Chapter 2

Immobilization of Horseradish Peroxidase

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2.1 Horseradish Peroxidase :

Peroxidases are distributed widely in both plant and animal kingdom. Perhaps one of the widely known to enzymologists is horseradish peroxidase (EC 1.11.1.7) because of its wide use as an indicator enzyme in standard spectrophotometric and immunoessay technique. According to their source and mode of action they have been divided into three super families :

- plant peroxidase
- animal peroxidase
- catalase.

Peroxidases (E.C.1.11.1.7) are heme proteins which catalyze dehydrògenation of aromatic substrates through reaction with hydrogen peroxide. They are extensively used in clinical biochemistry and immuno assay [1,2] . Horseradish peroxidase is a monomeric protein consisting of a single polypeptide chain arranged in two domains and glycosylated at eight specific sites [3,4] . It contains one heme prosthetic group , two calcium ions and four disulphide bridges [5].

Enzymatic oxidation is preferred over chemical oxidation process because of

* Need for replacement of oxidation process involving stoichometric amounts of heavy metal salts by cleaner catalytic alternatives .

*Due to increasing demand for high chemo- , region- , stereo selectivity in chemical reactions in order to improve chemical yields to minimize ballast. Redox enzymes are potentially suitable for meeting these goals .

Peroxidases have tremendous applications in environmental and health care sectors and in the pharmaceuticals , chemicals and biotechnological industries. Peroxidases have potential applications in many different areas. The most developed field for their commercial application is in analytical diagnostics, for example in biosensors and immunoassay . Moreover peroxidases are being extensively studied as bleaching inducer in , the detergent and pulp industries [6] , in anti-microbial applications, for removing aromatics from waste streams [7,8] .

2.2 Literature Survey on Horseradish Peroxidase

Considerable work has been carried out in the area of enzyme immobilization . Among the enzymes studied for immobilization horseradish peroxidase is one of the most widely used enzyme. Work carried out in past has been classified as per the methods used for immobilization.

2.2.1 Entrapment

The entrapment of horseradish peroxidase was first carried out by Ugarova et al [9-10] . They entrapped horseradish peroxidase in poly (AAm) gels . The thermostability of entrapped enzyme was observed to decrease by three fold and 17 fold at 20°C and 56°C operation temperatures . To overcome the loss of enzyme activity horseradish peroxidase was first crosslinked with glutaraldehyde then entrapped into poly (AAm) gel . This could lead into improved thermostability depending upon cross-linking agent concentration. The maximum entrapment and retention was observed to be at 40 % poly (AAm) using 10 % crosslinking agent bis-AAm, previously crosslinked with 9 % glutaraldehyde. The values of Michaelis constant (K_m) were observed to remain unchanged on entrapment whereas V_{max} decreased 3 fold times at pH 7.0.

Horseradish peroxidase was also entrapped in graft copolymers of natural polysaccharides such as cellulose, sepharose , sephadex , and starch using glycidyl methacrylate corsslinking agent by D'Angiuro et al [11-13]. They have observed no change in the values of kinetic parameters K_m and V_{max} of the free and entrapped enzyme indicating no conformational change during entrapment.

Soluble-gel matrices of alkoxysilane derivatives have been used for entrapment of horseradish peroxidase by El-Essi et al. [14]. The sol-gel-immobilized peroxidase was used for the determination of glucose in serum. The type of soluble gel matrix was observed to affect optimum temperature for maximum enzymatic activity .However, entrapped enzyme did retain its activity for two months when stored at room temperature.

Horseradish Peroxidase has been effectively entrapped in a novel composite membrane of poly vinly alcohol (PVA) and regenerated silk fibroin (RSF) by Liu et al. [15]. New methylene blue mediating sensor based on the composite membrane of immobilized matrix of horseradish peroxidase for hydrogen peroxidase was developed . The performance and characteristics of the sensor were evaluated with regard to response time, detection time, selectivity and dependence on temperature and pH as well as operating and storage stability. The sensor was reported to exhibit good sensitivity, rapid-response time and low detection limit of 5.0μg.

Horseradish peroxidase has been entrapped in tributyl methylphosphonium chloride polymer bound anionic exchange resin , at a pyrolytic graphite electrode by Tommaso et al. [16] It was observed that the immobilization enhanced the electron exchange between the protein and the electrode surface . Moreover the embeded protein showed native like structural properties and increased stability. These results are of special significance as they represent a first step for engineering a novel solid state electrode system which is useful for basic and applied biochemistry.

Entrapment of horseradish peroxidase in uv-polymerizable screen-printable paste was carried out by Schumacher et al [17] They further used it in flow injection analysis systems. They checked the effect of different additives on immobilized system.

A novel reagentless hydrogen peroxide sensor fabricated by the entrapment of horseradish peroxidase into poly (thionine) film on a thionine self assembled monolayer modified gold electrode was developed by Xiao et al [18] . The sensor displayed excellent electrocatalytic response to the reduction of H_2O_2 . Moreover it showed good stability and reproducibility within the linear range of H_2O_2.

2.2.3.Covalent Binding :

Horseradish Peroxidase was covalently bound onto different supports and further used in synthesis of quinone and benzoquinone derivatives by Bartling and coworkers[19-23]. The cellulose acetate fiber of 0.4 mm inner diameter was activated using CNBr and poly(4-methacryloxy benzoic acid) support was activated using N-ethoxy carboxyl-2-ethoxy 1,2, dihydroquinoline . No change in kinetic parameters of free and immobilized enzyme was reported. The polymer – enzyme complex was reported to show better thermal and storage stability . They have also used methylene chloride for immobilization of horseradish peroxidase after crosslinking with N-N' carboxyldiimedazol. The immobilized system was reported to show better stability towards denaturation in the aqueous mixtures of methanol , ethanol and 1,4 dioxane.

Schell et al. [24] immobilized HRP on CNBr activated cellulose a commonly used natural support . They observed that the pH for the optimum activity of immobilized enzyme remains unchanged on immobilization . However , immobilized enzyme was reported to lose 50 % of its activity at room temperature after 2 weeks .

Berezin et al. [25] covalently bound AH-sepharose 4 B through the enzyme carbohydrate residue . They observed that the kinetic parameters (K_m) did not change on immobilization . But V_{max} decreased two fold when o-dianisidine was used as substrate.

Miller et al. [26-27] covalently bound HRP to poly styrene beads . 90% loss in the initial activity was observed after 30 days of storage. They also used it for detection of low concentrations of H_2O_2 by using a packed bed reactor.

Covalent coupling of HRP to CNBr activated cellulose was done by Ceremonesi et al. [28-29] .They observed that immobilized HRP lost all its activity after 2 cycles . Moreover maximum coupling was achieved within 40 minutes coupling time at pH 7.0.

Gray et al. [30] covalently bound HRP to diazotized m-diaminobenzene and studied its properties . They studied the effect of sorbitol on the free and immobilized enzyme and observed that sorbitol has more protective effect for thermo-stability of immobilized HRP at $60^{\circ}C$ in phosphate buffer at pH 8.0

Chin and Lanks [31] developed a new method for covalent coupling of peroxidase to polystyrene. They observed that this new method was effective only when nitration of polystyrene with HNO_3 –H_2SO_4 was followed by reduction with Na hydrosulfite and simultaneous incubation of HRP.

Covalent coupling of horseradish peroxidase to poly(AAm) prepared by free radical polymerization using bis-acrylamide as crosslinking agent in the presence of enzyme protecting reagent as dithiothreitol was carried out by Adalsteinsson et al [32]. The monomer concentration and cross-linking time were found to be critical in retention of enzyme activity.

Covalent coupling of horseradish peroxidase on poly(acryloyl-morpholine) gel network was carried out by Epton et al. [33,34]. The activity of the immobilized horseradish peroxidase was compared with that of the native enzyme in aqueous buffer and in buffered mixtures of dimethyl-formamide/water , ethane diol /water , methanol /water and tetrahydrofuran /water of varying ratios at pH 6.1. The authors observed that with increasing organic solvent concentration in the solution , the active immobilized enzyme retained its activity much better than an equivalent amount of the native enzyme . Moreover , the immobilized enzyme exhibited superior resistance to thermal denaturation.

Minamoto et al. [35] used polymethylglutamate(PMG) for covalent coupling of HRP and other enzymes . They observed that PMG coated glass beads retained more than 90% enzyme activity. Improved thermostability was observed in case of immobilized enzyme compared to free ones due to increase in hydrophilicity of the polymer and the multipoint binding of enzyme by covalent and ionic binding.

Para tolyl-sulphonyl chloride has been used as a coupling agent for activation of free hydroxyl group of agarose by Nilsson and coworkers [36,37]. They observed 70 mg /g loading of HRP of dry support with 19% retention of enzyme activity. Loading of 6-10 μmol HRP /g wet gel was obtained in phosphate buffer of pH 8.0 at room temperature for 10 h.

Immobilization of peroxidase was carried out on two different types of inorganic supports such as aliphatic amino silica activated with glutaraldehyde and aromatic amino silica activated with nitrite by Thibault et al.[38]. The immobilized enzymes exhibited maximum activity at pH 7.0 . However , peroxidase immobilized on glutaraldehyde activated aliphatic amino silica was more resistant to various concentrations of 10 to 20 % ethanol than free aromatic silica enzyme conjugate.

Taylor [39] carried out immobilization of peroxidase and other enzymes on various commeriacially available , activated supports such as Sepharon HEMA activated with epicholorohydrin , CNBr activated agarose and sepharose . The coupling of peroxidase onto sepharon-HEMA activated supports at pH 7.0 and

9.0 was found to be 7.1 and 5.9 U/g with upto 30 % retention of enzyme activity. While in case of sepharose 5.9 and 5.6 U/g coupling was observed with 59 and 50% retention of activity respectively.

Sepharose 4B has been used for covalent coupling of HRP by D'Angiuro et al [40] using two different functional monomers bisacryloyl piperazine and triazine derivatives. Optimum coupling was observed at enzyme/polymer ratio of 0.111 and 0.22 respectively . There was no change in kinetic parameters K_m and V_{max} for free and immobilized enzyme.

Horseradish Peroxidase has been immobilized by covalent binding on different carriers by Ermekbae et al. [41]. They observed that CNBr-agarose forms a wider spectrum of reaction products with quercelin as compared with soluble peroxidase .Moreover the purification of the compounds was also studied by using HPLC.

Husain [42] immobilized HRP on CNBr-activated sepharose and studied heat and urea-induced inactivation and ability to regain activity after denaturation .All the immobilized preparations were reported to exhibit higher stability against heat induced inactivation as compared to native HRP.

HRP has been covalently attached to the surface of the carbon particles via cyanuric chloride by Cardosi [43] . Electrochemical studies on these carbon particle electrodes indicated that they were stable for extended periods and the bound HRP was capable of mediating the electrocatalytic reduction of hydrogen peroxide.

Peroxidase monolayer-modified electrode coated with conducting polymer was prepared by Tatsuma et al. [44] The conducting polymers used are poly (alkyl 3-methyl pyrrole –4- carboxylate) and poly (3-dodecyl pyrrole) . This electrode was further used for amperometric response to H_2O_2 .

Dasilva et al. [45] covalently bound HRP on TiO_2 coated cellulose microfibres and used it for oxidation of pyrogallol to purpurogallin by using a flow injection system. They observed that the value of kinetic parameters K_m and V_{max} for immobilized HRP was 47 mM and 22 μmol /min whereas in case of free enzyme it was 11 mM and 53 μmol/min . They also observed that activity of immobilized enzyme is reduced presumably due to its interaction with the oxide surface.

Yotova and Ivanov [46] immobilized HRP onto acetylcellulose beads. The immobilized enzyme manifested no change in its pH and temperature optima but slight increase in the value of K_m compared to free enzyme was reported.

HRP has been immobilized onto chitosan beads activated with glutaraldehyde for detection of microamounts of H_2O_2 in environmental samples by Sakuragawa et al.[47]. The HRP immobilized on chitosan beads was reported to be useful for seven days when stored in solution containing BSA and NaCl at 4°C.

Rao et al. [48] immobilized HRP through covalent binding to polystyrene beads activated through water soluble carbodiimide. They observed that the kinetic parameters for free and immobilized HRP were unaltered. They also observed that immobilized HRP retained 81 % of its initial activity when stored at 5°C for 30 days.

Horseradish peroxidase (HRP) was modified chemically with p-formyl –phenyl ferrocene (FPF) by Wang and Yagin [49] . The modified HRP and pyrrole were electrochemically polymerized and glucose oxidase was immobilized chemically with glutaraldehyde onto platinium electrode to form HRP/GOD biosensor. The biosensor was used for detection of the concentration of hydrogen and glucose , which resulted in largely improving the response of the enzyme sensor to glucose.

Alencar et al. [50] immobilized HRP on poly(propylene –g- poly(acrylic acid) prepared by using radiation –induced graft copolymerization of acrylic acid onto polypropylene spheres characterized by TGA and SEM . They observed that there was no change in the enzyme affinity for the substrate on immobilization .

Immobilization of HRP on self assembled monolayer modified gold electrode for the detection of hydrogen peroxide was carried out by Sun et al. [51] .The resulting electrode exhibits a high sensitivity to hydrogen peroxide in presence of a mediator. The optimum pH of the solution for enzyme electrode is 6.0 . A decrease in current response is observed as the concentration of hydrogen peroxide increases above 6.0 mM , which might be due to deactivation of the enzyme electrode at high concentration of the substrate .

Non porous cross-linked poly (2-hydroxyethyl methacrylate-co-ethylene imethacrylate) (poly hema-co-edma) micro-spheres were prepared by dispersion polymerization in toluene/2-methy propan-1-ol medium in the presence of dibenzoyl peroxide initiator by Horak et al.[52] The particle size was controlled by controlling the toluene : methyl propan-1-ol ratio and polymerization temperature . Adipohydrazide was attached to the microspheres activated with 2,4,6,-trichloro-1,3,5, s-triazine . After periodate oxidation of its carbohydrate moieties , HRP was coupled to the hydrazine functionalized poly (HEMA-co-EDMA) microparticles upto 7.8 μg/g of carrier without a significant loss of its activity . Immobilized peroxidase was found to be stable , retaining more than 97 % of its initial activity when stored for 23 days after the preparation .

Horseradish peroxidase has been immobilized on different supports by Muginova et al [53] . They have used it for oxidation of o-phenylenediamine, 3,3'-dimethoxybenzidine and 3,3',5,5'-tetramethyl benzidine with H_2O_2 The kinetics and pathway of the enzyme catalyzed reaction was observed to depend on use of native free enzyme or enzyme immobilized on support. .

Bindhu et al. [54] immobilized HRP on chitosan by covalent coupling . The influence of the substrate and solvent hydrophilicity , or hydrophobicity and support aquaphilicity on the catalytic activity of immobilized enzyme in non aqueous medium was examined . They observed that the catalytic efficiency was greatest when o-phenylene diamine was used as substrate and least when guaiacol was used . The aquaphilicity of the support played an important role in the kinetics of immobilized HRP in water –miscible solvents. The results were found to be promising for future development of chitosan –immobilized enzyme for use in organic media.

Horseradish peroxidase has been covalently bound onto chemically synthesized polyaniline by Fernandes et al. [55] by using glutaraldehyde as a activating agent. The authors observed that the immobilized enzyme showed better performance than free enzyme when subjected to concentration of organic solvents such as ethanol , acetone and acetonitrile .The immobilized enzyme was found to be better when subjected to heat treatment with a plateau of activity range from 30 to 60 ^{o}C . Moreover the pH activity profile revealed a similar behavior .

Covalently bound horseradish peroxidase onto poly(ethylene terphthalate) – poly (aniline) composite was reported by Samantha and coworkers [56] .The composite was characterized by photomicrography and multiple reflectance infrared spectrometry . The immobilized HRP showed 40 % enzyme activity retention when immobilization was carried at $4^{o}C$, at pH 4.5 using enzyme concentration of 0.01 mg ml^{-1} for 90 min. The immobilized enzyme showed better storage stability than free enzyme.

2.2.3. Adsorption :

Adsorption is another immobilization technique used for immobilization of enzymes. Adsorption is a surface phenomena . A few references of adsorption are given below.

HRP has been adsorbed on pyrographite for preparation of amperometric electrode by Wollenberger and coworkers [57]. They used it further for mediatorless hydrogen peroxide detection at a potential of –0.010.V

Silin et al [58] adsorbed HRP on chemically modified Al and Cu surfaces and characterized by FTIR spectroscopy . The pretreatment of Al surface with $TiCl_4$ increased the activity of adsorbed HRP 7 times . An additional pretreatment of the surface with s-trichlorotriazine solution led to 2-3 fold increase in enzyme activity and a subsequent pretreatment with polyetheleneimine merely increased the stability of the enzyme .

HRP and other enzymes have been used for preparation of enzyme electrode by adsorbing horseradish peroxidase to colloidal gold by Crumbles et al.[59]. This was used for cholesterol detection in human serum and whole blood.

Friedman et al. [60] adsorbed HRP on carbon matrices. They further used cyclic voltametry to check the electrochemical transformation of peroxidase adsorbed on carbon materials for the purpose of detecting the mechanism of electron transport and optimal conditions for enzyme immobilization.

HRP was immobilized on carbon by adsorption and covalent binding and was used in amperometric biosensor by Cardis et al.[61] They further studied the distribution of active population of peroxidase molecules on the carrier by fluorescence microscopy and microphotography

Wang et al. [62] used Celite , alumina , , XAD-7 and Florisil for adsorption of HRP. It was observed that celite had most and Florsil had the least effect on enzyme adsorption . However, immobilized HRP retained high activity on silanized Florisil . BSA and PEG were used as protective agents and were used to enhance the activity of immobilized HRP in organic solvents.

Zamora [63] adsorbed peroxidase onto the anion exchanger Amberlite IRA 400. He obtained maximum enzyme coupling at pH 7.0 at 4°C within 2 h coupling time. The enzyme-immobilized resin was further used to remove phenols from paper and pulp manufacturing effluents.

A simple method of immobilization of HRP on the inner wall of PTFE tubing by physical adsorption was described by Li and Townshed [64] . The horseradish peroxidase immobilized tubing was used to form a reaction coil that was inserted into a flow system. Various conditions influencing the reaction , immobilization , adsorption and stability of the reaction coil were evaluated by a flow injection method based on HRP –catalyzed fluorescent reaction of H_2O_2 with p-hydroxy phenyl propionic acid as flurogenic substrate . They observed that immobilized reaction coil was stable for at least a month if it is kept in buffered phosphate solution at pH 5.8 in a refrigerator. They observed a linear relationship between fluorescence intensity and H_2O_2 concentration in the range of 4-80 ng mL^{-1} with HRP solution and with the immobilized reactions coil system . The standard deviation for 4 to 80 mol/L was 5 % and < 1% respectively.

Adsorption of horseradish peroxidase on cinnamic carbohydrate esters was carried out by Melgarejo et al.[65] The polymerization and cross-linking of the derivates initially obtained by irradiation in the ultraviolet region , where these prepolymers showed maximum sensitivity . They observed that the immobilized enzyme was much more resistant to inactivation by hydrogen peroxide and to heat at neutral pH. The stability and durability were also described for the immobilized HRP.

2.2.4. Crosslinking :

Lei et al. [66] made a hydrogen peroxide sensor using coimmobilized methylene green and HRP in the same Montinorillonite-modified Bovine serum albumin –

glutaraldehyde matrix on a carbon glass surface. They observed that the immobilization on methylene green from the dye mother solution is due to adsorption on montmorillonite, whereas immobilization of HRP was attributed to the cross-linkng function of BSA-glutaraldehyde . The sensor responded rapidly to low H_2O_2 concentration and achieved 95 % of the steady state current in < 205, with a detection limit of 4.0×10^{-7} M H_2O_2

Immobilization of HRP in crosslinked phyllosilicates was carried out by Shen et al.[67] They observed that the immobilized enzyme exhibited high activity compared to free one. Different factors including drying method , particle size, surface cations of the pyyllosilicates and ratio of phyllosilicates and orthosilicate were investigated to optimize the immobilization system .

Work Undertaken :

HRP has been immobilized on many supports for covalent coupling and entrapment. We have carried out entrapment of *HRP* on K -carrageenan by entrapment and carried out its covalent coupling on copolymer of AAm-HEMA and exploited the optimum conditions for immobilization by both methods.

K-Carrageenan is a marine polysaccharide obtained from red algae Rhodophyceae. K-carrageenan is alternating copolymer of 1,3,-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-D-galactose. The three major types are designated by greek letter kappa(κ), lambda(χ), iota (ι), according to the relative number and position of sulfate ester substituents on these sugars and the extent to which the1,4-linked residues exist as the 3,6-anhydroderivatives.

They are produced on commercial scale in Argentina , Chile , Philippines , Indonesia , Morocco , France , Canada and North Atlantic region. Carrageenans are used in a wide variety of applications , especially in food products , such as frozen desserts, chlocolate milk , cottage cheese, whipped cream , yoghurt , jellies , and sauces. In addition the carrageenan is used in pharmaceutical and cosmetic formulations and in other industrial applications such as in oil-well drilling fluids.

The use of carrageenan for food application started from 600 years ago. Due to this long and safe use carrageenan is generally recognized as safe by experts of the US food and Drug administration and is approved as a food additive . Since , the 19^{th} century , carrageenan has been used for industrial applications.

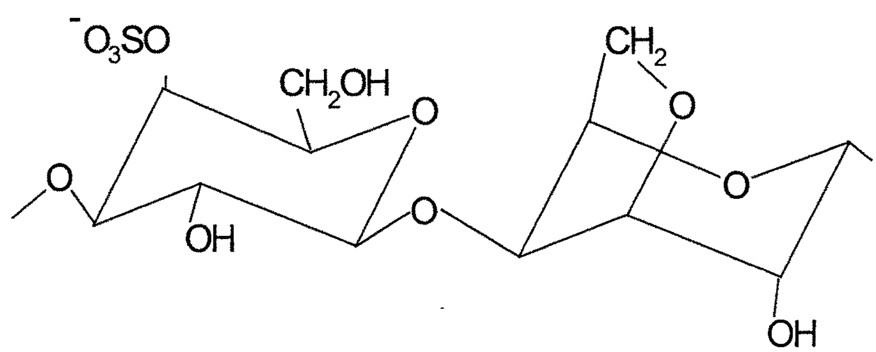
In the 1970's the use of carrageenan for the immobilization of enzyme was introduced through the pioneering work of Chibata and coworkers as stated by Fred [68] .

However , much work has not been carried out on K -carrageenan and hence we have taken it as a support for our study. The immobilization on K-carrageenan can be carried out by four different techniques such as gel method , droplet method, emulsion method and dehydration method. The droplet method is most widely used method among them.

K -carrageenan is soluble in water at 80 °C but at 20°C only Na salts of it are soluble and K, Ca, NH_4 salts show swelling . K -carrageenan forms strongest gel with K^+ but the gel formed is brittle . The gel is stable in acid solution but has poor stability in salt solution.

We have taken K -carrrageenan which is a natural support for entrapment while copolymer of AAm-HEMA has been used for covalent coupling . Immobilization in poly(AAm) and poly(HEMA) is well known . Moreover the use of copolymer of AAa-HEMA has also been reported for entrapment but the copolymer of AAm-HEMA has not been used for covalent coupling .

The structure of K-carrageenan is given below



Poly (HEMA) is a non toxic , biocompatible and hydrophilic in nature . It is very inert towards microbial contamination and resistant to attack by many chemicals whereas Poly (AAm) is more hydrophobilc in nature. Many coworkers have used Copolymer of AAm- HEMA for entrapment of different enzymes but no report is available for the covalent coupling of HRP on Poly(AAm--HEMA) .

The main aim of present work is to immobilized *HRP* on a natural polysaccharide and synthetic support and check the effect of both on enzyme stability . *HRP* has been entrapped in natural polysaccharides such as K -carrageenan where as is has been covalently bound to a copolymer (AAm-HEMA).

2.3 Experimental

2.3.1 Chemicals used :

Horseradish Peroxidase (strength 200 U /g solid)	*Sigma Chemicals ,St.Louis , MO , 63103, USA.*
Pyrogallol	*E. Merck, Mumbai , India.*
H_2O_2	
K-carrageenan	*As a gift from Central Salt and Marine Chemical Research Instittute, Bhavnagar, India.*
Acrylamide	*Qualigens , Mumbai , India.*
Folin's Reagent	
2-hydroxy ethyl methaacrylate (HEMA)	*Fluka Chemie , AG CH-9470 Buchs Switzerland*
Ascorbic Acid	*Loba Chemie , Mumbai , India.*
di-potassium hydorgen orthophosphate	*Spectrochem , Mumbai , India.*
Potassium Dihydrogen orthophosphate	
Sodium Carbonate	*Sulab , Baroda , India.*

2.3.2.Preparation of support material and Methodology

(a). Entrapment of HRP in K -carrageenan beads :

The entrapment of horseradish peroxidase (HRP) in K --carrageenan beads was done by modifying the method reported by Jose et al .[69] K -carrageenan (3%w/v) solution was prepared in water at 60°C and cooled below 35°C for 30 min .To 2 cm^3 of this solution 1 mg of HRP in 1 cm^3 of water was added and mixed thoroughly . The resulting solution was dropped in 2% w/v PEI solution using syringe. The beads were allowed to harden in PEI for 2 h and then washed several times with water for removal of PEI .

(b). Covalent coupling of HRP to AAm-HEMA copolymer .

Preparation of AAm-HEMA copolymer :

Acrylamide-2-hydroxy ethyl methaacrylate (AAm–HEMA) copolymer was synthesized through bulk polymerization technique using aqueous solution containing 1.0 g of acrlyamide and 2.0 g of HEMA . The mixture of solutions was stirred for 10 min and 0.3 cm^3 of 30% w/v hydrogen peroxide and 0.1 g of ascorbic acid dissolved in 0.5 cm^3 of water were added to it. The reaction mixture on stirring at 35°C for 10-15 min turned into gel . The gel was washed with water to remove the unreacted monomer and was dried and converted into uniform sized particles of diameter 0.20 $\pm$ 0.05 mm. AAm –HEMA copolymer gels were prepared by using 1:1 , 1:2, 2:1, 1:3 , 3:1, 2:3 and 3:2 ratios of AAm – HEMA.

Covalent coupling of HRP to copolymer :

The synthesized copolymer contained free hydroxyl and amine groups. The activation of amine groups by using glutaraldehyde was carried out as per the procedure reported by Rejikumar and Devi [70]. Activation of hydroxyl groups was done by using p-tolyl sulphonl chloride (PTS) [71] and also by using p-benzoquinone (PBQ) [72] as per the procedure reported earlier. In the process of immobilization hydroxyl groups of AAm-HEMA were activated first by using PBQ and the activation of $-NH_2$ groups was carried out by using glutaraldehdye . This was followed by the coupling of enzyme . Alternatively AAm-HEMA copolymer was first activated wit glutaraldehdye followed by coupling of enzyme and then activating the support with PBQ and coupling with enzyme . The process was examined by reversing the order of activation of support . The mechanism of activation of copolymer and coupling of the enzyme is given in **Figure 2.1.**

2.3.3. Assay of HRP :

The activity of free and immobilized enzyme was determined by using pyrogallol as a substrate as described by Worthington [73].The enzyme catalyzed reaction was monitored in 0.1 M phosphate buffer of pH 6, using 0.147 M 30 % (w/v) of H_2O_2 and 5% (w/v) pyrogallol solution at 35°C .The amount of liberated purpurogallin was estimated spectrophotometrically at 420 nm.

2.3.4. Entrapment of Horseradish Peroxidase in K-carrageenan :

Entrapment is a mild immobilization method and does not involve any modification or binding of the enzyme and hence the effect of hardening agent concentration and enzyme loading are checked for the K-carrageenan beads.

(a) Effect of Polyethyleneimine concentration

The effect of PEI strength on hardening of beads and retention of entrapped enzyme activity was studied by using 1-4 % PEI solution . The K-carrageenan beads containing enzyme were prepared as described earlier and they were allowed to harden in 1-4% w/v solution of PEI for 2 h. The protein content in supernatant liquid was calculated according to Lowry's method [74]. The activity of entrapped enzyme was calculated as per the procedure described by Worthington [73] . From this percentage retention of activity of entrapped enzyme was calculated with respect to protein content in the bead. The effect of hardening time on enzyme retention in the beads was studied by varying the time of contact from 1-4 h.

(b)Effect of Enzyme concentration :

The effect of enzyme concentration on enzyme loading in 3 % K -carrageenan beads was studied by using 200-1500 U enzyme solution under the optimized entrapment conditions . Different parameters during loading such as active protein in the beads and percentage retention of enzyme activity were calculated.

2.3.5.Covalent coupling of Horseradish peroxidase to copolymer

Horseradish peroxidase was covalently coupled to acrlyamide-2-hydroxylethylmethaacrylate (AAm-HEMA) copolymer. Coupling conditions were optimized as follows .

a).Effect of AAm-HEMA concentration :

AAm-HEMA copolymers were synthesized using 1:1,1:2 ,2:1,1:3,3:1, and 2:3 ratios ıf AAm and HEMA . The functional groups –OH and NH_2 were activated using p-tolyl sulphonyl chloride , p-benzoquınone and glutaraldehdye.

(b) Concentration of activating agent :

The activation of free NH_2 and OH groups of the copolymer was carried out using three different activating agents.NH_2 groups were activated by using glutaraldehdye (0.5 - 2 % v/v) solution .The OH groups were activated by using p-benzoquinone (PBQ) 0.05-0.1 g and p-tolyl-sulphonyl chloride (PTS) 0.015 - 0.1g . Both protein content and active protein were examined.

c). Activation time :

The effect of different activating agents on activation time was carried out . The NH_2 groups of the support were activated by using 10 ml of 2 % w/v glutaraldehyde solution and 50 μg of enzyme .The activation time was varied from 30 min –4 h .While OH groups were activated by using 10 ml of 2.5 mg/ml p-benzoquinone solution and 50 μg of enzyme for 2 hr and the activation time was varied from 1 - 8 h. While in case of simultaneous activation of both groups the glutaraldehyde optimized time was taken and the p-benzoquinone activation time was varied from 1 – 9 h.

d) Effect of pH of medium on activation of support :

The effect of pH of coupling medium on the covalent binding of enzyme was examined by using solutions of pH 4-9 and following the procedure described in **section 2.3.2.b** using 25 mg of support and 50 μg of enzyme. Coupling was carried out in 0.1 M phosphate buffer of different pH.

e) Effect of enzyme concentration on K -carrageenan beads :

Effect of enzyme concentration on 1 g of support was examined using 25 mg of AAm-HEMA copolymer and varied concentration of enzyme (50-3000 mg) in 0.1

M phosphate buffer of pH 7 . Coupling time was 12 h. Optimum concentration of enzyme required for coupling was determined by measuring the activity of enzyme coupled to support.

f) Effect of enzyme coupling time :

The effect of coupling time on enzyme immobilization was studied for all three modes of activation . In case of glutaraldehdye 10 cm^3 solution of 2 % w/v concentration was used for the activation of support for 2 h which was followed by coupling of 50 μg of enzyme for various time intervals (1- 20 h) .Procedure was repeated for activation of support by p-benzoquinone . The support was activated for 1 h using 25mg of p-benzoquionone and then coupled with 50 μg of enzyme for different time intervals from 1-10 h.

2.3.6 Comparative study of free and immobilized Horseradish Peroxidase :

Comparison of free and immobilized enzyme is very important parameter to be studied as from the comparative study we can know whether any change in pH , temperature , storage , reusability , kinetic parameter has taken place after immobilization . From these studies improvement in thermal stability on immobilization can be examined.

1. pH Profile :

Enzymatic reactions are sensitive to microenvironment . Hence pH of the medium plays important role in enzyme catalyzed reaction. If enzyme undergoes some structural changes its dependence of activity is likely to vary. Therefore pH activity profile for free and immobilized enzyme was carried out. The pH activity profile of free and covalently bound enzyme was obtained by incubating the enzyme at pH 5 to 9 at 35°C for 30 min in 0.1 M phosphate buffer medium

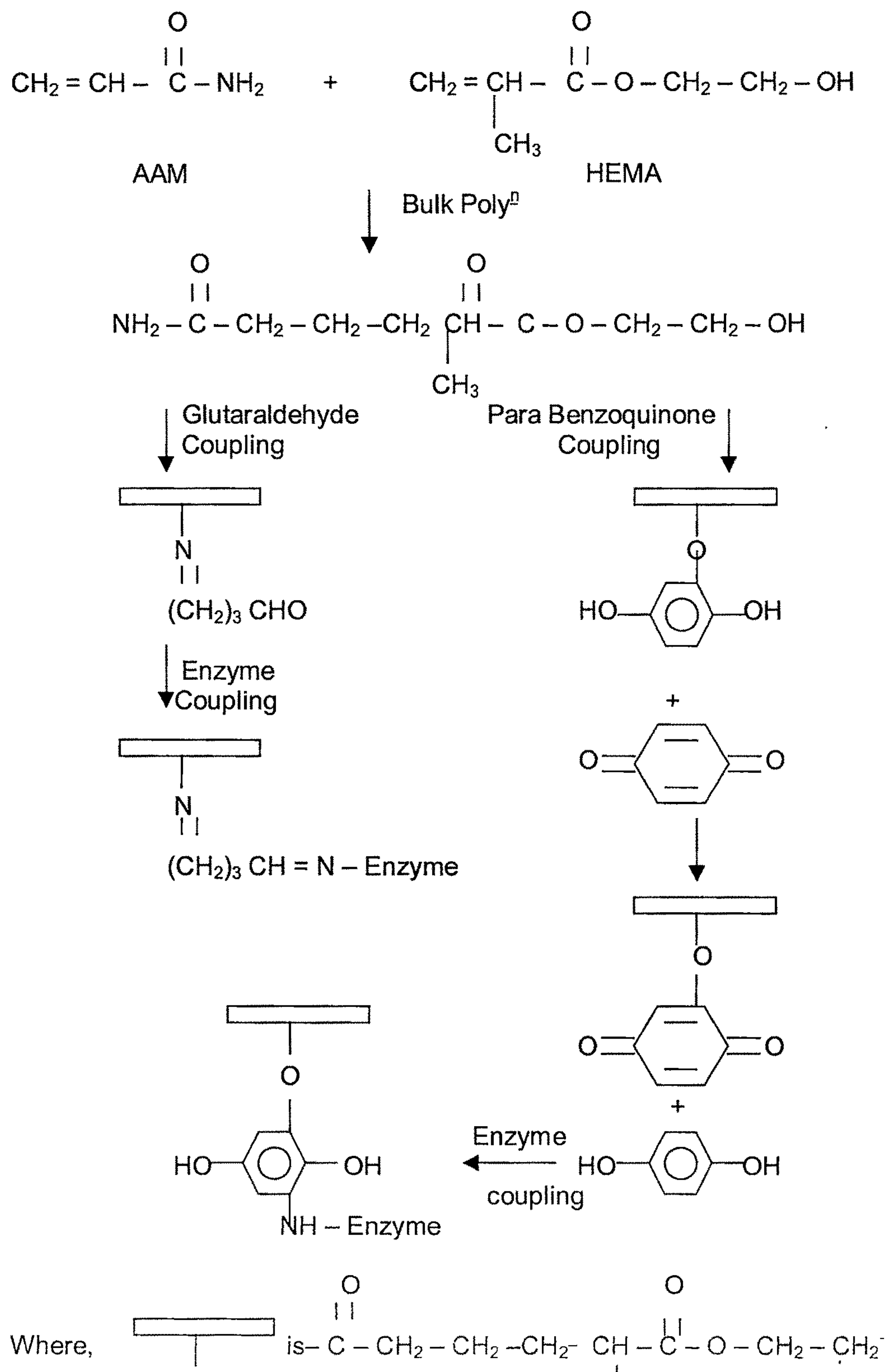

AAM
HEMA
Bulk Poly^n
Glutaraldehyde Coupling
Para Benzoquinone Coupling
Enzyme Coupling
$(CH_2)_3$ CHO
$(CH_2)_3$ CH = N – Enzyme
Enzyme coupling
NH – Enzyme
Where,
is–

Figure 2.1

using pyrogallol as a substrate . The purpupogallin obtained by oxidation of the pyrogallol was measured by using the procedure given in **section 2.6.**

2. Thermal Stability :

Another important factor which can deactivate enzyme is temperature . Hence thermal stability of free and immobilized enzyme was carried out by placing it in buffer solution of optimum pH at various temperatures ranging from 35°C to 55°C for different time intervals. The thermodeactivation constant (k_d) was calculated by using following equation .

$$ln\ A_t = ln\ A_o - K_d\ (t)$$

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

3.Storage Stability :

One of the proposed advantage for immobilized enzyme is improvement in its storage stability . Therefore storage stability of free and immobilized enzyme was examined by storing the enzyme at 35°C for various time intervals and measuring its activity.

4.Reusability :

Reusability is an important parameter in industrial applications of enzymes. Reusability studies were carried out using pyrogallol as substrate for both entrapped and covalently bound enzyme . 0.3 cm^3 of 5 % w/v pyrogallol was taken and the activity of the immobilized enzyme was checked till it lost 50 % of its initial activity.

5. Determination of Kinetic Parameters :

Enzymatic reactions are controlled by substrate and enzyme concentration. Therefore the kinetic parameters such as maximum reaction velocity *(V_{max})* and Michaelis constant *(K_m)* were calculated by varying the substrate concentration from 0.39 M/L to 1.9 M/L and keeping the enzyme concentration constant at $4x10^{-7}$ M/L and also by varying enzyme concentration from 2.5 x 10^{-7} to 12.5 x 10^{-7} M/L and keeping substrate concentration 0.39 M/L and using Lineweaver Burk plots.

2.3.7. Oxidation of Hydroquinones :

In 1967 Taylor and Battersky [75] used horseradish peroxidase for preparation of p-benzoquinone from hydroquinone . In present study oxidation of hydroquinone by using free and immobilized beads is carried and by batch process. Effect of various parameters such as enzyme concentration , pH and reaction time on the process of oxidation was examined by using both free and entrapped enzyme.

2.3.8. Enzymatic oxidation of phenol

Oxidation of phenol was carried out by Zamora [63] using horseradish peroxidase immobilized on the anion exchanger Amberlite IRA 400. Maximum coupling was reported to be at pH 7 and at 4°C within 2 hrs coupling time. The immobilized enzyme was further used for removal of phenol from paper and pulp manufacturing industry effluent . Sun and Payne used tyrosinase immobilized onto chitosan film for the removal of phenol [76]. Barton and Russell used polyphenol oxidase immobilized onto nylon for the detection of phenol from polluted water [77] . We have tried to remove phenol from industrial effluent through its oxidation by using horseradish peroxidase covalently bound to

AAm-HEMA copolymer . The schematic diagram of the fixed bed reactor used in the study is given in **Figure 2.2.**Operational conditions of reactor such as flow rate , substrate concentration , reactor temperature and l/d ratio were optimized .Turnover number of the enzyme catalyzed process was calculated.

2.3.9 Optimization of the reactor parameters :

1.Effect of Flow rate :

Dependence of extent of oxidation on flow rate was studied using 0.5 – 2 mM phenol solution and a fixed bed reactor containing covalently bound enzyme . The flow rate was varied from 0.5 cm^3/min - 2 cm^3/min.

2. Effect of Phenol Concentration :

At optimum flow rate of 0.5 cm^3 / ml using reactor of dimension 17 x 1 containing covalently bound enzyme , effect of variation in phenol (substrate) concentration on oxidation was examined . 0.5 to 2 mM phenol solution was passed through the reactor at 0.5 cm^3/min flow rate.

3. Effect of l/D ratio.

The determination of optimum l/d ratio is an important parameter in reactor studies as it affects the contact time with substrate and hence the extent of oxidation of phenol. The effect of l/d was checked by using 0.1 mM phenol concentration and flow rate 0.5 cm^3/min by varying l/d ratio from 2 – 8 .

3. **Effect of Temperature** :

Enzymes do get inactivated at a particular temperature . Higher the thermal stability of enzyme better its stability Hence, phenol oxidation was carried out at different temperatures from 35 °C – 65 °C using 1 mM phenol solution at a flow rate of 0.5 cm^3/min and l/d ratio of 6.

2.4 Results and Discussion :

2.4.1. Optimization of conditions for entrapment of enzymes:

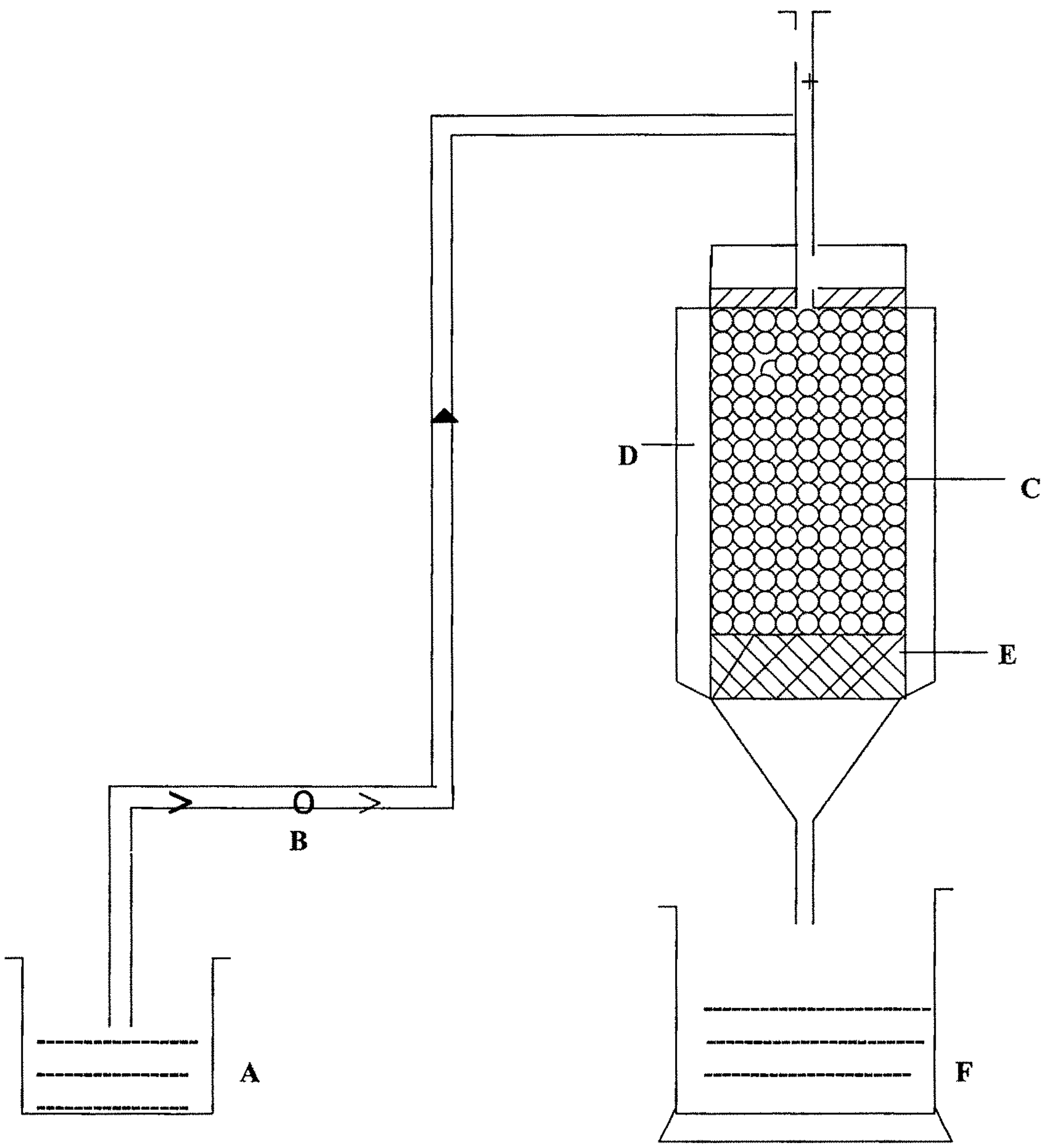
Entrapment of enzymes in materials such as poly(acrylamide) [78], polyacrylic acid [79], silica resin [80], polystyrene [81], nylon [82] has been extensively studied. For the application purpose entrapped material needs to have mechanical strength . This is achieved by hardening the material using KCl or PEI . This gives strength to the beads which can be used at elevated temperature and also can be used effectively in the reactors.

a. Effect of PEI concentration.

Hardening of the beads was done partially using 0.3 M KCl solution. However, beads did not show mechanical strength and were fragmented in water . Hence PEI has been used as a hardening agent. The PEI concentration was varied from 1-4 % and 2% PEI concentration was found to be best. The beads hardened in 2 % PEI showed maximum activity and further increase in PEI concentration did not show any effect on enzyme activity . Hence 2 % concentration was taken for further studies .The results are given **figure 2.3.**

b. Effect of enzyme concentration

Enzyme loading was carried out using enzyme concentration 200 – 1500 U and it was observed that with increasing enzyme concentration amount of protein and activity of enzyme in beads increases but the percentage of retention of



A = Reactant
B = Peristaltic Pump
C= Immobilized Enzyme
D= Temperature Controller
E= Porous Glass Frit
F = Product

Figure 2.2 Schematic diagram of Fixed bed Reactor

enzyme activity remains constant. **Table 2.1.** gives information about total protein content in beads , active protein and also the percentage retention.

2.4.2. Comparative studies of Free and Entrapped Enzyme :

a. Effect of pH on enzyme activity

pH profile for the free and entrapped enzyme is given in **Figure 2.4.** It was observed that free enzyme exhibits maximum activity at pH 8 whereas entrapped enzyme shows maximum activity at pH 7 . As the enzyme is immobilized through entrapment, it is not expected to observe any conformational changes.

b. Thermal Stability

Free enzymes often suffer from the problem of poor thermal stability which can be overcome by immobilization of enzymes which generally improves thermal stability and which is desirable for the reactions to be carried out at elevated temperature. From the results illustrated in **Figure 2.5.** It is observed that entrapped enzyme has better thermal stability compared to free enzyme .Thus indicating the advantage of immobilization of enzyme over free enzyme.The thermo deactivation constants are given in **Table 2.2.**

c. Reusability of Entrapped Enzyme

Free enzyme suffers from a drawback of non reusability and this drawback is overcome by immobilizing it. In the present study it has been observed that immobilized enzyme retained 50% of its activity after 4 cycles **(Figure 2.6.)** and about 25% of its activity after 8 cycles, thus showing an advantage over free enzyme and making it useful in large number of applications. The turnover number of entrapped enzyme was observed to be 51.6 x 10^6.

d. Kinetic Parameters

The kinetic parameters *(K_m)* and *(V_{max})* for *HRP* entrapped in K-carrageenan were calculated from Lineweaver-Burk Plot of 1/V vs 1/S **(Figure 2.7a,7b)**. The results are given in **Table 2.3.** No change in K_m and V_{max} for free and entrapped enzyme was observed when substrate concentration or enzyme concentration was kept constant. However, both V_{max} and K_m values were observed to be lower when the kinetics was controlled by concentration of substrate diffusion at a fixed enzyme concentration condition.

e. Storage Studies

On storage at 35 °C **(Figure 2.8)** free enzyme was observed to lose about 50 % activity in one week's time whereas the immobilized enzyme retained 50 % of its activity for more than one month.

2.4.3. Oxidation of Hydroquinone

The oxidation of hydroquinone was carried out by using both free and immobilized enzymes. It was observed that the initial rate of oxidation of hydroquinone by immobilized enzyme was lower than by free enzyme .But after 1 h it showed reverse order. This could be due to the diffusional limitation of the substrate in the beads which can be seen in the **Figure 2.9.** Effect of enzyme concentration on oxidation of hydroquinone was studied by using 25, 50, 75, 100 μg of free and immobilized enzyme. The results are illustrated in **Figure 2.10.** The rate of oxidation increases with increase in enzyme concentration for both free and entrapped enzymes. It was observed that use of 100 μg of enzyme resulted into 90% of oxidation of hydroquinone in 1 h. The oxidation was also

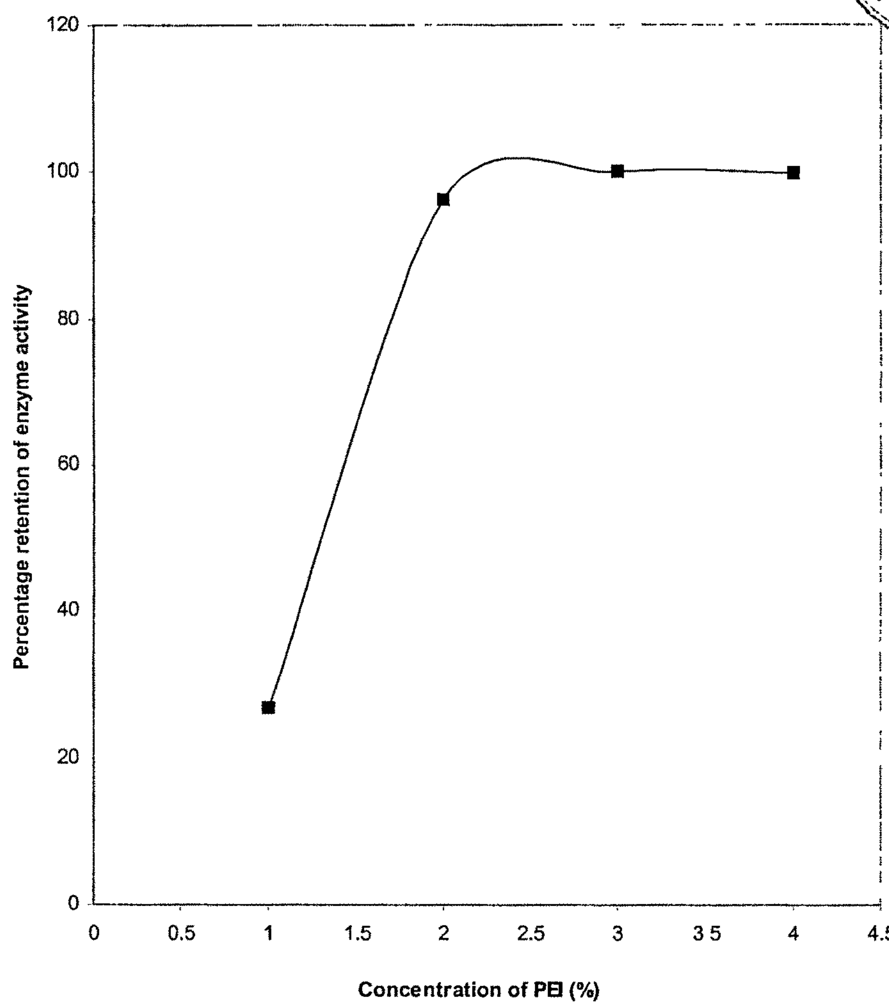


Figure 2.3 Effect of PEI concentration on the retention of enzyme activity at temperature 35°C ,pH 6 and time 1 h.

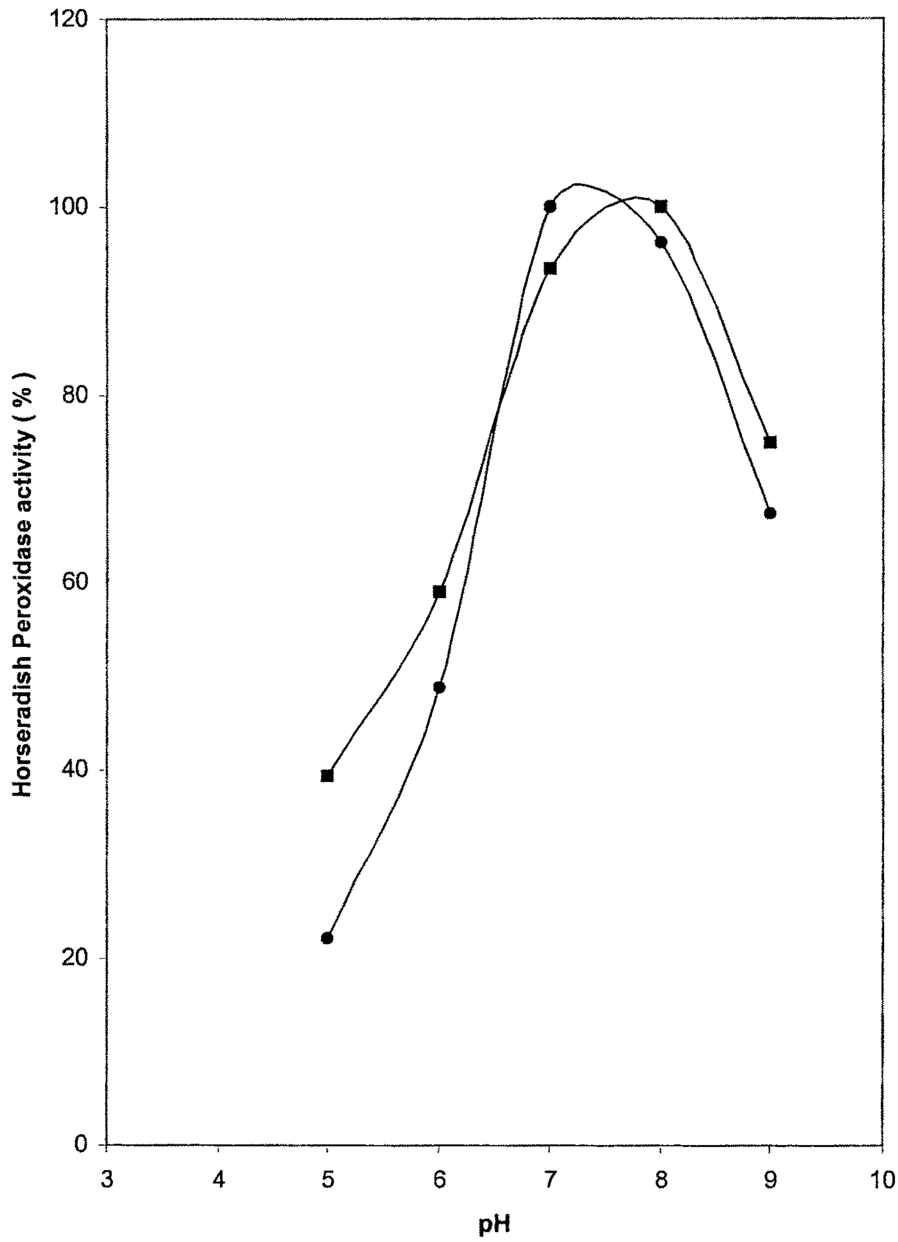


Figure 2.4 . pH activity Profile of Horseradish Peroxidase at 35°C. Free (●) and Entrapped (■) at 35°C.

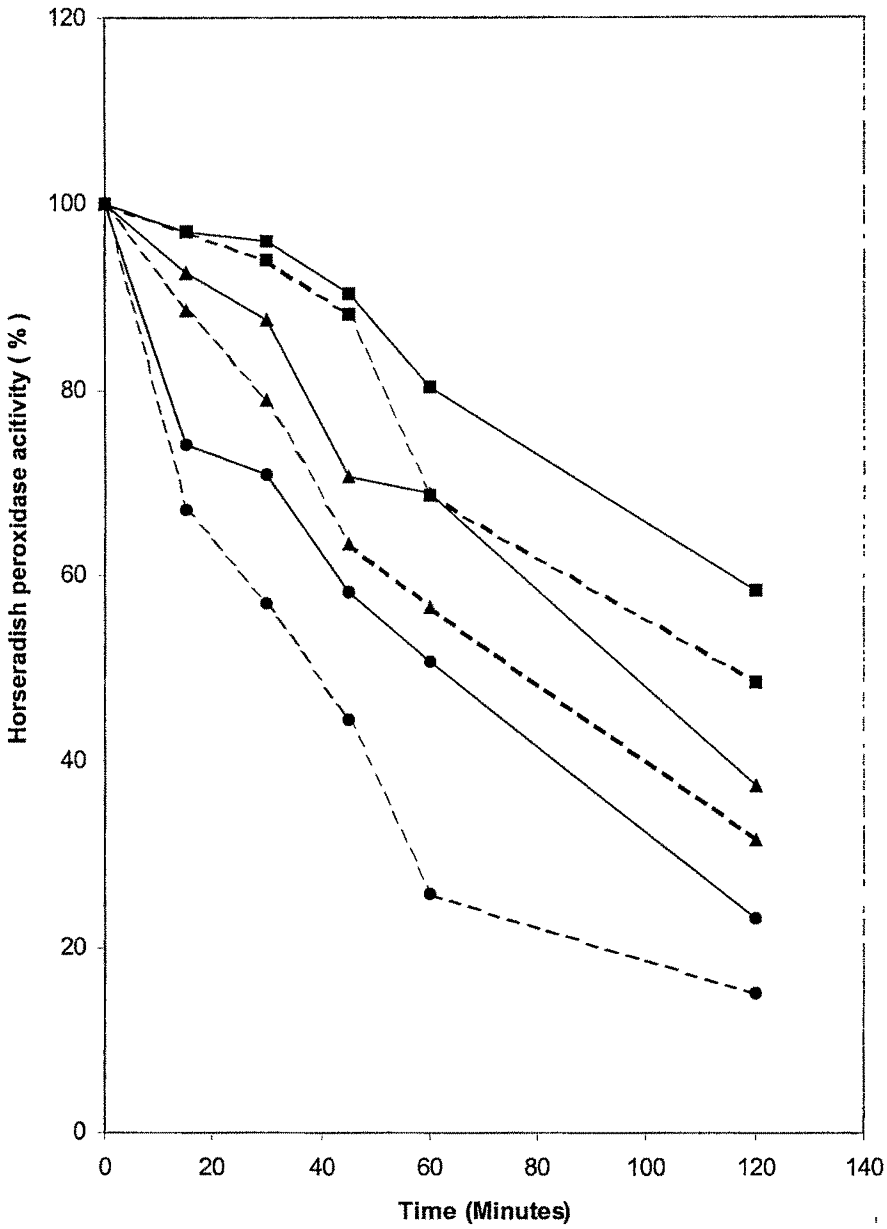


Figure 2.5 Effect of temperature on the activity of free and entrapped HRP at pH 6 at 35°C (■), 45°C (▲),55°C (●). Free enzyme (---) and Entrapped enzyme (_).

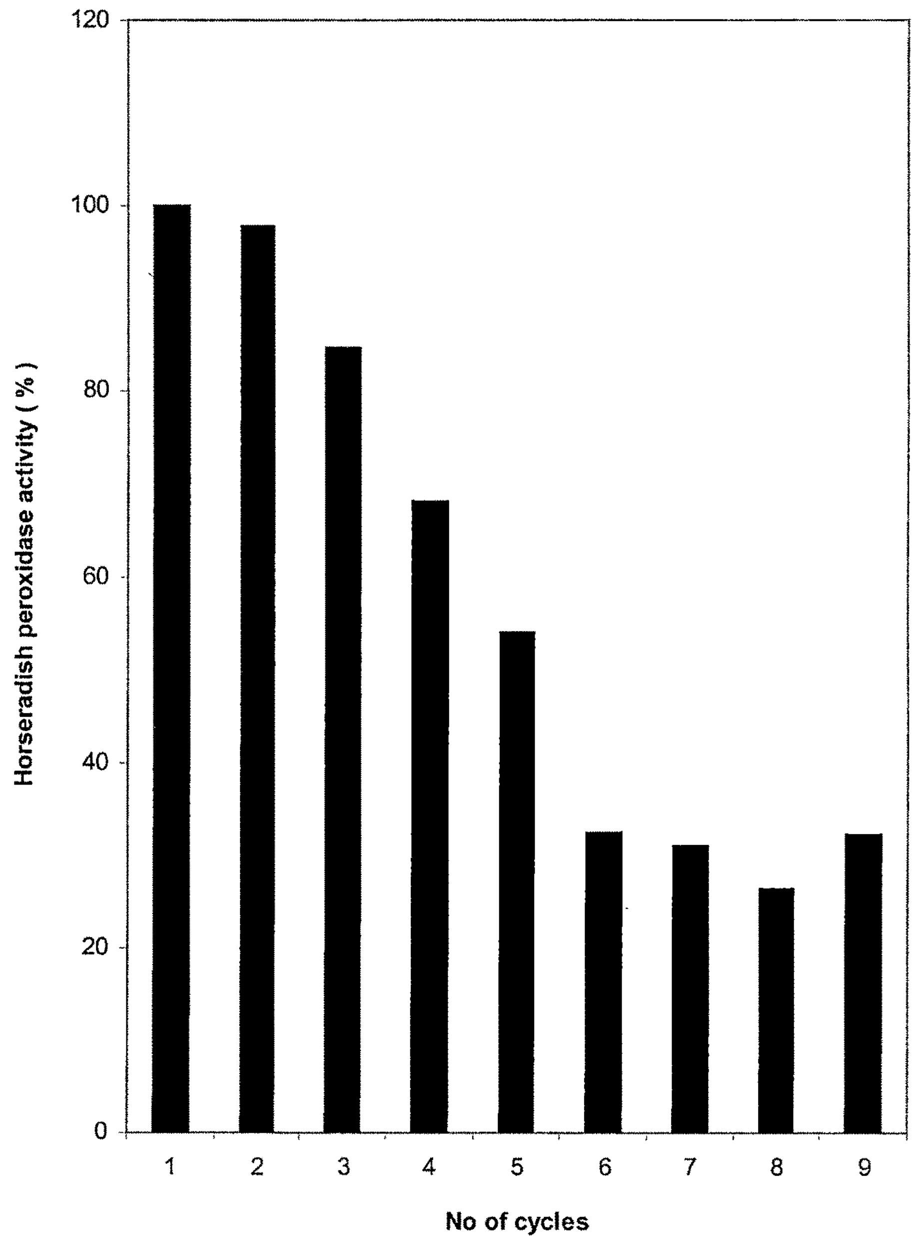


Figure 2.6 Reusability of Entrapped enzymes at 35°C and pH 6.

Table 2.1. Effect of enzyme concentration on enzyme loading.

Enzyme taken for entrapment in mg (U)	Total Protein Content in the Beads mg	Active Protein in beads Mg	Percentage retention of enzyme activity in beads
1 (200)	0.125	0.0429	34
3 (600)	0.893	0.260	29
5 (1000)	1.469	0.551	38
7.5 (1500)	1.907	0.609	32

Table 2.2.Thermodeactivation constant of free and entrapped enzyme.

Time In min	Thermodeactivation constant X 10^3 at					
	35 °C		45 °C		55 °C	
	Entrapped	Free	Entrapped	Free	Entrapped	Free
15	0.206	0.206	0.510	0.811	1.900	2.600
30	1.330	2.060	4.380	7.890	11.400	18.700
45	4.060	4.810	7.730	10.000	11.900	18.700
60	3.630	6.250	6.210	9.500	11.200	22.500
120	4.480	6.025	8.160	9.500	12.200	25.700

Table 2.3.Kinetic parameters of free and entrapped enzyme.

*Conditions	Free enzyme		Entrapped enzyme	
	K_m (mM)	V_{max} (mM/min)	K_m (mM)	V_{max} (mM/min)
When substrate concentration is varied and enzyme concentration is kept constant.	$8 X 10^{-5}$	1.53	$8.3 X 10^{-5}$	2.18
When enzyme concentration is varied and substrate concentration is kept constant.	$4 X 10^{-11}$	0.45	$4.5 X 10^{-11}$	0.58

*At 35 °C, pH 6 for 1 min.

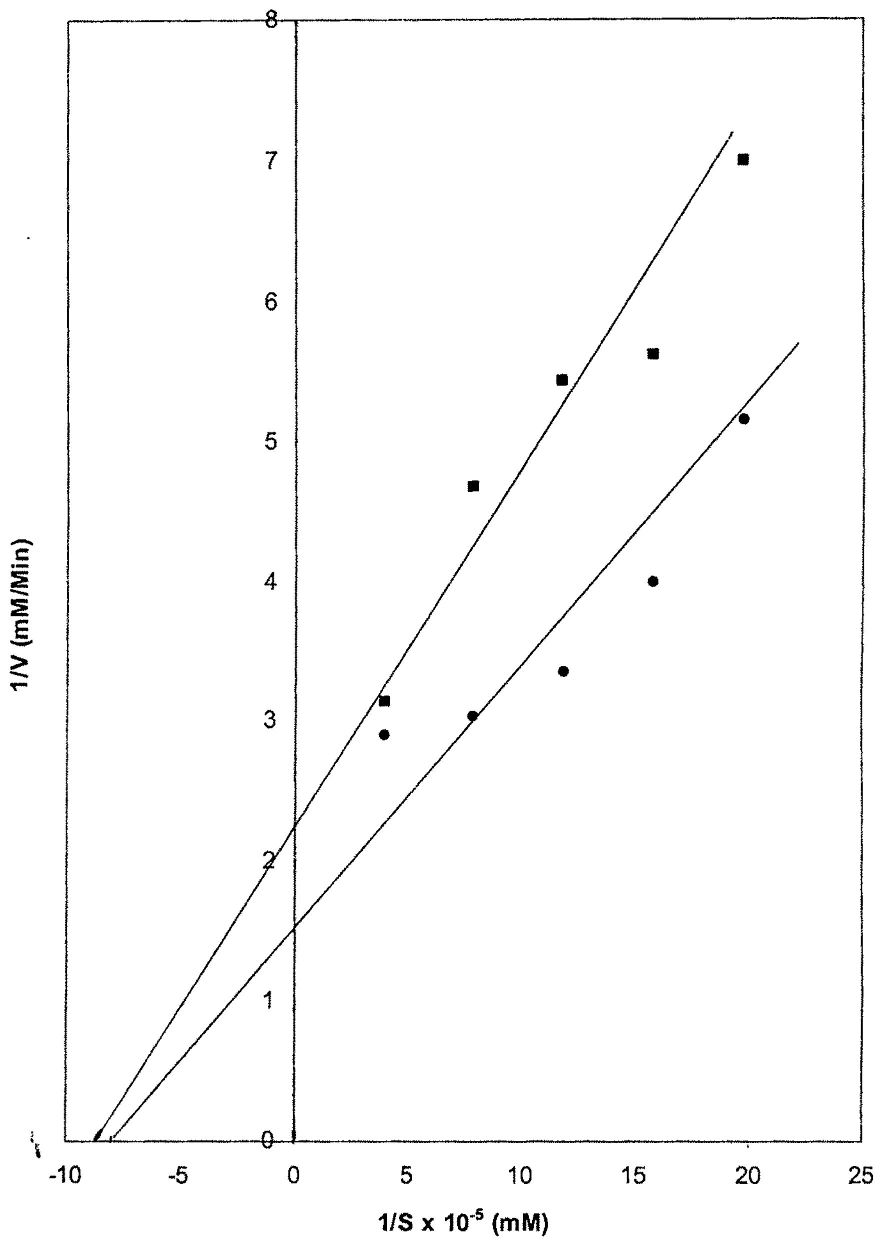


Figure 2.7. (a) Lineweaver burk plots at 35°C ,pH 6 , and 1min reaction time. When enzyme concentration is constant and substrate concentration is varied .

Free enzyme (•), Entrapped enzyme (■).

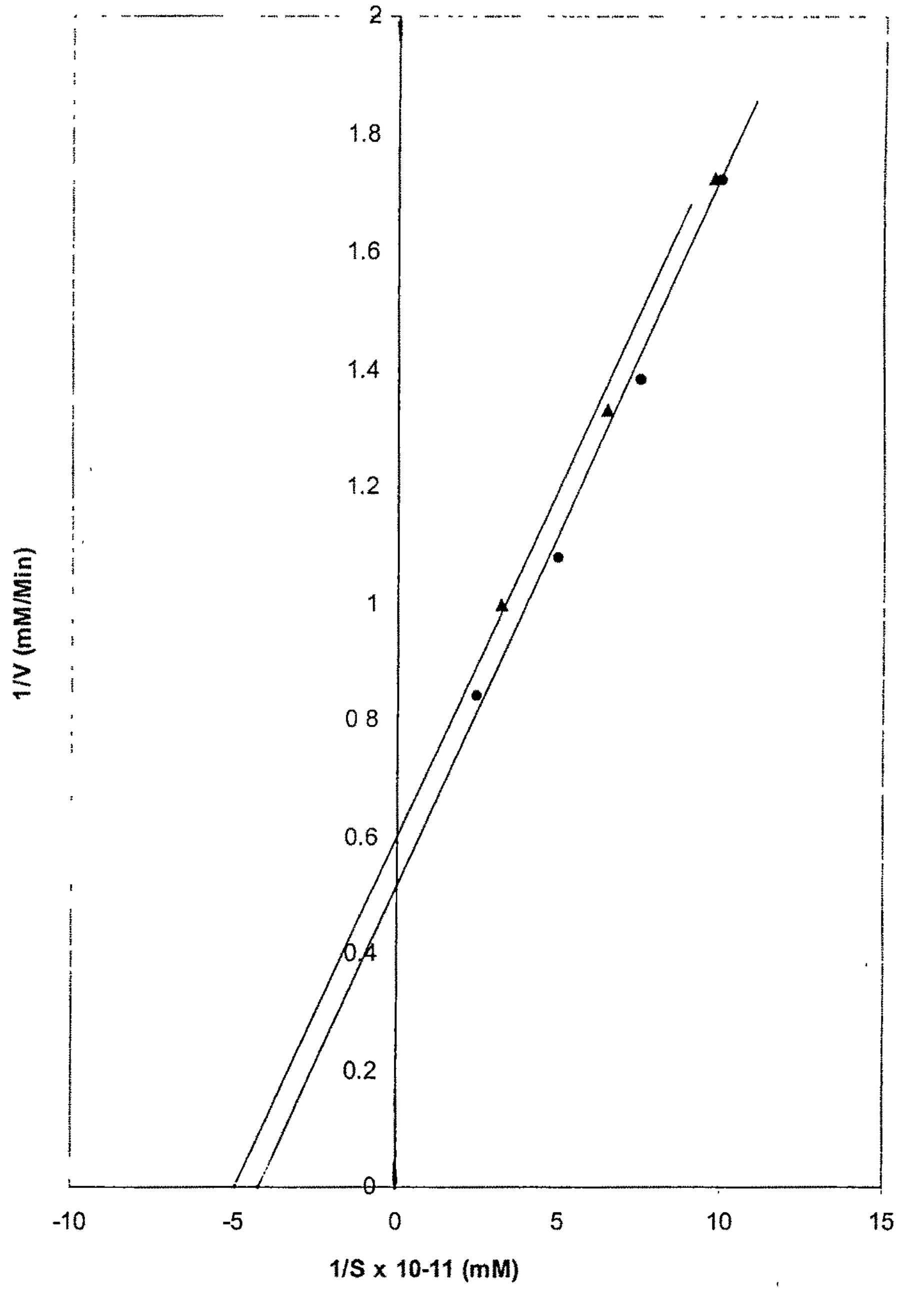


Figure 2.7 (b) Lineweaver burk plots at 35°C ,pH 6 , and 1min reaction time. When substrate concentration is constant and enzyme concentration is varied .

Free enzyme (●), Entrapped enzyme (■).

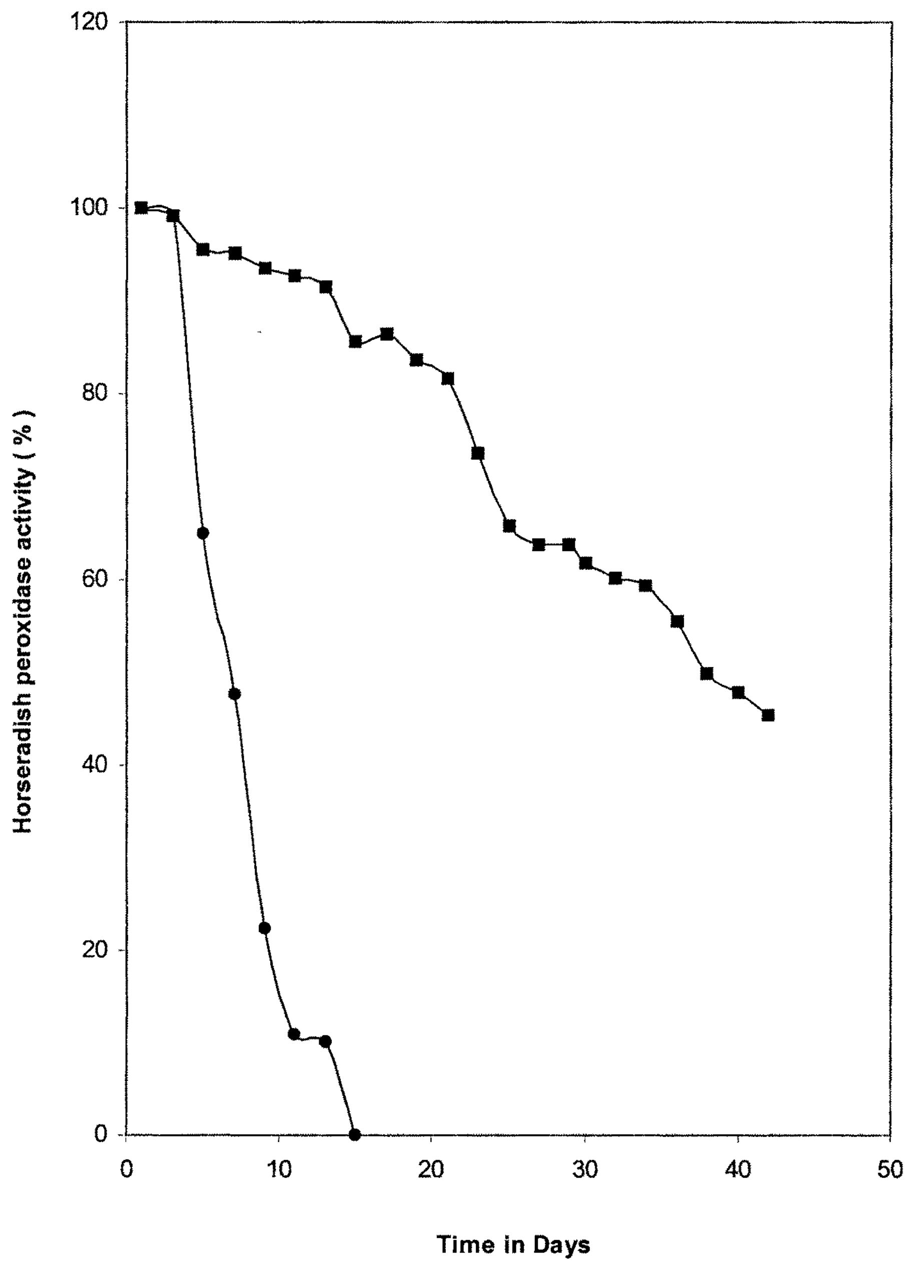


Figure 2.8 Storage Stability of free (●) , and entrapped enzyme (■), at 35°C and pH 6.

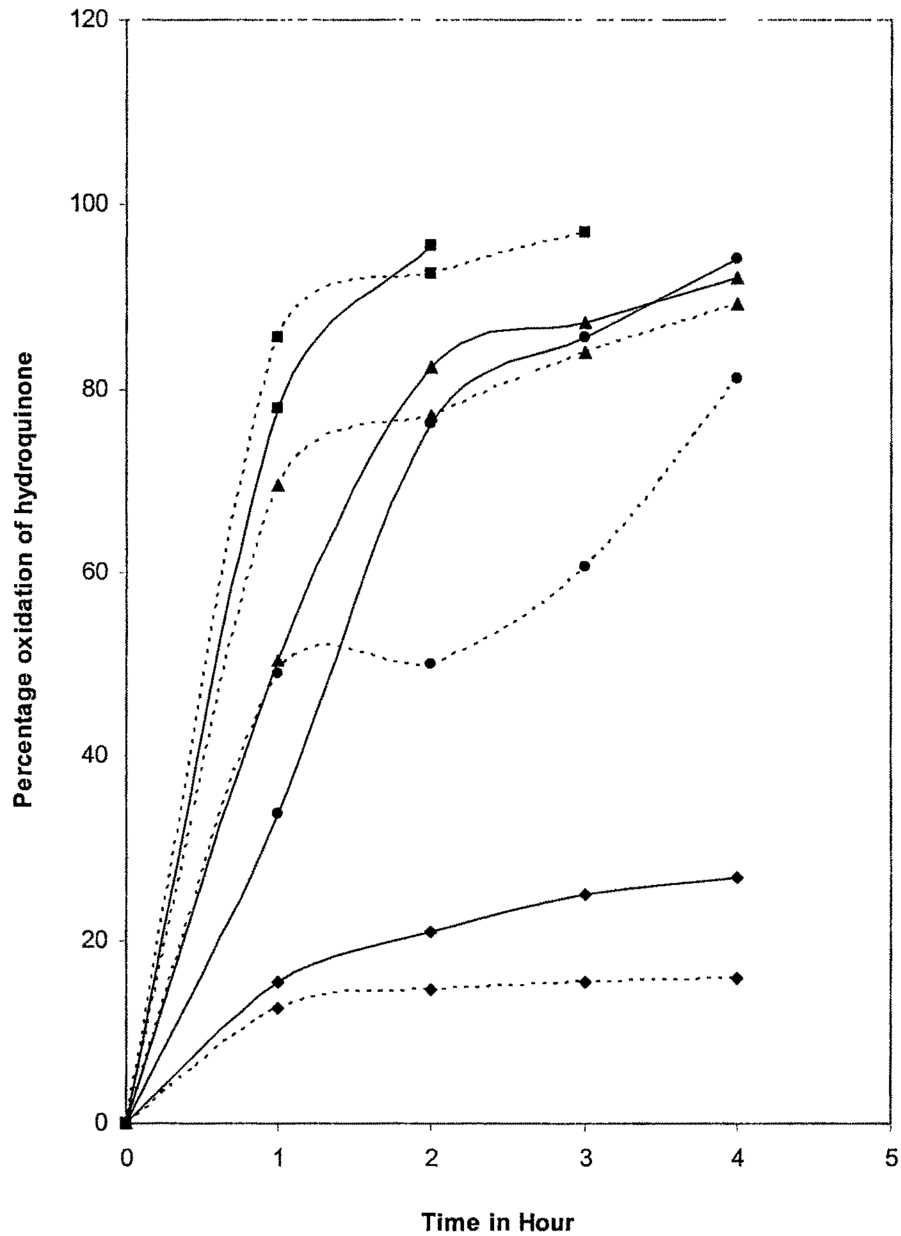


Figure 2.9.Effect of concentration of hydroquinone on the rate of oxidation at 35°C and enzyme concentration 50 µg .

Free enzyme (---) and entrapped enzyme (_) . 1mM (■), 2mM (▲), 3mM (●), 4 mM (◆).

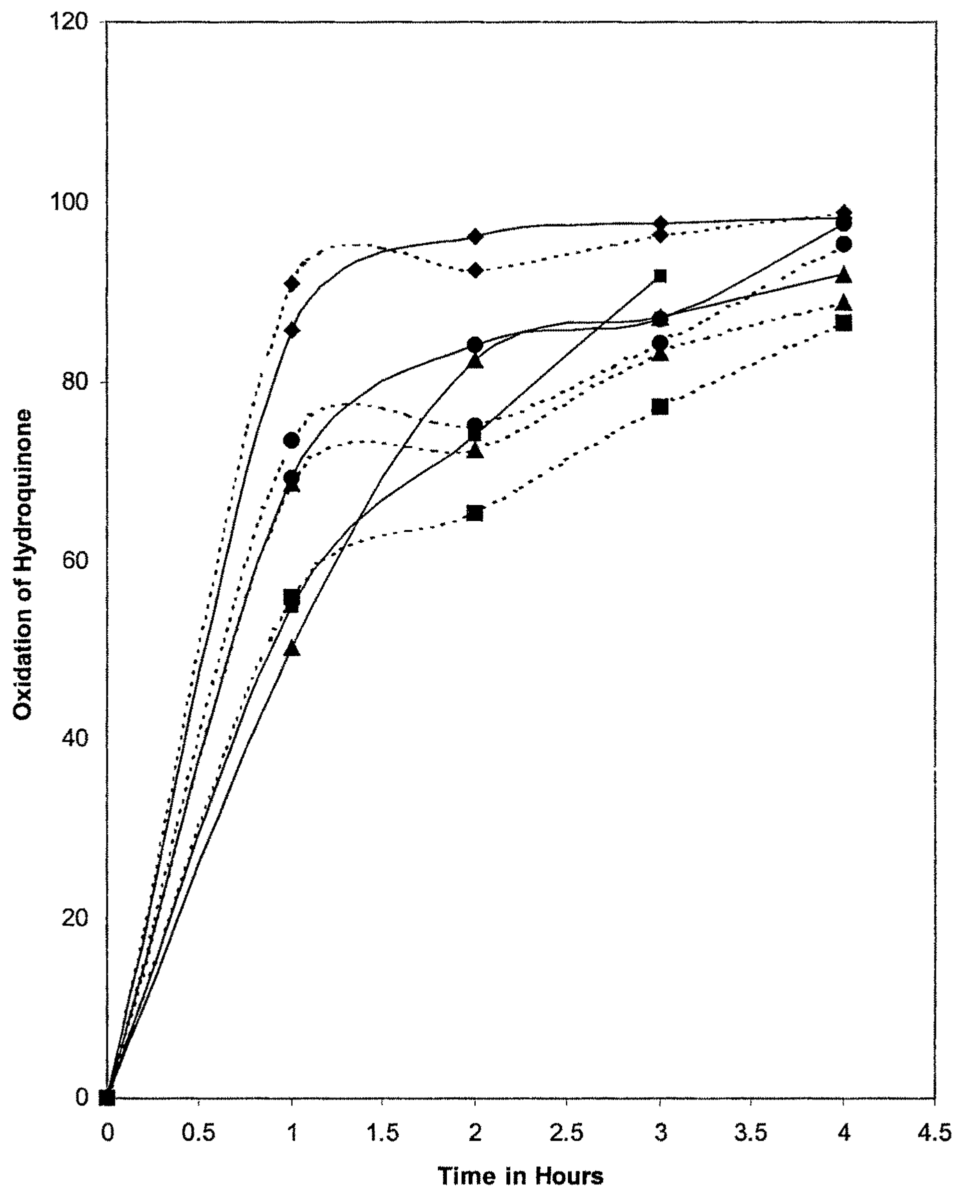


Figure 2.10. Effect of enzyme concentration on the rate of oxidation of 2mM hydroquinone concentration .

Free enzyme (---) , and entrapped enzyme (_). 25μg (■),50 μg (▲),75 μg (●) , 100 μg (◆).

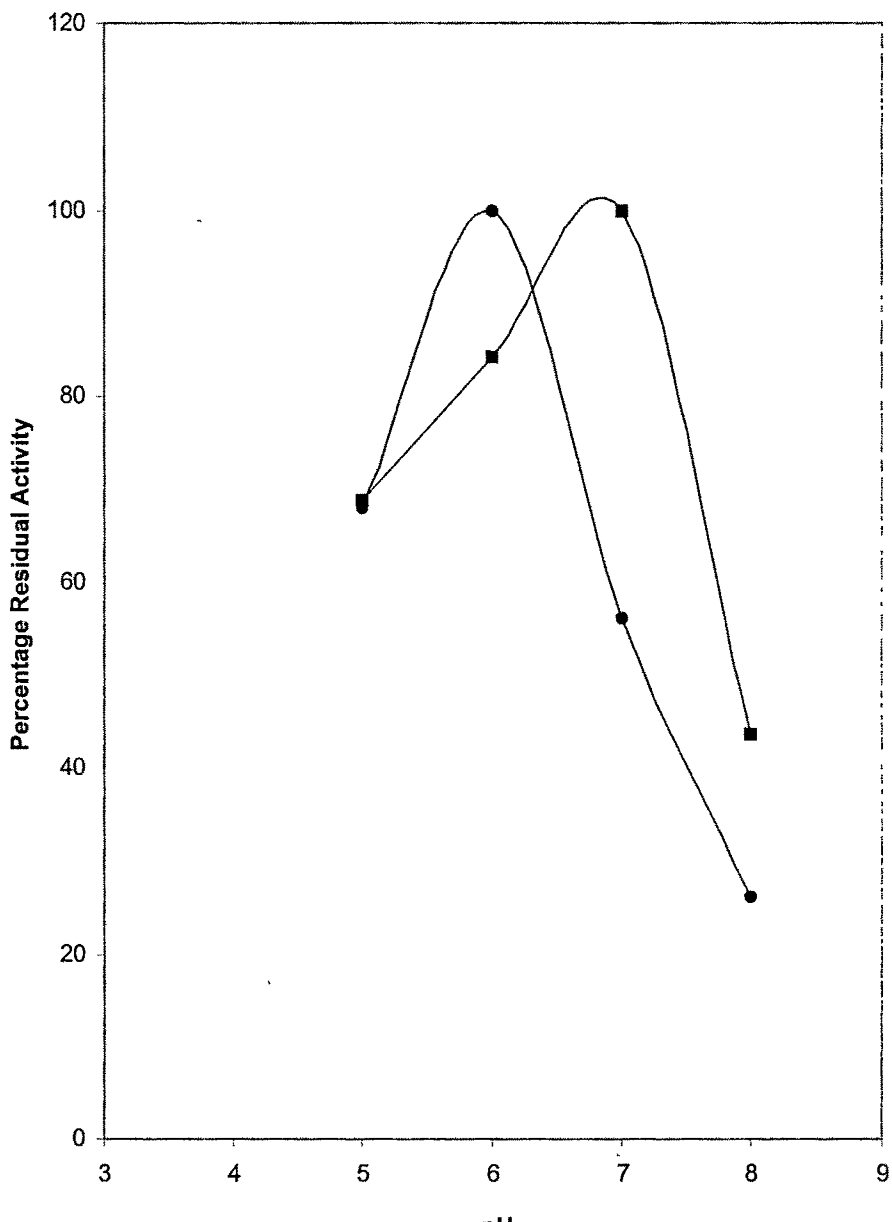


Figure 2.11Effect of pH on the rate of oxidation of hydroquinone at 35°C for 4 h.

Free enzyme (●) and Entrapped enzyme (■).

carried out at different pH and it was found that in case of free enzyme the optimum pH for the oxidation is 6 where as for entrapped enzyme it was 7(**Figure 2.11**). Hence the enzymatic reaction for the preparation of p-benzoquinone can be carried out at pH 7 and at room temperature whereas chemical process needs acidic medium and less than 10^{o}C temperature as the reaction is exothermic.

2.4.4. Optimization of covalently bound enzyme conditions

Covalent coupling of enzyme to activated support was examined by varying concentration of activating agent concentration , activation time , coupling time and enzyme concentration . These all parameters play an important role in covalent coupling of enzyme to the functionalized support .

a. Optimization of Copolymer ratio :

The copolymers of different compositions were prepared and it was found that maximum coupling was observed at 1:1 monomer ratio as shown in **figure 2.12.** The copolymers were characterized by FTIR analysis . The FTIR spectra of the copolymer showed bands at 3700-3200 cm^{-1} corresponding to –NH stretching and –OH stretching , at 1710 cm^{-1} due to –C=O stretching , at 1155 cm^{-1} due to C-N stretching.

b. Activation agent concentration :

One gram of support was taken for the activation of both OH and NH_2 groups of the copolymer of AAm-HEMA . Activation of NH_2 group was carried out using glutaraldehyde .It was observed that with increase in glutaraldehyde concentration the protein coupled increased upto 2 % of glutaraldehyde but after that it does not show much increase . The results are shown in **figure 2.13** . The activation of OH group was carried out using two activating agents p-benzoquinone and p-tolyl sulphonyl chloride . p-benzoquinone was found to be more effective compared to p-tolylsulphonyl chloride . Hence activation of –OH group was carried out using p-benzoquinone and it was observed that 25 mg of p-benzoquione is sufficient to activate the one gm of the support . The results are shown in **figure 2.14.**

Simultaneous activation of both NH_2 and OH group was also been carried out . Optimized conditions for both NH_2 and OH were used for this. Maximum activity was found when the support was activated with 10 cm^3 of 2 % w/v glutaraldehdye for 2 h and with 10 cm^3 of 2.5 mg/ml p-benzoquinone for 4 h. A two fold increase in the activity was observed by following this method. But when the order of activation was reversed the enzyme activity decreased considerably.

c. Effect of activation time on enzyme coupling :

Extent of coupling and retention of enzyme activity depends on the activating agent and activation time . Hence the effect of activation time was checked first using glutaraldehyde as an activating agent for NH_2 group and it was observed that maximum activation was achieved at 2 hr of activation and further increase in activation time did not show any increase in enzyme coupling.

In case of p-benzoquinone it was observed that maximum activation is achieved by using 10 cm^3 of 2.5 mg/ml p-benzoquinone solution for 6 h. Hence simultaneous activation of both groups was carried out using 20 cm^3 of 2 % w/v solution of glutaraldehdye and activation time of 2 h followed by enzyme coupling. Then activation of hydroxyl group by using 10 cm^3 of 2.5 mg/g of PBQ for 2 h , followed by enzyme coupling. The results are given in T**able 2.15** .

d. Effect of coupling medium

Coupling of activated support at different pH was carried out and it was found that maximum coupling was done at pH 7 . The results are given in **figure 2.16 .**

e. Effect of Enzyme concentration :

Maximum extent of enzyme loading was determined by using 50 –3000 μg of enzyme and activating the support by glutaraldehdye . It was observed that though 18.42 U of protein was coupled only 22 μg of protein was active.

We have also carried out the activation of 1 g of support using p-benzoquinone . Maximum activity was found to be 16.5 μg and maximum coupling was found to be10.15 U/.g of support using 25 mg in 10 cm^3 p-benzoquinone at 6h activation followed by 8 h coupling at pH 7.

Simultaneous activation of both NH_2 and OH groups was also carried out using 50 – 2000 μg/g of enzyme .First the amino group was activated followed by enzyme coupling followed by activation of OH group by p-benzoquinone which was again followed by enzyme coupling . A synergistic effect was found and the enzyme coupling increased by more than two fold and maximum coupling was found to be 21.80 U/ g of the support with maximum activity of 58.12 μg/gm of support .

Optimized conditions of all the three activating agents are given in **Table 2.4**. to give a overall view of how the support behaved against different activating agents.

f. Effect of enzyme coupling time :

Enzyme coupling time is another important parameter to be checked during covalent coupling of the enzyme . It was observed that with the increase in the coupling time the enzyme coupling increased upto a certain limit but beyond that there was no further increase as shown in **figure 2.17.** When glutaraldehdye

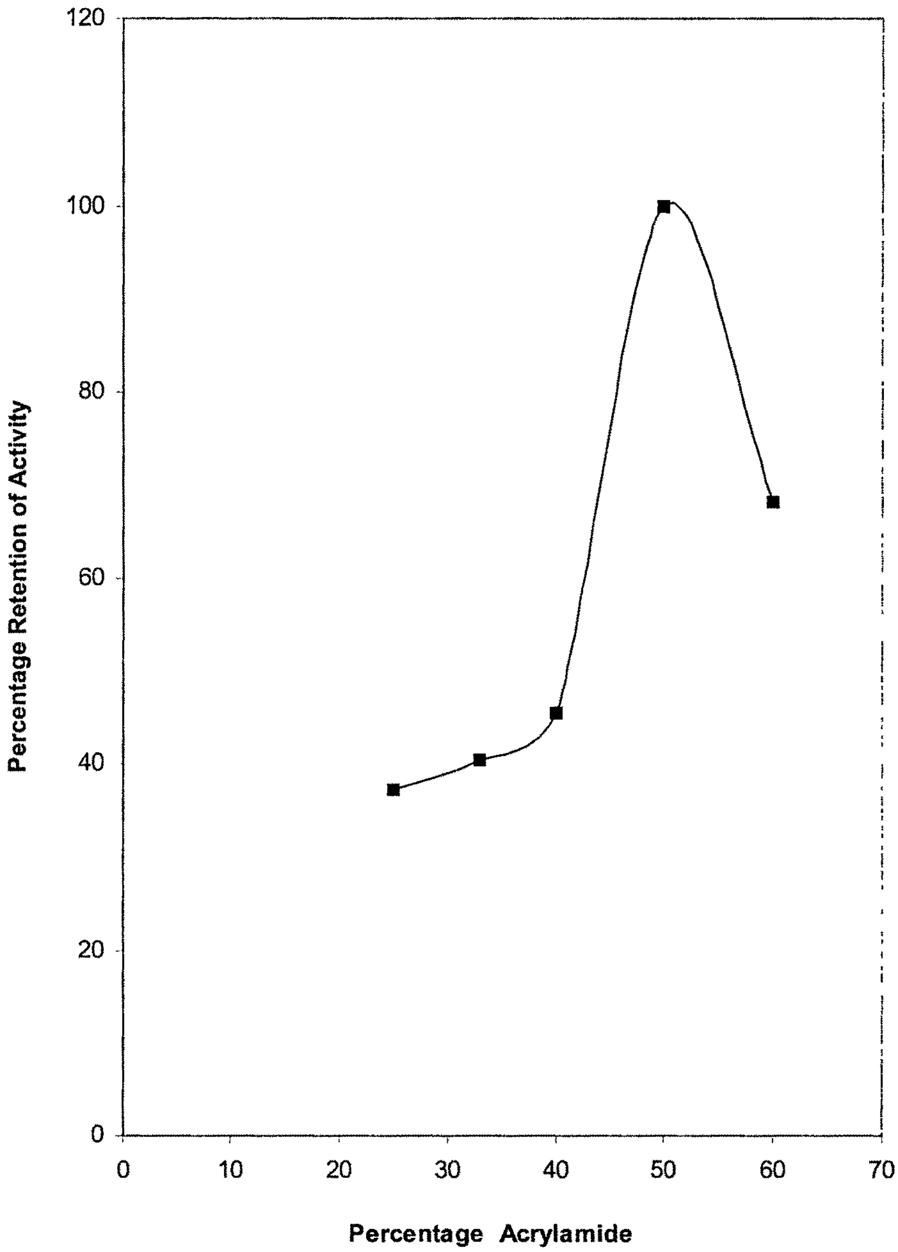


Figure 2.12.Effect of Acrylamide percentage on the retention of enzyme activity.

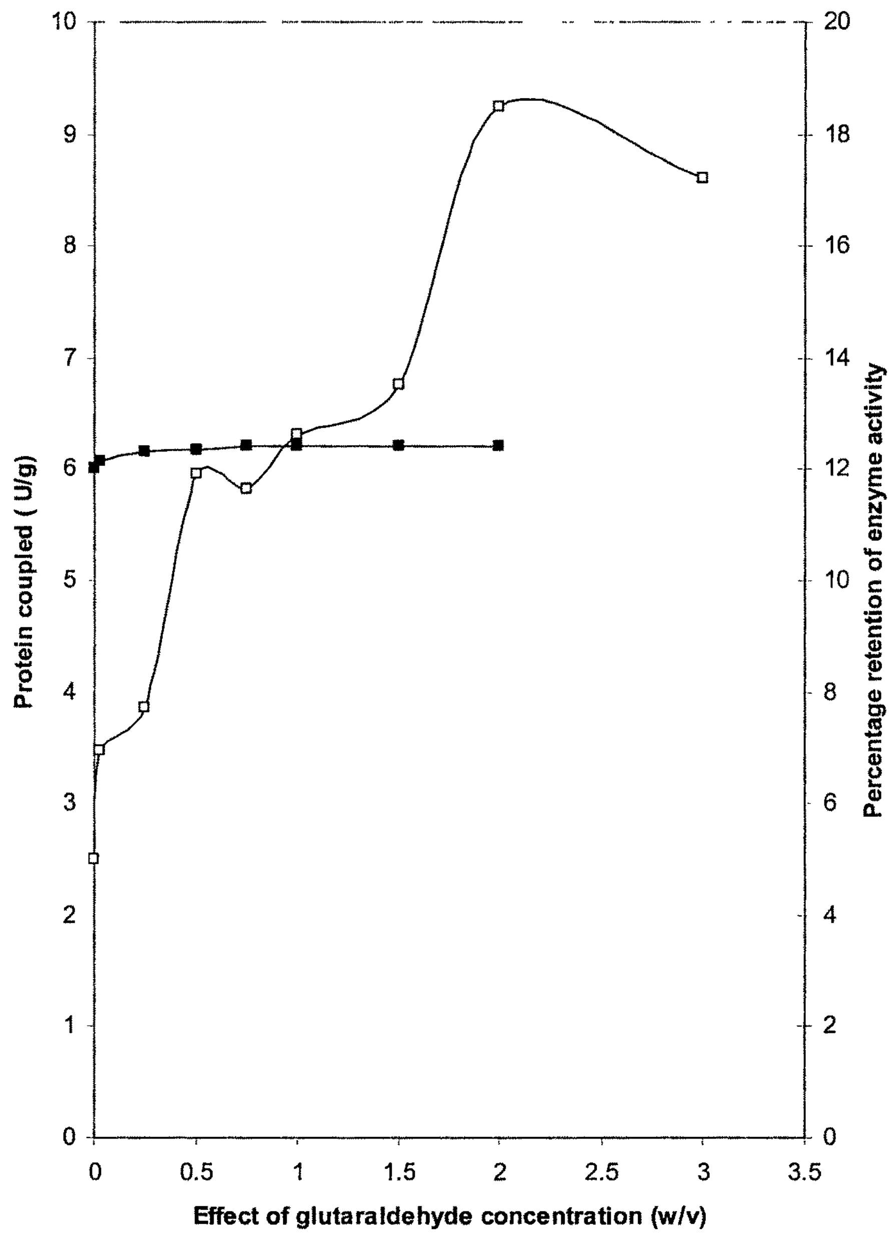


Figure 2.13 Effect of glutaraldehyde concentration on retention of enzyme activity.

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

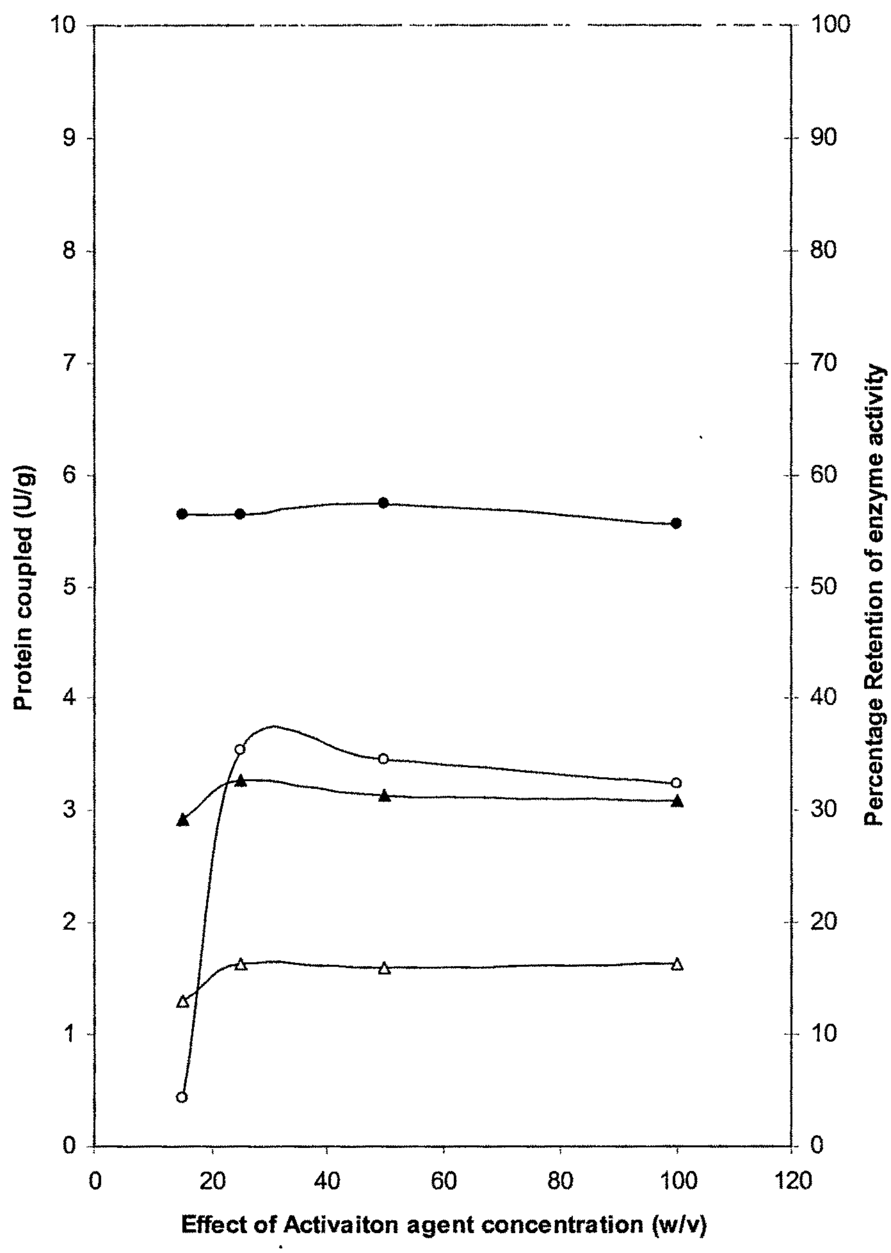


Figure 2.14 Effect of p-benzoquinone and p-tolyl sulphonyl chloride concentration on retention of enzyme activity.

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

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

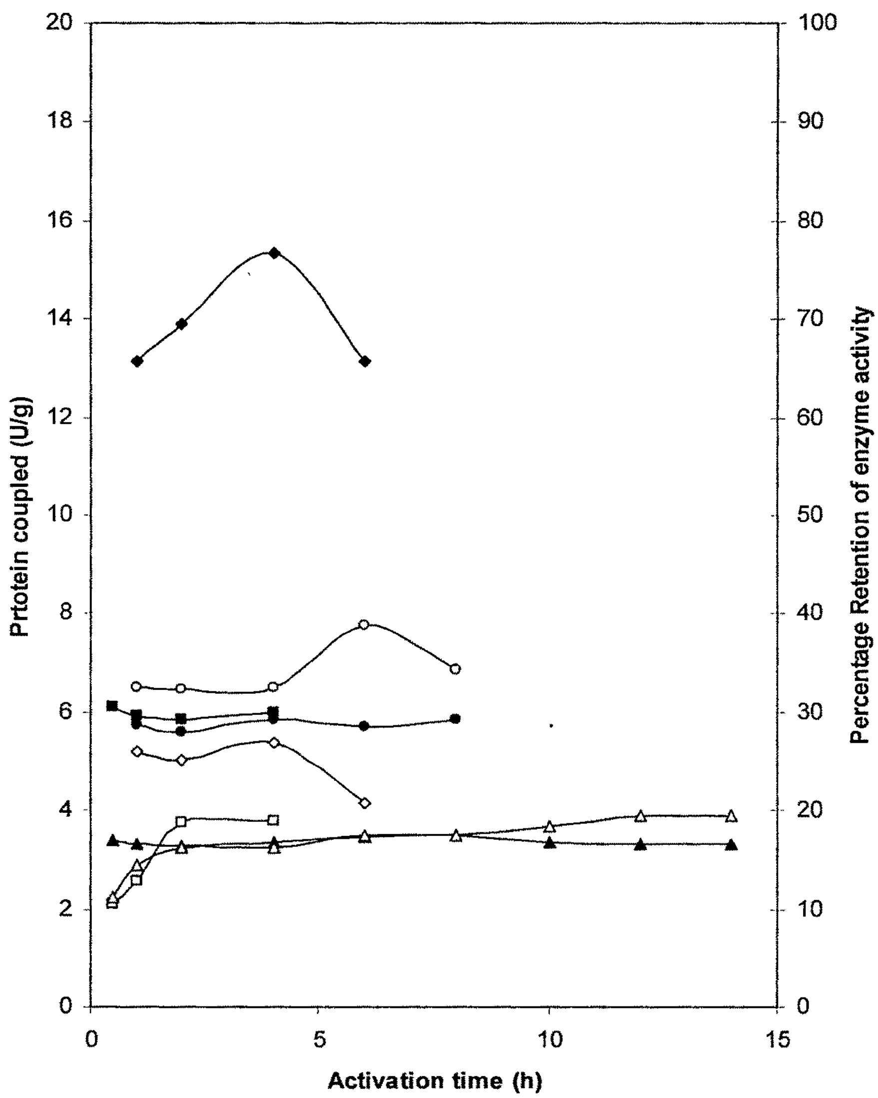


Figure 2.15 Effect of activation time on retention of enzyme activity.

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 (◇).

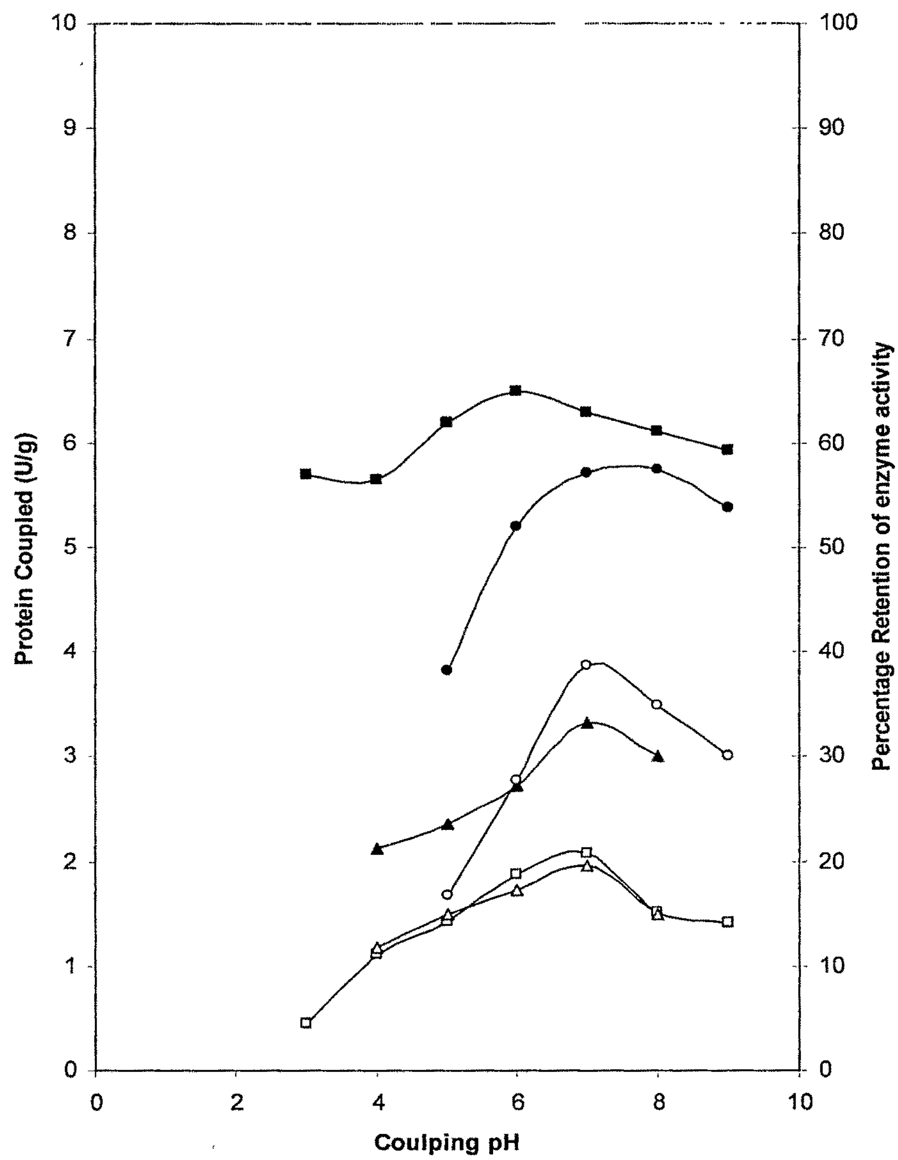


Figure 2.16 : Effect of enzyme coupling pH .

GLU : Protein Coupled (■), Retention(□)

PBQ : Protein Coupled (●), retention (○).

PTS : Protein Coupled (▲), retention (△).

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

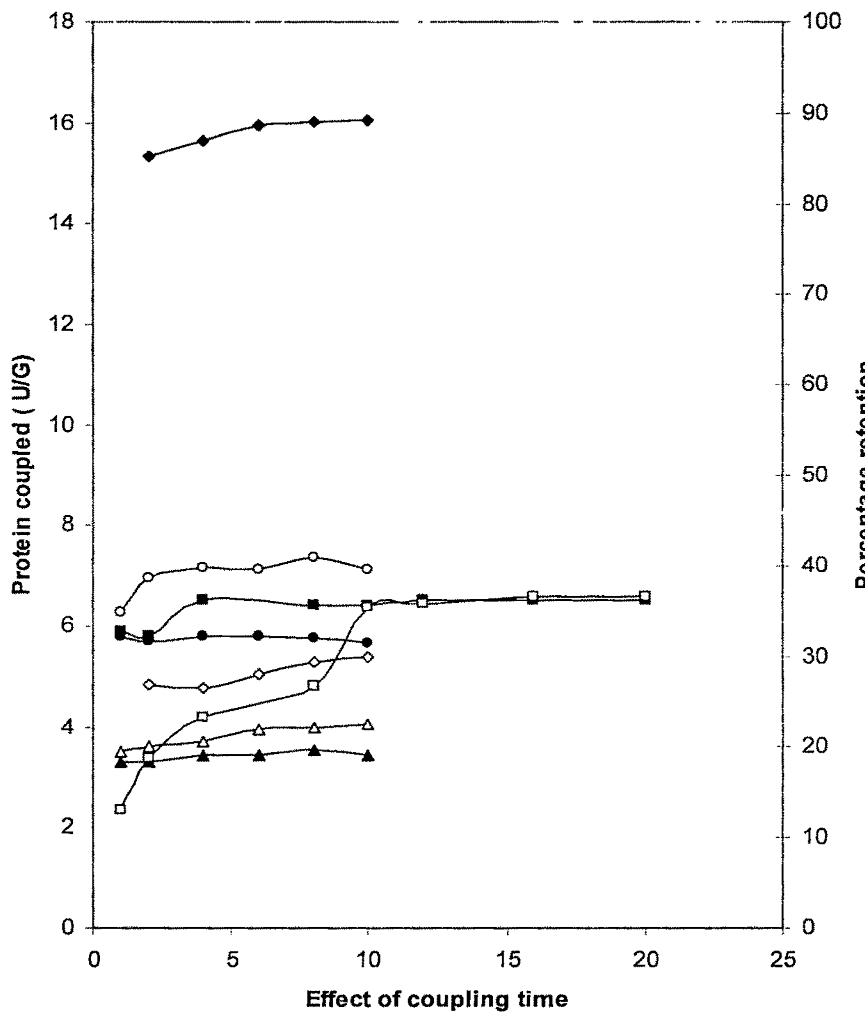


Figure 2.17 Effect of enzyme coupling time on retention of enzyme activity.

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 (◇).

and p-benzoquinone were used as activating agents the maximum coupling time was found to be 8 h while in case of simultaneous activation of both groups maximum coupling time was found to be 10 h.

2.4.5.Comparative study of free and covalently bound enzyme :

1.pH stability :

pH plays an important role in enzymatic reactions. We have observed that free enzyme showed maximum activity at pH 8 where as covalently bound enzyme showed maximum activity at pH 7 . For comparison the results are given in **Figure 2.18.**

2. Thermal Stability

Thermal stability of enzymes is important for it's industrial applications . We have observed that the covalently bound enzyme showed better thermal stability than free enzyme **Figure 2.19.** The thermodeactivation parameters are given in **Table 2.5.** The rate of deactivation of free enzyme was more compared to covalently bound enzyme . Thus indicating that immobilized enzyme is thermally more stable than free enzyme. Thus showing the advantage of immobilization.

3.Storage Stability

Storage stability of free , covalently bound and earlier studied entrapped enzyme was compared. The covalently bound enzyme showed 60% activity after 21 days whereas the free enzyme lost all its activity after 15 days **(Figure 2.20)** It is likely that due to dry storage covalently bound enzyme at the surface loses activity whereas that in the inner surfaces retains it's activity Whereas in entrapped system as enzyme is entrapped in polymer gel it is not affected by the temperature and retains its activity.

4.Reusability

Reusability of covalently bound enzyme was found to be better than entrapped enzyme. Entrapped enzyme lost 50% of it's activity after 2 cycles where as covalently bound enzyme retained 50% activity even after 8 cycles **(Figure 2.21)**. Entrapment being a physical process leaching of enzyme is expected to be more as compared to covalently coupled system.

5 .Kinetic parameters

The value of the kinetic parameters K_m and V_{max} for free enzyme were found to be 8 x 10^{-5} (mM) and 1.53 (mM / min) whereas for covalently bound enzyme they were 8.14 x 10^{-5} (mM)and 2.0 (mM / min) when substrate concentration was varied and enzyme concentration was kept constant .When enzyme concentration was varied and substrate concentration was kept constant K_m and V_{max} were 4 x 10^{-11} (mM) and 0.45 (mM/ min) for free enzyme and 4.25 x 10^{-11} (mM) and 0.55 (mM / min) for covalenlty bound enzyme **Table 2.6.** and **Figure 2.22. (a,b)** . This indicates that there is no conformational change during immobilization .

2.4.6.Optimization of reactor conditions :

a. Effect of flow rate :

Oxidation of phenol using 0.5 – 2 mM aqueous solution was carried out at different flow rates using compact bed reactor . It was observed that with increase in flow rate the percentage oxidation of phenol decreased due decrease in time of contact of enzyme and phenol **Figure 2.23.**

Table 2.4.Effect of different activating agents on covalent coupling parameters.

Coupling Condition	Glutarald-aldehdye	Benzo-Quinine	Glutaraldehyde + p-Benzoquinone	p-Benzoquinone + Glutaraldehyde
Activating Agent Concentration	10ml 2% (w/v)	2.5 mg/g of support	10ml 2% (w/v) + 25mg/g support	2.5mg/g support + 10ml 2% (w/v)
Activating Agent. Coupling Time (h)	2	6	2+4	4+2
Coupled enzyme Concentration (μg/g)	22	16.5	58.12	6.33
Enzyme coupling time (h)	12	7	7	7
Coupling pH	7	7	7	7

b. Effect of Phenol concentration

The effect of phenol concentration was checked and it was observed that maximum oxidation was carried out at 1 mM phenol concentration at a flow rate of 0.5 cm^3 /min . The results are shown in **figure 2.24.**

c. Effect of I/D ratio

The I/D ratio was varied from 2- 8 and it was observed that with the increase in I/D ratio the extent of percentage oxidation increases and attains a limit with no further increase. The results are given in **figure 2.25.**

d. Effect of temperature :

The temperature effect on phenol oxidation was examined at 35°C to 55°C . It was observed that with the increase in temperature initially the oxidation increases and there after it decreases.. **Figure 2.26.** shows the results .

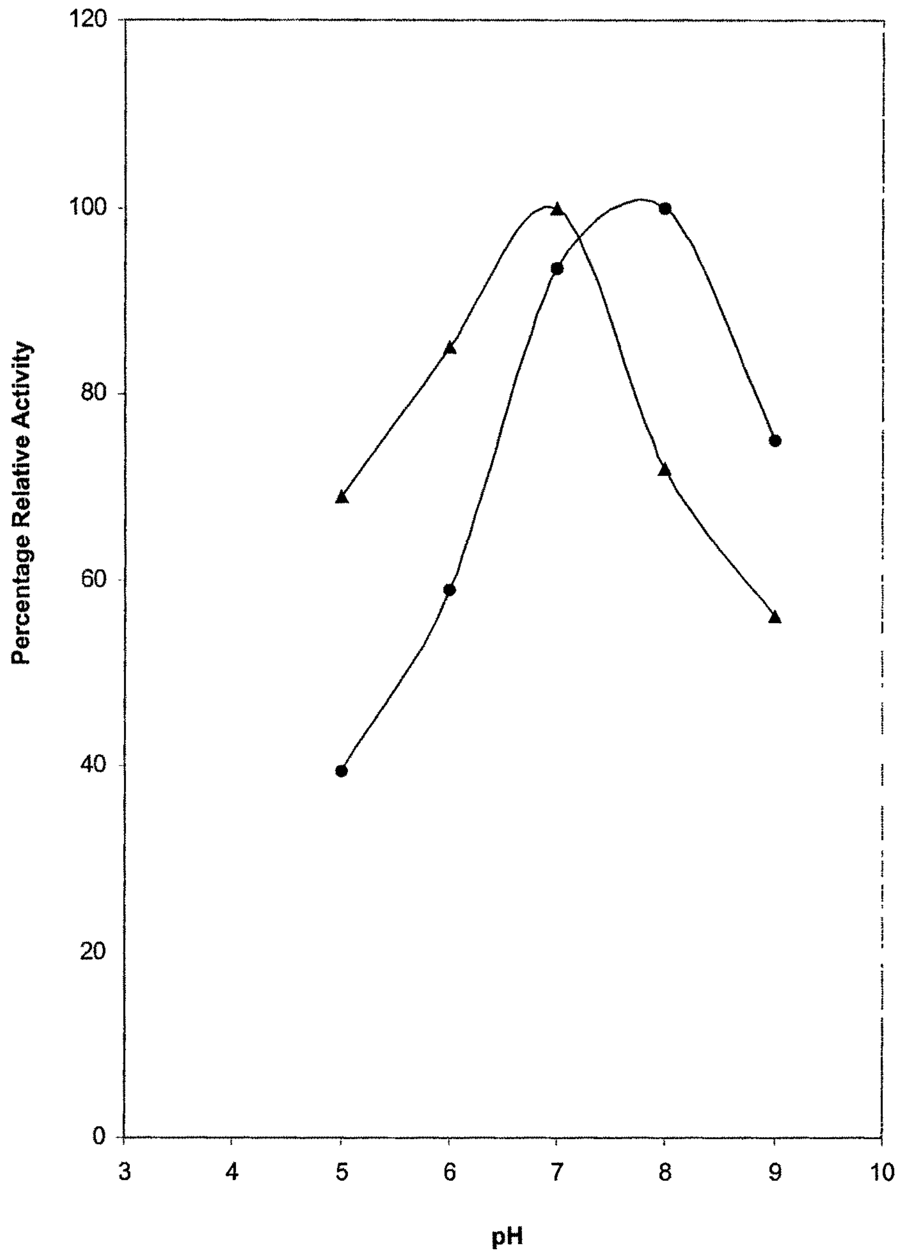


Figure 2.18 : pH dependence of Free (●) and Covalently bound enzyme (▲).

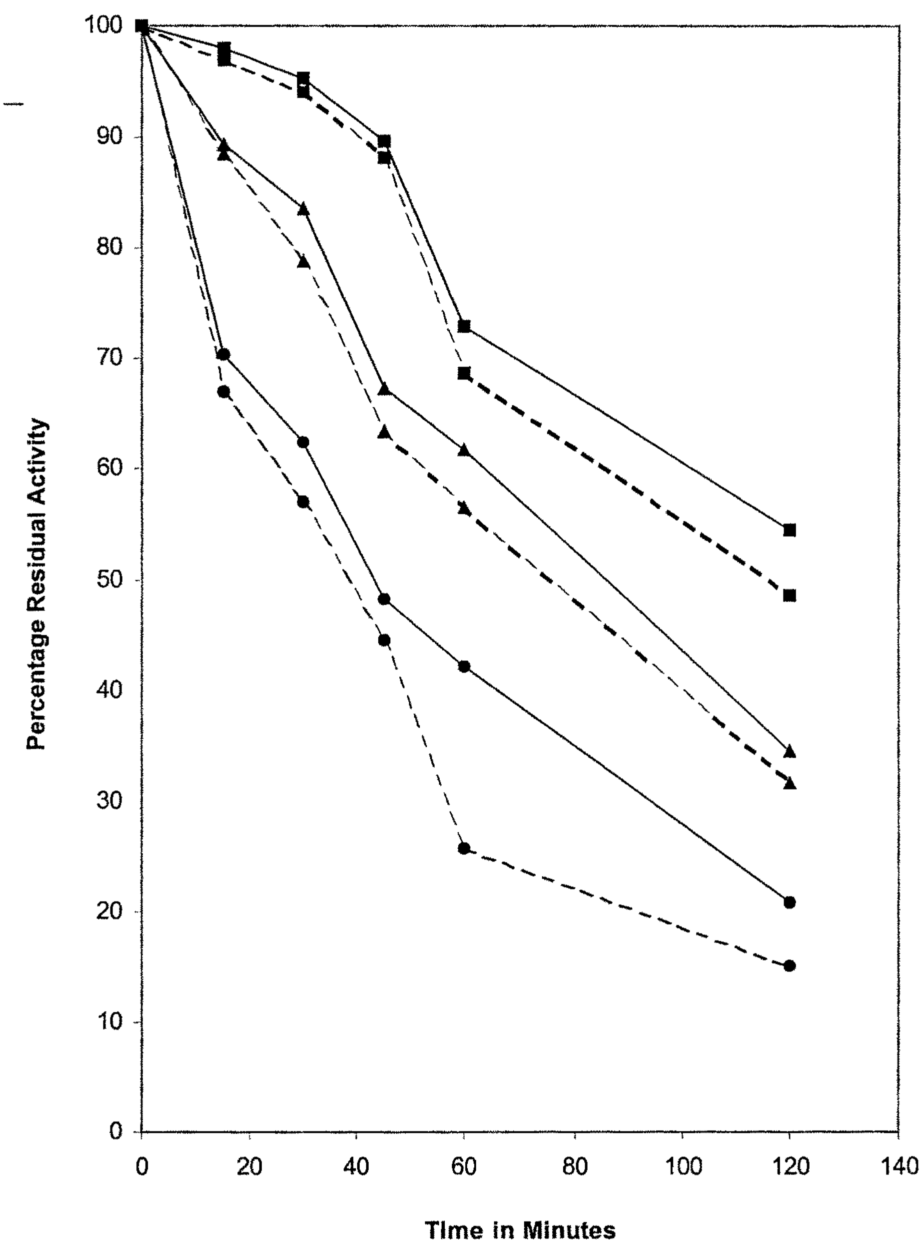


Figure 2.19 : Temperature dependence of free and covalently bound enzyme at 35°C(■),45°C(▲),55°C(●).

Free enzyme (--) and covalently bound enzyme (—).

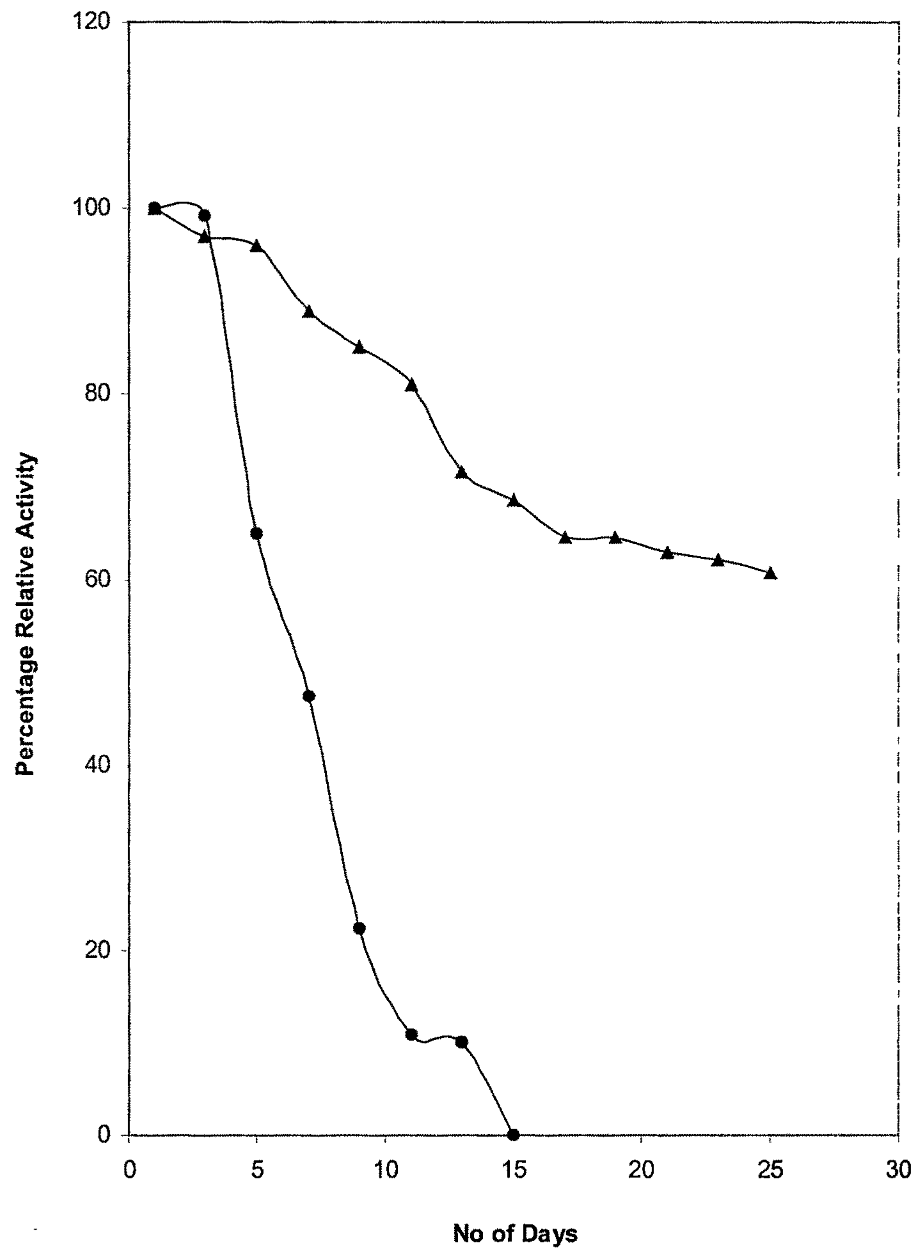


Figure 2.20 Effect of storage time on free (●) and covalently bound (▲) enzyme.

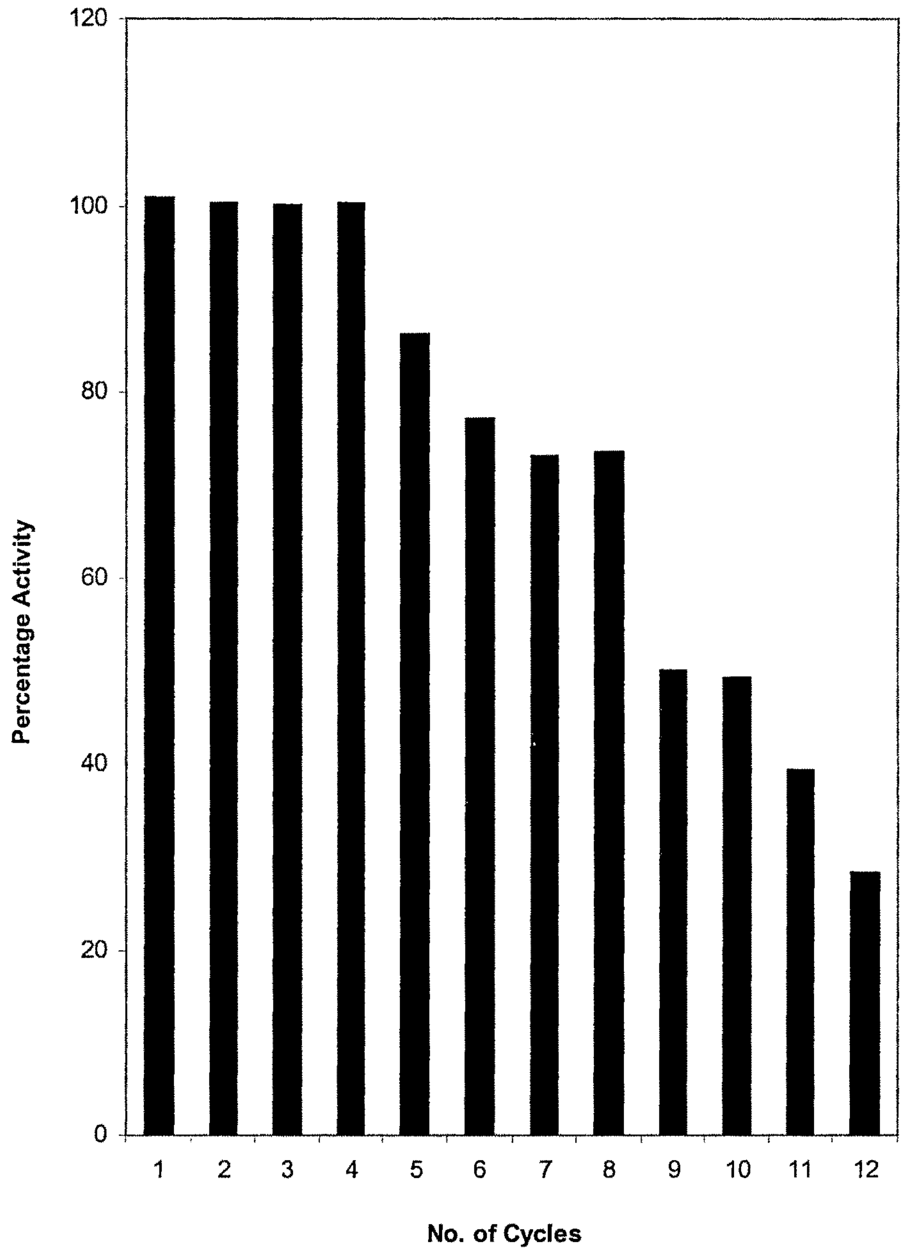


Figure 2.21 Reusability of covalently bound enzyme.

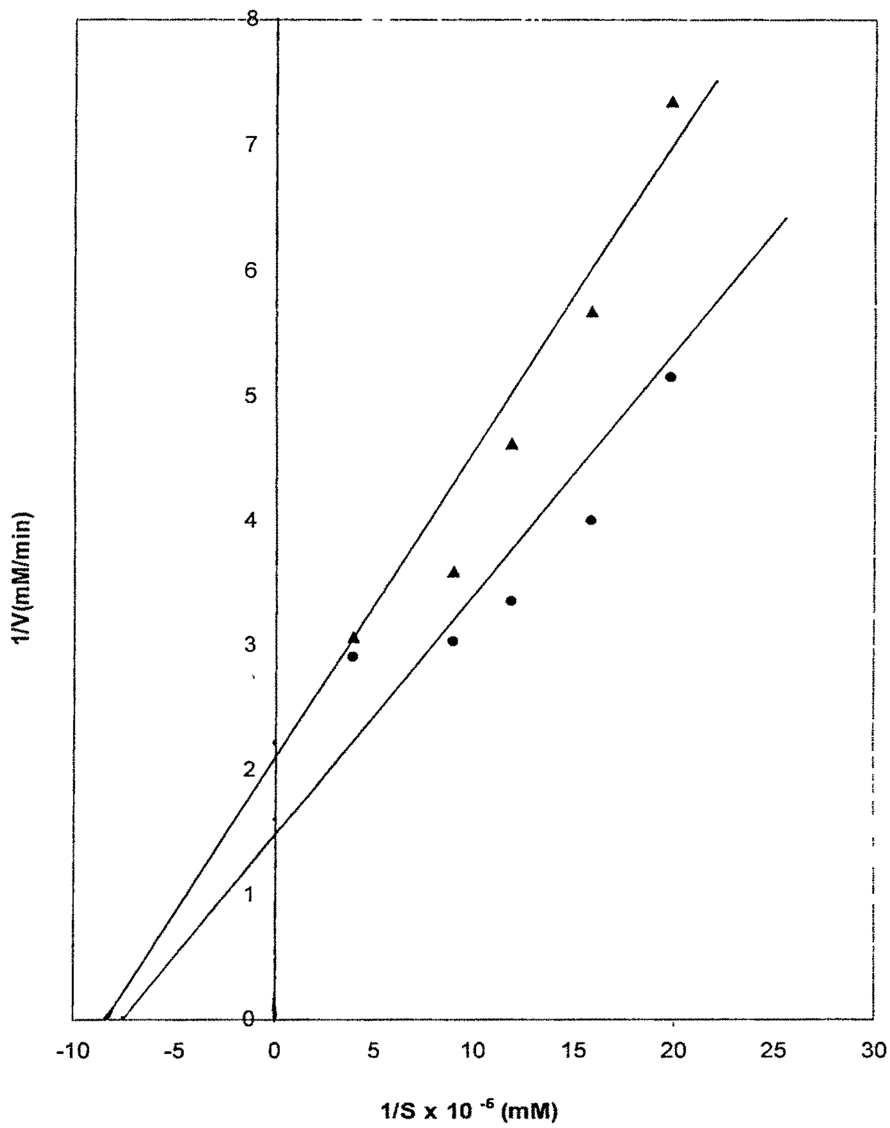


Figure 2.22 (a) Lineweaver Burk plot at 35°C , pH 6 for 1 min .When enzyme concentration is constant and substrate concentration is varied .

Free *enzyme (●) , covalently bound enzyme (▲).*

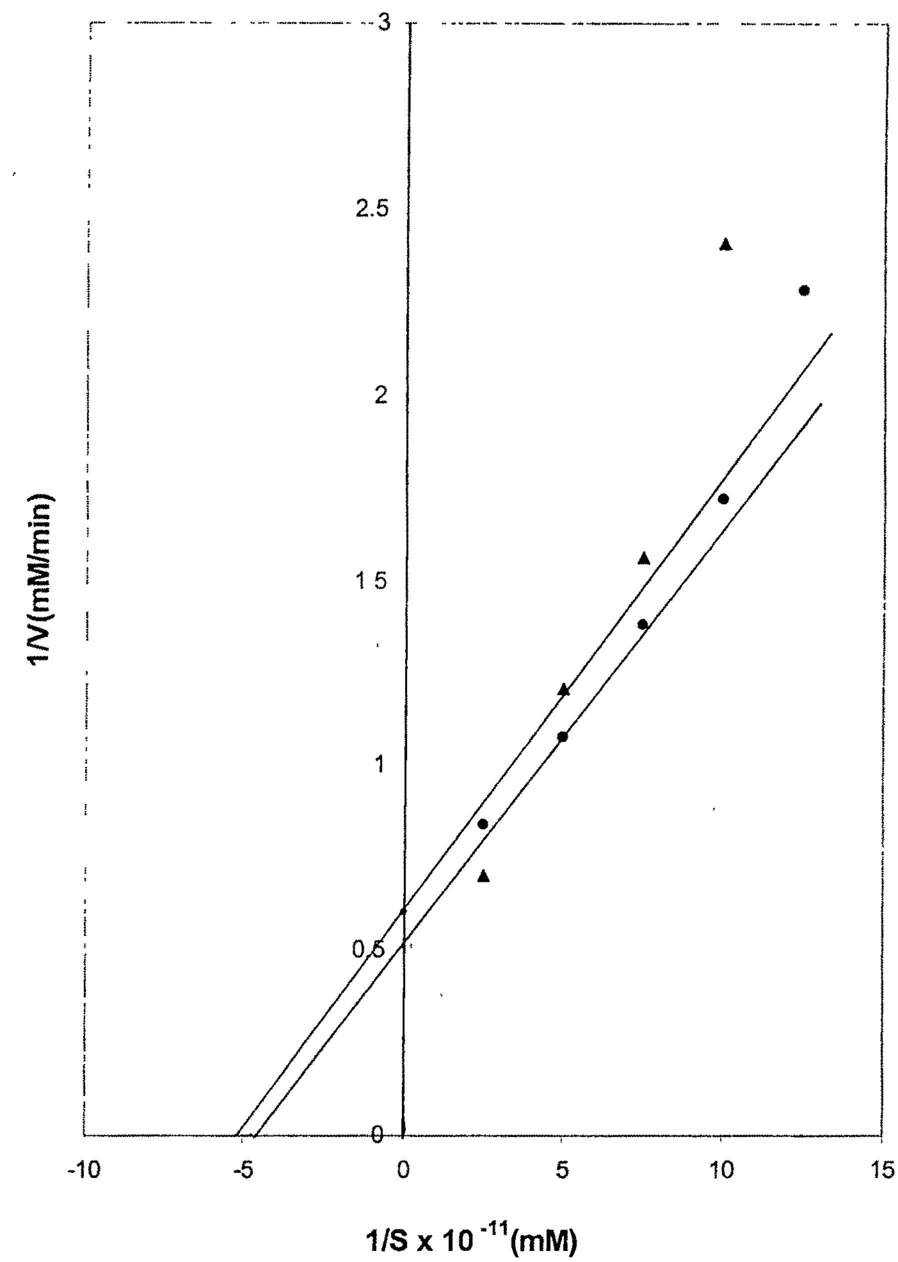


Figure 2.22 (b) Lineweaver Burk plot at 35°C , pH 6 for 1 min .When substrate concentration is constant and enzyme concentration is varied .

Free enzyme (•) , covalently bound enzyme (▲).

Table 2.5.Thremodeactivation constant of Free and covalently bound enzyme.

Time in min	Thermodeactivation constant X 10^3 at					
	35 °C		45 °C		55 °C	
	Covalent	Free	Covalent	Free	Covalent	Free
15	0.206	0.206	0.510	0.811	1.900	2.600
35	1.330	2.060	4.380	7.890	11.40	18.700
45	2.060	4.810	7.730	10.000	11.90	18.700
60	3.630	6.250	6.210	9.500	11.200	22.500
120	4.480	6.025	8.160	9.500	12.200	25.700

Table 2.6 .Kinetic parameters of free and covalently bound enzyme.

Conditions	Free enzyme		Covalently bound enzyme	
	K_m (mM)	V_{max} (mM/min)	K_m (mM)	V_{max} (mM/min)
When substrate concentration is varied and enzyme concentration is kept constant .	8×10^{-5}	1.53	8.14×10^{-5}	2.0
When enzyme Concentration Is varied and substrate concentration is kept constant.	4×10^{-11}	0.45	4.25×10^{-11}	0.55

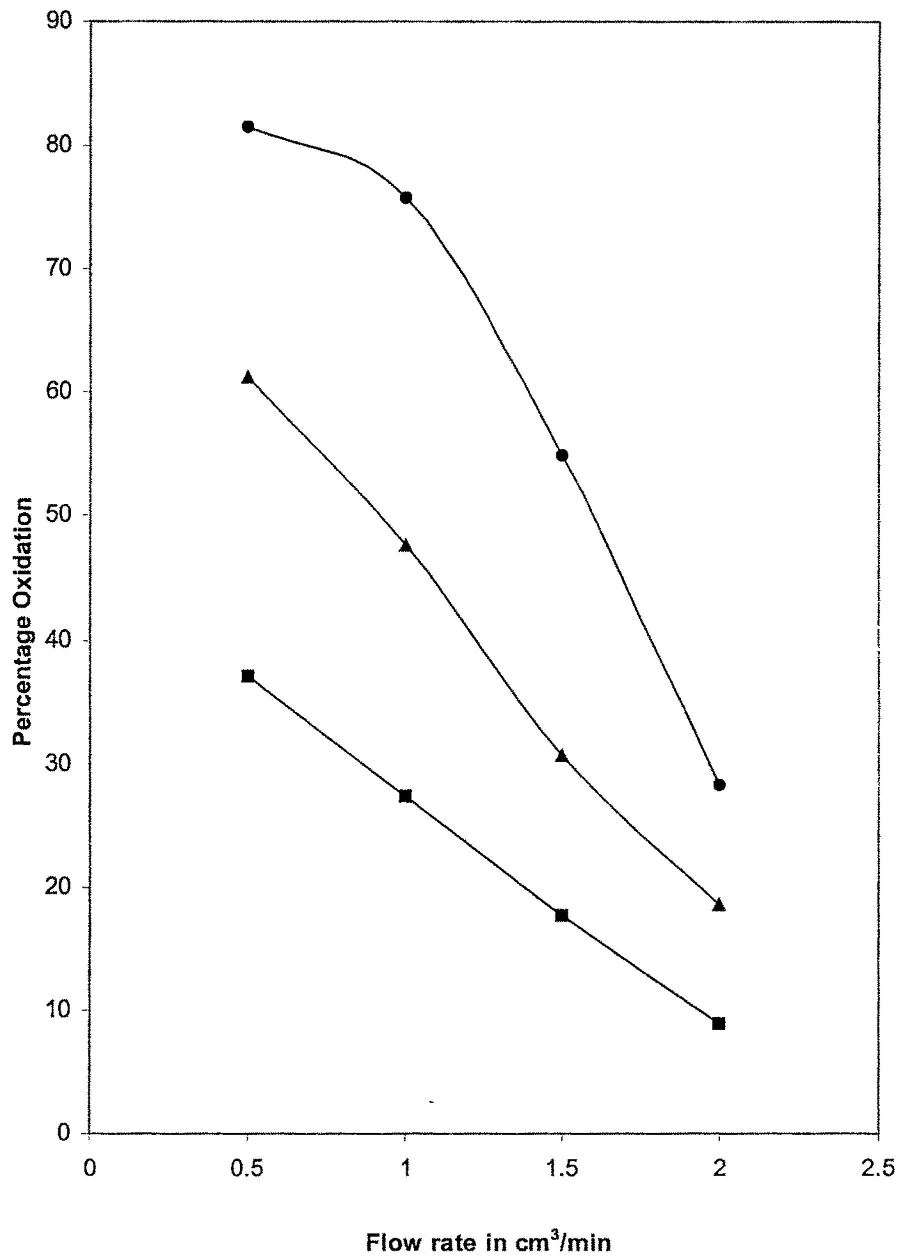


Figure 2.23 : Effect of flow rate on oxidation of phenol at 35°C at 1mM phenol concentration .

First Cycle (■), Second cycle (▲), Third Cycle (●).

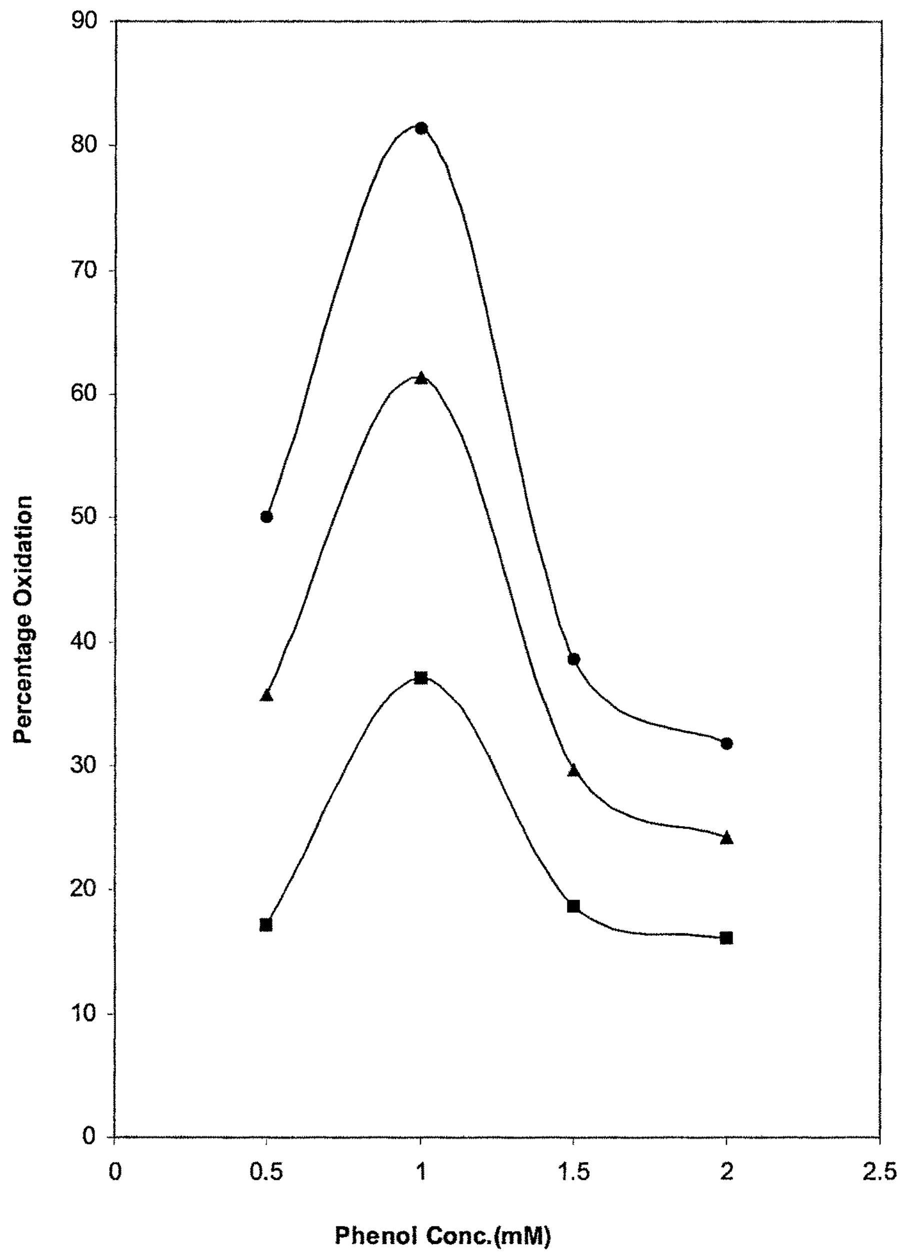


Figure 2.24 : Effect of phenol concentration on oxidation at 0.5 cm^3/min flow rate at 35°C

First Cycle (■), Second Cycle (▲), Third Cycle (●).

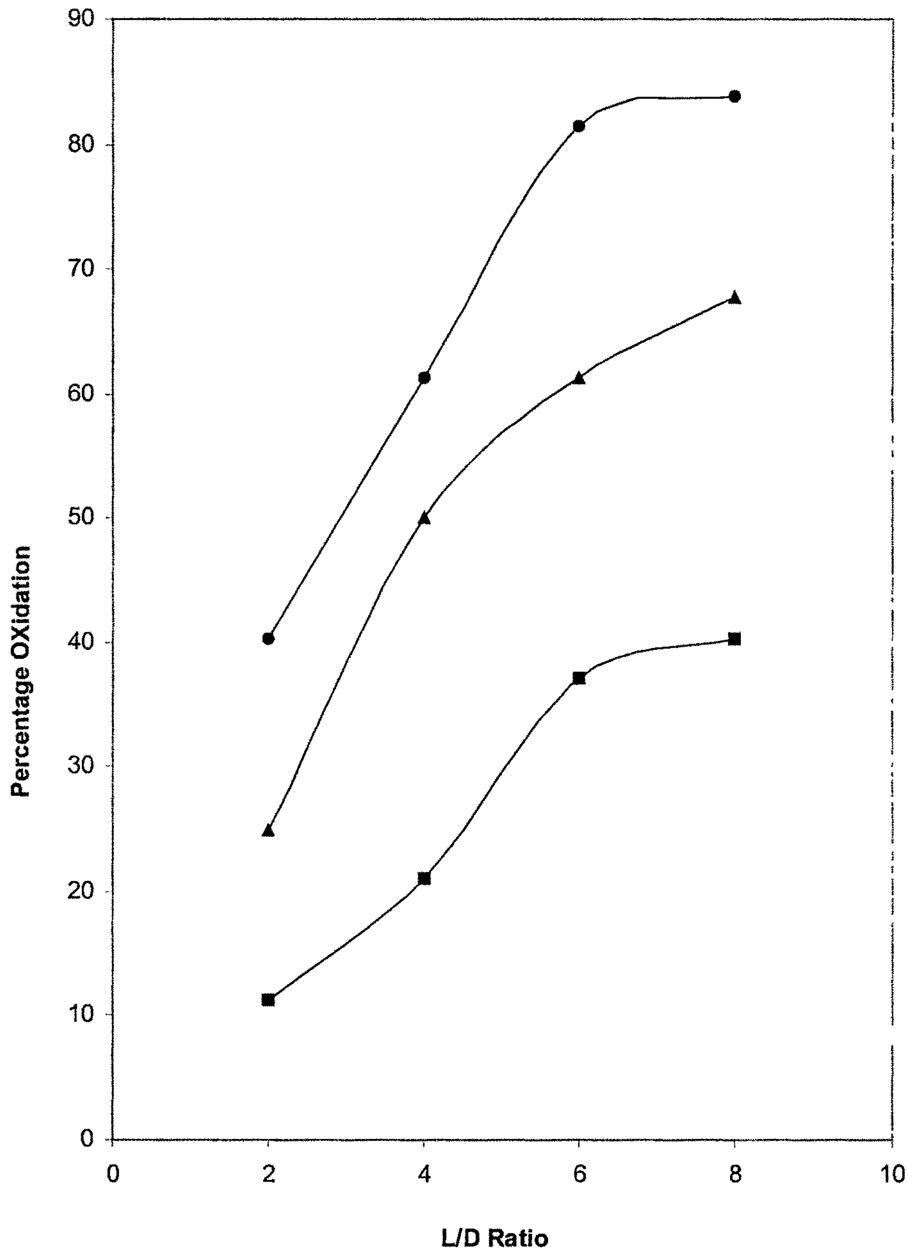


Figure 2.25 Effect of L/D ratio of phenol oxidation at 1mM solution for 0.5 cm^3/min flow rate at 35°C.

First Cycle (■), Second Cycle (▲), Third Cycle (●).

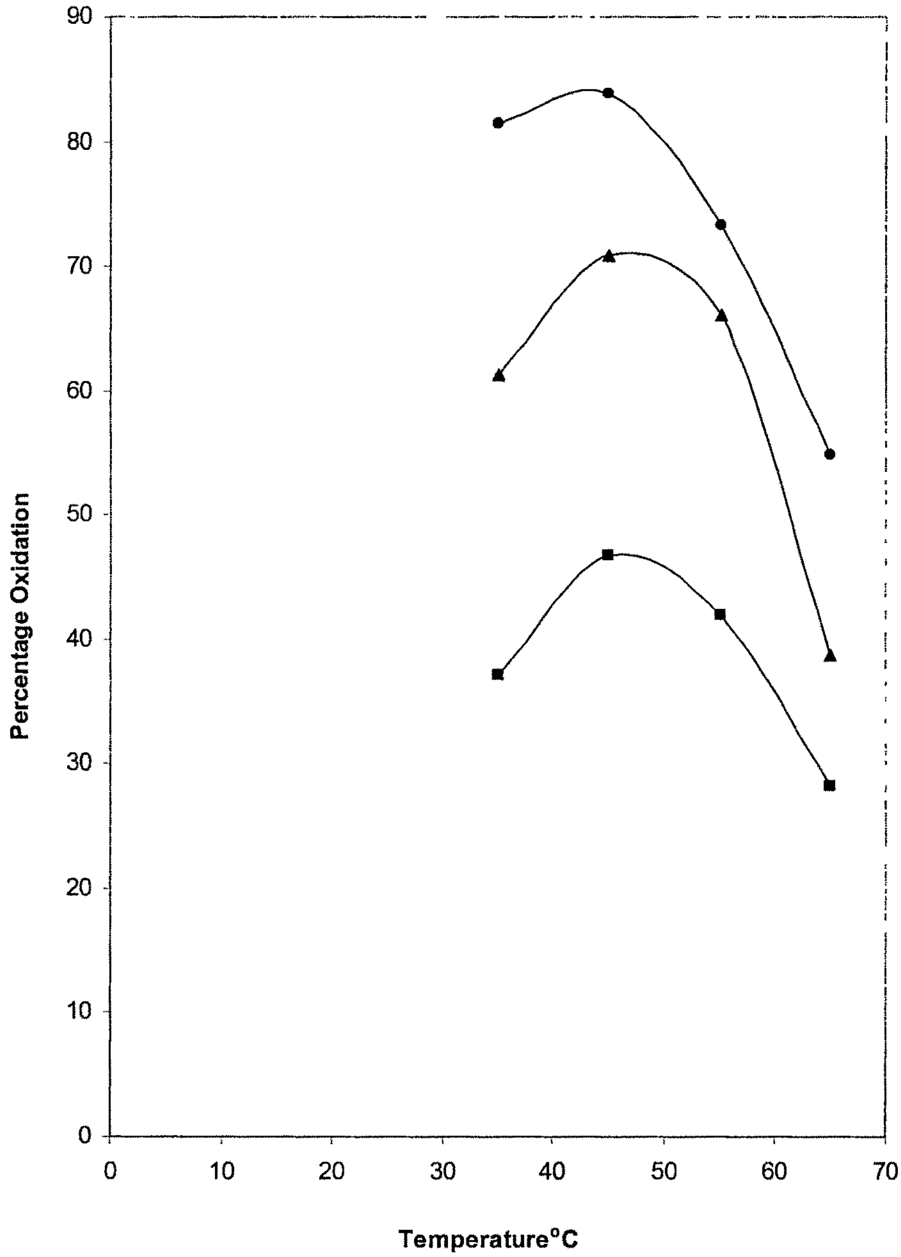


Figure 2.26 Effect of temperature on oxidation of phenol for 1 mM phenol concentration at 0.5 cm^3/min flow rate .

First Cycle (■), Second Cycle (▲), Third Cycle (●).

2.5. Summary and conclusion :

- Horseradish peroxidase was successfully entrapped in K-carrageenan beads using polyethyleneimine as hardening agents. The entrapped enzyme retained 50 % of its activity after 4 cycles and 50 % activity on storage after one month. The thermal stability of entrapped enzyme was found to be more compared to free enzyme. 90 % of oxidation of hydroquinone was observed with entrapped enzyme.

- The covalent coupling of HRP on copolymer of AAm-HEMA showed some interesting results . 1:1 ration of copolymer was taken for the studies and OH and NH_2 groups were activated by different activating agents and also simultaneous activation of both groups was done . Maximum coupling and maximum activity with glutaraldehyde as activating agent was observed to be 4.428 U/g and 22 μg/g of support , in case of PBQ it was 3.30 U/g and 16.5 μg/g , and for PTS maximum coupling was found to be 0.794 U/g while on simultaneous activation of both groups the maximum coupling and activity were observed to be 25.52 U/g and 58.12 μg/g respectively and then it was used for phenol and it was found that 90 % of oxidation was obtained at 0.5 cm^3/min flow rate and temperature 45°C at l/d ratio of 6..

- The comparative study of free and covalently bound enzyme showed that covalently bound enzyme showed 60 % of retention of activity after 3 weeks and retained 50 % activity after eight cycles.

-Thus immobilization of *HRP* on both natural and synthetic supports was carried out and all conditions were optimized. A synergistic effect was observed on simultaneous activation of both groups while entrapment procedure was simple and retention of around 30 % was observed . The value of K_m and V_{max} in both cases showed that there is a liking of the enzyme for the support. Though the activity increased almost to two fold during simultaneous activation of both groups the protein coupled nearly remained constant.

Table 2.7. Comparison of properties of free and immobilized enzyme.

Properties	Free enzyme	Entrapped enzyme	Covalently bound enzyme
Optimum pH	8	7	7
Optimum Temperature (° C)	35	35	35
Storage stability at room temperature till 50% loss in activity (days).	7	40	60
Durability (Cycles)	-	4	8
Thermodeactivation constant (K_d) at 45 °C.	0.811 x 10 ^{3}	0.510 x 10 ^{3}	0.510 x 10 ^{3}
Michaelis constant (K_m)(mM)	8 x 10^{-5}	8.3 x 10^{-5}	8.14 x 10^{-5}
Maximum velocity (V_{max})(mM/min)	1.53	2.18	2.0
Turnover number	-	51.6 x 10^{6}	10.3 x 10^{7}

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