in the optimum pH of the enzyme activity [23]. SBA-15 was synthesized in the acidic medium and contained no Al in the framework, whereas Nano-ZSM-5 was prepared in basic medium and contained Al in the framework, therefore both these matrices will interact with the protease enzyme differently. Due to the different matrix–enzyme chemical interaction, optimum pH was found to differ slightly in the two cases. It may further be noted that an increase in optimum pH was observed for the enzyme immobilized in the solid matrix [24].

Fig. 3B shows the effect of temperature on protease immobilized on SBA-15 and Nano-ZSM-5. Soluble protease from brinjal exhibited an optimum temperature of 70 °C, whereas immobilized protease exhibited the optimum temperature of 75 °C. A

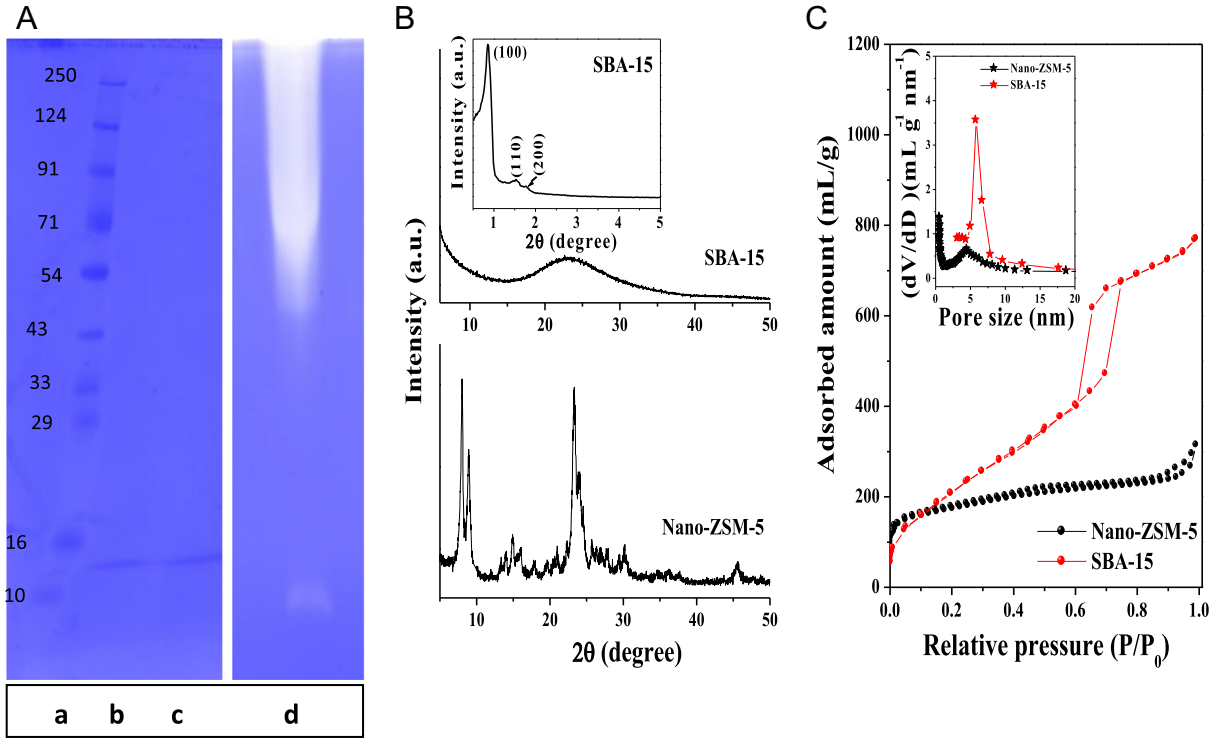


**Fig. 1.** (A) Electrophoresis pattern of brinjal protease. Molecular weight markers in (lane A), Coomassie staining of SDS-PAGE (lane B, C), Zymogram activity of purified alkaline protease (lane D); (B) XRD patterns and (C) N2-adsorption isotherms of Nano-ZSM-5 and SBA-15. Inset of (C) shows pore size distribution.

**Table 3**
Textural properties of materials investigated in this study.

| S.No. | Catalyst | Total surface area ($m^2$/g) | External surface area ($m^2$/g) | Total pore Volume (cc/g) |
|---|---|---|---|---|
| 1. | Nano-ZSM-5 | 492 | 246 | 0.412 |
| 2. | SBA-15 | 823 | 629 | 1.268 |

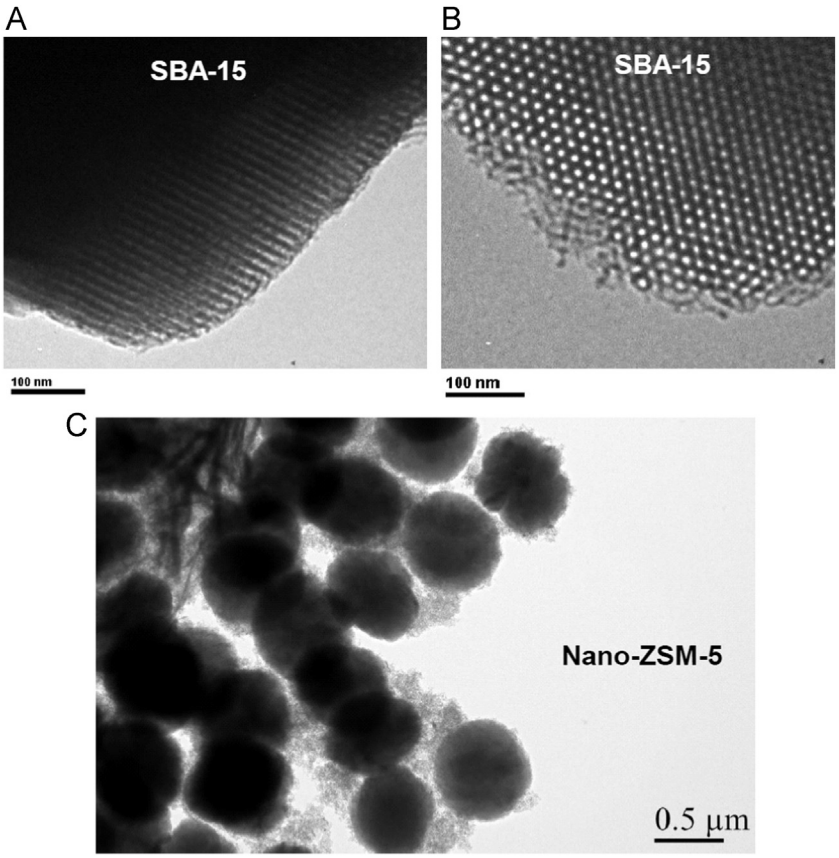


**Fig. 2.** TEM micrographs of SBA-15 observed with the electron beam, perpendicular (A) and parallel (B) to the axis of the hexagonal arranged mesopores and (C) Nano-ZSM-5 materials.

small increase in the optimum temperature for both the matrices may arise from changing the conformational integrity of the enzyme structure after entrapment. Though, the change in optimum temperature is subtle (5 °C), it may reflect a much significant gain in catalytic effect. Observed change optimum temperature is consistent with reported literature [25].

*3.3.2. Kinetics s of free versus immobilized enzyme*

$K_m$ for soluble and protease immobilized on SBA-15 and Nano-ZSM-5 were calculated using Lineweaver–Burk plot with casein as substrate (Fig. 3C). The $K_m$ was found to be 6.66 mg/mL and 3.33 mg/mL for protease immobilized on SBA-15 and Nano-ZSM-5, respectively. For the soluble protease, $K_m$ was found to be 1.66 mg/mL. An increase in $K_m$, could be due to either conformational changes in tertiary structure of enzyme or due to hindered accessibility of active site of the enzyme towards its substrate casein compared to the soluble protease and hence there was reduction in catalytic efficiency and an increase of $K_m$.

*3.3.3. Storage stability and reusability*

The storage stability of the alkaline brinjal protease and protease immobilized on SBA-15 and Nano-ZSM-5 was studied to assess its long term usage potential. Immobilized protease enzyme stored at 4 °C showed practically no leaching of enzyme over a period of two weeks. The percentage residual catalytic activity for free protease after 60 days of storage at 4 °C was 50%. Whereas, percentage residual activity was found to be 58% and 74% for SBA-15 and Nano-ZSM-5 immobilized protease, respectively, during the same period (Fig. 4A). The later represents a much significant gain in the stability of the enzyme. It may further be noted that the protease immobilized on SBA-15 showed a residual activity of 70%, whereas protease immobilized on Nano-ZSM-5 showed residual activity of 80%, after 12 reuses (Fig. 4B). The strength of binding between the matrix and enzyme is weakened in due course of repeated use, leading to leaching of enzyme from the matrix and loss in activity.

SBA-15 possess only surface silanol groups, whereas Nano-ZSM-5 possess surface silanol groups and Al acidic sites. Since enzyme has both -$NH_2$ and –COOH groups, therefore two different types of interaction sites (Al and surface –OH) are available in Nano-ZSM-5 whereas only one type of interaction sites (surface –OH) was present in SBA-15. Due to this reason, protease could bind more strongly to the Nano-ZSM-5 matrix than SBA-15 and thus Nano-ZSM-5 immobilized protease was found to exhibit more stability and residual activity than SBA-15 immobilized protease.

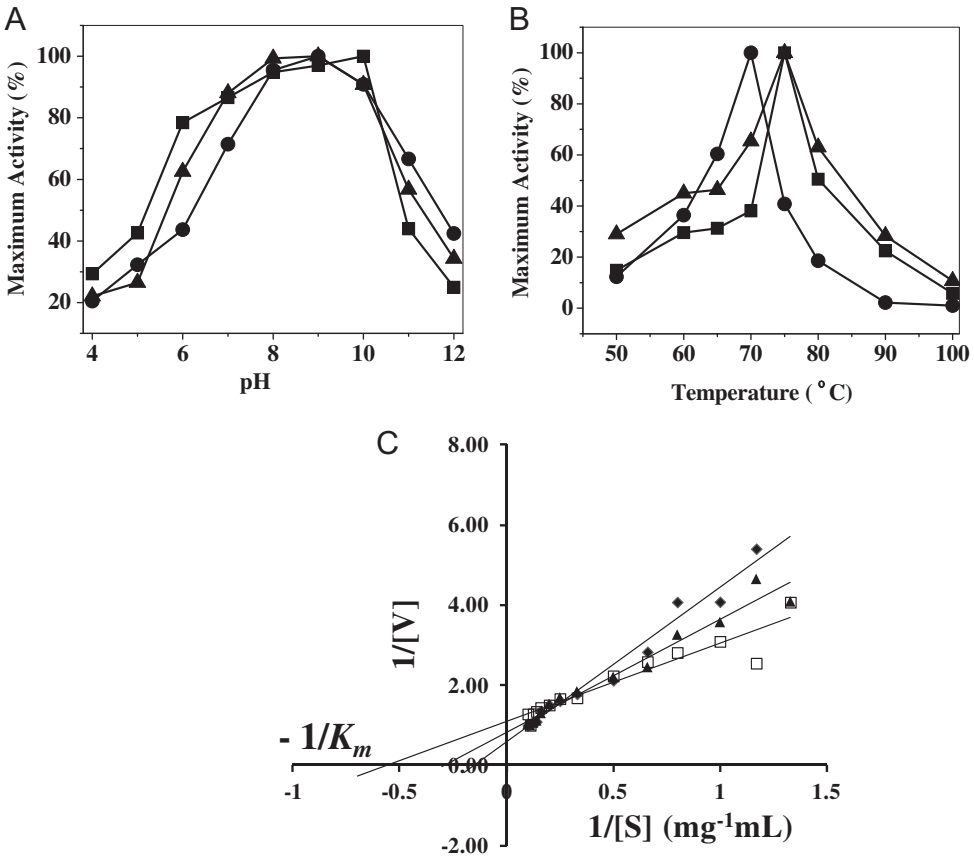


**Fig. 3.** **(A)** Effect of pH; **(B)** Effect of temperature for soluble proteases(●), proteases immobilized SBA-15 (■), and proteases immobilized Nano-ZSM-5 (▲).**(C)** Determination of $K_m$ for soluble proteases (●) proteases immobilized on SBA-15 (■), and proteases immobilized Nano-ZSM-5 (□), by Lineweaver–Burk plot method.

### *3.4. Application of soluble and immobilized enzyme for the cheese production from milk*

In general, milk coagulation, in cheese-making processes, is performed by specific hydrolysis of the peptide bond Phe-$_{105}$–Met-$_{106}$ in k-casein. This leads to in casein micelle destabilization and subsequent aggregation and finally the transformation of the milk into curd or cheese. Purified alkaline protease from brinjal leaf having high milk clotting activity, by using soluble protease, 25 mL of milk was coagulated within 40 min. Lower clotting time prevents unspecific proteolytic activity of the enzyme and hence bitter taste peptides were not produced. For industrial and economical point of view, immobilized protease was also investigated for the cheese production. For this purpose, immobilized samples (having bounded enzyme specific activity as given in Table 2) were packed in small cloth (same as the commercial tea-bag) and were dipped in 25 mL of milk. Immobilized enzymes successfully converted milk to cheese in less than 90 min. It may be noted that even though immobilized protease took 90 min for cheese production but its intrinsic activity was similar to soluble protease. Once the process was completed, immobilized samples stored in cloth bag were washed several times with water, followed by buffer and then were used in next cycle. Immobilized enzyme was successfully reused three times in the cheese formation. In these recycling experiments, cheese was produced approximately in 90 minutes, which confirms that enzyme was stable in the matrix and it was not leached to the milk solution during the cheese

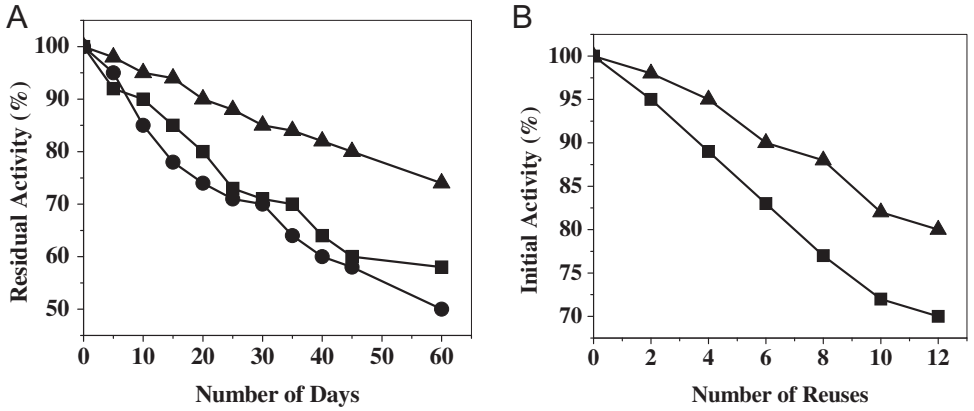


**Fig. 4.** (A)Storage stability of soluble proteases (●), protease immobilized on SBA-15 (■), and proteases immobilized on Nano-ZSM-5 (▲), for 60 days at 4 °C. (B) Reusability (12 uses) soluble protease (●) and protease immobilized on SBA-15 (■),and protease immobilized on Nano-ZSM-5 (▲), at 4 °C.

formation. Control experiment showed that Nano-ZSM-5 alone was found to be inactive in milk clotting. Further study about the quality of the cheese formed is underway in our laboratory to explore the utility of the product in dairy. This protocol serves as a novel approach for bioprocesses, mainly as milk coagulant. In addition, the isolated proteases could be used for the production of different protein hydrolysates of milk and whey protein that would be used in designing of new dietary products, as well as to obtain potentially bioactive peptides. Further the strength of binding of enzyme with Nano-ZSM-5 matrices was strong, so that the activity of the immobilized enzyme was restored and no leaching of enzyme took place. The reaction can be scaled up for industrial applications.

SBA-15 exhibited high surface area, well defined hexagonal mesopores, and large pore volume, which is good for the physical entrapment of bio-molecules. This is the reason; a large number of studies are devoted to physical adsorption of bio-molecules in mesoporous silica matrix such as SBA-15, MCM-41, KIT-6 etc [11,26]. For the immobilization of bio-molecules, both textural properties as well as chemical properties play crucial role. It may be noted that to improve the adsorption capacity of mesoporous silica, several organic functional groups such as $–NH_2$, –COOH, –SH were functionalized in the pores [27–29]. Nano-ZSM-5 exhibited comparatively less surface area and pore volume when compared to SBA-15 chosen in this study. However, the chemical environment provided by Al in the porous architecture imparts high adsorption capability. Through this study, we have shown here that Nano-ZSM-5 could be a good nanoporous material for such applications, which are not explored much.

## 4. Conclusions

Protease from brinjal was purified to homogeneity and was successfully immobilized in SBA-15 and Nano-ZSM-5 using entrapment method. Solid matrix was quite stable, less expensive, nontoxic, and preparation of biocatalyst involves mild conditions, which is an added advantage. Nano-ZSM-5 immobilized protease exhibited good activity in the cheese production and found to be reusable, which are very important with respect to immobilized enzymes for applications in dairy industry.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.05.009.

## Appendix B. Transparency document

Transparency document associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.05.009.

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