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Carboxymethyl tamarind gum–silica nanohybrids for effective immobilization of amylase

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ABSTRACT

Carboxymethyl tamarind gum initiated and catalyzed sol–gel polymerization of tetramethoxysilane produced polysaccharide nanohybrids which were efficient in immobilizing α-amylase for starch hydrolysis. The nanohybrids have been characterized by FTIR, XRD, SEM, TGA and BET analyses. The optimum nanohybrid sample (C) in terms of α-amylase immobilization was synthesized when water (H_2O), tetramethoxysilane (TMOS) and methanol (MeOH) were used in 17:1:1 ratio at fixed template amount (0.5 g of carboxymethyl tamarind gum). Enzyme immobilization efficiency of "C" was further enhanced on calcination in nitrogen atmosphere, the optimum calcination temperature being 400 °C (C4). Nanohybrid (C4) impregnated with 40 μg of α-amylase was used for hydrolyzing soluble potato starch to glucose syrup at different temperatures, pH values and enzyme concentrations as these parameters affect the kinetics of the studied reaction. The optimum pH and temperature for the hydrolysis reaction were pH 5 and 40 °C, respectively. The kinetic parameters, K_m (4.261 mg mL^{-1}) and V_{max} (2.55 mol mL^{-1} min^{-1}) for the immobilized amylase were found favorable over the respective values obtained for the free enzyme (K_m = 6.269 mg mL^{-1}, V_{max} = 1.53 mol mL^{-1} min^{-1}). The enzyme activity remained unchanged up to 90 days inside the nanohybrid matrix. The immobilization at the nanohybrid improved the overall stability, affinity and catalytic property of α-amylase.

1. Introduction

Enzymatic transformations are highly selective [1] and can be performed under ambient reaction conditions; however their use in large-scale operations and chemical processing applications sometimes require improvement in their catalytic efficiency and stability. Two different enzyme types are known; monomeric, containing only one polypeptide chain or multimeric having more than one polypeptide chains. Certain working environments have denaturing effect on the enzymes, while the change in the tertiary structure is the main reason behind the deactivation of most of the monomeric enzymes; inactivation of multimeric enzymes takes place either by their dissociation to subunits or due to disorder in the assembly of their structures. Enzyme dissociation in multimeric enzyme system may result due to the disruption in the subunit interactions and this dissociation often lead to the product contamination [2]. Thus stability is the most sought property in an enzyme system for its wide application and utility. Several procedures are known for the stabilization of multimeric enzymes, among them are medium engineering, chemical crosslinking, protein engineering or enzyme immobilization [2].

Immobilized enzymes are being increasingly used as catalysts in many commercial applications due to their ease of separation and high thermal or pH stability [3,4]. Immobilization of enzymes on a solid support is useful technique for enhancing enzyme stability [5,6] and the performance of an immobilized enzyme depends greatly on the character and structure of the carrier materials [7]. The immobilized enzymes may have improved functional properties compared to the corresponding soluble enzymes. Multipoint and multisubunit covalent immobilization are the well established methods for the stabilization of monomeric or multimeric enzymes [3]. Many efforts have been concentrated on modifying the carriers, in order to make the carriers more suitable for enzyme immobilization and catalysis, such as rendering biocompatibility, hydrophilicity, etc. [8–10]. Nanostructured materials can provide a large surface area for the attachment of enzymes [11] and thus are the attractive candidates for enzyme immobilization through adsorption. Adsorption is the most general, easiest to perform and oldest protocol of physical immobilization methods [12]. This method of enzyme insolubilization is quite attractive as it usually does not involve any reagent and requires minimum activation steps. It is cheap, easy to carry out and tends to be

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less disruptive to the enzymic protein than chemical means of attachment. It involves only hydrogen bonds, multiple salt linkages and van der Waals forces and in this respect, the method bears the greatest similarity to the situation found in biological membranes *in vivo* and has been used to model such systems. The enzyme molecules adhere to the surface of carrier matrix due to a combination of hydrophobic effects and the formation of several salt links per enzyme molecule. Ion exchange matrices, porous carbon, clays, hydrous metal oxides, glasses, and polymeric aromatic resins are the commonly used immobilization supports. Ionic exchange immobilizes biomacromolecules only when several enzyme–support ionic bridges are established, that is easier when using large proteins and much activated supports [13]. Amylase acts as large substrate; therefore a correct enzyme orientation is a key point to get an active immobilized enzyme [14–16]. However as a main problem, enzyme leakage may be produced during operation, driving to product contamination and biocatalyst inactivation. Enzyme leakage has been prevented by designing a proper support having a very high activation such as polymeric beads of polyethylenimine [17].

Many organic and inorganic supports [18,19] like clay/modified clays, silica, zeolite and amorphous aluminium phosphate have been studied for the immobilization of enzymes by adsorption. Impregnation of enzymes on polysaccharide–silica or protein–silica [20,21] hybrids is of current interest as these hybrids have desired properties such as biocompatibility, porosity, high surface area, rich functionality and thermal stability.

A new composite microsphere support fabricated from tamarind gum and chitosan has been reported for efficient immobilization of β-galactosidase [22]. There are recent reports on the immobilization of β-galactosidase on alginate/silica biocomposites [23] and glucose oxidaze on chitosan–silica gel [24]. Two different types of *O*-glycoside hydrolyses $1 \rightarrow 3$-β-D-glucanase and α-D-galactosidase have also been immobilized on polysaccharide-silica nanocomposites [25]. The sol–gel synthesis and structure of silica nanohybrids, containing carrageenan and their application as carriers for cell immobilization, have been reported [26]. In all these syntheses, the polysaccharides not only template the silica but also catalyze the sol–gel reaction [27]. The template can be later on removed to obtain porous matrix having high adsorption sites as demonstrated in our recent studies [28]. Performances of such hybrid nanocomposites were tailored by changing the size and functionalities of the templates and by choosing the appropriate calcination temperatures.

Tamarind (*Tamarindus Indica*) is a leguminous tree abundantly grown in many tropical countries and its seeds or kernels are the byproduct of tamarind pulp industry [29]. Though some uses of the seeds are reported in the literature, on a whole this natural resource remains under-utilized [30]. Its seed gum has a $\beta(1 \rightarrow 4)$ linked glucopyranosyl backbone which is frequently branched at *O*-6 position with short chains of one or two D-xylopyranosyl capped with D-xylopyranosyl, D-galactopyranosyl or L-arabinofuranosyl units. Carboxymethylated derivative of tamarind gum has better solubility in aqueous medium [31] as compared to native tamarind gum and is widely used in industry as stabilizer or emulsifier and also for the controlled drug release. Though chemically modified carboxymethyl cellulose [32,33] has been used for enzyme immobilization through covalent binding; no report is available on the use of carboxymethyl cellulose or carboxymethyl tamarind gum–silica hybrids for the enzyme immobilization. In an attempt to utilize the abundant and low cost tamarind seed polysaccharide for enzyme immobilization applications, in the present study, we have targeted the synthesis of novel nanohybrids through sol–gel route using carboxymethyl tamarind gum as template.

2. Experimental

2.1. Reagents

Tetramethoxysilane (98% TMOS; Merck, India) and methanol (Merck; GR) were used as silica precursor and as co-solvent respectively. Tamarind kernel powder was obtained from M/s Sooraj Trading Co., KGF, Karnataka, India and was used as supplied. Monochloroacetic acid (MCA), sodium hydroxide and hydrochloric acid were of laboratory grade (SD Fine-Chem. Ltd., Mumbai, India). Analytical grades of amylase (Lobachem), 3,5-dinitrosalicylic acid (Merck, India) and phosphate salts (Merck, India) were used. Dialysis of α-Amylase (Lobachem) was done using 10 KDa dialysis bags (Banglo Genei, Bangalore, India) that were obtained as gift from Dr. Anjana Pandey, Centre for Biotechnology, University of Allahabad, India.

2.2. Methods

2.2.1. Carboxymethylation of tamarind gum

Carboxymethyl tamarind (CMT) gum was synthesised and purified using the reported procedure [34]. The product was initially dried at room temperature and then in vacuum oven at 40 °C for 4 h.

2.2.2. Dialysis of enzyme

5 mL of 400 μg mL^{-1} enzyme solution contained in a dialysis bag (1″ width) was hanged in 200 mL of 0.02 M phosphate buffer solution for 2 h. The dialysis bag was then dipped in a sugar solution bed (0.02 M) for 1 h for the dialysis.

2.2.3. Nanocomposite synthesis

CMT gum (500 mg) was dissolved in 17 ml of distilled water. Separately a known volume of TMOS was dissolved in a known of volume of methanol and the two solutions were mixed well and stirred for a known time period at ambient temperature. The resulting gel (C) was thoroughly washed with distilled water and dried under reduced pressure. Using different ratios of H_2O, TMOS and MeOH at fixed CMT amount (0.5 g), a series of hybrids (A–E) were synthesized. The optimum hybrid sample "C" (in terms of immobilizing amylase) thus obtained was calcined in N_2 atmosphere (at 200 °C, 400 °C and 600 °C for 2 h at each temperature) to obtain heat treated nanohybrids (C2, C4 and C6, respectively) which were evaluated for the immobilization of α-amylase under identical conditions. The activity of the immobilized enzyme was monitored for the hydrolysis of soluble starch.

2.2.4. Immobilization of amylase onto the nanohybrids

0.1 mL of amylase (0.4 mg mL^{-1}) was adsorbed on 100 mg of the C4 and left for 2 h at 40 °C. The enzyme impregnated C4 was washed well with deionized distilled water until no protein was detected [35] in the washings. The impregnated nanohybrid thus obtained was dried in vacuum at 40 °C and was used for the characterization and evaluation of the biological activity [14]. These enzyme loaded nanohybrids were used for soluble starch hydrolysis. The immobilization of the enzyme was done at a definite pH range (pH of the enzyme solution was adjusted using phosphate buffers).

2.2.5. Characterization

The synthesized nanohybrids have been characterized using different analytical techniques such as FTIR (Perkin Elmer-RX1), XRD at Cu/K_α-source (Bruker AXS, Analytical Instrument Pvt. Ltd.), FESEM (FE-SEM, Quanta 200F, The Netherlands) and by TGA/DSC (TGA analyzer, Mettler Toledo TGA/SDTA 851) in nitrogen atmosphere. The material was heated from 25 to 800 °C at a constant heating rate of 10 °C min^{-1}. Nitrogen adsorption–desorption

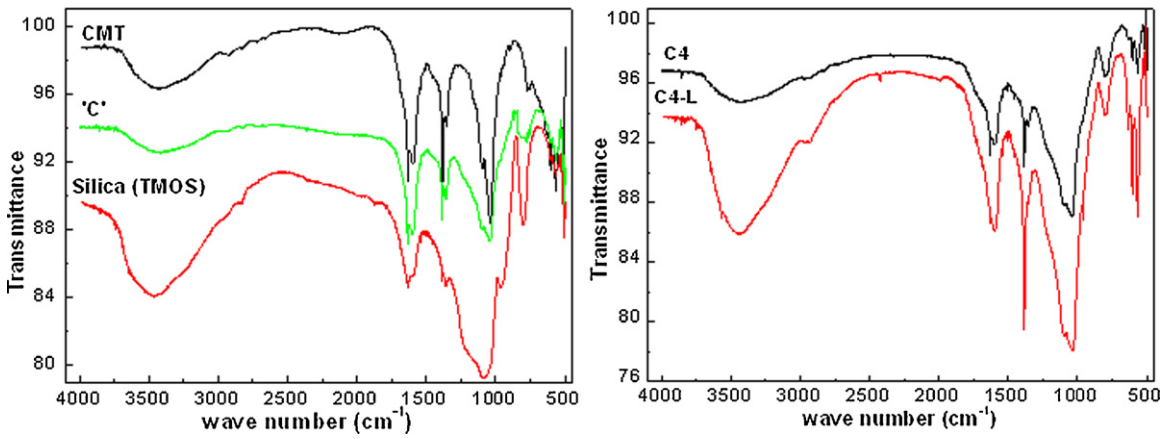


Fig. 1. FTIR spectra of (a) CMT, CS, hybrid 60 °C ('C'), hybrid 400 °C (b) (C4) and amylase loaded (C4-L).

isotherms were obtained using a BEL Japan Inc. Belsorp-HP surface area analyzer at 77 K. Prior to gas adsorption, all the samples were degassed for 4 h at 423 K.

2.2.6. Evaluation of enzyme activity

The hydrolysis of starch was monitored using the free and immobilized enzyme at different substrate concentrations, temperature and pH values. The activity was assayed for 1 mL of the reaction mixture (containing 0.5 mL of 1% soluble starch in 0.02 M phosphate buffer at pH 5, 0.1 mL of 0.4 mg mL^{-1} free amylase and 0.4 mL H_2O) after 10 min incubation at 40 °C. Under identical conditions, the hydrolysis of soluble starch was also monitored using enzyme impregnated nanohybrid (100 mg nanohybrid containing 40 μg of amylase) with 0.5 mL of H_2O, and 0.5 mL of 1% (w/v) starch solution at 40 °C. To interrupt the enzyme reaction, 1 mL of 3,5-dinitrosalicylic acid was added as coupling reagent [36]. The tube containing this mixture was heated for 4 min in boiling water and then cooled in running tap water. After addition of 10 mL of water, the optical density of the solution containing the brown reduction product was determined spectrophotometrically at λ_{max} 540 nm. A blank was prepared in the same manner without enzyme. Amylase activity was expressed in terms of micromoles of the reducing sugar. The determination of the released sugar during the reaction was estimated using maltose standard curve [37].

To study the thermal stability of the enzyme, 0.1 mL of 0.4 mg mL^{-1} free enzyme solution was incubated for 10 min at different temperatures ranging from 293 to 353 K using a temperature controlled incubator shaker at 200 rpm and was used for hydrolyzing 0.5 mL of 1% starch solution. For the immobilized enzyme, a known weight of enzyme impregnated nanohybrid was incubated for 10 min at different temperatures ranging from 293 to 353 K with 0.5 mL of 1% (w/v) starch and 0.5 mL of H_2O. Enzyme activity was tested as described above. One unit of enzyme was defined as the amount of enzyme capable of producing 1 mL reducing sugar from 1% soluble starch as substrate in 1 min at 40 °C. To study the pH effect on enzyme stability, 0.1 mL of 0.4 mg mL^{-1} enzyme was added to 0.5 mL of starch (1% w/v, pre adjusted at different pH values) and after the addition of 0.5 mL H_2O, the reaction mixture was incubated for 10 min. For immobilized enzyme, a known weight of immobilized enzyme was used and the enzyme activity was determined as above. For recycling of the nanohybrids after the first cycle, the reaction mixture was centrifuged to separate the used hybrids which were reused in the next cycle after washing with distilled water. For the kinetic study, concentration of starch was varied from 0.25 to 2% (w/v) at pH 5, keeping the other conditions same as described above.

2.2.7. Enzyme stability and recycling

The immobilized enzyme was used for ten consecutive cycles after repeated washings with distilled deionized water as described above. Enzyme impregnated nanohybrid was stored (after using it up to ten cycles) for 90 days at 4 °C and used again as described above under optimum conditions

3. Results and discussion

3.1. Characterization of the composite

3.1.1. FTIR

The IR spectrum of the pure control silica gel obtained from condensation polymerization of TMOS in absence of CMT is shown in Fig. 1. This spectrum includes a broad and intense absorption band between 1080 cm^{-1} and 1145 cm^{-1} due to the symmetric valence vibrations of Si–O–Si links in the transverse and longitudinal modes, respectively [38]. The peaks at 1080 cm^{-1} and 802 cm^{-1} correspond to stretching and bending motions of the oxygen atom in Si–O–Si networks, respectively. The spectrum also contained a band at 469–467 cm^{-1} due to Si–O–Si rocking vibrations [39]. The hybrid (C4) exhibited collective IR characteristics as shown in Fig. 1b. The strong peaks at 1631 cm^{-1} and ~1400 cm^{-1} are due to the presence of –COOH groups in CMT [40]. All the silica peaks merge with CMT peaks and the 1080 cm^{-1} peak shifts at lower wave number (1030 cm^{-1}) due to the involvement of Si–O–Si network in H-bonding interaction with carboxylic acid (COOH) groups in CMT gum. As per reported in the literature, there are two characteristic IR peaks for amylase enzyme, amide I band exists at 1650 cm^{-1} due to the υ(C=O) in plane stretching vibration of the protein backbone and the amide II band exists at 1550 cm^{-1} due to the δ (N–H) in plane bending mode Fig. 1b [41]. The peak at 1650 cm^{-1} is a combination peak due to α helix, β sheet, β turn and random coil [42] of the enzyme and this peak is known to be sensitive to the environment. Our observations suggest that after impregnation of enzyme onto the nanohybrid, amide II band disappears and amide I band shifts towards lower wavelength (1600 cm^{-1}), similar observations have been made by Sharma et al. [43].

3.1.2. X-ray diffraction

All samples scanned on XRD instrument were analyzed for the molecular arrangement from 10 to 80° glancing angle. The XRD data illustrate the amorphous nature of silica and the nanohybrid samples (Fig. 2A). The patterns of all the samples are almost identical except the shifting of humps at different angle centre (2θ). The peaks of the nanohybrids are observed between the

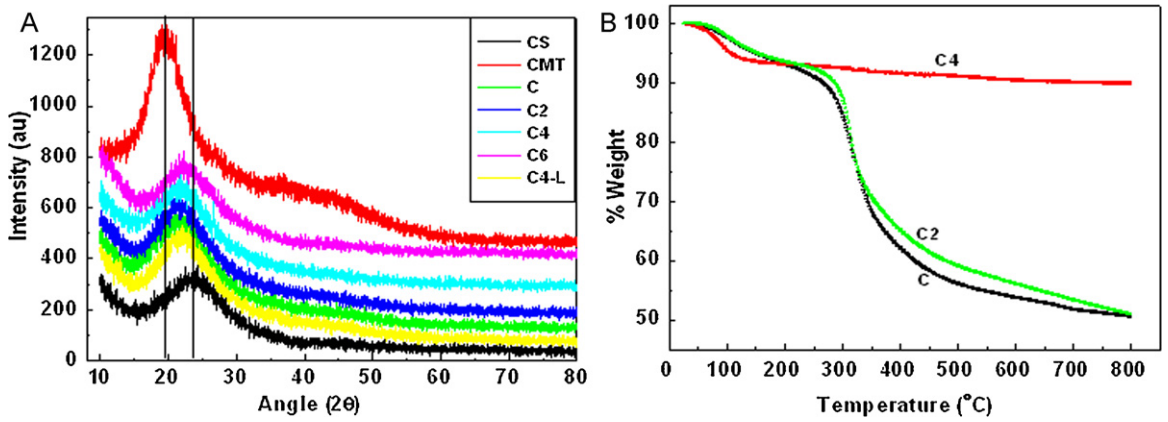


Fig. 2. (A) XRD patterns of control silica (CS), CMT, hybrid at 60 °C (C), C2 (hybrid at 200 °C), C4 (hybrid at 400 °C), C6 (hybrid at 600 °C) and C4-L (enzyme impregnated hybrid C4). (B) Thermograms (TGA) of C, C2 and C4 samples.

peak centers of CMT (19.37°) and control silica (CS) (32.87°). The XRD patterns of the hybrid (C4) and enzyme loaded C4 (C4-L) are almost similar indicating surface immobilization of the enzyme with unchanged internal molecular arrangement and the adsorption being the impregnation mode.

3.1.3. Thermal stability

The thermal gravimetric analyses of the materials were performed in the temperature range of 25–800 °C under nitrogen atmosphere at a constant heating rate 10 °C min^{-1}. The thermal stability of calcined samples is higher than the hybrid 'C'. Thermal stability of C and C2 are comparable, C2 being slightly more stable as is seen in Fig. 2B. C4 shows significant thermal stability as it show only ~5% loss up to 100 °C (due to loss of bound and adhered water) followed by gradual loss up 6% up to 800 °C (due to the loss of leftover organics) since it has already lost most of the organic contents during calcination at 400 °C.

3.1.4. Scanning electron microscope (FE-SEM)

SEM pictures reveal the molecular homogeneity of the hybrid gel prepared at 60 °C which is seen as globular structure in Fig. 3. After calcination at 400 °C (under nitrogen atmosphere) the gel results into porous silica structure on partial loss of the CMT which is evident by higher silicon content in C4. Incorporation of the enzyme is evident in the C4-L where 2.35% weight of nitrogen content is noticed. Enzyme incorporation did not affect the texture of the hybrid much except its porous matrix.

3.1.5. BET analysis of the nanocomposite material

The surface area, pore size and particle size distribution were analyzed by BET method using nitrogen adsorption–desorption isotherms. The data depict the surface area, pore size and pore volume of the hybrid adsorbent (C4) to be 11 m^2 g^{-1}, 6.1 nm and 0.0178 cc/g, respectively (Fig. 4). Pore volume of 0.0178 cc/g in the hybrid material indicates total voids of 0.0178 cc in 1 g of the sample. From BET results cylindrical pore shape is indicated, on the other hand the surface area of the free enzyme is negligible (<0.2 m^2 g^{-1}). Since the surface to volume ratio increases due to the immobilization of the enzyme, the activity of the immobilized enzyme particles in the nanohybrid material exceeded than that of the free enzyme. Average size of the pore for the nanohybrid is 6.1 nm and this is large enough to overcome the diffusion limitation of the porous gel.

3.1.6. Biocatalytic evaluation of enzyme impregnated nanohybrid

The effect of pH on the enzyme activity (free and impregnated) towards hydrolyzing starch was investigated in the range of pH 4.0–9.0, and results are shown in the Fig. 5A. It was observed that enzyme activity varied with the solution pH and maximum activity was noticed at pH 5.0 in both free and impregnated environments. At all pH values, the impregnated sample exhibited higher activity than free enzyme (Fig. 5A). The improved enzyme activity may be attributed to the fact that configuration of enzyme was altered under immobilized environment. Such variations of improved activity under immobilized conditions were observed with other microbial systems and enzymes [37,44].

Further, the amylase enzyme immobilized on the hybrid was evaluated for starch hydrolysis at different incubation temperatures (from 303 to 353 K) at pH 5.0. Impregnated enzyme showed higher activity as compared to free enzyme at all studied incubation temperatures suggesting that on impregnation at the nanohybrid, thermal stability of the enzyme was much enhanced (Fig. 5B). It was observed that although optimal *T* did not change, the residual *T* at 60 °C is much higher for the immobilized enzyme as the hybrid

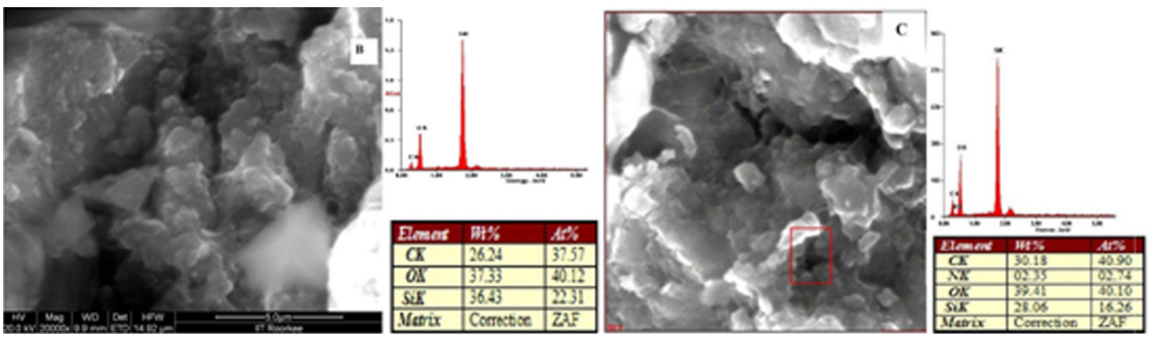


Fig. 3. SEM and EDX pictures of (A) composite C4; (B, C) C4-L samples.

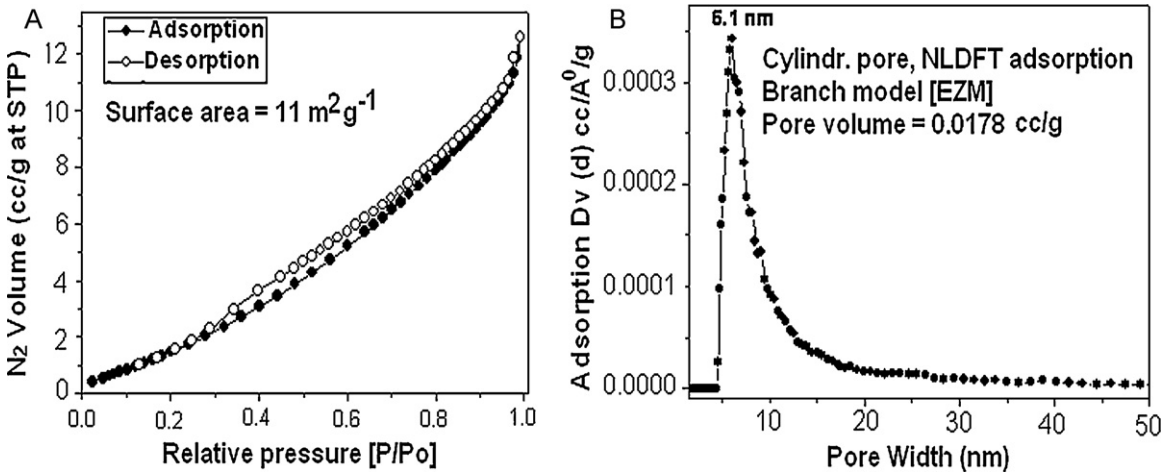


Fig. 4. BET isotherm (A) and pore size distribution (B) of CMT–Si composite C-4.

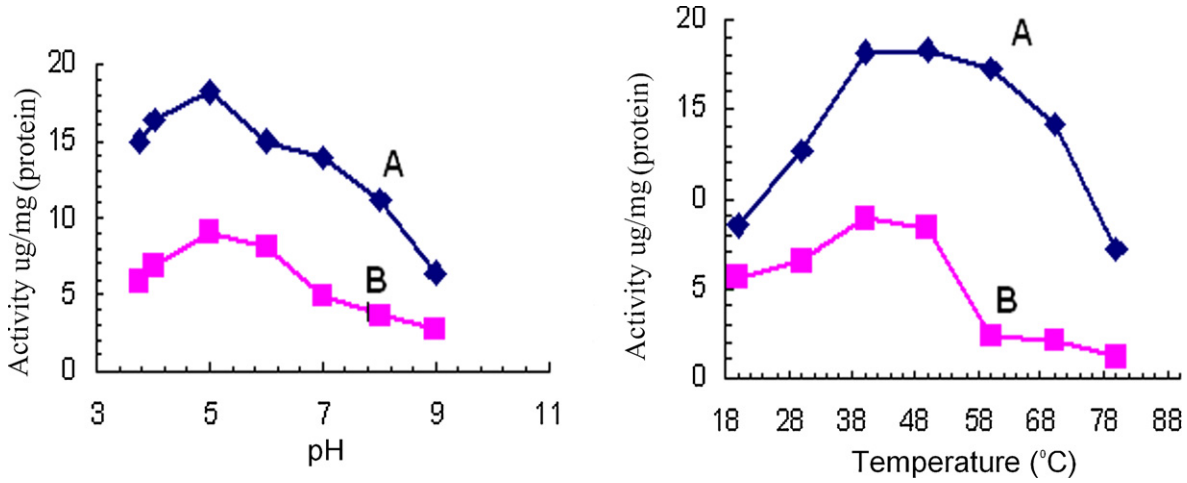


Fig. 5. Effect of pH and temperature on the activity of impregnated (A) and free enzyme (B).

silica cage protects the immobilized enzyme from dissociation as the nanoenvironment of the hybrid results into the rigidification of the enzyme structure.

These results were in accordance with data obtained using various other matrices [26,37] where enhanced activity was observed under immobilized conditions. Incubation temperature dependent catalytic activity for free and immobilized enzyme was also noticed as shown above. The immobilized enzyme exhibited good stability and catalytic activity in broad temperature range (from 20 to 80 °C), being most at 60 °C. This type of low temperature activity is rarely observed for the enzymes from thermophilic organisms. Present immobilization substrate is attractive as it is derived from a biomaterial which is abundant in nature (see Table 1).

3.1.7. Kinetic parameters

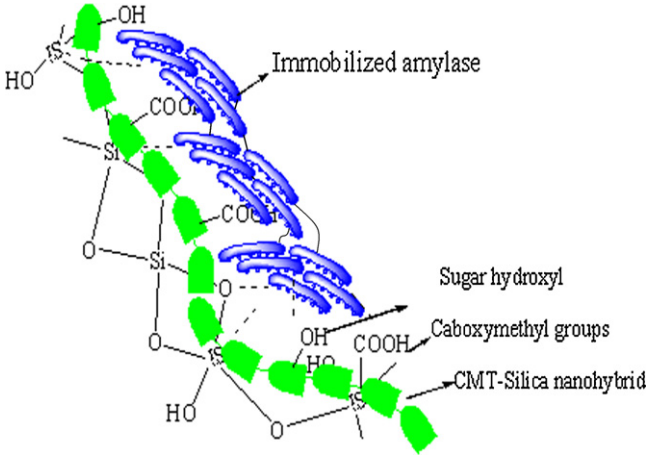
The kinetic parameters of starch (0.5–3.0%, w/v) hydrolysis using free and immobilized amylase have been determined. The relation between substrate concentration and rate of enzymatic reaction can be described by Michaelis–Menten (MM) equation-1, [45] which can be represented into linear form as Lineweaver–Burk equation [46] which helps in computation of V_{max} (the rate of reaction in mg mL^{-1} min^{-1}), and K_m (Michaelis constant in mg mL^{-1}) values. The K_m value for an enzyme give an idea about the affinity of enzyme towards the substrate, whereas the value of V_{max} provides the maximum rate of enzyme reaction when the enzyme is saturated by the substrate. K_m and V_{max} values obtained in this study and some other stud-

Table 1
Optimization of the composite synthesis in respect of gelation time and maximum enzyme activity at 100 mg hybrid loaded with 40 μg of enzyme, temperature 40 °C, reaction time 10 minutes, rpm 200, reaction pH 5.0 and wavelength 540 nm.

S. No.	Sample	TMOS (ml)	MeOH (ml)	Gelling time (min)	Yield (mg)	Calcination temperature (°C)	Activity (U/mg)
1.	A	0.25	0.25	–	622	–	–
2.	B	0.5	0.5	480	745	–	3.67
3.	C	1.0	1.0	20	985		4.72
	C2	–	–	–	–	200	11.09
	C4	–	–	–	–	**400**	**18.07**
	C6	–	–	–	–	600	9.57
4.	D	1.5	1.5	18	1235	–	3.87
5.	E	2.0	2.0	15	1450	–	4.01

Table 2
Kinetic parameters for the hydrolysis of starch using α-amylase in the present work and other studies.

Enzyme	K_m (gL^{-1})	V_{max}	References
Immobilized enzyme	6.68	4.97 (mol mL^{-1} min^{-1})	[45]
Immobilized enzyme	24.03	0.83 (mol mL^{-1} min^{-1})	
Immobilized enzyme	23	0.94 (mol mL^{-1} min^{-1})	
Free enzyme	5.69	5.86 (mol mL^{-1} min^{-1})	
Immobilized enzyme	37.1	3.25 (mol mL^{-1} min^{-1})	[49]
Immobilized enzyme	31.2	3.91 (mol mL^{-1} min^{-1})	
Immobilized enzyme	15.9	4.91 (mol mL^{-1} min^{-1})	
Immobilized enzyme	12.8	5.44 (mol mL^{-1} min^{-1})	
Free enzyme	4.5	6.63 (mol mL^{-1} min^{-1})	
Immobilized enzyme	15.3	21.0 (mg mL^{-1} s^{-1})	[50]
Free enzyme	37.9	22.4 (mg mL^{-1} s^{-1})	
Immobilized enzyme	13.8	–	[51]
Immobilized enzyme	5.9	–	
Free enzyme	25.5	–	
Impregnated onto hybrid	4.261	2.55(mol mL^{-1} min^{-1})	This work
Free	6.269	1.53(mol mL^{-1} min^{-1})	



Scheme 1. Schematic diagram for immobilization of α-amylse at CMT-silica nanohybrid

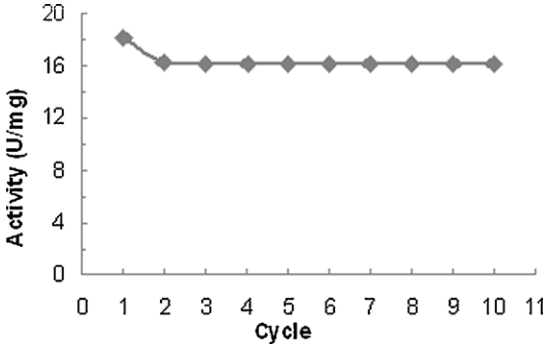


Fig. 6. Recycling of enzyme impregnated hybrid (40 μg enzyme loaded on 100 mg hybrid) at pH 5.0, temperature 40 °C, reaction time 10 minutes, rpm 200 at wavelength 540 nm.

ies for the free and immobilized α-amylase are reported in Table 2.

The lowering of K_m and elevation in V_{max} as compared to free enzyme indicated a positive distortion of the enzyme assembly. The nanohybrid support probably offers an environment which prevents large conformational changes in the enzyme structure which are the usual cause of enzyme protein deactivation. Unstrained non-covalent interactions can be assumed between the functionalities available at the nanohybrid and enzyme surface. The increased activity of the enzyme on immobilization at the nanohybrid indicates that the substrate is not sterically hindered from diffusing to the active site of the immobilized amylase. The improved affinity of the enzyme under immobilization conditions has been observed in few other studies [47,48]. Though most of the CMT in the nanohybird is lost upon calcination at 400 °C, some leftover of organics is still indicated by 6% weight loss in the TGA study of C4. Carboxymethyl groups at tamarind gum offer noncovalent interactions with the protein functionalities at the amylase. Moreover silica matrix have positively polarized silicon atom that can have noncovalent interactions to the negative centers at the enzyme protein.

3.1.8. *Effect of small additives present in the commercial solution on enzyme properties*

6% weight loss in TGA pattern of C4 indicates that some CMT escapes from being lost on calination of the "C". This is also evident from high C and O content in EDAX of C4. The polymeric nanohybrid also has carboxymethyl groups besides other sugar functionalites and electron deficient silicon centers. These sites by multipoint interaction with enzyme's active sites tightly entangle the enzyme in the desired shape for its maximum activity and stability. A plausible mechanism for the immobilization has been proposed in Scheme 1. To understand the possible role of the enzyme stabilizers (that may be present in the free enzyme), the study was repeated with dialyzed enzyme. The stabilizers may be responsible for the higher activity of the enzyme in the immobilized state as in the process of impregnation the stabilizers are likely to be removed. With dialyzed enzyme, the obscuring effect of the stabilizer is eliminated and the advantage of impregnation onto the hybrid becomes evident (Table 3). Even with the dialyzed enzyme, higher activity is observed for the immobilized enzyme as compared to the free enzyme. It is the nano-environment of the hybrid, which provides

Table 3
Activity of free and immobilized enzyme on C4 (before and after dialysis) with 40 μg of enzyme, temperature 40 °C, reaction time 10 min, rpm 200, reaction pH 5.0 and wavelength 540 nm.

S. No.	Activity of free enzyme (U/mg)		Activity of immobilized enzyme (U/mg)	
	Before dialysis	After dialysis	Before dialysis	After dialysis
1.	8.95	10.40	18.07	18.82

extra stabilization and activity to the enzyme in the immobilized state (Fig. 6).

4. Conclusions

The CMT catalyzed the gelation of TMOS sol to form a monolithic silica nanohybrid (CMT–silica) which proved to be a good candidate for immobilization of amylase. The immobilization improved the affinity and catalytic properties of amylase, besides the enzyme under immobilized state was more stable thermally. The enzyme impregnated hybrid material was easily separated by simple centrifugal method from the reaction cell. The immobilization at the nanohybrid doubled the enzyme activity besides improving the working conditions. The enzyme was stable in the hybrid matrix and its activity did not change even after 90 days on storing at 4 °C. The present study demonstrates a promising application of enzyme in immobilized stage on a novel hybrid support.

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