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Enhanced pH and thermal stabilities of invertase immobilized on montmorillonite K-10

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Abstract

Invertase was adsorbed onto micro-porous acid-activated montmorillonite clay (K-10) by two procedures, namely adsorption and covalent binding. The immobilized enzymes were characterized by XRD, surface area measurements and ^{27}Al NMR. XRD measurements revealed an expansion of clay layers due to immobilization which suggests that intercalation had taken place. Surface area measurements also support this observation. ^{27}Al NMR showed that interaction of enzyme with tetrahedral and octahedral Al changes with the immobilization procedure. Sucrose hydrolysis was performed in a batch reactor. The immobilized enzymes showed enhanced pH and thermal stabilities. Optimum pH and temperature were found to increase upon immobilization. The effectiveness factor (η) and Michaelis constant (K_m) suggest that diffusional resistances play a major role in the reaction. The immobilized invertase could be stored in buffer of pH 5 and 6 at 5 °C without any significant loss in activity for 20 days.

Keywords: Invertase; Immobilization; Montmorillonite; Sucrose hydrolysis; Adsorption; Covalent binding

1. Introduction

The separation and purity of products resulting from biotechnology processes are assuming greater commercial importance. For this reason, the topic of bioactive molecules immobilized on a great variety of supports with various organic and inorganic chemical compositions and shapes is of increasing current interest (De Queiroz, Vargas, Higa, Ribeiro, & Vitolo, 2002). Invertase or 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 high concentrations, finds numerous applications in the food industry. Its use in confectionery ensures that the products remain fresh and soft, even when kept for long 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 (Arica, Senel, Alaeddinoglu, Patir, & Denizli, 2000; Bayramoglu, Akgol, Bulut, Denizli, & Arica, 2003). Invertase has been immobilized on a variety of support materials. The most important among these are polymers. Immobilization of invertase on polyamines has been reported (Tumturk, Arslan, Disli, & Tufan, 2000). The authors have suggested an increase in K_m and a decrease in V_{max} upon immobilization. Optimum pH was increased but optimum temperature was the same for free and immobilized systems. Conducting polymers have also been used for immobilizing invertase (Cirfan, Alkan, Toppare, Hepuzer, & Yagci, 2003). Even though the K_m was higher than for free enzyme, it was of the same order of magnitude, suggesting that the conformation of enzyme was not affected much on immobilization. In addition, a 10 °C increase in optimum temperature was seen. Invertase has been immobilized on graft polymers with various applications (Chen, Kang, Neoh, & Tan, 2000;

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Gurcel, Alkan, Toppare, & Yagci, 2003). The major problem with polymer supports is lower pH and thermal stabilities. Inorganic supports provide better stability features and hence there is immense scope for research in this area. Porous glass (Ooshima, Sakimoto, & Harano, 1980), alginate gels (Tanriseven & Dogan, 2001) and rice husk (D'Souza & Godbole, 2002) are some of the supports on which invertase immobilization has been carried out. Even though these supports demonstrate a broader pH and thermal stability profile compared to free enzyme, along with appreciable storage capability, their rates of decrease in activity with respect to pH and temperature are high. The use of naturally occurring materials as immobilization supports is preferred to synthetic ones, which finally led us to use clays. 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 (Pinnavaia, 1983). Montmorillonite, a well-studied smectite clay, is a 2:1 dioctahedral clay and has been widely used as a catalyst as well as a support. The speciality 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; the acid sites can serve as centres of binding through the $-NH_2$ group of enzymes. Hence, simple adsorption of enzymes onto clays is enough to hold them on the surface and permit heterogeneous catalysis. Also, the clay can be suitably activated with amino silane and coupled with glutaraldehyde which can covalently bind the enzyme.

We have immobilized invertase on acid-activated montmorillonite clay (K-10) using two techniques – adsorption and covalent binding. The immobilized enzymes were characterized by XRD, surface area measurements and ^{27}Al NMR. Activity of free and immobilized systems for sucrose hydrolysis was tested in a batch reactor. Influences of pH and temperature were studied and the stabilities were evaluated. The initial rates were measured and effectiveness factor and Michaelis constant were determined. The storage stability at 5 °C was also evaluated.

2. Materials and methods

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. All other chemicals were of the highest purity commercially available.

2.2. Immobilization of invertase

For adsorption, montmorillonite K-10 was mixed with equal volumes of 0.1 M phosphate buffer and invertase solution and 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 1 °C for 1 h. Enzyme protein was estimated by the spectrophotometric method (Lowry, Rosebrough, Faar, & Randall, 1951) using the Folin-Ciocaltaue phenol reagent and measuring the absorption at 640 nm in a Shimadzu 160A UV–Vis spectrophotometer. In the case of covalent binding, the clay was first activated by stirring 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 at 5 °C.

2.3. Characterization of immobilized invertase

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.

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°.

Solid-state ^{27}Al MAS NMR experiments were carried out in 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 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 on free and immobilized invertase

Enzyme assay was performed in a batch reactor. One millilitre of free enzyme solution (0.1 g of immobilized enzyme) was mixed with buffered 10% aqueous sucrose

solution (w/v) and shaken in a water bath shaker. After 30 min of reaction, 1 ml of product mixture was mixed with 5 ml of DNS reagent and heated in a boiling water bath for 5 min. It was cooled to room temperature and the amount of reducing sugars was measured spectrophotometrically at a wavelength of 500 nm. One unit of enzyme was defined as the amount required to hydrolyze 1 mg of sucrose per minute under the assay conditions. All results were presented in a normalized form with the activity under optimum conditions being assigned a value of 100%.

2.5. Determination of pH stability

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 pH values (4, 5 and 6) and allowed to pre-incubate for different time intervals from 15 to 300 min at 30 °C. After the pre-incubation period, 10% aqueous sucrose solution (w/v) was added and the mixture incubated for 30 min. Product was analyzed as described earlier. Each result is an average of three estimations.

2.6. Determination of thermal stability

Optimum temperature was determined by performing the reaction at temperatures in the range 30–90 °C. Thermal stability was ascertained at 60 °C. One millilitre of free enzyme solution (0.1 g immobilized enzyme) was mixed with 0.1 M phosphate buffer and allowed to pre-incubate for various time intervals from 15 to 90 min. After the pre-incubation period, 10% aqueous sucrose solution (w/v) was added and reaction continued for 30 min. The product was analyzed as mentioned earlier. Each result is an average of three estimations.

2.7. Kinetic constants

The Michaelis–Menten kinetics were established from a study of effect of substrate concentration on the reaction rate. The reaction was conducted with various sucrose concentrations (5–25%) and the rate of reaction at each concentration was determined. The Michaelis constant, K_m, was obtained from the Hanes–Woolf plot. Effectiveness factor was taken as the ratio of the rate of reaction for immobilized enzyme to rate of reaction for free enzyme at a sucrose concentration of 10% (w/v) at 30 °C. Each result is an average of three estimations.

2.8. Storage stability

The catalysts were stored in 0.1 M phosphate buffer at 5 °C and their activities were tested for 30 days. The residual activities were calculated as percentage of initial activity.

3. Results and discussion

3.1. Characterization studies

Surface area, as well as pore volume, decreases 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}. This 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 the 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 $d_{0\,0\,1}$ plane, analogous to the inter-layer spacing. After functionalization with 3-APTES and glutaraldehyde, this peak shifted partly to a lower 2θ with a d spacing of 1.531 nm, demonstrating an expansion of layers, thereby indicating that the activation took place within the clay layers. The intensity of the new peak was very high, which shows that most of the clay layers are intercalated with silane and glutaraldehyde molecules. Adsorption with invertase did not change the intensity of the $d_{0\,0\,1}$ peak. There was a slight shifting of the peak to lower values (d spacing

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 subscripts represent enzyme loadings in mg g^{-1} clay.

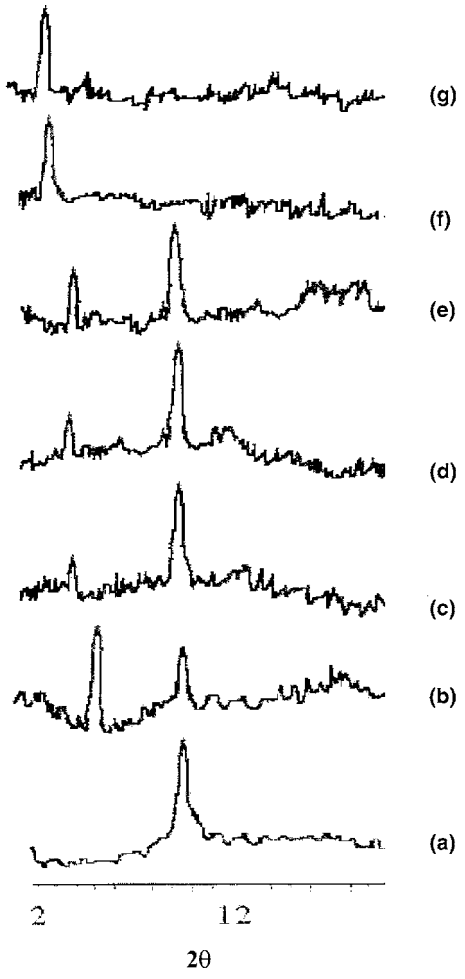

Fig. 1. XRD patterns of: (a) montmorillonite K-10, (b) silane glutaraldehyde activated K-10, (c) adsorbed invertase (10 mg^{-1} g^{-1} clay), (d) adsorbed invertase (50 mg^{-1} g^{-1} clay) and (e) adsorbed invertase (50 mg^{-1} g^{-1} clay) (f) covalently bound invertase (10 mg^{-1} g^{-1} clay) (g) covalently bound invertase (50 mg^{-1} g^{-1} clay).

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 increased, the intensity of the new peak also increased, confirming the intercalation of enzyme into the clay layers. Covalent binding with invertase also led to enzyme intercalation. The d spacing increased in the range 2.354–2.625 nm. Complete shifting of the $d_{0\,0\,1}$ peak to lower values suggested that intercalation of enzyme had taken place entirely. Covalent binding showed a complete shift even at a loading of 10 mg of enzyme. Enzymes are highly polymeric species of very high molecular size; hence the possibility of attachment of the whole enzyme within the inter lamellar space can be ruled out. It is reported that, in the case of polyaniline–montmorillonite nanocomposites, the $d_{0\,0\,1}$ spacing of parent montmorillonite peak shifts to lower values, indicative of intercalation (Kim, Jung, Kim, Choi, & Joo, 2001; Lee & Char, 2002). 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 intercalation. The polypeptide backbone does not enter the interlayer space, but is situated at the periphery of the clay (Gougeon et al., 2003).

The ^{27}Al NMR spectrum (Fig. 2) showed two types of Al species (Lipsicas et al., 1984; Rocha, 1999), namely octahedral Al (with chemical shift at 0 ppm) and tetrahedral Al (with chemical shift near 70 ppm). For montmorillonite, the octahedral Al appeared at 2.8 ppm. This peak shifted to −1.7 ppm after activation with silane and glutaraldehyde and further to 3.8 ppm upon coupling with enzyme. The tetrahedral Al peak (69.8 ppm for K-10) did not show much change after covalent binding. This indicated that, during covalent binding, most of the octahedral Al was involved. For the adsorbed enzyme, the tetrahedral Al peak shifted to 61.3 ppm, indicating that this species was engaged during enzyme adsorption. The octahedral Al peak showed only negligible change.

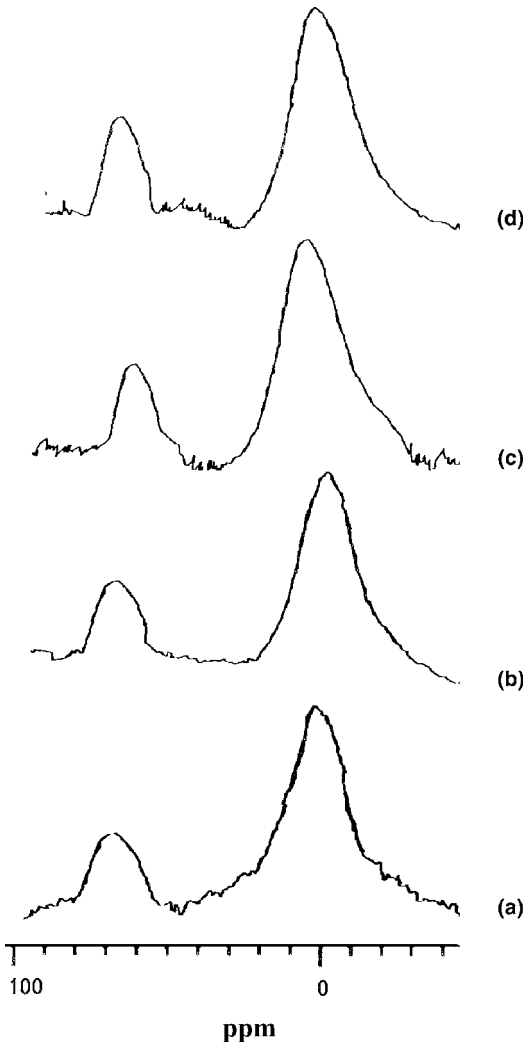

Fig. 2. ^{27}Al MAS NMR spectra of: (a) montmorillonite K-10, (b) silane glutaraldehyde activated K-10, (c) adsorbed and (d) covalently-bound invertase.

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

An enzyme's apparent response to pH may change dramatically when it is in a heterogeneous environment associated with organic/inorganic matrices. The study of such changes and the factors influencing them are of great interest to workers who need to be aware of all the features that may influence the activity of an immobilized enzyme preparation. Free invertase exhibits maximum activity at a pH of 5. Immobilization led to a broader pH profile (Fig. 3). Adsorbed invertase showed an increase in optimum pH that is an outcome of charge effects of the clay matrix. Negatively charged supports increase the optimum pH by means of charge interactions. Some authors have reported increase in optimum pH and broadening of pH profile after immobilization (Kizliyar, Akbulut, Toppare, Ozden, & Yagci, 1999; Nakane, Ogihara, Ogata, & Kurokawa, 2001). Covalently bound invertase, on the other hand, did not show any increase in optimum pH but demonstrated good activity at lower pH. This may be due to the changes inflicted on the enzyme on account of the strong covalent bond formed between the support and the enzyme. Strong interactions between enzyme and support will affect the intra-molecular forces responsible for maintaining the conformation of the enzyme that would lead to a change in activity. Immobilization brought about enhanced pH stability (Fig. 4). At the optimum pH, free invertase lost 35% activity after 300 min while the immobilized forms retained 90% of activity. Even though covalent binding showed good activity at lower pH, it was not stable and lost 60% of activity in 300 min at that pH. Therefore, the reaction pH values for free and covalently bound invertase were selected to be 5 whereas, for adsorbed invertase, pH was kept at 6. Other inorganic supports also demonstrated a shift in optimum pH but the profile was much narrower; only a 0.5 unit increase was seen in most cases. Montmorillonite provides a much broader pH profile and so invertase in the immobilized form can resist pH changes to a greater extent than can other supports.

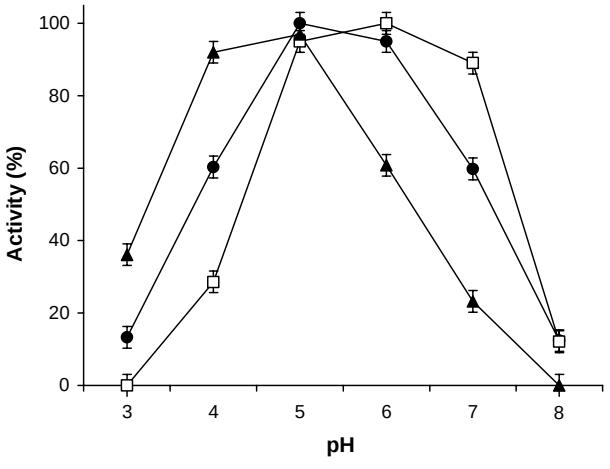

Fig. 3. Influence of pH on activity of (●) free, (□) adsorbed and (▲) covalently-bound invertase (three estimations each).

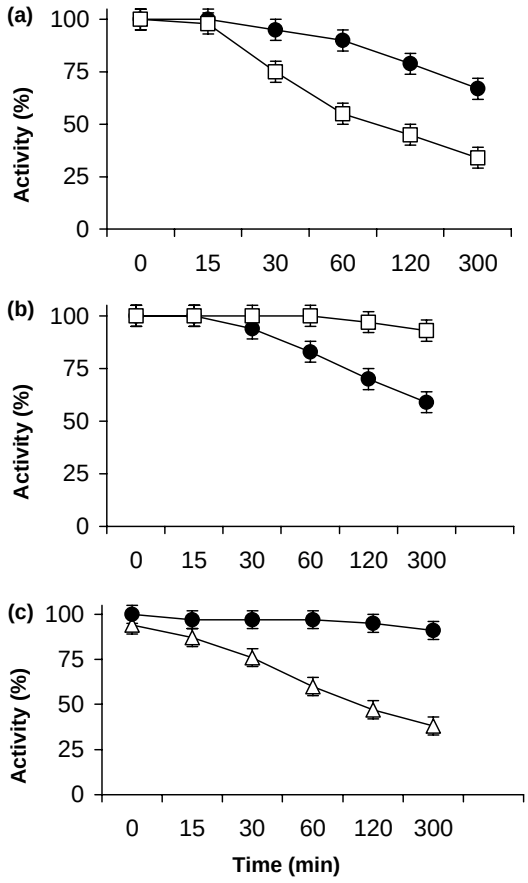

Fig. 4. Stability of: (a) free, (b) adsorbed and (c) covalently-bound invertase in a pH of (△) 4, (●) 5 and (□) 6 (three estimations each).

3.3. Optimum temperature and thermal stability

Fig. 5 gives the variation of activity with temperature for free and immobilized invertase. Immobilization increased the temperature optimum from 50 to 60 °C. A further increase in temperature led to lowered activity as a result of thermal inactivation of the enzyme. At higher temperatures, unfolding of enzyme protein is responsible for its inactivation. Immobilization improved thermal stability (Fig. 6). Covalent binding provides better thermal stability characteristics than does adsorption. While the free enzyme retained only 10% of initial activity after a 90-min pre-incubation at 60 °C, adsorbed and covalently bound invertase retained 40% and 75% of activity, respectively. Similar observations have been reported, previously, for various immobilized systems (Bayramoglu, Yilmaz, & Arica, 2004; Danisman, Tan, Kacar, & Ergene, 2004). In most of the cases of immobilized invertase, 60 °C is the optimum

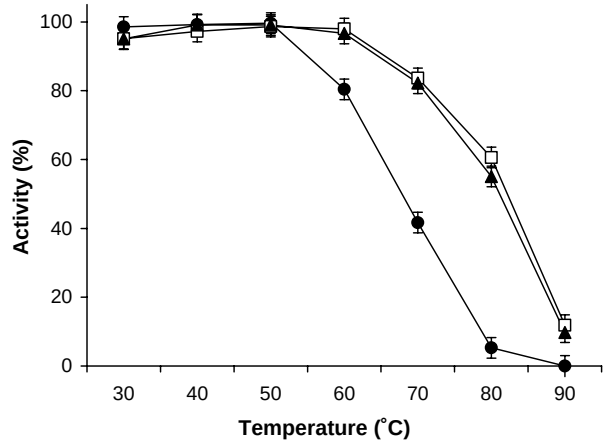

Fig. 5. Influence of temperature on activity of (●) free, (□) adsorbed and (▲) covalently-bound invertase (three estimations each).

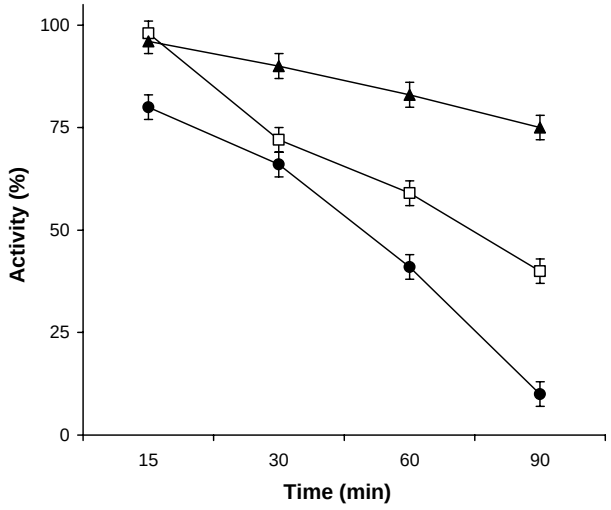

Fig. 6. Thermal stability of (●) free, (□) adsorbed and (▲) covalently-bound invertase (three estimations each).

temperature, even with other inorganic supports. But montmorillonite demonstrates higher stability. When invertase was immobilized on rice husk (D'Souza & Godbole, 2002), the activity dropped to 69% after a 30-min contact with a temperature of 60 °C. In our case, even after 90 min the activity retained was 75% at the same temperature. Montmorillonite was successful in reducing the rate of thermal inactivation of invertase. Covalent binding was more effective in this case.

3.4. Kinetic parameters

Table 2 gives an account of the kinetic parameters for free and immobilized invertase. The immobilized enzymes retained 29% and 36% of activity. K_m increased markedly upon immobilization. Adsorbed invertase showed a 14 times increase in K_m, while it was only eight times for covalent binding. The effectiveness factor, η, which is the ratio of rate of immobilized enzyme activity to rate of free enzyme activity, decreased sharply. These two facts reflect the involvement of diffusional resistances, due to which access of sucrose molecules to the active site was hindered, leading to a loss of substrate affinity. Diffusional resistances were greater for the adsorbed invertase, despite the fact that, during covalent binding, the enzyme was intercalated in the clay matrix and caused pore blockage. This may be because adsorption took place in close proximity to the active site of invertase and therefore access to these sites was diminished further.

3.5. Storage stability

All enzymes were stored in 0.1 M buffer of optimum pH at a temperature of 5 °C. The results are condensed in Fig. 7. Free invertase lost all activity in 15 days. Adsorbed enzyme retained 100% of initial activity up to 10 days and further lost 55% of activity in 30 days. Covalently bound invertase was stable up to 18 days and lost

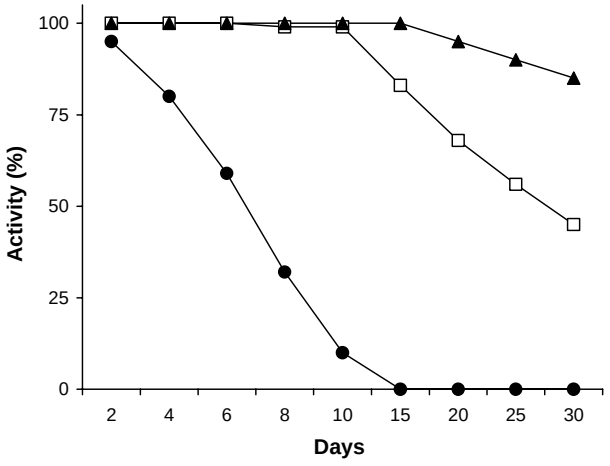

Fig. 7. Storage stability of (●) free, (□) adsorbed and (▲) covalently-bound invertase.

Table 2
Kinetic parameters for free and immobilized invertase

Catalyst	Rate[a] ($\times 10^{-5}$ mol ml^{-1} min^{-1})	Effectiveness factor η	Michaelis constant, K_m (mg ml^{-1})	V_{max} ($\times 10^{-4}$ mol ml^{-1} min^{-1})
Free	5.02	–	9.1	18.0
Adsorbed	1.46	0.29	130	5.14
Covalently-bound	1.81	0.36	70.4	6.57

[a] Rate of reaction at 10% sucrose concentration; used to calculate η.

only 15% of activity in 30 days. Immobilization improved storage stability.

4. Conclusions

Invertase was successfully immobilized on montmorillonite K-10 via two techniques – adsorption and covalent binding. Covalent binding led to enzyme intercalation within the clay matrix while adsorption occurs on the surface. Diminishing of the $d_{0\,0\,1}$ peak ($2\theta = 8.9°$) in the XRD profile, and reduction in BET surface area and pore volume, provided necessary proof of enzyme intercalation. ^{27}Al NMR suggested the involvement of octahedral Al species during covalent binding and tetrahedral Al during enzyme adsorption. The efficiency of immobilization was less than 40%. Adsorbed invertase showed a marked increase in K_m and a sharp decrease in η, indicating that diffusional resistances to mass transfer were very high. This may also result from adsorption of enzyme near to its active site. Comparatively, covalent binding shows less diffusional resistance. pH optimum was unchanged for covalently-bound invertase, while it increased for adsorbed species due to charge effects of the clay matrix. pH stability was also improved. A broader pH profile indicated the effectiveness of montmorillonite in providing resistance to wide changes in pH. Similarly, temperature optimum and thermal stability were enhanced upon immobilization. The rate of thermal inactivation was reduced on account of linkage to the clay matrix. The immobilized enzymes also exhibited improved storage stability. Even though the immobilization efficiency was low (40%), the combined effect of broader pH profile, reduced thermal inactivation rate and improved storage characteristics highlight the value of montmorillonite as a support for enzyme immobilization.

Acknowledgments

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