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Immobilization of α-amylase onto K-10 montmorillonite: characterization and comparison of activity in a batch and a fixed-bed reactor

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ABSTRACT: α-amylase was immobilized on acid-activated montmorillonite K-10 via adsorption and covalent linkage. The immobilized enzymes were characterized by X-ray diffraction (XRD), surface area measurements, ^{27}Al nuclear magnetic resonance (NMR) and scanning electron microscopy (SEM). Surface area measurements indicate pore blockage due to linking of the enzyme in the vicinity of the pore mouth. The XRD demonstrates intercalation of enzyme upon immobilization. The NMR studies indicate that, during adsorption, tetrahedral Al sites are involved, while covalent binding occurs exclusively on the octahedral Al sites. The SEM images depict the changed morphology of the clay surface due to immobilization. The efficiency of immobilized enzymes for starch hydrolysis was tested in a batch and a fixed-bed reactor and the performances were compared. The immobilized α-amylase showed a broad pH profile and improved stability characteristics in both reactor types when compared to the free enzyme. The effectiveness factor increased in the fixed-bed reactor, implying that diffusional restrictions to mass transfer operate in the heterogeneous reaction and the use of a fixed-bed reactor leads to a reduction in these diffusional resistances. In the continuous run, 100% initial activity was maintained for 72 h, and after 96 h, >80% activity was retained.

KEYWORDS: α-amylase, montmorillonite, adsorption, covalent binding, immobilized enzymes, starch hydrolysis.

The hydrolysis of starch to low-molecular-weight products is widely applied in the food, paper, textile, distillery and brewing industries (Ju *et al.*, 1995). α-amylase (EC 3.2.1.1; 1,4 α-D-glucan glucanohydrolase) catalyses the hydrolysis of α-1,4 linkages in amylose, amylopectin and glycogen in an endo fashion. It does not hydrolyse the α-1,6 linkages or any other branch points and so produces maltose and dextrin (Noda & Suda, 2001; Tanyolac *et al.*, 1998). Industrial production of maltose is generally performed in a batch reaction, which is economically disadvantageous since the enzyme can be used only once. To allow enzyme reuse, immobilization is currently used in industry. In order to reduce production costs further, the possibility of developing a continuous process with immobilized α-amylase is worth considering. Immobilization of α-amylase on various supports has been studied. Polymeric supports find wide applications in enzyme immobilization (Bayramoglu *et al.*, 2004; Aksoy *et al.*, 1998; Arica *et al.*, 1995; Chen *et al.*, 1997). The disadvantages of polymers are low pH and thermal stabilities. Inorganic supports are comparatively more stable. Porous silica is found to be a good

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medium for immobilization (Lomako *et al.*, 1982; Mody *et al.* 1999). Solid acid supports can be used to immobilize enzymes since the acidic sites act as centres of attachment via the amino group of enzymes.

In this study, we have immobilized *Bacillus subtilis* α-amylase on montmorillonite by two methods – adsorption and covalent binding. Montmorillonite, which is a 2:1 dioctahedral smectite, has been used successfully as a support as well as a catalyst for the past few decades (Laszlo, 1987). The swelling ability of these naturally occurring phyllosilicate minerals provide unusual properties and appreciable surface area for adsorption of organic molecules (Pinnavaia, 1983). A large number of modifications of clay that provide a variety of textural and catalytic properties are possible, including acid treatment, cation exchange, pillaring with robust metal ions, intercalation of polymeric organic moieties, etc. Depending on the type of modification, the properties of clay can be tuned in such a way as to suit specific applications. Clays have also been used as immobilization supports. Montmorillonite coated with varying levels of hydroxyaluminium was investigated for immobilization of tyrosinase via adsorption (Naidja *et al.*, 1997). Even though the optimal pH and temperature of immobilized enzyme were identical to the free enzyme, thermal and storage stabilities were improved. Aspartase immobilized on Ca-montmorillonite (Naidja & Huang, 1996) led to intercalation of the enzyme with a layer expansion to 2.65 nm. Garwood *et al.* (1983) reported similar results for immobilization of glucose oxidase on Na^+-montmorillonite. Montmorillonite, functionalized with 3-aminopropyl triethoxysilane and glutaraldehyde, showed better coupling properties with the enzyme, urease, compared to kaolinite and other soil minerals (Lai & Tabatabai, 1992). Other studies also demonstrate similar results (Sarkar *et al.*, 1989).

Here we have employed montmorillonite K-10, which is a commercially available acid-activated form. Since clays are aluminosilicates, they possess acid sites that are capable of interaction with amino groups of enzymes leading to ionic binding. This linkage is much stronger than mere physical binding and hence the enzyme will be retained on the support for a longer duration. Moreover, reports on immobilization studies over clays are seldom available. Covalent binding was performed after functionalizing the clay with 3-aminopropyl triethoxy silane (3-APTES) and glutaraldehyde. The immobilized enzymes were characterized using XRD, BET surface area measurements, solid-state NMR spectroscopy and SEM. The activities of free and immobilized enzymes were tested in batch and fixed-bed reactors and the kinetic constants were compared. The influence of pH and temperature on activity and stability of free and immobilized enzymes was ascertained in both reactor types. Operational stability in the continuous mode was also determined.

MATERIALS AND METHODS

Materials

Montmorillonite K-10, *Bacillus subtilis* α-amylase, 3-aminopropyltriethoxysilane (3-APTES) and glutaraldehyde were purchased from Sigma-Aldrich Chemicals Pvt Ltd, Bangalore. All other chemicals were of the highest purity commercially available.

Immobilization of α-amylase on montmorillonite

For adsorption, montmorillonite K-10 was mixed with equal volumes of 0.1 M phosphate buffer and α-amylase solution. It was shaken in a water bath shaker at the required temperature for 1 h and later centrifuged in a Remi C-24 model-cooling centrifuge at 1ºC for 1 h. Enzyme protein was estimated using the spectrophotometric method of Lowry *et al.* (1951) using Folin-Ciocaltaue's phenol reagent and measuring the absorption at 640 nm in a Shimadzu 160A UV-Vis spectrophotometer. In case of covalent binding, the method by Mody *et al.* (1999) was adopted. The clay was stirred with 10% 3-APTES solution in acetone (v/v) for 1 h at room temperature. It was filtered, washed with acetone, and dried. This was treated with 10% aqueous glutaraldehyde solution (v/v) for 1 h, filtered, washed and dried at ambient temperature. This activated clay was used to bind the enzyme as explained above. All immobilized systems were stored in 0.1 M phosphate buffer at 5ºC.

Characterization of immobilized enzymes

Powder XRD patterns of the immobilized enzyme systems and the support were collected using a Rigaku D/Max-C system with Ni-filtered Cu-$K\alpha$ radiation (λ = 1.5406 Å) within the 2θ range 2–15º.

A Micromeritics Gemini 2360 surface area analyser was used to measure the nitrogen adsorption isotherms of the samples at liquid nitrogen temperature. The specific surface area was determined from the BET plot (p/p_0 = 0.05–0.95). Prior to the measurement, the samples were degassed at room temperature for 12–16 h in nitrogen flow. Scanning electron microscopy images were obtained from a Cambridge Oxford 7060 scanning electron microscope connected to a 4-quadrant backscattered electron detector under a resolution of 1.38 eV. The samples were dusted on a double sided carbon tape, placed on a metal stub and coated with a layer of gold to minimize charge effects. Solid-state ^{27}Al MAS NMR experiments were carried out using a Bruker DSX-300 spectrometer at a resonance frequency of 78.19 MHz. For all experiments, a standard 4 mm double-bearing Bruker MAS probe was used. The sample spinning frequency was 8 kHz with a single pulse excitation corresponding to π/2 flip angle. The pulse length for the experiments was 10 μs whereas pulse delay was 2 s. The spectra were externally referenced with respect to a dilute solution of $AlCl_3$. XWINNMR software operating in a UNIX environment on a silicon graphics computer was employed to acquire and retrieve data.

Free and immobilized enzyme activity for starch hydrolysis

The activities of free and immobilized enzymes were tested in a batch and a fixed-bed reactor. In the batch reactor, 0.1 g of immobilized enzyme (1 ml of enzyme solution) was mixed with buffered 5% starch solution and shaken in a water bath shaker. A silica glass tube 1.2 cm interior diameter and 20 cm long was used as the fixed-bed reactor. 0.5 g of immobilized enzyme was packed into a bed in the middle of the reactor. The substrate was let into the reactor using a syringe pump. Space velocity was maintained at 3.26 h^{-1}. 1 ml of the product was mixed with 5 ml of iodine solution and the absorbance was read at 620 nm. One unit of enzyme is defined as the amount required to hydrolyze 1 mg of starch min^{-1} under the assay conditions. All results are presented in a normalized form with the activity under optimum conditions being assigned a value of 100%. The influence of substrate concentration was determined by estimating the rate of reaction at various starch concentrations (1–10% w/v) and the Hanes-Woolf plot was employed to calculate K_m and V_{max}. The effectiveness factor, η, was calculated by obtaining the ratio of rates of free and immobilized enzymes at 5% starch concentration.

Influence of pH

The effect of pH was studied at room temperature by varying the pH in the range 3–8. In order to determine pH stability, 1 ml of free enzyme solution (0.1 g immobilized enzyme) was mixed with 0.1 M phosphate buffer of various pHs and allowed to pre-incubate for different time intervals between 15 and 300 min at 30ºC. After the pre-incubation period, 5% aqueous starch solution (w/v) was added and incubated. Reaction time was kept constant at 30 min. In the fixed-bed reactor, 0.5 g of immobilized enzyme was packed into a bed and 0.1 M phosphate buffer was fed using a syringe pump at a flow rate of 5 ml h^{-1} for various time intervals between 1 and 24 h. After that, 5% aqueous starch solution was let into the reactor at a flow rate of 4 ml h^{-1}. Space velocity was kept at 3.26 h^{-1}. The product was analysed at intervals of 1 h. The time on stream (TOS) was kept at 3 h.

Thermal stability

The optimum temperature in the batch reactor was determined by performing the reaction at temperatures in the range 30–90ºC. Thermal stability was ascertained at 70ºC by mixing 1 ml of free enzyme solution (0.1 g immobilized enzyme) and 0.1 M phosphate buffer of optimum pH and allowing them to pre-incubate for various time intervals between 15 and 90 min. After the pre-incubation period, 5% aqueous starch solution (w/v) was added and the reaction continued for 30 min. The product was analysed as mentioned above. In the fixed-bed reactor, 0.5 g of immobilized enzyme was packed into a bed and pre-incubated at 40ºC for different time intervals between 15 and 90 min. After that, 5% aqueous starch solution in 0.1 M phosphate buffer of optimum pH was let into the reactor at a flow rate of 4 ml h^{-1}. Space velocity was kept at 3.26 h^{-1}. The product was analysed at intervals of 1 h with a time on stream of 3 h.

Determination of operational stability

In order to determine operational stability, 1 g of catalyst was packed into a bed in a silica glass

reactor of 1.2 cm interior diameter and 25 cm long. Buffered 5% aqueous starch solution (w/v) was added at a flow rate of 5 ml h^{-1} to yield a space velocity of 2 h^{-1}. The reaction was carried out for 96 h and the product was analysed at 8 h intervals.

RESULTS AND DISCUSSION

X-ray diffraction

Enzymes can be attached to clays either on the surface or within the interlayer space or both. In view of the fact that two independent techniques are employed for enzyme immobilization, it becomes absolutely essential to understand the effect of these two techniques on the clay matrix. Interaction with enzymes can cause significant structural changes in these clays and these are easily recognized by analysing the XRD patterns, which, for immobilized α-amylase, are shown in Fig. 1. For parent montmorillonite, there is a peak at 8.9º2θ corresponding to a *d* spacing of 0.998 nm. This peak represents the d_{001} plane analogous to the inter-layer spacing. After functionalization with 3-APTES and glutaraldehyde, this peak shifts partly to lower 2θ with a *d* spacing of 1.531 nm demonstrating an expansion of layers thereby indicating that the functionalization takes place between the clay layers. The intensity of the new peak is very high which shows that most of the clay layers are intercalated with silane and glutaraldehyde molecules. Adsorption with α-amylase does not change the intensity of the d_{001} peak. There is a slight shifting of the XRD peak to lower 2θ values (*d* spacing of 1.728−1.975 nm) but with very low intensity. This suggests that intercalation of enzyme takes place in the initial clay layers only. As enzyme loading increases, the intensity of the new peak also increases, confirming the intercalation of enzyme into the clay layers. Covalent binding with α-amylase also leads to enzyme intercalation. The *d* spacing increases to a value in the range 1.919−2.074 nm. A shift of the entire d_{001} peak to higher *d* spacing suggests that complete intercalation of enzyme has taken place. Enzymes are highly polymeric species with very large molecular diameter. α-amylase has a molecular diameter of 8 nm, hence the possibility of attachment within the interlamellar space can be ruled out. But a shift in the d_{001} peak to lower 2θ/higher *d* spacing is evidence for intercalation (Jia *et al.*, 2002; Kim *et al.*, 2001). Therefore, it is proposed that the whole enzyme does not intercalate between the clay layers and that it is the side chains of various amino acid groups that are responsible for intercalation. The polypeptide backbone does not enter the interlayer space but is situated at the periphery of the clay (Gougeon *et al.*, 2003). For α-amylase, an enzyme loading of 50 mg was necessary to bring about intercalation in all the clay layers.

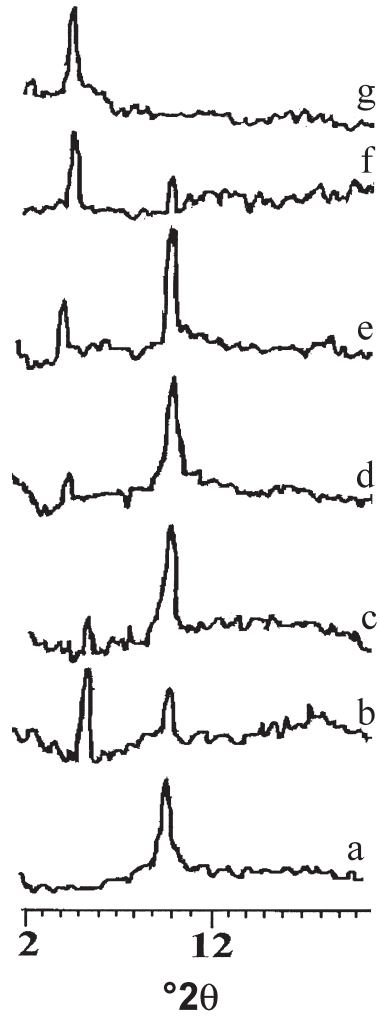

FIG. 1. XRD patterns of: (a) montmorillonite K-10; (b) silane glutaraldehyde activated K-10; (c,d,e) adsorbed α-amylase with enzyme loading of 10, 50 and 100 mg g^{-1} of clay, respectively; (f,g) covalently bound α-amylase with enzyme loading of 10 and 50 mg g^{-1}clay, respectively.

TABLE 1. Surface area and pore volume of immobilized α-amylase.

Catalyst system	Surface area(m^2 g^{-1}) Langmuir	BET	Pore volume ($\times 10^{-6}$ m^3 g^{-1})
M	352	201	0.2511
AM_{10}	258	162	0.2234
AM_{50}	219	134	0.2093
AM_{100}	189	108	0.1905
SGM	266	145	0.1745
CM_{10}	176	100	0.1056
CM_{50}	135	81	0.0765
CM_{100}	92	45	0.0456

M – montmorillonite K-10; AM – α-amylase adsorbed on montmorillonite K-10; SGM – montmorillonite activated with 3-aminopropyl triethoxy silane and glutaraldehyde; CM – α-amylase covalently bound to montmorillonite K-10. The values in subscript indicate enzyme loadings in mg g^{-1} of clay.

Surface area and pore-volume measurements

Surface area as well as pore volume decrease upon immobilization (Table 1). Activation of montmorillonite with aminopropylsilane and glutaraldehyde reduced the pore volume, suggesting that the binding of silane and glutaraldehyde molecules to the clay takes place between the layers. There is further proof of this since covalent binding of enzymes through the glutaraldehyde spacer reduces the pore volume to a greater extent. This is the case for surface area also. Adsorption of enzyme results in a reduction of surface area along with pore volume. The reduction is not as much as in case of covalent binding, thereby indicating that pore blockage is less and hence the enzyme is not concentrated in the porous network of montmorillonite.

Scanning electron microscopy

Scanning electron microscopy gives an idea of the morphology of the material. Since an enzyme is a highly polymeric material, immobilization into a solid matrix will lead to a change in the morphology. The revised morphology will depend on the type of immobilization and hence it is possible to differentiate between the two types of immobilization techniques. Adsorption with α-amylase does not change the porous structure of montmorillonite (SEM images not shown). Functionalization with 3-APTES and glutaraldehyde leads to a loss of porosity due to the linkage of enzyme at the pore entrance. Further covalent binding reduces porosity to a much greater extent. There is an increase in particle size due to the enzyme immobilization.

^{27}Al NMR studies

Table 2 presents the NMR spectra of the clay and the immobilized α-amylase. Montmorillonite exhibits octahedral Al resonance at 2.8 ppm and tetrahedral resonance at 69.8 ppm. A slight change in the octahedral chemical shift is due to the presence of Fe. Functionalization with silane and glutaraldehyde results in a shift of octahedral peak to −1.7 ppm while the tetrahedral peak remains unaltered, indicating that only the octahedral Al atoms are involved in binding with silane and glutaraldehyde. This further infers that the binding takes place within the clay interlayer space

TABLE 2. ^{27}Al MAS NMR data for clay and immobilized α-amylase.

Catalyst	Chemical shift (ppm) Octahedral	Tetrahedral
M	2.8	69.8
SGM	−1.7	69.5
AA_A	2.8	63.4
AA_C	1.3	69.3

M – montmorillonite; SGM – montmorillonite functionalized with silane and glutaraldehyde; AA – α-amylase; subscript A – adsorbed enzyme; subscript C – covalently bound enzyme.

and hence substantiates the results of XRD and surface-area measurements. Adsorption of α-amylase leads to a shift of the tetrahedral Al peak to 63.4 ppm; the octahedral peak is almost constant. Thus, during adsorption, the enzyme interacts with the tetrahedral Al alone. When α-amylase is covalently bound to the clay matrix, the tetrahedral Al resonance is unaltered while the octahedral peaks show a shift to 1.3 ppm. Covalent binding takes place on the glutaraldehyde spacer and not directly on to the Al species. In spite of this, there is sufficient shifting of the octahedral peak to signify secondary interactions between the enzyme and the octahedral Al layers. The side chains of the amino acid residues or other functional groups present in these side chains may involve electrostatic interactions with octahedral Al species, changing their chemical environment, thereby causing a shift in the NMR signal.

Immobilization efficiency and kinetic parameters

The kinetic parameters (Michaelis constant, K_m, maximum rate, V_{max}, and effectiveness factor, η) are the most important factors, the study of which reflects the activity of enzyme in the immobilized state. Evaluation of these factors imparts knowledge about changes in the conformation of enzyme on account of immobilization. The Michaelis constant, K_m, gives an indication of the affinity of the enzyme for the substrate; it is inversely proportional to affinity. V_{max} measures the extent of activity of the enzyme. The effectiveness factor, η, provides information on the role of diffusion in the reaction. $\eta = 1$ under conditions of complete diffusion, i.e. in case of homogeneous reaction with the free enzyme. Immobilization results in the use of enzymes in heterogeneous mode and therefore diffusional effects operate, the extent of which is given by measuring η. Another important parameter is the efficiency of immobilization, which is defined as the relative activity of free enzyme retained after immobilization. It is calculated as $\eta \times 100$. K_m and V_{max} values were obtained from the Hanes-Woolf plot. This plot was used because the distribution of errors is uniform in this case (Silverman, 2000) when compared to the Lineweaver-Burk or Eadie-Hoftsee plots and so the errors tend to cancel each other. The effectiveness factor was calculated by measuring the rate of reaction at a substrate concentration of 5% (w/v) starch. These data are shown in Table 3. The free and immobilized α-amylase obeys Michaelis-Menten kinetics. The Michaelis constant, K_m, increases upon immobilization, which shows that the activity of α-amylase is reduced (Balasubramaniam & Arasaratnam, 1989). The V_{max} values show a decrease that proves reduction in activity. Kinetic studies reveal that in the batch reactor, adsorbed and covalently bound α-amylase retain 49 and 59% activity of the free enzyme, respectively. This loss in activity may be attributed either to a loss in native conformation on account of immobilization or steric hindrances in the immediate vicinity of the enzyme molecule caused by the shielding effect of the matrix. In the packed-bed reactor, efficiency improves to 75 and 82%, respectively, for adsorbed and covalently bound enzymes. This shows that the native conformation is retained to a considerable extent during immobilization and the loss in efficiency in the batch reactor is mainly due to mass transfer restrictions. The decrease in K_m could be recognized as due to steric hindrances and diffusional limitations rather than to real changes in substrate affinity. Aksoy *et al.* (1998) obtained a 15-fold increase in K_m for α-amylase immobilized on

TABLE 3. Kinetic parameters of free and immobilized α-amylase.

Reactor	Catalyst	Michaelis constant K_m ($g\ l^{-1}$)	Maximum rate V_{max} ($\times 10^{-5}$ mol ml^{-1} min^{-1})	Effectiveness factor η
Batch	Free	4.5	6.63	–
	Adsorbed	37.1	3.25	0.49
	Covalently bound	31.2	3.91	0.59
Packed-bed	Adsorbed	15.9	4.91	0.75
	Covalently bound	12.8	5.44	0.82

polymer microspheres and related it to diffusional restrictions to mass transfer. In our case, the packed-bed reactor shows only a 4-fold increase which substantiates a reduction of diffusional restrictions. The reaction rate of enzyme bound on a porous particle can be affected by external or internal diffusional resistances, which correspond to the transport of substrate and products from the bulk solution to the outer surface of the enzyme particle, and to the internal transport of these species inside the porous system (Siso *et al.*, 1990). In this case, internal diffusional resistances can be avoided since the enzyme is too large to enter the pores of montmorillonite. Therefore, the only prevailing effect is external diffusional resistance. The effectiveness factor, η, is also increased in a packed-bed reactor. η values of 0.49 and 0.59 indicate that diffusion is important in the case of the batch reactor. When a packed-bed reactor is employed, this value increases to >0.75, demonstrating that diffusional effects are minimized. Therefore, during immobilization, the native conformation of enzyme is not altered much but activity in a batch reactor is reduced due to the operation of unwanted diffusional effects. Fixed-bed reactors are more efficient since they minimize diffusion-triggered mass-transfer limitations (Synowiecki *et al.*, 1982).

pH stability

The variation in activity with pH of free and immobilized α-amylase for starch liquefaction was tested in a batch and in a fixed-bed reactor (Fig. 2). Free enzyme demonstrates excellent activity at pH 6 and 7. The immobilized counterparts also show high activity at these two pH values. Additionally, adsorbed amylase has appreciable activity at pH 8 while covalently bound amylase shows good conversion at pH 5. pH affects the intra-molecular H-bonding responsible for maintaining the conformation of the enzyme. A change in pH will cause expansion or shrinkage of the protein structure leading to an altered conformation that will influence the activity of the enzyme. When an enzyme is linked to a support, some strain is enforced on the enzyme, which causes slight unfolding of enzyme protein. In this situation, contact with buffer solution will cause additional strain that may be beneficial or destructive. The nature of linkage between enzyme and support also decides the influence of pH. The adsorbed enzyme shows good activity in higher-pH regions. This is attributed to charge effects of the support matrix. Since the clay matrix is negatively charged, the pH in the microenvironment of the enzyme will be less than the bulk pH (Gaikwad & Deshpande, 1992). Therefore, a greater bulk pH is required to provide an optimum in the microenvironment of the enzyme and hence a shift to higher values is encountered. There are many reports about altering the optimum pH after immobilization (e.g. Tumturk *et al.*, 2000; Handa *et al.*, 1983). Covalently bound amylase shows high initial activity in the lower-pH regime. This may be due to secondary interactions between the enzyme and the clay matrix (Arica & Hasirci, 1993). At very high pH, enzyme inactivation occurs via oxidation of sulphur-containing amino acids and/or deamidation of glutamine residues (Hess & Kelly, 1999) leading to a loss in activity. The packed-bed reactor also shows similar results. The pH profile is broader, probably due to diffusional limitations or secondary interactions between enzyme and clay matrix (Arica *et al.*, 1995; Arica & Hasirci, 1993). Since the enzymes show very high initial activity at more than one pH, stability measurements have to be performed in order to

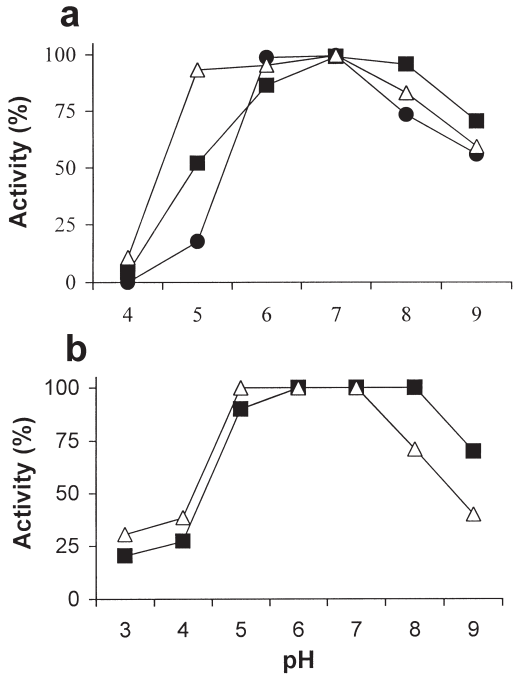

FIG. 2. Influence of pH on the activity of (●) free (■) adsorbed and (△) covalently bound α-amylase in (a) batch and (b) packed-bed reactors.

ascertain the optimum pH values. The results of stability experiments in batch reactors are shown in Fig. 3. Free α-amylase shows maximum stability at pH 6. As a general rule, an enzyme exhibits maximum activity and stability at its optimum pH; hence 6 can be considered as the optimum pH for free α-amylase. Covalently bound α-amylase also shows more stability at pH 6. Even though the initial activity is high, stability is lower for pH 5 proving that as time passes by, lower pH deactivates the enzyme by changing its conformation. Adsorbed enzyme shows increased stability at pH 7 compared to pH 6. This can be taken as evidence of the fact that adsorption causes a shift in the optimum pH to the basic side. Similar results are obtained for stability studies in a packed-bed reactor (Fig. 4). The optimum pH for free and covalently bound α-amylase is 6 while it is raised to 7 for the adsorbed counterpart. A change of reactor does not affect optimum values or stability.

Thermal effects

Temperature plays an important role in determining the activity of the enzyme. Immobilization brings about marked changes in the thermal stability of biocatalysts. The free α-amylase exhibits an optimum temperature of 50ºC in the batch reactor (Fig. 5). After immobilization, the temperature optimum is raised. The increase in optimum temperature is a direct outcome of immobilization; due to change in conformational integrity of the enzyme structure as a result of immobilization (Tumturk *et al.*, 2000; Lee *et al.*, 1993). Since the

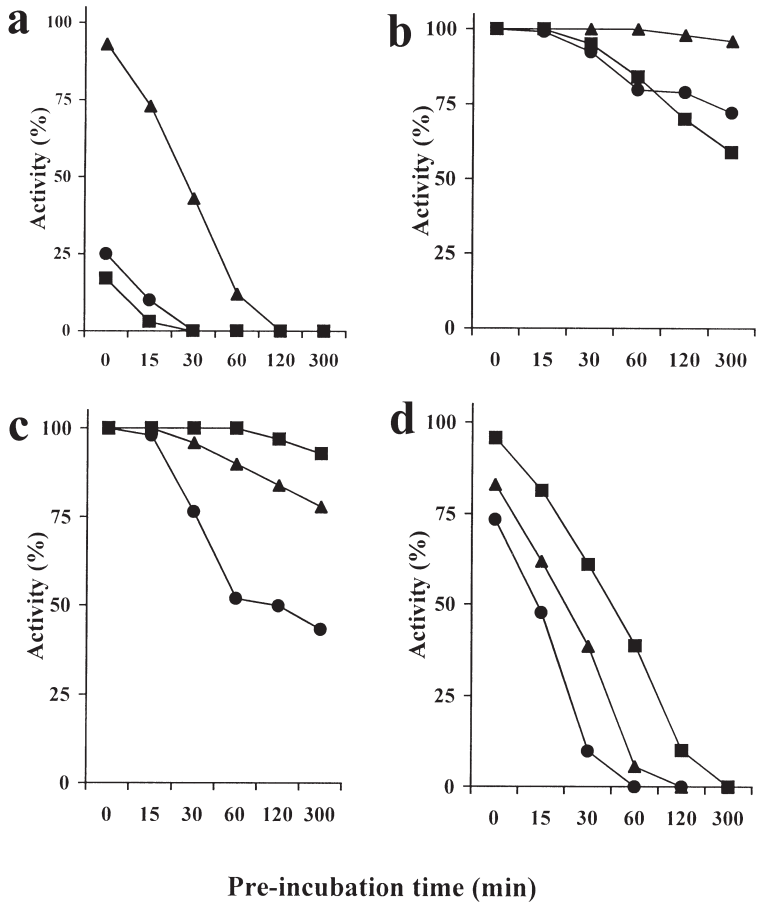

FIG. 3. Stability of (●) free (■) adsorbed and (▲) covalently bound α-amylase at pH (a) 5, (b) 6, (c) 7 and (d) 8 in a batch reactor.

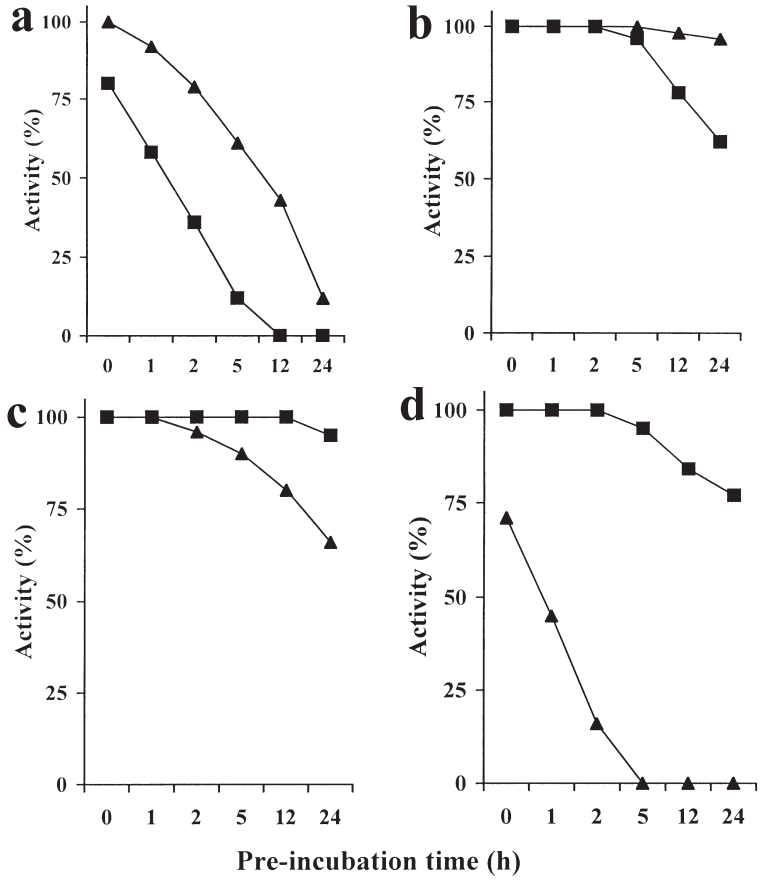

FIG. 4. Stability of (■) adsorbed and (▲) covalently bound α-amylase at pH (a) 5, (b) 6, (c) 7 and (d) 8 in a fixed-bed reactor.

mobility of enzyme is restricted upon immobilization, it retards the unfolding of protein, thereby maintaining activity. The activation energies were calculated from the Arrhenius plot as 10 kJ mol^{-1} for the free enzyme and 44 and 31 kJ mol^{-1} for the adsorbed and covalently bound enzymes, respectively. The adsorbed α-amylase demonstrates a higher optimum temperature compared to the covalently bound form. During adsorption, a direct interaction takes place between the enzyme and the clay matrix. Due to this, enzyme mobility is restricted to a greater extent than covalent binding and hence the enzyme is able to withstand higher temperature without denaturation. Thermal stability experiments were performed at higher temperatures in batch (70ºC) and packed-bed reactors (50ºC) (Fig. 6). The free enzyme became completely inactive after 90 min while the immobilized forms retained more than 50% activity after a 90 min treatment. Immobilized α-amylase is more stable at higher temperatures than the free enzyme (Chen *et al.*, 1997). In both cases, covalently bound α-amylase exhibited more stability than the adsorbed form. It is known that an enzyme will exhibit enhanced thermal stability when linked covalently to an insoluble matrix that may be explained by the reduction of mobility of enzyme after immobilization on the support (Ulbrich *et al.*, 1986). The adsorbed enzyme also exhibits a reduction in mobility but as time proceeds, this reduction in mobility is eliminated due to the weak nature of the enzyme support bond and therefore the protein unfolds leading to a loss of activity.

Operational stability

It is important for economical use of an enzyme, as a means for mass production of the desired

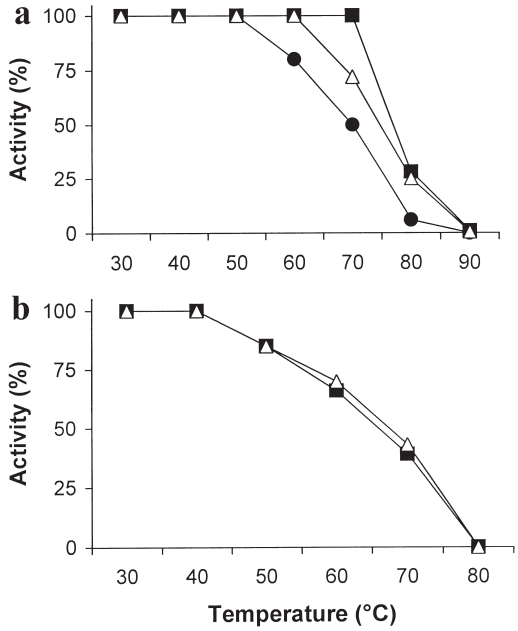

FIG. 5. Influence of temperature on activity of (●) free (■) adsorbed and (△) covalently bound α-amylase in (a) batch and (b) fixed-bed reactor.

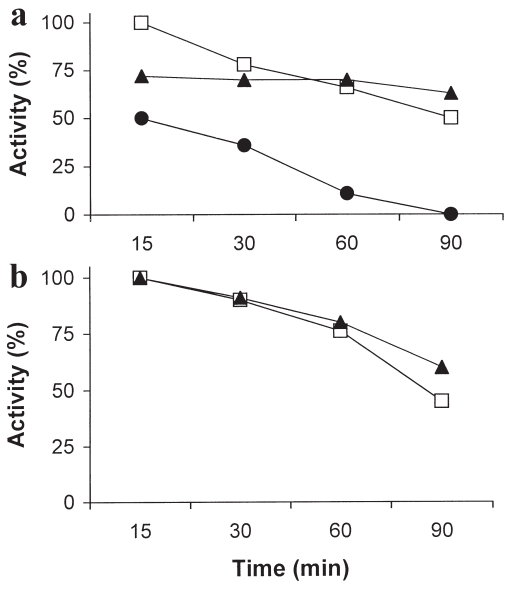

FIG. 6. Thermal stability of (●) free (□) adsorbed and (▲) covalently bound α-amylase in (a) batch and (b) fixed-bed reactor.

product, that the enzyme reaction is continuous. One of the problems in continuous enzyme reactions is the operational stability of the enzyme immobilized on the support (Akgol *et al.*, 2001). The fixed-bed reactor was operated continuously for 96 h at 30ºC and the activity of immobilized amylase was monitored. The immobilized enzymes retained 100% activity for 72 h of continuous operation (Fig. 7). Even after 96 h, more than 80% activity was retained. Loss in activity of enzyme may be attributed to deactivation upon use. Covalently bound enzymes exhibited better operational stability.

Conclusions

α-amylase was successfully immobilized on montmorillonite K-10 via two independent techniques, viz. adsorption and covalent binding. Surface area and pore volume decrease upon immobilization. The decrease is sharper for covalently bound enzyme, suggesting pore blockage. X-ray diffraction indicates that covalent binding leads to enzyme intercalation within the porous clay matrix while during adsorption the enzyme is not concentrated within the inter-lamellar space. The enzyme as a whole is not intercalated, but it is the side chains which take part in intercalation; the entire enzyme backbone is on the periphery of the clay surface. Scanning electron microscopy shows a decrease in the porous nature of clay upon covalent binding with amylase. Both immobilized enzymes demonstrate different optimal pHs and temperatures, with a broader profile compared to the free enzyme. The immobilized enzymes retain 49 and 59% activity in the batch reactor whereas use of fixed-bed reactor

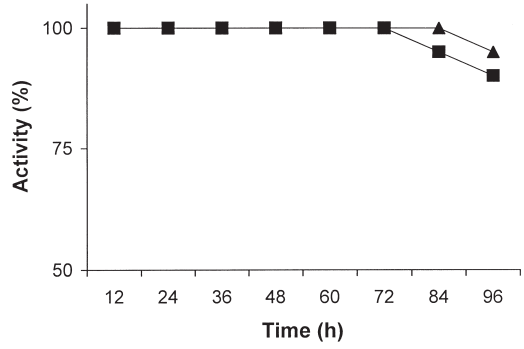

FIG. 7. Operational stability of (■) adsorbed and (▲) covalently bound α-amylase in a fixed-bed reactor.

improves efficiency to 76 and 82%, respectively. The effectiveness factor also increases, proving the greater efficiency in the fixed-bed reactor. Calclulated K_m values are higher than for free enzyme, which may be due to diffusional resistances encountered as a result of mass transfer restrictions due to immobilization. The pH and thermal stabilities are enhanced. The immobilized enzyme could be operated for 72 h in continuous mode without any loss in activity. Covalent binding demonstrates superior operational stability.

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