Chapter 3

Immobilization of Pepsin

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3.1 Properties of Pepsin

Pepsin belongs to the enzyme class protease . Protease enzymes are involved in a great variety of physiological processes and their action can be divided into two different categories .

* **Limited proteolysis** : In which a protease which cleaves only one or a limited number of peptide bonds of a target leading to the activation or maturation of the formerly inactive protein e.g. conversion of prohormones to hormones .

* **Unlimited proteoysis :** In which proteins are degraded into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecule of the polypeptide ubiquitin. Another pathway consists in the compartmentation of proteases e.g in lysosomes . Proteins transferred into this compartment undergo a rapid degradation.

Proteases are classified according to their source as

* plant proteases
*animal proteases
* microbial proteases

and according to the catalytic action as

* endopeptidases
* exopeptidases

The proteolytic enzyme pepsin (E.C.3.4.23.1) occurs in the gastric juices of all mammals [1,2] . Gastric mucosa of different forms of these hydrolases as well as of their zymogens is characteristics of this type of enzymes. The term pepsin is applied to the gastric proteinases active at acid pH values (pH 1-6) . The chief cells of the gastric mucosa secrete the inactive precursor pepsinogen , which is converted into proteolytically active pepsin by cleavage of peptide bond [3] which acts as an inhibitor of pepsin and other peptides. The available evidence indicates that , in the mammals stomach , the principal enzymatic component (pepsin A) is accompanied by minor components , denoted by pepsin B (parapepsin – I , gelatinase) , pepsin C (para pepsin I , gastricsin), and pepsin D (de-phosphopepsin A) .

Porcine pepsin has a molecular weight near 33,000 [4] and consists of a single peptide chain of 320 amino acid residues with isoleucine at the amino end and alanine at the carboxyl end .The chain is crosslinked by three disulfide bridges [5].

According to the nomenclature committee of the international union of biochemistry and molecular biology proteases are grouped under class 3 (hydrolases) and subclass 4 (peptide hydrolaes) [6]. Pepsin hydrolyzases N- and C – terminally blocked synthetic dipeptides like Glu-Try or Phe – Phe but with poor efficiency.

Pepsin acts on both natural and synthetic substrates such as Natural substrates : glelatin, casein, edestin, milk protein [7] , hemoglobin [8], serum albumin [9] And synthetic substrates : like N-acetyl –L- phenylananyl –L-phenyl alanine [10] , N-acetyl –L-phenylalanyl -L-3 , s-di-iodotyrosine [11]. Benzyloxycabonly-L-glutamyl-L-tyrosine , Acetyl-L-phenylalanyl-L-tyrosine , Benzyloxycarbonyl –L-histidyl-L-phenyl-L-phenylalanyl-L-phenylalanine methyl ester , Glycyl-glycyl-L-phenylalanyl-L-phenylalanine ethyl ester , Benzyloxycarbonyl-glycyl-glycyl-L-phenylalanyl-L-phenylalanine 4-picolyl ester [12].

3.1.1. Classification of Proteases according to their sources :

Plant proteases		
Enzyme	***Source***	***Application***
Papain	Latex of unripe yet mature papaya.	For preparation of highly soluble and flavored protein hydrolysates.
Bromelain	From stem and juice of pineapples	
Keratinases	Plants which produce enzyme which degrade hair	For production of essential aminoacids and for prevention of clogging of wastewater systems.
Ficin	Fig Tree	
Animal Proteases		
Trypsin	From Intestine	In food industry and in preparation of bacterial growth media ,
Chymotrypsin	Animal Pancreatic extract	For diagnostic and analytical applications , used in deallergizing of milk protein hydrolysates.
Pepsin	Stomach of all vertebrates	For cleavage of peptide bonds and in food industry.
Rennin	Stomach of nursing animals	In dairy industry for production of curd with good flavour.
Microbial Proteases		
Bacteria	Bacteria belonging to the geius bacillus	In detergent industry

3.2 Literature Survey on Immobilization of Pepsin.

Pepsin has been used since long for immobilization . The immobilized preparation further has good application in the industry. In 1960's experiments were carried out by Wieland to study the ability of pepsin to catalyze the condensation of well –defined synthetic peptides. After that it has been used in food industries. Both free and immobilized pepsin has been used in clotting of milk .Proteases execute large variety of functions extending from the cellular level to the organ and organism level. Protease have a long history of use in food applications. The important use of pepsin in food is in the form of a mixture with chymosin in rennet preparations. They are mainly used in clotting for eventual curd formation and casein hydrolysis . A detailed literature work of pepsin immobilized on natural and synthetic supports is given here.

3.2.1. Natural Support

Many natural and synthetic supports have been used for immobilization of pepsin . A detailed survey of the work is given below.

Glutaraldehyde has been used as an activating agent for covalent coupling of pepsin to chitosan by Hirano and Miura [13]. They observed that the immobilized enzyme showed retention of enzyme activity even after 4 cycles and 90 % of enzyme activity was retained after storage at 5°C for one month at pH 7.6 and at 40 °C for three days.

Pepsin has been immobilized on p-amino carbanilated cellulose derivatives and methylol derivative using diazo and glutaraldehye activating agents . High coupling extent was observed by Beddows et al [14].

N-[4-carboxy phenyl carbamoyl methyl] cellulose was used as a support for pepsin immobilization by Dumitriu et al. [15]They have reported that free enzyme showed higher values of kinetic parameters compared to immobilized one . Immobilized system was observed to loose 25 % of its activity after 3 cycles. Moreover , they observed that enzyme , support and activator - support ratio , pH and coupling duration influsdenced the coupling extent.

Chitin and chitosan have been used as matrix for immobilization of pepsin by Riccardo and Muzzarelli [16] . They used glutaraldehyde as an activating agent and discussed the preparation and performance of the immobilized enzyme. Chitosan and its derivatives have also been used for immobilization of protease by Huang et al. [17]

Pepsin has been immobilized on Triton X -100 substituted sepharose-4B by Gorgani et al. [18] They observed that the protein binding was independent of pH and salt concentration . They further discussed its use in continuous operation.

A new method for covalent binding of pepsin on a carboxy methyl dextran-modified gold surface intended for surface plasmon resonance sensor was described by Johnson et al. [19] The method involves two steps : first formation of N-hydroxy succinimide ester from a fraction of carboxyl groups of the carboxy methyl dextran matrix via reaction with N-hydroxyl-succinimide and N-ethyl –N'-(dimethyl aminoproyl) carbodiimide HCl in water .In the second step the protein is passed over the surface in a solution of low ionic strength with a pH value below the isoelectric point of the protein. Thereby concentrating the matrix by electrostatic attraction forces and a simultaneous reaction with the active ester. In a final step, the remaining active esters are transformed into amides via reaction with ethanolamine. Parameters such as protein concentration, were varied to control the immobilized amount of ligand. The biospecific interaction of the immobilized ligand with its biological counterpart was checked.

Succenilated chitosan has been used as a support for immobilization of pepsin by Jacqueline and Gustova [20] . They prepared insoluble active pepsin by covalent binding of the enzyme to succinylated chitosan through amide bond formation ,using carbodiimide as condensing agent . They observed that the enzyme retained 80 % specific activity and showed greater stability on storage than the soluble enzyme.

Sunitha and Saiprakash [21] carried out the immobilization of pepsin using two different techniques. They covalently bound the enzyme to egg shell using crosslinking agent glutaraldehdye and also by physical adsorption on synthetic zeolites. They observed that the bound pepsin possessed high retention of activity and high stability at high temperature and extreme pH than that of the pepsin adsorbed on zeolites. The nature and immobilized pepsin preparations were characterized in terms of their activity , thermal stability , pH , temperature optima and kinetic parameters.

The preparation of affinity sorbents containing immobilized iodinated derivatives of L-tyrosine for the affinity chromatography of porcine pepsin was described by Arnostova et al. [22] The ligand was coupled either to Sepharose 4B or beads of cellulose after the divinylsulfone activation or to sepharose 4 B after the activation with 2,4,6 – trichloro-1,3,5-triazine . The highest capacity for porcine pepsin was found to be in the case of 3,5-diiodo-L-tyrosine coupled to divinyl sulphone activated sepharose.

3.2.2. Synthetic Supports

Synthetic supports have also been used widely for immobilization of pepsin. Some of the widely used supports are discussed here.

Line et al. [23] have covalently bound pepsin to porous silica gels. They observed 96 % coupling of the enzyme on the support . The insoluble enzyme retained its activity for 30 days during continous operation and almost total activity was observed during an assay at high substrate concentration .

Immobilization of pepsin to alkylamine porous glass through covalent coupling using woodwards reagents or glutaraldehdye was carried out by Cheryan et al.[24-25] The immobilized enzyme was further used for milk clotting and the effect of different clotting parameters was examined . Ferrier et al. [26] used porous glass derivatives for immobilization of pepsin .

Crystaline Pepsin was bound to an alkylamino derivative by Valaris et al. [27] and they observed that the activity of such derivative was retained for 40 days . The pepsin content of the immobilized product was 3.7-4.4 % (on dry basis) with a binding efficiency of 72-89 % . They further carried out the proteolysis of casein at pH 1.6 in the presence of CM-cellulose (0.03 % solution) . They observed that V_{max} values decrease with the increase in CM-cellulose concentration .

Goldstein [28] used 1 : 1 copolymer of ethylene maleic anhydride and 1,6 diaminohexane (EMA : MMD) for the immobilization of pepsin through carboxyl groups activation using dicyclohexyl carbodiimide. Though the retention of immobilized enzyme activity was 20 % , it showed good thermal stability .

Styrene divinyl benzene copolymers with various structures have been used as polymeric carriers for the immobilization of pepsin by Makarova et al. [29] The effect of copolymer macromolecular structure and the halogen content on the process of immobilization of pepsin was studied .

A new polymeric carrier ,containing primary amino groups was prepared by coupling an ethylene maleic anhydride (1:1) copolymer (EMA) , with 1,6-

diaminohexane (HMD) in the presence of dicylcohexylcarbodiimide by Leon [30] . The resin was used for the immobilization of pepsin through its carboxyl groups .

Sand was used as a support for immobilization of pepsin by Puvanakrishnan and Boss .[31-32] The enzyme immobilized system was stable at 4°C upto 60 days . The kinetic parameters of immobilized system were found to be higher compared to free ones and the optimum temperature was found to be 40-45 °C.

Radiation induced graft copolymer of m and para fluro styrene onto nylon was used by Abdel-Hay et al [33] for covalent coupling of pepsin. They observed that only negligible enzyme was coupled to the support.

Lastra and Ortega [34] used four different types of polyvinly pyrrolidones (PVP) for covalent coupling of pepsin . The supports used were

* High –mol-wt PVP
* High-mol-PVP-bound to polyphenols extracted from the seeds of Myrtus communis
* Soluble PVP precipitated by addition of Myrtus extract and
* Soluble PVP on addition of tannic acid.

The authors investigated enzyme binding capacity , thermal stability and pH responses for all the four types of PVP and also checked its use in continuous operations.

A heterogenous catalyst was prepared by covalent binding of pepsin to wide porous and non porous ω-aminoalkyl derivatized inorganic supports by means of water soluble carbodimide by Ivanov et al. [35].The amount of attached enzyme and proteolytic activity of the catalyst were detected .Non porous catalyst , based on finely disperse pyrogenous silicon dioxide (carbosil), exhibited the maximum pepsin content (22 mol/g) and the highest proteolytic activity (5.6 units/g) . Wide-porous glass based catalyst showed the maximum retention of pepsin activity (92 %) . The immobilized pepsin content as well as proteolytic activity of catalysts was found to increase on increasing the chain length of the alkyl spacer from C_2 to C_6 where as thermal stability of catalyst (37°C) was independent of the alkyl spacer length. All the catalysts tested were stable for one month on storage at 8°C and pH 4.5 .

Radio-derivatized polymers (RDPS) were produced by contacting nonpolymerizable conjugates (e.g . aromatic amines) with radiolysable polymers in the presence of radiation by Varga and coworkers [36] The resulting PDP's were further linked with ligands for their immobilization. Depending on the types of conjugates used , the method produces functionalized or relative RDPS .The RDPS have improved adsorptive and ion-binding characteristics and can be used for cell/tissues culture substrate , in chromatography as sorbents , in immunoassays and in other binding assay .

Pepsin was used in both soluble and immobilized forms to study the effect of pH , calcium concentration and temperature on enzymatic coagulation by Mehaia [37] . It was observed that calcium appeared to affect only the secondary phase and not the primary phase. The activation energy and Q_{10} values for the primary phase were 10.6 kcal/mol and 1.8 , while for the secondary phase were 33.8 kcal/mol respectively . The mechanism of coagulation of camels milk was found to be similar to that of cows milk and it seemed to involve a charge – neutralization process together with a conformation change type reaction occurring in the secondary phase of the milk coagulation.

A cryogenic technique was used for studying the immobilized pepsin on poly (vinly alcohol) by Rozantseu et al.[38] They observed that introduction of 0.5 – 5 % pepsin into a 12 % aqueous solution at pH 1.7 – 2.2 resulted in a significant decrease in the mechanical strength . The cryogenic treatment at temperatures between –5 to –68 °C led to structure formation with lower network density than that observed in the absence of pepsin. Further it was found that immobilized pepsin showed improved thermal stability and widened pH range of pepsin activity.

Pepsin was adsorbed on silica gels by Khokhlova and Nikitin [39] They observed that the highest adsorption was observed in case of trimethyl –silylated silica gels , followed by dehydroxylated silica gel , while the lowest one was observed in case of hydroxylated silica gels.

Karasavova et al. [40] immobilized pepsin by covalent binding via hyroxymethyl group to ultrafilteration membrane , composed of acrylonitrile and acrylamide copolymer . The properties of the immobilized enzyme showed pH 2-3 and temperature optimum at 50^{o}C . Isolated wine proteins were treated with immobilized pepsin. The immobilized pepsin showed considerable advantage in protein hydrolysis in comparison with current methods. These advantages include : one step activation of membrane with formaldehyde, high relative activity of immobilized enzyme , controlled degree of hydrolysis , no introduction of additional substance in hydrolyzates

The property of electronic conductivity of polymers such as polyaniline exhibits a strong dependence on the redox and protonation state of the polymer was used for making a generic biosensor using enzyme by Contractor et al.[41] It was observed that the enzyme catalyzed reaction of the bio-molecule resulted in a change in redox potential and or pH of the microenvironment in the polymer matrix .Further it was found that the change in microenvironment triggered the change in the conductivity which was measured .

Pepsin catalyzed synthesis in organic media was studied with free and immobilized enzyme by Bemquerer et al.[42] Pepsin immobilized by adsorption onto the solid supports celite and chromsorb was employed to perform a study of secondary specificity of the enzyme in organic media through the coupling between Z-X-Phe-OH where X= ala , asp, glu , gly , phe , yal , trp , tyr and H-

Phe-OMe . The investigation was performed in two solvent system Et acetate citrate buffer pH 4.5 and acetonitrile :citrate buffer pH 4.5 .Reaction rate data showeed that pepsin had a preference for more hydrophilic substituents in the P_2 position performed in predominantly aqueous media .

The clotting and stabilization of milk using pepsin onto resinous material was studied by Shah et al.[43] They optimized different parameters like effect of concentration of enzyme as well as the crosslinking agent and pH of the coupling medium on the immobilization of enzyme . Styrene-resorcinol –formaldehdye resin bound pepsin showed better thermal and chemical stability . The maximum retention of activity was observed between 30 – 40 °C .

Marion et al. [44] immobilized pepsin covalently bound on the surface of new type of porous zirconia as well as a conventional porous silica activated with 3-isothiocyanato propyl triethoxy silane . They further checked the efficiency of immobilization onto the porous zirconia material in terms of the amount of enzyme attached to the particles and from the biological activity retained after immobilization step.Pepsin was assembled on PET-$NH_3{}^+$ surface by Xu et al. [45] They further characterized it by XPS and AFM .

Pepsin-collodial gold conjugates were prepared by a simple protein-friendly process and the enzymatic activity of the biocatalyst was reported by Gole et al. [46] The pepsin gold conjugates were obtained by mixing colloidal gold and protein solution at pH 3 and thereafter centrifugation , washing and dispersion of the pepsin gold conjugate material in water. The bio conjugates were characterized by uv-vis spectrophotometer, flouresence , biocatalytic activity measurement while films of bio conjugate materials obtained by solvent evaporation on suitable substrate were further analyzed by SEM , energy dispersion analysis of –rays , transmission electron spectroscopy. This clearly indicated that enzyme is stable in its natural state and was possibly stabilized by the colloidal gold particles. The enzyme in the pepsin-Au bio conjugate retained substantial bio catalyst activity and was more than the free enzyme in the solution.

Pepsin has been immobilized on gelatin using glutaraldehyde , formaldehyde and epoxides by Asai et al.[47] They further used it for measuring the solubilization of elastins.

Pepsin was immobilized on A-acetyl-phenylalanine and iodinated derivative of tyrosine , divinyl sulhone activated sepharose by Jana et al.[48]The ligand with blocked amino groups and free carboxyl ones were linked to sepharose via ethylene diamine spacer using carbodiimide reagent . They further optimized conditions of affinity chromatography of porcine pepsin and pepsinogen on the prepared carriers. The effect of pH and ionic strength was examined.

3.3 Experimental :

Materials	***Place of Purchase***
Pepsin (E.C. 3.4.23.1) (strength 3380 U/mg) Casein Molecular Weight (65000)	Sigma Chemical , St. Louis MO , 63103USA
k-carrageenan	*Central Salt and Marine Chemical Research Institute, Bhavnagar,India.*
2-hydroxyethyl methacrylate (HEMA)	*Fluka Chemie , AG CH-9470 Buchs , Switzerland*
Ascorbic Acid :	*Loba Chemie , Mumbai ,India.*

Tricholoro acetic acid *Follin Reagent* *Hemoglobin* *Acrylamide*	*Qualigens ,Mumbai ,India.*

3.3.1. Assay Method of Pepsin :

The activity of free and immobilized pepsin was determined by using 4 cm^3 of 2 % w/v hemoglobin solution prepared in 0.06 N HCl as a substrate as described by Anson [49] . Enzyme and substrate were incubated for 30 min at 37°C . The reaction was terminated using 5 cm^3 of 5 % w/v trichloroacetic acid. The product being soluble in trichloroacetic acid 5 cm^3 of the filterate was taken and to it 10 cm^3 of 0.5 N NaOH and 3 cm^3 diluted phenol reagent were added and the absorbance due to tyrosin produced during the reaction was measured at 750 nm against reagent blank.
The mechanism of pepsin assay is as follows

$$RCONHR'+E\text{-}OH \longrightarrow RCOOH + R'NH\text{-}E$$

3.3.2 Preparation of support material :

Immobilization of pepsin was carried out using two supports namely K -carrageenan and copolymer of AAM-HEMA . K -carrageenan beads and copolymer of (AAm-HEMA) were prepared as described in **section 2.3.2 a and b** respectively .

3.3.3 Immobilization of Enzyme :

Immobilization provides many advantages over free enzyme in respect to different conditions like pH , temperature , storage and reusability Hence entrapment of pepsin in K - carrageenan beads and also its covalent coupling to the copolymer of (AAm-HEMA) using different activating agents was carried out. Entrapment of pepsin was carried out as discussed in **sec 2.3.2(a)** and different conditions like effect of PEI concentration, enzyme loading and hardening time were optimized.

Covalent coupling of pepsin to copolymer of (AAm - HEMA) was carried out using the method discussed in **sec 2.3.2 (b)**. The activation of amino groups was carried out by using glutaraldehyde as activating agent. The glutaraldehdye concentration was varied from 0.1 –4.0 %, activation time was varied from 1-8 h, enzyme concentration was varied from 50 –7000 μg, enzyme coupling time was varied from 1- 20 h and coupling pH was varied from 1 - 6.

The activation of OH groups was carried out using two different activating agents like p-benzoquinone and p-tolyl sulphonyl chloride .In case of p-tolyl sulphonyl chloride the activation time was varied from 0.5 -10 h at pH 1 to 6 and varying enzyme coupling time from 2- 10 h and enzyme concentration (50 –10000 μg).

The activation of hydroxyl group was carried out by varying p-benzoquinone concentration from 15- 100 mg and activation time 1- 8 h. Coupling time was varied from 2 - 20 h at pH 1to 8 using 200 - 5000 μg enzyme concentration.

Simultaneous activation of both groups was also studied by using optimized conditions for glutaraldehyde and p-benzoquinone. For this optimized conditions for glutaraldehyde activation were taken followed by enzyme coupling and then activation of the same system with p-benzoquinone using optimized conditions followed by enzyme coupling. The order of activation of NH_2 group by glutaraldehyde and OH by p-benzoquinone was reversed and its effect on enzyme activity was examined.

3.3.4 Comparative study of free and immobilized enzymes :

A comparison of behavior of free immobilized enzyme is very important not only for the application of immobilized systems but also for the elucidation of structure function relationship and mechanisms of enzyme reaction.

1.pH

pH activity profile of both free and immobilized enzyme is an important parameter to be studied , as it helps in understanding changes taking place during immobilization . Pepsin is an acidic protease and shows maximum activity in the acidic range . Hence the pH stability of free and immobilized pepsin was studied at 1 – 7 pH at 35°C for 30 min using hemoglobin as a substrate . The residual activity was measured at different pH.

2.Thermal stability

The catalytic activity of enzymes is dependent on temperature , as in case of ordinary chemical catalysts, but the activity is lost at high temperature above a certain limit due to the denaturation of enzyme protein. Thermal stability of free and entrapped enzyme was studied by heating the enzyme at 35 – 65 °C for different time intervals from 15 – 120 min and determining its activity by using hemoglobin as a substrate .The thermodeactivation constant (k_d') were calculated by using following equation

$$\ln A_t = \ln A_0 - kd\,(t)$$

where A_o is initial activity of enzyme and A_t is the activity after heat treatment for t minutes.

3.Storage Stability :

Storage stability of enzyme increases on immobilization . The increased stability is important for industrial application of immobilized enzyme. The storage stability of free and immobilized enzyme was checked by storing both forms of enzyme at 35 °C and measuring the activity after different time intervals.

4.**Reusability**

Reusability is a very important parameter of enzyme study as it directly affects the cost. The reusability of entrapped pepsin was examined by using the same enzyme with a fresh aliquot of substrate solution till 50 % of enzyme activity is retained.

5.Kinetic Parameters

Kinetic parameters play an important role as conformational changes of the enzyme protein may occur on immobilization and the affinity between enzyme - substrate may change . The kinetic parameters for free and immobilized enzyme were examined by keeping the enzyme concentration constant and varying the substrate concentration and vice-versa.

3.3.5 Milk Clotting:

Milk clotting is an important application in industry as the clotted milk can be further processed to form hard cheese. Milk contains water, solids such as fats , proteins , carbohydrates and minerals.Milk clotting was carried out by using a fluidized bed reactor of dimension 17 x 1 cm as shown in **figure 3.1**.Milk clotting was carried out by using 5 cm^3 milk solution prepared by dissolving 10 g of milk powder in 100 cm^3 of 0.06 N $CaCl_2$ using free and entrapped enzyme by batch

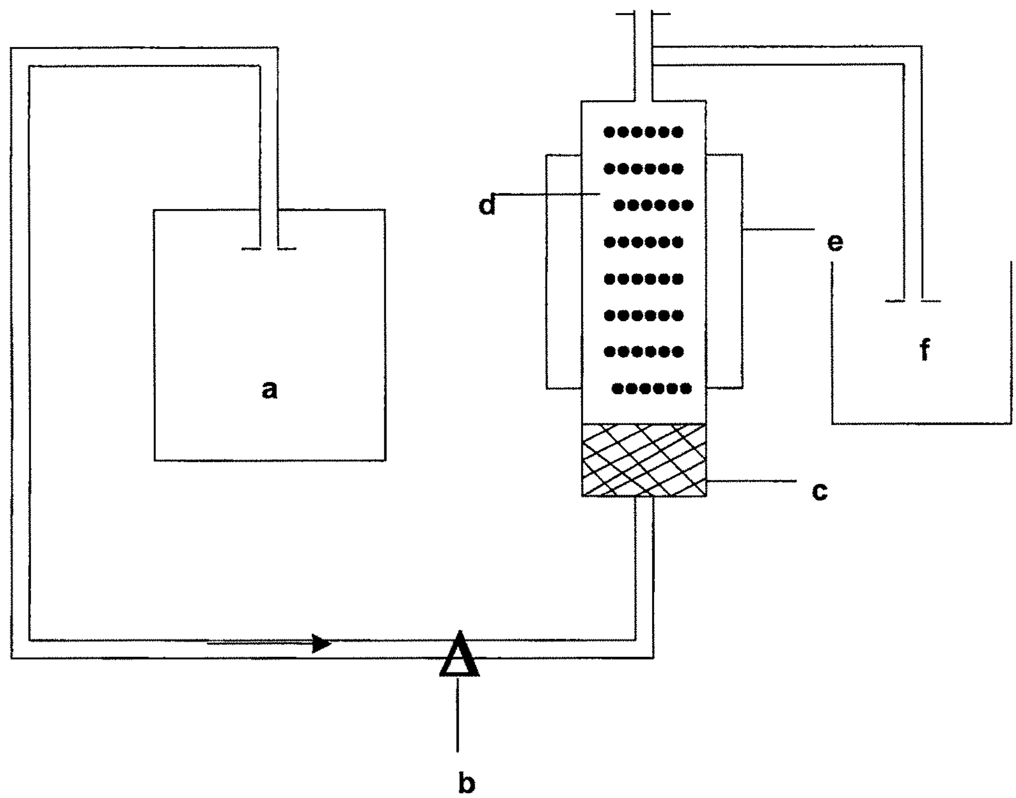

Figure 3.1.Schematic diagram of fluidized bed reactor.A. Milk reservoir B. Peristaltic pump C. Porous glass disc D. Suspended support E. Temperature controller F. Treated Milk.

process. The flow rate was adjusted to 1 cm^3/min and the reactor temperature was kept 20^o C . The effect of enzyme concentration on clotting was examined by using 5 – 50 μg free and entrapped enzyme in batch and also by using 5 – 50 μg entrapped enzyme in fluidized bed reactor . The milk was kept in contact with enzyme for 10 min and effect of enzyme concentration , effect of pH (1-5) and effect of dilution on clotting was checked.

3.3.6 Casein Hydrolysis :

Casein is the principal protein of cow's milk. It is most commonly used milk protein in the industry and contains 21 amino acids. Acid casein, a granular milk protein, is available in two types : edible and technical. Hydrolyzed proteins are added to food to serve various functions. They can be used as stabilizers, thickeners, flavor enhancers and as a nutrients. Dharmapuri and Saiprakash [50] have reported increased catalytic activity of pepsin in casein hydrolysis in aquo-alcoholic medium than in aqueous medium. The maximum activity of pepsin has been reported in the range of 20 to 40 % in 0.2 M aquo-alcoholic medium. Pepsin immobilized on egg shells and synthetic zeolites has been used for hydrolysis of casein by Sunitha and Saiprakash [21].They have covalenlty bound pepsin on egg shell by using glutaraldehyde crosslinking agent and have also physically adsorbed it on synthetic zeolites. They observed that covalently bound enzyme possessed higher retention of activity and stability at higher temperatures and extreme pH conditions than the pepsin adsorbed on zeolites. We have used pepsin covalently coupled to copolymer of AAm-HEMA using different activating agents for the hydrolysis of casein using a fixed bed reactor .Reactor conditions such as flow rate , substrate concentration , reactor temperature and I/D ratio were optimized .

3.4.Results and Discussion

Many natural and synthetic supports have been used for immobilization of enzymes. Entrapment and covalent coupling has been carried out in present case and different conditions have been optimized.

3.4.1. Optimization of conditions for entrapment of enzyme

Hardening of the beads is important if beads are being used in the reactor study. The effect of PEI on the hardening of the beads was checked and it was observed that maximum hardening and retention of activity of enzyme was found at 2 % PEI concentration. Moreover, the effect of hardening time was also checked and maximum hardening was found at 2 h. The results are given in **figure 3.2**.

The effect of enzyme loading was checked by varying the enzyme concentration from 1 to 12 mg and maximum of 998 μg /g loading was achieved .The enzyme activity was found to increase with increase in enzyme loading whereas the retention in enzyme activity increased initially but after that it showed a decreasing trend. The results are given in **table 3.1** .

3.4.2. Optimization of covalent coupling conditions

The copolymer was characterized as stated in sec **2.4.5.a** . The amino groups were activated by using glutaraldehdye as an activating agent using 10 cm^3 1 % (w/v) glutaraldehdye for 5 h activation time and keeping enzyme coupling time 8 h at pH 2 and maximum of 147μg/g of support .

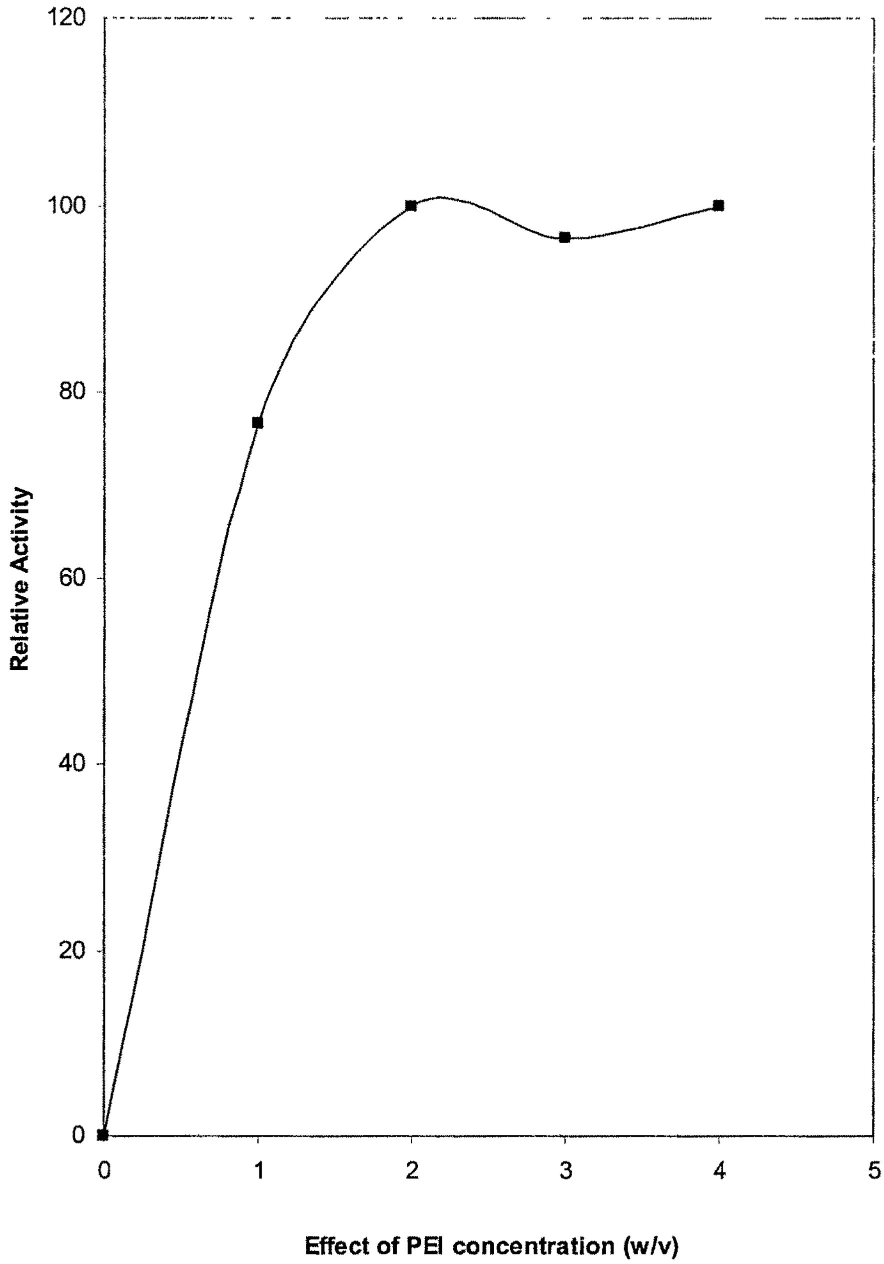

Figure 3.2. Effect of polyethyleneimine concentration on retention of enzyme activity at 37°C and pH 2 for 1 h.

Table 3.1. Dependence of enzyme concentration on retention of entrapped enzyme.

mg of enzyme taken	Total protein content in the beads(mg)	Active protein in the beads(mg)	Percentage protein in beads	Percentage retention of enzyme activity.
1000	197	105	20	53
2000	330	257	17	78
5000	1263	444	25	35
75000	1837	704	34	38
10000	3510	998	35	28
12000	3829	970	32	25

At 35°C and 2 % PEI as hardening agent and pH 2.

The OH groups of the copolymer were activated by both PTS and p-benzoquinone . The p-benzoquinone activated support showed better performance and the maximum concentration of PBQ required was observed to be 25 mg/g of support and maximum enzyme coupling was observed to be 52.8 μg/g after 16 h coupling time and at coupling pH 2. The results are given in **figure 3.3- 3.8**

A synergistic effect was observed upon activation of both the amino and hydroxyl groups . i.e first activating the support with glutaraldehdye and then coupled with enzyme and again activating with p-benzoquinone followed by enzyme coupling. The enzyme coupled was observed to be 559 .85 μg/g . On reversing the order of activation a reverse trend was observed , i.e. the activity was observed to decrease considerably . The results are given in **table 3.2.**

3.4.3.Comparison of behavior free and immobilized enzyme

1.pH

pH plays an important role in enzymatic reactions. Pepsin is a aspartic protease and shows optimum activity in acidic pH range. On examining enzyme activity at pH 1-7 it was observed that the free and covalenlty bound enzyme as well as entrapped enzyme showed maximum activity at pH 2 .However, a little broadening in pH range was observed for entrapped enzyme system. This shows that there is no change in the enzyme structure during either entrapment or covalent binding **(Figure 3.9)**.

2.Thermal Stability :

Thermal stability is an important parameter in enzymatic study . It was found that the thermal stability of entrapped and covalently bound enzyme was more than the free enzyme as shown in **figure 3.10** Moreover in case of covalently bound

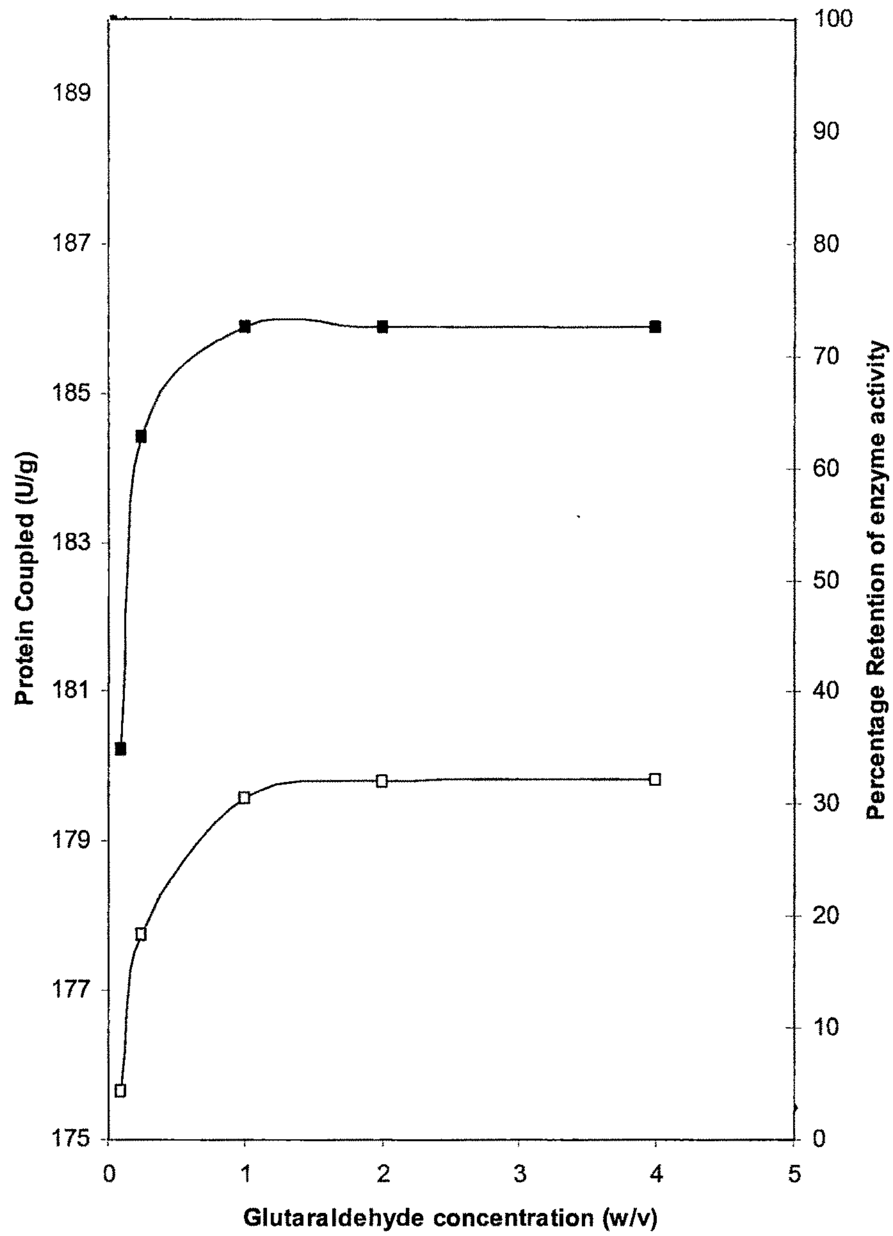

Figure 3.3. Effect of glutaraldehyde concentration on retention of enzyme activity.

Protein Coupled (■), Retention of enzyme activity (□) .

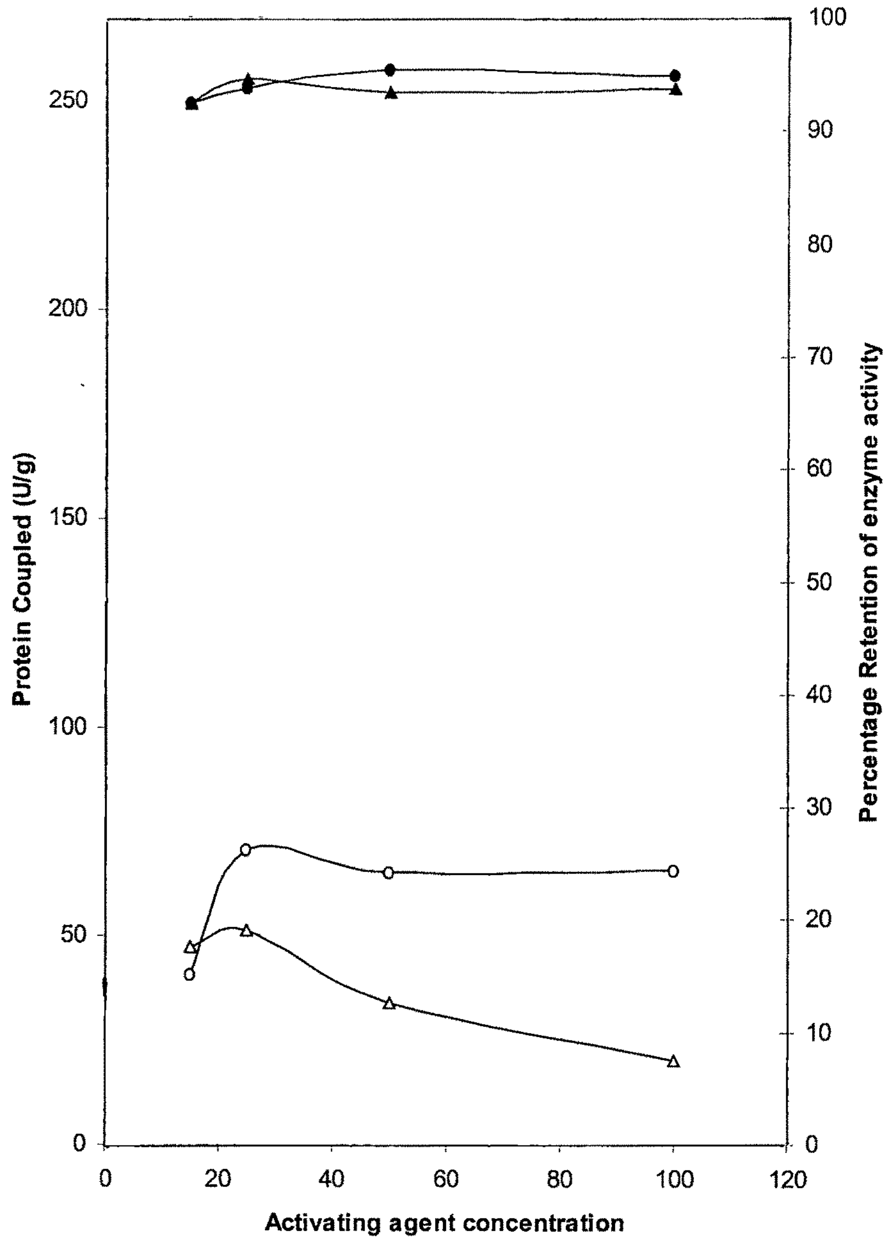

Figure 3.5 Effect of p-benzoquinone and p-tolyl sulphonyl chloride concentration on retention of enzyme activity.

PBQ : Protein Coupled (●), retention of enzyme activity (○).

PTS : Protein Coupled (▲), retention of enzyme activity (△).

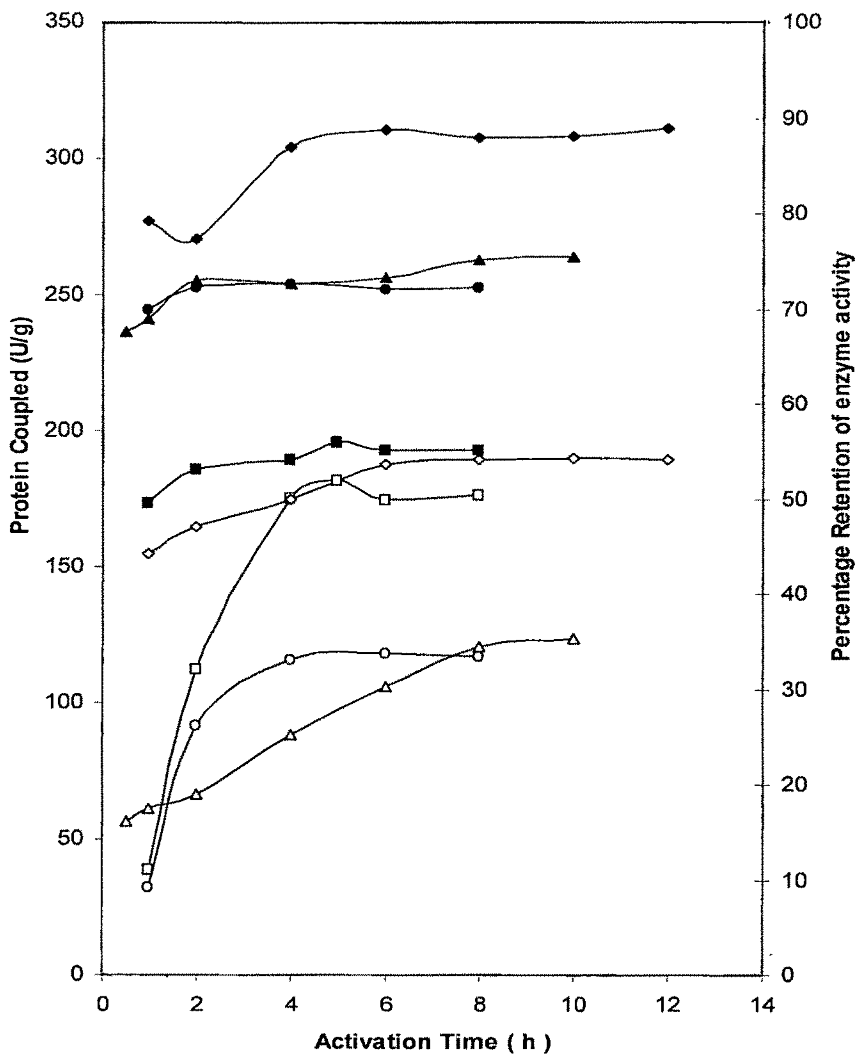

Figure 3.6. Dependence of enzyme activity on activation time.

GLU : Protein Coupled (■), retention of enzyme activity (□).

PBQ : Protein Coupled (●), retention of enzyme activity (○).

PTS : Protein Coupled (▲), retention of enzyme activity (△).

GLU+PBQ : Protein Coupled (◆), retention of enzyme activity (◇).

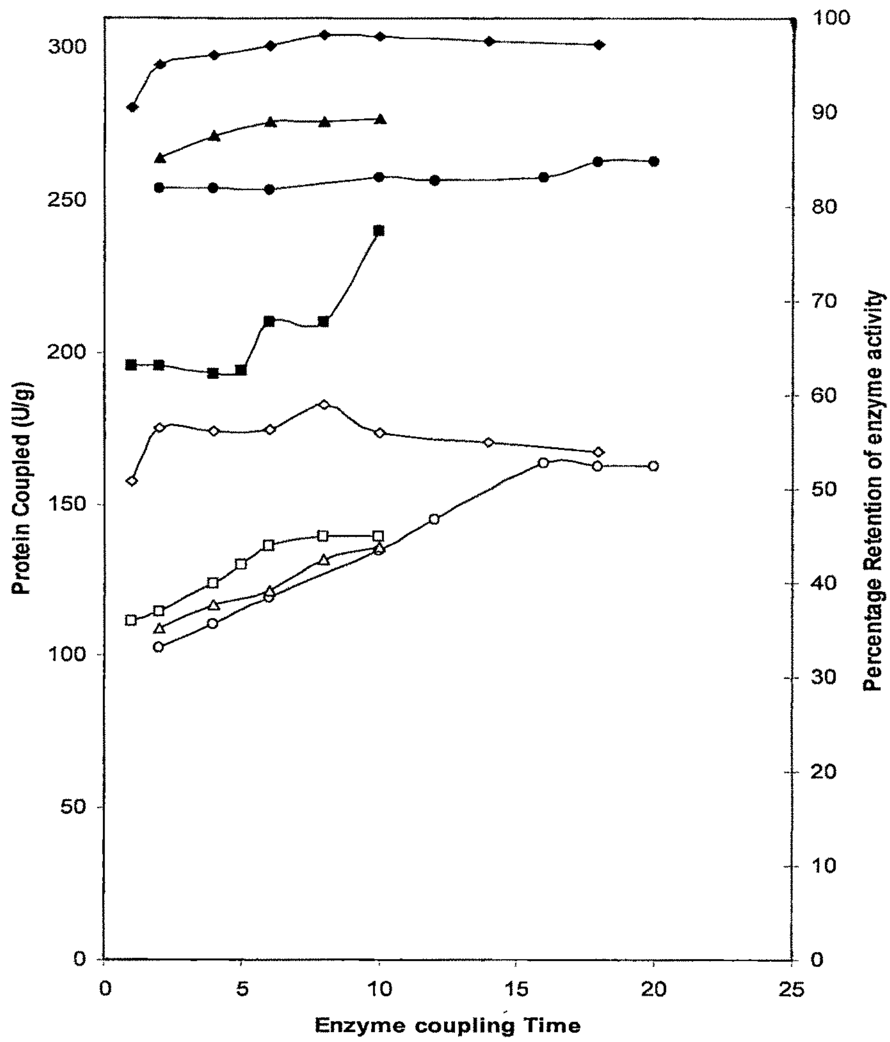

Figure 3.7. Dependence of enzyme activity on enzyme coupling time on retention of enzyme activity.

GLU : Protein Coupled (■), retention of enzyme activity (□)

PBQ : Protein Coupled (●), retention of enzyme activity (○)

PTS : Protein Coupled (▲), retention of enzyme activity (△).

GLU+PBQ : Protein Coupled (◆) , Retention of enzyme activity (◇).

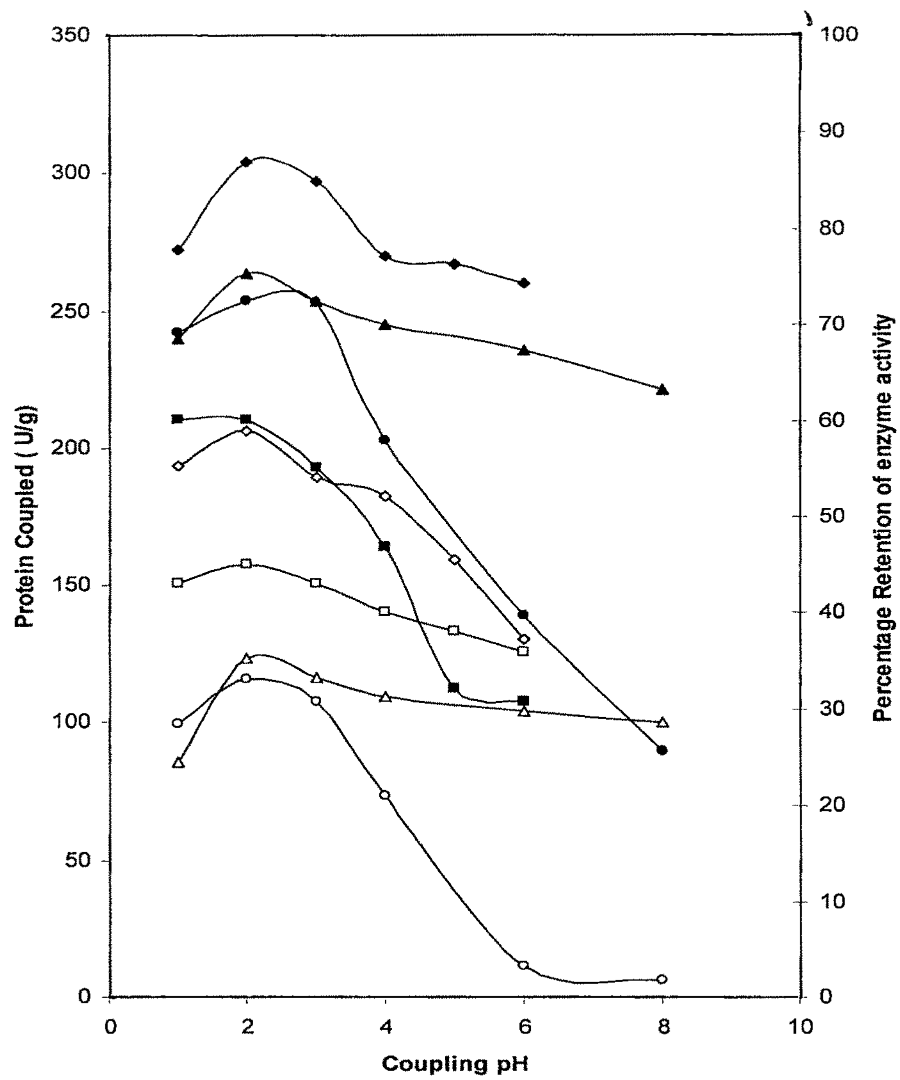

Figure 3.8. Dependence of enzyme activity on coupling pH.

GLU : Protein Coupled (■), retention of enzyme activity (□)

PBQ : Protein Coupled (●), retention of enzyme activity (○).

PTS : Protein Coupled (▲), retention of enzyme activity (△).

GLU+PBQ : Protein Coupled (◆), retention of enzyme activity (◇).

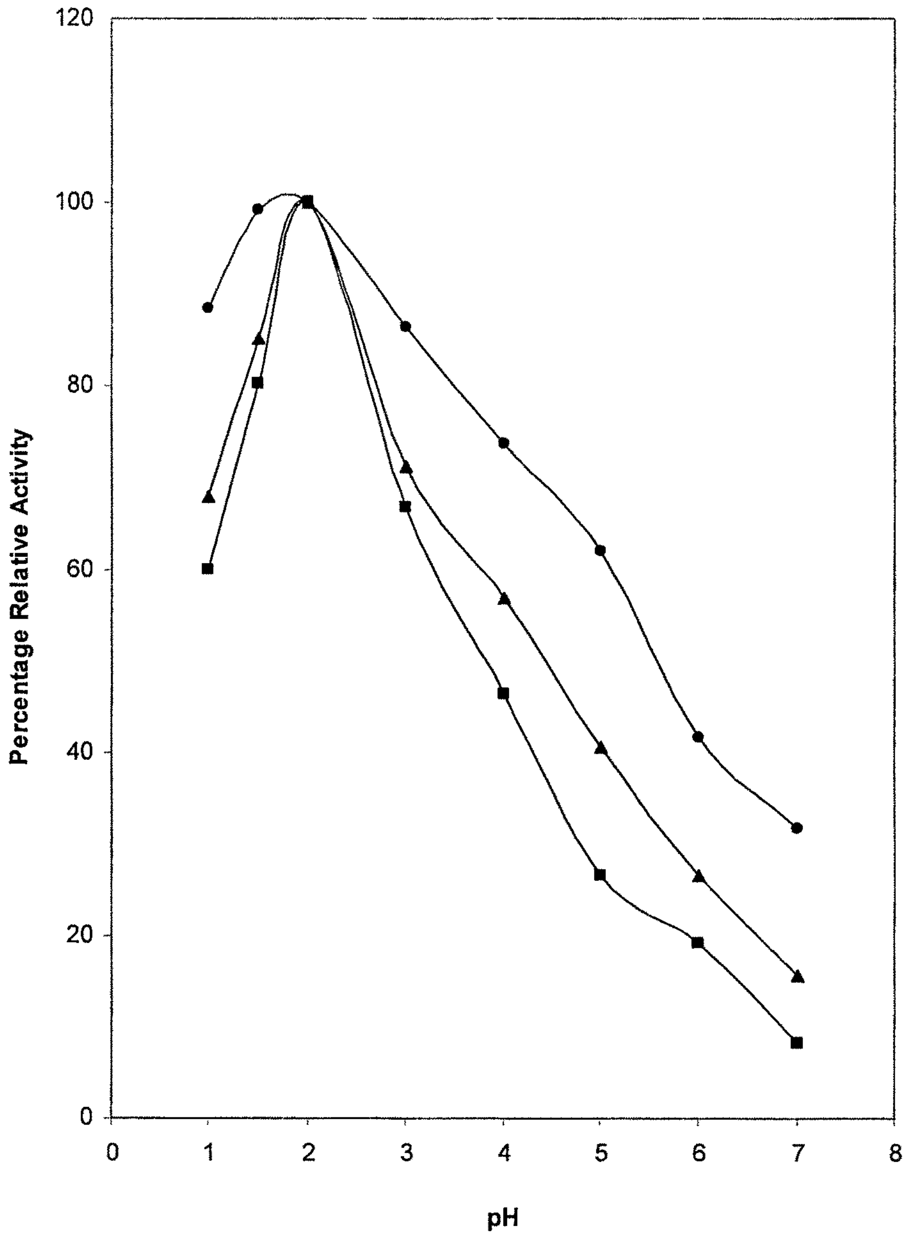

Figure 3.9.Dependence of enzyme activity on pH.

Free (●), entrapped (■), and covalently bound enzyme (▲) at 37°C

Table 3.2. Effect of different activating agents on different coupling parameters.

Coupling agents → / Coupling conditions ↓	Glutar-Aldehyde	p-Benzo-quinone	Glu+ PBQ	PBQ + Glu
Activating agent concentration	10 cm^3 1% (w/v)	25 mg /g of support	10 cm^3 1% (w/v) + 25 mg/g of support	25 mg /g +10 ml 1% (w/v)
Activation time (h)	5	4	5+8	8+5
Bound Enzyme Concentration (μg/g) of support	147 μg/g	52.9 μg/g	559.85 μg/g	151.24 μg/g
Enzyme Coupling Time (h)	8	16	8	8
Coupling pH	2	2	2	2

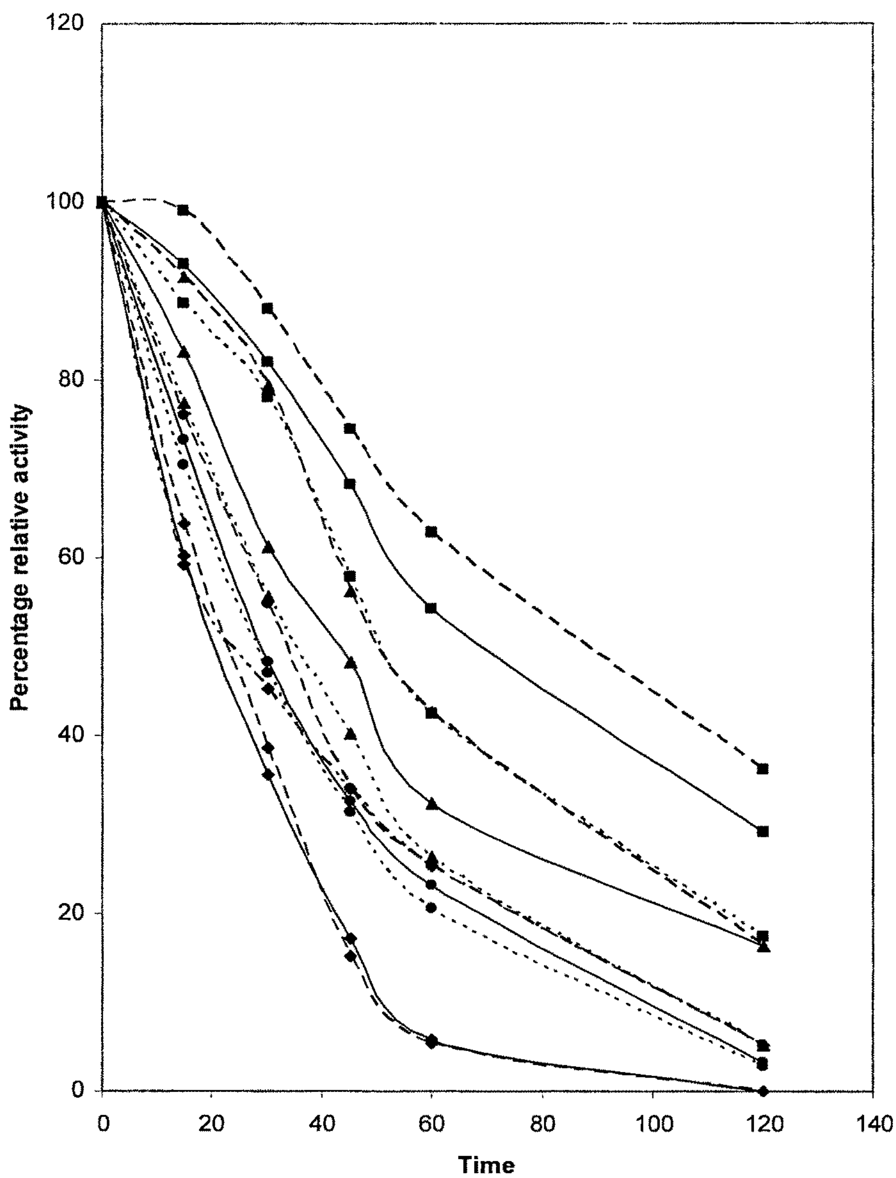

Figure 3.10.Temperature dependence on activity of free, entrapped and covalently bound enzyme at pH 2 at 35°C (■), 45°C (▲), 55°C (●), 65 °C (◆).

Free enzyme (---), entrapped enzyme (--) and covalently bound enzyme (___).

enzyme it was observed that the difference in the deactivation rate goes on decreasing with increased temperature indicating that at higher temperature free and entrapped enzyme lose activity to the same extent.

3.Reusability :

Reusability and turnover number of the catalyst govern the economics of the process . **Figure 3.11** shows that the entrapped enzyme lost 50 % activity after 5 cycles whereas covalently bound enzyme lost 55 % of its activity after 6 cycles . The turn over no. was found to be 500 for entrapped and 650 for covalently bound enzyme.

4 Kinetic Parameters :

The kinetic parameters K_m and V_{max} for free enzyme were 1.1×10^{-6} and 0.876 , whereas for entrapped enzyme 1.33×10^{-6} (mM) and 1.03 (mM/ min) and for covalenlty bound enzyme they were 1.32×10^{-6} (mM) and 0.988 (mM / min) when enzyme concentration was kept constant and substrate concentration was varied .Similarly when enzyme concentration was varied and substrate concentration was varied K_m and V_{max} for free and entrapped enzyme were found to be 6.73×10^{-11} (mM) and 0.47 (mM / min) and for entrapped enzyme 8.69×10^{-11} (mM) and 0.545 (mM / min). Thus indicating no conformational change during immobilization .**(Figure 3.12 a and b).**

5. Storage stability :

The storage stability of free and immobilized enzyme was examined at 35°C . It was observed that the free enzyme lost all its activity within 10 days whereas entrapped enzyme retained 80 % of its activity for one month **(Figure 3.13)**. This may be due to the shielding effect of K-carrageenan shell formed around the

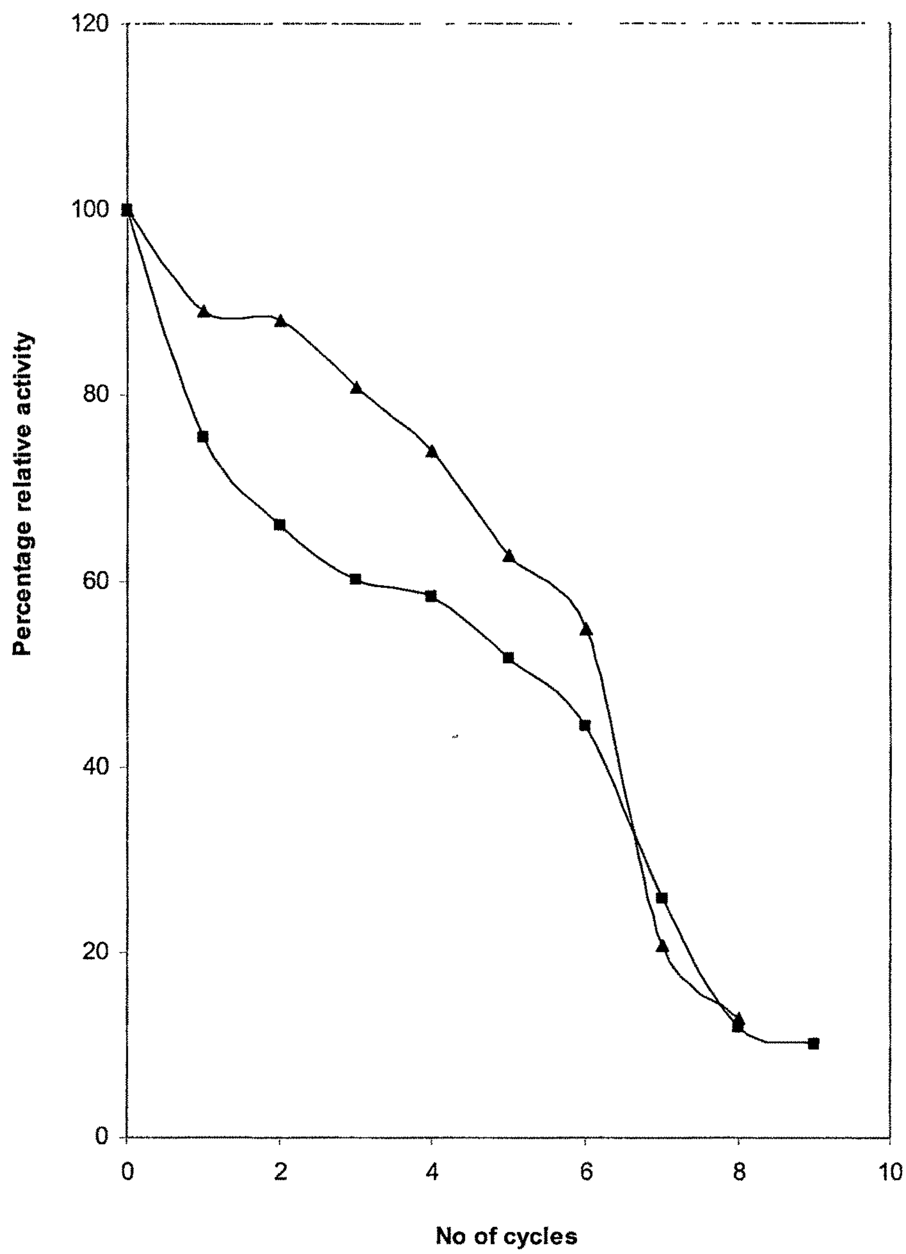

Figure 3.11.Reusability of entrapped (■) and covalently bound enzyme (▲) at 37°C and pH 2.

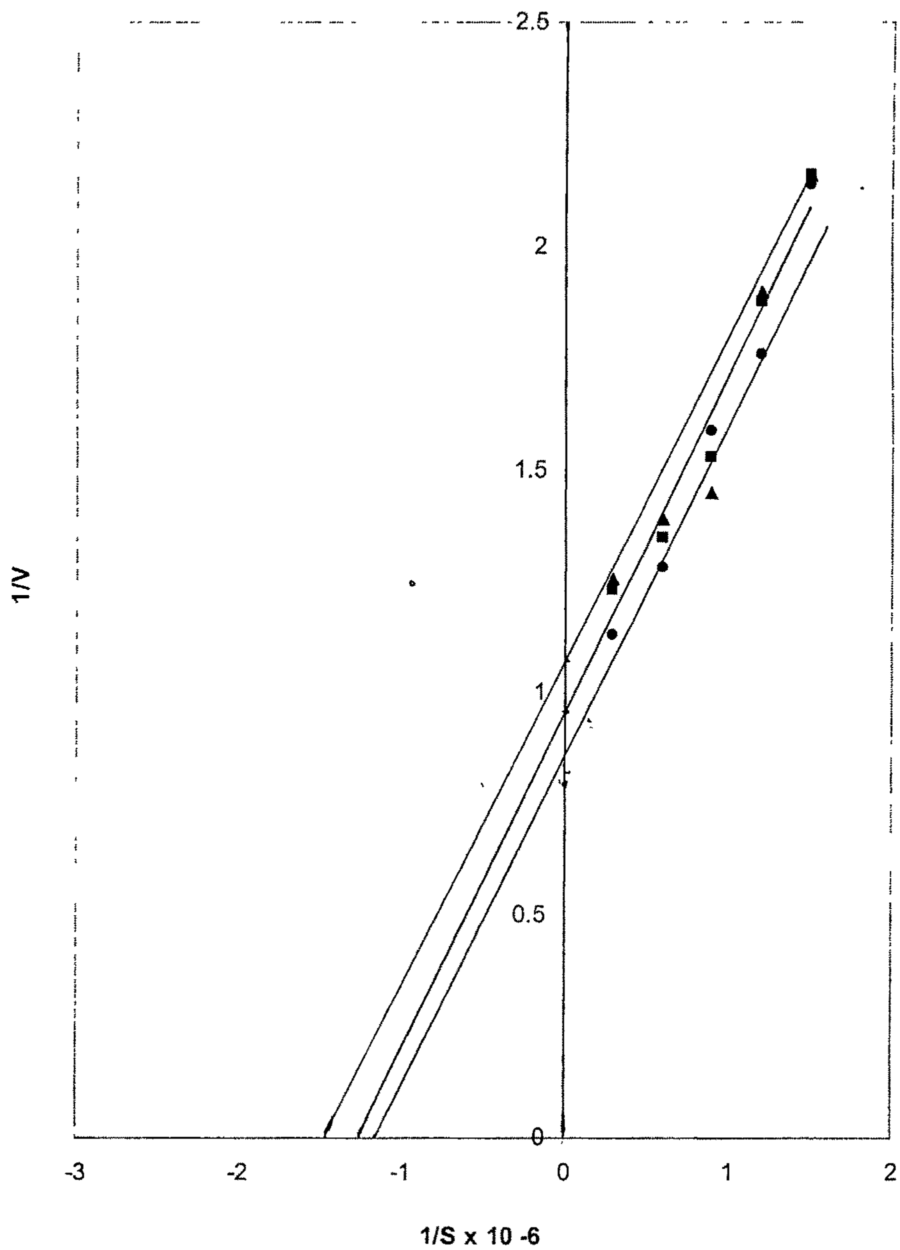

Figure 3.12.a. Line- weaver burk plot at 35°C , pH 2 and 1 min reaction time. When enzyme concentration is kept constant and substrate concentration is varied.

Free enzyme (●), entrapped (■) and covalently bound enzyme (▲).

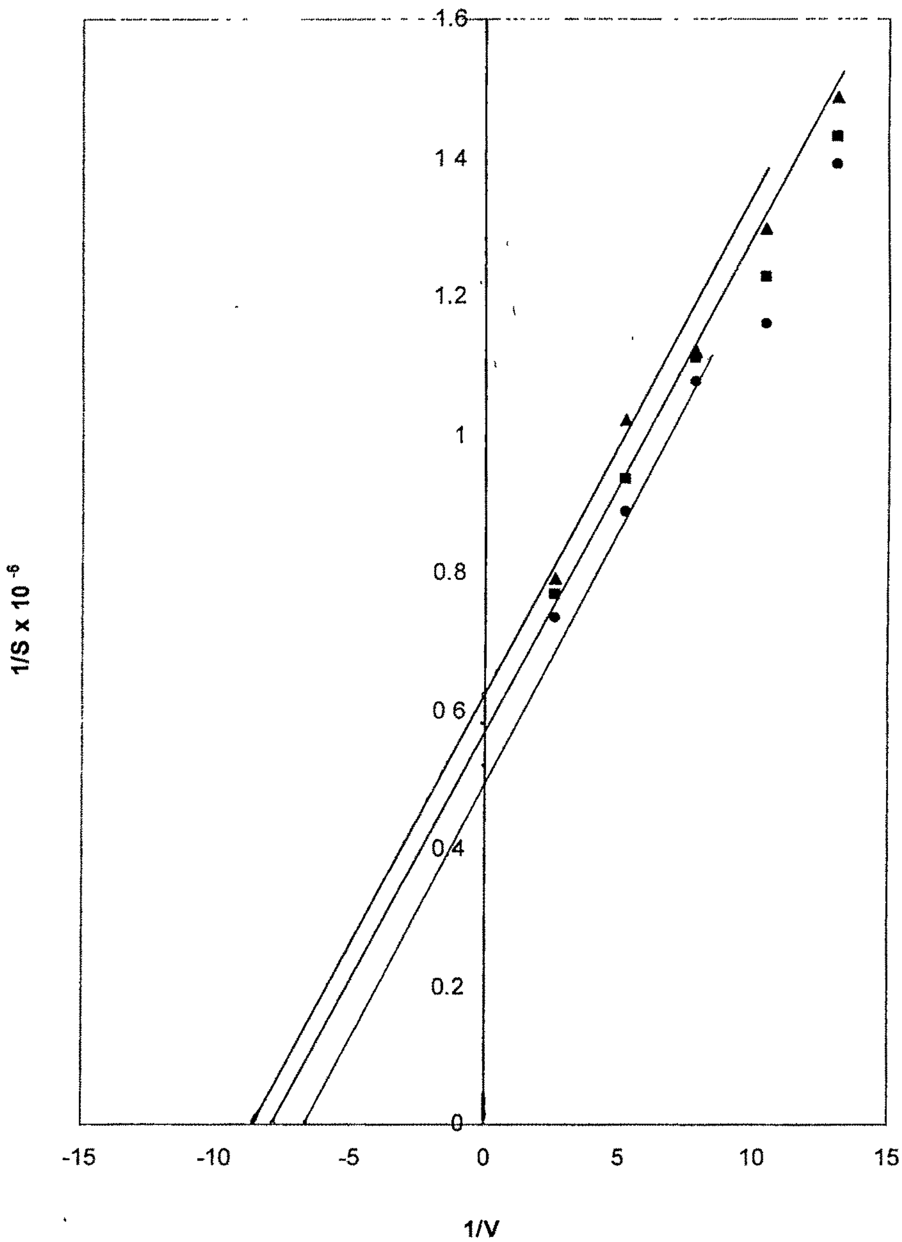

Figure 3.12.b. Line- weaver burk plot at 35°C , pH 2 and 1 min reaction time. When substrate concentration is kept constant and enzyme concentration is varied.

Free enzyme (●), entrapped (■) and covalently bound enzyme (▲) .

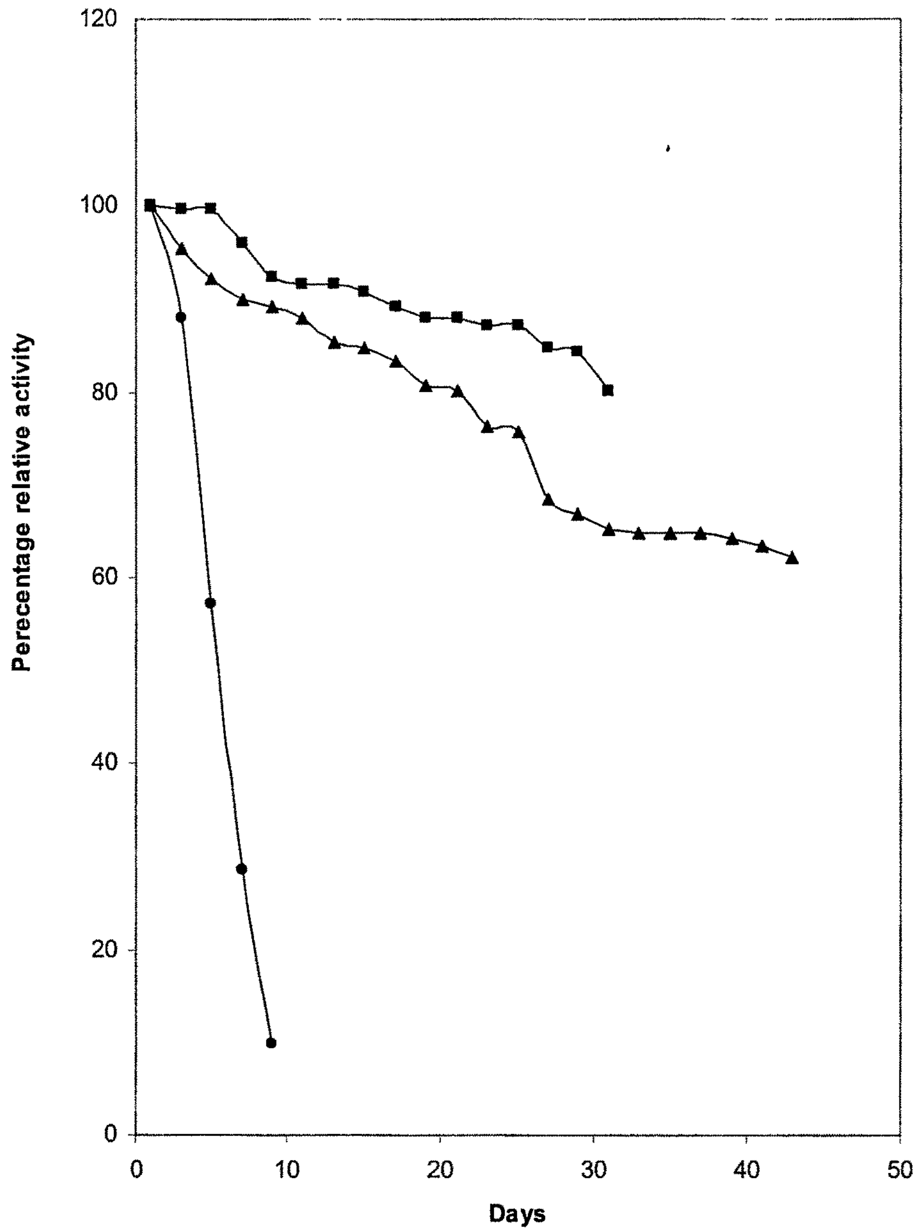

Figure 3.13. Storage stability of Free enzyme (●), entrapped (■) and covalently bound enzyme (▲).at 35°C.

entrapped enzyme protecting it form thermal deactivation whereas the covalently bound enzyme lost 60 % of its activity after one month. support which is more stronger where as free enzyme has no such bond and it can be easily affected by environmental changes during use.

3.4.4. Milk Clotting

Milk clotting was carried out by using 5 cm^3 milk solution prepared by dissolving 10 g of milk powder in 100 cm^3 of 0.06 N $CaCl_2$ as reported by Arima et al [51] using free and entrapped enzyme by batch process and also by using a fluidized bed reactor of dimension 17 x 1 cm . The flow rate was adjusted to 1 cm^3 / min and the reactor temperature was kept at 20°C. The enzymatic treatment of milk was carried out at 20°C for 10 min using 5-50 μg of enzyme and then the time required for the clotting was measured. It was observed that with increase in enzyme concentration the milk clotting activity (MCA) increased as shown in **Figure 3.14**. Milk clotting time was observed to decrease with decrease in pH **(Figure 3.15).** Effect of dilution on milk clotting was examined by taking 25,50,75,100% of milk and found that as dilution increases the milk clotting activity decreases **(Figure 3.16)**. In a blank experiment carried out at pH 2 without using enzyme milk clotting activity was found to be 0.78 indicating a little dependence of acidity of milk on clotting efficiency. The milk clotting activity in soxhlet unit was calculated using equation given by Arima et al [51].One SU is defined as the amount of enzyme which clots one ml of milk substrate in 40 min at 35°C.

$$\text{Milk clotting activity in SU} = \frac{M \times 35^{\circ}C \times 2400(s)}{E \times t \times T(s)}$$

Where M is volume of milk , E is volume of enzyme , t is temperature of the reaction ,T= time for clotting (s).

3.4.5. Casein Hydrolysis

Hydrolysis of casein was carried out by using a fixed bed reactor of dimension 17 x 1 cm. The casein solution was prepared in 0.05 M lactic acid as described by Ohtsuru et al. [52]The effect of flow rate on hydrolysis of 1mM casein solution was checked by varying the flow rates from 2 to 8 cm^3/min .It was observed that with the increase in flow rate the percentage hydrolysis decreased as the time of contact of covalently bound support and casein solution decreased. Moreover 90 % of hydrolysis was achieved after 4 cycles at 2 cm^3/min flowrate **(Figure 3.17)**.Percentage hydrolysis of casein was observed to decreased when casein concentration was increased from 1 to 4 mM and flow rate was maintained at 2 cm^3/min at 35°C **(Figure 3.18)**. Similar trend was observed with increase in reaction temperature .Percentage hydrolysis decreased with increased temperature **(Figure 3.19)**. This may be due to the fact that some enzyme would have been deactivated at higher temperature. With increase in l/d ratio upto 4 the casein hydrolysis increased but on further increase in l/d ratio it did not show much change in casein hydrolysis. Maximum of 90 % hydrolysis was achieved after 4 cycles at the l/d ratio of 4**(Figure 3.20)**.

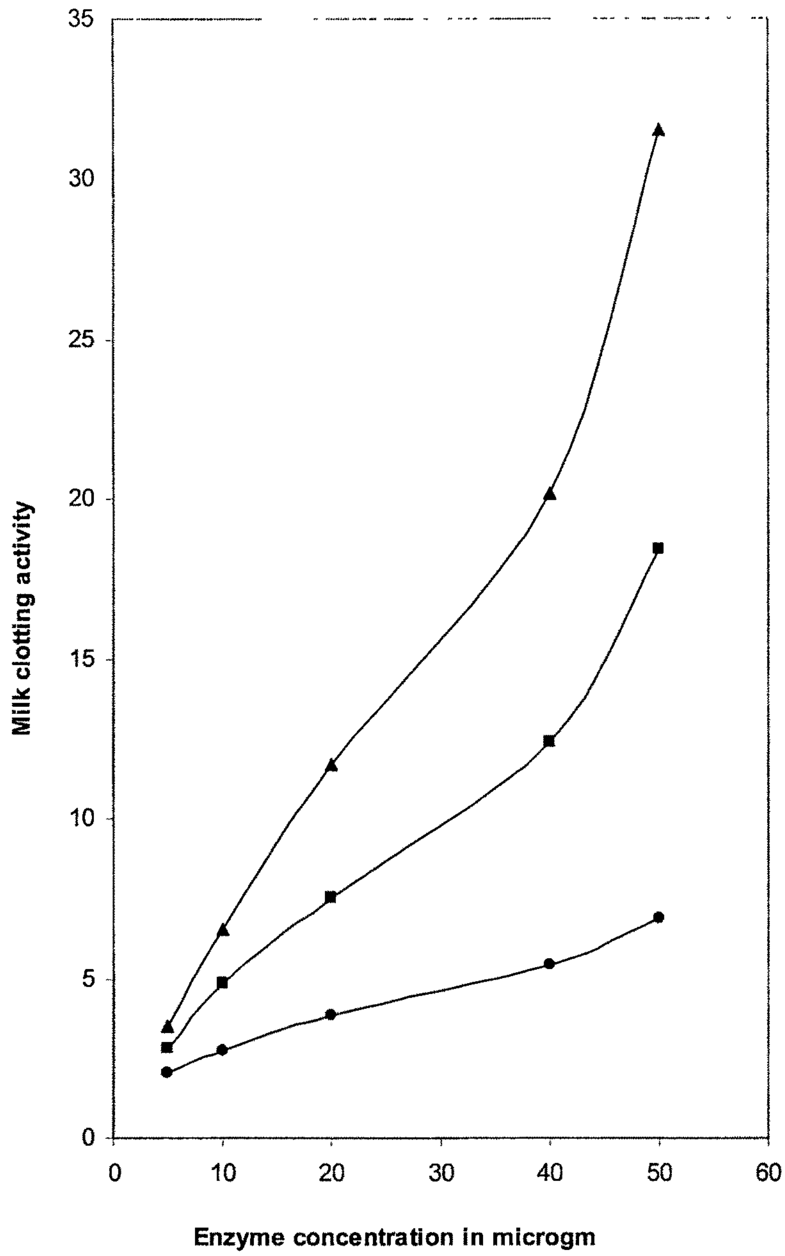

Figure 3.14.Effect of enzyme concentration on milk clotting activity at pH 2. Free enzyme (●), entrapped enzyme (■) by batch process and by fluidized bed reactor (▲) reactor

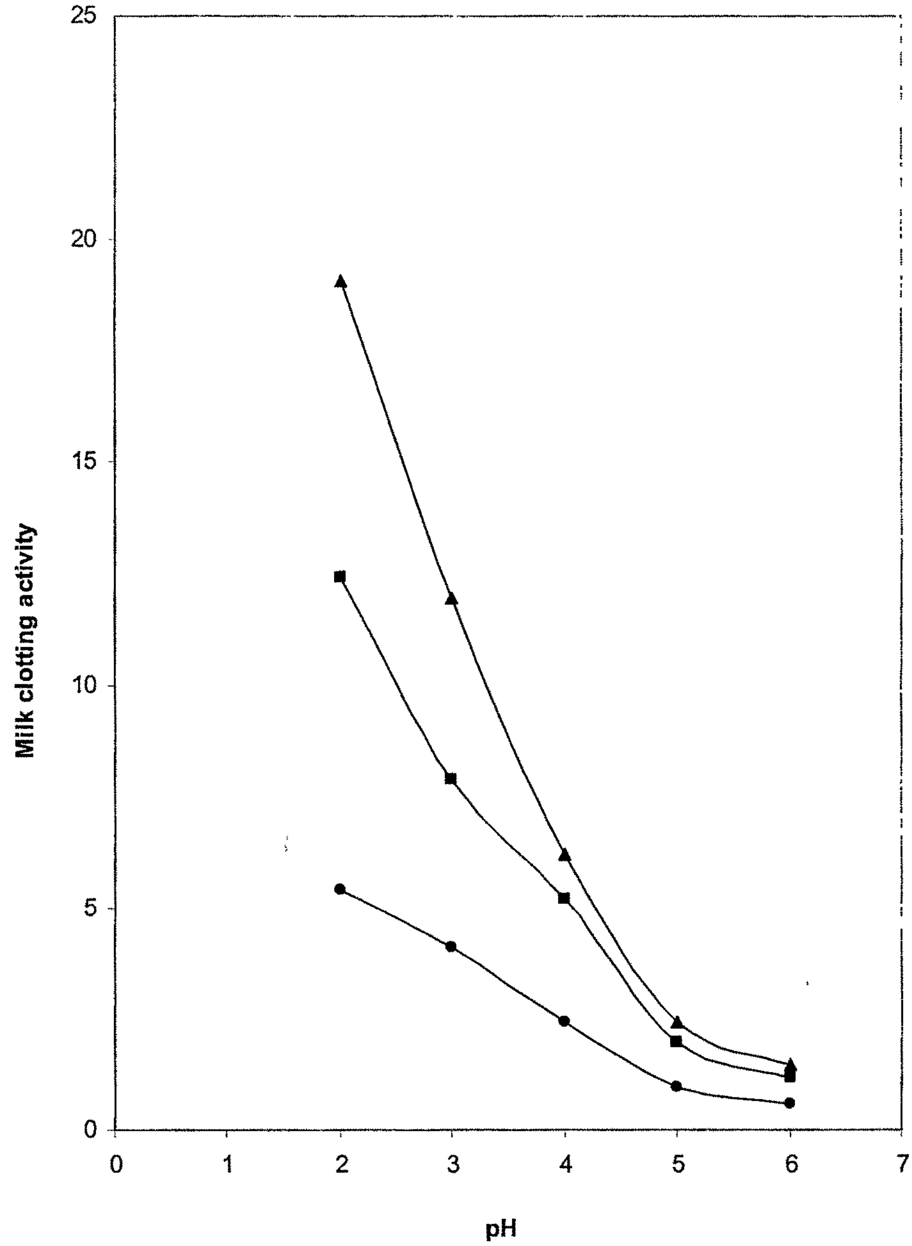

Figure 3.15. Effect of pH on milk clotting activity using 40 μg of enzyme. Free enzyme (●), entrapped enzyme (■) by batch process and by fluidized bed reactor (▲) reactor.

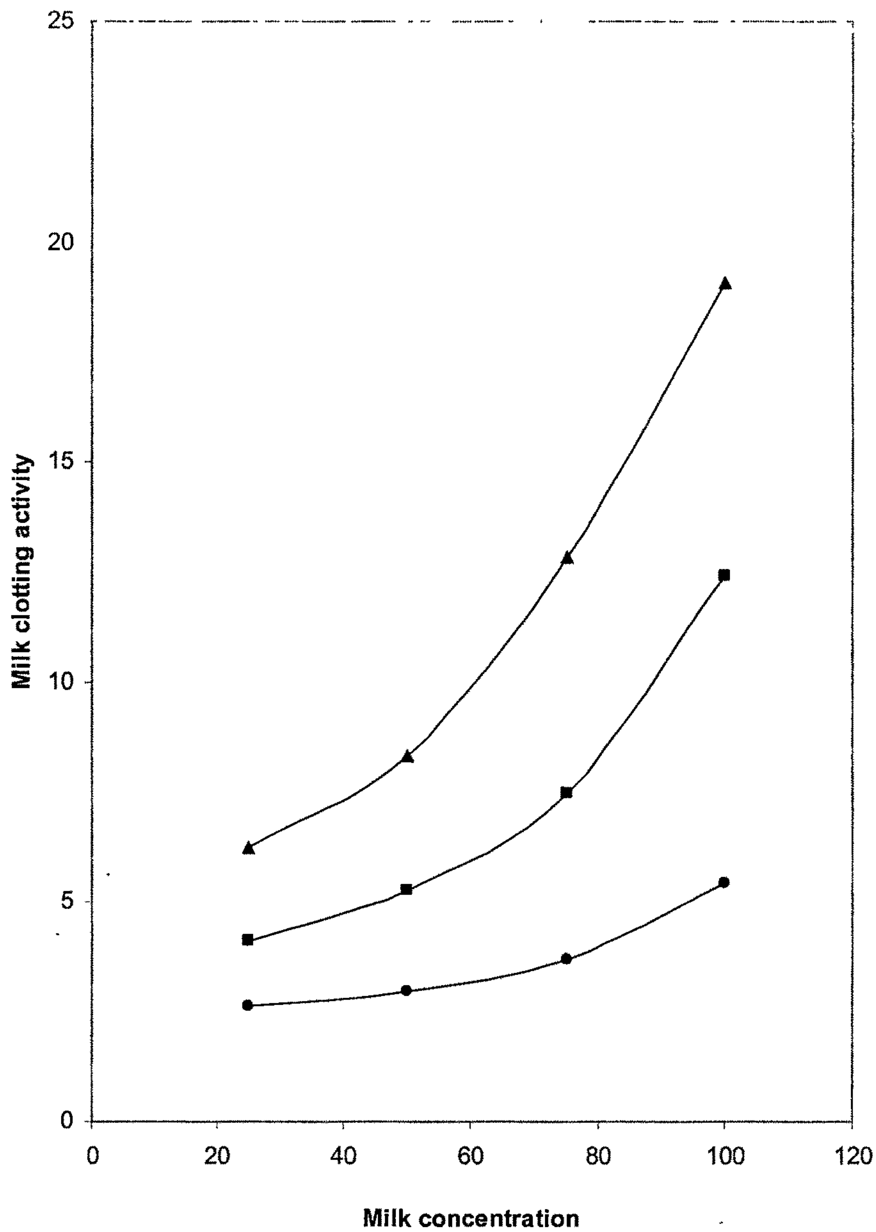

Figure 3.16. Effect of dilution on milk clotting activity.

Free enzyme (●), entrapped enzyme (■) by batch process and by fluidized bed reactor (▲) reactor.

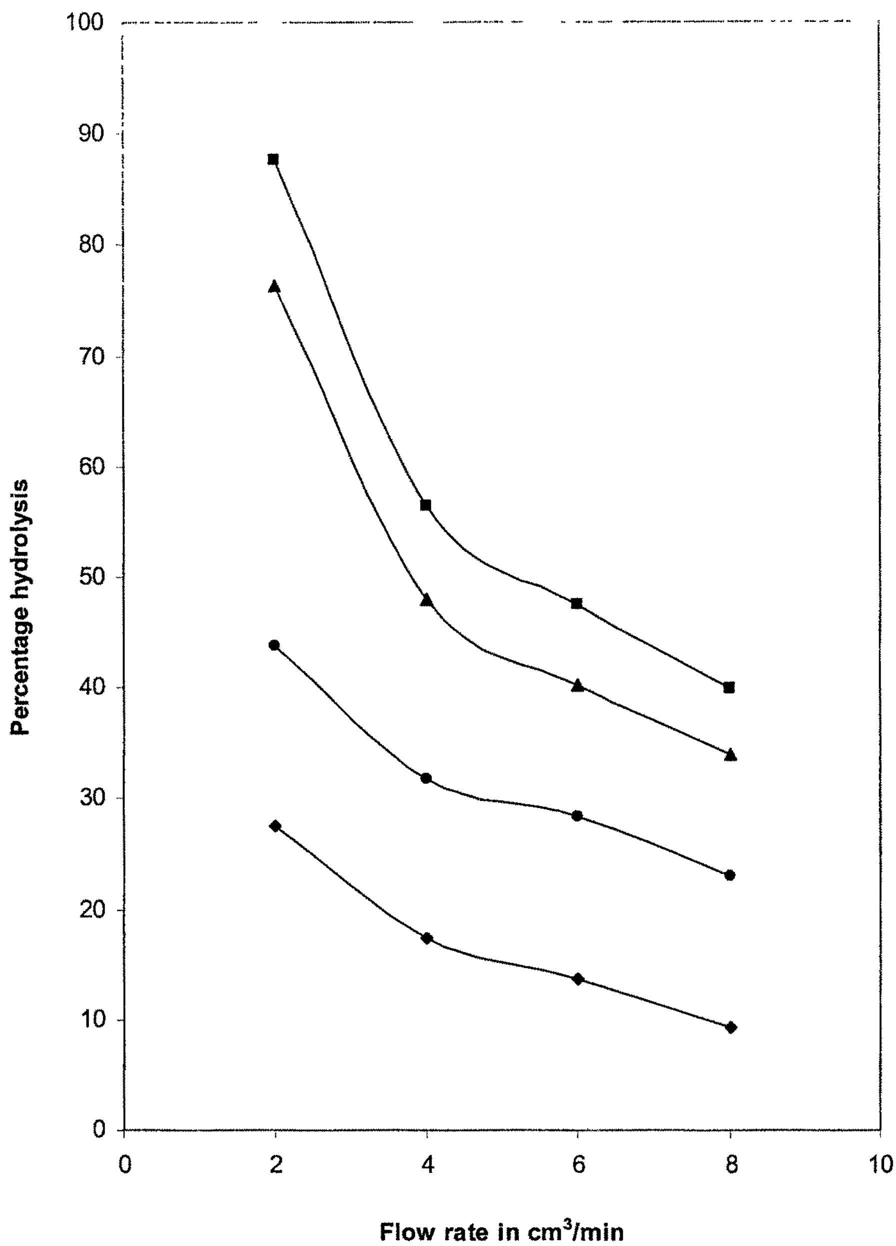

Figure 3.17. Effect of flow rate on casein hydrolysis at 35°C and 1 mM casein solution.

First cycle (■), second cycle (▲), third cycle (●), fourth cycle (◆).

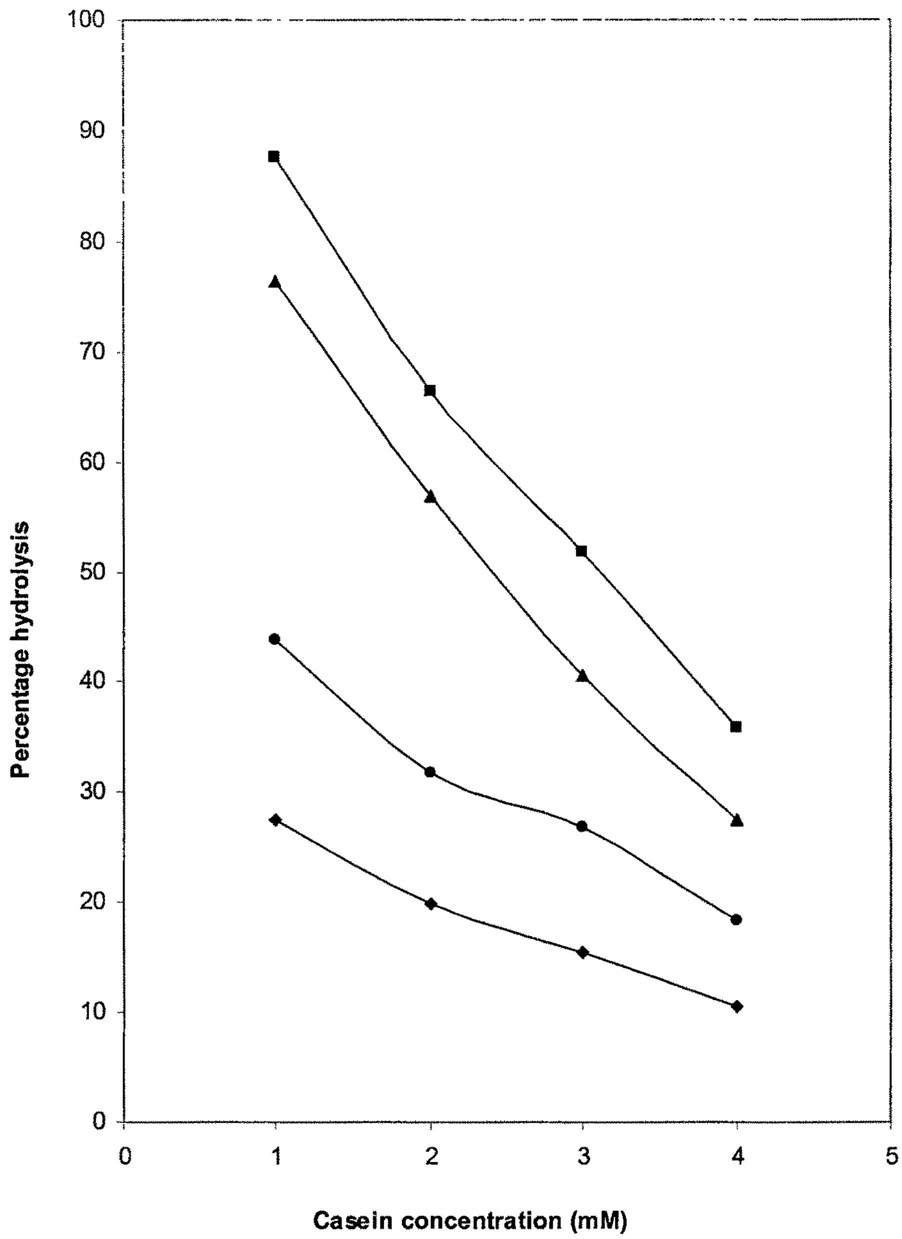

Figure 3.18. Effect of casein concentration on hydrolysis of 2 ml / min flow rate at 35°C.

First cycle (■), second cycle (●), third cycle (▲), fourth cycle (◆).

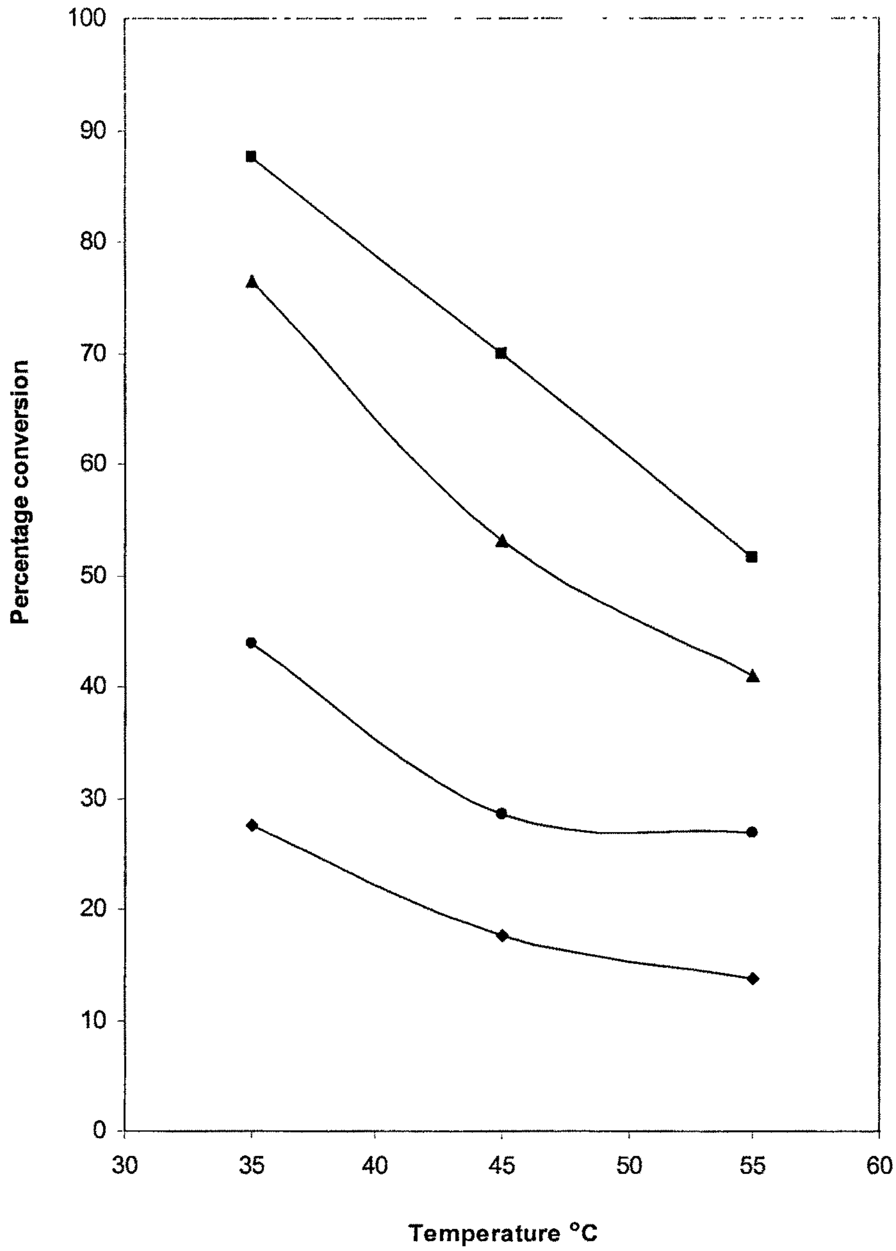

Figure 3.19 Effect of temperature on hydrolysis of casein at 2ml/min flow rate and 1 mM casein concentration.

First cycle (■), second cycle (●), third cycle (▲), fourth cycle (◆).

3.5. Summary and Conclusion :

Immobilization of pepsin was successfully carried out on K -carrageenan and copolymer of AAm-HEMA .

We have observed that under all tested conditions such as pH , temperature , storage , kinetic parameters, the immobilized enzyme was superior to free one.

On entrapment the percentage retention increased initially and then decreased. Moreover a synergistic effect was observed in case of covalent coupling to the copolymer of AAm-HEMA . The activity of the enzyme increased upto 3 fold.

The milk clotting was carried out using K -carrageenan and it was found that with decrease in pH the milk clotting activity increased.

The hydrolysis of casein was carried out using copolymer of AAm-HEMA and it was observed that nearly 90 % hydrolysis is obtained using 1mM casein solution at a flow rate of 2 cm^3/min at 35°C.

Table 3.3. Comparison of properties of free and immobilized enzyme.

Conditions	Free	Entrapped	Covalently bound
Optimum pH	2	2	2
Optimum Temperature (^{o}C)	35	35	35
Storage stability at RT after one month (days)	-	80	60
Reusability	-	5	6
Thermodeactivation constant at 45°C.	1.66×10^{2}	0.551×10^{2}	1.12×10^{2}
Michaeli's constant , K_m (mM)	1.1×10^{-6}	1.3×10^{-6}	1.2×10^{-6}
Maximum velocity , V_{max} (mM/min)	0.88	1.03	0.98
Turn over number	-	500	650

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