UREASE IMMOBILIZATION

CHAPTER 3

CONTENTS

3.1 Urease

Urease is a hydrolytic enzyme and the isolation procedure developed for obtaining crystals of urease has been reported first time in 1926 by Sumner [1]. Possible sources of enzyme urease are bacteria, yeast, fungi and large number of higher plants. The richest plant source known is the Jackbean [2,3] (*Canavalia ensiformis, C. obtusifolia*) which contains 0.15% urease. This has about sixteen times the urease content of the soybean[4]. The richest known source of urease is *Bacillus pasteurii* which contains upto 1% of its dry weight of urease. The jackbean urease having molecular weight of 480,000 was first reported by Sumner and coworkers [5,6]. The reaction of urease with urea has been studied by a number of workers. Reaction mechanism is neither as simple nor as well understood as glucose oxidase. Sumner *et al* [7] were the first to demonstrate the formation of ammonium carbamate as one of the products during urease catalyzed hydrolysis of urea. Further this was supported by Wang and Tarr [8] in 1955 by Gorin in 1959 [9], by Janecks in 1963[10] and by Blakely *et al* in 1969[11]. Reaction product carbamate further decomposes into carbondioxide and ammonia. The carbondioxide thus formed further reacts with water to form carbonic acid.

The mechanism of urea hydrolysis by urease has been discussed well by Laidler[12]. The noteworthy point in this reaction is that unlike other enzymatic reactions, the rate of the urease catalyzed reaction is not directly proportional to the enzyme concentration regardless of the type of buffer used. Wall and Laidler [13] ascribed this fact to the inhibitory action of the ammonium ions produced during reaction at higher concentrations. This is in addition to the reaction being inhibited by the substrate urea.

Substrate concentration has been found to have a strong effect on reaction rate. Wall and Laidler [13] have examined the kinetics of urea hydrolysis in THAM-sulfate (Trishydroxymethyl aminomethane) while Laidler and Hoare [14] have described it in phosphate buffer. However, among the buffer systems that have been investigated, only trihydroxymethyl aminomethane-maleate and maleic acid-maleate have been found to have no influence on the urease reaction [15], whereas phosphate buffer was observed to change the intermediate route [16].

3.2 Properties of urease

Urease is a highly efficient enzyme that has been described as specific for converting only urea to ammonia and carbondioxide. For the urea hydrolysis several possibilities are discussed in the literature. The carbondioxide-ammonium mechanism runs without intermediates to the end products, but the carboxylic acid and carbamine acid

mechanism proceeds via intermediate steps [8]. Various investigations have proved the formation of carbamate intermediate [10,11].

The effects of temperature, pH and substrate concentration on the rate of the urease-catalyzed hydrolysis of urea are complex and interrelated. Various types of acid ureases and their properties have been thoroughly studied and reported by Kakimoto *et al* [17]. Acid ureases were first found in *Lactobacillus sp.* and *L. fermentum* by Moreau *et al* [18] and Suzuki *et al* [19]. The optimum pH values for these enzymes were found to be 3.0 and 4.0 respectively.

The another important property is enzyme inhibition. The relative effectiveness of various metal ions as inhibitors for urease shows the order[20] : $Ag^{+}>Hg^{2+}>Cu^{2+}>Cd^{2+}>Co^{2+}>Ni^{2+}>Mn^{2+}$ with pb^{2+} and Fe^{2+} unassigned, but less than Cu^{2+}. This order correlates with the relative insolubility of the sulphides of these metals, which may have formed due to the interaction with -SH groups of urease. The various activators or protectors such as proteins, amino acids and gum arabic function by binding heavy metals, thereby protecting the urease —SH groups. The other inhibitors are p-chloromercuribenzoate and acetohydroxamate.

Urea is one of the major toxic wastes in the dialyzate of hemodialysis. Since no efficient sorbent is available for urea [21,22] the most effective method for the removal of urea from aqueous solution may be the utilization of urease. However ammonium ions are toxic above 10^{-4} mol. dm^{-3} concentration in blood. Therefore, the utilization of urease in the dialyzate regeneration system must be coupled with effective ammonium ion removal process. Hence in the present work immobilization of urease has been attempted on the cheaper polymeric materials some of which have good biocompatibility. The immobilized urease has been thoroughly characterized and its performance is compared with free enzyme. In addition immobilized urease is used in the packed bed column reactor for the continuous hydrolysis of urea.

3.3 Selected literature survey for the immobilization of urease

Numerous synthetic and natural polymeric supports have been used for urease immobilization and their uses in medicinal and technical fields have been reported [23]. Most commonly performed clinical analysis is of urea determination in urine and blood. Consequently the enzyme urease has been immobilized by a variety of methods. Weetall and Hersh [24] used covalent coupling of urease to aminoaryl glass which was further used in the packed bed reactor for the assay of urea. They have reported precise and accurate

assay of urea upto 0.17 M concentration, but concentrations exceeding 0.34 M were showing inhibitory action. Over 30 days period with intermittant use, no loss of enzyme activity was observed. Reisel and Katchalski [25] also used immobilized urease to determine urea in blood serum.

In 1970, Sundaram and Hornby [26] developed a method for the attachment of enzymes onto the inner surface of nylon-6 tubing. The linking of amino groups present in the enzyme takes place through the bifunctional reagent glutaraldehyde. Urease was successfully immobilized by this method and the immobilized derivative was reported to show substantial resistant to thermal inactivation and a superior storage stability compared to the free enzyme. Nylon-6 in the form of membrane is a preferred support material to polystyrene for the immobilization due to better mechanical properties and more hydrophilic nature. The only major drawback encountered in the use of nylon membrane as a support material for the immobilization was the limited number of free amino groups present on the inner surface of tubing available for the attachment of glutaraldehyde. Sundaram and Hornby [26] alleviated this problem to a certain extent by exposing nylon tubing to a carefully controlled hydrolytic procedure using HCl. This resulted in the cleavage of amide bonds in the nylon backbone releasing free amino groups. However, over exposure of the tubes to this hydrolytic process could result in a complete degradation of the tube structure.

Filippusson *et al* [27] have reported attachment of urease to the inside surface of the nylon tubing which was further used in a Technicon Auto Analyzer for the determination of urea.

Dinelli *et al* [28] described fibre entrapped urease and its use in continuous flow automated analysis. The enzyme fibres were also introduced into silicon plastic tubing and when introduced into continuous flow automated systems in this form, did not disrupt the bubble patterns and exhibited satisfactory flow characteristics. Fibre entrapped urease was used for 20 days for the determination of urea levels in both aqueous solutions and in biological fluids, without loss of enzyme activity.

The work on immobilization of urease on various supports carried out during 1960 to 1980 mainly involves polyacrylamide and cellulose and their derivatives. Different techniques such as entrappment [29,30], and covalent binding such as crosslinking [31,32], diazo coupling [33], cynogen bromide coupling [31] etc have been reported for the immobilization of urease on polyacrylamide and its derivatives.

Derivatives of cellulose were used for the immobilization of urease through covalent binding techniques [34,35] and microencapsulation [36,37]. Microencapsulation techniques were widely used for the immobilization of urease. Encapsulated urease systems and their applications are well described by Chang and coworkers [38-44].

In 1971, Gardner *et al* [37] used phase separation method for urease encapsulation using cellulose derivative. During immobilization stabilized urease lost ~70-90% of the enzyme activity. Chang *et al* [39] also reported 80% loss in enzyme activity after encapsulation of urease. Presumably this decrease represents a diffusion barrier to urea and denaturation of the active enzyme sites by the organic solvents, which inevitably have trace solubility in the aqueous phase. However, even with the loss occur ing during encapsulation, the enzyme capsule contains sufficient residual activity for urea conversion. In addition, results showed no loss of enzyme activity for enzyme capsules stored in a sealed jar at room temperature for 2 weeks but 25% decrease in the activity was observed after 6 months. Some other organic supports such as derivatives of nylon[45-48], methacrylate [49], agarose [50], sephadex [51], collagen [52-54], chitin [55] and poly(vinyl alcohol) [56] have also been reported for urease immobilization.

Other inorganic supports such as derivatives of porous glass [57,58], sand [59] and aluminium hydroxide [60] are also reported. However, with many of these carriers, immobilization has resulted into considerable change in enzyme activity as well as its binding capacity.

The more recent works are discussed here in somewhat details. In 1980 Kitano *et al* [61] used three kinds of hollow fibres made from poly(vinyl alcohol) and cellulose diacetate for the entrapment of two kinds of ureases from jackbean. The apparent Michaelis menten constants for the entrapped urease were reported to be larger than those for free enzymes, because of permeation resistance of substrate across the hollow fibre membrane. The half-lives of the free enzymes from Merck and PL-Biochem, stored at 25° C in phosphate buffer of pH 6.8 were reported to be 5 and 16 days respectively, whereas the half-lives of Merck and PL-Biochem urease entrapped in the hollow fibre reactor in the continuous reaction system were reported to be 3 and 12 days respectively. Activation energies for the urease entrapped in hollow fibres were smaller than those for the free enzyme.

Immobilization of urease on polyacrylamide by using entrappment method was studied by Sada *et al* [62]. The amount of urease loaded in the dry and wet beads was 7.1 and 3.6 mg g^{-1} respectively. The Michaelis menten constant for soluble and immobilized

urease were 2.2 mM and 6.6 mM respectively. The retention of enzyme activity on immobilization was about 35% and no loss of urease binding capacity was noted during storage for weeks.

Immobilization of urease on copolymers of N.N-dimethyl aminoethyl methacrylate, poly(vinyl chloride) and poly(acrylonitrile) was reported by Miyma *et al*[63]. The immobilized systems were observed to be very stable in 0.05 M phosphate buffer of pH 7.5 at 4° C. The immobilized systems also exhibited higher heat stability than the native urease.

Another type of graft copolymer containing collagen-poly(glycidyl-methacrylate) was used for the immobilization of urease by Raghunath *et al*[64]. In this work, the authors described the immobilization of urease onto collagen-glycidyl methacrylate graft copolymer through the reaction of enzyme with epoxy groups of the copolymer. Urease showed considerable retention of activity even after immobilization. Improvement in the physicochemical properties of immobilized urease were also reported.

In 1986, Sharma [65] reported the immobilization of urease on polyacrylamide gel by the entrappment method. Various conditions such as urea concentration, pH, temperature and response time were studied for the activity of immobilized urease. The optimum pH 8.5 at 37° C temperature with 2 min response time was achieved for the immobilized system. He also developed urea specific electrode from immobilized system and this electrode was used for the determination of urea in blood.

Sugii *et al*[66] synthesized macroporous hydrophilic poly(vinyl pyridine) beads by suspension polymerization of 4 or 2-vinyl pyridine and hydrophilic crosslinking reagents such as ethylene glycol dimethacrylate and tetraethylene glycol dimethacrylate. Their physicochemical properties were studied and polymers were further used for the immobilization of urease by adsorption. The immobilized system was used for the determination of blood urea from human serum.

Barbara *et al*[67] used $TiCl_4$ for covalent coupling of enzyme. Several parameters were studied in order to optimize the residual activity upon immobilization and during operation. The kinetic constants K_m and V_m for immobilized enzyme were observed to be much higher than those for the free form. Immobilized enzyme was reported to show good temperature stability, operational stability, pH stability and good retention of activity. The optimum pH of the immobilized enzyme was shifted from 7.4 to 8. The optimum temperatures were reported to be 75 and 65° C for IME and free enzyme. The activation energy of the IME was observed to be 4-fold smaller than that of free indicating less

sensitivity to temperature. In another article Lynbinskii *et al* [68] have reported silica as a support material for the immobilization of urease from *Staphylococcus seprophyticus* (*S. sephrophyticus*). They observed that the value of specific activity of urease grafted to silica depends not only on the type of the enzyme-carrier bond but also on the protein to silica distance. Only 26% of enzyme activity was observed to be retained after immobilization.

Hsine and Chou [69] synthesized a copolymer with balanced hydrophilicity and hydrophobicity by grafting butyl acrylate onto poly(vinyl alcohol). Films made from it showed good urea permeability after immobilization of the enzyme urease on the surface of the polymeric film. The film was attached to the tip of an ammonia gas sensing electrode to form an enzyme sensor. The sensor was able to detect urea in solution in the range of 6 to 600 mg/dL with a response time of about 5 min. The immobilized system was reused over 100 times and the reproducibility was reported to be very good.

Ogawa and coworkers [70] proposed a method for continuous preparation of relatively large and uniform crosslinked beads. They prepared four kinds of macroreticular and gel types of polymer beads with a mixture of maleic-anhydride, styrene, and divinyl benzene. Urease was immobilized on these beads and its properties after immobilization were evaluated. The immobilized system showed the highest enzymic activity and the system was successfully applied for the determination of blood urea nitrogen in human serum.

Urease entrapped onto the film of photo crosslinked poly(vinyl alcohol) was used for the hydrolysis of urea by Jing *et al* [71]. Urease from jackbean was immobilized on granular active calcium by entrapping it between two sheets of photo crosslinked film of PVA having 2% mole of stilbenzolium group (PVA-SbQ). The PVA-SbQ films containing immobilized urease were observed to be suitable for the urea removal in hemodialysis.

The new economical method was suggested by Ivanov *et al* [72] for the determination of urea content using immobilized urease. The suggested technique needs very little reagent and the same reagent can be used up to 500 times. The Sepharose-2B was used as a support material and the immobilized system did not lose its activity for ≥6 months. Substituted sepharoses (DTNB-thiol-thiopropyl or hydroxy urea-sepharose) are also reported in the literature [73] for this purpose. The immobilized urease activity was observed to be between 14-54%.

Recently Demirel *et al*[74] have reported a comparative study of the performance of solid supported and soluble urease for the enzymatic hydrolysis of urea. The authors used petroleum-based spherical activated charcoal of size 0.4 to 1 mm diameter and of surface area around 1000-1300 $m^2\ g^{-1}$. The immobilization method has been physical attachment by simple adsorption of enzyme molecules onto the support material. Kinetic reaction showed that the kinetic data are consistent with the proposed model of inhibition on specific growth rate for the free enzyme solution.

Huang and Chen[75] described a five compartment electrodialyzer with immobilized urease which was developed for the removal of urea from aqueous solution. Urease supported on polyurethane foam was placed in the central (dilute) compartment where urea was hydrolyzed and the products NH_4^+ and CO_3^{2-}/HCO_3^- were removed simultaneously by electrodialysis. The system was studied both under constant current and constant voltage. The removal of urea by enzymatic reaction was observed to be not affected by the electric field. In another article the authors reported kinetic study of urea hydrolysis by using urease immobilized with BSA and glutaraldehyde on polyurethane foam[76]. The residual activity of urease after immobilization was observed to be ~50%. The activity of urease at low pH was improved on immobilization. Storage and operational stabilities were good for the immobilized urease. The method for immobilization was very simple and practical. Polyurethane as a support material for urease immobilization was also reported by Vasudevan *et al*[77]. They reported urease immobilization on polyurethane and its use in packed bed and CSTR for the hydrolysis of urea. Deactivation kinetic parameters were established by separate batch experiments. The activity of the immobilized urease was 3.4 ± 0.6 IU/g of support.

Kobayashi *et al*[78] reported immobilization of urease and glucose oxidase on positively charged dimethyl amino nylon gel. The stabilities of the immobilized enzymes after preheating and incubation in organic solvents were measured and were compared with those of the native enzymes. The pH activity profile for the immobilized enzymes shows shift towards a low pH compared with that of for the native one. Using the pH difference the electrostatic potential of the positively charged nylon gel was estimated. The estimated values of electrostatic potential of positively charged gel were +90 and +120 mv for the immobilized urease and glucose oxidase respectively.

Carboxymethyl cellulose gelatine was also used for the immobilization of urease by Sungur *et al*[79]. Immobilization was based on the formation of insoluble salts of CMC and gelatine with chromium (III) ions. Cr (III) acetate and Cr (III) sulphate were used for this purpose and their effect on urease activity was investigated. Reusability, pH, enzyme

concentration and concentration of cross linking reagent and incubation period were optimized for better retention of the enzyme activity by using Berthlot reaction method. They observed that the immobilized systems were stable for at least 2 months and for 16-24 usages. The immobilization yield was reported to be 40%.

Moynihan *et al* [80] investigated urea hydrolysis by urease immobilized onto Amberlite IRP-64-A ion exchange resin in a fixed bed reactor. A modified Michaelis menten rate expression was used to describe the pH dependence of substrate product inhibited kinetics and ionic equilibria of product. Buffer species were discussed to account for pH changes observed during reaction. Immobilized urease system was studied both theoretically and experimentally with an isothermal heterogeneous fixed bed reactor.

In another article Wang *et al* [81] studied urea sensor with single poly propylene membrane. In this study urease was immobilized on the aminated plasma surface of a hydrophobic membrane. This matrix with urease on one side maintained its original hydrophobic property and was used to construct the urea sensor. They observed that enzyme electrode with a single membrane gave a shorter response time when compared to the commercial electrode.

Recently, Steffen and Stande [82] studied the covalent attachment of urease, to polyamide or polysulphone-based membranes. The original membrane polymers are not chemically reactive; hence diazo, acyl-acid, carbodiimide and methyl bromide activation methods were used. The enzyme was fixed within the porous substructure of the membrane. The amount of enzyme immobilized at the membrane was found to be negligible. The kinetics was described according to the Michaelis-Menten model. Compared to the free urease the activity of the membrane bonded urease was very low.

In 1993, Leszko and Zaborska[83] reported the influence of sodium fluoride inhibitor on the activity of urease in native form and covalently bound on glutaraldehyde pretreated chitosan membrane. They observed that sodium fluoride is a competitive slow-binding inhibitor of urease. Hydrolysis of urea at the standard conditions of pH 7.0 at 25°C and a constant ionic strength and variable concentration of substrate and of inhibitor was studied.

Immobilized urease was prepared by adsorption or glutaraldehyde crosslinking techniques onto soybean polysaccharide by Chen[84]. Adsorbed urease showed good stability and activity at pH 6-7 and at temperature 65-75°C. The Michaelis constant (K_m) for the immobilized urease was observed to be 46 mM at 30°C. The activity and stability

of the column reactor prepared by adsorption were lower than that prepared by glutaraldehyde crosslinking.

Jackbean urease immobilized on PVA membrane was reported by Chen *et al* [85]. Immobilized urease was further used for the hydrolysis of urea in a reactor-separator combining with an anion-exchange membrane. The urea in the feed solution was passed through the anion exchange membrane and urease immobilized on PVA membrane. The experimental results showed that no ammonium ion was found in the feed solution under either phosphate or citrate buffer systems at 0.05-0.2 mol dm^{-3} and pH 6-9 and various initial concentrations of urea in the feed solution (20-200 m mol dm^{-3}). The properties of the urease immobilized PVA membrane were also examined.

Epoxide polymer as carrier material for the immobilization of urease was used by Schwedt *et al*[86]. Immobilized urease was further used for the determination of urea in foods by Flow Injection Analysis.

Husiniye *et al*[87] have reported immobilization of urease onto copolymer of 2-hydroxy ethyl methacrylate (HEMA) and N-vinyl pyrrolidone (VP). The activities of immobilized urease stored in phosphate buffer of pH 7 at 4°C were examined periodically upto 90 days. They have also observed the higher the VP/HEMA mole ratio in the structure, higher the volume increase and enhanced enzyme activity.

In 1995, Campanella and Lin[88] reported the immobilization of urease on silica. The major steps involved for the process are amination, activation and immobilization. Silica-3-aminopropyl methoxy silane and glutaraldehyde were used as support and activating reagent respectively. On the basis of the study the authors performed the characterization of the NH_3 electrode with immobilized urease in standard solution of urea.

Immobilization of urease on acrylamide grafted starch by radiation method was established by Nguyen anh *et al*[89]. They observed that the immobilized urease retained its activity even after 7 repeated runs.

Immobilization of urease on modified sulfone membrane was investigated by Gryzelda *et al* [90]. The properties of the immobilized and free urease were examined. The Michaelis constant (K_m) was 4.4 times higher for the immobilized urease than that for free enzyme.

Recently, urease was immobilized on composite cellulose by using adsorption and crosslinking method by Jumei and Wenbin [91]. The immobilized urease possesses high activity and good stability.

It is observed from the literature that various types of supports have been used for the immobilization of urease. The nature of the support and the distance of the enzyme from support after immobilization plays important role in determining the enzyme activity. Hence we have undertaken a study of two types of supports: synthetic and natural polymers having hydroxy groups and amino groups for the coupling of the enzyme. The spacers with various chain lengths were also used for the activation and coupling of enzyme. The details are discussed in the following sections.

3.4 Experimental

Polymeric supports used in the present study are well described in the Experimental Section 2.4.1. All the experiments were done in duplicate to ensure reproducibility and the chemicals used in the present study are listed below.

► Chemicals used

Crude crystalline urease (EC 3.5.1.5) from jackbeans [strength 1 unit/mg]	:	TRIZMA Chemical Co. (India)
Urea	:	BDH (India)
Sodium hypochlorite (4% available chlorine)	:	BDH (India)
Sodium nitroprusside	:	Ranbaxy (India)
Phenol	:	BDH (India)
Ammonium sulphate	:	BDH (India)
Glycerol	:	Qualigens (India)
Methanol	:	Qualigens (India)
Propanol	:	Qualigens (India)
Acetone	:	Qualigens (India)
p-tolylsulphonyl chloride	:	Merck (Germany)

Cynuric chloride	:	Fluka (Switzerland)
Glutaraldehyde 25% (w/w) (India)	:	S.D. Fine Chemicals
Disodium hydrogen phosphate	:	BDH (India)
Monosodium hydrogen phosphate	:	BDH (India)

Phosphate buffer (0.2 M) was prepared from disodium hydrogen phosphate and mono sodium hydrogen phosphate. All solvents were distilled before use. All the other reagents used were of Analytical grade and double distilled deionised water was used throughout the work.

3.4.1 Polymeric matrix

The polymeric supports used for the immobilization of urease were the same which were used for the immobilization of lactase. The details of the preparation of these supports are given in Section 2.4.1. For urease immobilization three different polymeric materials which are already discussed in Chapter-2 namely salicylic acid-formaldehyde-resorcinol (SRF) resinous material in powder form of 60-100 mesh size; chitosan beads of ~ 1.5 mm diameter and crosslinked poly(vinyl alcohol) in the form of beads of ~ 2 mm diameter were used.

3.4.2 Immobilization procedures

Activation of SRF-resinous material and crosslinked poly(vinyl alcohol) was done by using p-benzoquinone, p-tolyl sulphonyl chloride and cynuric chloride spacers and the activation procedures as reported in Section 2.4.2. Glucosamine poly saccharide chitosan was activated through glutaraldehyde treatment as described earlier in Section 2.4.2.

The activated polymeric samples were washed with 0.2 M phosphate buffer of pH 8 and were further used for the immobilization of 2 ml of 7.5 mg ml^{-1} urease at temperature 278° K and reaction time 18 h. Low temperature shaker bath INSREF was used for the reaction to reduce the external diffusion barrier due to formation of Nernst layer. The immobilized product was washed with appropriate buffer and water. Lowry assay procedure was adopted to determine the protein content of supernatant liquid as mentioned in Section 2.4.2.e and the activity of the coupled protein was measured by using phenol-hypochlorite method[92,93] and urea as a substrate.

3.4.3 Assay of Enzyme Activity

Urease solution was prepared in 50% glycerol and stored at 4° C. The activity of the free and immobilized urease was determined by phenol-hypochlorite method[92,93] using urea as a substrate in 0.2 M phosphate buffer of pH 8 and incubating the mixture at 37° C for 30 min in presence of sodium nitroprusside as a catalyst. The resulting indophenol product was measured spectrophotometrically at 580 nm for the activity of urease. One unit of activity was defined as the amount of enzyme that liberates 1 μmole of ammonia under the conditions of assay. Reactions involved in the urease activity measurements are given here in Fig 3.1.

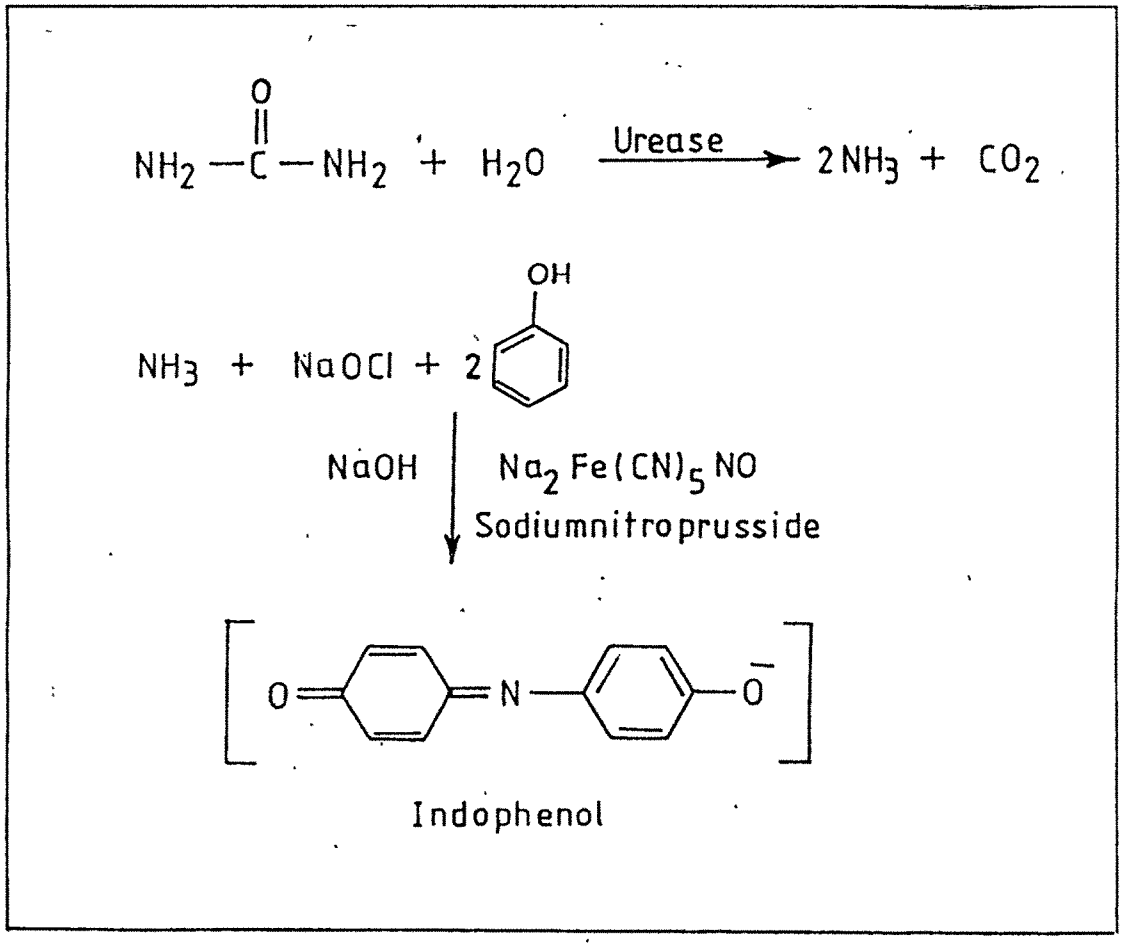

Fig. 3.1 Reactions involving urea–urease

3.4.4 Optimization of Conditions for Immobilization of Urease

Effects of pH of coupling medium, coupling time, type and concentrations of crosslinking reagent and enzyme concentration on the extent of enzyme immobilization and on the retention of enzyme activity were studied through appropriate experiments as described for ß-galactosidase in Section 2.4.5.

3.4.5 Stability of Immobilized and Free Urease

pH, temperature, kinetics, storage, and solvent stability of immobilized and free enzyme were studied as follows :

(i) pH stability studies were carried out for the free and immobilized urease by determining the enzyme activity at various pH values in 0.2 M phosphate buffer as discussed earlier in Section 2.4.6.a.

(ii) Effect of temperature on enzyme activity of free and immobilized urease was studied at various temperatures ranging from 30-70° C as discussed in Section 2.4.6.b. From the data energy of activation (ΔEa) was calculated for the free and immobilized urease by using Arrhenius equation as described in Chapter-2.

(iii) The thermal stability of the free and immobilized urease was evaluated by measuring the residual activity of the system exposed to various temperatures (60-70° C) for different time periods. Thermodeactivation study was carried out and evaluated as described in Section 2.4.6.c for ß-galactosidase.

(iv) Solvent stability for the free and immobilized urease was determined by measuring the residual activity of the system in 1-5 M water miscible organic solvents such as methanol, ethanol and propanol as described in Section 2.4.6.e for lactase.

(v) Storage stability of the free and immobilized urease was measured by storing the enzyme in 0.2 M phosphate buffer of pH 8 at room temperature for various time intervals.

vi) Kinetics of enzymatic reaction; Michaelis constant (K_m) and maximum reaction velocity (V_m) for free and immobilized urease was calculated from Lineweaver-Burk plots as discussed earlier in Section 2.4.6.f by using various concentrations of urea as substrate.

3.4.6 Hydrolysis of Urea by Immobilized Urease Using Fixed Bed Reactor

The schematic diagram of a fixed bed reactor as given in Fig 2.2 and was used for the study of continuous hydrolysis of urea by immobilized urease. The compact column of 0.6 x 10 cm dimensions containing urease immobilized on PVA-F was prepared by placing a porous disc at the bottom of the reactor. All fixed bed reactor experiments were carried out at 40-70° C controlled temperature by circulating water through the outerjacket. The packed bed column was washed with 0.2 M phosphate buffer of pH 8 before operation. The substrate urea was passed through the column at different velocities using a peristaltic pump. The extent of urea hydrolysis was measured at different concentrations of urea and flow rates. Estimation of NH_3 was done by following phenol-hypochlorite method and incubating the test solution for 30 min at 37° C and measuring the absorbance at 580 nm as reported earlier in Section 3.4.3.

Effect of temperature on urea hydrolysis by immobilized urease in fixed bed reactor was studied at different temperatures ranging 40-70° C. From the study column efficiency in the hydrolysis of urea at different temperatures and energy of activation (ΔEa) was calculated by using the Arrhenius relationship as discussed earlier.

To examine the effect of substrate concentration, column temperature and flow rates on urea hydrolysis, 10 ml of urea solutions of 5 mM to 25 mM concentrations prepared in 0.2 M phosphate buffer of pH 8 were passed through the reactor at flow rates of 0.5 to 2 ml min^{-1}. The percentage hydrolysis of urea to ammonia was also studied at different temperatures. From the results, the space time of the reactor (W/F) was calculated at different temperatures, where W = Weight of immobilized enzyme (Urease-PVA-F) (g) and F = Volumetric Flow rate (ml min^{-1}).

Operational stability of the immobilized enzyme reactor was determined by operating the reactor continuously at constant temperature (50° C) and 0.5 ml min^{-1} flow rate for varying time periods and measuring the residual activity of the immbolized enzyme periodically.

The thermal deactivation of the immobilized enzyme reactor was examined by monitoring the reactor performance at constant temperatures (40-60° C) for longer periods ranging from 30 min to 3 h incubation time. The deactivation rate constant (K_d) and deactivation energy (ΔE_d) of the system was calculated by using the method described in Section 2.4.8.e.

3.5 Results and Discussion

Synthesis and characteristic properties of the polymeric supports used for the immobilization of urease are already discussed in detail in Chapter-2.

3.5.1 Activation of Support

Among the various enzyme immobilization techniques covalent coupling of the enzyme to the support is popular due to higher accessibility, reusability and stability of the bound enzyme[94]. In addition, no leaching of enzyme takes place during repeated uses as binding forces are stronger than those in adsorption or ionic binding.

In this Chapter, the preparation of immobilized urease, characterization of immobilized system and its use in continuous hydrolysis of urea using fixed bed reactor are discussed. For the preparation of immobilized urease, we have used covalent coupling of urease to SRF, PVA-F and chitosan supports. The results obtained are given in Table 3.1. It was observed that ~ 2.78 u/g urease was coupled to SRF through cynuric chloride, p-benzoquinone and p-tolylsulphonyl chloride activation. But % retention of immobilized enzyme activity was negligible, whereas the PVA-F system showed good coupling with greater retention of urease activity through cynuric chloride activation. However, chitosan activated through glutaraldehyde did not show any activity for the coupled enzyme urease. This may be due to the reaction of glutaraldehyde with the urease - SH group, which is essential for catalytic activity. Hence further studies were carried out by activating PVA-F with cynuric chloride. The urease PVA-F system retained 70-80% enzyme activity from 8.2 u/g urease which was coupled to PVA-F at pH 8 in 0.2 M phosphate buffer. In the previous Chapter we have observed that ß-galactosidase could retain its activity after immobilization on PVA-F, chitosan and SRF resin. This indicates that there may be a structure property relation between enzyme and the support . However, it is difficult to make any definite remarks about the possible pattern observed in our study.

3.5.2 Optimization of Conditions for Immobilization of Urease

Various coupling conditions were utilized for the better coupling and retention of urease activity after immobilization. Different parameters such as pH, coupling time and concentration of enzyme were optimized accordingly for the PVA-F system.

The results obtained in the study of effect of pH of coupling medium on the extent of enzyme immobilization and retention of its activity are given in Fig 3.2. Maximum

Table 3.1 Immobilization of urease on polymeric supports

Method of Activation	SRF		(PVA - F)			Chitosan	
	Protein coupled (u/g)	Active protein (u/g)	Protein coupled (u/g)	Active protein (u/g)	Retention of activity (%)	Protein coupled (u/g)	Active protein (u/g)
p-Benzoquinone	2.78	Inactive	13.9	2.9	21	-	-
Cynuric chloride	2.78	Inactive	8.2	6.3	76	-	-
p-tolyl sulponyl chloride	2.78	Inactive	8.3	2.5	30	-	-
Glutaraldehyde	-	-	-	-	-	4.1	Inactive

Coupling time 18h at 278° K in phosphate buffer of pH 8: amount of supports used 200 mg, enzyme used 15 u (15 mg).

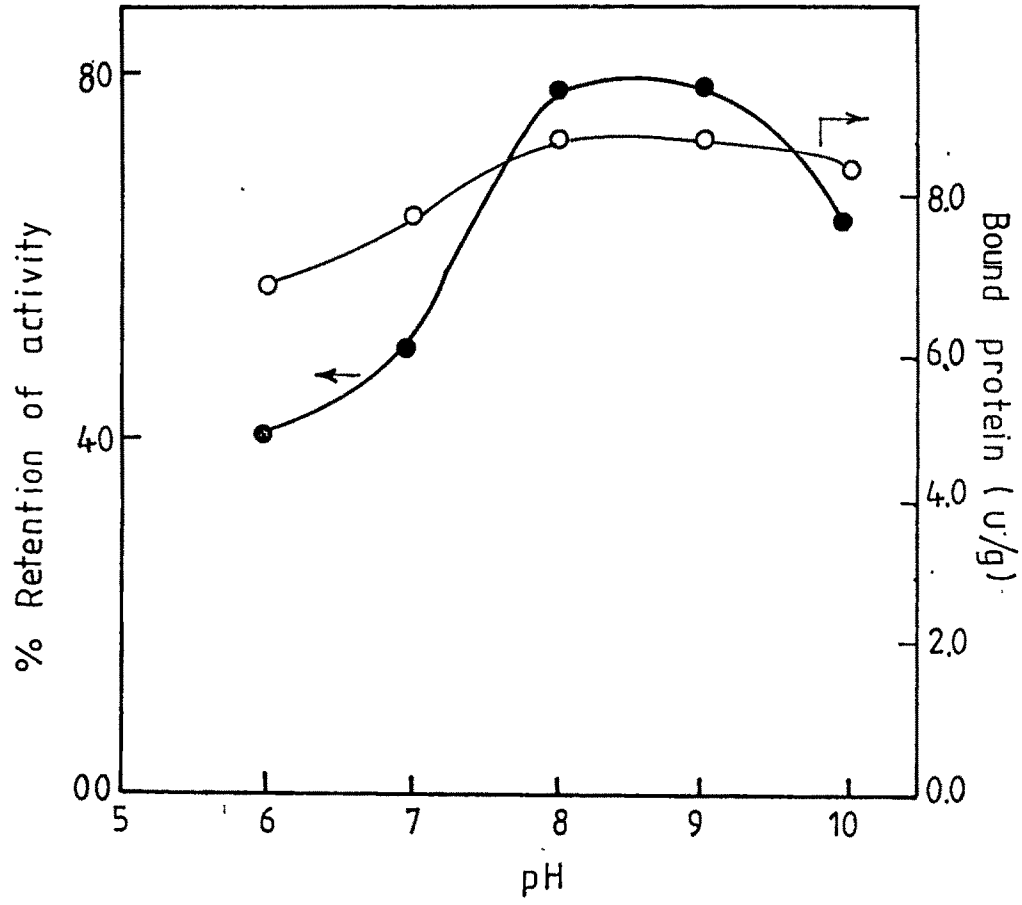

Fig. 3.2 Effect of pH of the coupling medium on immobilization
(○) Bound protein, (●) % retention of activity.
Temperature 278°K, time 18 h, enzyme concentration 15 u(15 mg), support -PVA-F 0.2 g

coupling and retention of enzyme activity was observed to be at pH 8-9 for PVA-F support activated with cynuric chloride.

The results obtained in the study of effect of coupling time on the extent of enzyme immobilization are given in Fig 3.3. It was observed that almost quantitative coupling of urease takes place within 3-4 h time with 70-80% retention of activity.

The effect of enzyme concentration on immobilization was studied by using 25-150 units enzyme concentration per gm of support. From the results obtained (Fig 3.4), it was observed that enzyme loading capacity goes on increasing as the concentration of enzyme increases up to 75 units/g support. Further increase in urease concentration does not increase the extent of coupled protein but decreases the retention of enzyme activity. This may be attributed to the substrate diffusion limitation as well as with increasing concentration of enzyme on support, some of the active sites are either utilized in the coupling process or become inaccessible due to coiling of enzyme. Similar trend was observed by us when pepsin was immobilized on polymeric supports[95,96].

The immobilized enzyme was further compared for its pH, thermal, storage, solvent, and kinetic stability with the free enzyme.

3.5.3 Stability of immobilized and free urease

(a) pH activity profile

pH activity profile is an important criteria in the application of immobilized enzymes. Change in the enzyme structure on immobilization results into change in the microenvironment and hence nature of pH activity profile of the enzyme. However, in the present study of pH dependence of activity of free and immobilized urease it was observed that (Fig 3.5) no change in the pH range takes place indicating that the enzyme has not undergone any structural changes during immobilization. However, retention of enzyme activity showed improvement on immobilization particularly at lower and higher pH. Whereas Dumitru *et al*[91] have reported that coupled urease loses its activity intensively when compared to the free enzyme. They also observed that even a little change in pH results in change in enzymatic activity of free as well as immobilized enzymes. In our study the pH change is less critical.

(b) Temperature activity profile and thermal stability

The effect of temperature on the activity of free and immobilized urease was studied over 30-70° C. From the results given in the Fig 3.6, it was observed that at lower temperature

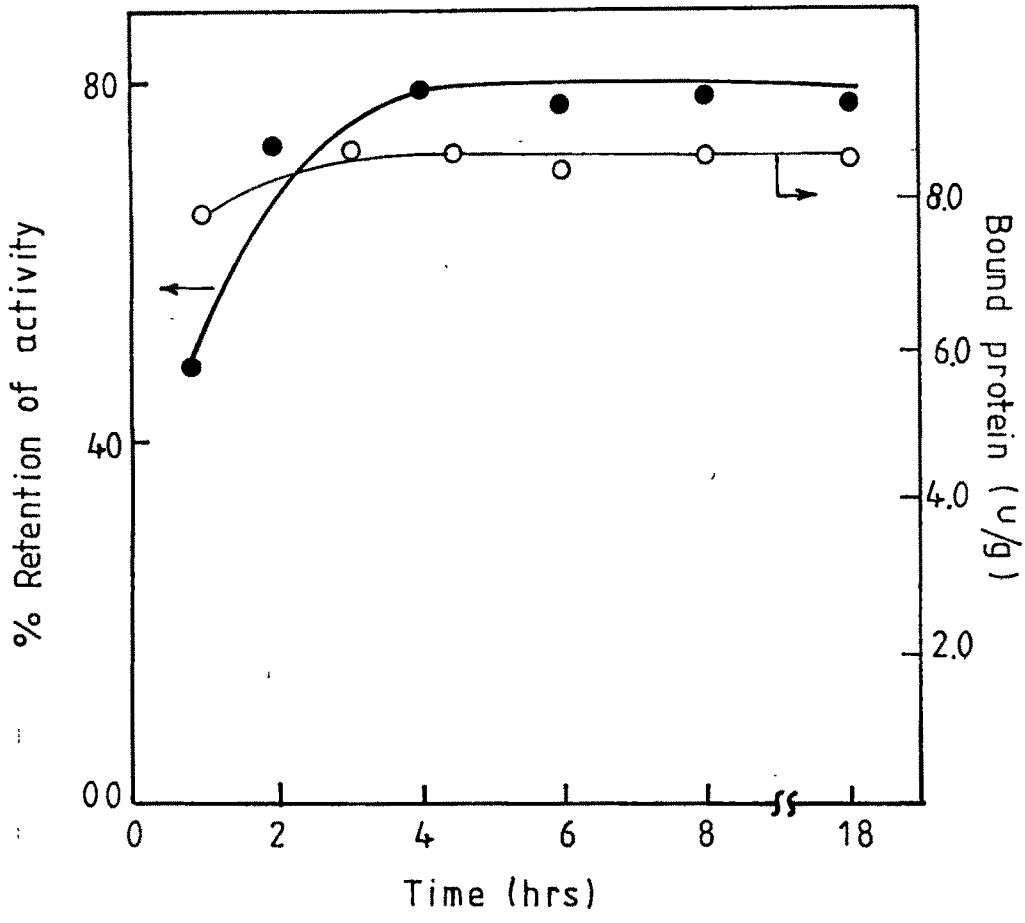

Fig. 3.3 **Effect of time on extent of immobilization**
(○) Bound protein, (●) % retention of activity
Temperature 278°K, enzyme concentration 15 mg, support -PVA-F 0.2 g, pH- 8,

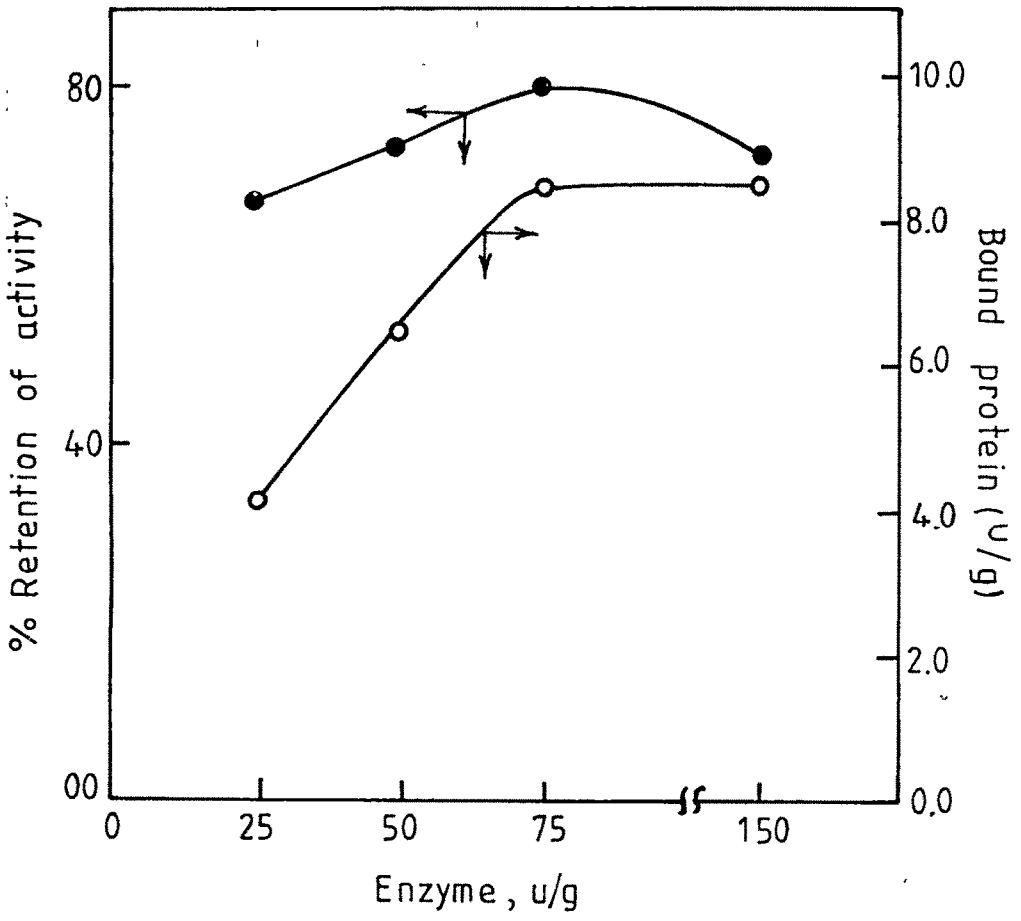

Fig. 3.4 **Effect of enzyme concentration on immobilization Temperature 278°K, Time 18 h, pH 8.**

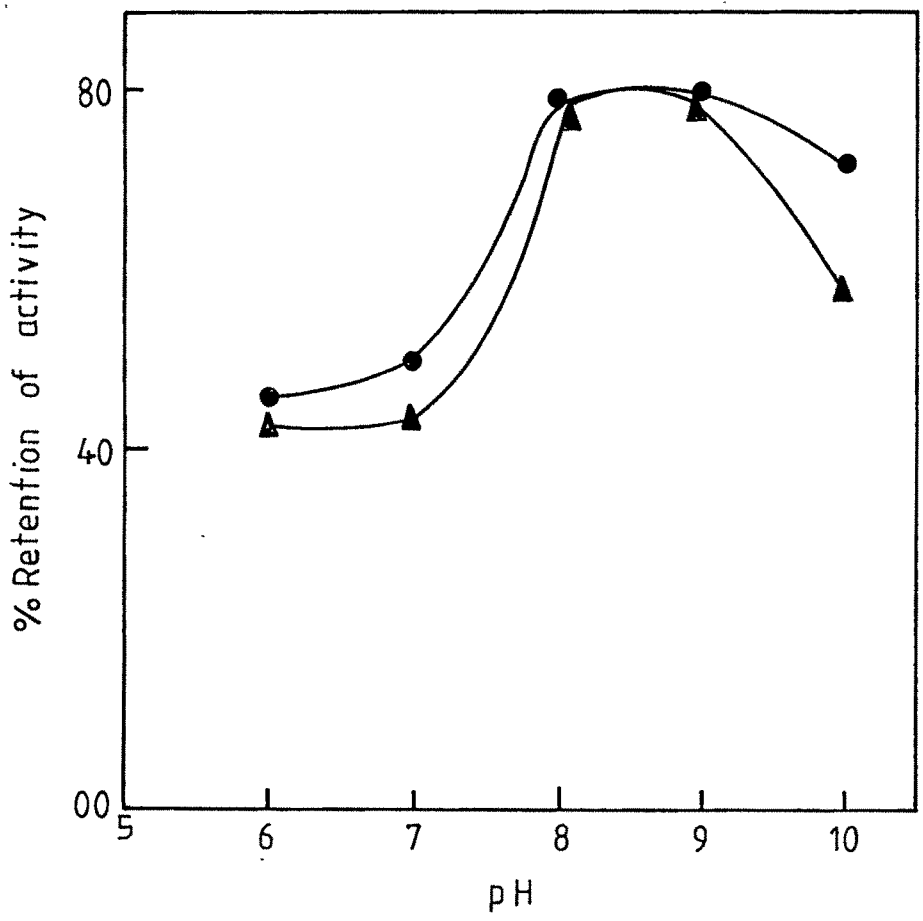

Fig. 3.5 pH activity profile for soluble and immobilized urease (●) Urease on PVA-F, (▲) Free Urease.

there is no significant difference in the activity of free and coupled enzyme. However, free enzyme loses its activity very rapidly above 50° C whereas urease PVA-F system was thermally stable up to 70° C. The improved stability of the immobilized urease can be attributed to the covalent binding of enzyme to the matrix. From temperature dependence data energy of activation (ΔEa) of free and immobilized urease was calculated by using the method described in Chapter-2 and the results are tabulated in Table 3.2. It was observed that activation energy for the free urease is less than that for the urease immobilized on PVA-F. This may be because in the free enzyme system reaction is not diffusion controlled whereas in the case of immobilized one reaction being diffusion controlled activation energy was observed to be higher.

To study the thermal deactivation of the free and immobilized systems they were further heated at 60° C and 70° C for a longer time durations. The plot of A/Ao vs time as described in Chapter-2 (Fig 3.7) indicates that free enzyme loses its activity completely after 150 min incubation. Whereas immobilized enzyme retained its activity over 3 h incubation time. From the results, thermodeactivation constant (K_d) was calculated by using an equation described earlier [97]. The results are given in Table 3.2. An overall decrease in thermodeactivation was observed for the immobilized system even at 60° C and 70° C.

(c) Kinetics of enzymatic reaction

The rate of hydrolysis in all enzymatic reactions is expected to be the first order. The initial reaction rates were determined at different urea concentrations ranging from 0.5 mM to 4 mM. Michaelis constant (K_m) and maximum reaction velocity (V_m) for the free and immobilized enzymes were calculated from Lineweaver-Burk plots (Fig 3.8.a and 3.8.b) as discussed earlier. The K_m value for the immobilized enzyme was found to be lower when compared to the native enzyme (Table 3.2). The decrease in the K_m value for IME indicates the limitation of diffusion resistance to the reaction or partially kinetically controlled reactions. The decrease in the K_m values on immobilization of enzymes on polymeric materials is known to be frequently observed phenomenon [98]. However, the reaction velocity of immobilized urease was observed to be lower than that of free urease. Angiuro *et al* [99] have also observed a decrease in K_m when various enzymes were immobilized onto polysaccharide - GMA graft copolymers.

(d) Stability towards repeated use

Stability of immobilized urease in the continuous reactions is very important for the

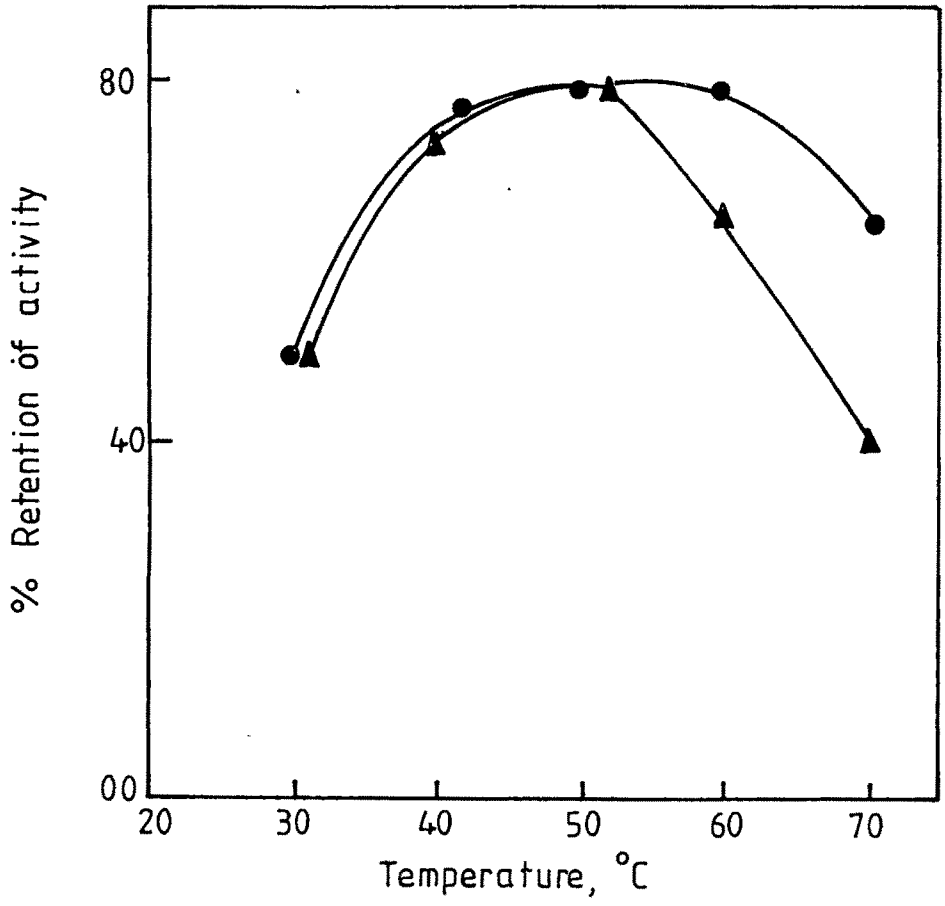

Fig. 3.6 **Temperature activity profile for free and immobilized urease (●) Urease on PVA-F, (▲) Free Urease**

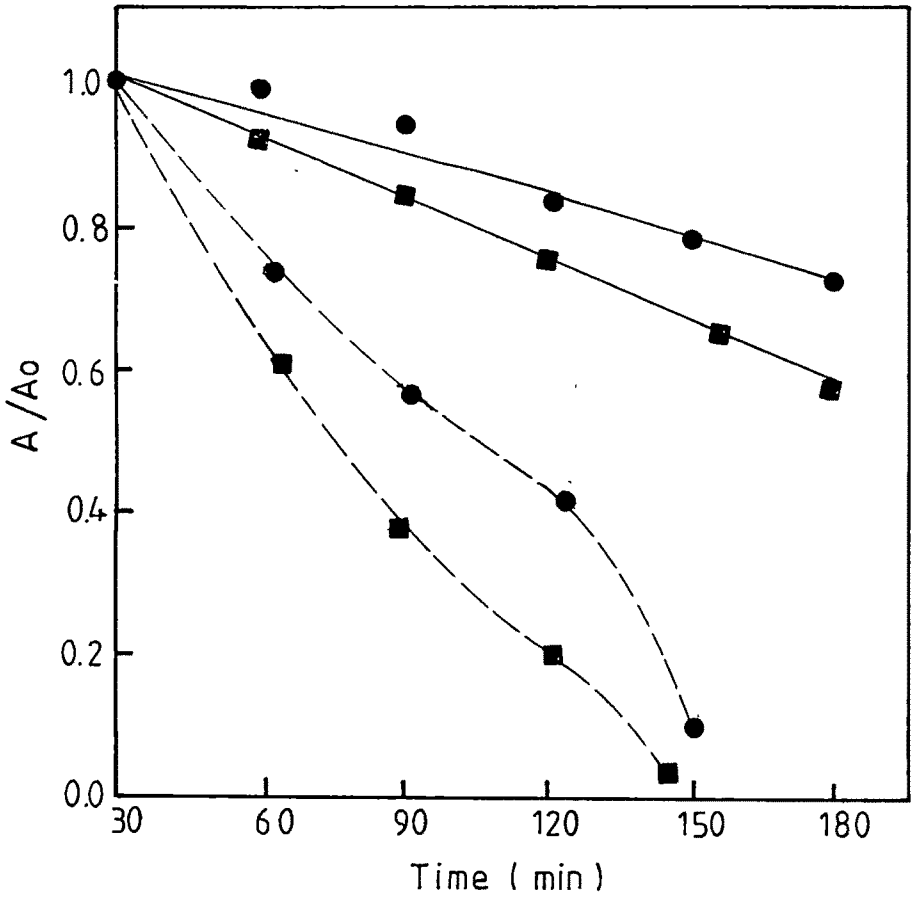

Fig. 3.7 Kinetics of thermal deactivation of free and immobilized urease At 60°C (●——●) Urease on PVA-F, (●-----●) Free Urease At 70°C, (■——■) Urease on PVA-F, (■-----■) Free Urease.

Table 3.2 Kinetics and thermodynamic parameters for free and immobilized Urease

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}]
			60° C	70° C	
Urease	2 60	0.38×10^{-4}	2.11×10^{-3}	3.40×10^{-3}	3.70
Urease-PVA-F	0.71	0.24×10^{-4}	1.98×10^{-3}	2.04×10^{-3}	4.95

Table 3.3 Energy of activation (ΔE_a) for immobilized Urease in fixed bed reactor.

System	Substrate Concentration [mM]	Energy of activation ΔE_a [Kcal $mole^{-1}$]
Urease-PVA-F	05	3 69
	10	4 20
	15	4.50
	20	6 50
	25	6 69

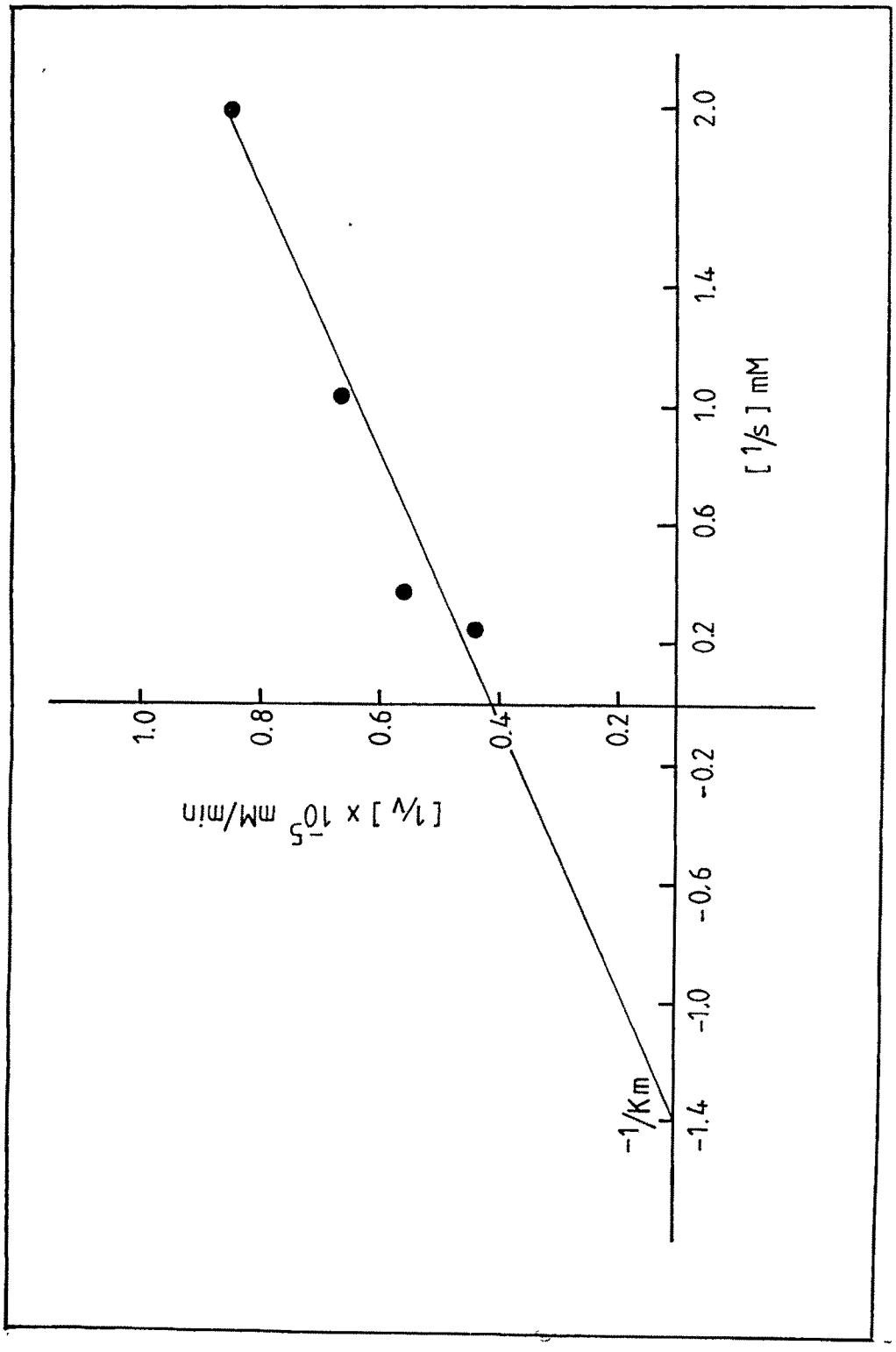

Fig. 3.8.a Lineweaver-Burk plot for immobilized urease
Temperature 37°C, pH 8, Time 30 min.

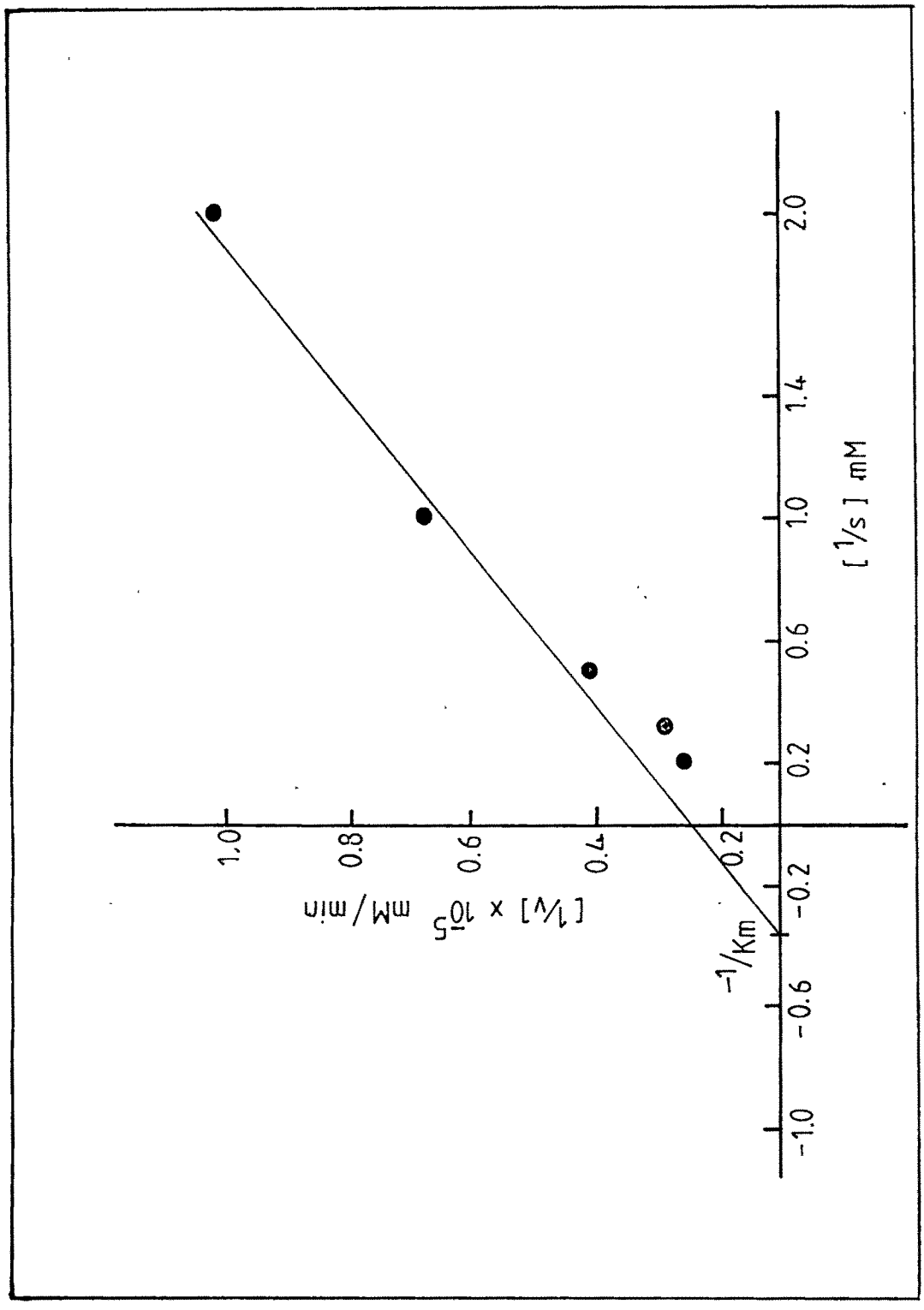

Fig. 3.8.b Lineweaver-Burk plot for free urease
Temperature 37°C, pH 8, Time 30 min,

applications as it is subjected to continuous hydrolysis reactions. The results from Fig 3.9 show that ~ 50% residual activity of urease was retained evenafter 5 repeated runs.

(e) Solvent stability

The practical importance of solvent stability studies for the immobilized enzyme is described in Chapter-2. Accordingly we have studied, the hydrolysis of urea by the immobilized enzyme system in 1-5 M methanol, ethanol and propanol. The results are summarized in Fig 3.10. It was observed that free and immobilized urease have similar stability towards water miscible solvents.

(f) Storage stability

Storage stability of soluble and insolubilized urease was determined by storing them at room temperature (30° C) in 0.2 M phosphate buffer of pH 8. From the results given in Fig 3.11 it was observed that after 45 days free enzyme loses its activity completely whereas immobilized urease still retains ~ 50% of its activity.

3.5.4 Continuous Hydrolysis of Urea by Using Urease-PVA-F System in a Fixed Bed Reactor

Various types of polymeric supports with immobilized urease, and different types of biochemical reactors have been used for the continuous hydrolysis of urea as reported earlier[100]. The types of reactors such as batch reactor[101], continuous stirred tank reactor[102], packed bed reactor[80] are widely used. Because immobilized enzymes can be reused and operated in a continuous process, enzyme immobilization and immobilized enzyme reactors have attracted considerable attention in industrial applications[77].

In the present study thorough investigation of the hydrolysis of urea using immobilized urease was carried out. As discussed earlier we have observed that the PVA-F system shows very good retention of coupled enzyme activity, stability and durability. Other two supports under study, chitosan and SRF showed comparatively inferior properties in terms of retention of urease activity and hence were not used for the study of hydrolysis of urea using packed bed reactor.

► Column Operation for Hydrolysis of Urea

Different parameters were optimized for column operation for the continuous hydrolysis of urea in a fixed bed reactor.

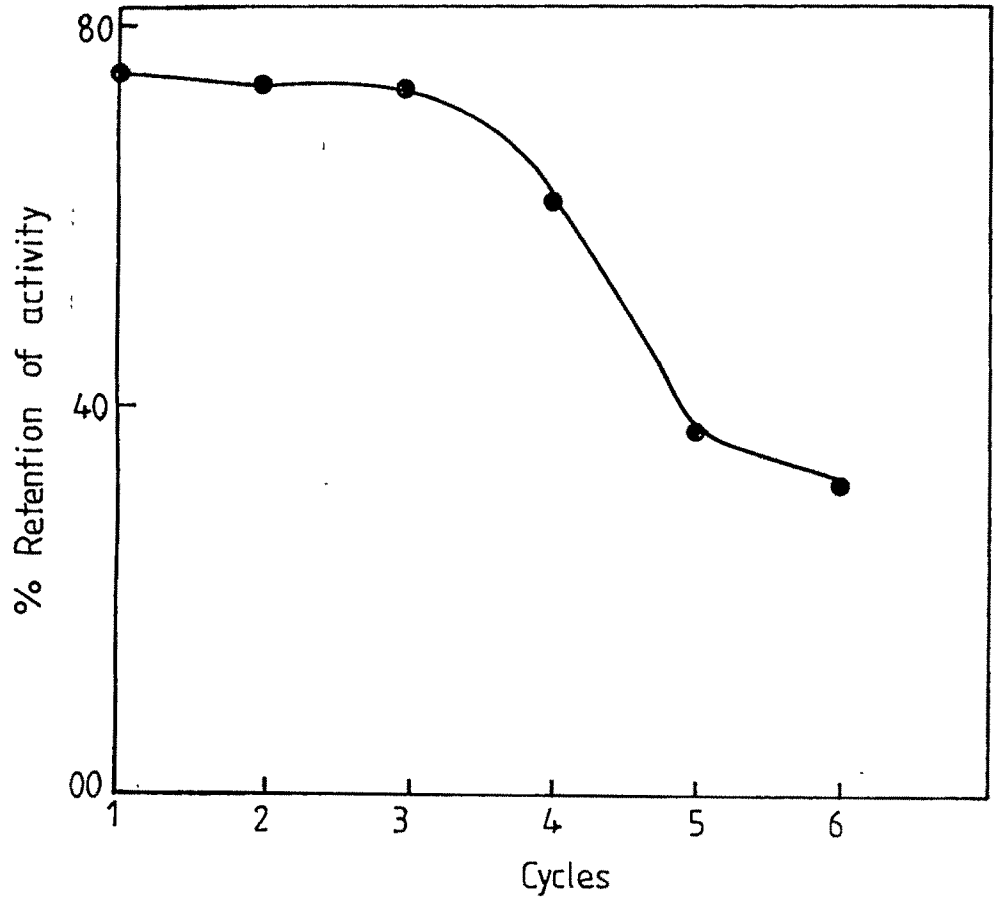

Fig. 3.9 Reusability of Immobilized urease
Temperature 37°C, pH 8, Time 30 min

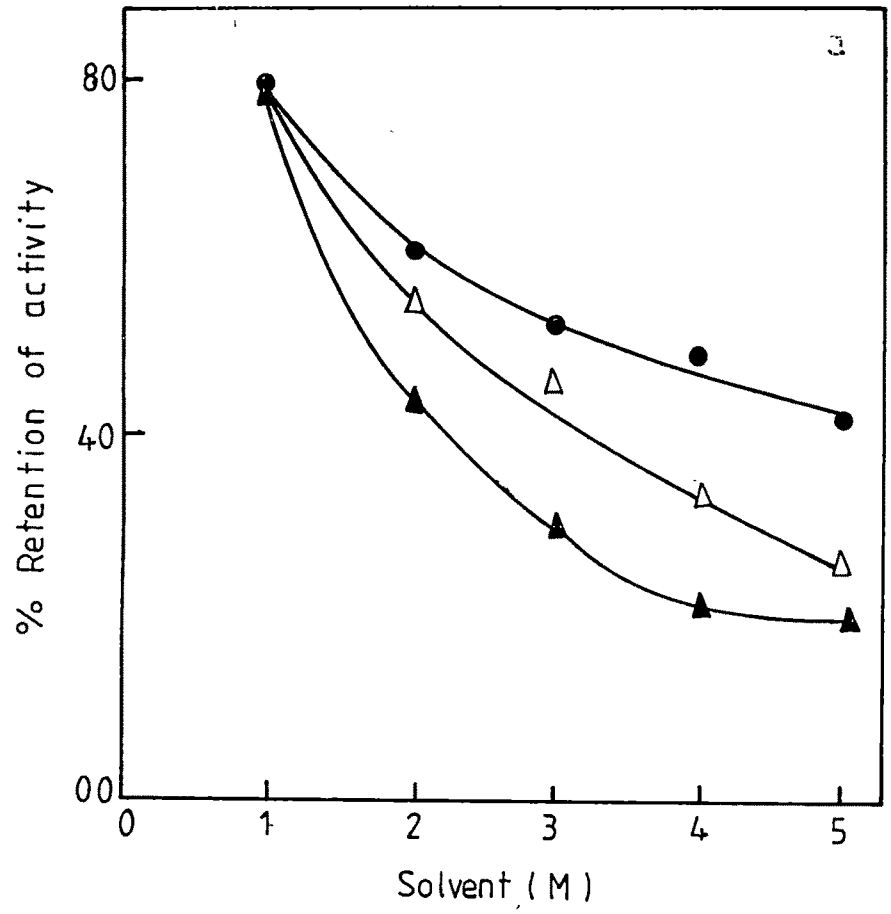

Fig. 3.10.a Effect of solvent on the activity of Immobilized Urease (●) Methanol, (Δ) Ethanol, (▲) Propanol

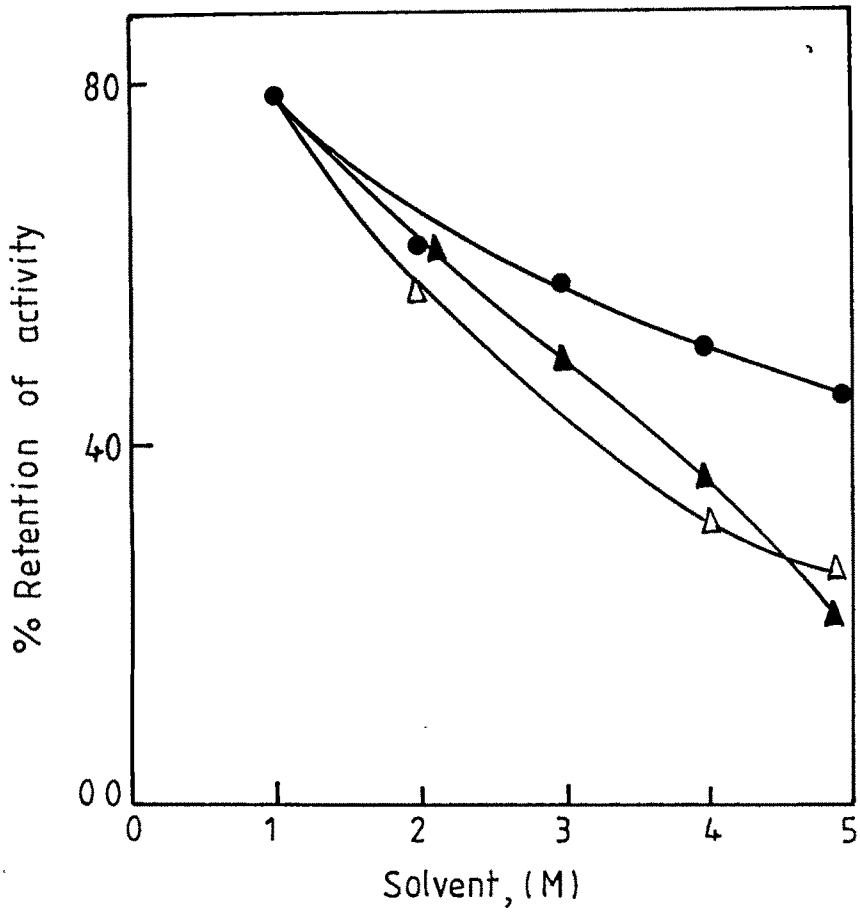

Fig. 3.10.b Effect of solvent on the activity of free Urease (●) Methanol, (Δ) Ethanol, (▲) Propanol

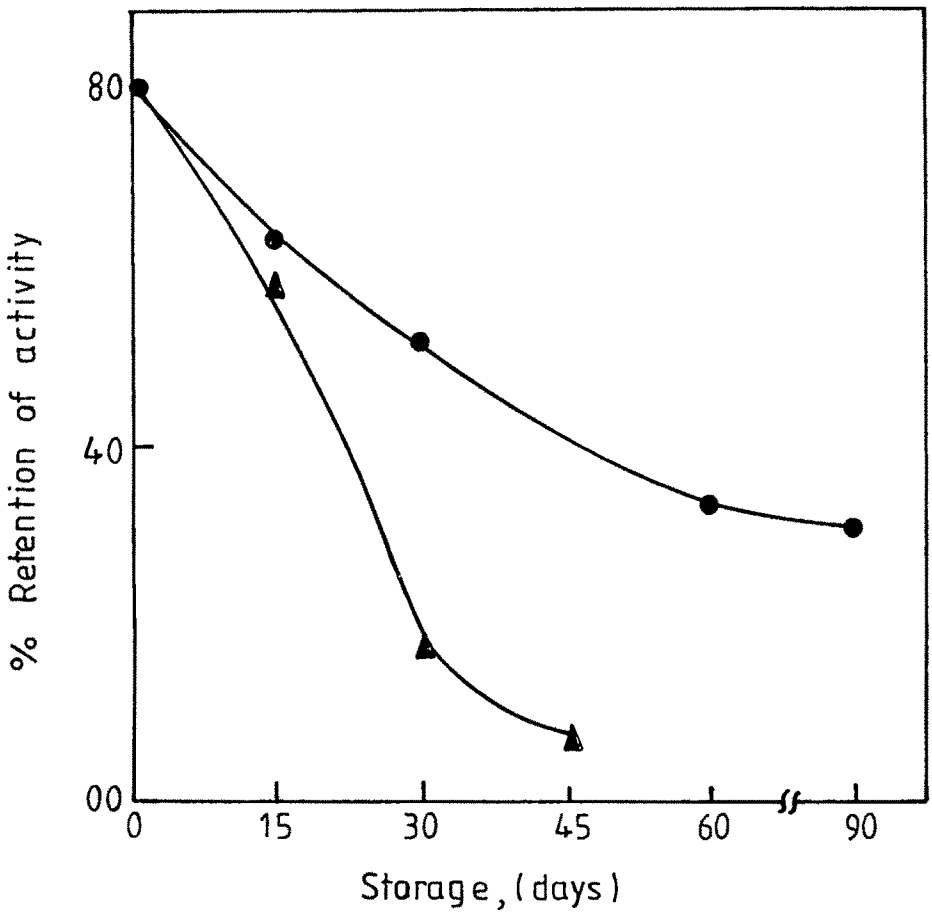

Fig. 3.11 Storage stability of free and immobilized urease
Temperature 37°C pH 8
(●) Urease coupled to PVA-F, (▲) Urease

(a) Effect of temperature

Effect of temperature on hydrolysis of urea using immobilized urease in a fixed bed reactors was studied at various temperatures from 40-70° C. Different urea concentrations (5 mM to 25 mM) were used for the hydrolysis and the hydrolysis rates were compared. The results obtained at different temperatures and flow rates are illustrated in Fig 3.12-3.17. It was observed that rate of hydrolysis does not change considerably with increasing temperature. However, it can be seen that optimum temperature of the hydrolysis was 60° C at 0.2 M phosphate buffer of pH 8. It was also observed that with increasing flow velocity of the substrate % hydrolysis decreases rapidly due to decreased contact time with the immobilized enzyme.

From temperature study, we have calculated energy of activation (ΔEa) as discussed earlier in Section 2.4.6.b. The results obtained are summarized in Table 3.3. It was observed that activation energy increases with increasing substrate concentration. Lower activation energy at the lower substrate concentration indicates pore diffusions in an immobilized urease with the increasing temperature. In 1980 Kitano *et al*[61] used urease immobilized on PVA hollow fibres for urea hydrolysis. The activation energy of the system was found to be smaller than that of the free enzyme. In our system also activation energy of immobilized enzyme was observed to be smaller than that of free enzyme. However, the value of activation energy of the urease immobilized on PVA-F support was observed to be smaller than that reported for PVA hollow fibres. This can be attributed to the difference in nature and surface area of the polymeric supports used for the immobilization.

(b) Effect of urea concentration on hydrolysis

The results obtained in the study of variation in urea concentration on the hydrolysis extent are given in Fig 3.18. The rate of hydrolysis was studied at 40, 50 and 60° C. It was observed that % conversion goes on decreasing with the increasing substrate concentration. Maximum urea conversion was obtained at 5-10 mM concentration of urea. Increase in substrate concentration decreases enzymatic activity due to the inhibiting action of excess of ammonium ions produced during reaction[77].

(c) Substrate flow

The effect of substrate flow on the hydrolysis of urea by the immobilized urease was studied in the packed bed column reactor using 0.2 M phosphate buffer of pH 8. Hydrolysis of urea was studied at different flow rates (0.5 ml mn^{-1} to 2 ml min^{-1}) and

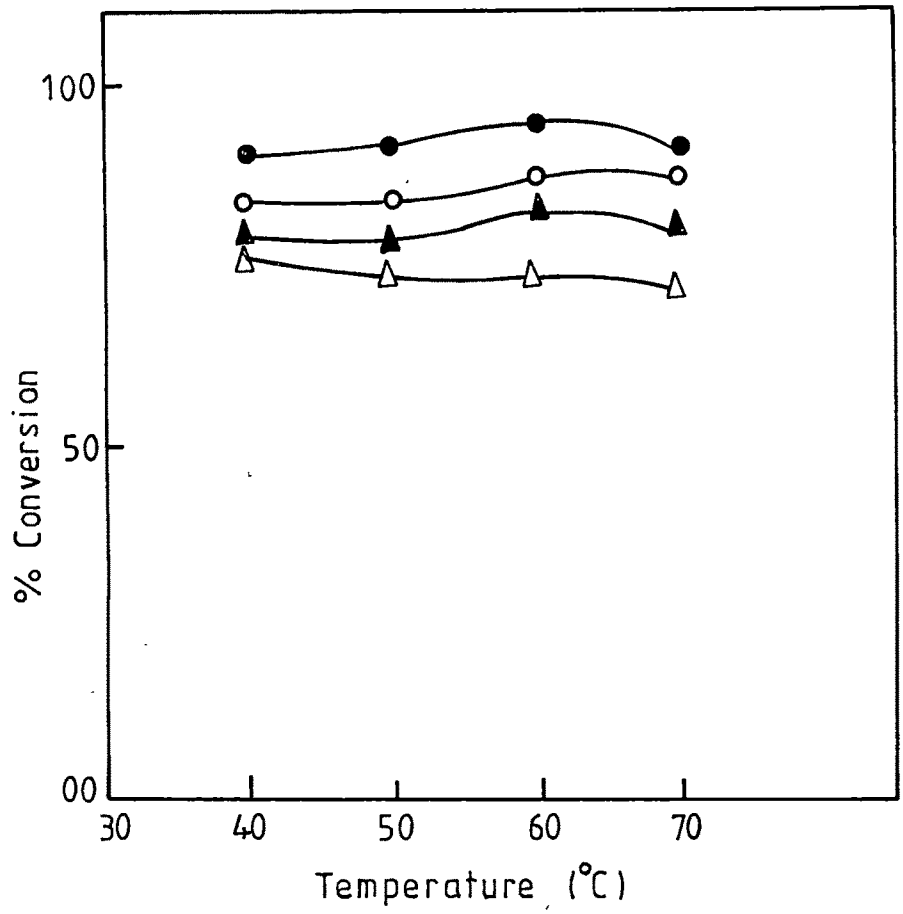

Fig. 3.12 Effect of temperature on urea hydrolysis in packed bed reactor Flow rates (●) 0.5 ml min^{-1}, (○) 1 ml min^{-1}, (▲) 1.5 ml min^{-1}, (Δ) 2 ml min^{-1}. Urea concentration 5 mM, pH 8.

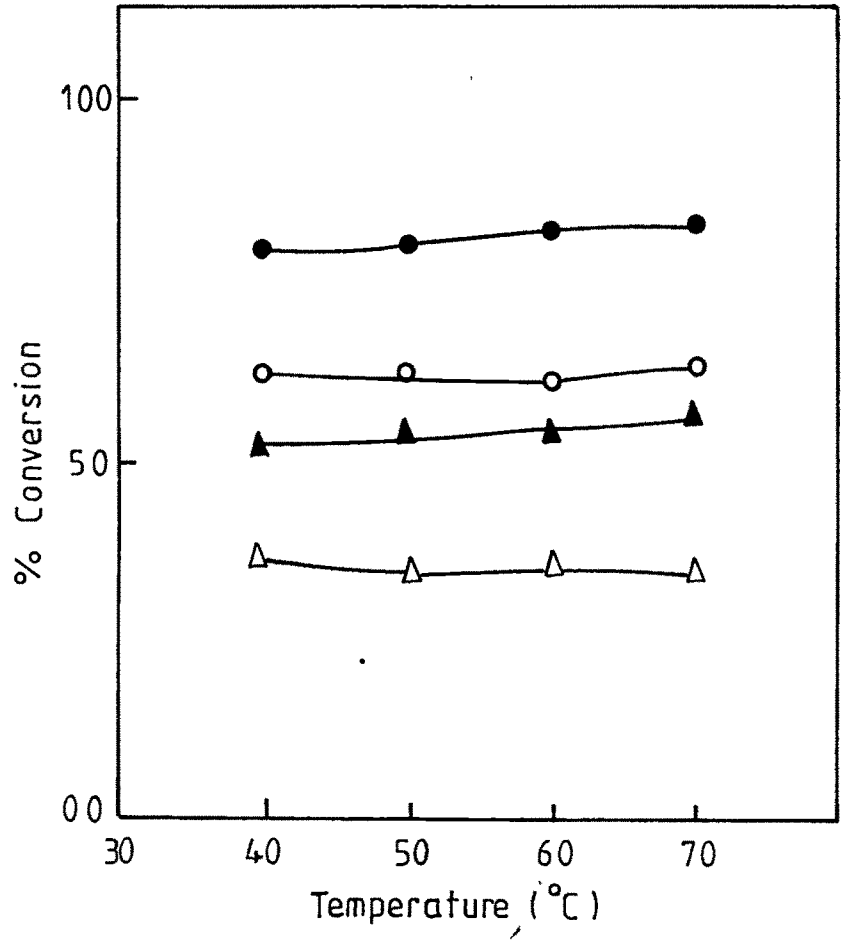

Fig. 3.13 Effect of temperature on urea hydrolysis in packed bed reactor
Flow rates (●) 0.5 ml min^{-1}, (○) 1 ml min^{-1}, (▲) 1.5 ml min^{-1}, (Δ) 2 ml min^{-1}
Urea concentration 10 mM, pH 8, .

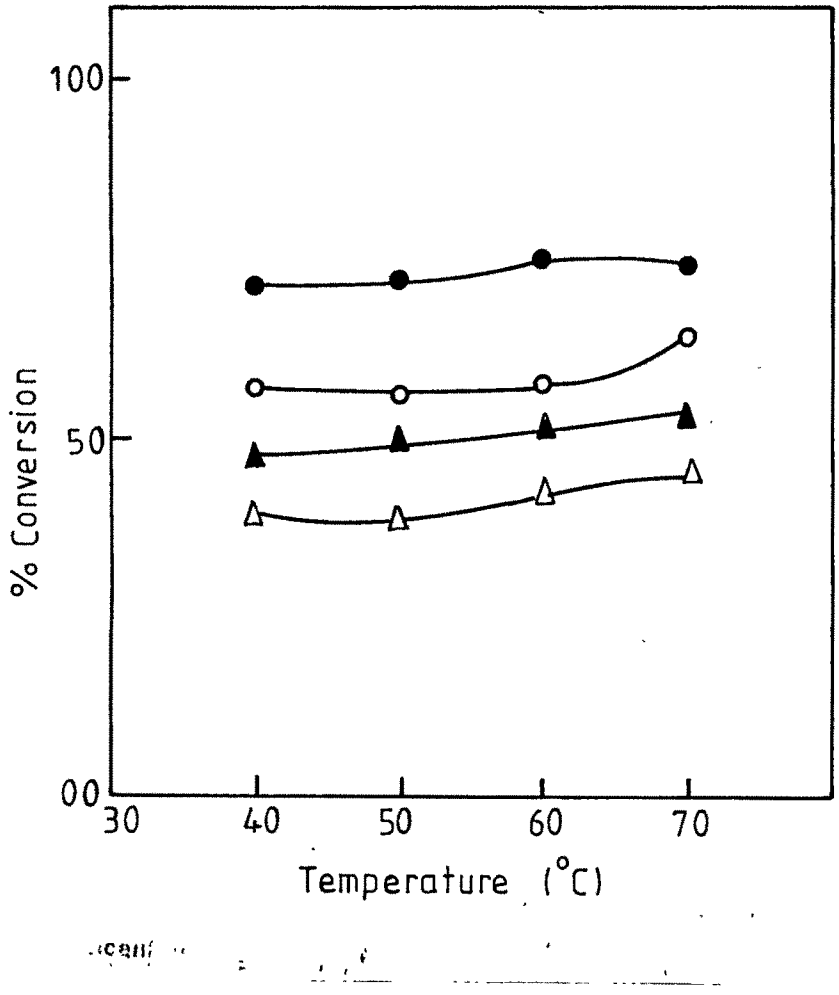

Fig. 3.14 Effect of temperature on urea hydrolysis in packed bed reactor
Flow rates (●) 0.5 ml min^{-1}, (○) 1 ml min^{-1}, (▲) 1.5 ml min^{-1}, (Δ) 2 ml min^{-1}
Urea concentration 15 mM, pH 8

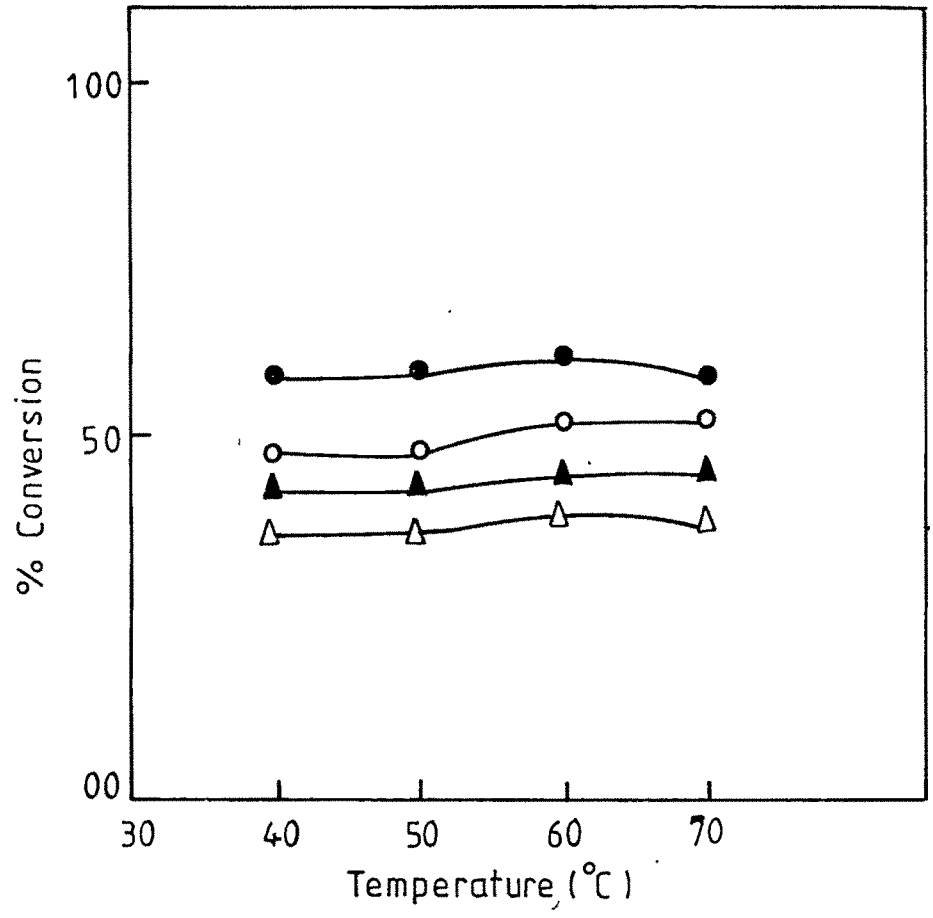

Fig. 3.15 Effect of temperature on urea hydrolysis in packed bed reactor
Flow rates (●) 0.5 ml min^{-1}, (○) 1 ml min^{-1}, (▲) 1.5 ml min^{-1}, (Δ) 2 ml min^{-1}
Urea concentration 20 mM, pH 8

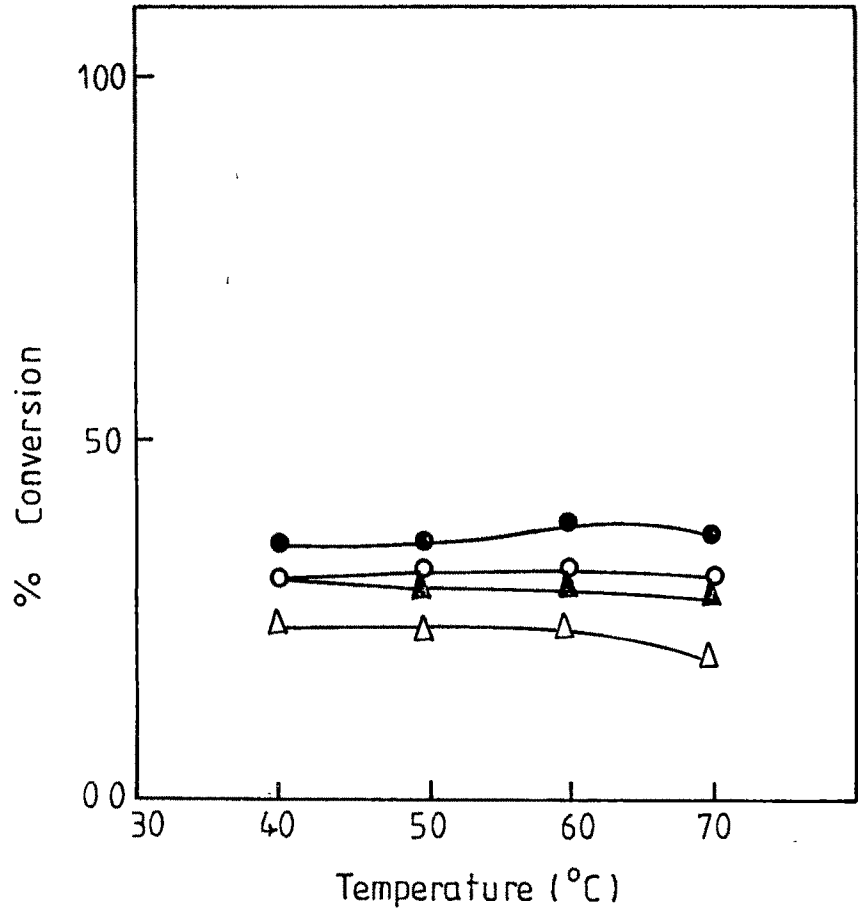

Fig. 3.16 Effect of temperature on urea hydrolysis in packed bed reactor
Flow rates (●) 0.5 ml min^{-1}, (○) 1 ml min^{-1}, (▲) 1.5 ml min^{-1}, (Δ) 2 ml min^{-1}
Urea concentration 25 mM, pH 8

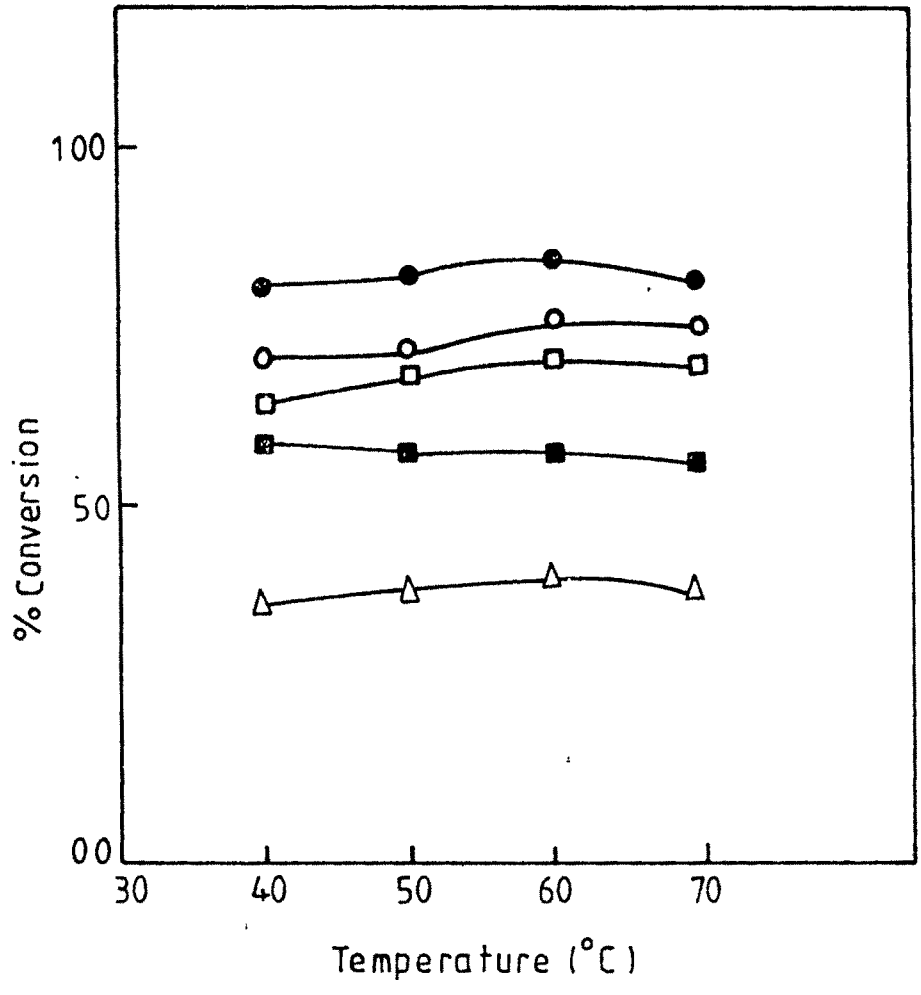

Fig. 3.17 Effect of temperature on urea hydrolysis at various concentrations of urea using free urease
(●) 5 mM, (○) 10 mM, (□) 15 mM, (■) 20 mM, (Δ) 25 mM.

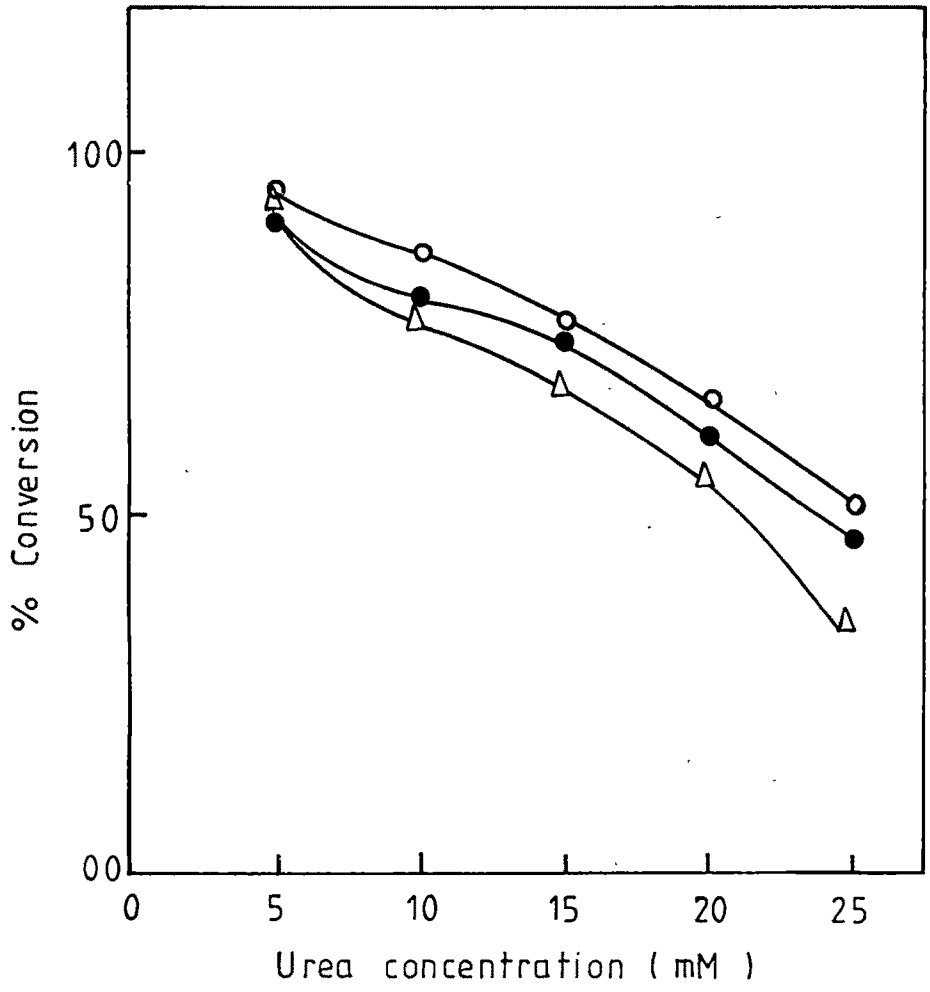

Fig. 3.18 Effect of urea concentration on hydrolysis
Flow rates 0.5 ml min^{-1}
At (Δ) 40, (●) 50, (○) 60° C.

different temperatures. The variation in conversion with space time is given in Figures (3.19-3.22). The space time of the reactor (W/F) was calculated from the weight of immobilized enzyme present in the reactor divided by the flow rates of the substrate. From the results we have observed that high space time results into high conversion of substrate, urea at all temperatures of the reactor except, at lower concentration of the substrate.

(d) Kinetics of urea hydrolysis

The hydrolysis of urea by immobilized urease in the packed bed reactor was conducted with various concentrations of substrate urea (5 mM to 25 mM). From the study, the Michaelis constant (K_m) and maximum reaction velocity (V_m) for urease immobilized on PVA-F was calculated from Lineweaver-Burk plots of 1/v vs 1/s as described earlier in Section 2.4.6.f. The reaction velocity 'V_{max}' was measured at different urea concentrations. From Lineweaver-Burk plots (Fig 3.23), K_m and V_m were calculated and the results are given in Table 3.4. It was observed that somewhat lower value of K_m for IME may be due to strong electrostatic attractions between the support polymer and the protein chain as well as a protein-substrate interaction.

(e) Thermal deactivation

Data from enzyme deactivation studies at 40, 50 and 60° C show a first-order reaction mechanism for thermal deactivation of immobilized urease. The plot of A/Ao (Fig 3.24) as described earlier, indicates the thermal deactivation of the immobilized urease in the column reactor at different temperatures (40-60° C) and various time intervals. From the data we have calculated deactivation rate constant (K_d) and energy of deactivation (ΔE_d) as described in Chapter-2 and the values obtained are given in Table 3.5. The deactivation rate constant (K_d) was found to increase with temperature and moderate value of deactivation energy shows immobilized urease is less critical of the temperature particularly at 40° C. Hence column operation is possible at this temperature for longer time periods without much deactivation of enzyme.

(f) Operational stability of immobilized urease in fixed bed reactor

The operational stability of the urease PVA-F system was determined by operating the reactor continuously at constant temperature 40° C and flow rate 0.5 ml min^{-1} for various time intervals and estimating urease activity periodically. The results obtained from the study are given in Fig 3.25. It is observed that urease PVA-F system is efficient even

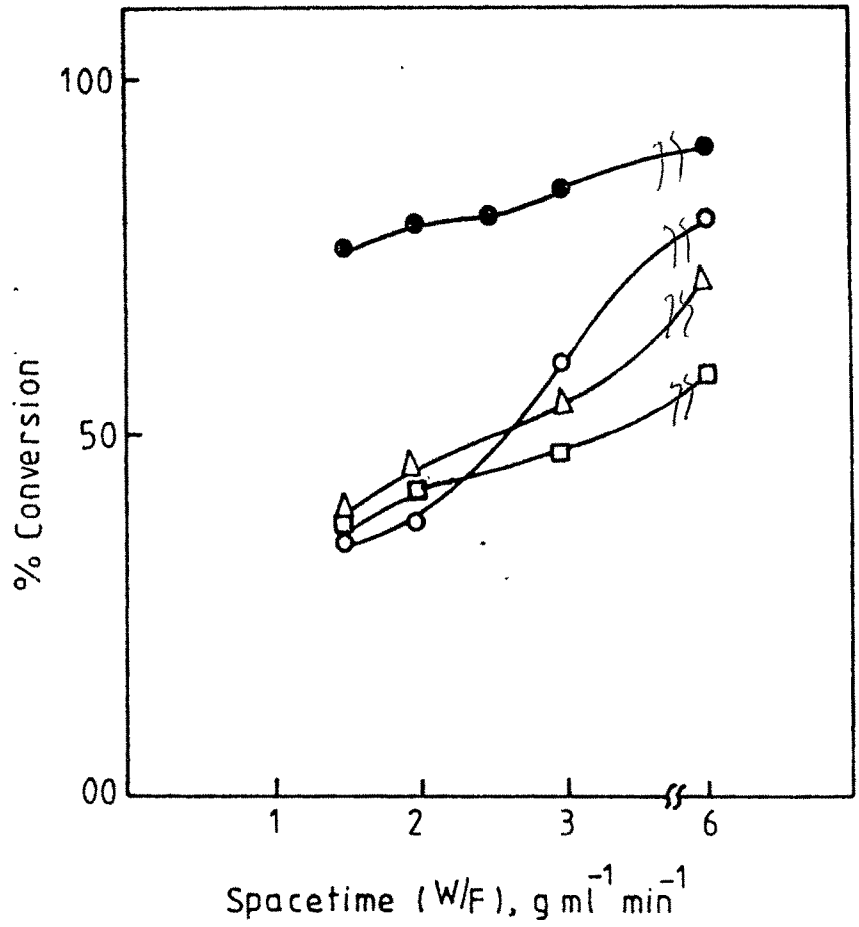

Fig. 3.19, Effect of substrate flow on the hydrolysis of urea at 40°C (●) 5 mM, (○) 10 mM, (Δ) 15 mM, (□) 20 mM.

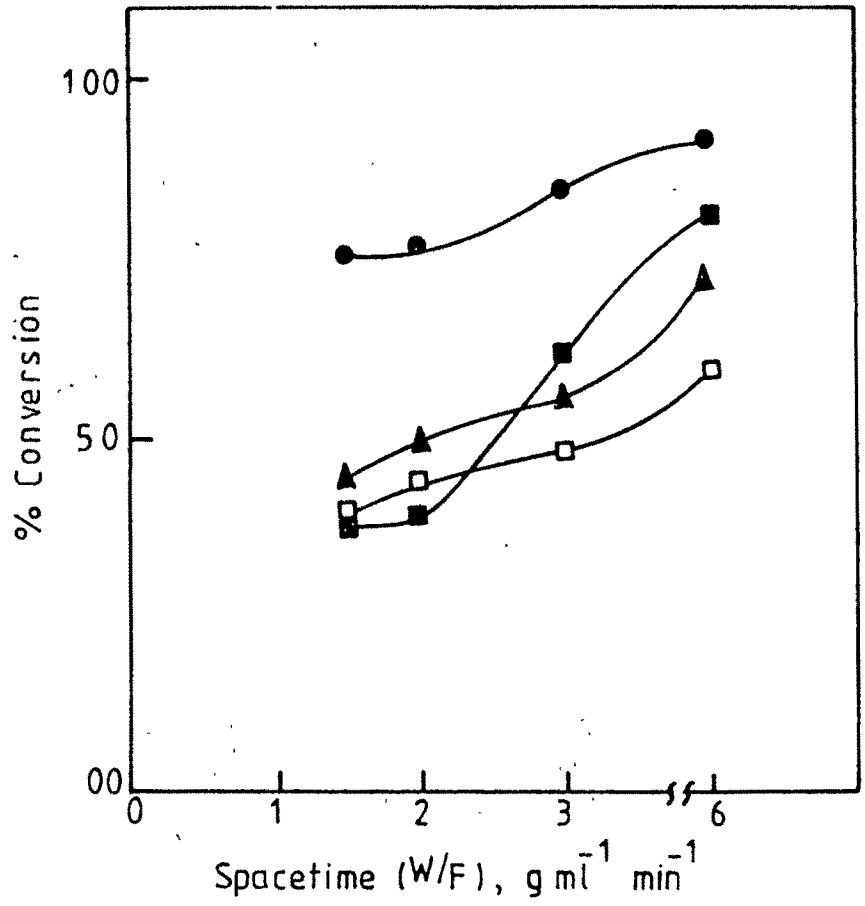

Fig. 3.20 Effect of substrate flow on the hydrolysis of urea at 50°C (●) 5 mM, (■) 10 mM, (▲) 15 mM, (□) 20 mM.

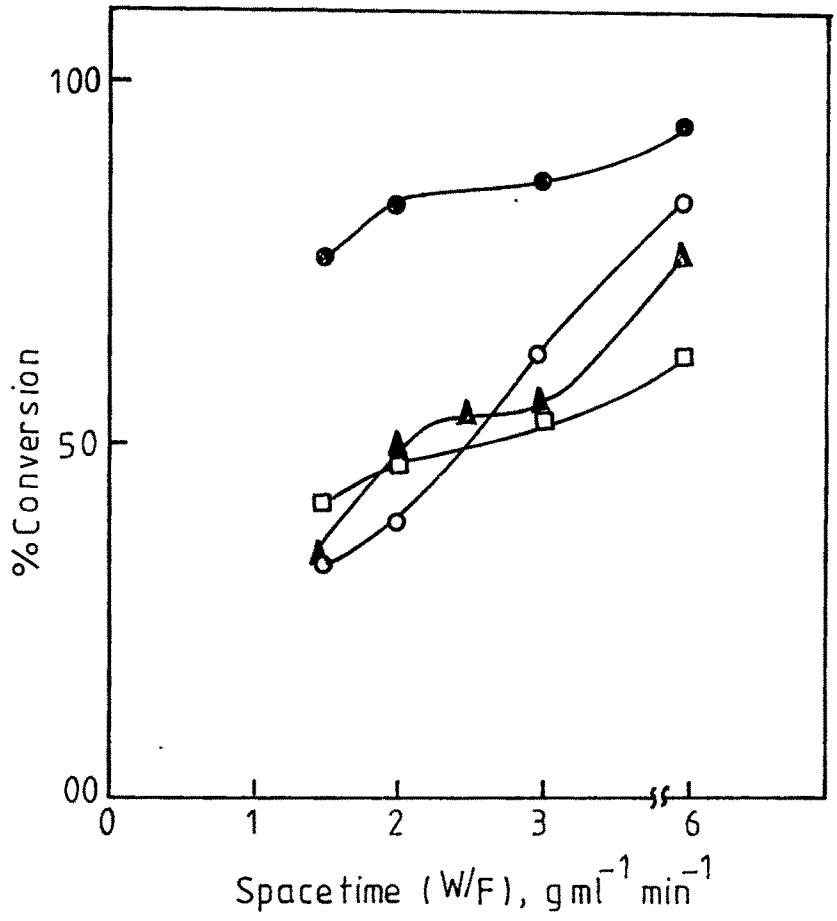

Fig. 3.21 Effect of substrate flow on the hydrolysis of urea at 60°C (●) 5 mM, (○) 10 mM, (▲) 15 mM, (□) 20 mM.

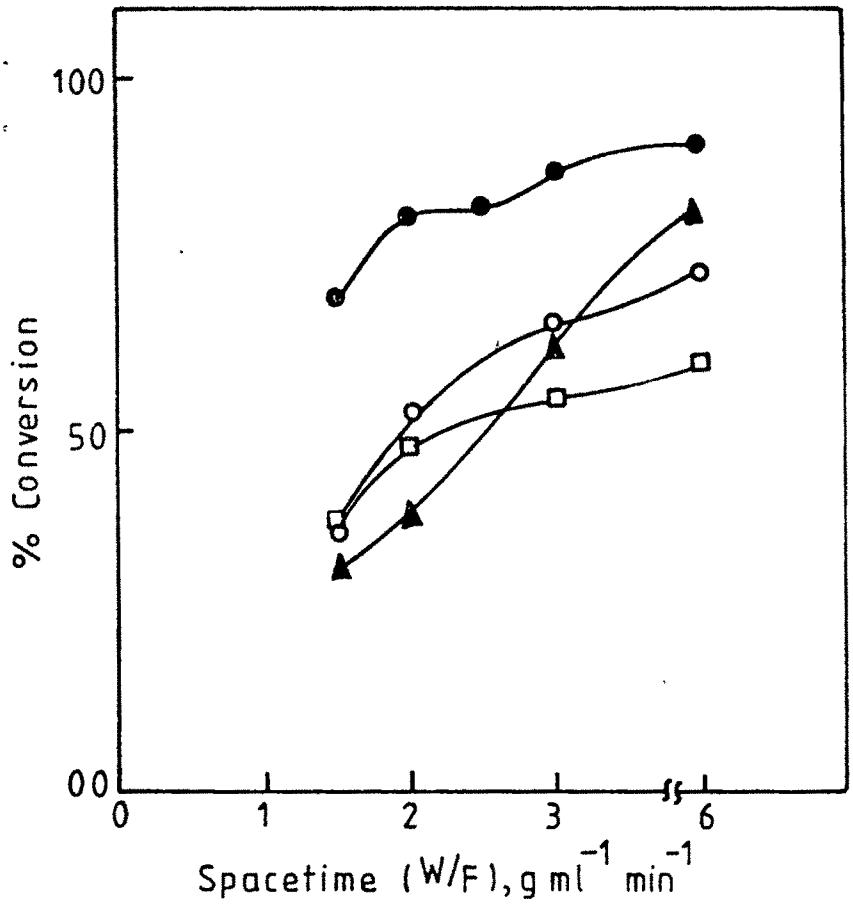

Fig. 3.22 Effect of substrate flow on the hydrolysis of urea at 70°C (●) 5 mM, (▲) 10 mM, (○) 15 mM, (□) 20 mM.

Table 3.4 Michaeli's constant (K_m) and maximum reaction velocity (V_{max}) for free and immobilized Urease for Urea hydrolysis in column process.

Systems	Michaeli's constant (K_m) [mM]	Maximum Reaction Velocity (V_{max}) [mM min^{-1}]
Urease-PVA-F	55	12.5×10^{-2}
Urease	71	14.2×10^{-2}

Table 3.5 Thermal deactivation rate constant (K_d) and deactivation energy (ΔE_d) for Urease-PVA-F in column process.

System	Temperature [° C]	Deactivation constant (K_d) [min^{-1}]	Deactivation energy (ΔE_d) [Kcal $mole^{-1}$]
Urease-PVA-F	40	3.2×10^{-3}	0.48
	50	3.6×10^{-3}	
	60	3.9×10^{-3}	

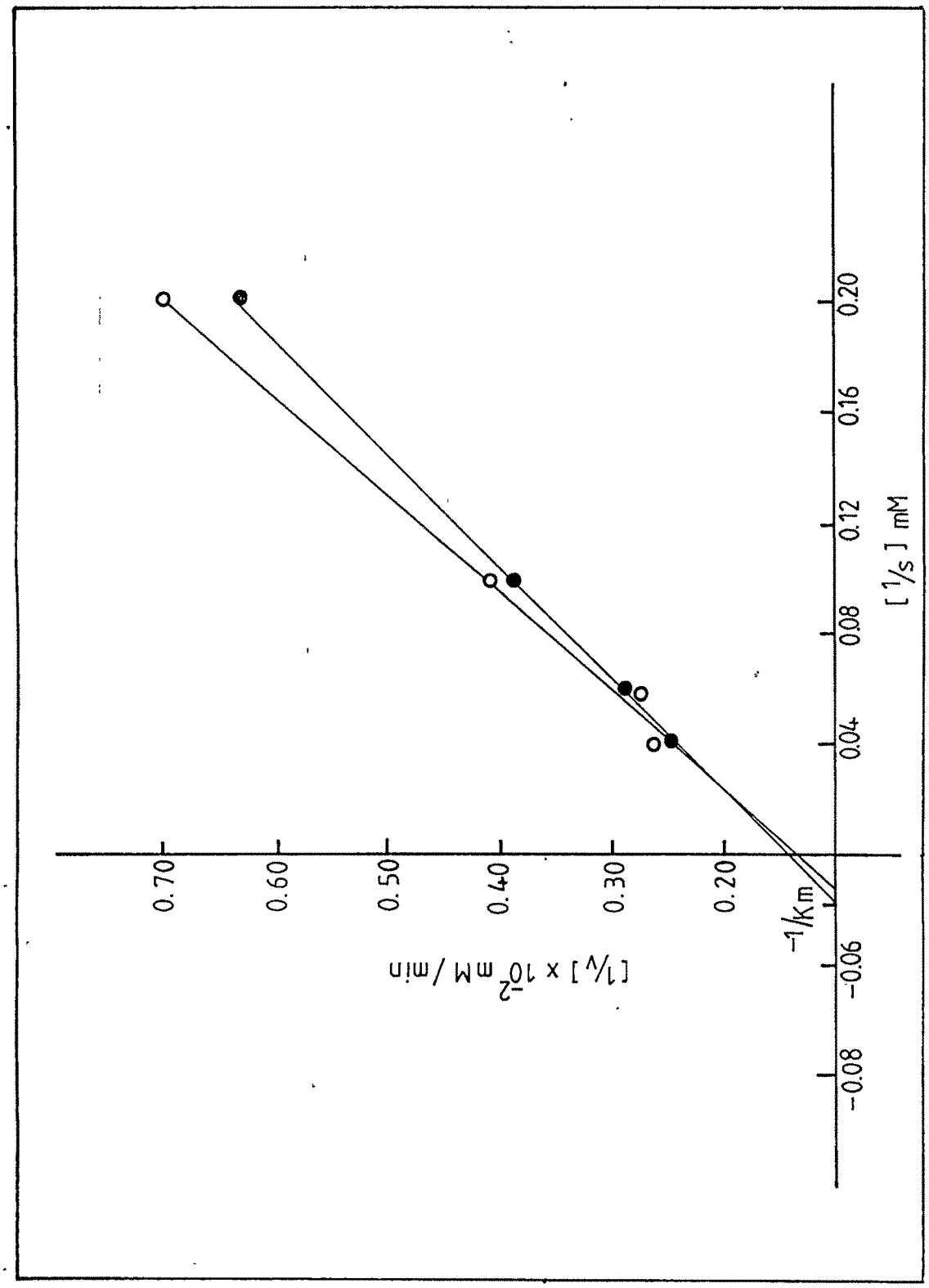

Fig. 3.23 Lineweaver-Burk plots for free and immobilized urease
(●) Urease, (○) Immobilized urease.

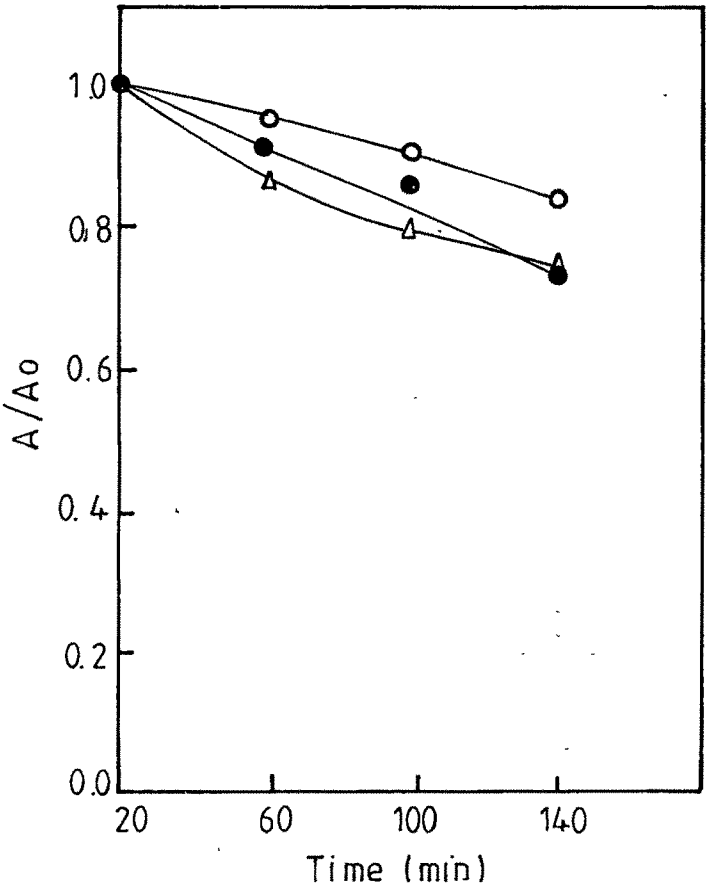

Fig. 3.24 Thermal deactivation of urease-PVA-F
At (○) 40, (●) 50, (Δ) 60°C

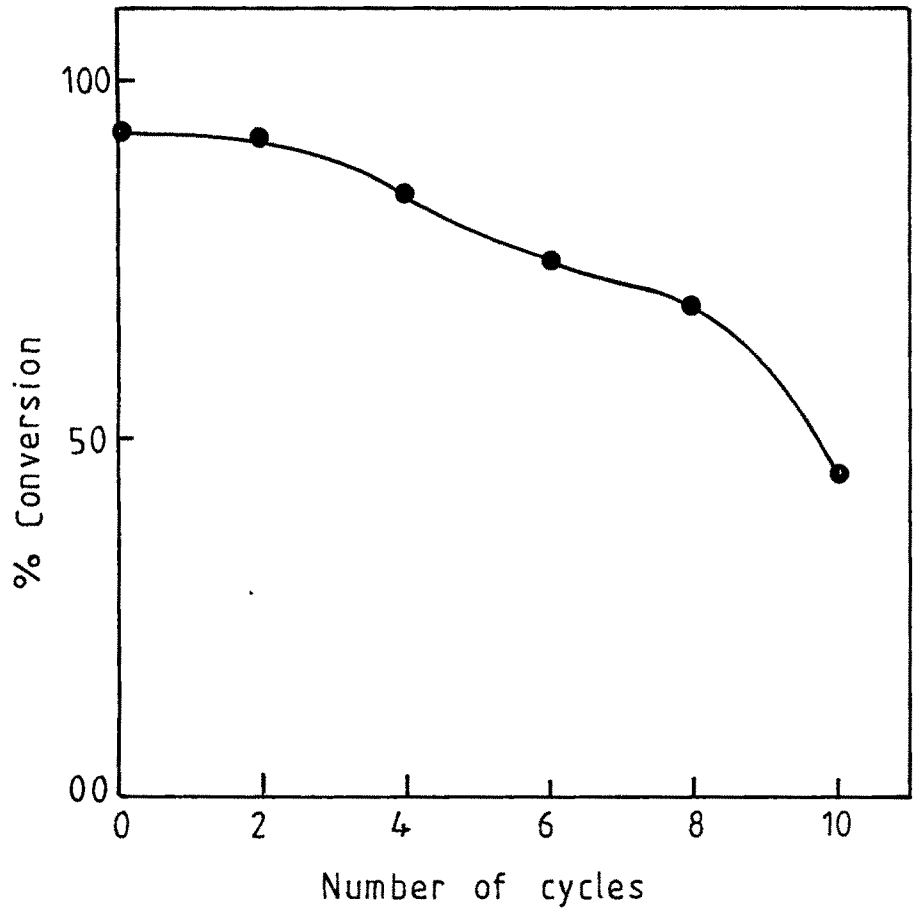

Fig. 3.25 Operational stability of immobilized urease

after 7-8 cycles (160 min) operation and only 30% reduction in enzyme activity was observed in urease (PVA-F).

3.6 Conclusion

Among the polymeric supports studied urease PVA-F system was observed to give a better performance in the immobilization of urease. The retention of enzymatic activity on PVA-F system was observed to be 70-80%. In addition, the thermal, storage, pH and solvent stability of the urease immobilized on PVA-F was found to be higher than that of the free enzyme.

Furthermore, the immobilized urease was successfully used in packed bed column reactor for the continuous hydrolysis of urea.

3.7. REFERENCES

1. Sumner, J.B., *J. Biol. Chem.*, 1926, **69**. 435.
2. Damodaran, M. and Sivaramakrishnan, P.M., *Biochem. J.*, 1937, **31**, 1041.
3. Tai, K., Science Repts., *Shiga Agri. Cell Serv.*, 1953, **4**, 57 (CA 50, 6598 g, 1956).
4. Mateer, J.G. and Marshall, E.K. Jr., *J. Biol. Chem.*, 1916, **25**, 297.
5. Sumner, J.B., Graten, N and Eriksson-Quensel, J.B., *Science*, 1938. **87**, 395.
6. Sumner, J.B., Graten, N and Eriksson-Quensel, J.B., *J. Biol. Chem.*, 1938, **125**, 37.
7. Sumner, J.B., Hand, D.B. and Holloway, R.S., *J. Biol. Chem.*, 1931, **91**, 333.
8. Wang, J.H. and Tarr, D.A., *J. Am. Chem. Soci.*, 1955, **77**, 6205.
9. Gorin, G., *Biochem. Biophys. Acta.*, 1959, **34**, 268.
10. Jenecks, N.P., *Methods Enzymol.*, 1963, **6**, 914.
11. Blakeley, R.L., Hinds, J.A., Webb, E.C. and Zerner, B., *Biochemistry*, 1969, **8**, 1991.
12. Laidler, K.J., *The Chemical Kinetics of Enzyme Action*, Oxford University Press, London, 1958.
13. Wall, M.C. and Laidler, K.J., *Arch. Biochem. Soc.*, 1953, **43**, 312 & 299.
14. Laidler, K.J. and Hoare, J.P., *J. Am. Chem. Soc.*, 1949, **71**, 2699.
15. Lynn, K.R. and Yankwich, R.E., *Biochem. Biophys. Acta.*, 1962, **56**, 512.
16. Jesperson, N.D., *J. Am. Chem. Soci.*, 1957, **97**, 1662.
17. Kakimoto, S., Okazaki, K., Sakane, T., Imni, K., Sumino, Y., Akiyama, S. and Nakao, Y., *Agr. Biol. Chem.*, 1989, **53**, 1111.
18. Moreau, M.C., Ducluzeau, R. and Raiband, P., *Infect. Immun.*, 1976, **13**, 9.
19. Suzuki, K., Benno, Y., Mitsuoka, S., Takeba, S., Kobashi, K. and Hase, *J., App. Environ. Microbiol.*, 1979, **37**, 319.

20. Show, W.H.R., *J. Am. Chem. Soci.,* 1954, **76**, 2160.

21. Mueller, W.A., *Dialysis System, US Patent* 1978, **4**, 094775.

22. Ohshima, Y., Shirane, K. and Funakubo, H., *Ann. Rep. Eng. Res. Inst. Fac. Eng. Uni., Tokyo,* 1980, **39**, 81.

23. Chang, T.M.S., *Biomedical Applications of Immobilized Enzymes and Proteins,* Plenum Press, New York (1977).

24. Weetall, H.H. and Hersh, L.S., *Biochem. Biophysi. Acta.,* 1969, **185**, 464.

25. Reisel, E. and Katchalski, E.J., *J. Biol. Chem.,* 1964, **239**, 1521.

26. Sundaram, P.V. and Hornby, W.E., *FEBS Lett.,* 1970, **10**, 325.

27. Filippusson, H., Hornby, W.E. and McDonald, A., *FEBS Lett.,* 1972, **20**, 291.

28. Dinelli, D., Marconi, W., Cecere, F., Galli, G. and Morisi, F., In:*Enzyme Engineering,* Plenum Press, New York, 1978, **vol. 3**, 477.

29. Shipe, W.F., Senyk, G. and Weetall, H.H., *J. Dairy Sci.,* 1972, **55**, 647.

30. Gestrelins, S., Matiasson, B. and Mosbach, K., *Eur. J. Biochem.,* 1973, **36**, 89.

31. Johansson, A.C. and Mosbach, K., *Biochem. Biophys. Acta.,* 1974, **370**, 339.

32. Biodi, P.A., Pace, M., Benna, O. and Pietta, P.G., *Eur. J. Biochem.,* 1976, **61**, 171.

33. Shimer, L, Granot, R., Freeman, A., Sokolovsky, M. and Goldstein, *Biotechnol. Bioeng.,* 1979, **21**, 1607.

34. Katchalski, E.J. and Bar-Eli, A., *Israel Patent,* 1960, 13950.

35. Sundaram, P.V. and Joy, K., *J. Solid Phase Biochem.,* 1978, **3**, 223.

36. Kitajema, M., Miyano, S. and Kondo, A., *Kogyo, kageku, zasshi (Japanese)* 1969, **72**, 493.

37. Gardner, D.L., Falb, R.D., Kim, B.C. and Emmerlang, D.C., *Trans. Amer. Arti. Int. Org.,* 1971, **17**, 239.

38. Chang, T.M.S., *Science,* 1964, **146**, 524.

39. Chang, T.M.S., Johnson, L.J. and Ransome, O.J., *Can. J. Physiol. Pharmacol.*, 1967, **45**, 705.

40. Chang, T.M.S., Gonda, A., Dirks, J.H. and Malave, N., *Trans. Am. Soci. Artifi. Organs,* 1968, **14**, 163.

41. Chang, T.M.S., Maclutosh, F.C. and Mason, S.G., *Can. J. Physiol. Pharmacol.,* 1966, **44**, 115.

42. Chang, T.M.S. and Maclutosh, F.C., *Pharmacologist,* 1964, **6**, 198.

43. Chang, T.M.S. and Malave, N., *Trans. Am. Soci. Artifi. Organs.,* 1970, **16**, 141.

44. Chang, T.M.S. and Poznonski M.J., *J. Biomed. Mater. Res.,* 1968, **2**, 187.

45. Hornby, W.E., Filippusson, H. and Inman, D.J., *Anatom. Anal. Chem. Technicon. Symp.,* 1974, p. 56.

46. Inman, D.J. and Hornby, N.E., *Biochem. J.,* 1972, **129**, 255.

47. Leving, S.N. and Lacoarse, W.C., *J. Biomed. Mater. Res.,* 1967, **1**, 275.

48. Ngo, T.T., *Can. J. Biochem.* 1976, **54**, 62.

49. Brown, E., Racois, A. and Guemifaay, H., *Tetrahedran. Lett.,* 1970, **25**, 2139.

50. Axen, R., Carlsson, J., Sammson, J.C. and Porath, *J., Enzymologia.* 1971, **41**, 359.

51. Sundaram, P.V., *Biochem. Biophy. Research Commun.,* 1974, **61**, 667.

52. Coulet, P.R., Julliard, J.H. and Gantherson, D.C., *Biotechnol. Bioeng.*, 1974, **16**, 1055.

53. Nakamoto, Y., Karuba, J. and Suzuki, S., *J. Ferment. Technol.,* 1975, **53**, 595.

54. Nakamoto, Y., Karuba, I. and Suzuki, S., *Biotechnol. Bioeng.,* 1975, **17**, 1382.

55. Iyenger, I. and Rao, A.V.S.P., *Biotechnol. Bioeng.,* 1979, **21**, 1333.

56. Blaedel, W.J., Kissel, T.R. and Bognslaski, R.C., *Anal. Chem.,* 1972, **44**, 2030.

57. Ramachandran, K.B. and Perlmatter, P.P., *Biotechnol. Bioeng.,* 1976, **18**, 685.

58. Mattiasson, B., Danielson, B., Hermansson, C. and Moshach, K., *FEBS Lett.,* 1978, **85**, 203.

59. Brotherton, J.E., Emerg, A. and Rodwell, V.W., *Biotechnol. Bioeng.,* 1976, **18**, 527.

60. Grunwald, P., Gunssen, W., Heiker, F.R. and Roy, W., *Anal. Biochem.,* 1979, **100**, 54.

61. Kitano, H., Yashijima, S. and Ise, W., *Biotechnol. Bioeng.,* 1980, **22**, 2643.

62. Sada, E., Katoh, S. and Terashima, M., *Biotechnol. Bioeng.,* 1980, **22**, 243.

63. Miyama, H., Kobayashi, T. and Nosaka, Y., *Biotechnol. Bioeng.,* 1982, **24**, 2757.

64. Raghunath, K., Panduranga Rao, K. and Thomas Joseph, K., *Biotechnol. Bioeng.,* 1984, **26**, 104.

65. Sharma, K.K., *J. Ind. Inst. Sci.,* 1986, **66**, 609.

66. Sugii, A., Ogawa, N. and Matsumoto, K., *J., App. Poly. Sci.,* 1986, **32**, 4931.

67. Barbara, M., Martins, F., Cruz, M., Juaquim, M.S. and Kennedy, J.F., *J. Chem. Technol. Biotechnol.,* 1987, **39**, 201.

68. Lynbinskii, G.V., Yamishpolskii, V.V., Tertykh, V.A., Juodvalkite, D. and Glemza, A., *Ukr. BioKhim. Zh.,* 1987, **59**, 35.

69. Hsine, G.H. and Chou, Z.S., *J. App. Poly. Sci.,* 1987, **34**, 319.

70. Ogawa, N., Kawauchi, Y., Nishimura, K. and Sugii, A., *J. App. Poly. Sci.,* 1988, **35**, 1283.

71. Jing, S., Yoshikawa, K. and Yamaguchi, T., *Nippon Kagakukaishi,* 1989, **11**, 1933.

72. Ivanov, I., Danes, S., Palaschev, Ch. and Dimitrov, D., *Lab. Delo.,* 1989, **8**, 29.

73. Kabziuski, Andrzej, K.M., Parygczak, Tadeusz., *Pvezem. Chem.,* 1992, **71**, 309.

74. Demirel, G., Akovalia, G., Tanyolae, A. and Hasiri, N., *J. Chem. Technol. Biotechnol.,* 1992, **55**, 319.

75. Huang, T.C. and Chen, D.H., *J. Chem. Technol. Biotechnol.,* 1992, **55**, 191.

76. Huang, T.C. and Chen, D.H., *J. Chem. Eng. (Japan)*, 1992, **25**, 458.

77. Vasudevan, P.T., Raggiano, L. and Weiland, R.H., *Biotechnol. Bioeng.*, 1990, **35**, 1145.

78. Kobayashi, T., Miyama, H., Kawate, S., Nosaka, Y. and Fuji, N., *J. App. Poly. Sci.*, 1992, **46**, 2183.

79. Sunger, S., Ekin, M. and Akbulut, U., *Macromol. Reports,* 1992, **3**, 251.

80. Moynihan, R.J., Lee, C.K., Clark, W. and Wang, W.H.L., *Biotechnol. Bioeng.*, 1989, **34**, 951.

81. Wang, Y.J., Chen, C.H., Hsine, G.H. and Yu, B.C., *Biotechnol. Bioeng.*, 1992, **40**, 446.

82. Steffen, A.S. and Stande, E., *Biotechnol. Bioeng.*, 1992, **39**, 725.

83. Leszko, M. and Zaborske, W., *J. Chem. Technol. Biotechnol.*, 1993, **57**, 113.

84. Chen, X., *Lizi jiaohuan Yu Xifu*, 1993, **9**, 481.

85. Chen, D.H., Leu, C.J. and Huang, C.T., *J. Chem. Tech. Biotechnol.*, 1994, **61**, 351.

86. Schwedt, G., Shi, R. and Stein, K., *Dtsch. Lebensm-Randsch*, 1994, **90**, 178.

87. Husniye, D., Haluk, B., Abdurrahman, T. and Nesrin, H., *Poly. Int.*, 1994, **35**, 321.

88. Campanella, L. and Lin, J., *Forum for Applied Biotechnology*, 1995, **1**, 2047.

89. Nguyen anh. D., Nguyen dinh. H., Nguyen duy. H. and Trantich, C., *Radiat. Phys. Chem.*, 1995, **46**, 4.

90. Gryzelda, P., Barbara, K. and Witold, T., *Biomaterials*, 1995, **16**, 129.

91. Jumei, W. and Wenbin, W., Xianweisu kexue yu Jishu, 1996, **4**, 46.

92. Chaney, A.L. and Marbach, E.P., *Clin. Chem.*, 1962, **8**, 130.

93. Berthlot, E., *Report Dechim. App.*, 1959, **282**.

94. Zaborsky, O.R., *Immobilized Enzymes*, Chem. Rubber Publ. Co., Cleveland, Ohio, 1973, 5-60.

95. Rejikumar, S. and Devi, S., *J. Macromol. Sci. Pure App. Chem.,* 1995, **32**, 2035.

96. Dumitriu, S., Popa, M., Artenic, V. and Dan, F., *Biotechnol. Bioeng.,* 1989, **34**, 283.

97. Hayashi, T. and Ikada, Y., *J. App. Poly. Sci.*, 1991, **42**, 85.

98. Melrose, G.T.H., *Res. Pure App. Chem.,* 1971, **21**, 83.

99. Angiuro, L.D., Cremonesi, P., Cantali, G. Mazzole and Veichio, G., *Angew. Macromol. Chem.,* 1980, **91**, 161.

100. Messing, R.A., *Immobilized Enzymes for Industrial Reactors*, Academic Press, New York, 1975.

101. Huang, T.C. and Chun, D.H., *Biotechnol. Bioeng.,* 1992, **40**, 1203.

102. Brown, T., Narratil, J.D. and Faray, A.B., *Polyurethane foam. Sorbarts in Separation Science,* CRC Press, Broc. Raton. F1, 1985.