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Invertase immobilised on montmorillonite: reusability enhancement and reduction in leaching

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Abstract

Invertase was immobilised on microporous montmorillonite K-10 via adsorption and covalent binding. The immobilised enzymes were tested for sucrose hydrolysis activity in a batch reactor. K_m for immobilised systems was greater than free enzyme. The immobilised forms could be reused for 15 continuous cycles without any loss in activity. After 25 cycles, 85% initial activity was retained. A study on leaching of enzymes showed that 100% enzyme was retained even after 15 cycles of reuse. Leaching increased with reaction temperature. Covalent binding resisted leaching even at temperatures of 70 °C.

Keywords: Immobilisation; Immobilised enzymes; Montmorillonite; Adsorption; Microporous; Sucrose hydrolysis

1. Introduction

Enzymes are protein molecules that serve to accelerate the chemical reactions of living cells. They speed up (bio)chemical reactions by lowering the energy of activation. Immobilised enzymes are preferred over native ones owing to their multiple and repetitive use. In addition, the reaction product is not contaminated with the enzyme. Furthermore, immobilised enzyme has longer half-life and predictable decay rate [1,2]. Invertase (β-fructofuranosidase; E.C. 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. Immobilisation of invertase on corn grits [3], polymer matrices [4–7], calcium alginate [8], composite gel fibres [9] etc., has already been reported. Studies on reusability of the immobilised enzymes are also reported. Arica et al. [10] have reported reusability up to 10 successive batches with 100% retaining of initial activity. Immobilisation on polymer supports gives much better reusability [2,11]. Very little attention has been provided to leaching studies on immobilised enzymes.

In our present work, we report the results of reusability and leaching studies on invertase immobilised 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 in general) is that the properties can be tailor made to suit the need by

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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 [12]. 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. All studies were conducted in a batch reactor. Influence of temperature, successive usage and sucrose concentration on enzyme leaching was investigated.

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 SD Fine Chemicals, Mumbai. All other chemicals were of highest purity commercially available.

2.2. Immobilisation 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 [13], using Folin–Ciocaltaue'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 immobilised 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 using X-ray diffraction

Powder XRD of the immobilised 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°.

2.4. Activity assays of free and immobilised invertase

Enzyme assay was performed in a batch reactor. One ml free enzyme solution (0.1 g immobilised enzyme) was mixed with 0.1 M phosphate buffer and 10% sucrose solution and shaken in a water bath shaker. After the required time, 1 ml solution was withdrawn, mixed with 5 ml 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 an absorbance of 500 nm [14]. The products of sucrose hydrolysis (glucose and fructose) reduce dinitrosalisylic acid to form a red brown solution with absorption maxima at 500 nm. One unit of enzyme is defined as the amount required hydrolyzing 1 mg sucrose per minute under the assay conditions. The influence of substrate concentration was studied by performing the reaction with different sucrose concentrations (2–25%) and calculating the rate. Michaelis constant K_m was determined from the Hanes–Woolf plot. Reusability studies were performed with immobilised invertase. After each reaction, the product mixture was centrifuged and the separated immobilised enzyme was subjected to further reaction. At the end of the day, the enzyme was stored in 0.1 M phosphate buffer at 5 °C. The entire process was carried out within five days. In order to study leaching of enzyme, the product mixture after reaction was tested for presence of protein. The effect of temperature and sucrose concentration on leaching was also investigated similarly.

3. Results and discussion

3.1. XRD analysis

The XRD patterns for immobilised invertase are shown in Fig. 1. 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 functionalization 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. Covalent binding with invertase also leads to enzyme intercalation. The d spacing increases to a maximum of 2.25 nm. Complete shifting of the d_{001} peak to lower values suggests that intercalation of enzyme has taken place entirely. It is reported that in case of polyaniline–montmorillonite nanocomposites, the d_{001} spacing of parent montmorillonite peak shifts to lower values that is indicative of intercalation and pillaring [15,16]. We propose that the whole enzyme does

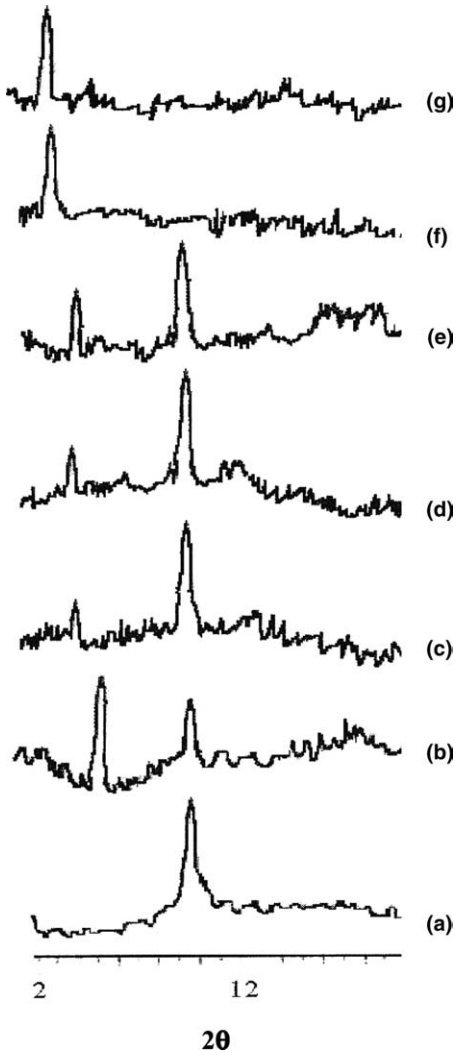

Fig. 1. XRD patterns of support and immobilised invertase. (a) montmorillonite, (b) silane–glutaraldehyde activated montmorillonite (c), (d), (e) adsorbed invertase with enzyme concentrations 10, 50 and 100 mg g^{-1} clay (f), (g) covalently bound invertase with enzyme concentrations 10 and 50 mg g^{-1} clay.

not get intercalated into the clay layers. It is the side chains of various amino acid residues that are responsible for intercalation. The polypeptide backbone does not enter the interlayer space, but is situated at the periphery of the clay [17–19].

3.2. K_m of free and immobilised invertase

The Hanes–Woolf plot is shown in Fig. 2. The plot is linear for the free as well as immobilised invertase depicting that the enzyme kinetics obeys Michaelis–Menten equation. The apparent Michaelis constant K_m showed an increase on immobilisation (Table 1). This is as expected, since immobilisation may bring about a drop in the affinity for substrate due to conformational changes of the enzyme. Maximum rate V_{max} of immobilised enzymes is much lower than the free enzyme. Upon adsorption, K_m shows an abnormal increase (about 14 times that of free enzyme) while after covalent binding, the increase is about eight times only. It is therefore clear that diffusional restrictions to mass transfer operate strongly for immobilised invertase. Due to these limitations, the sucrose molecules cannot effectively access the active sites of invertase and hence formation of enzyme-substrate complex becomes more difficult leading to a decreased affinity for substrate and consequently a high K_m value [20]. Other authors have also obtained similar results [21,22]. Efficiency of immobilisation is less than 40% that is an additional proof for diffusional limitations.

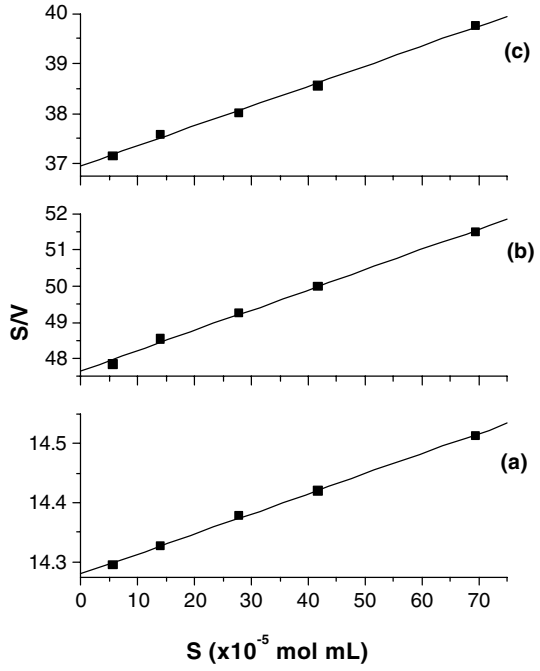

Fig. 2. Hanes–Woolf plot for (a) free (b) adsorbed and (c) covalently bound invertase.

Table 1
Kinetic parameters of free and immobilised invertase

Catalyst	Michaelis constant K_m (mg ml^{-1})	Maximum rate V_{max} ($\times 10^{-4}$ mol ml^{-1} min^{-1})	Immobilisation efficiency (%)
Free	9.1	18.04	–
Adsorbed	130.24	5.14	29
Covalently bound	70.36	6.57	36

3.3. Reusability of immobilised invertase

The major advantage of immobilisation is easy separation and reusability. Reusability studies were performed at room temperature. Results are in Fig. 3.

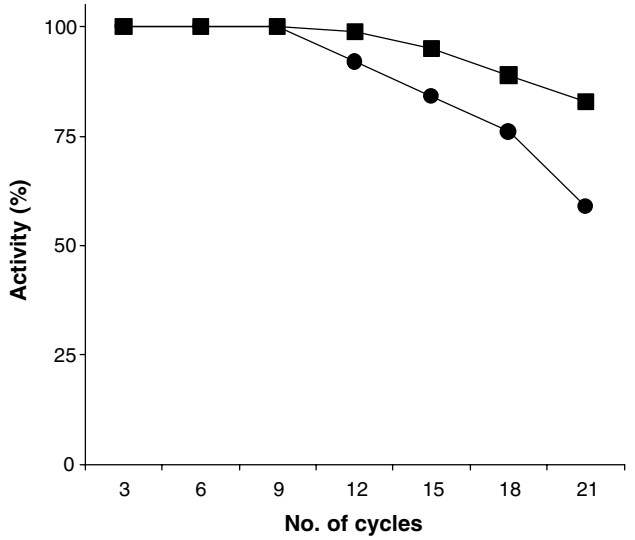

Fig. 3. Variation of activity with reuse for (●) adsorbed and (■) covalently bound invertase. Reaction conditions: Sucrose concentration 10% w/v, invertase concentration 10 mg g^{-1} clay, immobilized enzyme 0.1 g, reactant volume 20 ml, temperature 30 °C, pH 5 (covalently bound) and 6 (adsorbed), time 30 min.

Both immobilised systems showed 100% reusability for 10 cycles after which a drop in activity was encountered. After 21 continuous cycles, the covalently bound invertase retained 80% initial activity, while adsorbed form lost 40% initial activity. The loss in activity was attributed to inactivation of enzyme due to continuous use. Nakane et al. [9] have immobilised invertase on composite gel fibre of cellulose acetate and zirconium alkoxides and found that enzyme activity dropped to 90% after 10 repeated runs. Similar result was observed by Arica et al. [10] for invertase immobilised on spacer-arm attached poly(hydroxyethyl methacrylate) membrane. Bahar and Tuncel [7] found that the activity of invertase immobilised onto crosslinked poly(*p*-chloro methyl styrene) decreased by 20% after 25 batch experiments. Our results demonstrate better reusability characteristics for the covalently bound invertase.

3.4. Enzyme leaching studies on immobilised invertase

At a low enzyme loading of 10 mg g^{-1} clay, no leaching was observed for both immobilised enzymes (Table 2). As enzyme loading increased, the adsorbed invertase showed some amount of leaching, while covalently bound enzyme resisted leaching. In case of the adsorbed enzyme, it is the physically adsorbed species that leaches out. At very high enzyme loadings, some amount of physical adsorption may also take place along with covalent binding. It is this physically adsorbed enzyme that comes out during reaction. The variation in leaching with temperature was investigated in the range 30–70 °C with an enzyme loading of 100 mg g^{-1}. The adsorbed invertase showed very high leaching at higher reaction temperatures (Table 3). During adsorption, the amino groups of enzyme interact with acid sites of clay leading to an ionic bond formation. The strength of this bond depends on the nature of acid sites (weak, medium or strong). Weak acid sites interact weakly with the enzyme and a slight increase in temperature will result in breakage of this bond leading to enzyme leaching. So at higher temperatures the weakly bound enzyme leaches out, while the strongly bound ones remain intact. In case of covalent binding, a strong bond is formed between the enzyme and clay that cannot rupture under the temperatures employed and hence leaching should not occur. But some leaching is seen at higher enzyme loadings, which may be due to physically adsorbed enzyme being present. The effect of sucrose concentration on enzyme leaching was studied (Table 4). Different sucrose concentrations between 2% and 25% (w/v) in water were employed. The reactions were carried out at 30 °C with an enzyme loading of 50 mg g^{-1} clay. At higher concentrations, the adsorbed invertase showed little leaching while the covalently bound enzyme was retained completely. Leaching occurred due to the presence of physically adsorbed enzyme. The influence of

Table 2
Variation of leaching with enzyme loading for immobilised invertase

Enzyme loading (mg g^{-1} clay)	Immobilized enzyme (%)	
	Adsorbed	Covalently bound
10	100	100
50	88	100
100	75	92

Table 3
Influence of temperature on enzyme leaching for immobilised invertase

Temperature (°C)	Remained enzyme (%)	
	Adsorbed	Covalently bound
30	75	92
40	53	89
50	41	85
60	37	81
70	32	77

Table 4
Variation of enzyme leaching with concentration of sucrose for immobilised invertase

Sucrose concentration (%)	Remained enzyme (%)	
	Adsorbed	Covalently bound
2	100	100
5	96	100
10	88	100
15	80	96
25	67	92

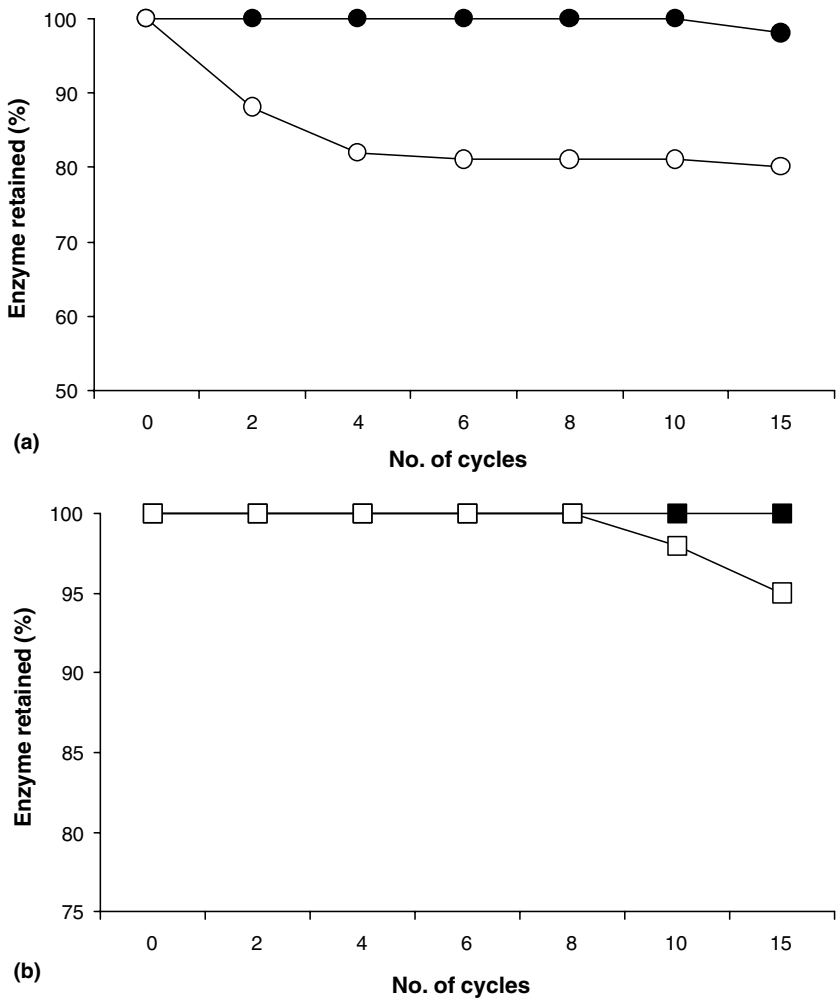

Fig. 4. Influence of repetitive cycles on enzyme leaching for (a) adsorbed invertase (●) 10 mg g^{-1} clay, (○) 50 mg g^{-1} clay and (b) covalently bound invertase (■) 10 mg g^{-1} clay, (□) 50 mg g^{-1} clay. Reaction conditions: Sucrose concentration 10%, immobilized enzyme 0.25 g, reactant volume 50 ml, temperature 30 °C, pH 5 (covalently bound) and 6 (adsorbed), time 30 min.

repetitive usage on enzyme leaching was studied (Fig. 4). At an enzyme loading of 10 mg g^{-1} clay no leaching was seen even after 15 continuous cycles. As enzyme loading increased, the adsorbed invertase showed some leaching while covalently bound invertase resisted leaching. In case of adsorbed invertase an initial leaching was observed for the first two cycles after which enzyme retained became constant. This is due to the fact that physically bound enzyme is removed first after which further removal does not take place as a result of the stronger interaction between enzyme and support (ionic binding). This result is similar to our earlier reports on immobilised α-amylase [23].

4. Conclusions

Invertase was successfully immobilised on montmorillonite K-10 via two methods, i.e., adsorption and covalent binding. The efficiency of immobilisation was calculated to be below 40%. XRD demonstrates the intercalation of enzyme into the inter-layer space of montmorillonite through the side chains of amino acid residues. The linear nature of Hanes–Woolf plot suggests that immobilized invertase obeys Michaelis–Menten kinetics. An evaluation of Michaelis constant K_m showed that the affinity for substrate was lowered upon immobilisation. This can be attributed to restricted access to the enzyme active site on account of diffusional resistances to mass transfer. The immobilised enzymes could be well reused without any loss in activity for 10 continuous cycles. Covalent binding demonstrated retaining of activity up to 18 cycles. Leaching of enzymes was practically nil at an enzyme loading of 10 mg g^{-1} clay. At higher loadings, adsorbed species demonstrated leaching, while the covalently bound forms did not show any appreciable leaching. It is the physically adsorbed enzymes that leach out easily. At higher temperatures, leaching was increased. Sucrose concentration induced leaching in adsorbed enzymes alone.

Covalently bound invertase is much more resistant to leaching due to the strong covalent bond formed between enzyme and support.

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