Fixed bed reactor performance of invertase immobilized on montmorillonite

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Abstract

Invertase was immobilized on acid activated montmorillonite via two independent procedures, adsorption and covalent binding. The immobilized enzymes were characterized by XRD, NMR and N_2 adsorption measurements and their activity was tested in a fixed bed reactor. XRD revealed that the enzyme was situated on the periphery of the clay and the side chains of different amino acid residues were involved in intercalation with the clay matrix. NMR demonstrated that tetrahedral Al was linked to the enzyme during adsorption and the octahedral Al was involved during covalent binding. Secondary interaction of the enzyme with Al was also observed. N_2 adsorption studies showed that covalent binding of enzymes caused pore blockage since the highly polymeric species were located at the pore entrance. The fixed bed reactor proved to be efficient for the immobilized invertase. The optimum pH and pH stability improved upon immobilization. The kinetic parameters calculated also showed an enhanced efficiency of the immobilized systems. They could be used continuously for long period. Covalently bound invertase demonstrated greater operational stability.
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1. Introduction

Immobilized biocatalysts (enzymes and whole cells) are at the heart of innovative biotechnological processes as alternatives to traditional chemical technologies [1]. Independent of the application, the common point in the use of immobilized biocatalysts is that their kinetic properties should be known [2]. This is valid in the stage of screening and design since the specification of operational conditions in which they should be used is important. Invertase (β-fructofuranosidase; EC 3.2.1.26) is produced from commercial yeast strains grown on molasses. It is mainly used to hydrolyze sucrose in the production of invert sugar, which has a lower crystallinity than sucrose at higher concentrations employed. Its use in confectionary thus 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. Immobilization of invertase on corn grits [3] polymer matrices [4–7], calcium alginate [8], composite gel fibres [9,10], etc. has already been reported. Studies on reusability of the immobilized enzymes are also reported. Arica et.al [11] has reported reusability up to 10 successive batches with 100% retaining of initial activity. Immobilization on polymer supports gives much better reusability [2,12]. 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 our present work, we report the results of activity studies of invertase immobilized over montmorillonite K-10 (the commercially available acid activated form) by adsorption and covalent binding. 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. The speciality of montmorillonite (smectites

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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 [13]. Moreover, these acid sites can serve as centers of binding through the –NH$_2$ group of enzymes during adsorption. This ionic binding is much stronger than mere physical adsorption and therefore the chances of enzyme leaching are prevented. The immobilized enzymes were characterized by XRD, surface area measurements and NMR spectroscopy. All activity studies were conducted in a fixed bed reactor. Influence of pH on activity and conformational stability of invertase was investigated. The kinetics and operational stability were also determined.

2. Experimental

2.1. Materials

Bakers yeast invertase, montmorillonite K-10, 3-aminopropyltriethoxy silane (APTES), glutaraldehyde and 3,5-dinitro salicylic acid (DNS) were purchased from Sigma–Aldrich Chemicals Pvt. Ltd., Bangalore. Sucrose, glucose and fructose were from S.D. Fine Chemicals, Mumbai.

2.2. Immobilization of invertase onto montmorillonite K-10

Montmorillonite K-10 (particle size ~0.2 μm) was mixed with equal volumes of 0.1 M phosphate buffer and invertase solution. It was shaken in a water bath shaker at required 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 by the spectrophotometric method [14] using Folin–Ciocalteau's phenol reagent and measuring the absorption at 640 nm in a Shimadzu 160A UV–Vis spectrophotometer. Bovine serum albumin was used as the standard. In case of covalent binding, 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 80 °C. 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 described above. All immobilized systems were stored in 0.1 M-phosphate buffer of pH 5 and 6 (for covalently bound and adsorbed forms, respectively) at 5 °C.

2.3. Characterization of immobilized invertase

Powder XRD of the immobilized enzyme systems and support were taken on a *Rigaku* D/Max-C X-ray diffractometer with Ni filtered Cu Kα radiation ($\lambda = 1.5406$ Å) within the 2θ range 2–15°.

A *Micromeritics* 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 ($p/p_0 = 0.05$–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 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.

2.4. Activity studies

A silica glass tube of 1.2 cm i.d. and 25 cm length was used as the reactor. Provision for hot water circulation was also made available. The immobilized enzyme (0.5 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 syringe pump and the products were collected at the bottom. Product was analyzed using the method described in the previous section. The reactor was operated at a space velocity of 3.26 h^{-1}. After the reaction time, an aliquot (1 mL) of the product was removed from the reaction mixture and analyzed colourimetrically. Colour was developed using 2% 3,5-dinitro salicylic acid solution in 1 M NaOH (w/v) and absorbance was read at 490 nm [8,15,16]. A standard calibration curve was constructed using glucose solution and the total reducing sugar content was estimated. One unit of enzyme is defined as the amount required to hydrolyze 1 mg sucrose per minute under the assay conditions. 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 1–24 h.

The kinetic parameters (Michealis constant K_m and maximum rate V_{max}) were calculated by measuring the rates of reaction at various substrate concentrations. These 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 (10% w/v).

Operational stability was tested in a packed bed reactor. One gram of the immobilized enzyme was packed into the reactor and the substrate was introduced at a particular flow rate so as to get a space velocity of 2. The reactor was operated continuously for 96 h. The activity is represented as percentage of initial activity retained.

3. Results and discussion

3.1. Characterization studies

Surface area as well as pore volume decrease upon immobilization (Table 1). Activation of montmorillonite with aminopropylsilane and glutaraldehyde lowers the pore volume suggesting that the binding of silane and glutaraldehyde molecules to the clay takes place within the inter-lamellar space. This fact is proved further since covalent binding of enzymes through the glutaraldehyde spacer leads to a drastic decrease in pore volume. An enzyme loading of 10 mg g^{-1} clay lowers the pore volume to 0.054 cm^3 g^{-1}. It is the case for surface area also where a sharp decrease from 145 to 16 m^2 g^{-1} is encountered. Adsorption of enzyme also results in a lowering of surface area along with pore volume but the reduction is not as much as in case of covalent binding. This implies that during adsorption the enzyme is less concentrated in the inter-lamellar space of montmorillonite.

Fig. 1 illustrates the XRD patterns for the various systems. For parent montmorillonite, there is a peak at $2\theta = 8.9°$ 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 activation takes place within 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 invertase does not change the intensity of the d_{001} peak. There is a slight shifting of peak to lower values (d spacing 1.952–2.175 nm) with very little intensity. This suggests that the enzyme intercalates in a very few of the layers. As enzyme loading increases, the intensity of the new peak also increases confirming the intercalation of enzyme into the clay layers. Covalent binding with invertase also leads to enzyme intercalation. The d spacing increases in the range 2.354–2.625 nm. Complete shifting of the d_{001} peak to lower values suggests that intercalation of enzyme has
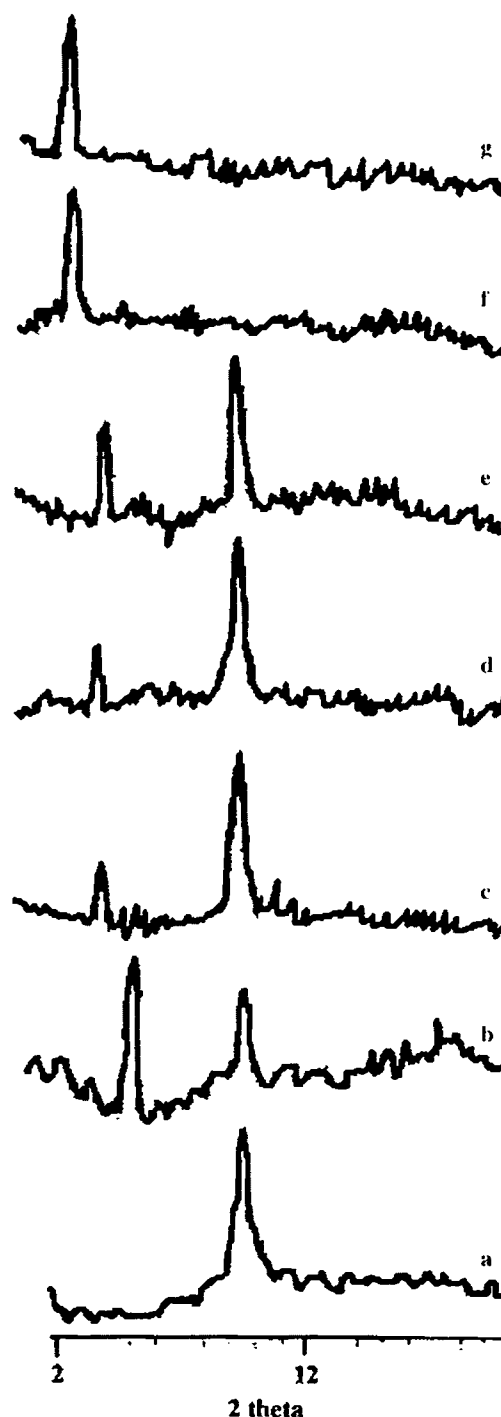

Fig. 1. XRD patterns for immobilized invertase: (a) montmorillonite K-10, (b) functionalized montmorillonite K-10, (c), (d) and (e) adsorbed enzymes with enzyme loading 10, 50 and 100 mg g^{-1} clay, (f) and (g) covalently bound enzymes with enzyme loading 10 and 50 mg g^{-1} clay.

Table 1
Surface area and pore volume of parent clay and immobilized invertase

Catalyst	Surface area (m^2 g^{-1})		Pore volume ($\times 10^{-6}$ m^3 g^{-1})
	Langmuir	BET	
M	352	201	0.2511
SGM	266	145	0.1745
AI$_{10}$	278	152	0.2153
AI$_{50}$	270	148	0.2025
AI$_{100}$	255	135	0.1928
CI$_{10}$	39	16	0.0536
CI$_{50}$	35	15	0.0524
CI$_{100}$	23	10	0.0398

M – montmorillonite K-10; SGM – silane glutaraldehyde activated montmorillonite K-10. AI – adsorbed invertase. CI – covalently bound invertase. Values in subscript represent enzyme loadings in mg g^{-1} clay.

taken place entirely. Covalent binding shows a complete shift even at a loading of 10 mg enzyme. Enzymes are highly polymeric species of very high molecular size hence possibility of attachment of the whole enzyme within the inter-lamellar space can be ruled out. It is reported that in case of polyaniline–montmorillonite nanocomposites,
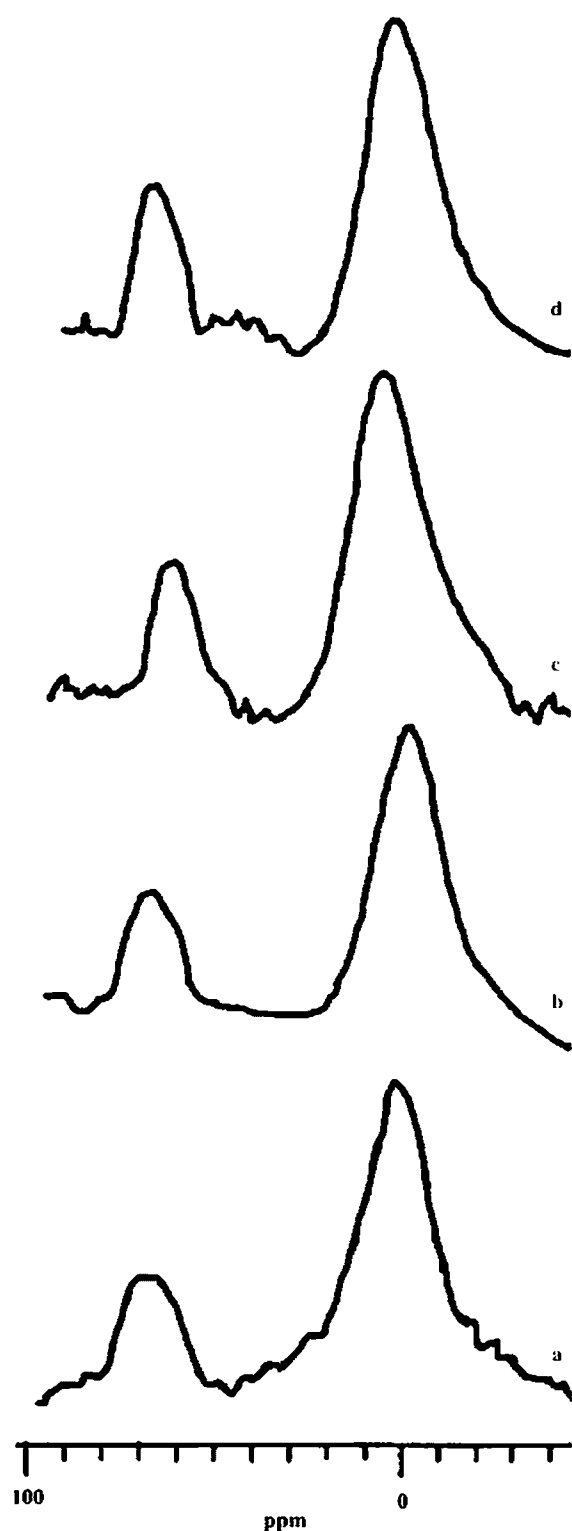
Fig. 2. ^{27}Al MAS NMR spectrum of: (a) montmorillonite K-10, (b) functionalized montmorillonite K-10, (c) adsorbed invertase and (d) covalently bound invertase.

the d_{001} spacing of parent montmorillonite peak shifts to lower values that is indicative of intercalation [17,18]. Therefore, it is proposed that the whole enzyme does not get intercalated into the clay layers. It is the side chains

of various amino acid groups that are responsible for inter-calation. The polypeptide backbone does not enter the inter-layer space but is situated at the periphery of the clay [19].

^{27}Al NMR spectrum (Fig. 2) shows two types of Al species [20,21] i.e. octahedral Al (with chemical shift at 0 ppm) and tetrahedral Al (with chemical shift near 70 ppm). Montmorillonite exhibits octahedral Al resonance at 2.8 ppm and tetrahedral resonance at 69.8 ppm. A slight change in 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 at 69.5 ppm indicating that only the octahedral Al atoms are involved in linkage 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. Adsorption of invertase leads to a shift of tetrahedral Al peak to 61.3 ppm keeping the octahedral peak almost constant (2.7 ppm). 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 (69.7 ppm) while the octahedral peaks show a shift to 3.8 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 that signifies secondary interaction 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 it
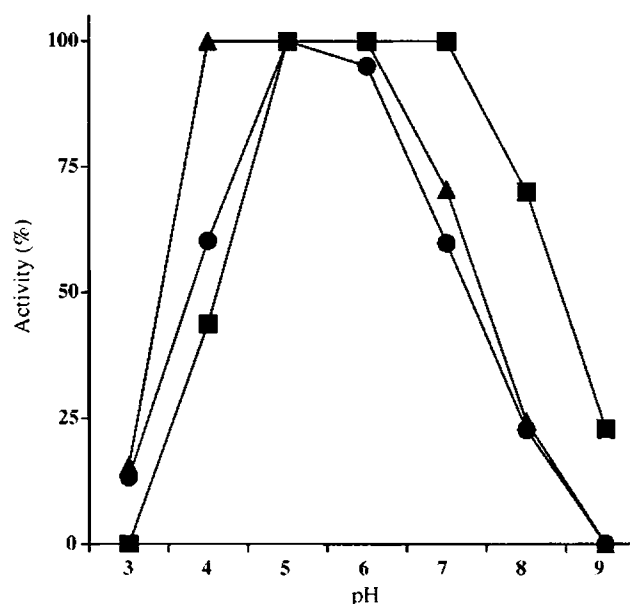

Fig. 3. Variation of activity with pH for (●) free (■) adsorbed and (▲) covalently bound invertase. Reaction conditions: Temperature 30 °C, catalyst 0.5 g, space velocity 3.26 h⁻¹, sucrose concentration 10% w/v, enzyme loading 10 mg g⁻¹ clay.

chemical environment thereby causing a shift in NMR signal.

3.2. Influence of pH on activity and stability of immobilized invertase

The free invertase shows an optimum pH of 6 (Fig. 3). For the adsorbed invertase, the optimum pH shifts to the basic side while it is unchanged for the covalently bound one. Shift to the basic side is an outcome of charged effects of the support. Clays are negatively charged supports; hence the pH felt at the microenvironment of the enzyme will be lower than the bulk pH. Consequently, a larger pH is required in the bulk so that the pH experienced in the microenvironment will be optimum. In addition to that, the pH profile is much broader for the immobilized invertase in comparison with the free form. This indicates that retention of inactivation from changing pH increases upon immobilization. There are reports on marginal broadening of pH profiles after immobilization presumably by restricting the unfolding of the enzyme [9,16,22]. In our case, the use of fixed bed reactor has led to an increased broadening.

Therefore, it can be visualized that change of reactor improves retention to inactivation on account of changing pH. The immobilized invertase demonstrated greater stability than the free form (Fig. 4). Even though the covalently bound invertase shows high initial activity in pH 4, it lacks stability. As time passes by, pH mediated inactivation of enzyme occurs and hence activity drops. The stability of immobilized invertase is maximum at the optimum pH. The use of packed bed reactor is very much beneficial and it provides retention to inactivation on account of change in pH. Thus, the suitable choice of reactor is important in determining stability to pH.

3.3. Effect of immobilization of invertase on the kinetic parameters

K_m and V_{max} were calculated from the Hanes–Woolf plot and all kinetic constants are represented in Table 2. This plot was used because the distribution of errors is uniform in this case [30] when compared to the Lineweaver–Burk or Eadie–Hoftsee plots and so the errors tend to cancel each other. The free and immobilized invertase
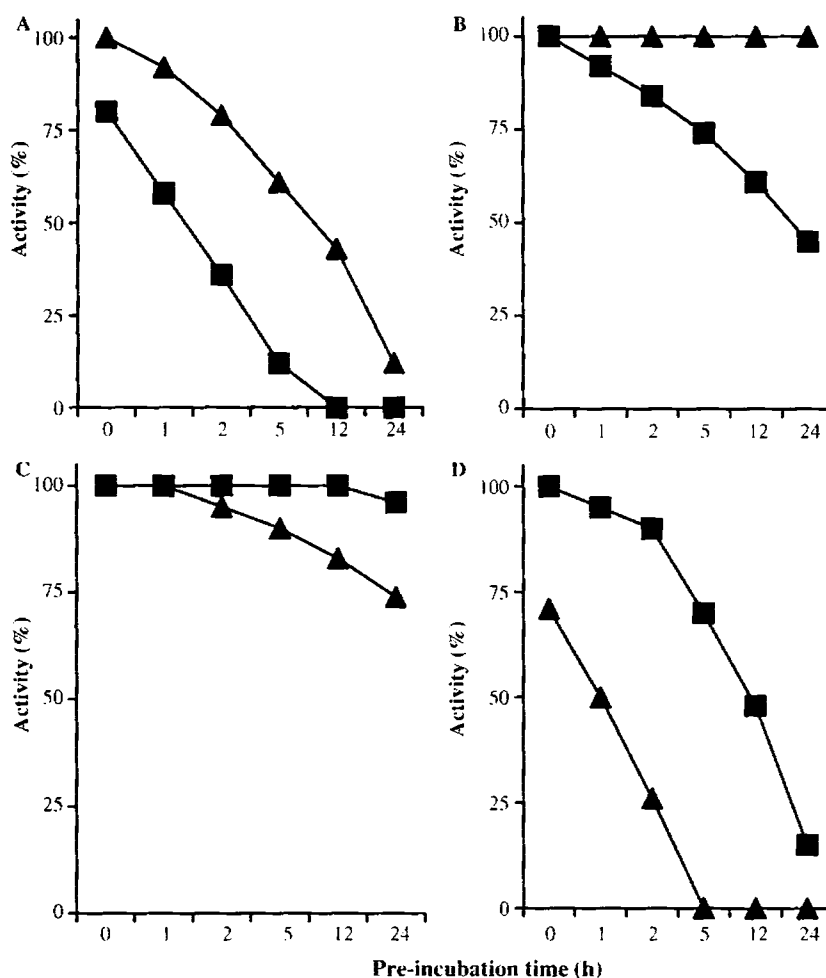

Fig. 4. Variation of activity with time for (■) adsorbed and (▲) covalently bound invertase at pH of: (A) 4 (B) 5 (C) 6 and (D) 7. Reaction conditions: Temperature 30 °C, catalyst 0.5 g, space velocity 3.26 h⁻¹, sucrose concentration 10% w/v, enzyme loading 10 mg g⁻¹ clay.

Table 2
Kinetic constants for free, adsorbed and covalently bound invertase

Mode of reaction	Catalyst	Michaelis constant, K_m (g L^{-1})	Maximum rate, V_{max} ($\times 10^{-6}$ mol mL^{-1} min^{-1})	Effectiveness factor, η
Batch	Free	9.11	5.02	–
Packed	Adsorbed	57.52	2.96	0.59
Bed	Covalently bound	12.83	3.86	0.77

follows Michaelis–Menten kinetics. The efficiency of immobilization was 59% and 77% in the packed bed reactor. Efficiency of immobilization is the percentage activity of free enzyme, which is retained by the immobilized forms. Since the effectiveness factor η is the ratio of rates of immobilized enzyme to free enzyme, efficiency of immobilization becomes equal to $\eta \times 100$. Immobilization leads to an increase in K_m, which suggests a lower affinity for the substrate. Immobilization can bring about changes in the conformation of the enzyme, which leads to a lowered substrate affinity, and hence lower activity. V_{max} values show a decrease that confirms that the activity is reduced. A decrease in V_{max} may be caused by the difficulty of substrate diffusion owing to the rigid structure of the enzyme-entrapped matrices. This is in agreement with other authors also [6,23,24]. Covalently bound invertase exhibits better activity than the adsorbed counterpart [8,15,25]. Effectiveness factor η is a measure of diffusional effects on the reaction. In the packed bed reactor, η was 0.59 and 0.77 for adsorbed and covalently bound invertase, respectively. This indicates that diffusional resistances are a minimum in case of covalently bound invertase. The adsorbed invertase shows an abnormally high value of K_m and low η. Loss in enzyme efficiency, in this case, is due to a combined effect of change in native conformation of the enzyme as well as diffusional resistances to mass transfer. These resistances can include both internal and external transport of the substrate to the clay surface [7,26,27]. In our case, external resistances are of more importance since the clay is bound on the surface and it is the side chains of different amino acid residues which extend to the inter-layer space. At high substrate concentrations, invertase is known to undergo substrate inhibition that may also contribute towards lowering of η. But in our case the adsorbed and covalently bound enzymes show entirely different characteristics which lead to the conclusion that at the substrate concentration employed (10% w/v) inhibition effects are negligible. If inhibition were present, both the immobilized preparations should show a very low activity.

3.4. Operational stability of immobilized invertase

In the packed bed reactor, the covalently bound enzyme showed excellent stability (Fig. 5). It retained complete activity up to 64 h of continuous reaction. Even after
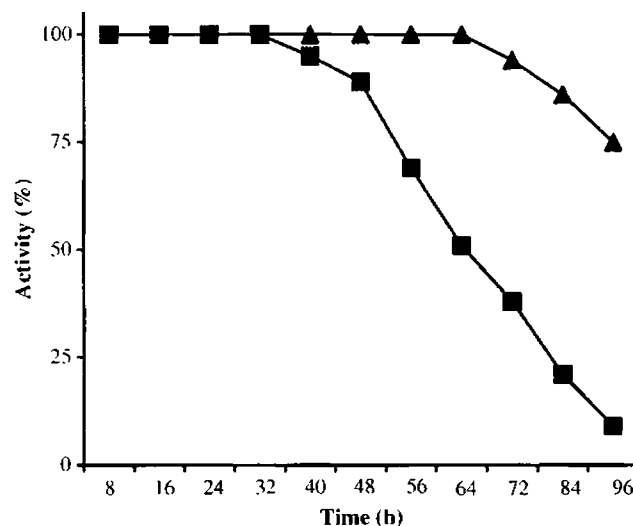

Fig. 5. Operational stability of (■) adsorbed and (▲) covalently bound invertase. Reaction conditions: Temperature 30 °C, catalyst 1 g, space velocity 2 h^{-1}, sucrose concentration 10% w/v, enzyme loading 10 mg g^{-1} clay.

96 h, 75% activity was retained. The adsorbed invertase did not show promising results. It was active only for 32 hours after which inactivation started and in 96 h almost complete activity was lost. From the effectiveness factor measurements it is clear that adsorption of invertase on montmorillonite leads to a loss of conformation. Even though packed bed reactor provides resistance against the unwanted effects of diffusional resistances, there is a change in the native conformation of the enzyme and upon long term usage further inactivation occurs. This explains the rapid loss of activity during continuous use. The immobilized invertase, especially the covalently bound form, depicted excellent operational stability. Other authors have also obtained similar results [11,28,5,29].

4. Conclusions

Invertase was successfully immobilized over acid activated montmorillonite using two independent techniques, adsorption and covalent binding. Covalent binding leads to enzyme intercalation within the clay matrix while adsorption occurs on the surface. Diminishing of the d_{001} peak ($2\theta = 8.9°$) in the XRD profile and reduction in BET surface area and pore volume provide necessary proof for enzyme intercalation. Al NMR suggests the involvement of octahedral Al species during covalent binding and tetrahedral Al during enzyme adsorption. The efficiency of immobilized invertase is 59% and 77%, respectively, for the adsorbed and covalently bound forms. K_m increases upon immobilization. The difference in η for both the immobilized systems shows that adsorption leads to a conformational loss in addition to effects of restricted diffusion of support through the heterogeneous catalyst. The optimum pH shifted towards the basic side for the adsorbed invertase, which was, attributed to charge effects of the support. The immobilized invertase demonstrated enhanced pH stability also. The operational

stability for covalently bound invertase was very high although the adsorbed one did not show promising results. This might be on account of a loss in enzyme conformation due to immobilization.

Acknowledgements

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