Immobilization of β-Galactosidase onto Polymeric Supports

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SYNOPSIS

Poly(vinyl alcohol) cross-linked with *para*-formaldehyde (PVA–F) and natural polysaccharide–chitosan in bead form and salicylic acid–resorcinol–formaldehyde polymeric resin (SRF) in powder form were used for immobilization of β-galactosidase through covalent linkages. Various activation processes and conditions were optimized. Immobilized enzyme showed very good storage stability at room temperature. Durability of the enzyme was also improved on immobilization. On repeated use of enzyme immobilized on chitosan beads, no loss was observed in enzyme activity even after 10 batches. Michaelis constant K_m and maximum reaction velocity V_m were calculated for free and immobilized enzyme systems. Effect of pH and temperature on enzyme activity was estimated and energy of activation (E_a) and inactivation constant (K_i) for free and immobilized enzyme were calculated.

INTRODUCTION

Lactose in milk and milk byproducts is undesirable for many people due to deficiency in intestinal lactase causing lactose malabsorption as well as impairment of normal digestive processes.[1] Hence, immobilized lactase (β-galactosidase) is finding importance in the removal of lactose from dairy products as well as in the recovery of sugars from milk whey. β-Galactosidase has been entrapped in polyacrylamide gel,[2] bound to nylon,[3] collagen,[4] DEAE–cellulose,[5] phenolic resin,[6] Sephadex,[7] corn grits,[8] fructogel derivative,[9] and porous glass.[10] However, with many of these supports, immobilization has resulted in considerable reduction in enzyme activity as well as in binding capacity.

Effects of polymer supports on the activity of this enzyme have not been studied in detail except for porous chitosan beads[11] to some extent. Hence, in the present work, we compared the activity and loading capacity of β-galactosidase on polymeric supports in the powdered form and in the bead form. For this study, we used a resinous material synthesized in our laboratory[12] containing salicylic acid, resorcinol, and formaldehyde (SRF), poly(vinyl alcohol) cross-linked with *p*-formaldehyde (PVA–F), and natural glucosamine chitosan. Various activation processes were used during immobilization and stabilities and durabilities of immobilized β-galactosidase were compared with those of free enzyme.

EXPERIMENTAL

Materials

β-Galactosidase (E.C. 3.2.1.23) from *Aspergillus oryzea* and *o*-nitrophenyl β-D-galactopyranoside (ONPG) were obtained from Sigma Chemical Co., USA. Bovin serum albumin (BSA) was purchased from Sisco Research Lab. (India). Chitosan [α-(1 → 4)2-amino-2-deoxy-β-D-glucan], a natural polysaccharide, was obtained from Central Institute of Fisheries Technology (CIFT) Cochin, India, as a gift material. All other reagents used were of analytical grade and double-distilled deionized water was used throughout the work.

Preparation of Polymeric Supports

Resinous material SRF synthesized in our laboratory[12] was used in the 60–100 mesh size form. Chitosan beads were prepared by using 2–4% (w/v) solution of chitosan powder in 2% acetic acid. This

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was added dropwise to 3:1 (v/v) mixture of 1*N* sodium hydroxide and methanol with the help of a microsyringe. The beads formed were separated and washed with hot water several times to remove the excess of alkali. They were stored moist at 30°C room temperature.

Cross-linking of PVA with *p*-formaldehyde and conversion of it into bead form was done per the process described by Aleyamma and Sharma.[13] One hundred milliliters of 12% (w/v) poly(vinyl alcohol) with ~ 77,000 molecular weight and 98–99% hydrolysis mol in water was added to 35 mL of 20% (w/v) *p*-formaldehyde in 5*N* sodium hydroxide and kept overnight at room temperature for cross-linking. This cross-linked solution was added dropwise with a microsyringe to 10*N* sodium hydroxide and beads formed were separated and washed with hot water for removal of excess of alkali and were stored moist at room temperature. The extent of cross-linking was not determined.

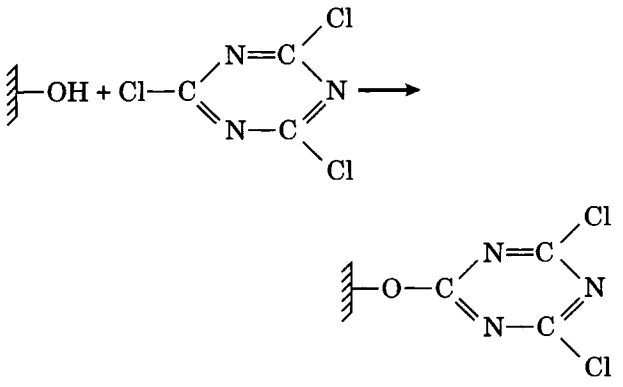
Immobilization of β-Galactosidase

Activation of SRF–resinous material and cross-linked PVA was done by using *p*-benzoquinone, cynuric chloride, and *p*-tolylsulfonyl chloride spacers, whereas that of glycosamine poly(saccharide)–chitosan, using glutaraldehyde. The activation procedures for these supports are described here briefly.

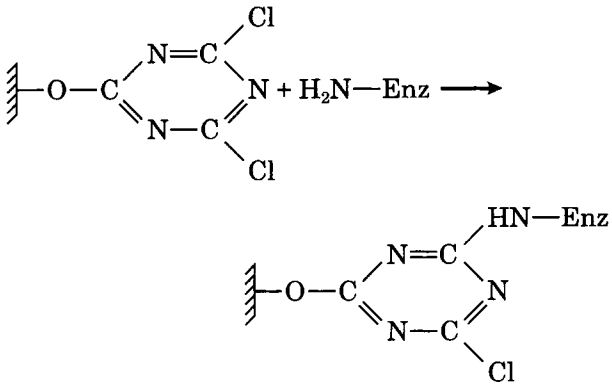
Activation Through Cynuric Chloride

The polymer SRF/PVA–F, 0.2–0.4 g, was activated with 1 g of the cynuric chloride in 10 mL dioxane at room temperature for 10 min and then with 25 mL of water and 25 mL of 20% acetic acid for 5 min. The activated product was washed with water, acetone, and MacIlvaine buffer of pH 4.5 before immobilization of β-galactosidase.

Activation step



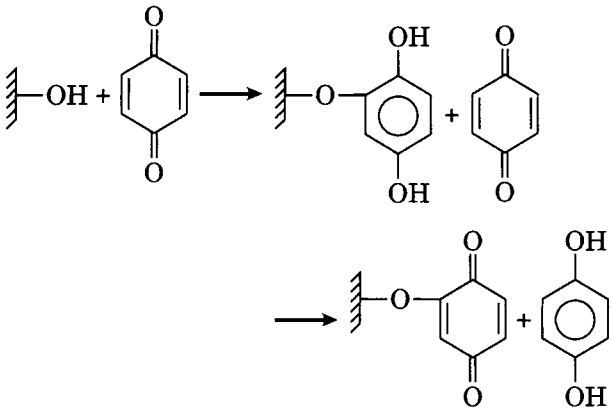
Coupling step



Activation Through *p*-Benzoquinone

The polymeric support, SRF/PVA–F (0.2–0.4 g), was stirred with 0.05 g of *p*-benzoquinone in 10 mL of 20% ethanol in 0.1*M* phosphate buffer of pH 8 at room temperature for 2 h. The support was washed with ethanol, an ethanol–water mixture, 1*M* NaCl solution, water, and MacIlvaine buffer of pH 4.5.

Activation step



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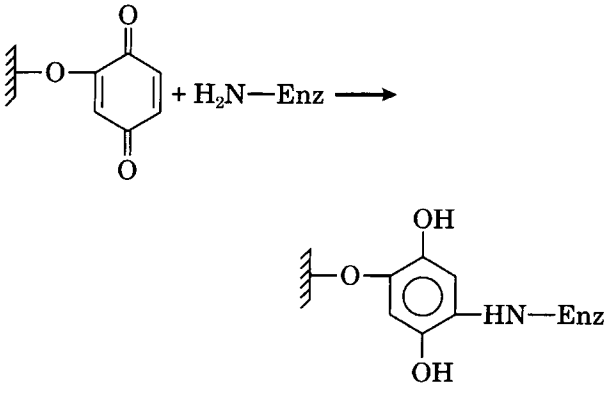
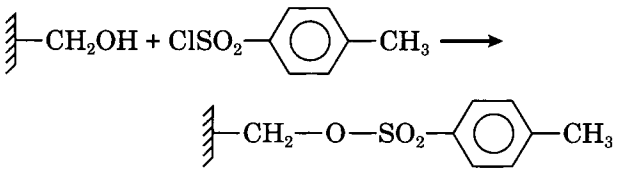


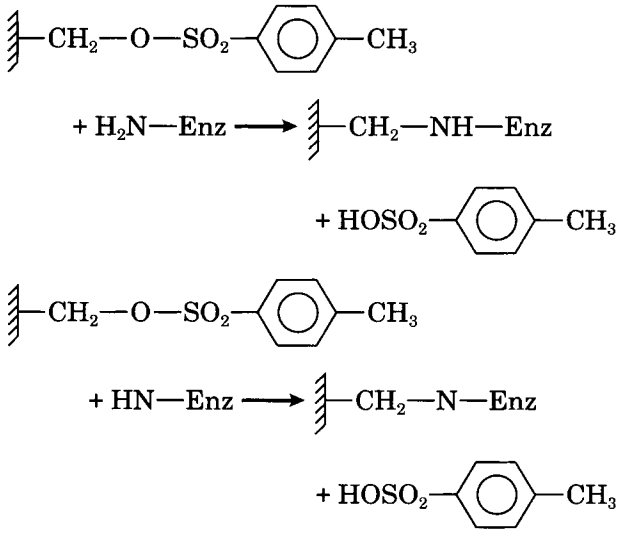
Activation Through *p*-Tolylsulphonyl Chloride

Polymeric supports PVA-F/SRF, 0.2–0.4 g, were activated for 1 h using 0.1 g *p*-toluene sulfonyl chloride in 10 mL dry dioxane at room temperature. One milliliter of pyridine was added dropwise over 1 min. The support was washed with dry acetone, then with acetone–water (1:3) and citrate–phosphate buffer of pH 4.5.

Activation step



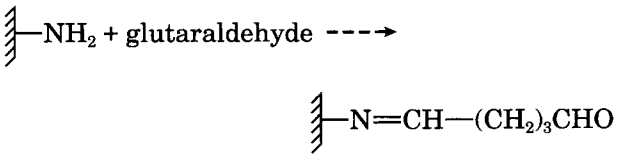
Coupling step



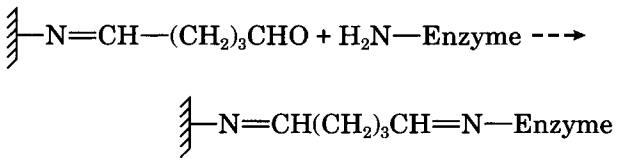
Activation Through Glutaraldehyde

Chitosan beads, 0.2–0.5 g were activated by 4 mL of 6% aqueous glutaraldehyde solution at room temperature for 1 h. Excess of glutaraldehyde was washed with water and citrate–phosphate buffer of pH 4.5.

Activation step



Coupling



The activated supports were used for the immobilization of β-galactosidase using 0.2*M* MacIlvaine buffer at 278 K and 19 h, reaction time. A low-temperature shaker bath (INSREF) was used for the reaction. The immobilized protein content was estimated by determining the protein from the supernatant liquid by Lowry process.[14]

The activity of free as well as immobilized β-galactosidase was determined using Craven's method[15] and ONPG substrate at 40°C and pH 4.5. The reaction was stopped after 10 min by adding 2 mL of 1*M* sodium carbonate. The amount of *o*-nitrophhenol liberated was measured at 410 nm for the activity of the enzyme. One unit of activity was defined as the amount of enzyme that liberated 1 μmol of *o*-nitrophenol/min under the condition of the assay.

Chitosan beads were also treated with bovin serum albumin (BSA) for albumination of the support and then were used for immobilization of β-galactosidase to reduce the linking of enzyme to support through its active sites. About 0.2 g dry chitosan beads were treated with 200 mg of BSA in 0.1*M* phosphate buffer of pH 7.4 for 3 h. The albuminated beads were washed with water and buffer and then treated with 4 mL of 6% glutaraldehyde before using for immobilization of β-galactosidase.

On immobilization, supernatant liquids did not show any protein content and, hence, it was assumed that the enzyme used for immobilization (1.932 U/200–500 mg of support) was quantitatively coupled with the activated supports. The immobilized enzyme was washed thoroughly with water/buffer and was stored at 30°C in a small quantity of 0.2*M* MacIlvaine buffer of pH 4.5.

Stability Measurements

The thermal stability of the free and immobilized β-galactosidase was evaluated by measuring the residual activity of β-galactosidase exposed to various temperatures for 10 min. The kinetics and thermoinactivation constant were investigated by using the following equation:[16]

$$\ln A = \ln A_0 - K_i t$$

where K_i is the first-order inactivation rate constant; A_0, the initial activity, and A, the activity after t min of temperature effect. Heating of free and immobilized enzyme was also carried out at 65°C for a longer time period and residual activity was measured by quickly cooling the enzyme to room temperature. The residual activities were related to the original activities prior to heating.

The pH stability of free and immobilized enzyme was determined by measuring the activity of enzyme at various pH values. The storage stability of the free and immobilized β-galactosidase was determined by storing the enzyme at 4 and at 30°C room temperature for various time intervals and measuring the residual activity periodically. Solvent stability was determined by measuring the activity of the free and immobilized β-galactosidase in the 1–6*M* methanol, ethanol, and propanol.

The reusability of the immobilized β-galactosidase was determined by using a reactor composed of immobilized enzyme and using the same enzyme for number of cycles after washing it thoroughly every time. Leakage of the enzyme, if any, was determined by measuring the enzyme activity in the washings.

RESULTS AND DISCUSSION

Effect of Coupling Method

Chitosan powder has a very low density and, hence, was not useful for immobilization of β-galactosidase. Therefore, chitosan was converted into beads of ~ 1.5 mm diameter size as described earlier. Salicylic acid–resorcinol–formaldehyde resin was used in the powder form of 60–100 mesh as it was not possible to convert it into bead form. Poly(vinyl alcohol) cross-linked with *p*-formaldehyde was used in the bead form of ~ 2 mm diameter size. The results obtained by using various activating methods are given in Table I. With SRF, quantitative coupling of 6.4 U β-galactosidase/g was observed but only 28% of coupled protein was observed to be active after immobilization, whereas in the case of PVA–F beads, on activation with *p*-benzoquinone, 6.4 U β-galactosidase was immobilized quantitatively and showed 100% retention of enzyme activity. Activation of chitosan beads by glutaraldehyde resulted into quantitative coupling and retention of enzyme activity with 9.66 U/g loading. However, lower activity was observed by Weetall et al.[17] when β-galactosidase was covalently bound to glass through glutaraldehyde. Higher retention of activity in the present study can be assigned to the nature of the polymeric supports.

Covalent coupling of BSA with the support before immobilization of β-galactosidase did not show better retention of activity and, hence, was not used for further studies.

Optimization of Coupling Conditions

In the present study of effect of pH, coupling time, and enzyme concentration on immobilization of β-galactosidase, it was observed that maximum coupling and retention of activity was observed at pH 4.5–6.5, 3.5–4.5, and 6.5–7.5, respectively, for chitosan, PVA–F, and SRF supports. It was also observed that within 2–4 h almost quantitative coupling of β-galactosidase takes place with all the three supports (Fig. 1).

Determination of Michaelis Constant

The rate of hydrolysis in all enzymatic reactions is expected to be the first order. The initial reaction

Table I Effect of Various Activation Methods on Immobilization of β-Galactosidase

	SRF		PVA–F		Chitosan	
Methods	Active Protein (U/g)	% Retention of Activity	Active Protein (U/g)	% Retention of Activity	Active Protein (U/g)	% Retention of Activity
p-Benzoquinone	1.0	15	6.44	100	—	—
Cyanuric chloride	0.9	14	2.76	43	—	—
p-tolyl sulfonyl chloride	1.84	28	1.67	26	—	—
Glutaraldehyde	—	—	—	—	9.66	100

Time 19 h at 278 K in MacIlvaine; amount of supports used: 200 mg of dry chitosan beads at pH 4.5; 300 mg of SRF and PVA-F–beads at pH 6.5 and 3.5, respectively, Enzyme used: 0.7 mg.

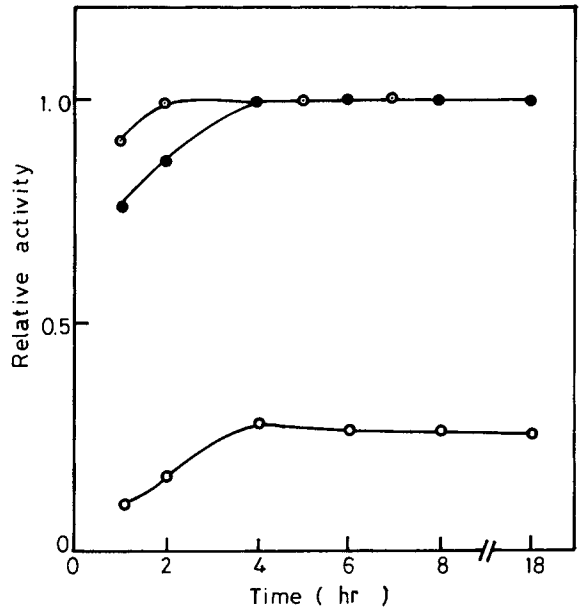


Figure 1 Effect of time on immobilization of β-galactosidase: (⊙) β-galactosidase–chitosan; (●) β-galactosidase–PVA–F; (○) β-galactosidase–SRF.

rates for free and immobilized β-galactosidase were determined at different concentrations of ONPG ranging from 0.4 to 2.2 mM. The Michaelis constant (K_m) and maximum reaction velocity (V_m) were calculated from Lineweaver–Burk plots of $1/v$ vs. $1/s$, where v = velocity of the reaction and s = substrate concentration. From the graph (Fig. 2), the K_m and V_m were evaluated by extrapolating the plots. The intercept on the Y axis corresponds to $1/V_m$ and the intercept on the X axis corresponds to $-1/K_m$. The Michaelis constant K_m and the maximum reaction velocity V_m for free and immobilized β-galactosidase systems are given in Table II. Similar K_m values for free and β-galactosidase immobilized on chitosan show that the microenvironment of both the systems are identical, but decreased V_m in case of the immobilized system may be due to the increased resistance for the substrate diffusion. However, in the case of β-galactosidase immobilized on PVA–F, higher K_m and V_m were obtained, indicating a partially kinetically controlled reaction. Such types of varied results were also reported in earlier studies, which originate due to the difference in the method used, nature of support, and the source of the enzyme.

Effect of pH on Enzyme Activity

Figure 3 shows the pH activity profile for the free β-galactosidase and that immobilized on three poly-

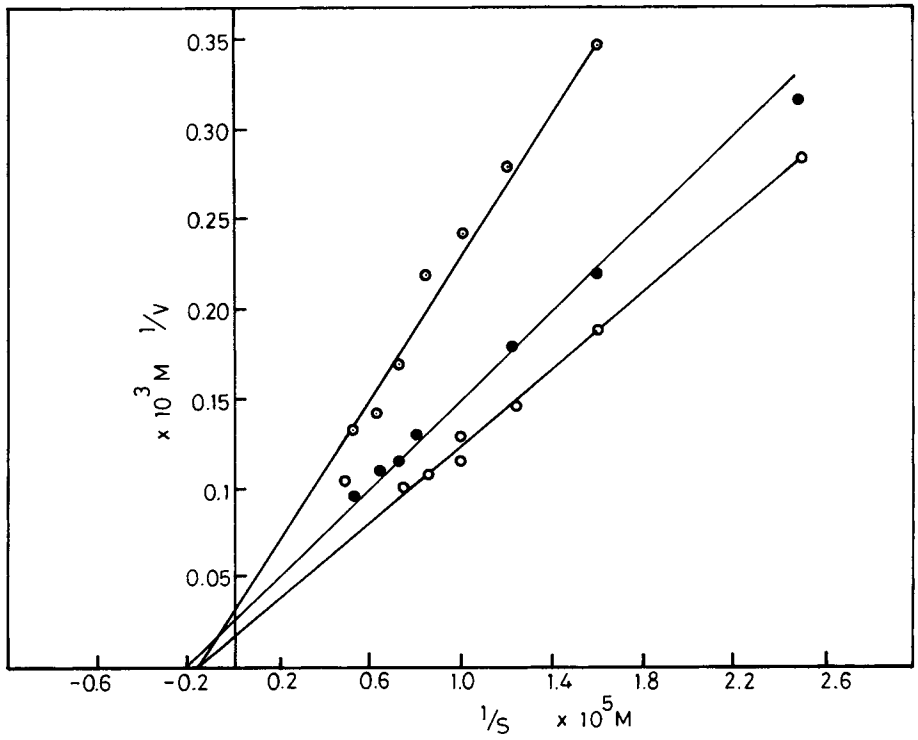


Figure 2 Lineweaver–Burk plots for soluble and immobilized β-galactosidase at temperature 40°C, pH 4.5, for 10 min: (⊙) β-galactosidase–chitosan; (●) β-galactosidase–PVA–F; (○) β-galactosidase.

Table II Kinetic and Thermodynamic Parameters of the Systems

Sample	Michaelis Constant K_m (M)	Maximum Reaction Velocity V_m (M)	Thermo Inactivation Constant (K_i)	Activation Energy (E_a) (kcal/mol)
β-Galactosidase	6.25×10^{-5}	57×10^{-3}	6.76×10^{-3}	72
β-Galactosidase–chitosan	6.25×10^{-5}	30×10^{-3}	5.59×10^{-4}	64
β-Galactosidase–PVA–F	5.0×10^{-4}	66×10^{-3}	7.05×10^{-4}	62

meric supports under study. In general, it was observed that on immobilization enzyme activity has a wide pH range. In case of the SRF support, enzyme activity is almost independent of pH in the range 3–8 but retention of activity was only 28% in this system.

Effect of Temperature on Stability of Enzyme

In case of β-galactosidase immobilized on all the three polymeric supports, enzyme activity was retained at higher temperatures when compared with the free enzyme (Fig. 4). Enzyme activity was determined by incubating the enzyme systems at various temperatures for 10 min and then quickly cooling to 40°C for the measurement of activity by using the ONPG substrate. For the study of thermal deactivation of the enzymes, free and immobilized enzymes were further heated at 65°C for a longer duration. The results are given in Figure 5 and Table II. It was observed that free enzyme loses its activity to 40% after 60 min incubation at 65°C, whereas chitosan and PVA–F-immobilized systems retained the activity over 5 h incubation at 65°C, indicating the improved thermal stability of the immobilized enzymes. However, the E–SRF system showed a decrease in activity to 30% after 1 h incubation and thereafter showed stabilization of enzyme activity even up to 5 h incubation time.

Activation energies for free and immobilized β-galactosidase were calculated by using the Arrehenius equation and a plot of log V_{max} vs. $1/T$. The results obtained are given in Table II. The correlation coefficients for the plots were within the 0.95–0.98 range. The observed decrease in apparent activation energy for immobilized enzymes confirms pore diffusion control of the process rather than kinetic control. The thermoinactivation constants calculated as discussed earlier are given in Table II. An overall decrease in thermoinactivation was observed

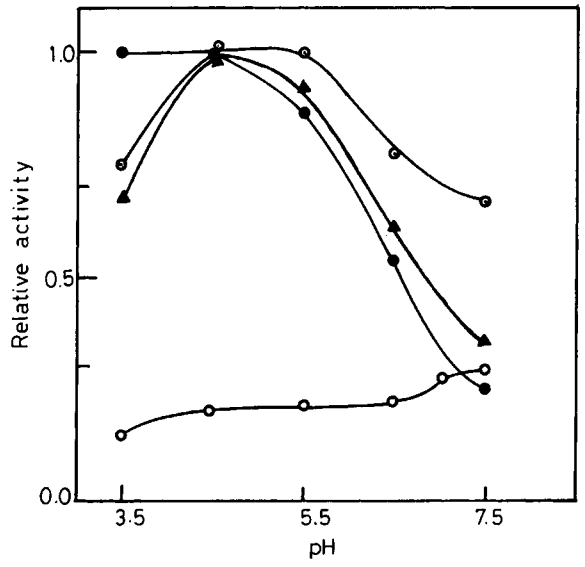


Figure 3 Effect of pH on the activity of free and immobilized β-galactosidase: (⊙) β-galactosidase–chitosan; (●) β-galactosidase–PVA–F; (○) β-galactosidase–SRF; (▲) β-galactosidase.

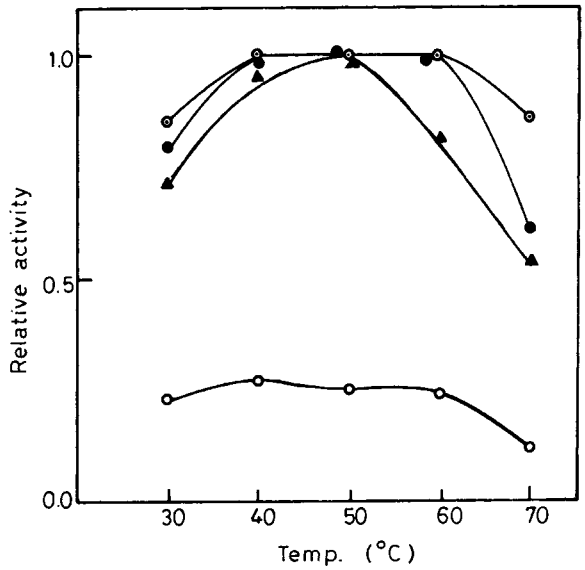


Figure 4 Effect of temperature on the activity of free and immobilized β-galactosidase at pH −4.5 for 10 min: (⊙) β-galactosidase–chitosan; (●) β-galactosidase–PVA–F; (○) β-galactosidase–SRF; (▲) β-galactosidase.

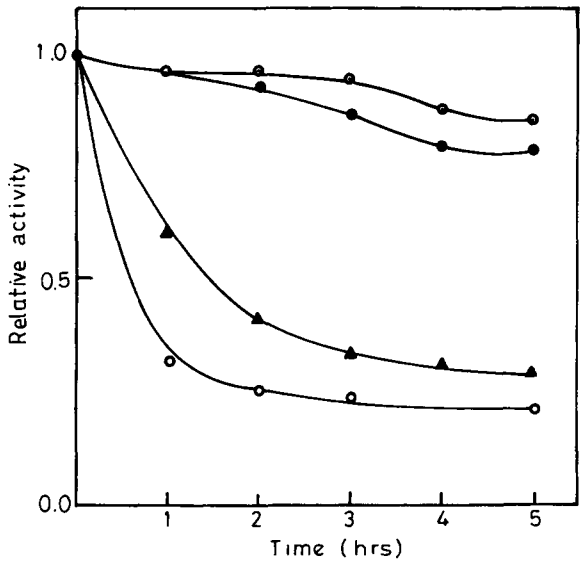


Figure 5 Kinetics of thermal inactivation at 65°C on ONPG hydrolysis at pH 4.5: (⊙) β-galactosidase–chitosan; (●) β-galactosidase–PVA–F; (○) β-galactosidase–SRF; (▲) β-galactosidase.

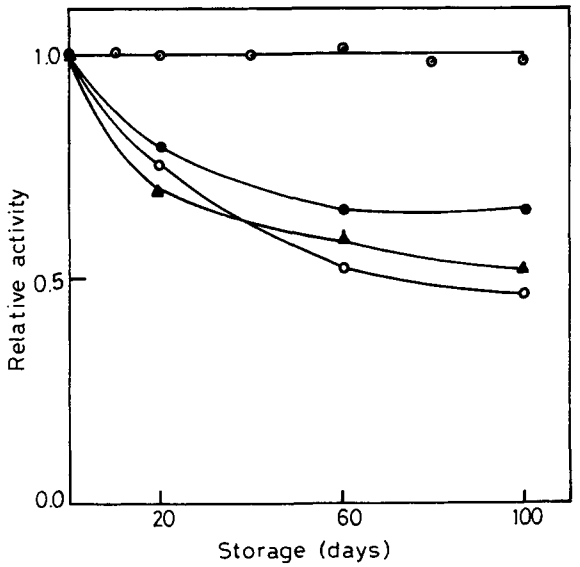


Figure 6 Storage stability of enzyme at 40°C in 0.2*M* McIlvane buffer: (⊙) at pH 4.5 for chitosan; (●) pH 3.5 for PVA–F; (○) pH 6.5 for SRF; (▲) pH 4.5 for free enzyme.

on immobilization; particularly, for chitosan and PVA–F systems, it was reduced by a factor >10.

Storage Stability

Immobilized β-galactosidase was stored at room temperature (30°C) in 0.2*M* MacIlvaine buffer. From the results illustrated in Figure 6, it is observed that activity of the enzyme immobilized on chitosan is retained without any loss even after 5 months storage at room temperature. But the other two systems PVA–F and SRF showed 35 and 50% loss in activity after 3 months storage at room temperature, respectively, whereas free β-galactosidase when stored at room temperature showed 40% loss of activity only after 60 days storage. Retention of activity was determined by considering the activity of the enzyme just after immobilization using the ONPG substrate.

Durability for Repeated Use

Durability of immobilized β-galactosidase is very important in applications as it is subjected to repeated hydrolysis reaction. Figure 7 shows the effect of repeated use on the residual activity of ONPG hydrolysis by immobilized β-galactosidase. The activity was observed to be retained without any loss even after 10 times repeated use of the enzyme immobilized on chitosan beads, whereas that on PVA–F and SRF, respectively, showed 20 and 70% loss in activity only after four cycles. No leakage of enzyme was observed in the repeated washings done during the study.

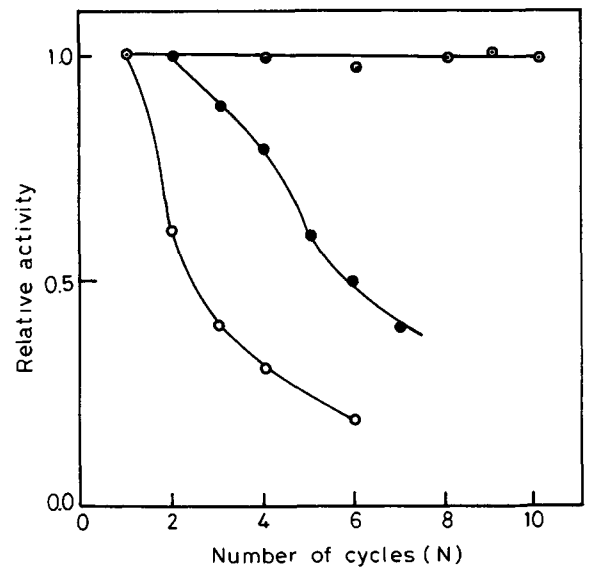


Figure 7 Reusability of immobilized β-galactosidase at 40°C at 4.5 pH: (⊙) β-galactosidase–chitosan; (●) β-galactosidase–PVA–F; (○) β-galactosidase–SRF.

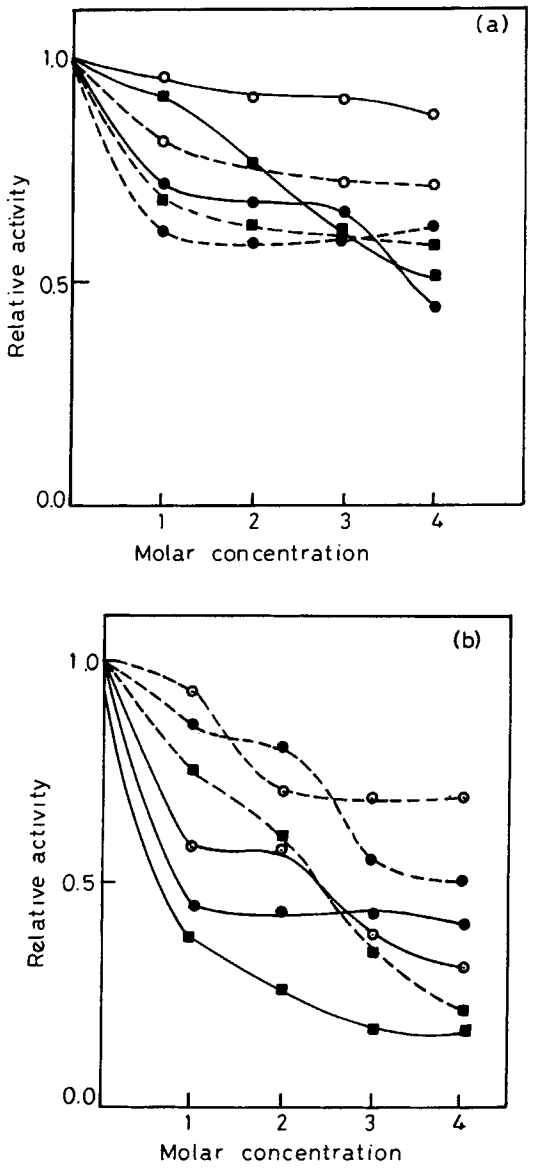


Figure 8 Effect of solvent on the activity of enzyme: (a) (-----) free enzyme; (——) *E*–PVA–F; (○) ethanol; (●) methanol; (■) propanol. (b) (-----) *E*–chitosan; (——) *E*–SRF; (⊙) ethanol; (●) methanol; (■) propanol.

Solvent Stability

The activity of immobilized enzyme in organic solvents is of significant importance in organic synthesis. Stability of free and immobilized β-galactosidase toward methanol, ethanol, and propanol was studied by the batch process. Hydrolysis of ONPG by these enzyme systems was carried out in 1–4 *M* methanol, ethanol, and propanol. From the results given in Figure 8, it is observed that free as well as immobilized systems show a 75 ± 5% retention of activity at 4*M* ethanol, except the E–SRF system. There is no particular trend toward solvent stability but it can be broadly said that β-galactosidase immobilized on chitosan and PVA–F beads show better solvent stability than does the free β-galactosidase.

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