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Acid activated montmorillonite: an efficient immobilization support for improving reusability, storage stability and operational stability of enzymes

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Abstract Three enzymes, α-amylase, glucoamylase and invertase, were immobilized on acid activated montmorillonite K 10 via two independent techniques, adsorption and covalent binding. The immobilized enzymes were characterized by XRD, N_2 adsorption measurements and ^{27}Al MAS-NMR spectroscopy. The XRD patterns showed that all enzymes were intercalated into the clay inter-layer space. The entire protein backbone was situated at the periphery of the clay matrix. Intercalation occurred through the side chains of the amino acid residues. A decrease in surface area and pore volume upon immobilization supported this observation. The extent of intercalation was greater for the covalently bound systems. NMR data showed that tetrahedral Al species were involved during enzyme adsorption whereas octahedral Al was involved during covalent binding. The immobilized enzymes demonstrated enhanced storage stability. While the free enzymes lost all activity within a period of 10 days, the immobilized forms retained appreciable activity even after 30 days of storage. Reusability also improved upon immobilization. Here again, covalently bound enzymes exhibited better characteristics than their adsorbed counterparts. The immobilized enzymes could be successfully used continuously in the packed bed reactor for about 96 hours without much loss in activity. Immobilized glucoamylase demonstrated the best results.

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1 Introduction

The swelling phyllosilicate minerals known as smectite clays constitute a naturally occurring class of inorganic catalysts. These ubiquitous minerals are components of many soils and sediments and often are found as large mineralogically pure deposits. Due to their small size and unusual intercalation properties, they afford an appreciable surface area for adsorption of organic and inorganic moieties [1]. Montmorillonite, the most famous member of smectite clays, is a 2:1 dioctahedral clay and has been widely used as a catalyst as well as support [2]. The specialty of montmorillonite (smectites in general) is that the properties can be tailor made to suit the need by simple methods such as acid activation, ion exchange, pillaring and intercalation with organics. Montmorillonite is acidic in nature that justifies its remarkable activity in Friedel–Craft's reactions [3].

Immobilization of biocatalysts is undertaken either for the purpose of basic research or for use in technical processes of commercial interest. Immobilization makes possible heterogeneous catalysis by which efficient separation of biocatalysts is made feasible and in addition the process can be carried out continuously [4]. The immobilized enzymes have been efficiently used in food technology, biotechnology, biomedicine and also analytical chemistry. Enzymes can be immobilized by a variety of methods covering adsorption, entrapment, ionic & covalent binding, cross-linking etc.

Covalent binding is very effective in retaining the enzyme and can achieve high activity after immobilization [5]. Many supports have been employed to immobilize enzymes, the most important being polymers. Polystyrene microspheres have found good application as an immobilization support [6]. Magnetic polystyrene particles [7] and activated pHEMA microspheres [8, 9] have also been used, the latter one employed in a packed bed reactor. Magnetic polyvinylalcohol microspheres were also used to immobilize enzymes [10]. Other polymers like poly (*p*-chloromethylstyrene) [11], polypropylene [12], polypyrrole [13, 14], graft co-polymers [15, 16] etc. have been used efficiently. The major problem with polymer supports is lower pH and thermal stabilities.

Inorganic supports provide better thermal stabilities and hence there is immense scope for research in this area. The most extensively studied inorganic support is porous silica. Mody et al. [17] have immobilized α-amylase (1,4-α-glucan glucanohydrolase, EC 3.2.1.1) on porous silica and they successfully applied it for starch hydrolysis in a packed bed reactor. Siso et al. [18] have immobilized α-amylase on porous silica. They found that external diffusional resistances to mass transfer were minimized when the immobilized enzyme was operated in a packed bed reactor. Clays are also used as supports for enzyme immobilization. The acid sites of clays can serve as centres of binding through the $-NH_2$ group of enzymes. Cellulase was sorbed on clay minerals and humic colloids in the soil environment [19]. The authors found that $Al(OH)_x$ intercalated K- and Ca-montmorillonite showed better sorption properties than the pure forms. Natural kaolin was also applied as a support for the immobilization of lipase [20]. deFuentes et al. [21] immobilized lipase on different pyllosilicates and found that laminar silicates were quite active for the immobilization for higher molecular weight proteins. Bentonites, sepiolites and other modified montmorillonites are also used for immobilization of different enzymes [22–25]. Most of the immobilization studies concentrate on the change in enzyme activity on account of immobilization. Variations in the surface properties of the support after immobilization are not well documented.

In this research paper we present the results of immobilization of three enzymes—α-amylase, glucoamylase and invertase on acid activated montmorillonite via two independent techniques i.e. adsorption and covalent binding. Amylases find wide application in the food; textile, distillery and brewing industries since they hydrolyze starch to low-molecular weight products e.g. maltose, glucose etc [26]. Invertase (β-fructofuranosidase, EC 3.2.1.26) catalyzes the hydrolysis of sucrose to glucose and fructose. This invert sugar, which has a lower crystallinity than sucrose at higher concentrations employed, finds numerous applications in the food industry. Its use in confectionary ensures that the products remain fresh and soft even when kept for longer periods of time. Soluble invertase is used in the sweet industry in the production of artificial honey. Enzyme catalyzed hydrolysis has the advantage of colourless products compared to the coloured version obtained through acid hydrolysis [27, 28].

The immobilized enzymes were characterized using XRD, N_2 adsorption measurements and NMR. Enzymes can be attached to clays either on the surface or within the inter layer space. 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. XRD can be conveniently used for this purpose. Clays are semi-crystalline in nature; hence there are some phases, which can be identified by XRD analysis. Interaction with enzymes can cause significant changes in these phases that are easily recognized by analyzing the XRD spectrum. Clays are highly porous in nature. Hence a measurement of surface area and pore volume using N_2 adsorption studies would be advantageous to understand the effect of immobilization on the porosity of the clay. Clays are aluminosilicates and there is ample scope for NMR analysis due to the presence of the NMR-active nucleus ^{27}Al. Clay minerals have been the subject of several NMR investigations [29–31]. Changes in the chemical environment of Al can easily be visualized with the help of ^{27}Al NMR. This technique is of particular interest in studying octahedral and tetrahedral sites in the Al framework and so can be suitably applied for the study of interaction of the enzyme with the clay mineral.

The most striking features of a heterogeneous catalyst that makes it diverse from homogeneous catalysis are easy separation, reusability and application to continuous process [32, 33]. These are important for the economical use of an enzyme, as a means for mass production of the desired product. The number of successive cycles for which a catalyst can be efficiently reused is an intrinsic property of the catalyst. To some extent, it also depends on the reaction conditions employed. In case of immobilized enzymes, reusability is a vital parameter especially for the reason that enzymes are costly. Hence efficient reuse is necessary to optimize the cost. One of the problems encountered in continuous reactions is the operational stability of the enzyme immobilized on the support [10]. Immo-

bilization of enzymes is also taken up for long-term storage of enzyme. In the free form, it has a short life and therefore its use is restricted. After immobilization, the life of enzyme improves and therefore it can be kept for long-term usage. Thus it is very much essential to evaluate these parameters, which will lead to effective economic utilization of the enzyme industrially. The reusability and operational stability of the immobilized enzymes were tested in a batch and packed bed reactor respectively. The storage stability of the immobilized enzymes was also estimated in a batch reactor.

2 Experimental

2.1 Materials

Bacillus subtilis α-amylase, *Aspergillus niger* glucoamylase, *Baker's yeast* invertase, acid-activated montmorillonite K 10 (CEC 60 meq/mg; main exchangeable cations Na^+, Ca^{2+}, K^+), 3 aminopropyl triethoxysilane, glutaraldehyde and 3,5-dinitro salicylic acid were purchased from Sigma-Aldrich Chemicals Bangalore. All other chemicals were of purest grade available commercially.

2.2 Immobilization of enzymes

Adsorption: Montmorillonite K 10 was first mixed with deionized water and vigorously stirred for 6 h. It was filtered, dried at 120°C for 12 h and calcined at 350°C for 12 h. This calcined clay was mixed with equal volumes of 0.1 M phosphate buffer solution (pH 4, 6 and 7 for glucoamylase, invertase and α-amylase respectively) and enzyme solution and shaken for 1 h in a Remi thermostated water bath shaker at room temperature. It was then centrifuged in a Remi C-24 cooling centrifuge for 1 h. The centrifugate was tested for presence of protein by developing colour using Folin Phenol Ciocaltaue's reagent [34] and measuring the absorbance at 640 nm in a Shimadzu 160A UV-VIS Spectrophotometer. A standard calibration curve was plotted using bovine serum albumin and the amount of un-adsorbed protein was calculated. The residue was washed several times with deionized water; each time the amount of protein in solution was measured. It was found that all added enzyme got adsorbed and the enzyme loading was 10 mg g^{-1} clay. The immobilized enzymes were stored in 0.1 M phosphate buffer of optimum pH (7 for adsorbed α-amylase and glucoamylase, 6 for adsorbed invertase) at 5°C for further use.

Covalent binding: Calcined montmorillonite K 10 was mixed with a 10% (v/v) solution of 3-amino propyl triethoxy silane in acetone and vigorously stirred for 3 h at room temperature [17]. It was filtered, washed several times with acetone until the washings became colourless and later dried at 80°C for 12 h. This silanized clay was treated with 10% aqueous solution (v/v) of glutaraldehyde and stirred vigorously for 3 h. It was filtered, washed free of excess glutaraldehyde and dried at 60°C for 12 h. This functionalized clay was used for immobilization as per the procedure described above. The covalently bound enzymes were stored in 0.1 M phosphate buffer of optimum pH (6 for covalently bound α-amylase and glucoamylase, 5 for covalently bound invertase) at 5°C for further use. The enzyme loading was kept 10 mg g^{-1} clay.

2.3 Characterization techniques

Powder XRD of the immobilized enzyme systems and the support were taken on a *Rigaku* D/Max-C system with Ni filtered CuK_α radiation (λ = 1.5406 Å) within the 2θ range 2°–15° at a scanning rate of 0.5°/min at room temperature. Solid-state ^{27}Al MAS-NMR experiments were carried out over 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 $\pi/2$ flip angle. The pulse length for the experiments was 10 μs whereas the 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. A *Micromeritics* model Gemini 2360 surface area analyzer 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 (10 point, p/p_0 = 0.05–0.3, adsorption isotherm). The pore volume was determined from the adsorption isotherm in a p/p_0 range 0.05–0.95. Prior to the measurement, the samples were degassed at room temperature for 12–16 h in nitrogen flow.

2.4 Activity measurements

Batch reactor: One ml of free enzyme solution (0.1 g immobilized enzyme) was mixed with 20 ml buffered substrate solution (5% w/v) at optimum pH and incubated in a water bath shaker at room temperature. After the reaction time (30 min for α-amylase and glucoamylase, 40 min for invertase), an aliquot of the product was removed from the reaction mixture and

analyzed colourimetrically. For starch hydrolysis, colour was developed using iodine solution [9, 35] and the absorbance read at 610 nm. The results were compared with absorbance of standard starch and the amount converted was calculated. In case of sugar hydrolysis, colour was developed using 2% 3,5-dinitro salicylic acid solution in 1 M NaOH (w/v) and absorbance was read at 490 nm [11, 36]. A standard calibration curve was constructed using glucose solution and the total reducing sugar content was estimated.

Packed bed reactor: A silica glass tube of 1.2 cm id and 25 cm length was used as the reactor. The immobilized enzyme (1 g) was packed into a bed at the middle of the reactor, which was filled with glass beads. The substrate was fed from the top of the reactor using a *Cole Palmer 74900 series* syringe pump and the products were collected at the bottom at 1 h intervals. Product was analyzed using the method described in the previous section. The reactor was operated at a space velocity of 2 h^{-1}.

Reusability of the immobilized enzymes was tested in a batch reactor. The reaction was continued several times; after each reaction, the mixture was centrifuged, catalyst separated and mixed with fresh substrate solution. Around 30 continuous cycles were performed. The whole reaction was carried out over a period of 4 days. Operational stability was tested in a packed bed reactor as described earlier. The reactor was operated continuously for 96 h and the activity was tested at 12 h intervals. The free and immobilized enzymes were stored in 0.1 M phosphate buffer solution of optimum pH at 5°C for a period of 30 days and the activity was tested every day in the batch mode. The results are presented as percentage of initial activity retained.

3 Results and discussion

3.1 Storage stability studies

Storage stability measurements were conducted in the dry form and in buffer solution of optimum pH. In the dry condition, immobilized enzymes exhibited very poor storage stability. After 36 h of storage, adsorbed α-amylase lost all activity while the covalently bound form retained 25% activity only. Similarly both forms of immobilized glucoamylase lost all activity in 12 hours. Adsorbed invertase was completely deactivated in 15 min whereas the covalently bound form lost all activity within 12 h.

When stored in 0.1 M buffer of optimum pH (Fig. 1), the free α-amylase lost all its activity within 10 days. The adsorbed form showed a gradual decrease

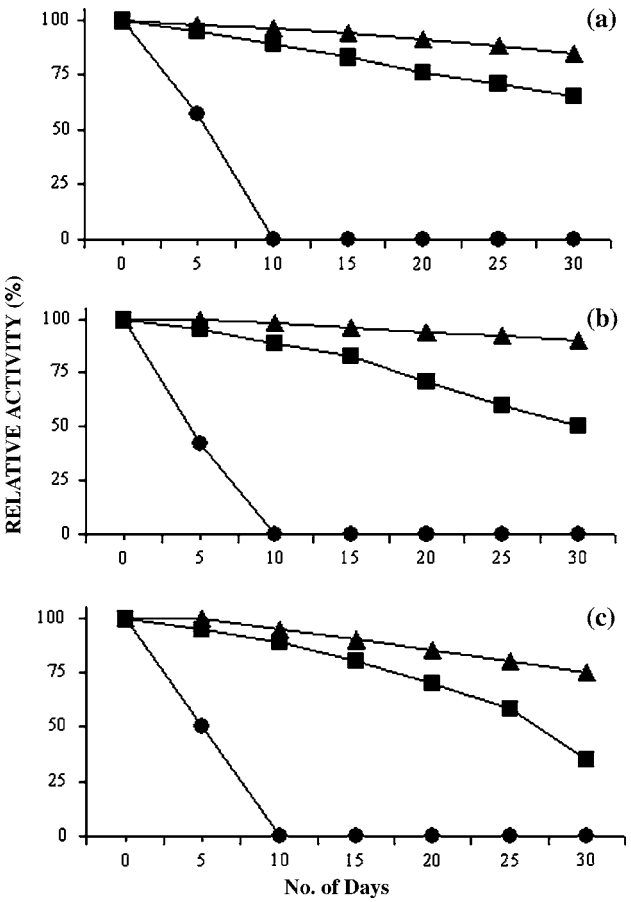

Fig. 1 Storage studies of (**a**) α-amylase (**b**) glucoamylase and (**c**) invertase in the (●) free (■) adsorbed and (▲) covalently bound forms

in activity and retained 65% initial activity after 30 days whereas the covalently bound counterpart retained 85% activity in the same period. Free glucoamylase lost complete activity in 7 days. The adsorbed form retained 50% activity in 30 days while the covalently bound glucoamylase was completely active for 5 days after which it showed a gradual decrease in activity (upto 85% in 30 days). Similar results have been reported earlier [37, 38]. Similar results were obtained for invertase. The free enzyme lost complete activity in 9 days. Adsorbed invertase retained 35% activity after 30 days while the covalently bound enzyme retained 75% activity in the same time. The improved storage stability of immobilized enzymes can be attributed to a reduction in the rate of denaturation of the enzyme as a result of fixation on the clay matrix [27, 39].

3.2 Reusability

The results of reusability investigations in batch reactor are shown in Fig. 2. Adsorbed α-amylase could be

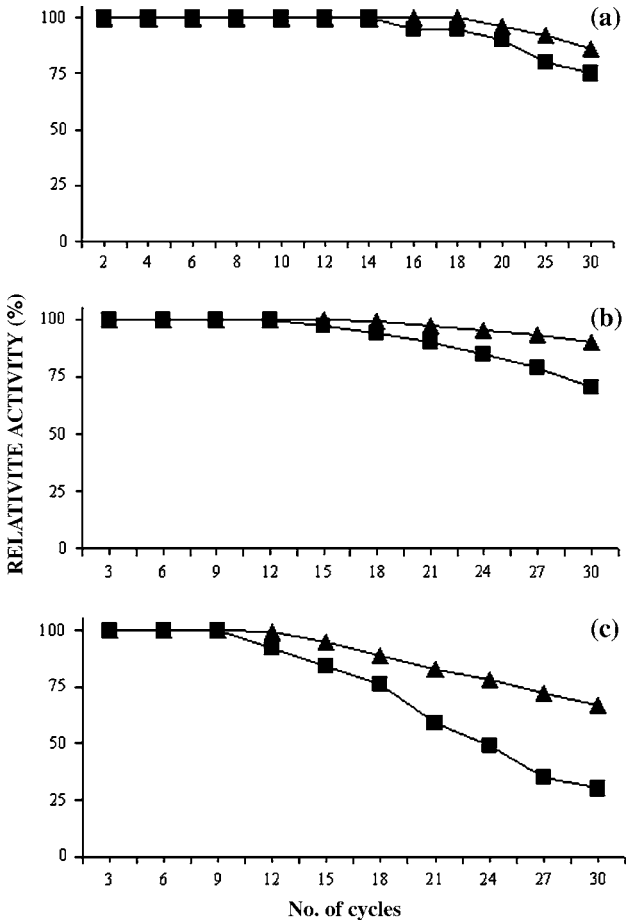

Fig. 2 Reusability of (**a**) α-amylase (**b**) glucoamylase and (**c**) invertase in the (■) adsorbed and (▲) covalently bound forms performed in a batch reactor

used without any loss in activity for 14 continuous cycles. After 30 cycles, it retained 75% of its initial activity. For the covalently bound enzyme, 100% activity was maintained for 18 cycles and at the end of 30 cycles, it retained 85% of its initial activity. Immobilization of α-amylase on montmorillonite clay has improved reusability of the enzyme. The loss in activity is due to the natural inactivation of enzyme as a result of time dependent denaturation of the enzyme protein. The results indicate the significance of clay as support for immobilization. There are various reports on reusability characteristics of immobilized α-amylase. Ju et al. [26] have reported 65% retained activity after 30 cycles in case of α-amylase immobilized on hollow fibre reactor. Tanyolac et al. [40] have reported complete reusability for α-amylase immobilized onto nitrocellulose membrane up to 10 successive cycles followed by a 35% reduction in activity. The immobilized glucoamylase demonstrate excellent reusability. The adsorbed glucoamylase could be reused without any loss in activity for 12 consecutive cycles. After 30 cycles it retained 70% activity. Covalently bound glucoamylase could be reused for 15 cycles with 100% activity and it retained 90% activity even after 30 cycles. These results are comparable to literature [41, 42]. The adsorbed invertase could be reused without any loss in initial activity for 9 continuous cycles after which there was a rapid drop in activity. The covalently bound invertase preserved all of its initial activity for 12 cycles and at the end of 30 cycles it retained 70% activity.

3.3 Operational stability

Figure 3 depicts the results of operational stability measurements on the immobilized enzymes. The adsorbed α-amylase preserved 100% initial activity for 72 h after which it showed a 10% drop in activity. At the same time, covalently bound α-amylase maintained 100% initial activity for 84 h after which a moderate loss in activity was observed. Immobilized glucoamylase displayed excellent operational stability. The

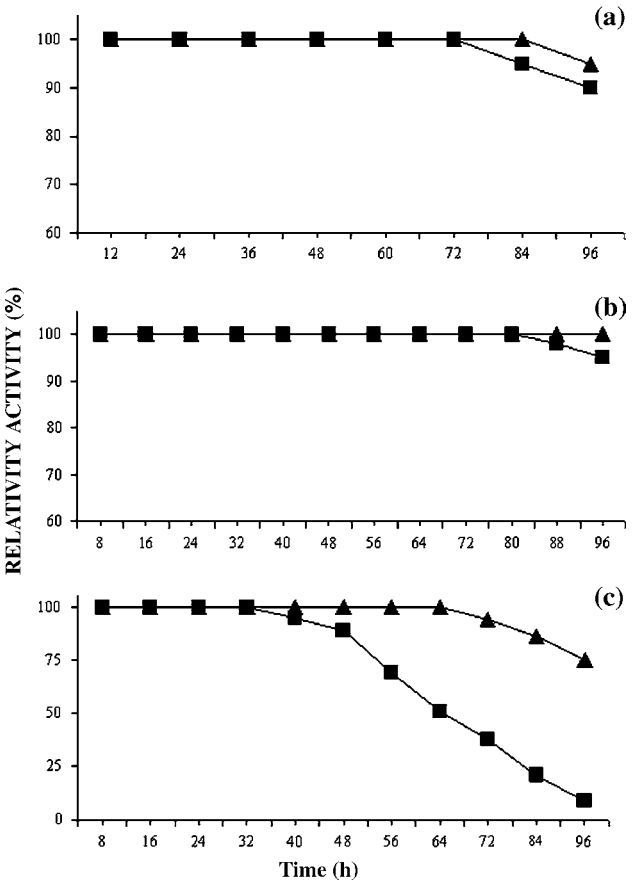

Fig. 3 Operational stability of (**a**) α-amylase (**b**) glucoamylase and (**c**) invertase in the (■) adsorbed and (▲) covalently bound forms performed in packed bed reactor

covalently bound enzyme could be used without any loss in activity for 96 h while the adsorbed form lost only 5% activity after 84 h. Covalently bound invertase retained complete activity up to 64 h of continuous reaction. Even after 96 h, 75% activity was retained. The loss in activity may be due to the natural inactivation of enzyme on account of time dependent denaturation of the enzyme-protein [43]. The adsorbed invertase did not show promising results. It was active only for 32 h after which inactivation started and in 96 h almost complete activity was lost. Even though the use of packed bed reactor improved the activity of immobilized invertase, there was a change in the native conformation of the enzyme rapid loss of activity occurred during continuous use. Other authors have also obtained similar observations [9, 28].

3.4 N_2 adsorption measurements

Table 1 gives the surface area and pore volume (from N_2 adsorption measurements) for the immobilized enzymes. Montmorillonite functionalized with 3-APTES and glutaraldehyde showed a decrease in surface area and pore volume. This may be because the silane and glutaraldehyde molecules were bound to the clay matrix within the inter lamellar space. Direct adsorption of α-amylase reduced the surface area, but the decrease is moderate and surface area reduced with increasing amounts of enzyme. It was the case with pore volume also. Covalent binding lead to a sharp decrease in pore volume as well as surface area. Covalent binding occurs via the glutaraldehyde spacer group which was present within the inter layer space. Hence linkages with enzyme lead to severe blockage of pores and so the surface area decreased sharply. Immobilized glucoamylase also exhibited comparable characteristics. For the adsorbed glucoamylase, the surface area and pore volume decreased slowly as in the case of α-amylase but the covalently bound counterpart demonstrated an abrupt decline in surface area from 145 to 25 $m^2\ g^{-1}$ even for a low enzyme loading of 10 mg g^{-1} clay. Analogous was the case for immobilized invertase. This could be related to the size of the enzyme. The molecular size of glucoamylase (Mol. wt. 180,000) and invertase (Mol. wt. 270,000) were much higher than α-amylase (Mol. wt. 50,000) and hence a small enzyme concentration (10 mg g^{-1} clay) was enough to bring about excessive pore blockage for the covalently bound systems.

3.5 X-ray diffraction analysis

The XRD results for immobilized enzymes are shown in Table 2. For parent montmorillonite, there was a peak at $2\theta = 8.9°$ corresponding to a d spacing of 9.98 Å. This peak represents the d_{001} plane analogous to the inter-layer spacing. After functionalization with 3-APTES and glutaraldehyde, a new reflection of increased intensity appeared at a d-value of 15.3 Å whereas the intensity of the original reflection decreased sharply. This demonstrated that inter-layer space expansion occurred during functionalization. Adsorption of α-amylase did not change the intensity of the d_{001} reflection. A new reflection appeared which was of low intensity. As enzyme loading increased, the intensity of the new reflection increased confirming intercalation of enzyme or a higher degree of ordering. Covalent binding of α-amylase lead to basal spacing in the range 19.2–20.7 Å. The original d_{001} reflection disappeared completely indicating that intercalation of enzyme reached a high degree or that the typical layer structure is reconstituted. Enzymes are polymeric species of very high molecular size. α-amylase has a molecular size of 80 Å, hence the possibility of

Table 1 Surface area and pore volume data for the immobilized enzymes

Catalyst	BET surface area ($m^2\ g^{-1}$)			Pore volume ($\times 10^{-6}\ m^3\ g^{-1}$)		
	AA	GA	I	AA	GA	I
A_{10}	162	156	152	0.2234	0.2196	0.2153
A_{50}	134	132	148	0.2093	0.2050	0.2025
A_{100}	108	105	135	0.1905	0.1919	0.1928
C_{10}	100	25	16	0.1056	0.0743	0.0536
C_{50}	81	19	15	0.0765	0.0582	0.0524
C_{100}	45	15	10	0.0456	0.0432	0.0398

AA = α-amylase, GA = glucoamylase, I = invertase.

A = adsorbed enzyme, C = covalently bound enzyme.

Values in subscript denote enzyme loading in mg g^{-1} clay.

Montmorillonite K 10: BET surface area = 201 $m^2\ g^{-1}$, pore volume = $0.2511 \times 10^{-6}\ m^3\ g^{-1}$. Functionalized montmorillonite: BET surface area = 145 $m^2\ g^{-1}$, pore volume = $0.1745 \times 10^{-6}\ m^3\ g^{-1}$.

Table 2 2θ and d spacing values for the immobilized enzymes

Catalyst	α-Amylase	Glucoamylase	Invertase
A_{10}	8.95[a] (9.87)	8.9[a] (9.93)	8.85[a] (9.98)
	5.1 (17.28)	4.9 (18.43)	4.55 (19.52)
A_{50}	8.9[a] (9.93)	8.95[a] (9.87)	8.9[a] (9.93)
	4.85 (18.68)	4.45 (19.75)	4.35 (20.54)
A_{100}	8.85[a] (9.98)	8.85[a] (9.98)	8.85[a] (9.98)
	4.45 (19.75)	4.25 (21.75)	4.25 (21.75)
C_{10}	8.85 (9.98)	4.35[a] (20.54)	3.75[a] (23.54)
	4.6[a] (19.19)		
C_{50}	4.35[a] (20.54)	4.0[a] (22.85)	3.35[a] (25.96)
C_{100}	4.3[a] (20.74)	3.95[a] (23.00)	3.3[a] (26.25)

A = adsorbed enzyme, C = covalently bound enzyme, Values in subscript denote enzyme loading in mg g^{-1} clay. Values in parentheses represent d spacing in Å.

[a] Peak intensity 100%.

The second number in the cells represents the additional peak.

attachment within the inter lamellar space could be ruled out. But a shift in the d_{001} peak to lower values was an evidence for intercalation [44, 45]. Therefore it was proposed that the whole enzyme does not get intercalated into the clay layers. It was the side chains of various amino acid groups that were responsible for intercalation. The polypeptide backbone did not enter the interlayer space but was situated at the periphery of the clay [46]. For α-amylase, an enzyme loading of 50 mg was necessary to bring about an elevated level of intercalation. Similar is the case for immobilized glucoamylase and invertase. The adsorbed enzymes show only slight shift of basal spacing to higher values indicative of non-uniform inter-layer expansion whereas covalent binding shows a complete disappearance of the original d_{001} reflection. The inter-layer spacing increases to 26.3 Å for covalently bound invertase and 23 Å for covalently bound glucoamylase. An enzyme loading of 10 mg g^{-1} clay was enough to bring about a high degree of intercalation. As in case of α-amylase, enzyme intercalation was through the side chains of the amino acid residues.

3.6 Nuclear magnetic resonance spectroscopy

Table 3 depicts the ^{27}Al MAS NMR chemical shift for the different materials. ^{27}Al NMR shows two resonances around 0 and 70 ppm representing Al in tetrahedral and octahedral co-ordination respectively [47, 48]. Montmorillonite exhibited an octahedral Al resonance at 2.8 ppm and a tetrahedral resonance at 69.8 ppm. A slight change in octahedral chemical shift was due to the presence of Fe. Functionalization with silane and glutaraldehyde resulted in a shift of octahedral peak to –1.7 ppm while the tetrahedral peak remained unaltered indicating that only the octahedral

Table 3 Chemical shift values of ^{27}Al NMR for the parent clay and the immobilized enzymes

	Chemical shift (ppm)	
Catalyst	Octahedral	Tetrahedral
M	2.8	69.8
SGM	–1.7	69.5
AA_A	2.8	63.4
AA_C	1.3	69.3
GA_A	2.6	62.1
		54.1
GA_C	2.3	69.2
		53.9
I_A	2.7	61.3
I_C	3.8	69.7

M = montmorillonite, SGM = montmorillonite functionalized with silane and glutaraldehyde, AA = α-amylase, GA = glucoamylase, I = invertase.

Subscript A = adsorbed enzyme, subscript C = covalently bound enzyme.

Al atoms were involved in binding with silane and glutaraldehyde. This further concluded that the binding takes place within the clay inter-layer space and hence substantiated the results of XRD and surface area measurements.

Enzyme adsorption lead to a shift of tetrahedral Al peak by 6–8 units keeping the octahedral peak almost constant. Thus during adsorption the enzyme interacted with the tetrahedral Al alone. When the enzyme was covalently bound to the clay matrix, the tetrahedral Al resonance was unaltered while the octahedral peaks showed a shift by 3–6 ppm. Covalent binding took place on the glutaraldehyde spacer and not directly on to the Al species. In spite of this, there was a sufficient shifting of octahedral peak that signified 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 in electrostatic interactions with octahedral Al species changing its chemical environment thereby causing a shift in NMR signal. This shift increased with increasing size (and molecular weight) of the enzyme (α-amylase < glucoamylase < invertase). This was because as the molecular weight increased, the number of side chains on the polypeptide backbone also increased providing more functional groups for secondary interactions with the support. Immobilized glucoamylase showed an additional tetrahedral resonance at 54 ppm, which was present for the adsorbed as well as the covalently bound forms. Glucoamylase contained the cystine residue that has the reactive –SH group in its side chain. This reactive group could interact with tetrahedral Al bringing about such a large chemical shift. The reason for a split in tetrahedral Al resonance may be that only a few of the Al are involved in interaction with cystine side chain and so they resonate at a different value than the remaining Al atoms.

4 Conclusions

The three enzymes, α-amylase, glucoamylase and invertase, were successfully immobilized on montmorillonite using two techniques i.e. adsorption and covalent binding. XRD demonstrated an increase in inter-layer spacing, which suggested intercalation of enzymes or a higher degree of ordering due to typical reconstitution of delaminated layer structure. The whole enzyme did not get intercalated. The protein backbone was situated at the periphery of the clay while the side chains were involved in intercalation. The extent of intercalation was restricted to the initial clay layers for adsorption whereas during covalent binding the amount of intercalation reached an elevated level. Surface area and pore volume of montmorillonite showed a decrease after immobilization. The decrease was more pronounced for the covalently bound enzymes suggesting excessive pore blockage. ^{27}Al NMR provided information on the interaction between Al species of clay and the enzyme. During adsorption, only the tetrahedral Al species were involved while covalent binding involved the octahedral Al exclusively. In case of immobilized glucoamylase, an additional tetrahedral resonance was seen which may be due to interaction of the reactive –SH group of cystine residue with Al. The immobilized enzymes demonstrated greater storage stability compared to the free counterpart. They could be reused for more than 15 cycles without any loss in activity. The operational stability of the immobilized enzymes was also very high. Covalently bound enzymes exhibited better characteristics than the adsorbed form.

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