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Glucoamylase immobilized on montmorillonite: influence of nature of binding on surface properties of clay-support and activity of enzyme

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Abstract Glucoamylase was immobilized on acid activated montmorillonite clay via two different procedures namely adsorption and covalent binding. The immobilized enzymes were characterized by XRD, NMR and N_2 adsorption measurements and the activity of immobilized glucoamylase for starch hydrolysis was determined in a batch reactor. XRD shows intercalation of enzyme into the clay matrix during both immobilization procedures. Intercalation occurs via the side chains of the amino acid residues, the entire polypeptide backbone being situated at the periphery of the clay matrix. ^{27}Al NMR studies revealed the different nature of interaction of enzyme with the support for both immobilization techniques. N_2 adsorption measurements indicated a sharp drop in surface area and pore volume for the covalently bound glucoamylase that suggested severe pore blockage. Activity studies were performed in a batch reactor. The adsorbed and covalently bound glucoamylase retained 49% and 66% activity of the free enzyme respectively. They showed enhanced pH and thermal stabilities. The immobilized enzymes also followed Michaelis–Menten kinetics. K_m was greater than the free enzyme that was attributed to an effect of immobilization. The immobilized preparations demonstrated increased reusability as well as storage stability.

Keywords Immobilization · Glucoamylase · Adsorption · Covalent binding · Montmorillonite

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1 Introduction

The hydrolysis of starch to low molecular weight products is widely applied in the food, paper, textile, distillery and brewing industries [1]. An enormous increase in interest in glycosidases has been observed in recent years, caused by the biotechnological potential of such enzymes. Amylases catalyze the hydrolysis of glycosidic linkages in starch and other related oligo- and polysaccharides. These enzymes are widespread among the higher plants, animals and microorganisms and some of these are commonly used in various industries like starch processing, paper manufacture and pharmacology [2]. Glucoamylase (α-1,4-D-glucan glucohydrolase, EC 3.2.1.3), an exo enzyme, acts on the 1,4-glucosidic linkages from the non-reducing ends of amylose, amylopectin and glycogen in a consecutive manner liberating D-glucose. It also hydrolyses α-1,6- and α-1,3-glucosidic linkages but at a much slower rate compared to its action on α-1,4-linkages. Glucoamylase is an industrially important enzyme and is used for large-scale saccharification of malto-oligosaccharides into glucose and various syrups required in the food and beverages industry [3]. Immobilized enzymes, as well as immobilized bacterial cells are at the heart of innovative biotechnological processes as alternatives to traditional chemical technologies and hybrid processes including biochemical stages to substitute some chemical ones. The main advantage of immobilization is the combination of unique biocatalytic properties of enzymes with heterogeneity that makes it possible to use them in continuous flow reactors [4]. Studies on immobilization of glucoamylase are in rapid progress and many supports have been utilized. These include ceramic membranes [5], polymer microspheres [6, 7],

magnetic supports [8] etc. Inorganic supports for enzyme immobilization are of great interest because of their durability and high mechanical strength for usage in packed or fluidized bed reactors and relatively low cost. In addition, immobilization by adsorption is economically feasible and attractive.

In the present study, we have immobilized glucoamylase on montmorillonite clay via two methods i.e. adsorption and covalent binding. Adsorption refers to the direct interaction of support and enzyme. Covalent binding is the attachment of enzyme via a spacer. Montmorillonite, which is a 2:1 dioctahedral smectite, has been successfully used as a support as well as a catalyst for the past few decades [9]. The swelling ability of these naturally occurring phyllosilicate minerals provide unusual properties and appreciable surface area for adsorption of organic molecules [10]. A large number of modifications on clay that provide a variety of textural and catalytic properties are possible which include acid treatment, cation exchange, pillaring with robust metal ions and intercalation of polymeric organic moieties. Depending on the type of modification, the properties of clay can be tuned in such a way as to suit specific applications. Here we have employed montmorillonite K-10, which is the acid activated form. Since clays are aluminosilicates, they possess acidic sites that are capable of interaction with amino groups of enzymes leading to adsorption. This linkage is much stronger than mere physical binding and hence the enzyme will be retained on the support for a larger duration.

The immobilized glucoamylase was characterized by X-ray diffraction, N_2 adsorption measurements and NMR spectroscopy and the activity for starch hydrolysis was tested in a batch reactor. 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 for which XRD can be conveniently used. Measurement of surface area using N_2 adsorption studies would be advantageous to understand the effect of immobilization on the porosity of the clay. Clay minerals have been the subject of several NMR investigations [11–13]. 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 enzyme immobilization on montmorillonite. The rates of reactions were studied at different substrate concentrations and the Hanes–Woolf plot was used to determine the kinetic constants K_m and V_{max}. The effectiveness factor η was determined from the ratio of rates for immobilized and free enzyme activity. The effect of pH and temperature on activity and conformational stability of glucoamylase was also investigated. In addition to that reusability, storage stability and leaching studies were also conducted and the results for adsorption and covalent binding were compared.

2 Experimental

2.1 Materials

Montmorillonite K-10, *Aspergillus niger* glucoamylase, 3-amino propyl triethoxy silane (3-APTES) and glutaraldehyde were purchased from Sigma-Aldrich Chemicals Pvt Ltd, Bangalore. All other chemicals were of the highest purity commercially available.

2.2 Immobilization of glucoamylase on montmorillonite

For adsorption, montmorillonite K-10 was mixed with equal volumes of 0.1 M phosphate buffer and glucoamylase solution. It was shaken in a water bath shaker at room temperature for 1 h and later centrifuged in a Remi C-24 model-cooling centrifuge at 1 °C for 1 h. Enzyme protein was estimated using the spectrophotometric method of Lowry [14] 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 [15] 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 at ambient temperature. This was treated with 10% aqueous glutaraldehyde solution (v/v) for 1 h at room temperature, filtered, washed and dried at ambient temperature. This functionalized clay was used to bind the enzyme as explained earlier. All immobilized systems were stored in 0.1 M phosphate buffer at 5 °C.

2.3 Characterization of immobilized enzymes

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 A°) within the 2θ range 2°–20°. A *Micromeritics* Gemini 2360 surface area analyzer was used to measure the nitrogen adsorption of the samples at liquid nitrogen tempera-

ture. 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. 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 2s. 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.

2.4 Activity measurements

About 1 mL of free enzyme solution (0.1 g immobilized enzyme) was mixed with 20 mL buffered 5% starch solution (w/v) and incubated in a water bath shaker at room temperature. After the reaction time, an aliquot (1 mL) of the product was removed from the reaction mixture and analyzed colourimetrically. Colour was developed using iodine solution and the absorbance read at 610 nm. The results were compared with absorbance of standard starch solution and the amount of starch converted was calculated. All the results are presented in a normalized form with the activity under optimum conditions given a value of 100.

The influence of pH on activity of free and immobilized enzymes was studied by varying the pH from 3 to 8. Stability of the enzyme preparations to various pH was estimated by calculating the activity with respect to pre-incubation time in the range 15–300 min.

Reactions were conducted at various temperatures between 30 and 90 °C and the activity measured at each temperature. Obtaining the rate at each temperature and substituting in the Arrhenius plot measured activation energy. Thermal stability experiments were conducted with respect to pre-incubation time in the range 15–90 min.

The kinetic parameters (Michaelis constant K_m and Maximum rate V_{max}) were calculated by measuring the rates of reaction at various substrate concentrations. The values were substituted into the Hanes–Woolf equation to obtain K_m and V_{max}. The effectiveness factor η was measured by obtaining the rates at a substrate concentration high enough to eliminate inhibition effects.

Reusability of the immobilized enzymes was tested in a batch reactor. The reaction was continued several times; each time after the reaction, the mixture was centrifuged, catalyst separated and mixed with fresh substrate solution. Around 30 continuous cycles were repeated each of 30 min duration. The whole reaction was carried out over a period of 4 days.

The free and immobilized enzymes were stored in 0.1 M buffer solution of optimum concentration at 5 °C for a period of 30 days and the activity was tested every 2–3 days. The activity was compared with first time activity and is represented as percentage initial activity retained.

A study on leaching of enzyme was investigated in the batch reactor. The immobilized enzyme was treated with pH buffer and shaken for 30 min. It was centrifuged and the centrifugate was estimated for protein. The influence of enzyme concentration on leaching was understood by carrying out the reaction with enzyme amounts of 10, 50 and 100 mg per g clay. The process was repeated continuously for 15 cycles and the influence of repeated use on leaching was analyzed. The reaction was conducted at various temperatures between 30 and 90 °C to estimate the influence of temperature on the extent of leaching. All results were expressed as percentage enzyme retained.

3 Results and Discussion

3.1 X-ray diffraction

Figure 1 illustrates the XRD pattern for the various immobilized systems. Activation of clay with silane and glutaraldehyde results in a lowering of the intensity of the (001) peak at $2\theta = 8.9°$. A new peak appears at lower 2θ (Table 1) suggesting that the silane and glutaraldehyde molecules are intercalated within the clay layers. Upon covalent binding with glucoamylase, the new peak shift further to lower value whereas the original peak disappears indicating that the enzyme is also intercalated within the clay layers leading to layer expansion. An increase in enzyme loading leads to a further expansion of the clay layers. Organic-clay intercalated nanocomposites demonstrate a shift of the d_{001} peak to lower values or occurrence of a new peak along with a decreased intensity of the actual one, and this is taken as an evidence for intercalation and pillaring [16, 17]. Glucoamylase is a very large molecule with molecular size over 100 Å, so it is impossible for the whole molecule to intercalate within the clay interlamellar space. At the same time, formation and further shifting of the new d_{001} peak to lower values is a conclusive evidence for intercalation. So it can be said that the entire enzyme molecule does not get interca-

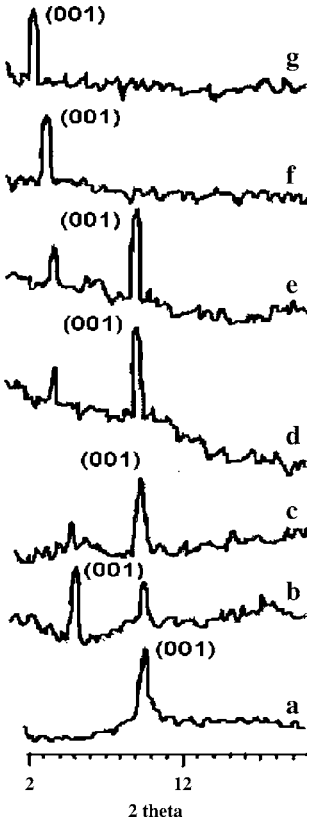

Fig. 1 XRD patterns of (**a**) montmorillonite K–10, (**b**) functionalized montmorillonite K–10, (**c**–**e**) montmorillonite adsorbed with glucoamylase at enzyme loadings of 10, 50 and 100 mg g^{-1} clay, (**f** and **g**) covalently bound glucoamylase at enzyme loadings of 10 and 50 mg g^{-1} clay

Table 1 Inter-layer spacing for parent and functionalized montmorillonite and glucoamylase immobilized clay

Catalyst	d_{001} (Å)
M	9.9
SGM	10.0
	15.3
A_{10}	9.3
	18.4
A_{50}	9.9
	19.8
A_{100}	10.0
	21.8
C_{10}	20.5
C_{50}	22.9

M—montmorillonite K-10; SGM—montmorillonite functionalized with silane and glutaraldehyde; A—adsorbed glucoamylase; C—covalently bound glucoamylase. Values in subscript depict enzyme concentrations in mg g^{-1} clay

lated. The side chains of different amino acid residues take part in intercalation while the major part of enzyme is situated outside the pores. Some researchers [18] have shown that polylysine adsorption over synthetic montmorillonite leads to intercalation of the side chains into the clay layers while the polypeptide backbone does not enter the interlayer space. In our case the enzyme is situated at the periphery of montmorillonite and covalent binding takes place between the side chains and the glutaraldehyde molecule. In case of adsorption also a small peak appears at lower 2θ whose intensity increases with amount of enzyme adsorbed. This points out that the enzyme gets intercalated during adsorption. The lower intensity of this peak demonstrates that intercalation takes place in a non-uniform fashion i.e. only in a few of the clay layers. Even in case of adsorption, intercalation occurs via the side chains of different amino acid residues while the entire protein backbone is situated at the pore entrance.

3.2 Surface area and pore volume from N_2 adsorption studies

Surface area as well as pore volume decrease upon immobilization (Table 2). Activation of montmorillonite with aminopropyltriethoxysilane and glutaraldehyde lowers the surface area suggesting that the binding of silane and glutaraldehyde molecules to the clay takes place within the layers (the silane molecule reacts with hydroxyl groups of the clay matrix with the release of ethanol; the –CHO group of glutaraldehyde condenses with the free $–NH_2$ group of silane). This fact is proved further since covalent binding of glucoamylase through the glutaraldehyde spacer reduces the surface area to a greater extent. It is the case for pore volume also. Adsorption of glucoamylase results in a lowering of surface area along with pore volume but the reduction is not as much as in case of covalent binding.

3.3 ^{27}Al NMR studies

^{27}Al NMR shows two resonances around 0 and 70 ppm representing Al in tetrahedral and octahedral co-ordination respectively [19–21]. Montmorillonite

Table 2 Surface area and pore volume data for parent and functionalized clay and glucoamylase immobilized clay

Catalyst	Surface area (m^2 g^{-1})		Pore volume ($\times 10^{-6}$ m^3 g^{-1})
	Langmuir	B.E.T.	
M	352	201	0.2511
SGM	266	145	0.1745
A_{10}	247	156	0.2196
A_{50}	215	132	0.2050
A_{100}	180	105	0.1919
C_{10}	53	25	0.0743
C_{50}	40	19	0.0582
C_{100}	30	15	0.0432

M—montmorillonite K-10; SGM—montmorillonite functionalized with silane and glutaraldehyde; A—adsorbed glucoamylase; C—covalently bound glucoamylase. Values in subscript depict enzyme concentrations in mg g^{-1} clay

exhibits octahedral Al resonance at 2.8 ppm and tetrahedral resonance at 69.8 ppm (Table 3). A slight change in octahedral chemical shift from standard value is due to the presence of Fe^{3+}. 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 inter layer space and hence substantiates the results of XRD and surface area measurements. Enzyme adsorption leads to a shift of tetrahedral Al peak by 6–8 units keeping the octahedral peak almost constant. Thus during adsorption the enzyme interacts with the tetrahedral Al alone. When the enzyme is covalently bound to the clay matrix, the tetrahedral Al resonance is unaltered while the octahedral peaks show a shift by 3–6 ppm. Covalent binding takes place on the glutaraldehyde spacer and not directly on to the Al species. In spite of this, there is a sufficient shifting of octahedral peak (from –1.7 to 2.3) that signifies 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. An additional tetrahedral resonance is seen at 54 ppm, which is present for the adsorbed as well as the covalently bound enzyme. Glucoamylase contains the cystine residue that has the reactive –SH group in its side chain. This reactive group can 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.

Table 3 ^{27}Al NMR chemical shifts of clay in the parent, functionalized and enzyme immobilized form

Catalyst	Chemical shift (ppm)	
	Octahedral	Tetrahedral
M	2.8	69.8
SGM	–1.7	69.5
GA_A	2.6	62.1
		54.1
GA_C	2.3	69.2
		53.9

M—montmorillonite K-10; SGM—montmorillonite functionalized with silane and glutaraldehyde; GA—glucoamylase; subscript A—adsorbed enzyme; subscript C—covalently bound enzyme

3.4 Influence of pH

Figure 2 gives an account of the variation of glucoamylase activity with respect to pH for the free, adsorbed and covalently bound forms. The optimum pH for free glucoamylase is 6. Adsorbed glucoamylase shows good activity at higher pH while the activity of covalently bound enzyme decreases after pH 6. The pH profile is broader than the free glucoamylase [5, 22] indicating that the sensitivity to pH is reduced as a result of immobilization. Adsorbed enzyme shows high activity at greater pH. This is attributed to the charge effects of the clay matrix. The negatively charged clay matrix causes partitioning effects whereby the pH in the microenvironment of the immobilized enzyme is lowered and therefore a higher bulk pH is required to obtain good activity. Covalently bound glucoamylase shows high activity in a lower (acidic) pH, which may be on account of the strain imposed on the enzyme due to strong interaction with support.

3.5 pH stability

The variation in activity with respect to time for different pH is shown in Fig. 3. Adsorbed glucoamylase exhibits maximum stability at pH 7 while the free and covalently bound glucoamylase show maximum stability at pH 6. In spite of the fact that there is 100%

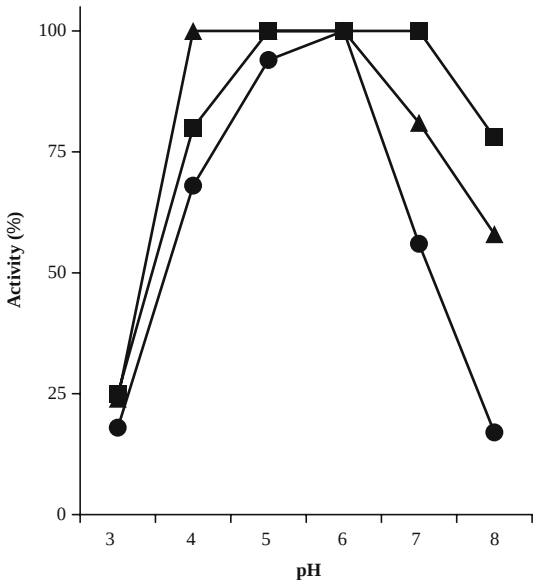

Fig. 2 Variation of activity with pH for (•) free (■) adsorbed and (▲) covalently bound glucoamylase

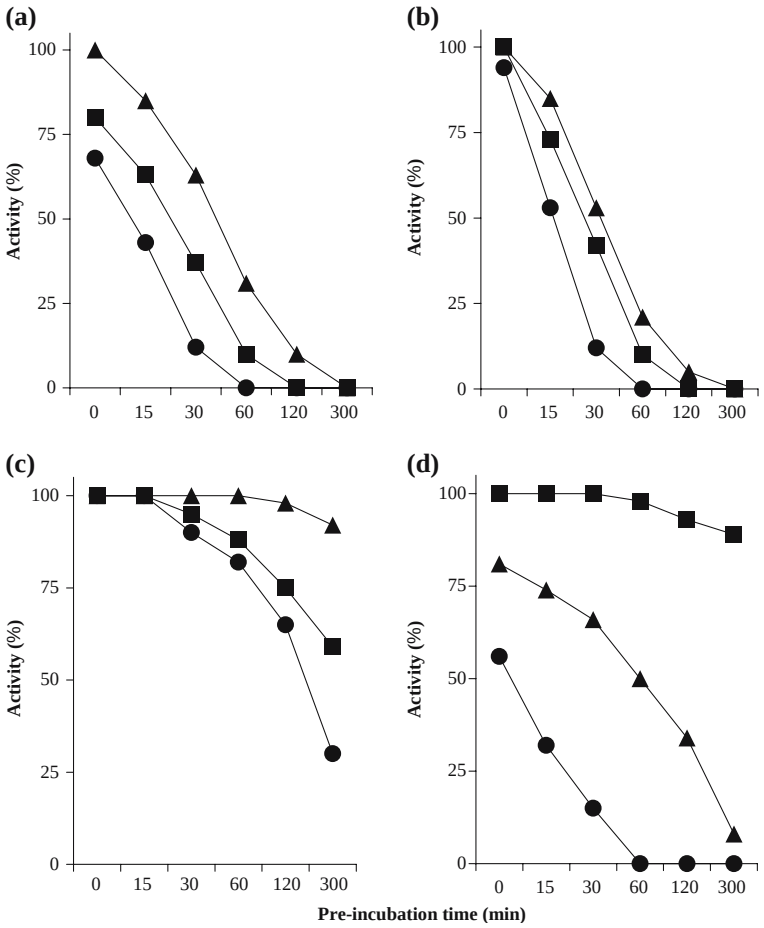

Fig. 3 Stability of (•) free (■) adsorbed and (▲) covalently bound glucoamylase towards various solutions of pH (**a**) 4, (**b**) 5, (**c**) 6 and (**d**) 7 in the batch reactor

activity at pH 4 and 5 in some cases, the stability is very less indicating that pH slowly affects the intramolecular interactions of the enzyme leading to a conformational loss and hence a loss in activity. The immobilized enzyme can endure sudden changes in pH. This is advantageous because there may be changes in the pH of the microenvironment of enzyme during the reaction. In the immobilized state, the enzyme can adjust to these changes thereby lowering the rate of inactivation.

3.6 Thermal effects

Influence of temperature on activity of immobilized glucoamylase is depicted in Fig. 4. In the batch reactor, free glucoamylase demonstrates an optimum temperature of 60 °C. Immobilization results in an increase of optimum temperature. The adsorbed glucoamylase retains all activity even at 80 °C while the optimum temperature for covalently bound enzyme is 70 °C. *Aspergillus niger* is a good source of thermostable enzymes. This explains the high activity of glucoamylase at 60 °C. Immobilization increase the temperature optimum [23] since in the bound state, enzymes are less mobile and so resist denaturation of protein. The Arrhenius plot (Fig. 5) was used to determine the activation energies for the free and immobilized

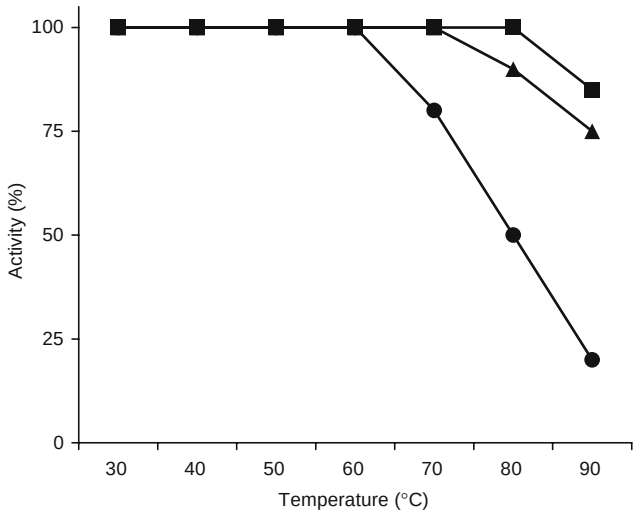

Fig. 4 Influence of temperature on activity of (•) free (■) adsorbed and (▲) covalently bound glucoamylase

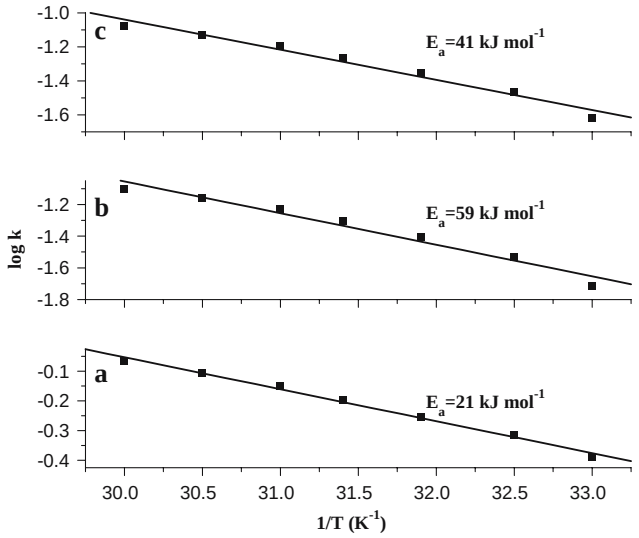

Fig. 5 Arrhenius plot for (**a**) free (**b**) adsorbed and (**c**) covalently bound glucoamylase

glucoamylase. Activation energy increases upon immobilization. This increase may either be the excess energy required to orient the enzyme into its native conformation or it may result due to improper transport of substrate molecules from the bulk to the enzyme active site on account of diffusional resistances to mass transfer [8, 24].

Figure 6 gives an account of the thermal stability of free and immobilized glucoamylase. The free glucoamylase demonstrates a sharp drop in activity with time indicating a high rate of inactivation. The immobilized enzymes show moderate decrease in activity, which emphasizes that the rate of inactivation is lowered upon immobilization. After a 90 min preincubation, the free enzyme loses almost all its activity while the adsorbed and covalently bound enzymes retain 40% and 75% activity respectively. Even though the adsorbed glucoamylase exhibits higher optimum temperature, it possesses lower stability compared to the covalently bound form. This can be attributed to the nature of attachment of the enzyme with montmorillonite.

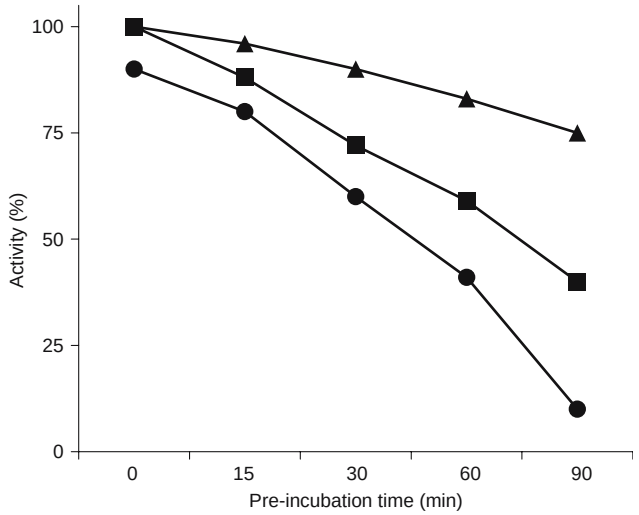

Fig. 6 Thermal stability of (•) free (■) adsorbed and (▲) covalently bound glucoamylase at 70 °C

3.7 Kinetic study

K_m and V_{max} were calculated from the Hanes–Woolf plot (Fig. 7). The η was calculated from the rate of reaction at 5% starch concentration. All the results are depicted in Table 4. The efficiency of immobilization in the batch reactor is 49% and 66% respectively for adsorbed and covalently bound glucoamylase. K_m increases and V_{max} decreases upon immobilization [4, 25]. This shows that immobilization leads to a decrease in substrate affinity of the enzyme thereby resulting in a loss of activity [26–28]. The η values decrease for the immobilized enzymes. This demonstrates that there is a significant role played by mass transfer restrictions due to diffusional limitations. The reduced substrate

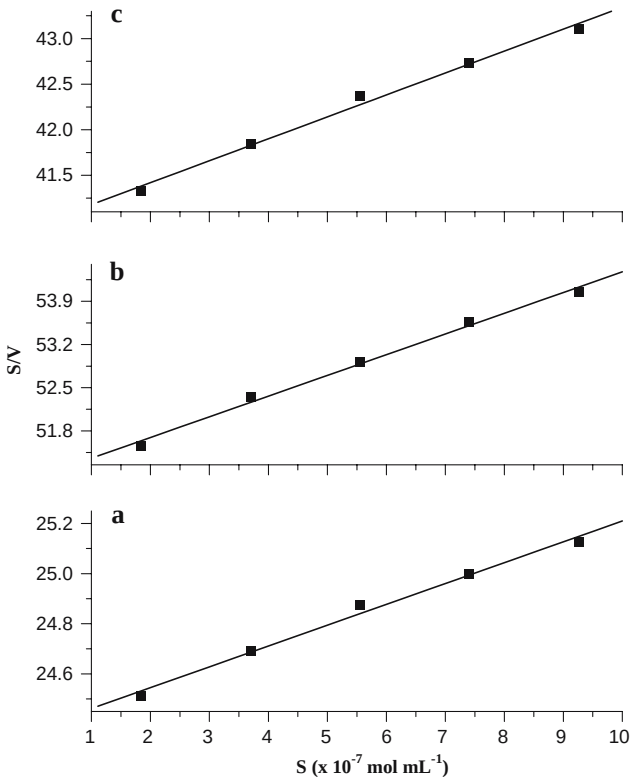

Fig. 7 Hanes–Woolf plot for (**a**) free (**b**) adsorbed and (**c**) covalently bound glucoamylase

Table 4 Kinetic constants for the free, adsorbed and covalently bound glucoamylase

Mode of reaction	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	1.58	1.20	–
	Adsorbed	7.94	0.59	0.49
	Covalently bound	6.49	0.79	0.66

affinity is not on account of changed native conformation of the enzyme but due to improper diffusion of the substrate to the active sites of enzyme [29].

3.8 Reusability

The immobilized glucoamylase demonstrate excellent reusability (Fig. 8). 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 completely reused for 15 cycles and it retained 90% activity after 30 cycles. Our results are comparable to other literature [30, 31]. The loss in activity may be due to the natural inactivation of enzyme on account of time dependent denaturation of the enzyme protein [32].

3.9 Storage stability

Storage stability measurements were conducted in the dry form and in buffer solution. In the dry form, the immobilized enzymes did not exhibit any stability. Both the forms lost complete activity in 12 h. When stored in buffer solution at 5 °C (Fig. 9), the free glucoamylase lost complete activity in 7 days. The adsorbed form

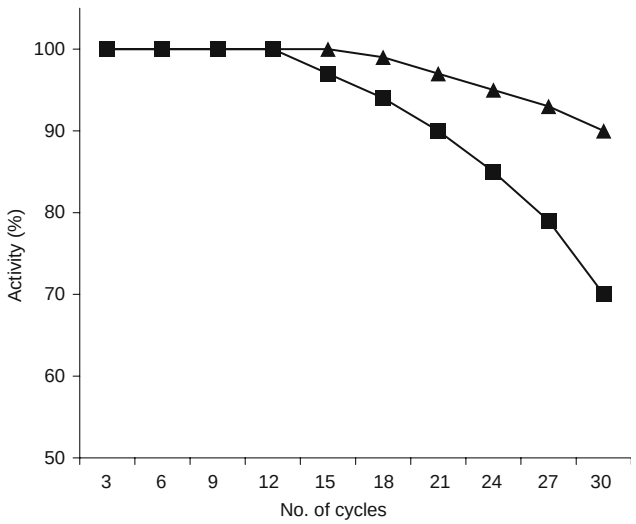

Fig. 8 Reusability of glucoamylase in the immobilized form. (■) adsorbed and (▲) covalently bound enzyme

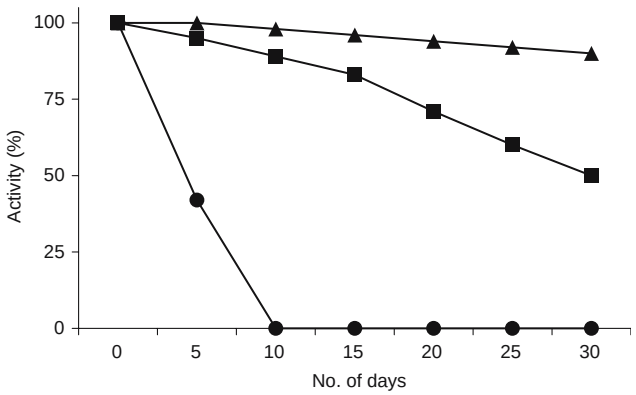

Fig. 9 Variation of activity with storage at 5 °C for (•) free (■) adsorbed and (▲) covalently bound glucoamylase

retained 50% activity in 30 days while the covalently bound glucoamylase retained 85% activity even after 30 days. Immobilization has improved long term storage ability of the biocatalyst. Storage in buffer of optimum pH at 5 °C is recommended. Other authors have also reported similar results [2, 33].

3.10 Study of enzyme leaching

Table 5 shows the results of leaching studies at different enzyme loadings. It can be seen that there is no leaching at lower loadings. As the loading increases leaching also increases. In all cases, the adsorbed glucoamylase was prone to more leaching whereas the covalently bound glucoamylase resisted leaching. The leaching occurs due to the physically adsorbed enzyme. Covalent binding leads to a very strong bond between the enzyme and the clay matrix and hence leaching occurs to a negligible extent even in the case of high enzyme loadings. Leaching can vary significantly with temperature of reaction (Table 6). At all temperatures,

Table 5 Amount of enzyme retained after reaction at various enzyme loadings for immobilized glucoamylase

Enzyme loading (mg g^{-1} clay)	Enzyme retained (%)	
	Adsorbed	Covalently bound
10	100	100
50	90	99
100	83	95

Table 6 Enzyme retained at various reaction temperatures for immobilized glucoamylase

Temperature (°C)	Enzyme retained (%)	
	Adsorbed	Covalently bound
30	90[a] (83)	99 (95)
40	83 (70)	96 (91)
50	74 (57)	92 (86)
60	66 (45)	88 (81)
70	65 (42)	80 (74)

[a] Enzyme loading 50 mg g^{-1} clay. Value in parenthesis depicts an enzyme loading of 100 mg g^{-1} clay

100% enzyme is retained for a loading of 10 mg g^{-1} clay (data not shown). When temperature increases, the thermal energy breaks the bond between enzyme and support. This affects adsorbed enzymes to a greater extent since in this case the bond is weak and easily broken. The strength of covalent bond is very high and therefore even at high temperatures, most of the enzyme is retained [34]. Leaching occurs with reuse of the immobilized systems also. Figure 10 gives an account of the extent of leaching after continuous reuse. Here again, adsorbed glucoamylase demonstrates more leaching compared to covalently bound one. This is due to the presence of physically adsorbed enzyme. Our earlier studies with α-amylase and invertase also showed similar results [35, 36].

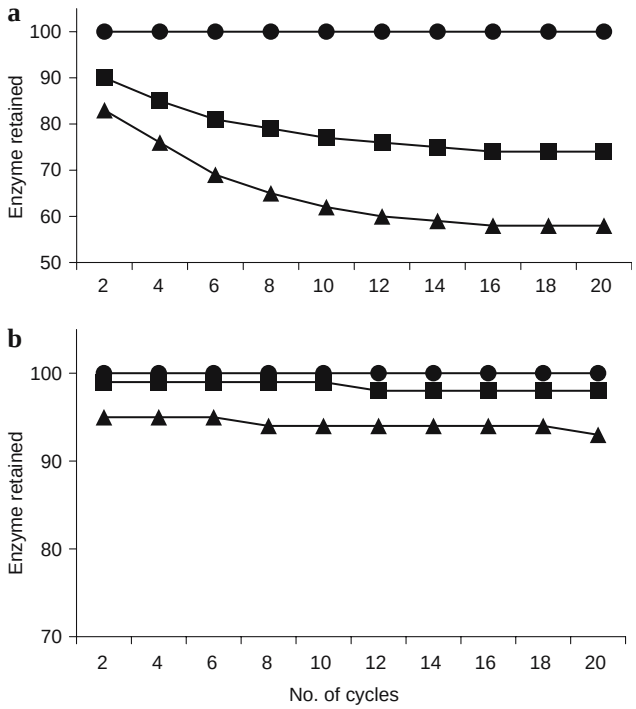

Fig. 10 Enzyme retained after continuous reuse at loadings of (•) 10, (■) 50 and (▲) 100 mg g^{-1} clay for (**a**) adsorbed and (**b**) covalently bound glucoamylase

4 Conclusions

Glucoamylase was successfully immobilized on acid activated montmorillonite by two techniques—adsorption and covalent binding. The immobilized enzymes were characterized by XRD, NMR and N_2 adsorption measurements. The inter layer spacing of the clay showed and expansion which indicated intercalation of the enzyme, as evident from XRD studies. The whole enzyme was not intercalated; it was only the side chains that were involved in intercalation. The entire polypeptide backbone was situated at the periphery of the clay matrix. Adsorption and covalent binding differ in the extent of immobilization alone, which was complete for the covalently bound glucoamylase. ^{27}Al MAS-NMR illustrated the involvement of different Al species during immobilization. The enzyme interacted with tetrahedral Al during adsorption and octahedral Al during covalent binding. Secondary interactions between enzyme and Al atoms were also evident. An additional tetrahedral resonance at 54 ppm was seen which was ascribed to be due to interaction with the cystine residue. Surface area and pore volume of montmorillonite decreased upon immobilization. During covalent binding, the decrease was very sharp which indicated extensive pore blockage. The immobilized preparations demonstrated 49% and 66% activity of the free enzyme. Immobilization led to a broader pH profile. Optimum pH increased for the adsorbed glucoamylase that was an outcome of charge effects of the support. Temperature optimum was also high indicating an improved performance. The higher energy of activation was due to the excess energy required either to orient the immobilized glucoamylase into its native conformation or due to diffusional restrictions to mass transfer when operated in a batch process. The K_m was lower than the free enzyme. The η values indicated that there was some role played by diffusional restrictions in the batch reactor. The immobilized glucoamylase demonstrated excellent reusability and storage stability. They were also very much resistant to leaching. Covalently bound glucoamylase showed better results than the adsorbed counterpart.

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