IMMOBILIZATION OF ß GALACTOSIDASE

CHAPTER 2

CONTENTS

2.1 ß-Galactosidase (Lactase)

Lactase is the common name for the enzyme ß-D galactosidase or formerly known as ß-D-galactoside galactohydrolase. It catalyses, among other reactions, the hydrolysis of lactose towards an isomolecular mixture of glucose and galactose. In practice depending on the conditions the isomolecular pattern is sometimes not followed because galactose can polymerize or join to lactose to form oligosaccharides [1,2]. The mechanism of lactose hydrolysis by lactase from *E. Coli* was first described by Wallenfels and Malhotra [3]. This mechanism was further examined in different review papers [2-6]. The important step in lactose hydrolysis is the formation of the enzyme galactosyl complex with simultaneous glucose liberation. The enzyme is also capable of catalyzing the synthesis of certain oligosaccharides via the galactosyl transfer reactions.

Later, when Jobe and Bourgeois [7] showed that allolactose is a primary transfer product in lactose hydrolysis, the enzyme was ascribed the additional role of being able to modify 1-4 to 1-6 binding. They suggested different binding sites on the enzyme for the 1-4 and 1-6 lactose isomers. Nisizawa and Hashimoto[4] found that the rates of hydrolysis for the different lactose isomers increased in the order 1-3, 1-4 and 1-6 binding. The influence of mutarotation of lactose and galactose on the rate of hydrolysis was reported by Huber *et al* [8] and Flaschel *et al* [9]. According to Huber, the lactase from *E. Coli* hydrolyzes α-lactose two times faster than the ß-form. When mutarotation is slower than hydrolysis, the α-form will eventually become exhausted and mutarotation becomes the rate determining step.

Galactose is generally considered to be a competitive inhibitor while it competes with lactose for the enzyme active sites. Flaschel and coworkers[9] found α-galactose to be a significantly stronger inhibitor than the ß-form.

In comprehensive reviews by Wallenfels and Weil[10] and Shukla [2] lactase sources, their purification, assay techniques, physicochemical properties, kinetics, inhibition and its uses in biological and industrial waste-disposal as well as in crystallization of lactose in dairy products are discussed extensively.

Possible sources for enzyme ß-galactosidase are plants, animal organs, bacteria, yeast (intracellular enzyme), fungi or moulds (extracellular enzyme) and are given in Table 2.1. The commercial sources for the availability of ß-galactosidase are given in Table 2.2. All enzyme sources are not acceptable or generally recognized as a safe (GRAS) when enzyme is going to be used in food systems[11].

Table 2.1. Sources of ß-galactosidase

Plants	: Peach, Apricot, Almond, Kefir grains, Tips of wild roses, Coffee berries, Alfalfa seed
Animal organs:	Intestine, Brain and Skin tissue
Yeast	: *Kluyveromyces (Saccharomyces) lactis, Kluyveromyces (Saccharomyces)fragilis, Candida Pseudotropicalis, Brettanomyces anomalus, Wingea roberstsii*
Bacteria	: *Escherichia coli, Bacillus megaterium, Thermus aquaticus, Streptococcus thermophilus, Streptococcus lactis, Lactobacillus bulgaricus, Bacillus sp., Bacillus circulans, Bacillus stearothermophilus, Lactobacillus sporogenes*
Fungi	: *Neurospora crassa, Aspergillus foetidus, Aspergillus niger, Aspergillus flavus, Aspergillus oryzea, Aspergillus phoenicis, Mucor pucillus, Mucormichei, Scopuloriopsis, Alternaria palmi, Curvularia inaegualis, Fusarium-moniliforme, Alternaria alternara.*

Table 2.2. Commercial sources for ß-galactosidase

Aspergillus niger

Baxter Laboratories, Chicago, USA
Dairyland Food Labs, Waukesha, USA
Kyowa Hakko Kogyo Co, Japan
Societe Rapidase, Seclin, France
GB Fermentation Industries Inc, USA

Kluyveromyces or Saccharomyces lactis

Gist-Brocades, Maxilact, Holland
Nutritional Biochemicals Co Ltd, Cleveland, USA
Tokyo Tenabe Co Ltd, Japan

Kluyveromyces or Saccharomyces fragilis

Kyowa Hakko Kogyo Co, Japan
Sigma Chemicals Co, St. Louis, USA
Novo A/S, Lactozym, Denmark

Escherichia Coli

CF Boeringer GmbH, Mannheim, Federal Republic of Germany
Worthington Biochemical Corp, Freehold, USA

Yeast preparations

BDH Ltd, London, England
DEBI Cassina de Pecchi, Milan, Italy
Sturge Enzymes Ltd, Hydrolact, England
Miles Laboratories, Gode, USA

Fungal preperations

Miles Laboratories Takamine, USA

Enzyme ß-galactosidase preparation from *Aspergillus niger* and *Aspergillus oryzea* from Saccharomyces (Lactis or Fragilis) is considered to be safe because these sources have been already tested exhaustively and are having a history of safe use[12] in food industry. *E. Coli* ß-galactosidase although most studied species is not used in food processing because of its cost and the fact that it gives toxicity problems with crude extracts califorms[13]. Hence we have used ß-galactosidase from *Aspergillus oryzea* for the immobilization purpose.

2.2 Properties of ß-galactosidase

ß-galactosidase is one of the better defined enzyme in the literature. Traditionally much of the work has been done on the *E. Coli* enzyme. The properties of the enzyme depend on its source. The largest molecular weights possessed by *E. Coli* lactase are 52,0000-85,0000 daltons whereas *S. fragilis* and *Aspergillus oryzea* enzymes have the molecular weights 21,0000 and 9,0000 daltons respectively[6]. The enzyme hydrolyzes substrates such as lactose and o-nitrophenyl ß-D galactopyranoside (ONPG) between 50-65 °C temperature[14,15].

The enzyme ß-galactosidase from different sources varies considerably in many of its properties, although the sp~~a~~ecificity of the enzyme remains essentially the same. Wasserman[16] and Kulp[17] havereported that the best commercial sources include *E. Coli, Aspergillus niger* and fermenting yeast *K.frgilis* and *K.lactis*. The optimum pH and temperature show variation for enzymes from different sources. Immobilization of enzymes and type of carrier can also influence temperature and pH dependence of enzyme activity[18]. In general fungal lactases have pH optima in the range of 2.5 to 4.5 whereas yeast and bacterial lactases in almost neutral pH of 6 to 7.5[11]. This pH optima property makes each ß-galactosidase suitable for a specific application. Thus fungal ß-galactosidases are used in acid whey hydrolysis while yeast and bacterial ß-galactosidases are suitable for milk (pH 6.6) and sweet whey (pH 6.1) hydrolysis[11]. Some of the sources belonging to thermophilic organisms are very interesting in respect of enzyme thermostabilities. Thermostable enzymes are able to retain their activity at temperatures 60 °C or above for prolonged periods. Hence they have advantages of high conversion and are less prone to microbial contamination[14].

Product inhibition by galactose during hydrolysis is another property which also depends upon the source of ß-galactosidase. The enzyme from *A. niger* is more strongly inhibited by galactose than that from *A. oryzea*[19]. An enzyme preparation from a *Bacillus sp.* showed less inhibition than that from *K. fragilis*[20].

Various methods are available for the determination of ß-galactosidase activity by using lactose as a substrate and glucose and galactose as products. Because of transfer reactions it may be advisable to determine both appearance of glucose and disappearance of lactose[17]. Alternatively ONPG can also be used as the substrate and progress of the reaction can be followed by estimating the chromogen o-nitrophenol[21] In 1972, Wallenfels and Weil[10] reported galactosyl transfer reaction mechanism for ß-galactosidase to some extent without discussing anything about active sites. However, in 1975 Shukla[2] proposed a reaction mechanism for the galactosyl transfer reactions by ß-galactosidase. He has also suggested the active sites of the enzyme to be at one sulphydryl and one imidazole group. Formation of a covalent intermediate involving a carbon-nitrogen bond was also suggested. Earlier Wallenfels and Malhotra[3] have also explained the enzymatic lactose hydrolysis through transfer reaction. This means that the enzyme transfers the galactose moiety of a ß-galactosidase to an acceptor containing a hydroxyl group. When this acceptor is water, galactose is formed and liberated from the active site. However, unhydrolysed lactose present in the solution can also serve as acceptors, and new sugars, oligosaccharides can be formed. In fact, Nisizawa and Hashimoto[4] considered the lactase hydrolysis to be a special case of a general transfer reaction with water acting as an acceptor. Hence they feel lactase should be classified as a transferase rather than hydrolase.

Some of the new products such as low lactose milk processing, low lactose dairy products, low lactose yogurt, sweetened yogurt, low lactose concentrate for ice-cream, lactose processing for acid whey and sweet whey, food syrups and sweetner and cheese have been prepared by using ß-galactosidase[2]. These products have a good market as they are undesirable for the persons having deficiency in intestinal lactase causing lactose malabsorption as well as impairment of normal digestive processes. Hence, the process of lactose hydrolysis must be economically feasible. This problem can be solved by using immobilized ß-galactosidase to avoid loss of unreacted free enzyme during hydrolysis. Hence, in this chapter some inexpensive polymeric materials have been used for the immobilization of ß-galactosidase. The immobilized ß-galactosidase has been thoroughly characterized and compared for various physicochemical properties with free enzyme.

2.3. Literature cited for immobilized ß-galactosidase

In the literature , Kilara and Shahani[22] reviewed in detail, the use of immobilized enzymes in the food industry and methods of immobilization for various enzymes including ß-galactosidase. Finocchiaro *et al*[23] discussed the use of immobilized lactase in milk systems. They have summarised more than 50 immobilized lactase systems for their sources, immobilization techniques, reactor types and applications.

In another review White and Kennedy[18] described some of more popular immobilized ß-galactosidase systems and chemistry of number of polymer matrices for enzyme immobilization. In some of early works on immobilized enzymes Kay *et al*[24] reported the attachment of ß-galactosidase onto chloroamino-S-triazinyl derivative of DEAE-cellulose paper. Morisi *et al*[25] reported a comparative study of free and entrapped ß-galactosidase from *E. Coli* and yeast. ß-galactosidase entrapped on cellulose acetate shows no shift in optimum pH for its activity. They also found that enzyme from yeast is a better catalyst for hydrolysing lactose in milk. However, continuous operation of the system showed reduced enzyme activity of entrapped enzyme due to microbial contamination. Dahlqvist *et al*[26] reported immobilization of yeast ß-galactosidase in crosslinked polyacrylamide by a bead polymerization technique. The system was then used for preparation of lactose-free milk. A maximum amount of 75% of the enzyme was immobilized with 20% (w/w) acrylamide and N-N'-methylene bis-acrylamide. The presence of cystein and serum albumine increased enzyme activity upto 30% and 15% respectively.

Wierzbieki *et al*[27] reported covalently bound ß-galactosidase to porous glass through diazotization. Stability and initial hydrolytic activity of immobilized enzymes towards lactose in skim milk and acid whey were examined. They have observed that some of the immobilized ß-galactosidases retain sufficient activity and stability for their use in the industrial applications. In another article Coughlin *et al*[11] used porous glass support for immobilization of ß-galactosidase from *A-niger* by using glutaraldehyde treatment. 5% Lactose and raw whey were used as substrate. They observed 50% binding efficiency and 70-85% conversion rates for the immobilized enzymes.

Okos and Harper [28] reported ß-galactosidase bound to acrylamide derivatives of ZrO_2 porous glass. They have reported partial irreversible inhibition of immobilized lactase activity when used for the hydrolysis of whey. Further addition of 0.25% of galactose in the feed solution was reported to produce 55% inhibition of lactose hydrolysis at a conversion rate equivalent to 25%.

Gilbert and Kleyn [29] have reported immobilization of ß-galactosidase from *A. niger* on collagen for the hydrolysis of lactose in acid whey. The complexes were prepared by interdiffusional penetration and macromolecular complexing processes. They observed partial irreversible enzyme inhibition in unfiltered acid whey when compared to lactose. Moreover, trace amounts of divalent cations in the whey were reported as a possible cause of inhibitory effect.

Hustard *et al* [30,31] have discussed immobilization of ß-galactosidase from *E. Coli* onto polyisocynate based polymer. Maximum enzymatic activity was obtained by immobilizing the enzyme in phosphate buffer of pH 5. Immobilized enzyme showed good stability and activity at pH 8.75. The immobilized enzyme was stable for long periods and no leaching was apparent. Michaelis constant (K_m) for free and immobilized enzyme at pH 6.5 was reported to be 13.1 mM and 2.1 mM respectively. After 82 days 65.6% of the original activity was reported to be retained [31]. In another article Richardson and Olson[32] reported immobilization of ß-galactosidase on the same polyisocynate carrier.

Stainless steel and other metals were used by Charles *et al* [33] for immobilization of lactase. Desirable characteristics of the systems included simple inexpensive synthesis of the system giving rise to inert solid support of excellent mechanical integrity, high specific activity and excellent stability.

Olson and Stanley[34] used tannic acid and phenol formaldehyde resins on glutaraldehyde activation to immobilize enzymes including ß-galactosidase. The immobilized ß-galactosidase was further used for the hydrolysis of lactose in milk and milk products. Continuous rinsing of column with water and dilute hydrogen peroxide for controlling the microbial contamination was also suggested. Hyrkas *et al* [35] have also studied phenol formaldehyde resin for immobilization of ß-galactosidase using adsorption and crosslinking techniques. Whole whey, whey ultrafiltrate permeate were used as substrates and showed 77% conversion for ultrafiltrate whey and 39% for whole whey.

Pye and Weetall [36] investigated the possibility of ß-galactosidase adsorption on porous ceramics. Some of the properties of the system studied were pH, coupling efficiency, mass transfer effect, enzyme stability and microbial contamination. Economics of the process was also presented.

Kilara *et al* [37] immobilized ß-galactosidase from yeast on agarose with an amino alkyl derivatized spacer arm. The optimum pH for lactase activity (pH 7) and stability (pH 7.5) were not altered during immobilization. The IME showed better storage stability than that of free enzyme. Agarose derivatives were also reported for ß-galactosidase immobilization [38-41].

Cellulose triacetate was used as a support material for the immobilization of ß-galactosidase[42,43] by using entrapment method. Skimmed sterile milk was used as substrate for the activity and the observed maximum percentage conversion was 71-81% within 2h reaction time.

Phenol formaldehyde resins have been widely used for the immobilization of ß-galactosidase [44-50] and these systems were used for various applications. Some other organic supports such as derivatives of polyacrylamide [51-54], sepharose [55], cellulose polyacrylic beads[56-58], DEAE-cellulose[59,60], polyurethane[61], nylon-acrylonitrile copolymer [62], acrylic acid-oxirane [63], poly(vinyl alcohol) gel [64], polyethylene imine and polyethylene imine coated gelatine[65], ion exchange resins[66], p-amino carbanilated cellulose [67], Agar gel [68], chitosan [69] have also been reported. The details are discussed in the Table given in the review article by Greenberg and Mahoney[13] In addition some inorganic matrices such as porous alumina [70] derivatives of silica [71,72], Mn-Zn-Ferrite non porous particles [73,74] have also been reported by them. However, with many of these supports, immobilization has resulted in considerable reduction in enzyme activity as well as its binding capacity.

In recent times in 1986 Ivanov *et al* [75] discussed immobilization of ß-galactosidase on amino silochrome S-80 with gossypol cross-linking reagent. The immobilized enzyme was 3-4% less active than the free enzyme. The immobilized system was used for 85 days in the continuous hydrolysis of cheese whey and 71 days in the hydrolysis of milk ultrafiltrate.

Another type of support gelatine was successfully used for the immobilization of ß-galactosidase from *Saccharomyces* yeast by using entrapment techniques [76]. The optimum temperature and pH of the yeast enzyme were very little affected on immobilization. The K_m values for free and immobilized enzymes were observed to be 102 mM and 132 mM respectively. Authors have reported enhanced enzyme activity in presence of calcium and magnesium ions and also good stability without a significant loss of enzyme activity on storage at 4 °C for 120 days.

Another common carrier for covalent coupling of enzymes is porous silica beads. Baret [77] reported immobilization of ß-galactosidase from *A. niger* and *A. oryzea* on controlled pore silica of 30/45 mesh size and 350 A° pore diameter. Lactose was used as a substrate and half life of the system was found to be 55 days. The immobilization process was scaled up from laboratory to an industrial applications.

In another interesting experiment Pilloton *et al* [78] examined lactose determination in raw skim-milk with two immobilized enzyme-based electrochemical sensors. A hydrogen peroxide selective electrochemical sensor with ß-galactosidase and glucose oxidase immobilized on its surface and covered with a cellulose acetate dialysis membrane was proposed for the determination of lactose from raw milk. The liberated hydrogen peroxide was measured with platinum electrode polarized at +650 mv vs Ag/AgCl. The procedure is very simple and rigid response time permits its use in assaying milk samples.

Batsalova *et al* [79] reported immobilization of fungal ß-galactosidase on a PVA gel formed in pores of cotton material. Temperature and pH effect on the activity of free and immobilized enzymes was studied. The optimum temperature for free and immobilized enzymes was reported to be 60 and 50 °C respectively. The optimum pH was observed to be 4.5 to 5.0. The thermal stability of the immobilized ß-galactosidase was observed to be higher and K_m values for free and immobilized enzymes were reported to be 1.9 mM and 2.8 mM respectively. The immobilized system was further used for lactose hydrolysis. The degree of conversion was observed to decrease to 50% after 30 repeated runs. The capacity of the immobilized enzyme to hydrolyze lactose in whey was also studied.

In 1988, Narinesingh *et al* [80] reported covalent binding of ß-galactosidase from *E. Coli* on fractogel support. The method of immobilization was proved to be very mild and facile. Detailed investigations of the flow kinetics, pH profile, temperature effects and thermal stability were reported for a plug flow ß-galactosidase bioreactor. The effects of various water miscible organic solvents such as methanol, ethanol, propanol, glycerol, DMSO and acetonitrile on the kinetics and stability of this bioreactor were also investigated. The alcoholic solvents, with the exception of ethanol, enhanced the rate of liberation of o-nitrophenol from the substrate ONPG whereas other solvents depressed the rate of liberation of o-nitrophenol.

Prenosil *et al*[81] observed the formation of oligosaccharides during enzymatic lactose hydrolysis. The enzymes were immobilized on ion exchange resin Duolite S-761. The immobilized systems were compared for their oligosaccharide production and conditions for minimizing oligosaccharide content were developed.

Ariga and Tetaki[82] used PVA hardened by freeze thawing for the immobilization of lactase and the stability of immobilized enzyme was investigated after freezing thawing procedures. Leakage of enzyme from PVA hydrogel beads and faster drop in activity of the gel beads were observed during prolonged incubation.

ß-galactosidase immobilized on chitosan beads was reported by Sakurai *et al*[83]. An acetic acid solution of chitosan degraded with sodium perborate was added to a NaOH/aq.MeOH solution to give porous structure. Immobilized systems prepared by incubating a mixture of enzyme and glutaraldehyde solution at pH 5 showed activity without any reduction after 8 uses.

In 1989 Bakken *et al*[84] reported immobilization of ß-galactosidase from *A. oryzea* on silica particles and its use in spiral flow reactor for the hydrolysis of lactose from skimmed milk. At 30 °C and a space time of 7 min 80% of the lactose present in the skimmed milk was converted to glucose and galactose.

Korde *et al*[85] used *E. Coli* ß-galactosidase for the immobilization in albumine crosslinked with glutaraldehyde. A mathematical model which considers the external diffusional limitations and the disperson of the substrate in the axial direction, has been developed by them to simulate the performance of immobilized enzyme reactors. The effect of enzyme denaturation on the first order kinetics was also studied in detail.

Recently another type of support calcium-alginate dicarboxy cellulose gel was used for the immobilization of ß-galactosidase[86]. The immobilized system exhibited high activity and broader pH profile. In another article Bakken *et al*[87] reported immobilization of ß-galactosidase from *A. oryzea* on a ribbed membrane made from PVC and silica. Immobilized system was further used to hydrolyze the lactose constituent of skimmed milk. Multi-response non-linear regression methods were employed to determine the kinetic parameters of rate expressions based on a proposed enzymatic mechanism which includes the formation of oligosaccharides. The concentrations of all species in the effluent stream was monitored through HPLC.

Microencapsulation technique was developed for the immobilization of ß-galactosidase in butter-oil[88]. The degree of deactivation of ß-galactosidase due to the microencapsulation process was reported to be 5.2% of initial activity.

Recently, Ariga and Tetsuya[89] reported effect of poly(vinyl alcohol) on kinetic and thermal properties of ß-galactosidase. For the study they have used poly(vinyl alcohol) with various saponification values. The enzyme activity was determined by using ONPG substrate and found to increase with increasing PVA concentration. The influence of the properties of PVA on Michaelis constant was observed to be significant.

Polyurethane foam was used as a support material for the immobilization of ß-galactosidase[90] from *A. oryzea*. Among several immobilization methods studied, the IME preparation by *in situ* copolymerisation between enzyme and prepolymer HYPOL-3000 showed the highest activity. The results showed that immobilization was predominantly through covalent binding of primary amino groups of ß-galactosidase and isocynate groups of the prepolymer.

Piesecki *et al*[91] reported immobilization of ß-galactosidase from *E. Coli* for its application in synthetic organic chemistry. The strong interaction of hexahistidine fusion proteins with metal chelate adsorbants was utilized to immobilize ß-galactosidase with a hexahistidine peptide at the N-terminus to the Ni^{+2} nitriloacetic acid adsorbants. The enzyme activity retained 64% of its initial activity when bound to the adsorbants. To demonstrate the potential of immobilized enzyme in organic chemistry allyl-ß-D-galactopyranoside was synthesized from lactose and allyl alcohol.

Recently, triacetate fibres were used for the immobilization of ß-galactosidase[92] and to study the kinetics of lactose hydrolysis in the presence of the biocatalyst. The equilibrium and kinetic constants of the processes were calculated using computerized data. It was found that the character of the enzyme inhibition changed with temperature. The immobilized systems were also used for the continuous hydrolysis of lactose in milk whey using CSTR.

Generally, hydrogel systems have good blood and tissue compatibility. TacGwan[93] studied, stabilization of immobilized ß-galactosidase in temperature sensitive hydrogels. Crosslinked poly (N-isopropyl acrylamide co-acrylamide) hydrogels exhibited LCST (Lower Critical Solution Temperature) behaviour. The temperature dependent phase transition was observed around 37°C. The stability of IME was investigated at different temperatures.

Thompkinson[94] reported immobilization of ß-galactosidase from *K. fragilis* on a macroporous anion exchange resin through a silanization process. Immobilized enzyme activity was determined by ONPG substrate and it was observed that 42% of enzyme activity was retained after immobilization. The immobilized system was further used in fluidized bed reactor for the hydrolysis of lactose in sweet whey.

Recently, the immobilized ß-galactosidase from bacteria and fungi are reported by Rogalski and coworkers[95]. They have used porous glass and polyamide as supports for the immobilization of ß-galactosidase using glutaraldehyde and bis-oxirane crosslinking reagent. The immobilized ß-galactosidase was further used for the hydrolysis of lactose in milk.

Gabriela and Francisco[96] reported immobilization of ß-galactosidase from K-lactis on Nickel (II) imino diacetate-agarose using adsorption techniques. They observed that 90% of enzyme activity was retained after immobilization. The immobilized derivatives were further used for lactose hydrolysis in whey permeate.

ß-galactosidase was immobilized onto a gelatin carrier system by glutaraldehyde and chromium (III) acetate crosslinking reagents by Sibel and Ural[97]. The effect of pH on enzyme activity, kinetic parameters and reusability of bound enzyme were investigated. Gelatin-glutaraldehyde system showed 68% of enzyme activity after immobilization.

Takao *et al*[98] have reported covalent coupling of ß-galactosidase onto a porous ceramic support SM-10. Among the supports tested, the highest residual activity after 3 h incubation time at 70°C was obtained for silanized SM-10 with 3-[2-(2-amino ethyl amino propyl] trimethoxy silane. The amount of enzyme immobilized was about 60 mg/g of this support. The immobilized enzyme properties were almost the same as those of the free enzyme.

ß-galactosidase was covalently coupled on agarose by using glutaraldehyde cross-linking and the immobilized system was further used for the synthesis of oligosaccharides as reported by Berger *et al.* [99]

In the present work, we have compared the activity and loading capacity of ß-galactosidase on the polymeric supports in the powdered form and in the bead forms. For this study we have used a resinous material synthesised in our laboratory containing salicylic acid, resorcinol and formaldehyde (SRF). Another supports poly(vinyl alcohol) crosslinked with para formaldehyde (PVA-F) and natural polysaccharide chitosan in bead forms were also used for immobilization of ß-galactosidase. Various activation processes have been used during immobilization and physicochemical properties of immobilized systems are compared with those of free enzyme. Literature survey reveals that hydrolysis of lactose in whey and milk to glucose and galactose is becoming an attractive process. Due to the high cost of lactase enzyme the most promising way of achieving economic feasibility of the processes is use of immobilized systems. Hence we have constructed a packed bed column reactor and carried out continuous hydrolysis of lactose by using immobilized ß-galactosidase.

2.4. Experimental

The details of the polymeric supports used for immobilization and chemicals used for the study are discussed below. All the experiments were done in triplicate to ensure reproducibility.

► **Chemicals used**:

ß-galactosidase (EC.3.2.1.23) (from *Aspergillus oryzea* of strength 2.76U/mg)	: Sigma Chemical Company, USA
O-nitrophenyl ß-D-galacto-pyranoside (ONPG)	: Sigma Chemical Company, USA
Lactose	: S.D. Fine Chemicals, India
Bovine Serum Albumine (BSA)	: Sisco Research Lab, India
Salicylic acid	: Qualigens, India
Resorcinol	: BDH, India
Formaldehyde	: Qualigens, India
Paraformaldehyde	: S.D. Fine Chemicals, India
Chitosan [a-(1-4) 2-amino-2-deoxy ß-D-glucan]	: Central Institute of Fisheries Technology, (CIFT), Cochin, India
Poly(vinyl alcohol) with Mol.wt.~74,800-78,200 and 98% mole-hydrolysis	: Loba, India
Methanol	: Qualigens, India
Propanol	: Qualigens, India
Acetone	: Qualigens, India

Cynuric chloride	: Fluka, Switzerland
p-tolyl sulphonyl chloride	: BDH, Germany
Glutaraldehyde (25% w/w)	: S.D. Fine Chemicals, India
1,4 Dioxane	: Qualigens, India
Citric acid	: BDH, India
Disodium hydrogen phosphate	: BDH, India

All solvents and reagents used were of Analytical grade and solvents were distilled before use. Double distilled deionised water was used throughout the work. Fresh whey of pH 4.5 containing 4.99 to 5.2% total solid, 0.5 to 0.6% lactic acid and minor quantities of minerals and proteins were collected from Baroda Dairy Sugam Plant, Vadodara (India). Lactose content present in the milk whey was determined by Lane and Eynon's titrimetric method[100] and was found to be 2.39%.

2.4.1. Preparation of polymeric supports:

(a) Synthesis of polymeric resin (SRF)

The macroporous resinous material was synthesized by a condensation polymerization technique following a procedure reported by Martin[101]. Salicylic acid (13.8 g), boric acid (3.1 g) and sodium hydroxide (2 g) were treated in 25 ml of deionised water until a clear solution was obtained. To this solution, 36 ml of 40% (w/v) formaldehyde and anhydrous sodium acetate (20 g) were added and heating was continued for 60 min after the addition of 5.5 g of resorcinol. The resulting orange coloured gel was cured at 110°C and was washed with distilled water and acetone prior to storage. Characterization of synthesised product was done through the measurement of cation exchange capacity, elemental analysis, thermal analysis and spectral analysis. The reactions involved in the synthesis are given in Fig. 2.1. Synthesised SRF in 60/100 mesh size was used for the immobilization of ß-galactosidase and other enzymes.

(b) Preparation of crosslinked poly(vinyl alcohol)

Crosslinking of poly(vinyl alcohol) with paraformaldehyde and conversion of it into the bead form was done as per the procedure described by Chandy and Sharma[102]. 100 ml of 12% (w/v) poly(vinyl alcohol) of ~77,000 molecular weight and 98-99% hydrolysis mole in water was added to 35 ml of 20% (w/v) paraformaldehyde in 5N sodium hydroxide and kept overnight at room temperature for crosslinking. This crosslinked solution was added dropwise with a microsyringe to 10N 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 crosslinking was not determined.

(c) Preparation of chitosan (a-[1-4]-2-amino-2-deoxy ß-D glucan) beads

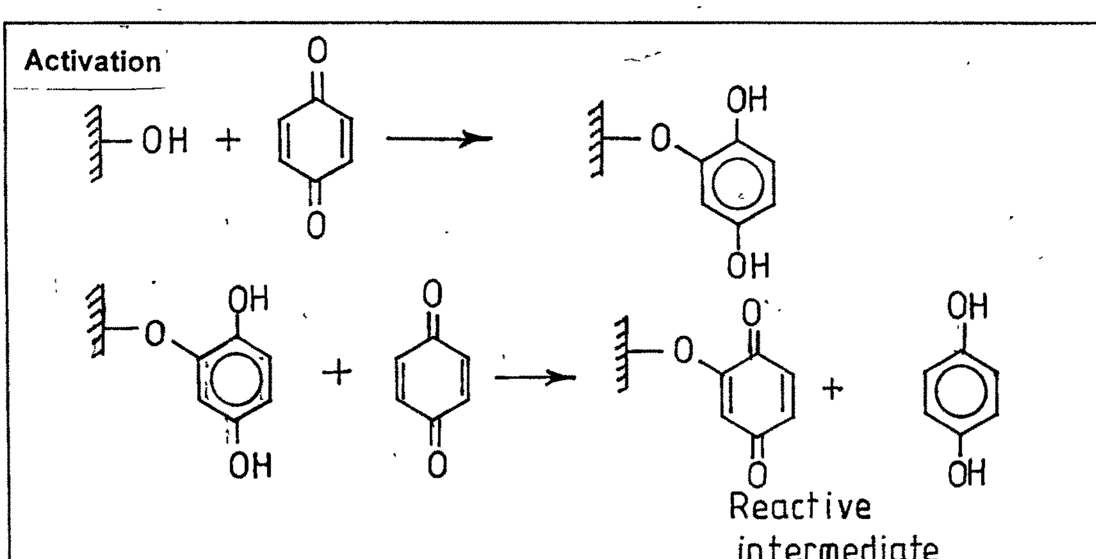
Chitosan beads were prepared by using 2-4% (w/v) solution of chitosan powder in 2% acetic acid. This was added dropwise to 3:1 (v/v) mixture of 1N sodium hydroxide and methanol with the help of microsyringe. The beads formed were separated and washed with hot water several times to remove the excess of alkali. They were stored moist at room temperature at 30 °C.

2.4.2 Immobilization of ß-galactosidase

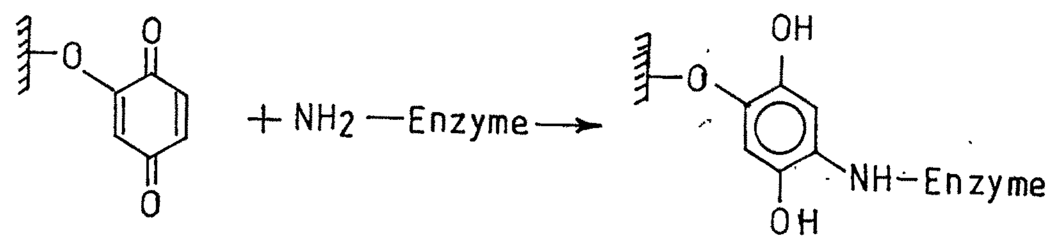
Various hydroxyl and amino group activation processes have been used for the immobilization of ß-galactosidase. The hydroxy activation methods such as p-benzoquinone[103], p-tolyl sulphonyl chloride[104] and cynuric chloride[105] were used for SRF-resinous material and crosslinked poly(vinyl alcohol). Whereas glucosamine polysaccharide chitosan was activated using glutaraldehyde treatment. The activation procedures for these supports are described here briefly.

(a) Activation through p-Benzoquinone

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



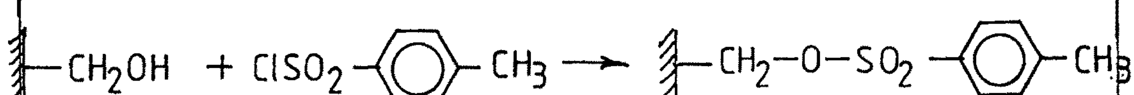
coupling



(b) Activation through p-tolylsulphonyl chloride

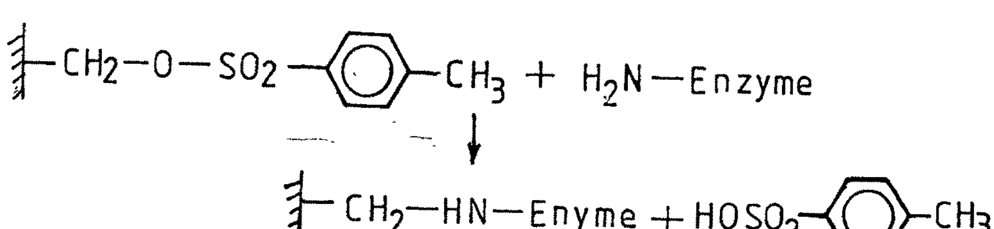
The polymers SRF/PVA-F (0.2-0.4 g) were activated for 1 h using 0.1 g p-tolyl sulphonyl chloride in 10 ml dry dioxane at room temperature. One millilitre of pyridine was added dropwise over 1 min. The support was washed with dry acetone then with acetone water (1:3) and citrate-phosphate buffer of pH 4.5.

Activation



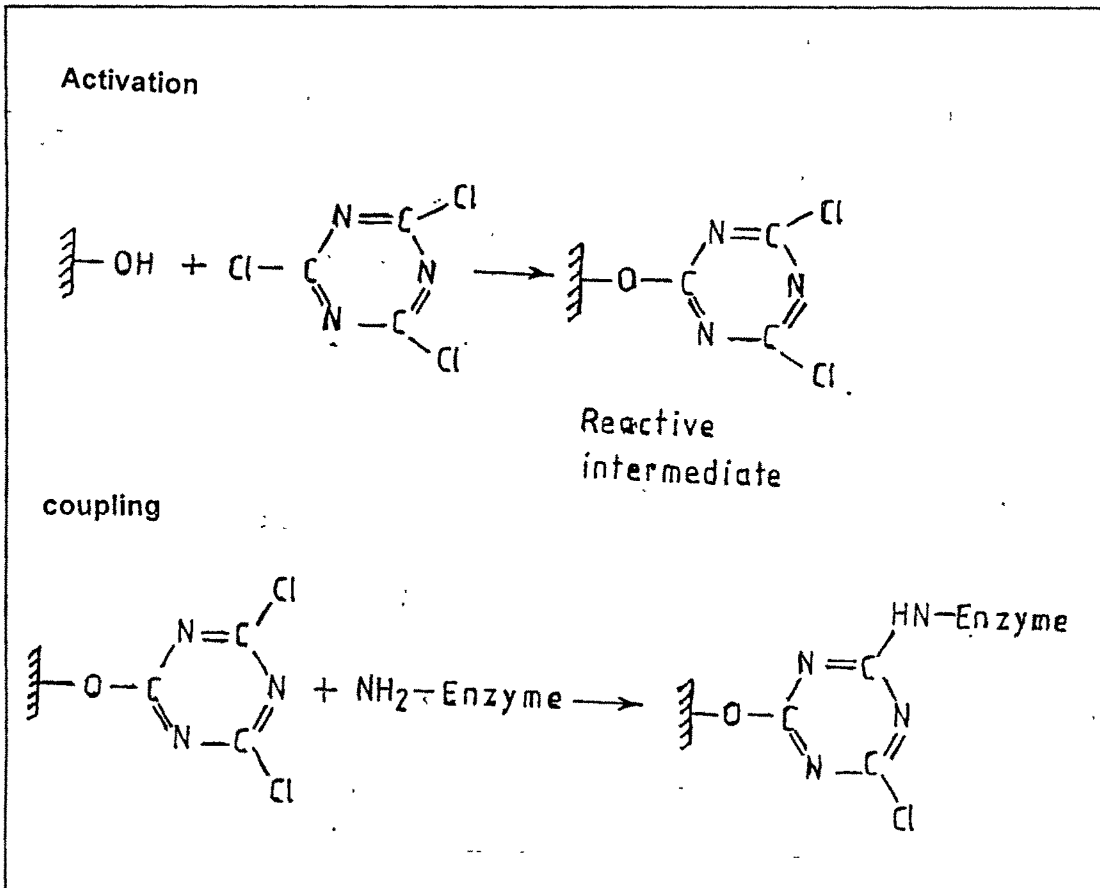
Tosylchloride

coupling



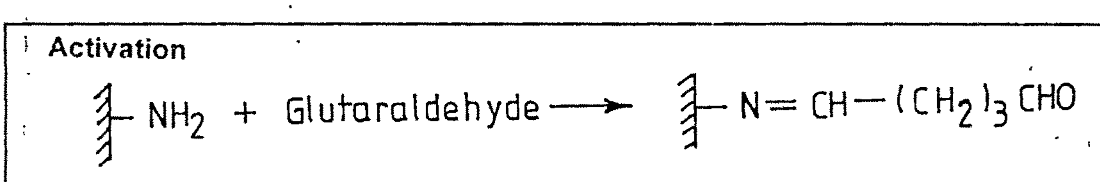
(c) Activation through cynuric chloride

The polymeric supports SRF/PVA-F, 0.2 to 0.4 g, were 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 citrate-phosphate buffer of pH 4.5 before immobilization of ß-galactosidase.

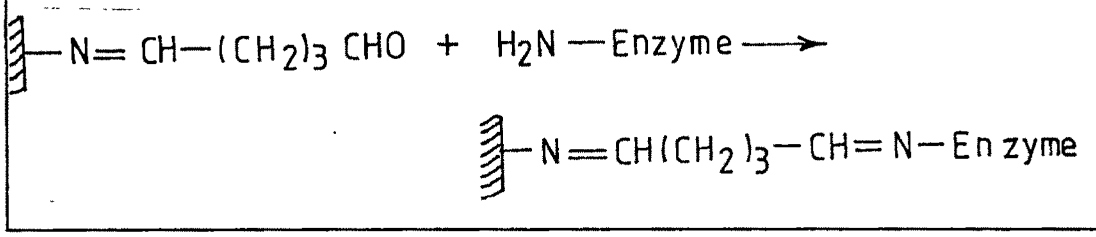


(d) Activation through glutaraldehyde

Polysaccharide chitosan beads 0.2 to 0.5 g, were activated by 4 ml of 6% (w/w) aqueous glutaraldehyde solution at room temperature for 1 h. Excess of glutaraldehyde was washed with water and citrate-phosphate buffer of pH 4.5.



coupling



e) Coupling of Enzyme

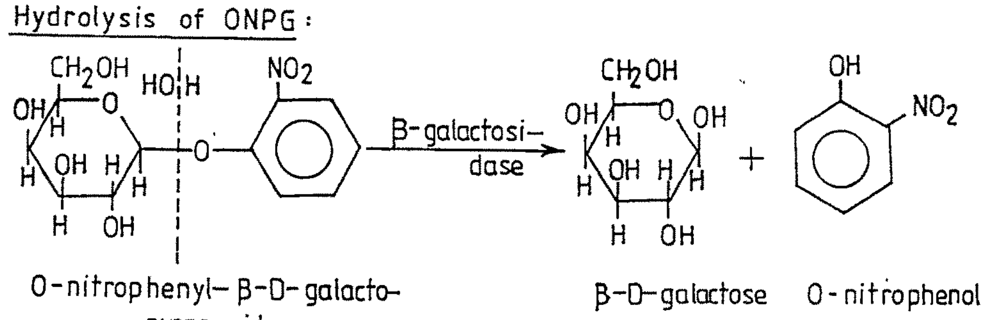
The activated polymeric supports were used for the immobilization of ß-galactosidase using 0.2 M citrate-phosphate buffer of pH 4.5 at 278 °K and 19 h reaction time. A low temperature shaker bath (INSREF) was used for the reaction to reduce the external diffusion barrier during immobilization process. The amount of protein bound to the supports was determined, using Lowry assay procedure[106] from the differences in protein concentration before and after immobilization.

2.4.3 Assay of enzyme activity

The activity of free and immobilized ß-galactosidase was determined using Craven's assay method[107] and o-nitrophenyl ß-D galactopyranoside chromogen as substrate (ONPG). 5 ml ONPG of 2 mM concentration was used for the activity of free and immobilized enzyme. The enzymatic activity was determined at 313 °K and pH 4.5 in 0.2M citrate-phosphate buffer. The enzymatic hydrolysis reaction was stopped after 10 min by adding 2 ml of 1 M sodium carbonate. The liberated o-nitrophenol was measured spectrophotometrically at 410 nm for the activity of enzyme. The reaction proceeds to essentially quantitative hydrolysis of the substrate. For each mole of o-nitrophenol liberated, 1 mole of reducing sugar i.e. galactose is liberated. The reaction is strictly a hydrolytic cleavage of the ß-D galactoside bond. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 micromole of o-nitrophenol per minute under

Hydrolysis of ONPG:

I



the condition of assay. The structural formulas for ONPG, o-nitrophenol and galactose are shown in the rendering of the hydrylysis reaction.

2.4.4 Albumination process for immobilization of ß-galactosidase

Glucosamine polysaccharide chitosan was treated with Bovine Serum Albumin (BSA) for albumination of the support and then was used for the immobilization of ß-galactosidase to reduce the linking of enzyme to the support through its active sites. For the preparation of albuminated matrix, about 0.2 to 0.4 g chitosan beads were treated with 200 mg of BSA in 0.2 M phosphate buffer of pH 7.5 for 3 h reaction time. The albuminated beads were washed with water and buffer solution and then treated with 4 ml of 6% (w/w) glutaraldehyde as described in Section 2.4.2 before coupling of ß-galactosidase.

2.4.5 Optimization of coupling conditions

Various coupling conditions and methods were optimized for the better retention of activity and stability of immobilized ß-galactosidase.

(a) Effect of type and concentration of crosslinking reagents

Activation of polymeric supports containing hydroxyl groups was done by using p-tolylsulphonyl chloride, p-benzoquinone and cynuric chloride activating reagents. Glutaraldehyde activator was used for the support containing amino group. During activation, different concentrations of these activating reagents ranging from 0.05 to 0.5 g of p-tolylsulphonyl chloride, 0.01 to 0.1 g of p-benzoquinone, 0.25 to 2 g of cynuic chloride and 2% to 10% (w/w) of glutaraldehyde were used.

(b) Effect of pH of coupling medium

The immobilization of ß-galactosidase on activated support was carried out by following the procedure described earlier in Section 2.4.2 but using coupling media of 0.2 M citrate- phosphate buffer of different pH ranging from pH 2-8.

(c) Effect of coupling time

The optimum time required for the coupling of ß-galactosidase to the activated supports was determined by measuring the amount of protein coupled on the support at various time intervals ranging from 30 min to 18 h reaction time.

(d) Effect of enzyme concentration

Effect of enzyme concentration on the extent of immobilization was studied by using various concentrations of enzyme ß-galactosidase during coupling of enzyme to the activated support.

2.4.6 Comparative account of free and immobilized ß-galactosidase

(a) pH profile

pH profile for the free and immobilized ß-galactosidase activity was determined by measuring the residual activity of enzyme at various pH values using 0.2 M citrate-phosphate buffer and ONPG substrate.

(b) Temperature profile

Effect of temperature on enzyme activity of free and immobilized ß-galactosidase was studied by measuring the activity of the enzyme exposed to the various temperatures ranging from 30 to 70 °C for 10 min. From the study energy of activation (ΔEa) was calculated by using Arrhenius equation

$$k = A.e^{-\Delta Ea/RT}$$

where k = Velocity constant of reaction A = Arrhenius constant, R = Gas constant, Δ Ea = Energy of activation, and T = Absolute Temperature

The velocity constant is not easy to obtain and since 'V' is directly proportional to 'k' the maximum velocity of hydrolysis ($\log_{10} V_{max}$) is plotted against reciprocal of the temperature (1/T) and from this Arrhenius plot, the activation energy of the immobilized and free enzyme was calculated.

(c) Thermal stability

The thermal stability of free and immobilized enzyme was determined at optimized temperature (65 °C) and longer exposure time periods. The residual activity was measured by quickly cooling the enzyme to room temperature. The residual activities were compared with the original activities prior to heating. The thermodeactivation constant (K_d) was evaluated by using an equation

$$ln\ A = ln\ Ao - K_d t$$

where 'Ao' is the initial activity and 'A' is the activity after heat treatment for 't' minutes[108]

(d) Storage stability

Storage stability of the free and immobilized ß-galactosidase was determined by storing the enzyme at 4°C and 30 °C (room temperature) for various time intervals and measuring the enzyme activity periodically.

(e) Solvent stability

Solvent stability of the system was determined by measuring the activity of the free and immobilized ß-galactosidase in the 1-4 M water miscible organic solvents such as methanol, ethanol, and propanol.

(f) Kinetic behaviour

Michaelis Constant (K_m) and maximum reaction velocity (V_{max}) for the free and immobilized ß-galactosidase were calculated from Lineweaver-Burk plots of 1/v vs 1/s where v = velocity of reaction and s = substrate concentration. Various concentrations of substrate ONPG (0.4 mM to 2 mM) and lactose (0.1 to 5%) were used for the kinetic study.

(g) Reusability

The reusability of the immobilized ß-galactosidase was determined by using a reactor composed of immobilized enzyme. The same enzyme was used 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.

2.4.7 Hydrolysis of lactose and milk whey

The schematic diagram of the fixed bed reactor used for the study of continuous hydrolysis of lactose and milk whey is given in Fig 2.2. The compact columns of 0.6 x 10 cm dimensions containing ß-galactosidase immobilized on PVA-F and Chitosan beads were prepared by placing a porous frit at the bottom of the reactor. The reactor was maintained at 40-60 °C controlled temperature by circulating water through an outer jacket. The packed column bed was washed with citrate-phosphate buffer of pH 5 before operation. The substrate was passed through the column at different flow velocities by means of a peristaltic pump. The extent of hydrolysis was measured at different concentrations of substrate and flow rates. The efficiency of the reactor was measured

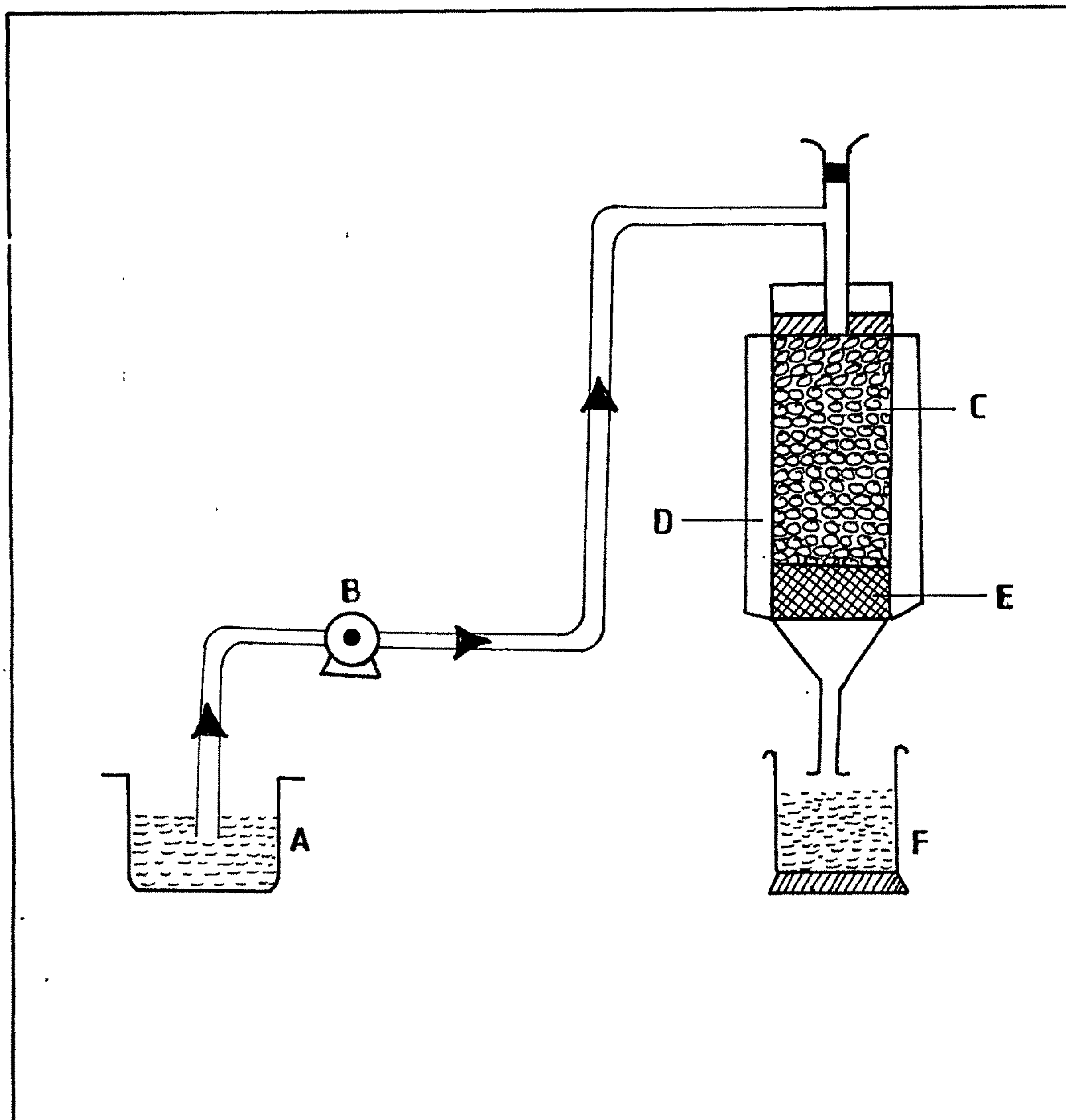
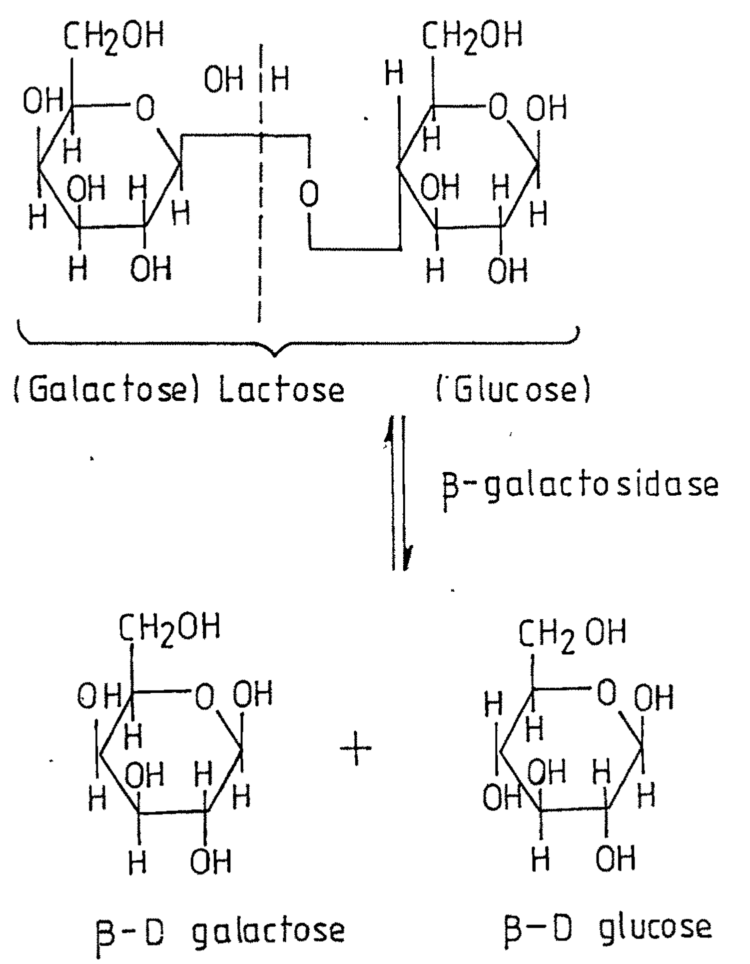


Fig 2.2 Schematic diagram of fixed bed reactor for the hydrolysis of lactose (A) Inlet, (B) Peristaltic pump, (C) Immobilized enzyme, (D) Temperature controller, (E) Porous glass frit, (F) Outlet.

through the amount of glucose liberated during hydrolysis of lactose by a free enzyme and immobilized enzyme. Estimation of glucose was done by using glucose oxidase - peroxidase method and incubating the test solution for 15 min at 37°C and measuring the absorbance at 510 nm[109,110].

During hydrolysis milk whey was denatured by heating to ~100 °C for 15 to 20 min and centrifuging at 6000 rpm for 10 min. For the hydrolysis of lactose from milk whey, 10 ml of supernatant milk whey of pH 4.5 was passed through the reactor containing immobilized ß-galactosidase. The amount of glucose generated at different flow rates and temperatures was measured by estimating glucose periodically as described earlier[109,110]. The hydrolysis reaction is illustrated below.

Hydrolysis of lactose



2.4.8 Optimization of column conditions

(a) Effect of temperature

Effect of column temperature on the continuous hydrolysis of lactose and lactose from milk whey by immobilized ß-galactosidase was studied at different reactor temperatures 40-60°C. From the results column efficiency in the hydrolysis of lactose at different temperatures and energy of activation (ΔEa) was calculated by using the Arrhenius equation as described earlier in Section 2.4.6.

(b) Effect of pH

Effect of pH on lactose hydrolysis was studied by passing 5% lactose solution of pH 4-7 through the reactor at flow rates of 0.5 ml min^{-1} and measuring the amount of glucose liberated during the process as reported earlier in Section 2.4.7.

(c) Flow rate

Hydrolysis of lactose was studied by varying the flow velocities from 0.5 ml to 2 ml min^{-1}.

(d) Effect of lactose concentration

Lactose solution of 0.1 to 5% concentration was prepared in 0.2 M citrate-phosphate buffer of pH 5 and 10 ml of the solution was passed through the reactor at a flow rate of 0.5 to 2 ml min^{-1}. The percentage hydrolysis of lactose was studied at different temperatures and flow rates. From the variation in substrate concentration study Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were calculated as described earlier in Section 2.4.6.

(e) Thermal deactivation

The thermal deactivation of the enzyme reactor was examined by monitoring the reactor performance at constant temperatures (40-60°C) for a longer time periods. The deactivation rate constant (k_d) and deactivation energy (ΔE_d) were calculated by using an equation [111].

$$\frac{da(t,T)}{dt} = -k_d a(t,T)$$

where a (t, T) is the activity of the enzyme at time 't' and temperature 'T' and 'k_d' is the deactivation rate constant.

The deactivation constant (k_d) is assumed to follow the Arrehenius relationship:

$$K_d = A_d.e^{-\Delta E_d / RT}$$

(f) Operational stability

The operational stability of the immobilized ß-galactosidase was determined by operating the reactor continuously at constant temperature (50 °C) and 0.5 ml min^{-1} flow rates for varying time intervals and measuring the residual activity periodically. Operational stability of the column was also examined by measuring the residual activity in the lactose and milk whey samples collected from the reactor outlets using ONPG substrates.

2.5 Results and Discussion

Activation of hydroxyl groups for covalent coupling of enzyme is a widely used process for enzyme immobilization. However, resins based on phenol show poisoning of enzymes. Hence, resinous materials derived from salicylic acid were used to overcome poisoning effect of phenolic compounds but retaining the advantage of hydroxyl group activation of the polymeric supports for the coupling of enyme. In addition for this support, the extent of crosslinking and the surface area can be controlled as required. The material synthesised has a low cost and has proved its potential as a good support for enzyme immobilization. The synthesised material is a mixture of various polymeric forms and the reactions involved in the synthesis are shown in Fig 2.1. Similar type of condensation is possible for the product I and III, resulting in the different forms of the resinous materials. Hence the final product will have a gross structure.

The synthesised SRF was characterized by IR -spectra and exhibited peaks at 3414 cm^{-1}, 1695 cm^{-1}, 1623 cm^{-1} and 1479 cm^{-1} respectively for -OH stretching, -C=O stretching, aromatic -C=C stretching and $-CH_2$ bending. The water regain capacity and specific density of the resin were 11.2% and 2.2 respectively. The resin showed stability towards organic solvents and acids of high strength (6M) and towards fungal activity on moist storage.

Crosslinked poly(vinyl alcohol) was prepared as described earlier in Section 2.4.1(b) This support is inexpensive, hydrophilic and exhibits excellent mechanical properties [112]. Hence we have tried this support for the immobilization of ß-galactosidase and its use in continuous flow reactor for the hydrolysis of lactose.

Another support material chitosan is an inexpensive, inert, hydrophilic, biocompatible support and thus is attractive for enzyme immobilization. The polymer can be casted into a transparent film from the polymer solution in dilute acetic acid. The

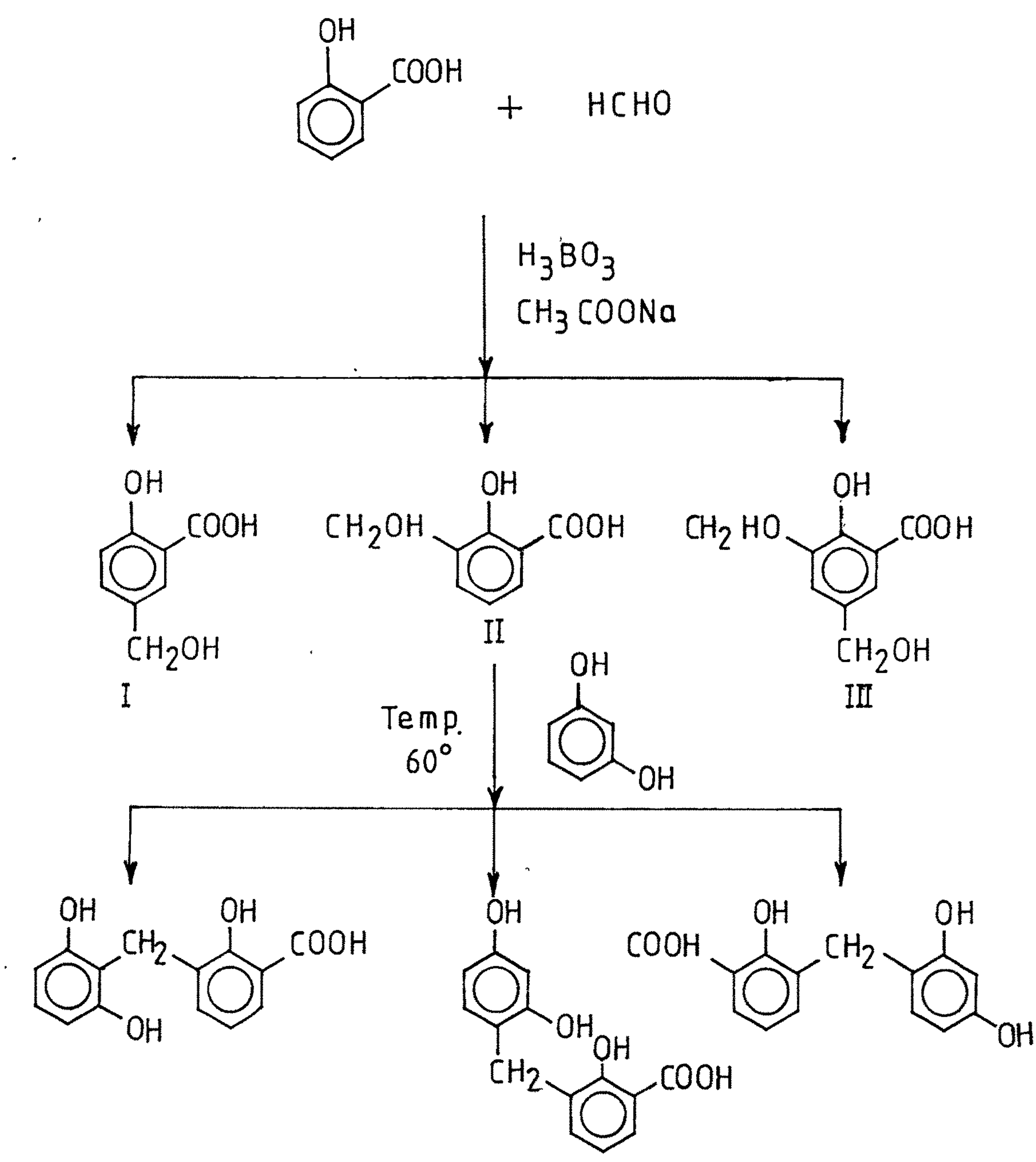


Fig 2.1 Reactions involving synthesized resin (SRF)

chemical structure of chitosan is a-(1→4)-2-amino 2-deoxy-ß-D glucan which is obtained by partially deacetylating chitin [113,114]. The presence of amino groups, facilitates covalent binding of enzymes and further derivatization of the polymer [115,116]. Immobilization can be carried out either by entrapment or by covalent binding by using chitosan beads or films.

2.5.1 Effect of coupling reagent

Chitosan powder has a very low density and hence was not useful for immobilization of ß-galactosidase. Therefore, chitosan was converted into beads of ~1.5 mm diameter size as described earlier. Salicylic acid/Resorcinol/Formaldehyde resin was used in 60/100 mesh size as reported earlier. Poly(vinyl alcohol) crosslinked with paraformaldehyde was used in the bead form of ~2 mm diameter. The results obtained by using various activating methods are given in Table 2.3. With SRF, quantitative coupling of 6.4 u/g ß-galactosidase was observed but only 28% 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 of immobilized ß-galactosidase showed 100% retention of enzyme activity. Activation of chitosan beads by glutaraldehyde resulted into quantitative coupling and retention of activity with 9.7 u/g enzyme loading. However, lower activity was observed by Weetall *et al*[117] when ß-galactosidase was covalently bound to glass through glutaraldehyde. Higher activity in the present study can be assigned to the difference in the nature of the polymeric supports and method of enzyme immobilization.

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

2.5.2 Optimization of coupling conditions

Different coupling conditions and techniques were optimized for the preparation of immobilized ß-galactosidase and are described below :

(a) Effect of type and concentration of crosslinking reagent

The amount of activating reagents used in the reaction significantly influences the amount of coupled enzyme and hence its activity. The results obtained in the study of effect of glutaraldehyde concentration for the activation of chitosan beads show maximum activity upto 4-8% of its concentration and then it decreases, whereas p-benzoquinone and p-tolyl sulphonyl chloride activation for PVA-F and SRF supports require 125 - 375 mg/g and 500-625 mg/g concentrations respectively. The results obtained from the effect

Table 2.3 Effect of various activation methods on immobilization of ß-galactosidase

Methods	SRF			(PVA - F)			Chitosan		
	Protein coupled (u/g)	Active protein (u/g)	Retention of activity (%)	Protein coupled (u/g)	Active protein (u/g)	Retention of activity (%)	Protein coupled (u/g)	Active protein (u/g)	Retention of activity (%)
p-Benzoquinone	6.4	1.0	15	6.4	6.4	100	-	-	-
Cynuric chloride	6.4	0.9	14	6.4	2.8	43	-	-	-
p-tolyl sulphonyl chloride	6.4	1.8	28	6.4	1.7	26	-	-	-
Glutaraldehyde	-	-	-	-	-	-	9.7	9.7	100

Time 19h at 278° K in citrate-phosphate buffer: 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 i.e. 1.9 u.

of concetration of crosslinking reagents are given in Fig 2.3. It is observed that with increasing concentration of crosslinking agent extent of coupled protein remains constant but activity of coupled protein increases only upto certain level and then decreases. A decrease in enzyme activity may be due to denaturation of enzyme with higher concentration of activator.

(b) Effect of pH

The effect of pH of the coupling medium on the extent of immobilization of ß-galactosidase was studied in the pH range 2 to 8 using 0.2 M citrate-phosphate buffer. From the results, it was observed that maximum coupling and retention of enzyme activity was exhibited at pH 4.5 to 6.5, 3.5 to 4.5 and 6.5 to 7.5 respectively for Chitosan, PVA-F and SRF-supports. Hence immobilization of ß-galactosidase was carried out within this pH ranges for all the supports. Chitosan shows relatively broader pH dependence.

(c) Effect of coupling time

The effect of time on the extent of immobilization of ß-galactosidase was studied for various time intervals of 30 min to 18 h. The bound protein and the active protein on the support was determined as described earlier. The results obtained are given in Fig 2.4. It was observed that within 2-4 h almost quantitative coupling of ß-galactosidase takes place with all the three supports indicating the fast coupling reactions with the activated supports.

2.5.3 Comparative account of free and immobilized ß-galactosidase

Various physicochemical properties such as pH, temperature, storage, kinetics and solvent dependence of enzyme activity of the immobilized and free ß-galactosidase have been studied.

(a) pH profile

Enzyme activity depends upon the microenvironment in which enzyme is acting. On immobilization microenvironment of enzyme changes due to the coupling of enzyme with support leading to the difference in the pH activity profile. Hence it is necessary to study the optimum pH for the free and immobilized enzyme activity.

In the present study Fig 2.5 shows the pH activity profile for the free ß-galactosidase and that immobilized on the polymeric supports under study. In case of E-SRF enzyme activity is almost independent of pH in the range pH 3-8 but % retention of enzyme activity was only 28%. E-PVA-F shows pH dependence but the activity retention

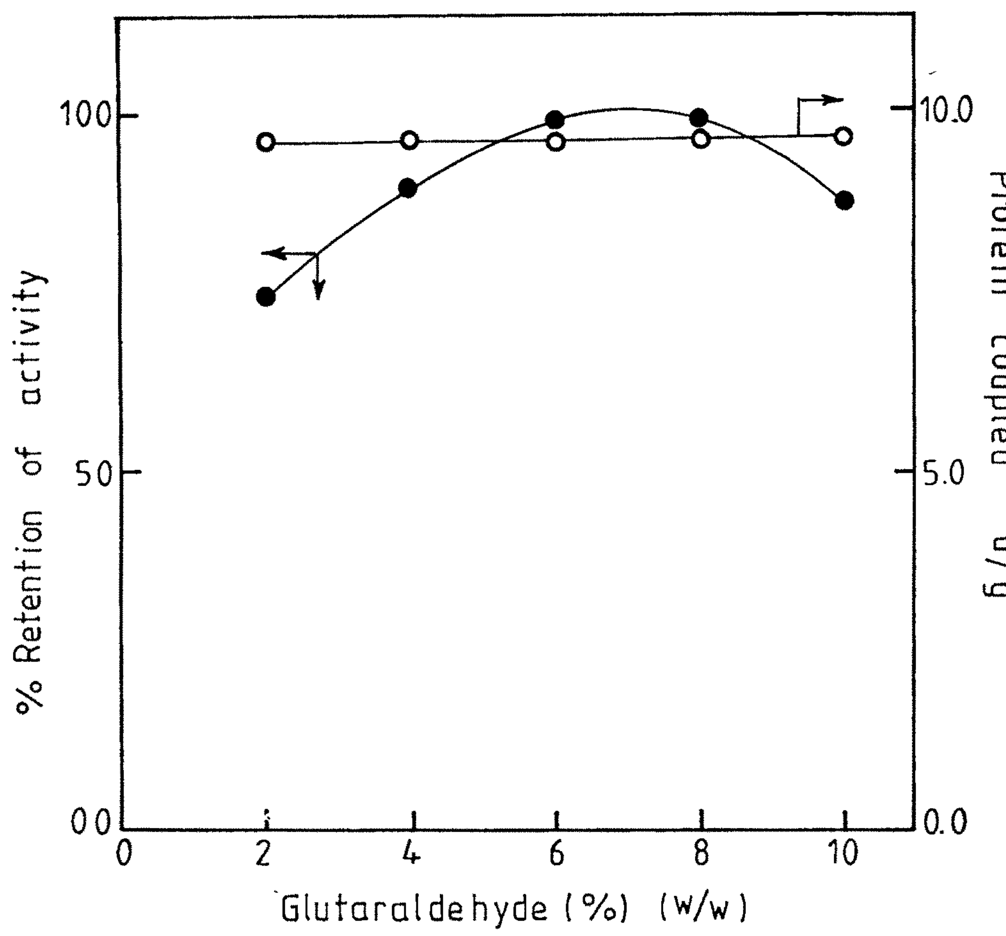


Fig 2.3.a Effect of concentration of glutaraldehyde
Chitosan 0.2 g support, Time 19 h at 278° K, pH 4.5, Enzyme 0.7 mg.

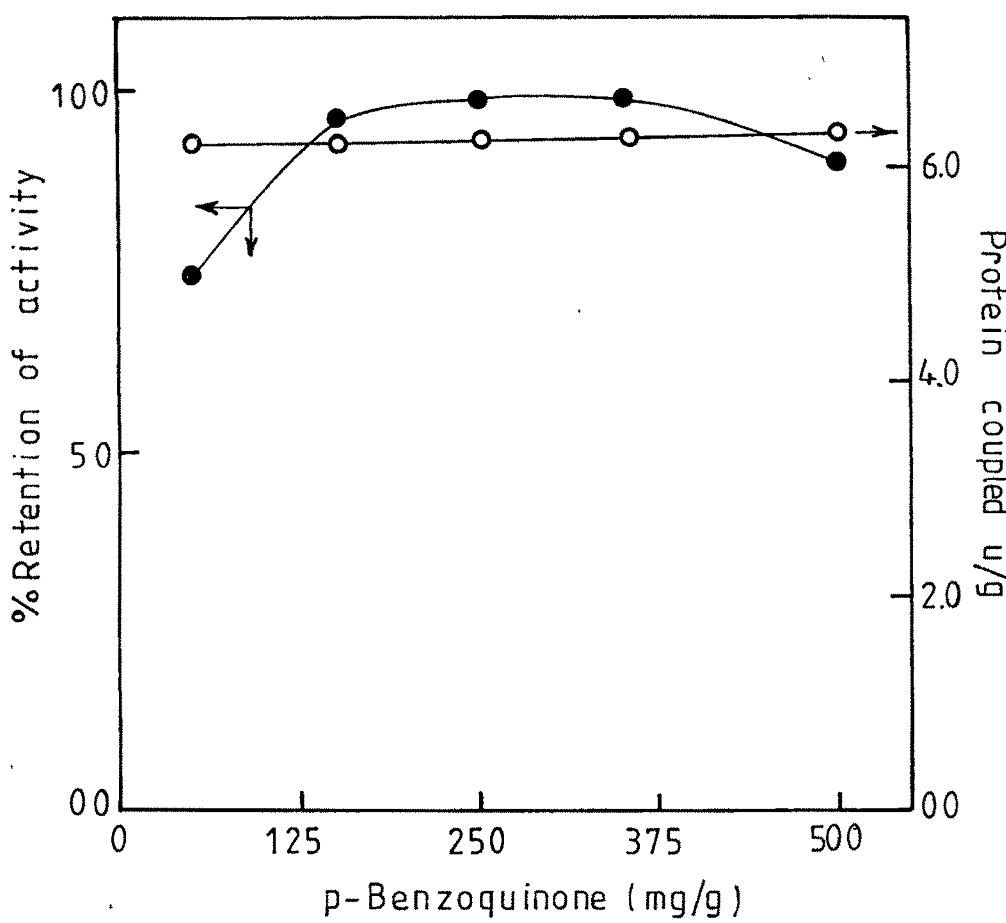


Fig 2.3.b Effect of concentration of p-benzoquinone
PVA-F 0.3 g, Time 19 h at 278° K in, pH 4.5, Enzyme 0.7 mg.

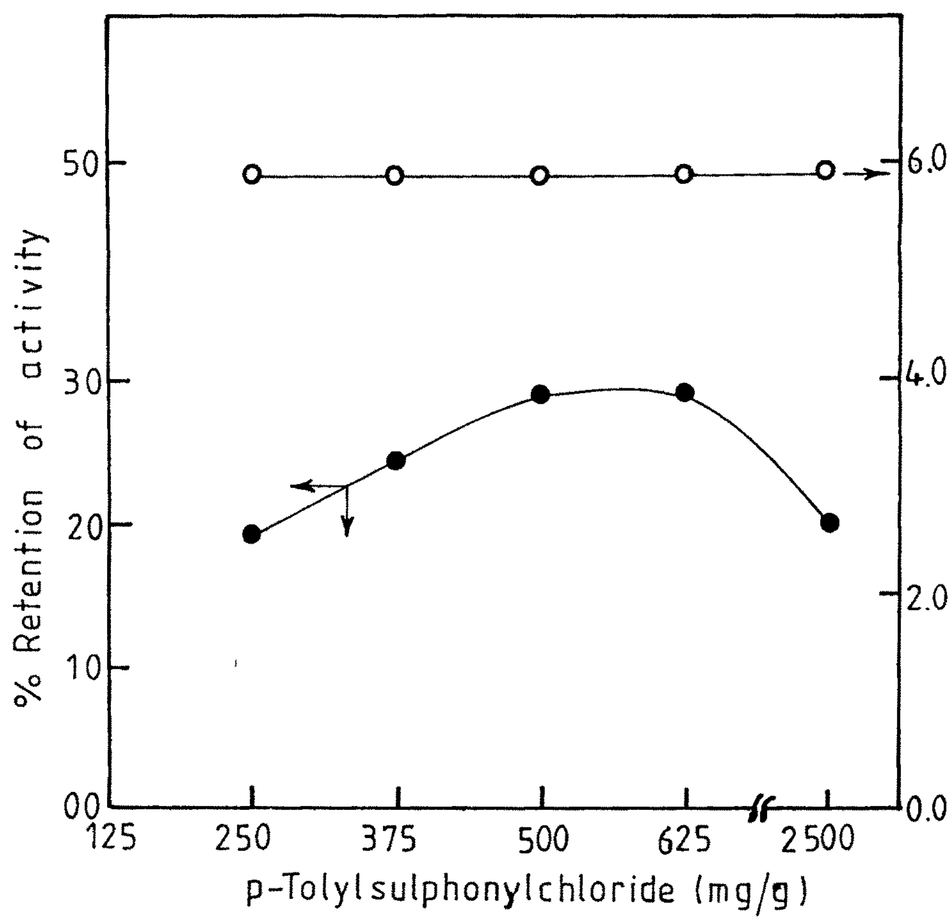


Fig 2.3.c Effect of concentration of p-tolylsulphonylchloride
SRF 0.3 g, Time 19 h at 278° K , pH 4.5, Enzyme 0.7 mg.

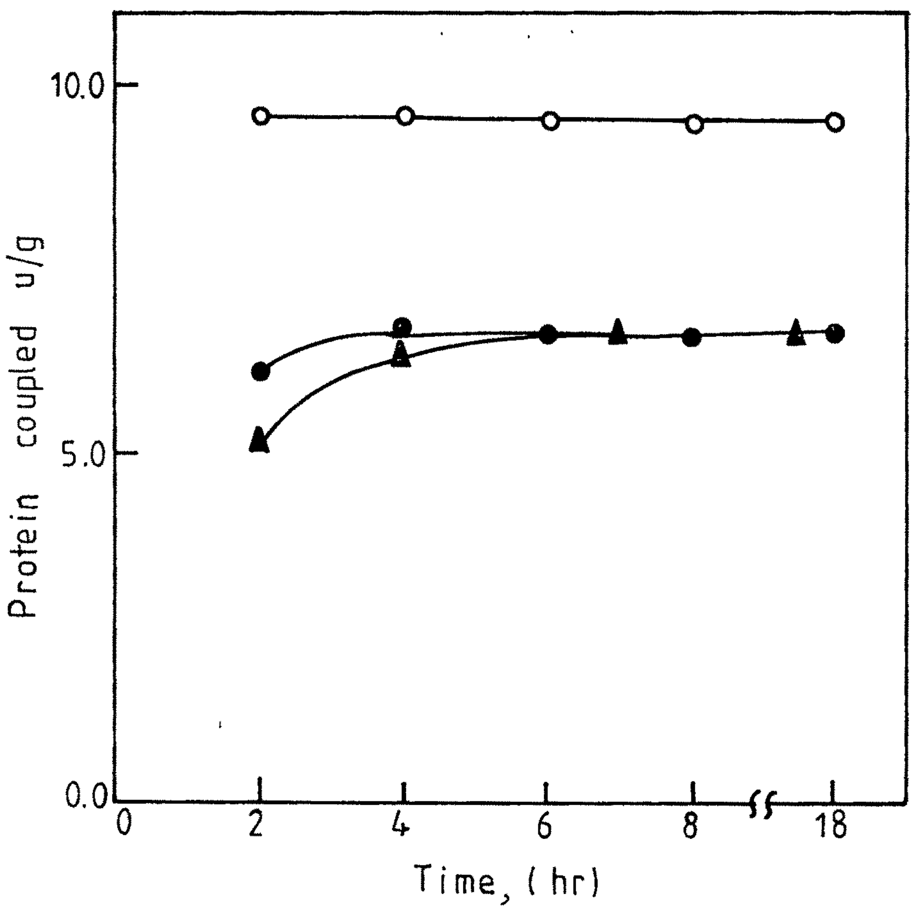


Fig 2.4.a Effect of time on protein coupled
(○) Chitosan 0.2 g, (●) PVA-F 0.3 g (▲) SRF 0.3 g, Time 19 h at 278° K , pH 4.5, Enzyme 0.7 mg

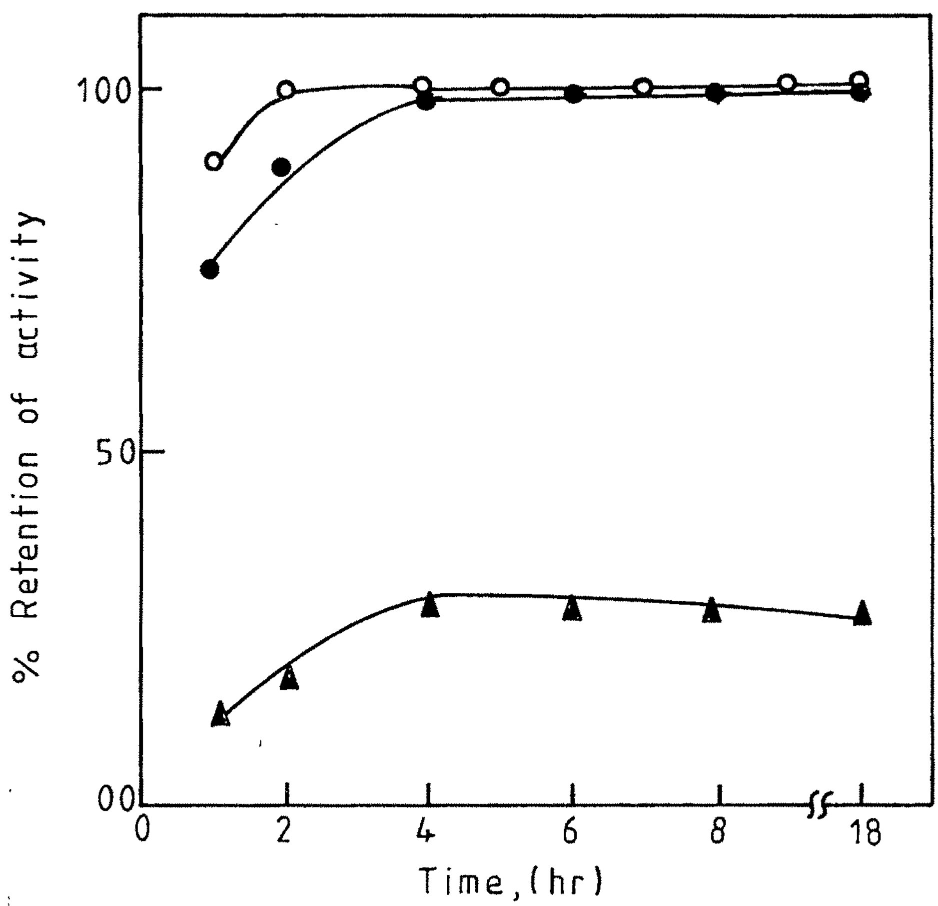


Fig 2.4.b Effect of time on percentage retention of coupled protein activity
(○) Chitosan 0.2 g, (●) PVA-F 0.3 g, (▲) SRF 0.3 g, Time 19 h at 278° K , pH 4.5, Enzyme 0.7 mg

was 100% between pH 3.5 to 4.5, whereas E-Chitosan system retained its activity quantitatively with pH 4-6. Free ß-galactosidase activity shows high pH dependence. The variation of enzyme activity and stability at different pH depends on the nature of the polymeric support used for the immobilization which ultimately affects the microenvironment.

(b) Temperature profile

Effect of temperature on enzyme activity is most important study for the enzymatic applications. In this study ß-galactosidase immobilized on all the three polymeric supports, shows high retention of enzyme activity at higher temperatures when compared with the free enzyme (Fig 2.6). Higher stability and retention of activity of the systems may be due to the covalent fixation of ß-galactosidase on polymeric supports. The spacers also act as arms keeping the enzyme molecules away from the support and freely moving into the substrate solution.

Activation energy data for soluble and insolubilized enzyme is most important parameter for their applications. From temperature study, activation energies for the free and immobilized ß-galactosidase were calculated by using Arrhenius equation as described earlier in Section 2.4.6.b and using a plot of log V_{max} vs 1/T. The results obtained are given in Table 2.4. The correlation coefficient for the plots were within 0.95 to 0.98 range. The observed decrease in the apparent activation energy for immobilized enzyme confirms pore diffusion control of the process rather than kinetic control [118].

(c) Temperature stability and thermodeactivation

In most immobilized enzyme preparations particularly in covalently bound enzymes, enzyme activity is more resistant against heat and denaturing agent than that of soluble form. Temperature stability studies were carried out for free and immobilized ß-galactosidase at temperature 65°C for a longer incubation periods. The results obtained are given in Fig 2.7. It was observed that free enzyme loses its activity (40%) after 60 min incubation at 65°C. Whereas E-Chitosan and E-PVA-F systems retained the activity over 5 h incubation at 65°C indicating the improved thermal stability of the immobilized systems. However, E-SRF system showed a decrease in activity to 30% after the incubation and thereafter showed stabilization of enzyme activity even upto 5 h incubation time. From the study thermodeactivation constant (k_d) was calculated as described earlier and the results are tabulated in Table 2.4. An overall decrease in thermodeactivation was observed on immobilization, particularly for Chitosan and PVA-F systems it was reduced by a factor >10.

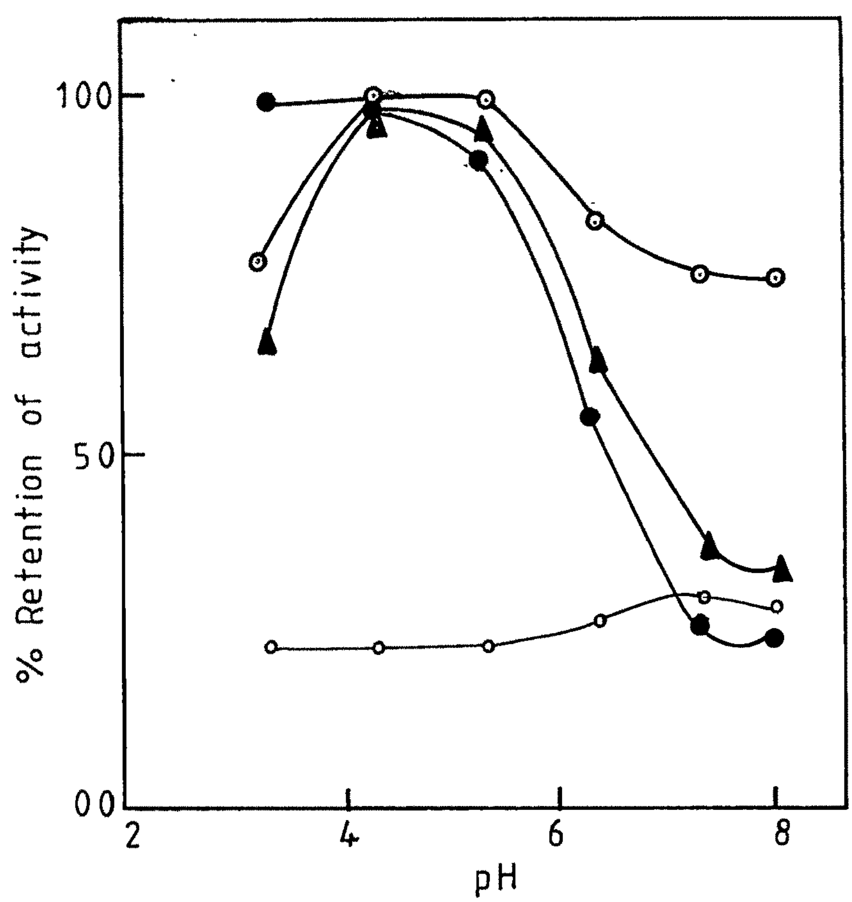


Fig 2.5 pH activity profile for free and immobilized ß-galactosidase
(⊙) ß-galactosidase-Chitosan, (●) ß-galactosidase-PVA-F,
(○) ß-galactosidase-SRF, (▲) ß-galactosidase

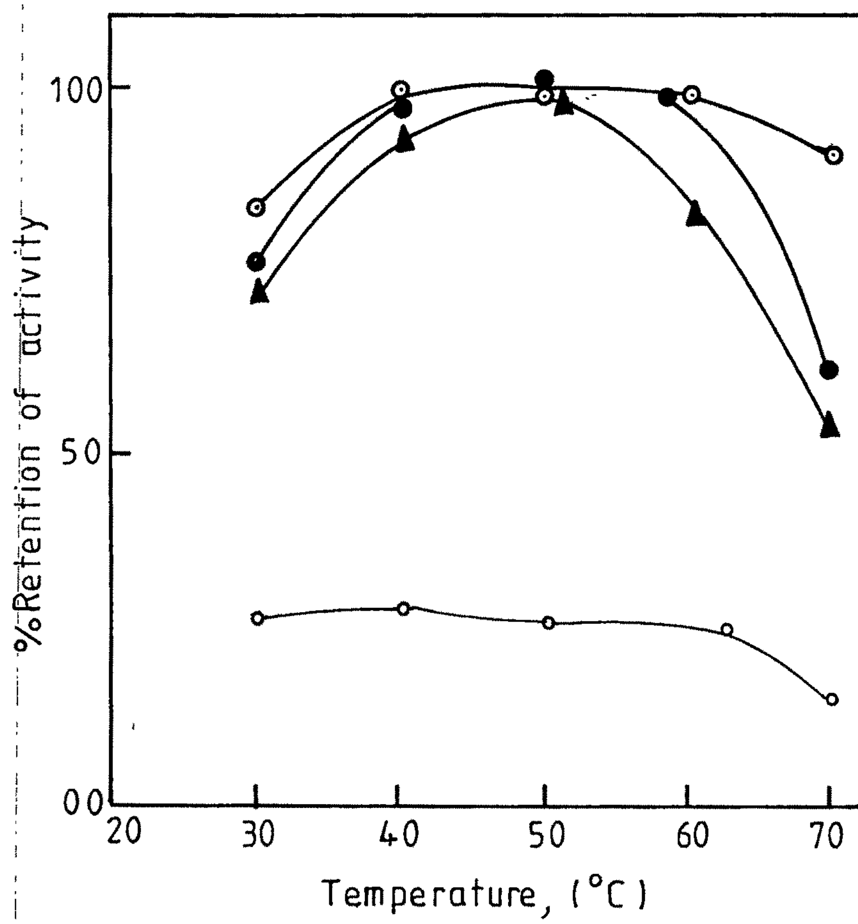


Fig 2.6 Temperature activity profile for free and immobilized ß-galactosidase
(◉) ß-galactosidase-Chitosan, (●) ß-galactosidase-PVA-F,
(○) ß-galactosidase-SRF, (▲) ß-galactosidase

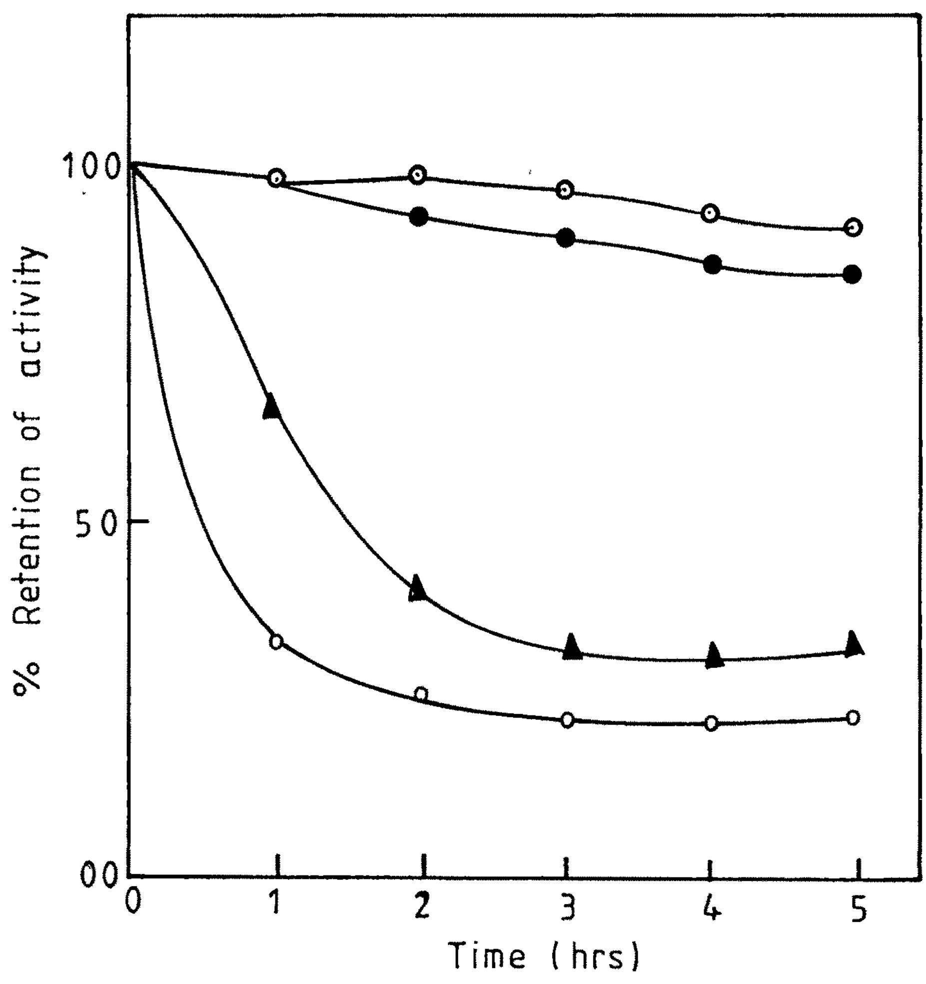


Fig 2.7 Kinetics of thermaldeactivation at 65°C on ONPG hydrolysis at pH 4.5
(⊙) ß-galactosidase-Chitosan, (●) ß-galactosidase-PVA-F,
(○) ß-galactosidase-SRF, (▲) ß-galactosidase

Table 2.4 Kinetics and thermodynamic parameters of the immobilized and free ß-galactosidase in batch process using ONPG substrate.

Sample	Michaeli's constant (K_m) [mM]	Maximum reaction velocity (V_{max}) [mM min^{-1}]	Thermodeactivation constant (K_d) [min^{-1}]	Activation energy (ΔE_a) [Kcal mol^{-1}]
ß-Galactosidase-Chitosan	6.3	3.0×10^{-2}	5.6×10^{-4}	3.6
ß-Galactosidase-PVA-F	5.0	4.0×10^{-2}	7.1×10^{-4}	3.5
ß-Galactosidase	6.3	5.7×10^{-2}	6.8×10^{-3}	4.1

Table 2.5 Energy of activation (ΔE_a) of free and immobilized ß-galactosidase for lactose hydrolysis in fixed bed reactor.

Systems	Substrate	Energy of activation ΔE_a [Kcal mole^{-1}]	Correlation coefficient (r) *
ß-Galactosidase-Chitosan	Lactose	1.8	0.983
	Milkwhey	2.4	0.998
ß-Galactosidase-PVA-F	Lactose	1.5	0.999
	Milkwhey	2.2	0.960
ß-Galactosidase	Lactose	2.7	0.970
	Milkwhey	2.5	0.962

* for 5 determinations

(d) Kinetic behaviour

The rate of hydrolysis in all enzymatic reactions is expected to exhibit the first order kinetics. The initial reaction rates for free and immobilized ß-galactosidase were determined at different concentrations of substrate ONPG ranging from 0.4 to 2 mM. The Michaeli's constant (K_m) and maximum reaction velocity (V_{max}) were calculated from Lineweaver-Burk plots of 1/v vs 1/s (Fig 2.8), where v = velocity of the reaction and s = substrate concentration, the K_m and V_{max} were evaluated by extrapolating the plots. The intercept on the X axis corresponds to $-1/K_m$ and the intercept on the Y axis corresponds to $1/V_{max}$. The K_m and V_{max} values for free and immobilized ß-galactosidase systems are given in Table 2.4. Similar K_m values for free and ß-galactosidase immobilized on chitosan show that the microenvironment of both the systems are identical, but decreased V_{max} in case of immobilized system may be due to increased resistance for the substrate diffusion. However, in the case of ß-galactosidase immobilized on PVA-F, lower K_m values were obtained indicating a partially kinetically controlled reactions. 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.

(e) Storage stability

Immobilized ß-galactosidase was stored at room temperature (30°C) in 0.2 M citrate-phosphate buffer of pH 4.5. From the results illustrated in Fig 2.9, it was 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 40% loss in activity after 3 months storage at room temperature, whereas free ß-galactosidase when stored at room temperature showed 50% loss of activity after 90 days storage. Retention of activity was calculated by considering the activity of enzyme just after immobilization using ONPG substrate.

(f) Solvent stability

The stability of immobilized enzymes in organic solvents is of significant importance in organic synthesis[119,120]. However, the reported stabilities of immobilized enzymes in such solvents are highly variable and have been found to be dependent upon the nature of the enzyme itself as well as the type of support and solvents[121]. In the present study stability of free and immobilized ß-galactosidase towards 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 in Fig 2.10 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 towards solvent stability

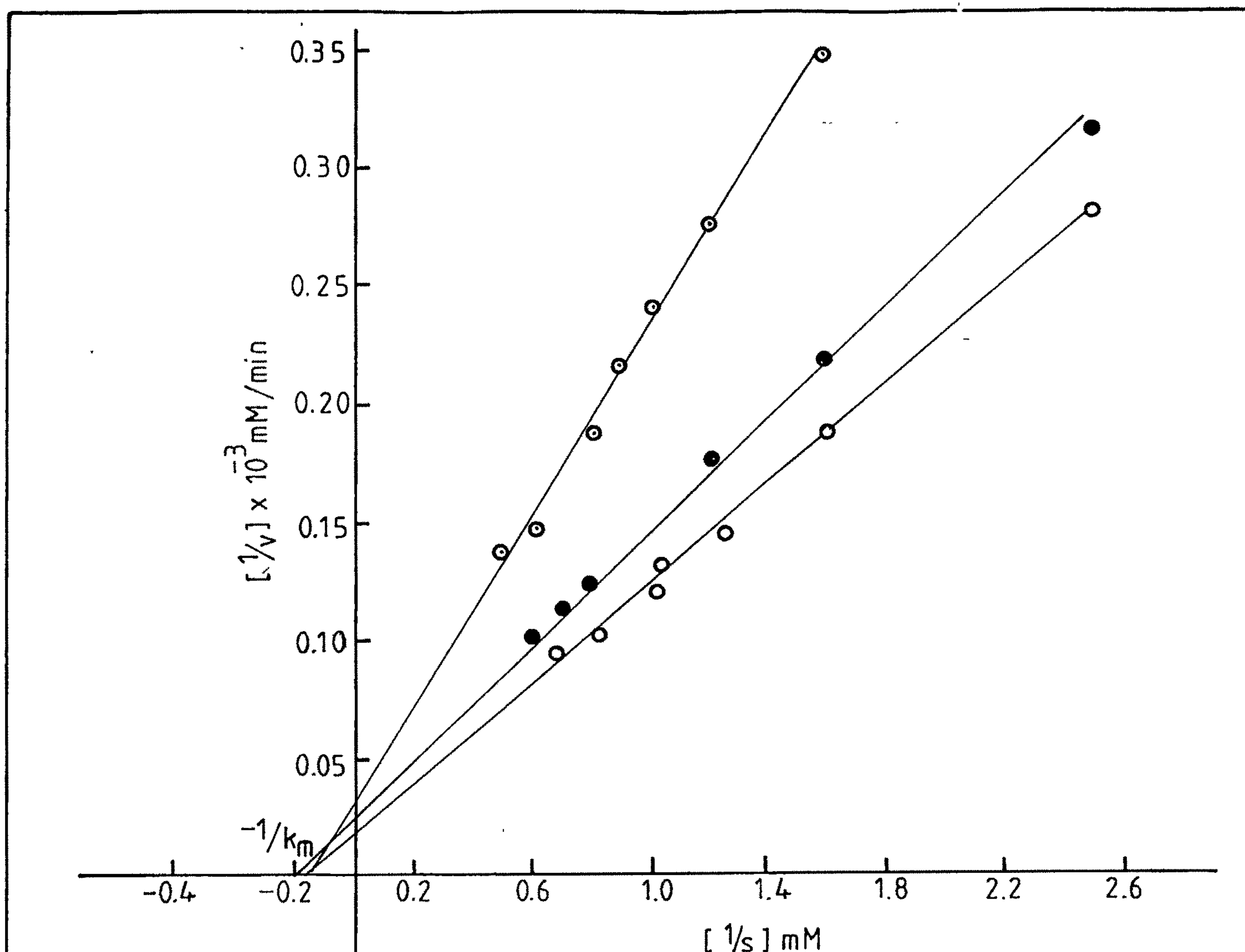


Fig 2.8 Lineweaver-Burk plots for soluble and immobilized ß-galactosidase at 40°C, pH 4.5, for 10 min
(⊙) ß-galactosidase-Chitosan, (●) ß-galactosidase-PVA-F, (○) ß-galactosidase

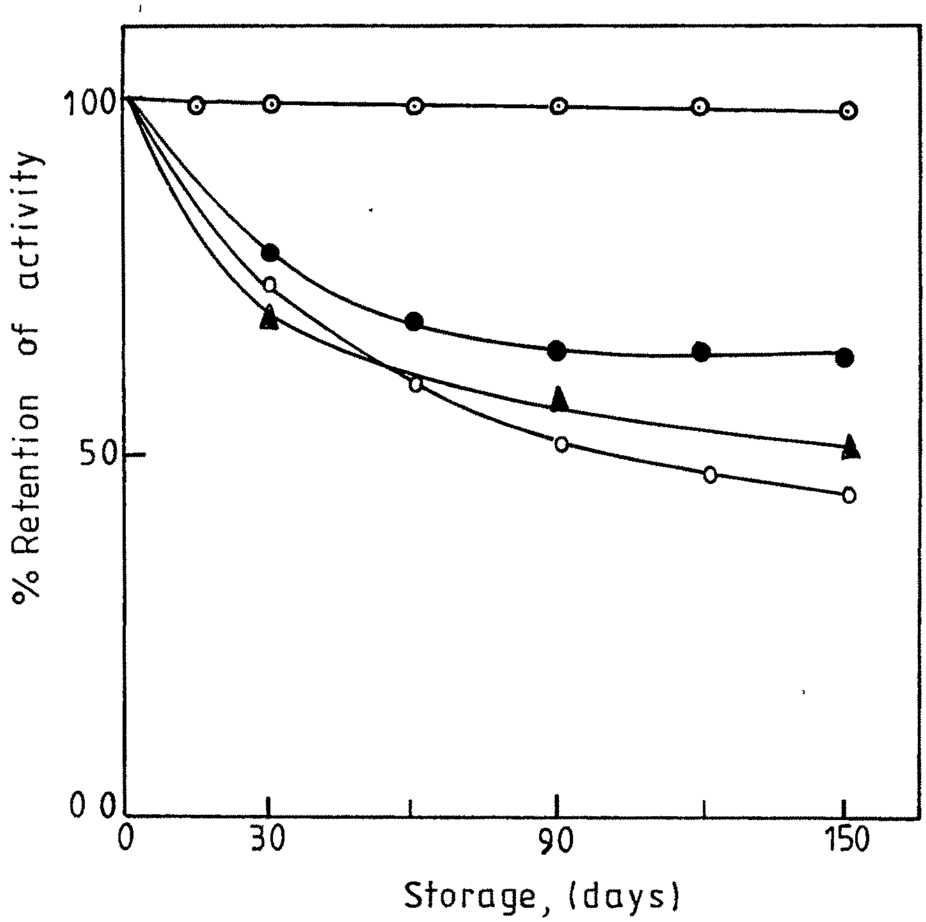


Fig 2.9 Storage stability of enzyme at 40°C in 0.2 M citrate-phosphate buffer (⊙) at pH 4.5 for ß-galactosidase-Chitosan, (●) at pH 3.5 for ß-galactosidase-PVA-F, (▲) at pH 6.5 for ß-galactosidase-SRF, (○) at pH 4.5 for ß-galactosidase

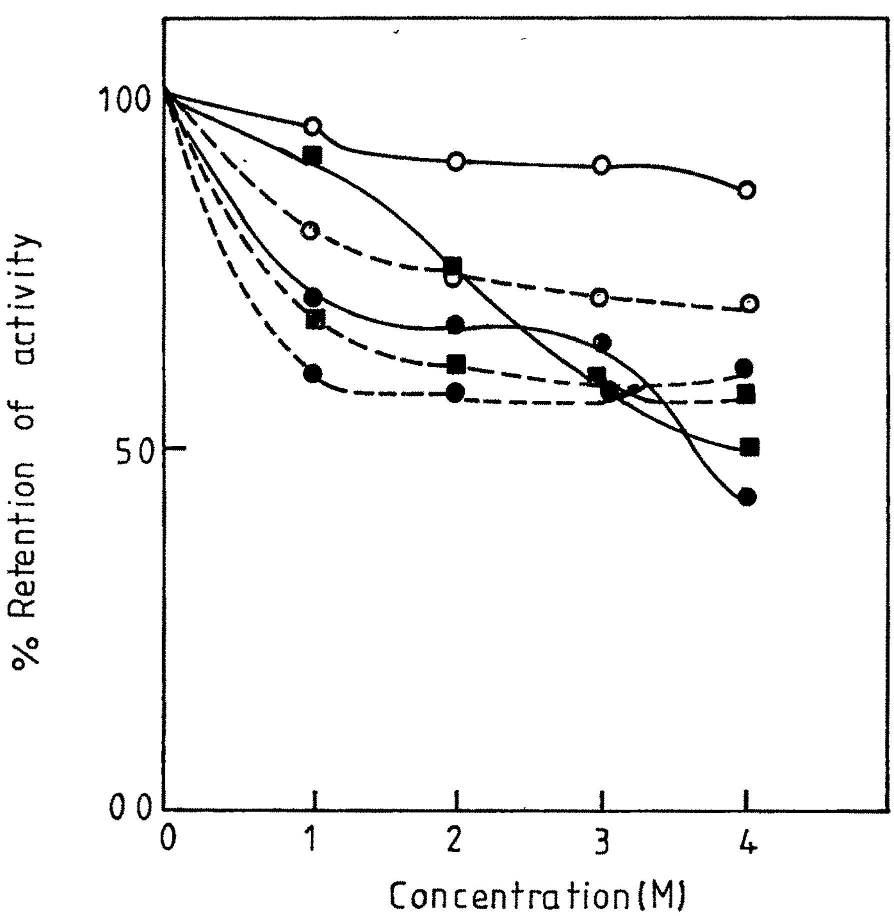


Fig 2.10.a **Effect of solvent on enzyme activity**
(—) Immobilized on PVA-F, (----) Free,
(○) Ethanol, (●) Methanol, (■) Propanol,

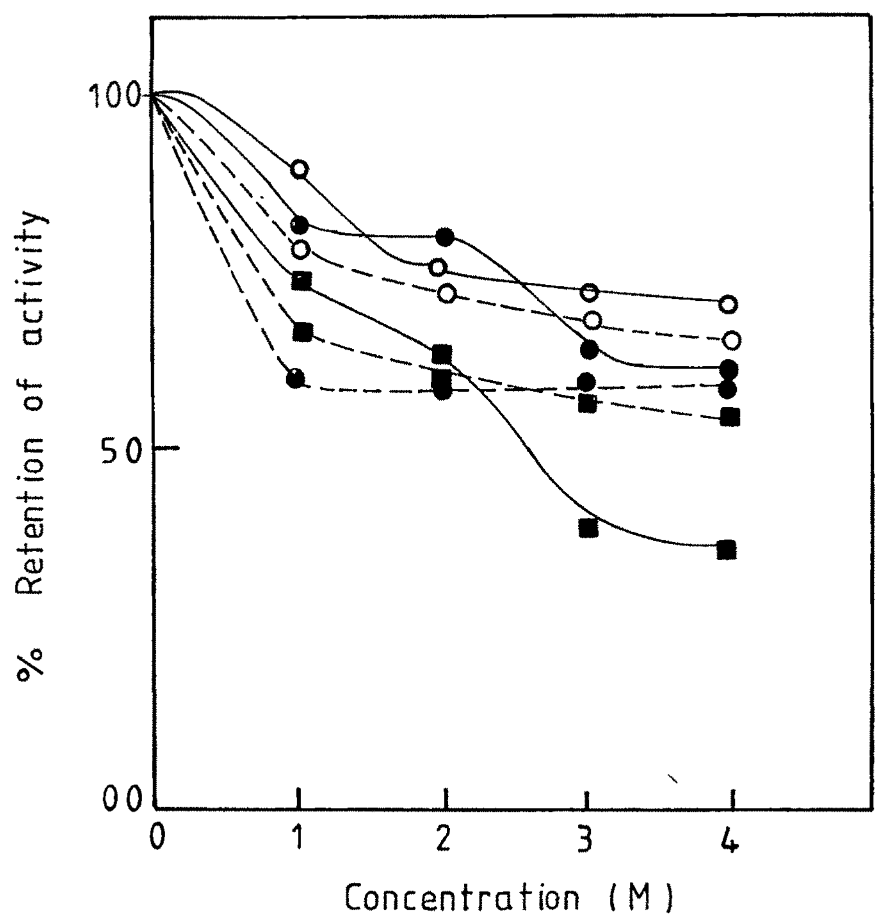


Fig 2.10.b Effect of solvent on enzyme activity
(—) Immobilized on chitosan, (----) Free,
(○) Ethanol, (●) Methanol, (■) Propanol,

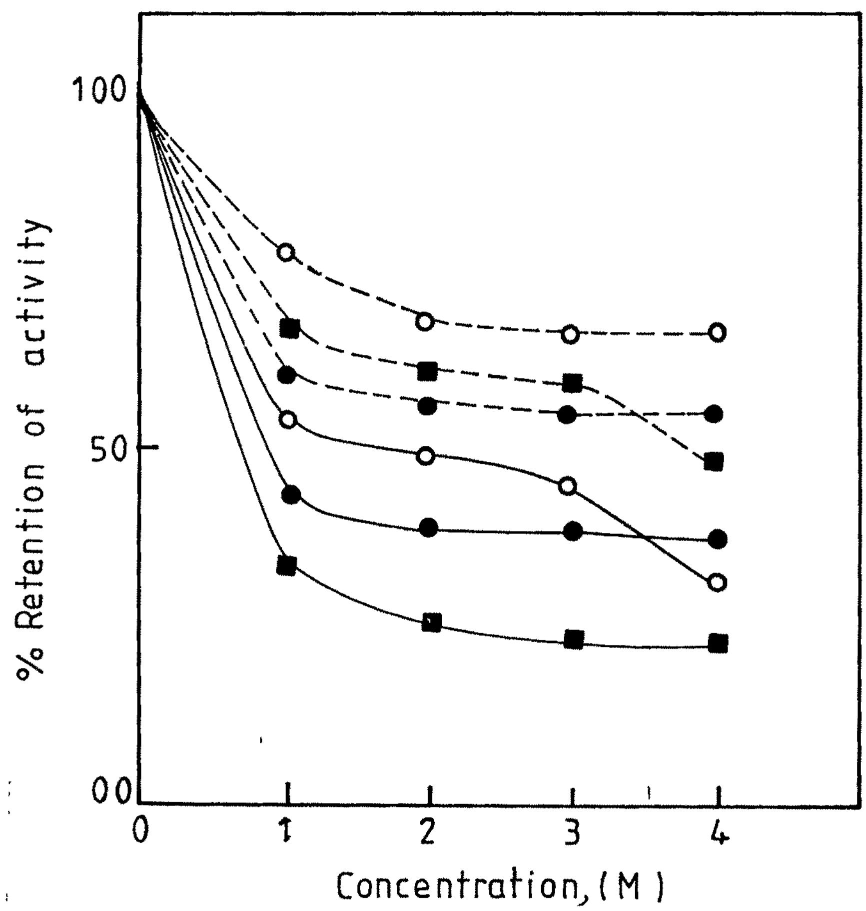


Fig 2.10.c Effect of solvent on enzyme activity
(—) Immobilized on SRF, (----) Free,
(○) Ethanol, (●) Methanol, (■) Propanol,

but it can be broadly said that ß-galactosidase immobilized on chitosan and PVA-F beads show better solvent stability than does the free ß-galactosidase.

(g) Stability towards repeated use

Stability of immobilized ß-galactosidase is very important in application as it is subjected to repeated hydrolysis reactions. Figure 2.11 shows the effect of repeated use on the residual activity of immobilized ß-galactosidase on ONPG hydrolysis. 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 of 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 during the study.

For industrial applications of immobilized enzymes various types of reactors are used. The type of the reactor influences the performances of the enzyme. A fixed bed reactor containing immobilized ß-galactosidase was used for the hydrolysis of lactose and milk whey. The conditions optimized at batch processes were also examined at the column operations and are discussed here.

2.5.4 Hydrolysis of lactose and milk whey using fixed bed reactor containing covalently coupled ß-galactosidase onto chitosan and crosslinked poly(vinyl alcohol)

In the previous section, we have thoroughly investigated the conditions for the immobilizaton of ß-galactosidase and the systems were compared for their activity with free enzyme. It was observed that E-Chitosan and E-PVA-F show maximum coupling and retention of their activity on glutaraldehyde and p-benzoquinone activation. With SRF-good coupling of protein was observed but % retention of activity was not satisfactory. Hence columns packed with E-Chitosan and E-PVA-F were used for the hydrolysis of lactose and milk whey. Higher coupling and retention of activity of ß-galactosidase immobilized on PVA-F through p-benzoquinone spacer may be due to the quinone moities generated during activation processes which may have higher affinity towards the enzyme under study. Similar observation was made by Ponnuchamy and Gupta[122].

(a) Loading capacity and coupling efficiency

In the study of effect of enzyme loading on the operational activity of the reactor, it was observed that with increasing enzyme loading enzyme activity is retained quantitatively upto 138 u/g of support (Fig 2.12). On further increase in the enzyme loading, it was observed that retention of enzyme activity decreases. This may be because the activity

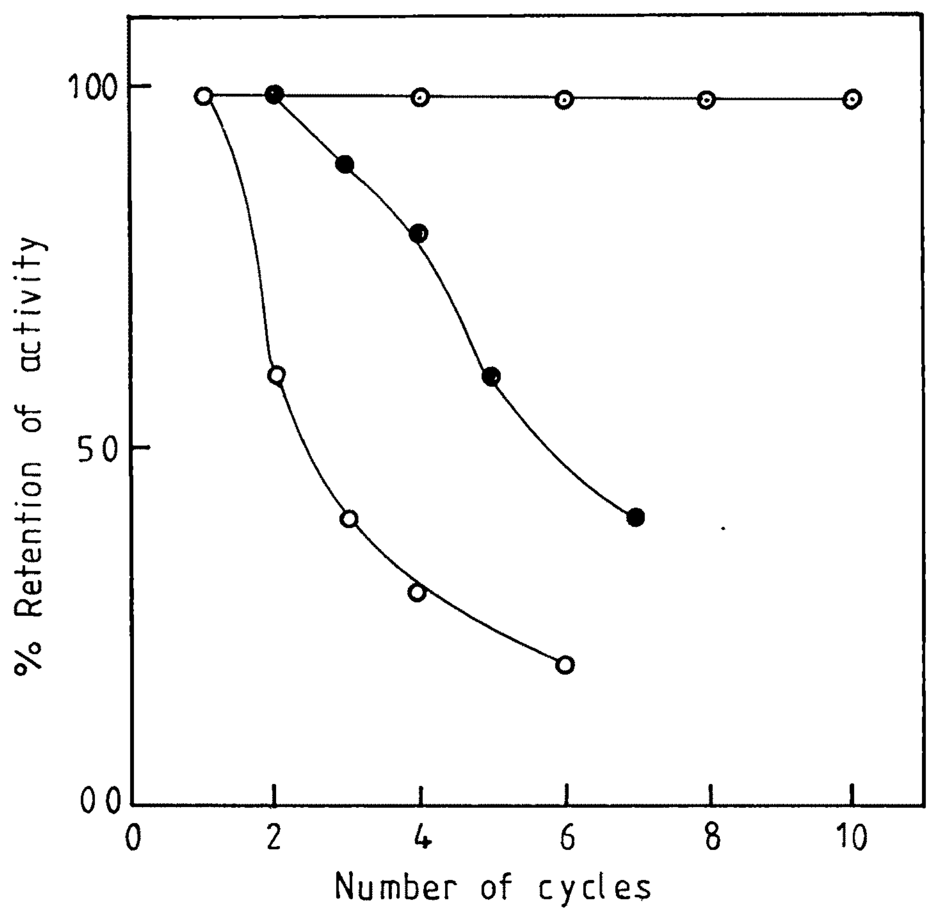


Fig 2.11 Reusability of immobilized ß-galactosidase
(⊙) ß-galactosidase-Chitosan, (●) ß-galactosidase-PVA-F,
(○) ß-galactosidase-SRF

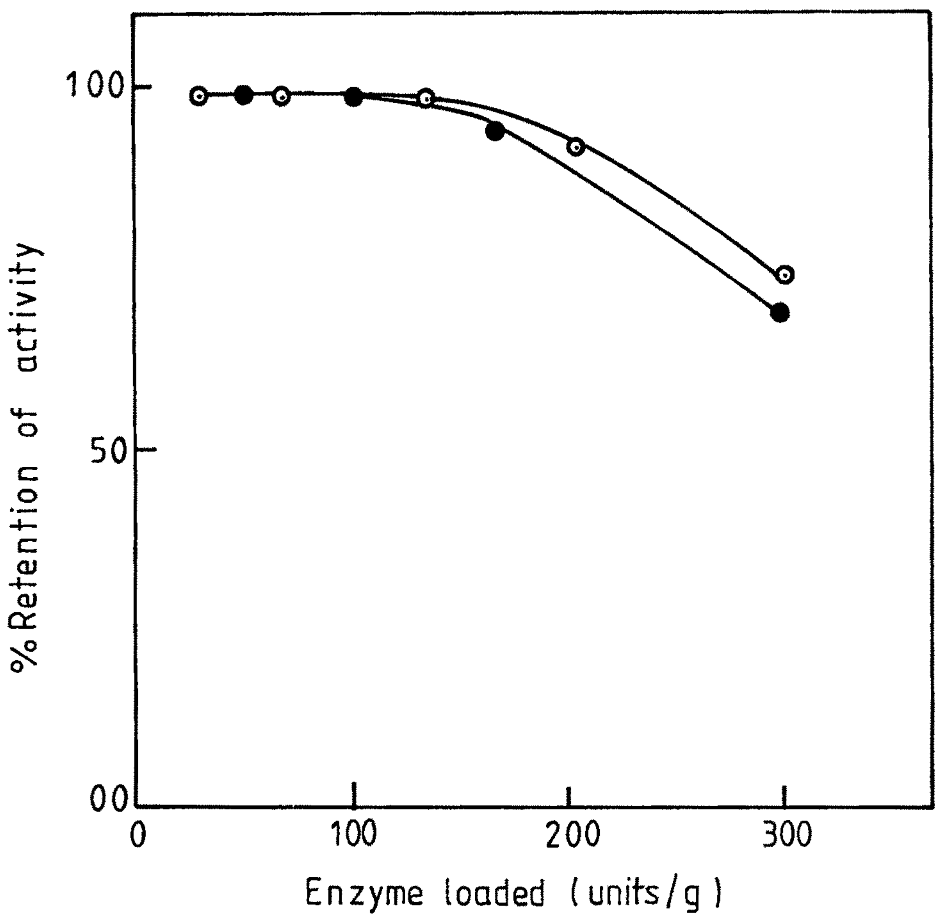


Fig 2.12 Effect of enzyme loading on enzyme activity
(◎) ß-galactosidase-Chitosan, (●) ß-galactosidase-PVA-F

of immobilized enzyme becomes subject to substrate diffusion limitations at higher enzyme loading.

(b) Effect of temperature

The results obtained in the study of effect of temperature on the hydrolysis of lactose and lactose from milk whey by using E-Chitosan and E-PVA-F systems are illustrated in Fig 2.13 and Fig 2.14. From the results (2.13.a and 2.13.b), it is observed that hydrolysis of lactose is temperature dependent and the immobilized enzyme offers maximum lactose conversion at 50-60 °C at pH 5.

Hydrolysis of lactose from milk whey by using E-Chitosan and E-PVA-F in a fixed bed reactor at different temperatures is illustrated in Fig 2.14.a and 2.14.b. It was observed that % hydrolysis is more critical of temperature at lower temperature (40-50°C) than at higher temperature (50-60°C). In the hydrolysis of lactose as well as lactose from milk whey, it was also observed that with increasing flow rate % hydrolysis decreases due to decreased contact time with the immobilized enzyme.

Temperature dependence of chemical reactions is most frequently modelled in terms of the Arrehenius equation as discussed earlier. Energy of activation (ΔEa) was calculated from temperature data and presented in Table 2.5. The interesting observation in the present study is that the activation energy for the hydrolysis of pure lactose is found to be less than that for the hydrolysis of milk whey. This is in fact consistant with the results reported by Hill and Huber[123] and Reithel and Kim[124]. A possible explanation for the results obtained is that bound enzyme is inhibited by trace quantities of divalent cations present in whey. Since the concentration of Ca^{2+} and Mg^{2+} ions in acid whey is considerably high (0.025 M and 0.0034 M respectively) as reported by Freeley *et al*[125] the deleterious effect of these cations may be affecting the results. Another important observation is that of activation energy of the immobilized enzyme is far less than that of the free enzyme. This may be due to the pore diffusion limitation in an immobilized enzyme system as suggested by Pitcher[118] leading to a decrease in the apparent activation energy with increased temperature.

(c) Effect of pH on lactose hydrolysis

Figure 2.15 indicates the effect of pH on the conversion of lactose by the immobilized ß-galactosidase at various temperatures. At all temperatures maximum extent of hydrolysis was observed at pH 5 for both the systems. However, E-PVA-F systems showed more critical behaviour at higher temperatures.

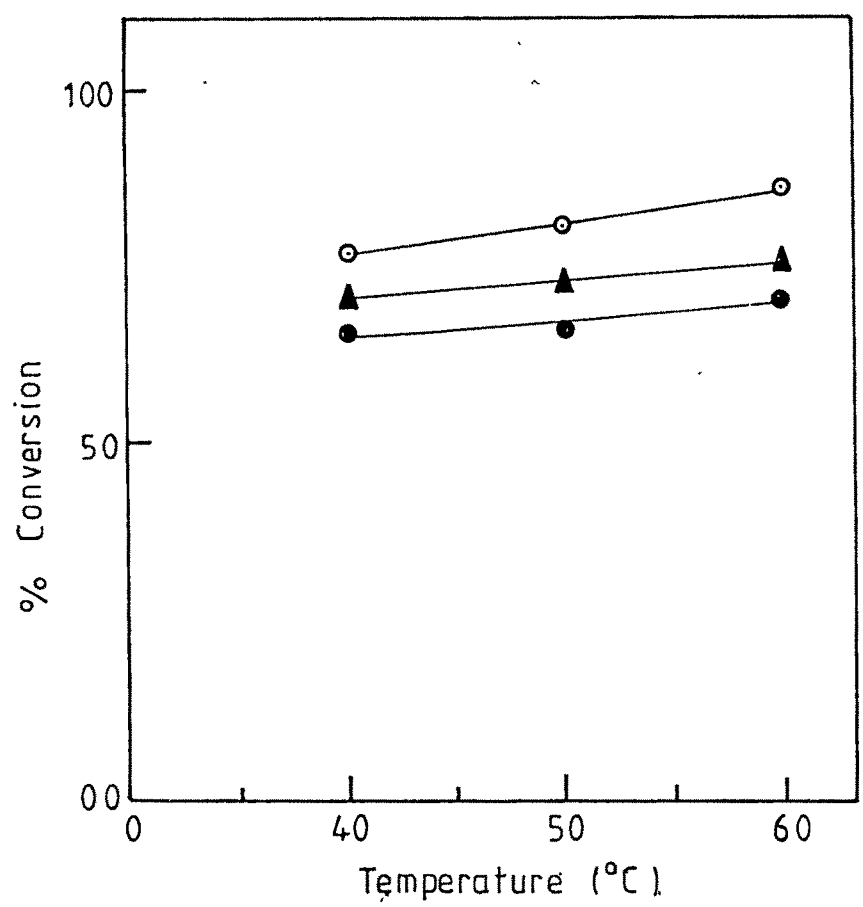


Fig 2.13.a Effect of column temperature on lactose hydrolysis
ß-galactosidase-Chitosan system, lactose 5%,
Flow rates: (⊙) 0.5 ml min^{-1} ,(▲) 1 ml min^{-1},(●) 1.5 ml min^{-1}

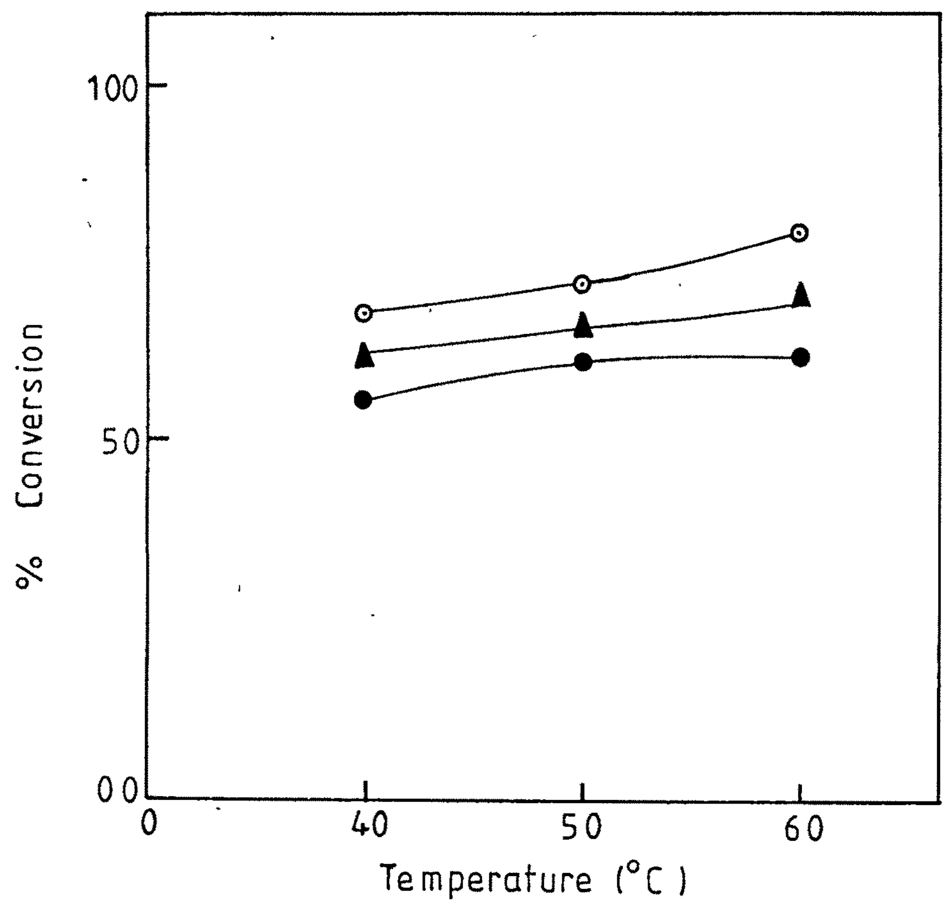


Fig 2.13.b Effect of column temperature on hydrolysis ß-galactosidase-PVA-F system, lactose 5%, Flow rates : (⊙) 0.5 ml min^{-1} ,(▲) 1 ml min^{-1},(●) 1.5 ml min^{-1}

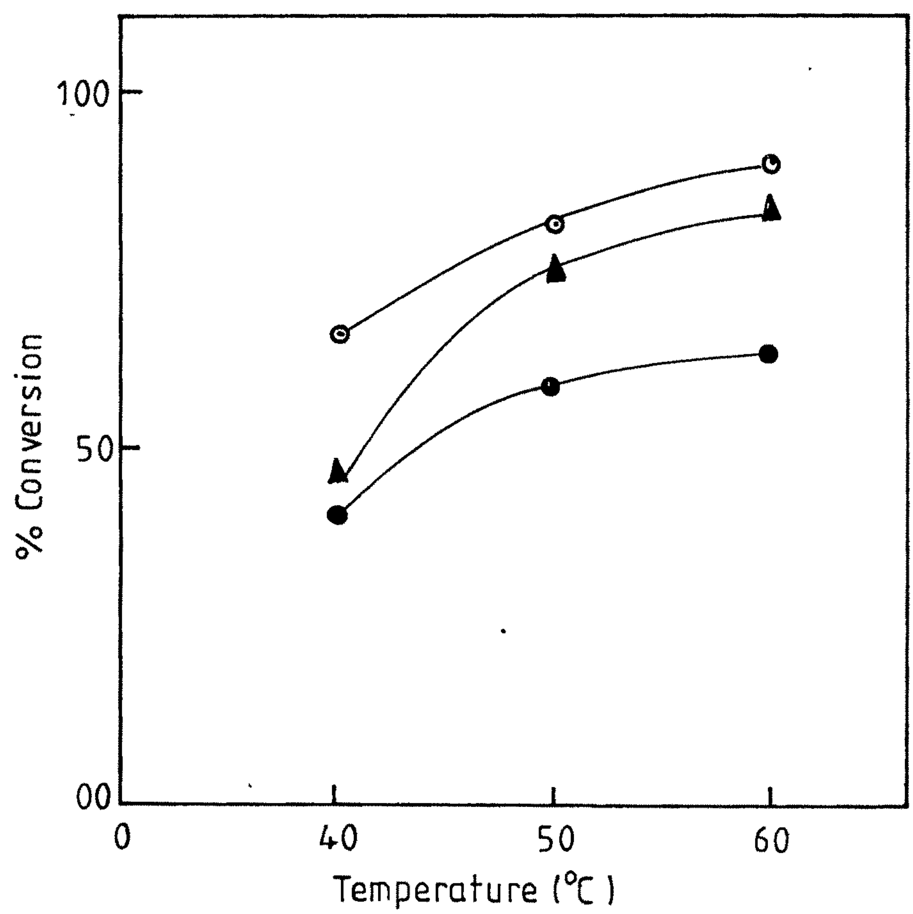


Fig 2.14.a Effect of column temperature on milk whey hydrolysis
ß-galactosidase-Chitosan system,
Flow rates : (◎) 0.5 ml min^{-1}, (▲)1 ml min^{-1}, (●)1.5 ml min^{-1}

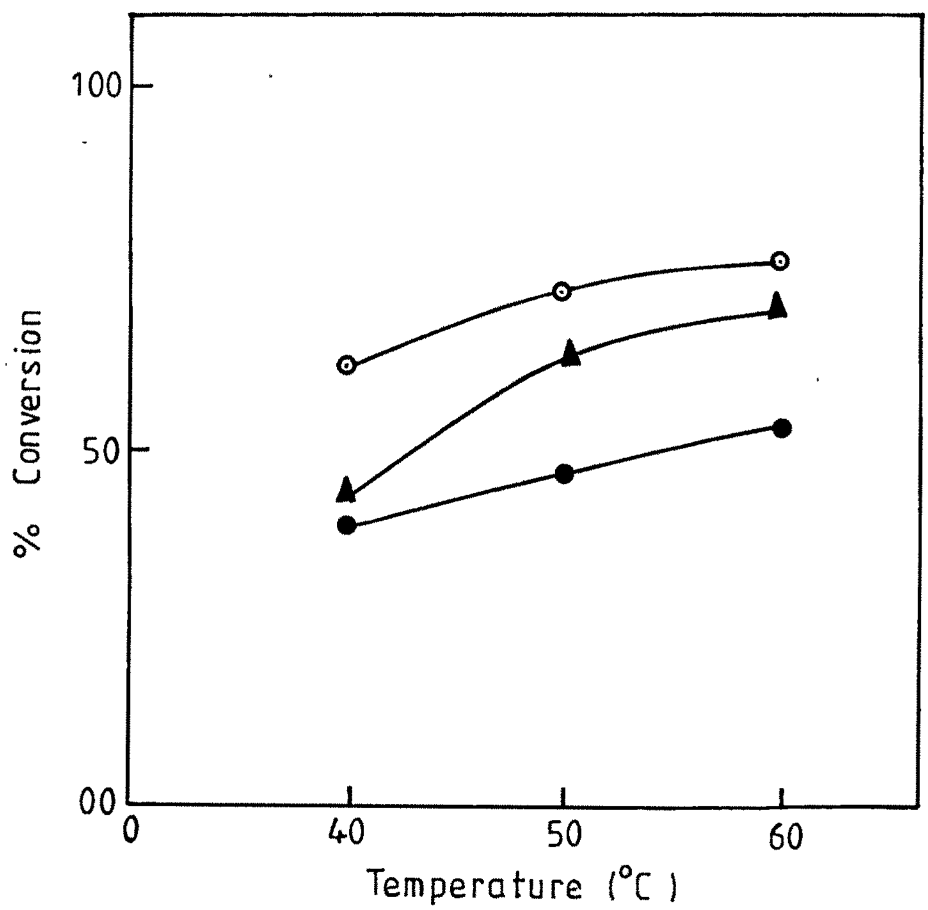


Fig 2.14.b Effect of column temperature on milk whey hydrolysis ß-galactosidase-PVA-F system,
Flow rates : (⊙) 0.5 ml min^{-1}, (▲)1 ml min^{-1}, (●)1.5 ml min^{-1}

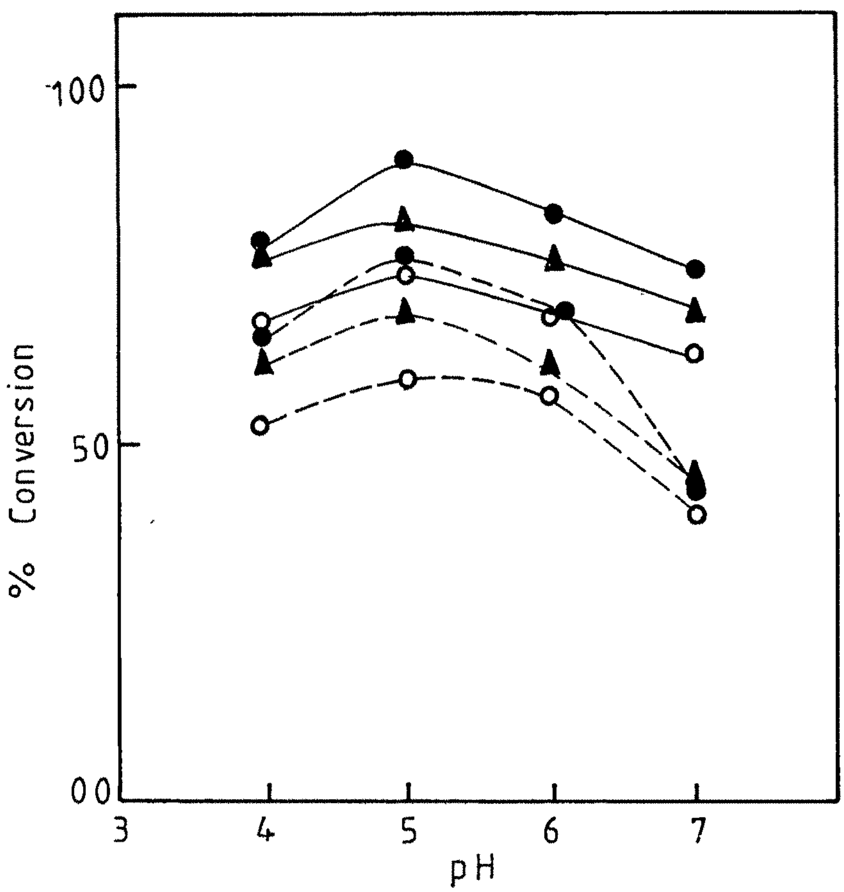


Fig 2.15 Effect of pH on lactose hydrolysis
(—) ß-galactosidase-Chitosan, (----) ß-galactosidase-PVA-F
At (●) 60°C, (▲) 50°C, (○) 40°C

(d) Effect of substrate concentration

Effect of lactose concentration on the extent of hydrolysis was studied by using various concentrations ranging from 0.1 to 5% (0.28 x 10^{-2} mM to 13.88 x 10^{-2} mM) lactose. The results obtained are given in Fig 2.16. It was observed that % hydrolysis goes on increasing with increasing substrate concentration upto 1% lactose concentration. However, further increase in substrate concentration results in the decrease of enzyme activity. This may be due to the increased amount of galactose generated acts as an inhibitor in the enzyme reaction and also the excess formation of some oligosaccharides at higher concentration of substrate retards the rate of hydrolysis[126].

(e) Flow rate

The effect of substrate flow velocity on the efficiency of the immobilized ß-galactosidase was studied in the packed bed column reactor at pH 5 for the hydrolysis of lactose and milk whey at different temperatures. Space time of the reactor was calculated as W/F, where 'W' is the weight of immobilized enzyme and 'F' is the flow rate of substrate. From the results presented in Fig 2.17 and 2.18 it was observed that with increasing flow velocity of the substrate percentage conversion decreases at all temperatures. Although higher flow rates reduce the tendency for column to plug, the enzyme activity is reduced disproportionately. On comparison with the results obtained for the hydrolysis of lactose and milk whey it was observed that hydrolysis of milk whey is more severely affected by the variation in space time of the reactor.

(f) Kinetic behaviour of lactose hydrolysis in reactor

The Michealis menten constant (K_m) and maximum reaction velocity (V_{max}) for ß-galactosidase immobilized on Chitosan and PVA-F for continuous hydrolysis of lactose were calculated from Lineweaver-Burk plots as discussed earlier in Section 2.4.6. The reaction velocity (V_{max}) was measured at different concentrations of lactose, keeping the amount of immobilized enzyme constant (Fig 2.19.a and 2.19.b). From Lineweaver-Burk plots K_m and V_{max} were calculated and the results are tabulated in Table 2.6. It was observed that the observed lower values of K_m for the immobilized systems are due to diffusional limitations of substrate in the system. Earlier K_m and V_{max} were calculated for the free ß-galactosidase using ONPG substrate. However, as milk whey was used as a substrate in column operation the results obtained for the kinetic study of free ß-galactosidase using milk whey substrate are also given in Table 2.6. Decreased rate of hydrolysis was observed in case of milk whey substrate. This is because of very low concentration <2.5% of lactose content in the whey.

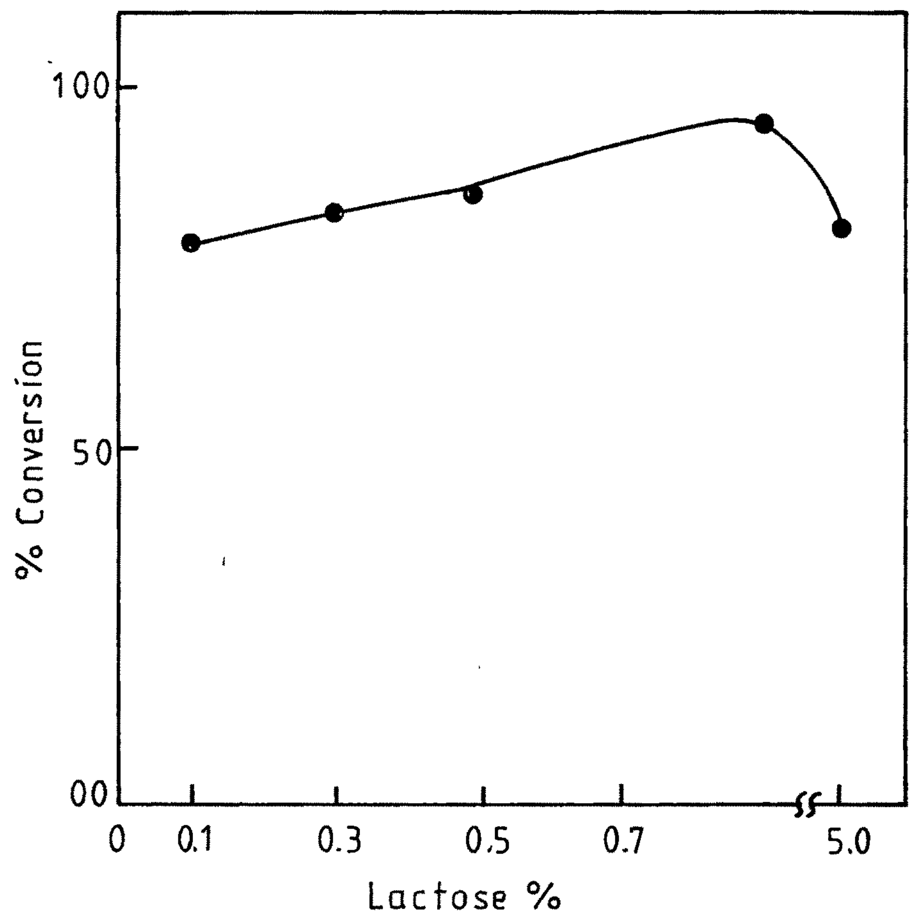


Fig 2.16 Effect of substrate concentration on % hydrolysis ß-galactosidase-Chitosan system
Flow rates: 0.5 ml min^{-1}, Temp. 50°C

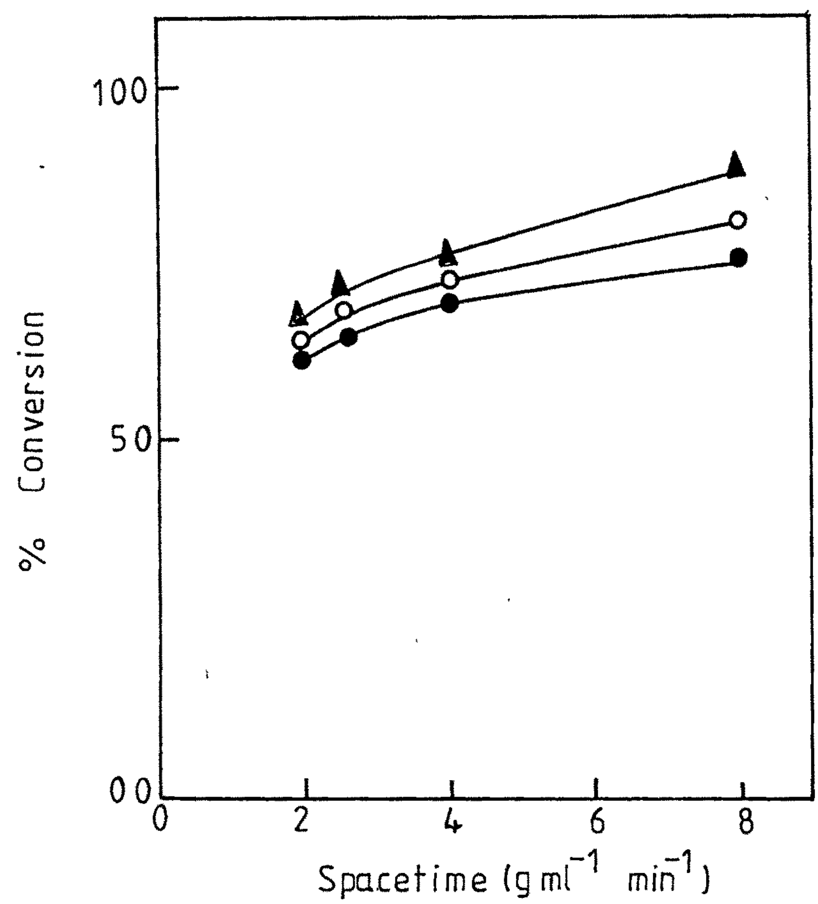


Fig 2.17.a Effect of substrate flow on lactose hydrolysis Column containing ß-galactosidase-Chitosan, At (▲) 60°C, (○) 50°C, (●) 40°C

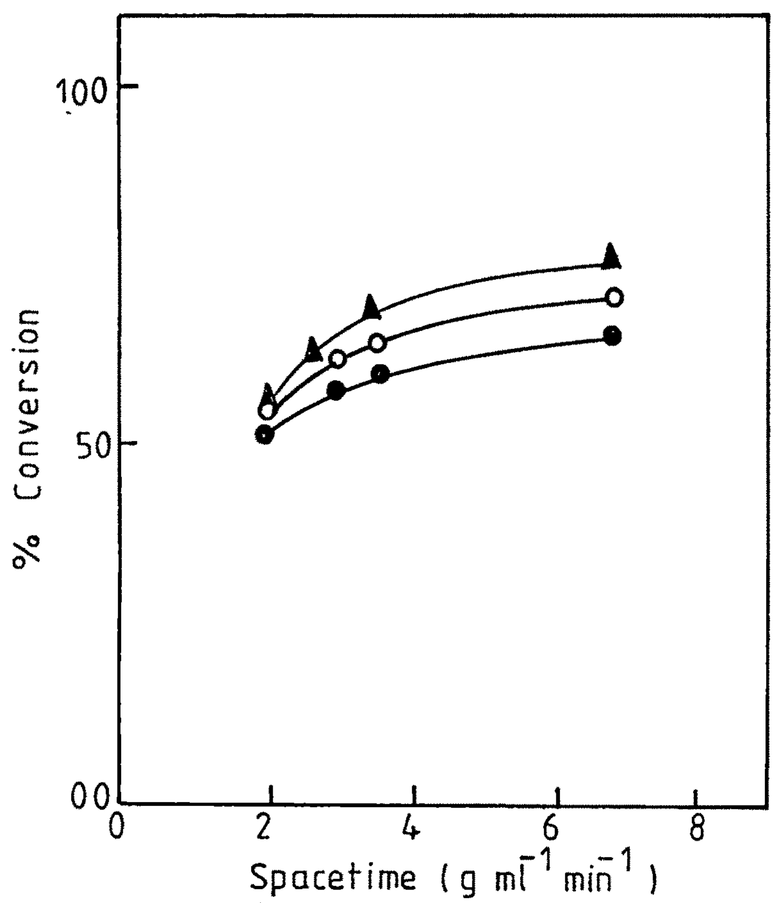


Fig 2.17.b Effect of substrate flow on lactose hydrolysis
Column containing ß-galactosidase-PVA-F,
At (▲) 60°C, (○) 50°C, (●) 40°C

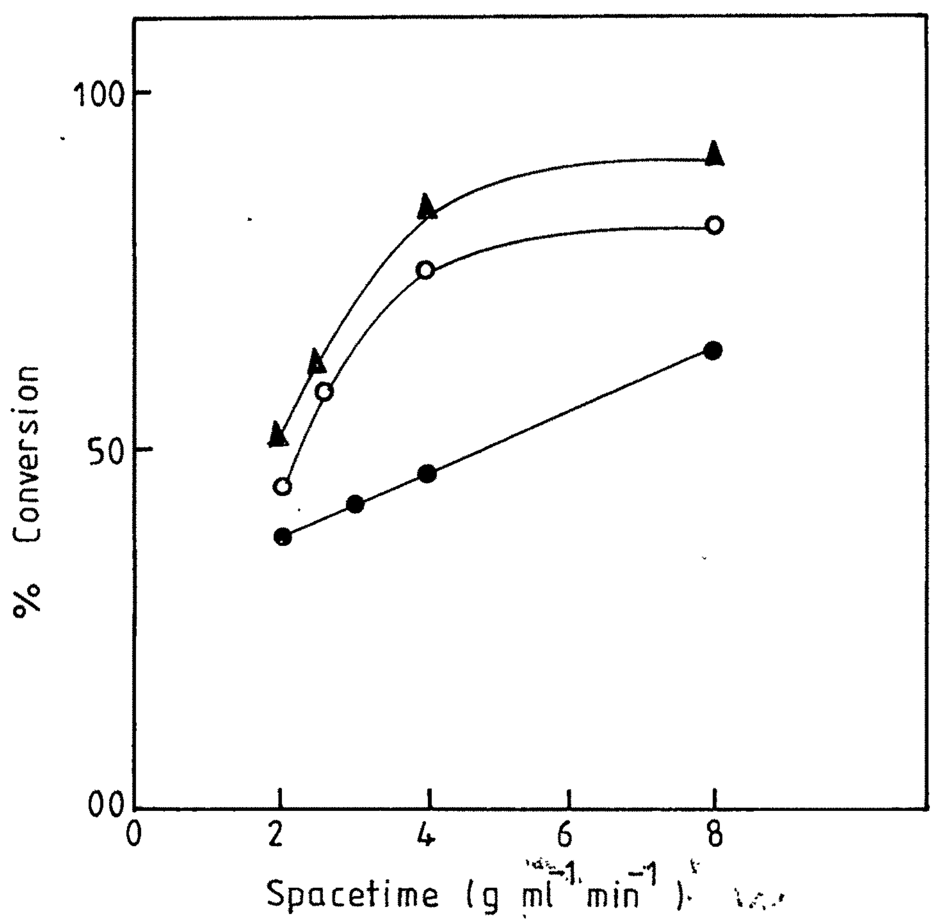


Fig 2.18.a Effect of substrate flow on milk whey hydrolysis
Column containing ß-galactosidase-Chitosan
At (▲) 60°C, (○) 50°C, (●) 40°C

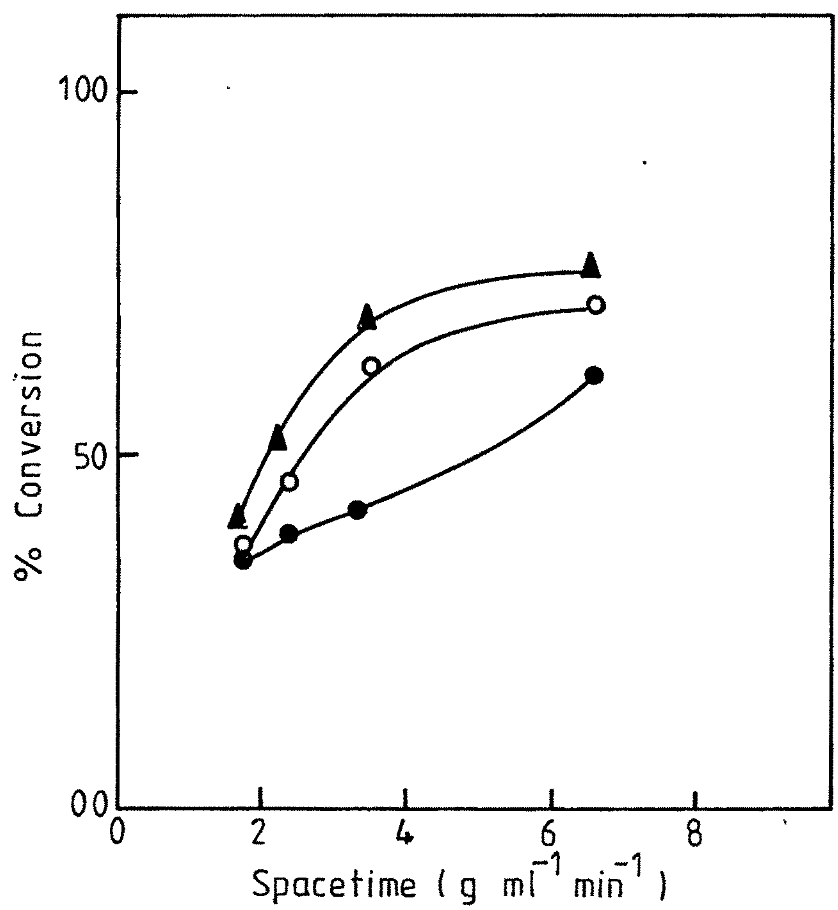


Fig 2.18.b **Effect of substrate flow on milk whey hydrolysis Column containing ß-galactosidase-PVA-F At (▲) 60°C, (○) 50°C, (●) 40°C**

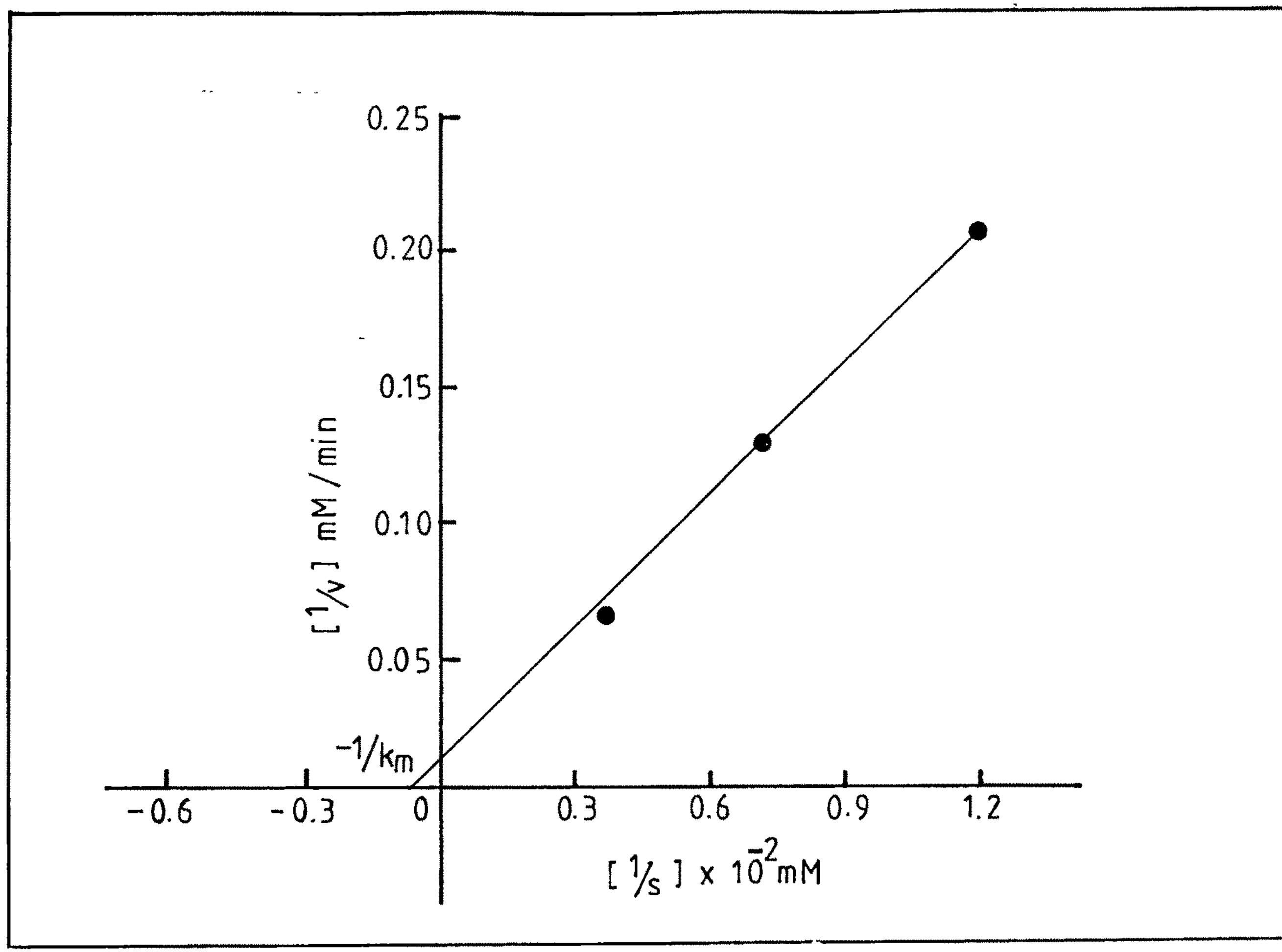


Fig 2.19.a Lineweaver-Burk plots for immobilized ß-galactosidase Reactor containing ß-galactosidase-Chitosan

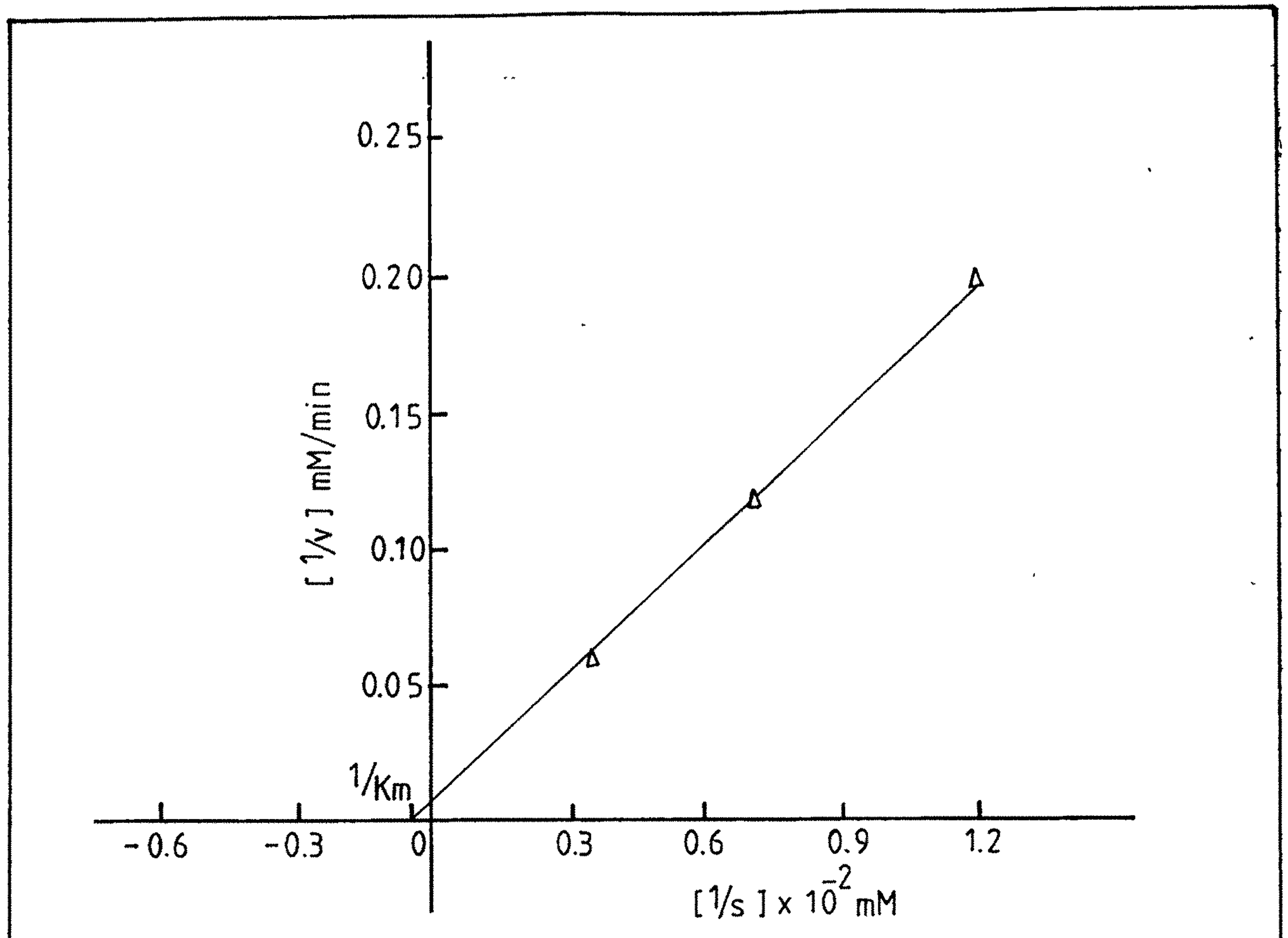


Fig 2.19.b Lineweaver-Burk plots for free ß-galactosidase

Table 2.6 Michaeli's constant (K_m) and maximum reaction velocity (V_{max}) for free and immobilized ß-galactosidase for lactose hydrolysis in column process.

Systems	Temperature [° C]	Michaeli's constant (K_m) [mM]	Maximum Reaction Velocity (V_{max}) [mM min^{-1}]
ß-Galactosidase-Chitosan	40	13.0×10^{-2}	57
	50	12.2×10^{-2}	50
ß-Galactosidase	50	15×10^{-2}	70

Table 2.7 Thermodeactivation rate constant (K_d) and deactivation energy (ΔE_d) for E-chitosan in column process.

System	Temperature [° C]	Deactivation constant (K_d) [min^{-1}]	Deactivation energy (ΔE_d) [Kcal $mole^{-1}$]
ß-Galactosidase-Chitosan	40	9.7×10^{-4}	1.1
	50	10.5×10^{-4}	
	60	10.7×10^{-4}	

(g) Thermal deactivation

The plot of A/Ao vs time, where 'Ao' is the initial activity of the enzyme and 'A' is the enzyme activity after time 't' min of temperature effect (Fig 2.20) indicates the thermal deactivation of the immobilized systems in the column reactor at different temperatures (40-60°C) and various time intervals. It was observed that at higher temperature as the time goes on increasing the activity decreases rapidly whereas at lower temperature the long term operation was possible without considerable deactivation. From the data we have calculated deactivation rate constant (k_d) and energy of deactivation (ΔE_d) by using an equation as discussed earlier and the values obtained are given in Table 2.7. As the operation time goes on increasing the percentage lactose conversion was decreased to some extent due to enzyme deactivation. Moderate value of deactivation energy indicates that the immobilized enzymes are less critical of the temperature and hence column operation is possible at these temperatures for longer time periods without much loss of activity of the immobilized systems.

(h) Operational stability

Operational stability of the immobilized ß-galactosidase was determined by operating the reactor continuously at constant temperature (50°C) and flow rates 0.5 ml min^{-1} for different time periods and measuring the activity periodically. From the results given in Fig 2.21 it is observed that immobilized enzyme systems E-Chitosan and E-PVA-F are highly stable and a little change is observed over the % hydrolysis of lactose after 12 cycles (240 min) operation. About 40% and 60% reduction in activity of immobilized systems (E-Chitosan and E-PVA-F) was observed after repeated use for 20 cycles (400 min).

2.6 Conclusion

In the present chapter, it has been observed that >94% conversion of lactose into glucose and galactose by the immobilized enzyme is achieved as against less than 90% conversion by the free enzyme. Hence it is expected that oligosaccharides likely to be formed during the hydrolysis of lactose by lactase as proposed by Stark model [81] are relatively in lower concentration due to the mass transfer effect as suggested by Prenosil *et al* [126].

Another important observation is that activation energy of the immobilized enzyme is far less than that of the free enzyme.

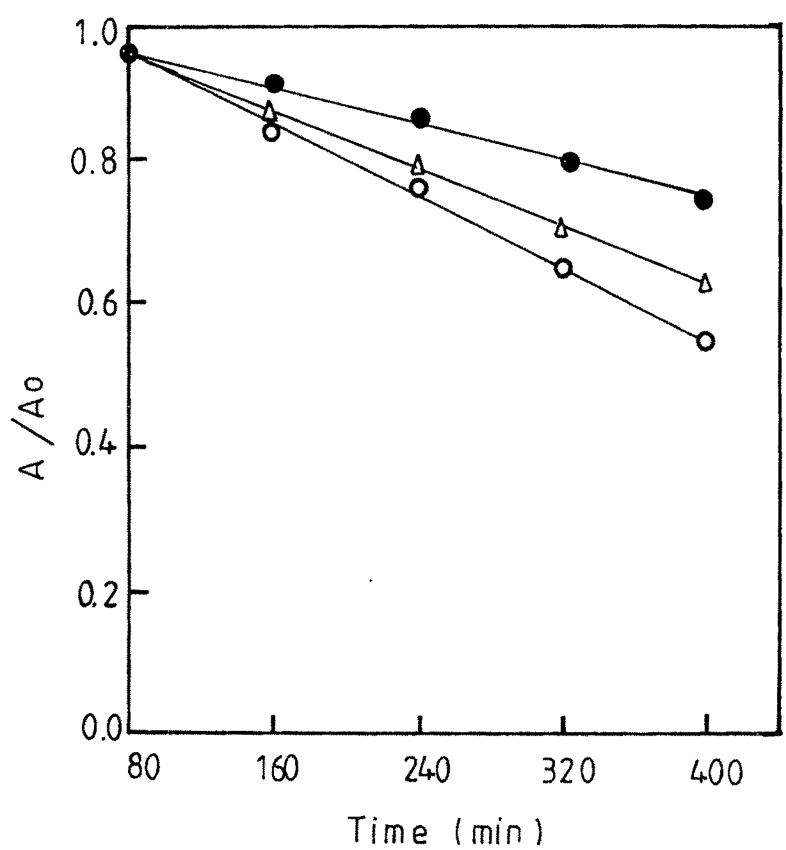


Fig 2.20 Thermal deactivation of enzyme reactorcontainingß-galactosidase-Chitosan
At (○) 60°C, (Δ) 50°C, (●) 40°C

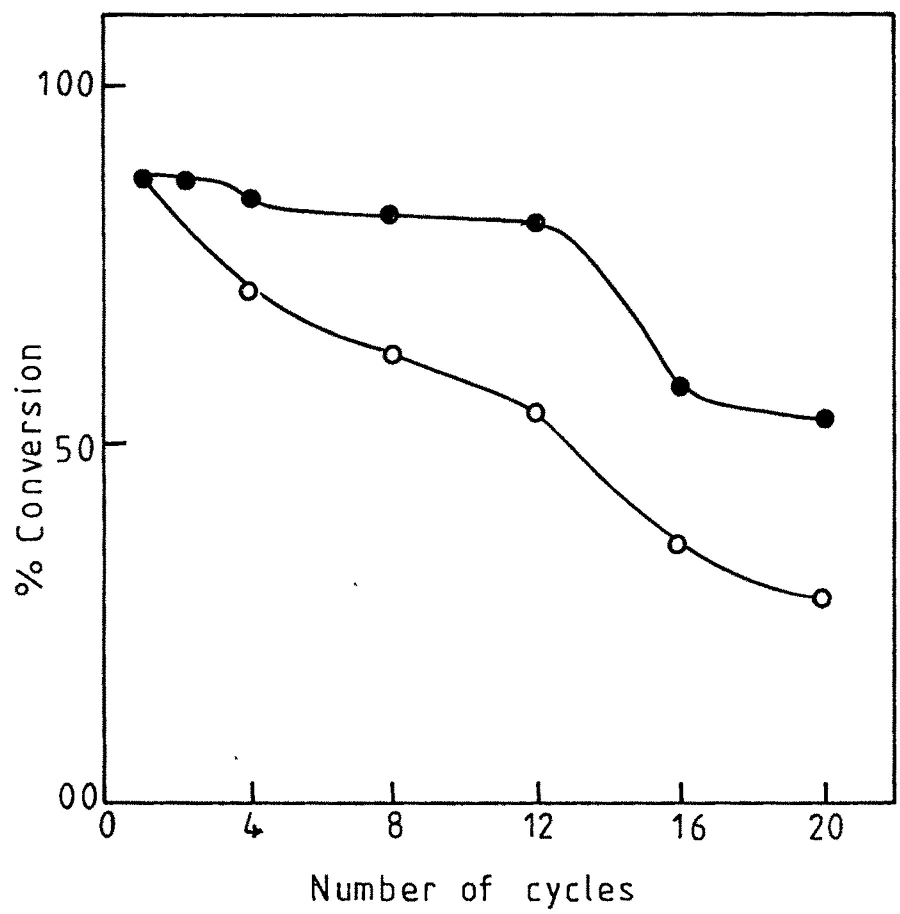


Fig 2.21 **Reusability of the column process**
(●) ß-galactosidase-Chitosan, (○) ß-galactosidase-PVA-F

Even though the deactivation rate is greater at higher temperature (60°C) the activity of immobilized enzyme was found to be better retained at 60°C. Hence the immobilized enzymes under study show good potential in reactions operating at high temperature.

Another important result of the immobilized enzyme is that it is less critical of the pH of the substrate. Therefore it can be successfully employed at a wide pH range of 4-7 without affecting the activity of the enzyme. Moreover it is noticed that the same results are obtained when the space time of the process are varied at different temperatures.

Immobilized ß-galactosidase was observed to have high storage, thermal, and solvent stability. Moreover the polymeric support materials used for the immobilization of the enzyme are very cheap and have higher retention potentials. Hence the immobilized enzyme systems under present study may find vast applications in the industrial processes.

2.7 REFERENCES

1. Aunstrup, K., *Annual Rep. Ferment. Processes*, 1983, **6**, 189.

2. Shukla, T.P., ß-galactosidase technology - a solution to the lactose problem. *CRC Critical Reviews in Food Technology*, 1975, **5**, 325.

3. Wallenfels, K. and Malhotra, O.P., ß-galactosidase in *The Enzymes,* ed. P.D. Boyer, Academic Press, New York, 1960, **vol. 4**, 2nd ed.

4. Nisizawa, K. and Hashimoto Y., In *The Carbohydrates,* ed. W. Pigman Academic Press, New York, 1970, **vol.IIA**, 241.

5. Nijpels, H.H., Lactases and their Applications in *Enzymes and Food Processing,* eds. G.G. Birch, N. Blakebrough and K.J. Parker, Applied Science Publishers, London 1981, p 89.

6. Richmond, M.L., Gray, J.I. and Strine, C.M., *J. Dairy Science*, 1981, **64**, 1759.

7. Jobe, A. and Bourgeois, S., *J. Mol. Biol.*, 1972, **69**, 397.

8. Huber, R.E., Hulbert, K.L. and Turner, C.L., *Can. J. Biochem.* 1981, **59**, 100.

9. Flaschel, E., Raetz, E. and Renken, A., Biotechnol. Bioeng., 1982, **24**, 2499.

10. Wallenfels, K. and Weil, R., ß-galactosidase *The Enzymes,* ed. P.D. Boyer, Academic Press, New York. 1972, **vol. 7**, p. 616.

11. Coughlin, R.W., Charles, M., In: Pitcher W. (ed.) *Immobilized enzymes for food processing*, CRC Press, Florida, 1980, **153**.

12. Parizia, M.W. and Foster, F.M., *J. Food Protection*, 1983, **46**, 453.

13. Greenberg, N.A. and Mahoney, R.R., *Proc. Biochem.* 1981, **16**, 2.

14. Park, Y.K., Desanti, M.S.S. and Pastore, G.M., *J. Food Sci.*, 1979, **44**, 100.

15. Pastore, G.M. and Park, Y.K., *J. Food Sci.,* 1979, **44**, 1577.

16. Wasserman, B., *Food Technol.*, 1984, **2**, 78.

17. Kulp, K., "Carbohydrates" In *Enzymes in Food Processing*, G. Reed (ed.) Academic Press, New York, 1975, **92**.

18. White, C. and Kennedy, J., *Enzyme. Micro. Technol.*, 1980, **2**, 82.

19. Sprosser, B. and Plainer, H., *Food Technol.*, 1983, **10**, 93.

20. MacBean, R.D., Hall, R.J. and Willman, N.J., *Aust. J. Dairy Technol.*, 1979, **34**, 53.

21. Kuby, S.A. and Lardy, H.A., *J. Am. Chem. Soc.*, 1953, **75**, 890.

22. Kilara, A. and Shahani, K.M., The use of immobilized enzymes in food industry; A review. *CRC Crit. Rev. Food Sci. Nutr.*, 1979, **12**, 161.

23. Finocchiaro, T., Olson, N.F. and Richardson, R., *Adv. Biochem. Eng.*, 1980, **15**, 71.

24. Kay, G., Lilly, M.D., Sharp, A.K. and Wilson, R.J.H., *Nature*, 1968, **217**, 641.

25. Morisi, F., Pastore, M., and Vigila, A., *J. Dairy Sci.*, 1973, **56**, 1123.

26. Dahlqvist, A., Mattiasson, B. and Mosbach, K., Biotechnol. Bioeng., 1973, **15**, 395.

27. Wierzbieki, L.E., Edwards, V.H. and Kosikowski, F.V., *Biotechnol. Bioeng.*, 1974, **16**, 397.

28. Okos, E.S. and Harper, W.J., *J. Food Science*, 1974, **39**, 88.

29. Gilbert and Kleyn D.H., *J. Food Science,* 1974, **39**, 751.

30. Hustard, G.O., Richardson, T. and Olson, N.F., *J. Dairy Sci.*, 1973, **56**, 1118.

31. Ibid, 1973, **56**, 1111.

32. Richardson, T. and Olson, N.F., Immobilized enzymes in milk systems. In: *Immobilized enzymes in food and microbial process.* A.C. Olson and C.L. Cooney eds., Plenum Press, New York, 1974, **19-40**.

33. Charles, M., Coughlin, R.W., Paruchuri, E.R., Allen, B.R. and Hesselberger, F.X. *Biotechnol. Bioeng.*, 1975, **17**, 203.

34. Olson, A.C. and Stanley, W.L., Immobilized enzymes In *Food and Microbial Processes.* A.C. Olson and C.L. Cooney (Eds.), Plenum Press, New York, 1974, **51-62**.

35. Hyrkas, K., Viskari, R., Linko, Y. and Linko, M., *Milchwissenschaft,* 1976, **3**, 129.

36. Pye, E.K. and Weetall, H.H., *Enzyme Engineering*, Plenum Press, New York, 1978, **483-96**.

37. Kilara, A., Shahani, K.M. and Wagner, F.W., *Lebeusm-wiss U-technology*, 1977, **10**, 84.

38. Cashion, P., Javed, A., Harrison, D., Seeley, J., Leutini, V. and Sathe, S., *Biotechnol. Bioeng.*, 1982, **24**, 403.

39. Cashion, P., Leutini, V., Harrison, D. and Javed, A., *Biotechnol. Bioeng.* 1982, **24**, 1221.

40. Kilara, A., Shahani, K.M. and Wagner, F,W. *J. Food Biochem.*, 1977, **1**, 261

41. Joshi, V.K., Kilara, A., Shahani, K.M. and Wagner, F,W. *J. Chromatogr.*, 1979, **176**, 11

42. Pastore, M., and Morisi, F., In: Mosback, K. (Ed.), Immobilized Enzymes *Methods in Enzymology*, Academic Press, New York, 1976, **44**, 822.

43. Dinelli, D., *Proc. Biochem.,* 1972, **7**, 9.

44. Harju, M., Heikonen, M., Kreula, M. and Linko, M., In: Linko, P. and Larin, K., *J. Food Proc. Eng.,* 1979, **137**.

45. Prenosil, J.E., Peter, J. and Bourne, J.R., *Verfhrensteehmik*, 1980, **14**, 392.

46. Prenosil, J.E., Peter, J. and Bourne, J.R., In: Pye, E.K. and Weetall, H.H. *Enzyme Engineering*, Plenum Press, New York, 1980, **5**, 317.

47. Peter, J., Prenosil, J.E. and Bourne, J.R., *J. Chem. Technol. Biotechnol.* 1981, **31**, 705.

48. Boer, R., DeRomijin, D. and Straatsma, J., *Neth. Milk Dairy J.*, 1982, **36**, 317.

49. Harju, M.., *Mejreiposten*, 1982, **71**, 43.

50. Nakanishi, K., Matsuno, R., Kazauyuki, T., Yamamoto, K. and Kamikubo, T., *Enzyme Micro Technol.*, 1983, **5**, 115.

51. Reimerdes, E.H., and Gottschick, W., *Lebensnitteltechnik*, 1981, **36**, 466.

52. Reimerdes, E.H., Gottschick, W. and Scholz, N., *Milchwissenschaft*, 1981, **36**, 664.

53. Reimerdes, E.H. and Scholz, N., *Miilchwissenschaft,* 1982, **36**, 308.

54. Makkar, H., Sharma, O. and Dawara, R., *Biotechnol., Bioeng.*, 1983, **25**, 867.

55. Friend, B.A. and Shahani, K., *Biotechnol. Bioeng.*, 1982, **24**, 329.

56. Chun, M., Dickopp, G. and Sernetz, M., *J. Solid Phase Biochem.*, 1980, **5**, 211.

57. Chun, M. and Sernetz, M., In: Weetall, H.H. and Royer, G.P. (Eds.), *Enzyme Engineering,* Plenum Press, New York, 1980, **5**, 457.

58. Hong, Y., Kwon, S., Chun, M. and Sernetz, M., In: *Enzyme Engineering* Plenum Press, New York, 1982, **6**, 217.

59. Griffiths, M.W. and Muir, D.D., *J. Sci. Food Agri.,* 1980, **31**, 397.

60. Park, Y. and Pastore, G.M., *J. Ferment. Technol.*, 1981, **59**, 165.

61. Thomas, G.V., Karla, M.S. and Singh, A., *Indian J. Exptal. Biol.*, 1980, **18**, 1020.

62. Beddows, C.G., Guthrie, J.T. and Abdel-Hay, F.I., *Biotechnol. Bioeng.*, 1981, **23**, 2885.

63. Hannibal-Friendrich, O., Chun, M. and Sernetz, M., *Biotechnol. Bioeng.*, 1980, **22**, 157.

64. Janesik, V., Beleznai, Z. and Keleti, T., *J. Mol. Cat.,* 1982, **14**, 297.

65. Torrey, S. (Ed.), *Enzyme technology, preparation, purification, stabilization, immobilization.*, Park Ridge, N.J., U.S.A., Noyes Data Corpn., 1983, **52**, 42.

66. Hirohara, H., Yamamoto, H., Kawano, E. and Nagase T., In: *Enzyme Engineering,* Plenum Press, New York, 1982, **6.**

67. Beddows, C.G., Miraur, R. and Guthrie, J.T., *Biotechnol. Bioeng.*, 1980, **22**, 311.

68. Miyata, N. in Report of Research Lab., Snow Brand Milk Products Co., 1981, **71**, 51.

69. Leuba, J.L. and Widmer, F., *J. Solid Phase Biochem.*, 1977, **2**, 257.

70. Coughlin, R.W., Charles, M. and Julkowski, K., *Alch E. Symposium Series*, 1978, **78**, 40.

71. Hung, M., Monsean, P., Mayer, R. and Roulean, D., *Can. J. Chem. Eng.*, 1983, **61**, 73.

72. Fanquex, D.F., Flaschel, E. and Renken, A., *Chinia.*, 1984, **38**, 262.

73. Halling, P. and Dunhil, P., *Biotechnol. Bioeng.*, 1979, **21**, 393.

74. Halling P. and Dunhil P., *Eur. J. App. Microbiol. Biotechnol.*, 1979, **8**, 27.

75. Ivanov, L.A., Bashirova, R.S., Stepanenko, N.P. and Ramanskya, N.V., *Molochu prom-st*, 1986, **7**, 26.

76. Banerjee, M., Chakravorty, A and Majumdar, S.K., *J. Microbiol. Biotechnol.*, 1986, **1**, 66.

77. Baret, J.L. (nov. Ind. Enzymes) SAF 75017 Paris, Fr., *Methods Enzymology*, 1987, **136**.

78. Pilloton, R., Maseini, M., Caselle, I.G., Festa, M.R. and Botleri, E., *Anal. letters*, 1987, **20**, 1803.

79. Bastalova, K., Kuncher, K., Popova, Y. and Kozhukharova, A., *App. Microbiol. Biotechnol.*, 1987, **26**, 227.

80. Narinesingh, D., Stoute, V.A. and Davis, G., *J. Mol. Cat.*, 1988, **45**, 285.

81. Prenosil, J.E., Stuker, E. and Bourne, J.R., *Biotechnol. Bioeng.*, 1987, **30**, 1026.

82. Ariga, O. and Tetaki, F.H., *J. Ferment. Bioeng.*, 1989, **68**, 293.

83. Sukurai, K., Kitade, K., Takahashi, T. and KogaKubon, *Kenkyn Hokoku*,1989, **37**, 173.

84. Bakken, A.P., Hill, C.G. and Amudson, C.H. Jr., *Biotechnol. Bioeng.*, 1989, **33**, 1249.

85. Korde, V.M., Kannan, K., Shankar, V. and Jayaraman, V.K., *Indian Chem. Eng.*, 1989, **31**, 71.

86. Sadoyasuo, M., Toshihide, M.A., Ishikawa K. Shikenjo Kenkyu, Hokoku, 1991, **39**, 17 (Japan).

87. Bakken, A.P., Hill, C.G. and Amudson, C.H. Jr., *App. Biochem. Biotechnol.*, 1991, **28**, 741.

88. Shinmyungson, Chang, Pannshicle, Min Bong, Ku, Kwarkttar Soo., *Anal. Sci. Technol.,* 1992, **5**, 471.

89. Ariga, O.S. and Tetsuya, S.Y., *J. Ferm. Bioeng.*, 1992, **14**, 120.

90. Huzhoug Chang, Korus Roger A. and Stormokeith E., *App. Microbiol. Biotechnol.,* 1993, **39**, 289.

91. Piesecki, S., Teng, W.Y. and Hochuli, E., *Biotechnol. Bioeng.*, 1993, **42**, 178.

92. Shul Chishin, V.A., Matveer, P.V. and Vivmik, A.D., *Biokhim. Mikrobiol.*, 1993, **29**, 108.

93. TacGwan, P., *Biotechnol. lett.*, 1993, **15**, 57.

94. Thompkinson, D.K., *Bull. Int. Dairy Fed.*, 1993, **289**, 23.

95. Rogalski, J., Dawidowicz, A. and Leonowicz, A., *J. Mol. Cat.* 1994, **93**, 233.

96. Gabriela, I. and Francisco, B.V., *Biotechnol. Tech.*, 1994, **8**, 233.

97. Sibel, S. and Ural, A., *J. Chem. Technol. Biotechnol.*, 1994, **59**, 303.

98. Takao, S., Yasuko, Y., Katsuya, K., Kong Hua, L., Sumio, M. and Takeshi, K. *App. Microbiol. Biotechnol.*, 1994, **40**, 618.

99. Berger, J.L., Lee, B.H. and Lacroix, C., *Biotechnol. Tech.*, 1995, **9**, 601.

100. Standard Analytical Methods of the Member Companies of the Corn Refiners Association, First Revision, 5-27-68, E.26.

101. Martin, R.W., *The chemistry of phenolic resins.*, John Wiley & Sons Inc., New York, 1956.

102. Chandy, T. and Sharma, C.P., *J. App. Poly. Science*, 1992, **44**, 2145.

103. Brandt, J., Anderson, L.O. and Porath, J., *Biochem. Biophys. Acta.*, 1975, **386**, 196.

104. Nilsson, K., Norlow, O. and Mosback, K., *Acta. Chem. Scand.*, 1981, **35**, 19.

105. Kay, G. and Croock E.M., *Nature*, 1967, **216**, 514.

106. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., *J. Biol. Chem.*, 1951, **193**, 265.

107. Craven, G.R., Steers, E.J. and Anfinsen, C.B., *J. Biol. Chem.*, 1965, **240**, 2468.

108. Hayashi, T. and Ikada, Y., *Biotechnol. Bioeng.*, 1990, **36**, 593.

109. Keilin, D. and Hartree, E.F., *Biochem. J.*, 1948, **42**, 230.

110. Trinder, P., *Anal. Clin. Biochem.*, 1969, **6**, 24.

111. Bakken, A.P., Hill, C.G. and Amudson, C.H. Jr., *Biotechnol. Bioeng.*, 1992, **39**, 408.

112. Aleyamma, A.J. and Sharma, C.P., *Poly. Mater. Sci. Eng.*, 1988, **59**, 692.

113. Brine, C.J., Chitin, accomplishments and perspectives, In: J.P. Zikakis(Ed.), *Chitin, chitosan and related enzymes*, Academic Press, Orlando, Fl.1984, xvii-xxiv.

114. Sandford, P.A. and Steinnes, A., Biomedical applications of high purity chitosan, In: Shalaby, S.W., McCormick, C.L. and Butler, G.B. (Eds.), *Water soluble polymers : synthesis, solution properties and applications* ACS Symposium Series no. 467, 1991, Washington, D.C.

115. Hirano, S., Sakaguchi, T. and Kuramitsu, K., *Carbohydrate polymers*, 1992, **19**, 135.

116. Pantano, P. and Kuhr, W.G., *Anal. Chem.*, 1993, **65**, 623.

117. Weetall, H.H., Havewala, N.B., Pitcher, W.H. Jr., Detar, C.C., Vann, W.P. and Yaverbaum, S., *Biotechnol. Bioeng.*, 1974, **15**, 689.

118. Pitcher, W.H. Jr., *Immobilized lactase system*. In: Proceedings of the Whey Production Conference, Chicago, 1974.

119. Jones, J.B., In: Mosbach (Ed.) *Methods in Enzymology*, Academic Press, New York, 1976, **44**, 831.

120. Whitesides, G.M., Applications of cell free enzymes, in enzymes In *Organic synthesis.* Ciba Foundation Symposium III, Pitman, London, 1985, **76**.

121. Klibanov, A.M., *Science*, 1983, **219**, 722.

122. Ponnuchamy, N.P. and Gupta, M.N., *Biotechnol. Bioeng.*, 1989, **33**, 927.

123. Hill, J.A. and Hubber, R.E., *Biochem. Biophy. Acta.*, 1971, **250**, 530.

124. Reithel, F.J. and Kim, J.C., *Arch. Biochem. Biophy.*, 1960, **90**, 271.

125. Freeley, R.M., Criner, P.E., Murphy, E.W. and Toepfer, E.W., Major mineral elements in dairy products, *Research*, 1972, **61**, 505.

126. Prenosil, J.E., Stuker, E. and Bourne, J.R., *Biotechnol. Bioeng.*, 1987, **30**, 1019.