CHAPTER - 3

IMMOBILIZATION OF ALCOHOL DEHYDROGENASE

CONTENTS

3.1 Properties of alcohol dehyrogenase

Primary alcohols are oxidized to aldehydes or acids and secondary alcohols are oxidized to ketones. Tertiary alcohols resist oxidation. Oxidation of alcohols can be achieved by catalytic dehydrogenation or by chemical oxidation.

Catalytic dehydrogenation of primary alcohols is achieved by passing vapours of the alcohols at high temperature over a catalyst, usually supported on asbestos, silica gel, pumice, etc. Ethyl alcohol is converted into acetaldehyde in 88% yield at 93% conversion by passing it over a mixture of oxides of copper, cobalt and chromium on asbestos at 275 °C.[1] Thus, vigorous conditions are required for oxidation of alcohols.

On the other side oxidoreductase class of enzymes can oxidize variety of alcohols at ambient conditions. Alcohol Dehydrogenase (ADH) is redox enzyme. ADH from yeast (YADH, EC 1.1.1.1) has been used as catalysts for biotransformations due to its broad substrate specificity and frequently higher steroselectivity for carbonyl reduction and sterospecificity for alcohol oxidation.[2,3]

Many enzyme-catalyzed redox processes involve the transfer of the equivalent of two electrons by either two one-electron steps or one two-electron step. The one-electron process is a radical process which very often involves the use of cofactors such a flavin, quinoid-coenzymes like vitamins C, E and K and coenzyme Q, and transition metals. The two electron processes can be hydride transfer or proton abstraction followed by two-electron transfer.[5]

Nicotinamide Adenine Dinucleotide (NAD) is involved in many two-electron oxidation catalysis by dehydrogenases. The nicotinamide ring is a redox active system, accepting a hydride or two electrons and a proton to form the 1,4-dihydronicotinamide derivatives. (NADH).[6]

YADH a typical redox enzyme, requires a coenzyme NAD^+, but is easily available and hence is widely studied. The *in vivo* role of YADH is to oxidize ethanol and reduce acetaldehyde. It readily oxidizes acyclic primary alcohols and reduces variety of aldehydes with stoichiometric consumption of NAD or NADH.[7]

$$RCH_2OH + NAD^+ \xrightleftharpoons{YADH} RCHO + NADH + H^+$$

The purified enzyme is available commercially as either a lyophilized powder or a buffered suspension. The enzyme is used in a whole cell preparation, either baker's or brewer's yeast, primarily *Saccharomyces cerevisiae*.[8,9] The properties of these whole cell oxidation and reductions are very different from the purified enzyme, presumably due to the action of other oxidoreductases, and may be superior for many applications.

The normal decrease in reaction rate with increasing chain length was observed for saturated alcohols. However, the decrease is less severe for ethylenic alcohols and even less for allenic alcohols. YADH can oxidize allenic alcohols to allenic aldehydes, which are difficult to obtain chemically.[10]

Another major dehydrogenase is horse liver alcohol dehydrogenase (HLADH), which can differentiate between prochiral groups or faces in symmetrical or meso compounds and make distinctions between enantiotropic groups or faces and geometric isomers. HLADH can oxidize primary alcohols. In addition the enzyme also reduces a significant number of aldehydes.

Other set of alcohol dehydrogenases that has been limited by their availability includes pig liver alcohol dehydrogenase, *Mucor javanicus* alcohol dehydrogenase, and *Curvularia falcata* alcohol dehydrogenase. All three enzymes require the phosphorylated nicotinamide cofactor, NADH.

3.2 Characteristics of YADH

YADH having molecular weight 141,000 daltons is comprised of four subunits of 35,000 each. Its Extinction coefficient is 12.6, while Isoelectric point is 5.4.

Composition : YADH is a tetrameric enzyme, with 36 free thiol groups and four catalytically essential zinc atoms per mole.[4] Per subunit, there are two distinct active site sulfhydryl groups which can be distinguished on the basis of differential reactivity with iodoacetate and butyl isocyanate.[11] A histidine residue is considered to have an essential role.[12]

Optimum pH : The optimum pH for YADH reaction is 8.0, but the enzyme is used in the pH range 6 to 8.5. For the oxidation of ethanol, pH 8.6 to 9.0, while for the reduction of acetaldehyde pH nearer to 7.0 is considered to be optimum.

Activators : Different activators of YADH are sulfhydryl activating reagents, mercaptoethanol, dithiothreitol, cysteine and heavy metal chelating reagents.

Specificity : Yeast enzyme has more narrow specificity than that of liver enzyme. It accepts ethanol, and is somewhat active on the straight chain primary alcohols, and also acts to a very limited extent on certain secondary and branched chain alcohols.[13]

Inhibitors : The enzyme is inhibited by heavy metals and SH reagents.[14] Substrate or product inhibition poses little or no problems with YADH.

Stabilizers : Dilute solution of the enzyme may be stabilized by serum albumin, glutathione or cysteine. At pH values below 6.0 and above 8.5 the enzyme is increasingly unstable. More concentrated solutions of the enzyme in high purity water, near neutrally, are stable for several days at 5 °C. Reactions are run at

temperature at or below 30 °C, above which the enzyme is unstable and inactivates quickly.

Stability : Lyophilized preparations stored refrigerated are stable for 6 to 12 months. Crystalline suspensions in ammonium sulphate have only 4 to 6 months storage stability at 5 °C. Organic solvents such as 20% acetone, 30% glycerol, and 30% ethylene glycol are tolerated by the enzyme.

3.3 Literature survey

The stabilization of biocatalysts remains one of the most actual problems in various fields of their applications. The immobilization of enzymes is one of the common methods used for this purpose. Immobilization leads to commercially viable applications of preparations in industry and many other spheres. ADH is an enzyme which is extremely interesting for industrial immobilization aimed at regeneration of coenzymes, fine organic synthesis and analytical determination of alcohol in various media. ADH from yeast and horse liver have been coupled to natural and synthetic carriers. Many studies have been reported on the stability of immobilized ADH towards heat, continuos operation, storage and different substrates. Different techniques like covalent bonding, cross-linking and entrapment of ADH are used for immobilization.

Immobilization of ADH on natural supports

Various natural supports are available for the immobilization of ADH. Among them naturally occurring polysaccharides such as sepharose is widely used as a support for the immobilization of ADH. Free hydroxyl groups of sepharose can be activated with cyanogen bromide (CNBr) and covalent attachment of enzymes can be achieved by optimizing the coupling conditions. Different workers have reported immobilization of ADH on sepharose activated with CNBr.

Koch-Schmidt and Mosbach[15] bound HLADH in the presence of various coenzymes to activated sepharose. They observed that the transition temperature for sepharose bound HLADH was increased by 12.5 °C in the presence of high concentration of NADH.

Ooshima et. al.[16] reported only 1.3 mg of ADH per g of wet sepharose. They studied the effect of butyraldehyde on the stability of free and immobilized enzymes and observed that free enzyme has less stability than immobilized enzyme when stored at 25 °C and pH 7.0. Mosbach et. al.[17] immobilized YADH by covalent coupling to sepharose activated with p-tolyl sulphonyl chloride. The amount of enzyme coupled was 112 mg g^{-1} dry particles and the coupling yield was 67%.

Carrea et. al.[18] also immobilized ADH on CNBr activated sepharose for the improvement in thermal stability in the presence of different additives. They observed that 1M phosphate ions and 0.5 M sulfate ions dramatically stabilize both free and immobilized enzyme against inactivation by temperature and urea. They also reported that high concentrations of phosphate and sulfate ions protect enzymes against urea, organic solvents (50% ethanol) and alkaline pH (9.5). Whereas Godbole et. al.[19] studied the effect of different concentrations of urea on ADH bound to sepharose. They reported that in the presence of 2 M urea concentration soluble ADH retains only 8% activity, while immobilized ADH retains 20% activity.

Half-life of the immobilized ADH is increased to almost twice compared to the soluble enzyme, when Schneider and Gorisch[20] immobilized ADH on CNBr activated sepharose. They also observed that in the presence of adenosine mono and diphosphate stability of immobilized ADH was more than free ADH. Nabi and Worsfold[21] immobilized ADH on sepharose and used it for the rapid and sensitive determination of ethanol by a flow injection technique.

Clark et. al.[22] prepared octyl-sepharose and CNBr-sepharose for the covalent immobilization of HLADH. They studied the properties of enzymes by EPR measurements and observed that selective inactivation of the less stable components results in an apparent increase in enzyme thermostability at 60 °C. Mori et. al.[23] immobilized ADH covalently onto sepharose 4B using CNBr. They further used immobilized enzymes for the low level detection of coenzyme NAD^+ and NADH. They observed that detection of coenzyme could be carried out in 6 min for the samples having less than 1μM concentration.

Das et. al.[24] used various natural supports such as agarose gel activated by CNBr, aminoethyl cellulose activated with glutaraldehyde and DEAE cellulose activated with an s-triazine derivative for the covalent immobilization of ADH. It was reported that the efficiency of coupling of ADH dropped from 94.5 to 72.2% and relative activity from 21 to 11% when ADH concentration increased from 18 to 54 mg g^{-1} activated agarose.

To the surface of a film of highly polymerized collagen ADH and other enzymes were bound using acyl-azide by Coulet et. al.[25] The activity of immobilized ADH was observed to be retained 100% after 5 months when stored in buffer at 4 °C.

YADH was immobilized on dextran in collodion-Hb microcapsules by Grundwald and Chang.[26] They observed that the stability of microcapsules containing YADH was increased several fold when purified Hb was used instead of crude Hb solutions. The improved stability of microcapsules by further cross-linking with glutaraldehyde was also reported. However, this treatment was reported to cause a considerable decrease in recycling activity of the enzyme.

Itozawa[27] immobilized HLADH on cross-linked chitosan beads and observed that the activity of enzyme decreases upon immobilization, but the specificity for alcohols was not altered.

Immobilization of ADH on synthetic supports

ADH was immobilized on nylon tube by Hornby et. al.[28] They used immobilized ADH for the continuous generation of NADH from NAD and observed 2-fold saving in the cost of coenzyme pyridine dinucleotide for analytical systems.

The preparation of various cross-linked acrylic copolymers in bead form, suitable for the immobilization of enzymes is described by Johansson and Mosbach.[29] ADH was either entrapped within poly(AAm) gel or covalently bound to poly(AAm) using glutaraldehyde. They also used copolymers of AAm-HEMA activated with CNBr and AAm-acrylic acid with water soluble carbodiimides. Attempts were made to optimize immobilization conditions for maximum retention of enzyme activity.

HLADH was treated with glutaraldehyde by Sodini et. al.[30] in which 40% of its 60 $-NH_2$ groups were modified under standard conditions. The cross-linked HLADH showed a specific activity three times higher than the native enzyme. The value of K_m for immobilized ADH was reported to be much higher than the free ADH.

A stable immobilized preparation of ADH was obtained by entrapment of ADH in poly(AAm) gel, polymerized using γ-rays (100 kR) by Godbole et. al.[31] The stability, pH activity profile and other properties of the entrapped ADH were compared with free ADH. The value of K_m for coenzyme NAD was observed to be four times higher than that for free enzyme, whereas for ethanol was not altered.

Ooshima et. al.[16] immobilized YADH ionically on anion exchanger Amberlite IRA-94 and into poly(AAm) beads by gel entrapment technique. They obtained maximum enzyme loading of 6.14 and 1.04 mg g^{-1} of wet carriers respectively.

Millis and Wingard[32] immobilized YADH in an albumin matrix cross-linked with glutaraldehyde. They observed that the enzyme half-life was almost doubled at pH 7.5 and 8.8 on immobilization. They also reported that the values of kinetic constants were increased with immobilization of enzyme for ethanol as well as for NAD.

YADH was covalently immobilized on nylon-polyethyleneimine microcapsules by Grunwald and Chang.[33] Efficient and stable recycling rates for ethanol oxidation were observed for 3 h continuous use of a column packed with immobilized YADH. They also observed that when column of microcapsules reused several times during a two weeks period retained 40% of its original activity.

Kover et. al.[34] immobilized HLADH by covalently binding it to glycidyl methacrylate and ethylene dimethacrylate copolymers treated with hexamethylenediamine using glutaraldehyde and carbodiimide activating reagents. HLADH immobilized on copolymer activated with glutaraldehyde showed 3.45 U active enzyme whereas copolymer activated with carbodiimide could show only 0.06 U of active enzyme g^{-1} of support.

Margolin et. al.[35] synthesized electrolyte complex by the reaction between poly(methacrylic acid) and poly(4-vinyl-N-ethylpipydinium bromide) for the covalent immobilization of HLADH using cyanuric chloride. The maximum activity of immobilized HLADH obtained was 24.5 U g^{-1} of support when they used 50 mM cyanuric chloride in 50% dioxane.

Danielson et. al.[36] used poly(chlorotrifluroethylene) particles for the immobilization of ADH and other dehydrogenases by adsorption which were further used in a packed column for the continuous detection of ethanol.

Novel support for the immobilization of YADH was prepared by Egerer et. al.[37] by esterifying poly(ethylene glycol) of molecular weight 800 with acrylic acid in the presence of toluene sulfonic acid and p-methoxy phenol, followed by reaction with isocyanato ethylmethacrylate in the presence of Desmorapid 50 to form a polymerizable resin. YADH was dissolved in this resin and the solution spotted in a mixture of silicon oil and paraffin oil in order to form entrapped enzyme beads.

Miyawaki et. al.[38] immobilized ADH in an ultrafilteration hollow fiber tube which was inserted in a fine nylon tube to form a hollow-fiber capillary reactor. They used it in a continuous bioreactor to detect ethanol.

YADH entrapped into poly(AAm) gel by Julliard et. al.[39] was reported to show 25% improvement in the storage stability after 28 days at 20 °C. The enzyme also retained its activity for 13 runs of 2 h.

YADH was adsorbed ionically onto porous anion exchangers Lewatit MP-64 of different particle sizes by Gerzina and Vasic-Racki.[40,41] They reported that by increasing the recirculation flow rate, the amount of protein attached to the small particles increased, but decreased on large particles. At the flow rate of 40 cm^3 min^{-1} maximum loading on the smallest support particles was obtained. During the study of the effect of glutaraldehyde cross-linking after adsorption thermal stability of the preparation was reported to be increased at 60 °C.

YADH was covalently bound to poly(AAm) gel activated with acryloyl chloride by Bille et. al.[42] They reported that the activation energy of the enzymatic reaction was significantly lowered upon immobilization. The thermal stability of the immobilized enzyme was however, greatly increased, even though it exhibited maximum activity at lower temperature. Mertens et. al.[43] entrapped HLADH into a poly(AAm) cross-linked with bis-AAm. They studied the influence of monomer

concentration, the degree of cross-linking and the polymerization technique on the extent of enzyme entrapment. They reported that a bead-polymerization process produced the most useful and stable immobilized enzyme preparation.

A new gas sensor for alcohol was fabricated by Ishizuka et. al.[44] by coimmobilizing ADH, NAD and an electron mediator in a conductive polymer. The sensor covered with a gas permeable membrane was used for alcohol detection in liquid as well as in gaseous phases.

YADH was immobilized on SH-silochrome by Voronina et. al.[45] They compared the catalytic properties and thermostablility of native and immobilized YADH. ADH was adsorbed on pyrroloquinoline quinone membrane by Ikeda et. al.[46] The immobilized ADH was used to develop electrodes from gold, silver and glassy carbon for the detection of alcohol.

Misra et. al.[47] immobilized ADH in a Langmuir-Blodgett film of stearic acid cross-linked with glutaraldehyde. They used it as an ethanol sensor on a conducting polypyrrole modified electrode. While, Goto[48] developed ethanol biosensor from ADH adsorbed on PVC.

HLADH was effectively immobilized by adsorption onto PVA and cross-linked poly(AAm) by Itozawa et. al.[27] They observed that the activity of HLADH decreased upon immobilization on PVA beads, but the specificity for various alcohols was not altered markedly.

ADH was immobilized in a redox polymer network of a polyvinylpyridine, partially N-complexed with osmium bis(bipyridine) chloride by Stigter et. al.[49] They reported that the electrode fabricated from immobilized enzyme had highest stability when the enzyme was immobilized at pH 10.0 and at 4 °C followed by

an additional cross-linking step with 1% glutaraldehyde. They also observed that the half-life time of the electrode was between 50 h and 200 h depending on the time the enzyme was in contact with the substrate. However, when the immobilized enzyme electrode was operated at temperatures above 37 °C the stability decreased considerably.

Immobilization of ADH on inorganic supports

Preparation of ADH attached to inorganic supports which are characterized by good mechanical property and higher stability in aqueous solutions was carried out by different workers. Coughlin et. al.[50] immobilized ADH onto less expensive inorganic support alumina using NAD coenzyme. Alcohol was added directly to the system while aldehyde leaves the system through an ultrafilteration membrane which prevents loss of the coenzyme.

Kelly et. al.[51] immobilized YADH on porous glass using different activating reagents such as p-nitrobenzyl chloride, steraroyl chloride, benzaldehyde and glutaraldehyde. YADH activated on glass through p-nitrobenzyl chloride had better results. They also observed that stabilization of YADH on glass activated with glutaraldehyde by further cross-linking with glutaraldehyde could not improve the enzyme stability.

Johnson[52] immobilized HLADH on Enzacryl-T1O. He studied the effect of bicarbonate and 2-mercaptoethanol on the stability of free and immobilized enzymes and found that immobilized enzyme had more protective effects than native enzyme. While Brougham and Johnson[53-55] immobilized YADH through covalent binding to various inorganic supports such as Enzacryl-10 and porous glass by using glutaraldehyde. The stability of the immobilized enzyme was observed to be greater in the pH range 6.5 to 8.0. However, the stability of immobilized enzyme was not improved by increasing the ionic strength of

storage solution as in case of free enzyme. They also immobilized YADH using silan-glutarldehyde technique using benzaldehyde. They observed that the pH and buffer concentrations used for immobilization have significant influence on enzyme coupling whereas the presence of 2-mercaptoethanol had no effect. They also studied the thermal stability of free and immobilized YADH bound to Enzyacryl-10 and hornblende and observed that free enzyme was more stable than any of the immobilized derivatives at 50 °C.

An inexpensive material carbon black was used by Yastrebova et. al.[56] for the immobilization of HLADH. Enzyme adsorption was observed only at pH < 8.7. With decreasing pH from 8.7 to 7.6 the affinity of ADH for carbon black increased. However, they obtained maximum adsorption at pH 7.8.

Mikelsone et. al.[57] immobilized HLADH by adsorption on silica gel and silica gel modified with cholesterol and bovine serum albumin. They reported that the maximum loading occurred within 3 to 5 h in phosphate buffer of 7.3. However, the affinity of enzyme to the gel decreased at and above pH 8.0.

Kovalenko et. al.[58,59] attempted to obtain stable and active immobilized YADH by selecting an optimal pore structure, easily available and inexpensive support alumina. To increase the stability of immobilized YADH it was further treated with condensing agents such as glutaraldehyde and water soluble carbodiimide. The maximum stability of YADH was observed when concentration of glutaraldehyde and carbodiimide was 0.1 and 2.5% respectively.

The potential of sand after alkylation as a support for immobilization of YADH was investigated by Brotherton et. al.[60] They compared the stabilities of free and covalently attached YADH to derivatized sand. Taya et. al.[61] immobilized ADH on a semiconducting TiO_2. They observed that immobilized enzyme had both the

activities, oxidation for NADH and reduction of NAD. They achieved the cyclic reaction with NAD regeneration by immobilized ADH.

The use of immobilized enzymes as selective adsorbents for the separation of organic compounds by HPLC is reported.[62] Recently, immobilized hydrolytic enzymes such as cellulase, protease and chymotrypsin were employed for the enantiomeric separation of various compounds.[63,64] HLADH covalently bound to glycerylpropyl-silica activated with tresyl chloride by Nilsson and Birnbaum[65] was reported to show 100% quantitative coupling and activity. They used immobilized ADH packed in a PTFE coated stainless steel column for the separation of various alcohols and ketones and for the chiral discrimination of optically active alcohols.

The enzyme electrodes made by using immobilized ADH were reported to be highly sensitive and specific for the detection of ethanol in biological fluids by Kanapieniene et. al.[66] However, they reported that the response time of the enzyme electrodes to substrate did not exceed 1 to 2 min. ADH was immobilized on the surface of glassy carbon by chemical cross-linking by Ji et. al.[67] The biosensor developed by using immobilized YADH was useful for the detection of alcohol at the concentration of 0.05 to 1.0 m mol L^{-1}. Amperometric biosensors for ethanol were also constructed by covalently binding YADH to carbon paste (graphite powder : paraffin oil) chemically modified with polyethyleneimine by Dominguez et. al.[68]

It is observed from the literature study that various types of supports have been used for the immobilization of ADH. The nature of the support and the techniques used for the immobilization play important role in enzyme activity. The present study has been aimed at immobilization of YADH by covalent coupling to chitosan and entrapment into poly(AAm-co-HEMA) copolymer. Optimization of the binding process and characterization of the immobilized

enzymes towards pH optima, various stabilities and kinetic behaviour were compared with free enzyme using alcohol as substrate.

Literature survey also reveals that oxidation of various primary alcohols to their respective aldehydes in continuous reactor is rarely investigated. Hence we have used immobilized YADH in a packed bed reactor for the oxidation of C_2 to C_5 alcohols.

3.4 Experimental

➢ Chemicals used

Yeast alcohol dehydrogenase (from Bakers Yeast of strength 450 U mg^{-1})	:	
Nicotinamide adenine dinucleotide (oxidized form)	:	**Sigma chemical Co. Ltd. USA**
Nicotinamide adenine dinucleotide (reduced form)	:	
Ethanol	:	**Alembic chemicals, Baroda, India**
Propanol	:	
Butanol	:	**S.D.Fine chemicals, Mumbai, India**
Pentanol	:	
Glycine	:	
Semicarbazide hydrochloride	:	**E.Merk, Mumbai, India**
Acetaldehyde	:	

3.4.1 Assay of YADH activity

The activity of the free and immobilized YADH, based on reduction of nicotinamide adenine dineucleotide (NAD^+) by ethanol in the presence of YADH

was determined spectroscopically by standard assay procedure of Colowick and Kaplan.[69]

$$C_2H_5OH + NAD^+ \underset{}{\overset{YADH}{\rightleftharpoons}} CH_3CHO + NADH + H^+$$

(oxidized) (reduced)

Reduced NAD (NADH) has an absorbance maxima at 340 nm, while NAD^+ has no absorption at this wavelength. Therefore all four reactants can be determined. The equilibrium lies to the left at pH 7.0. The reaction is completely displaced towards the right at alkaline pH. The amount of alcohol present can be measured by NADH formed.

The reaction mixture consists of free YADH or immobilized YADH, 0.2 mL of 200 mM ethanol and 0.1 mL of NAD^+ (6 mM). The reaction mixture is made upto 3.2 mL by adding semicarbazide-glycine buffer of pH 9.2 of the composition : 3 volume of 0.1 N NaOH, 7 volume of 0.1 N glycine in 0.1 N NaCl and 1 volume of 0.1 N semicarbazide in 0.1 N NaOH. The absorbance was measured at 340 nm after 30 min at room temperature (30 °C). The calibration plot was obtained by using the different concentrations of ethanol. The amount of aldehyde formed was measured from calibration plot.

One unit of YADH converts 1 μ mole of ethanol to acetaldehyde per minute at pH 8.8 at 27 °C.

3.4.2 Preparation of organic polymeric supports

The polymeric supports previously used for the immobilization of α-chymotrypsin were used for the immobilization of YADH. The details of the preparation of these supports are given in **section 2.3.1**. For the immobilization of YADH

copolymer of AAm-HEMA of mesh size 400 - 250 μ and chitosan beads of ~2.0 mm diameter were used.

3.4.3 Immobilization of YADH

Studies of the stability or activity of immobilized enzymes have shown that immobilization leads to deviation from their native form. Hence we have undertaken immobilization of YADH through covalent coupling (CB-YADH) and entrapment (ENT-YADH) on natural polymer chitosan and synthetic polymer poly(AAm-co-HEMA) to study their behaviour towards oxidation of ethanol.

(a) *Insitu* entrapment of YADH

To obtain maximum entrapped enzyme activity (EEA) for YADH, optimization of *insitu* entrapment was done by varying enzyme addition time and cross-linking agent bis-AAm as discussed in **sections 2.3.1** and **2.3.5**.

(b) Covalent coupling of YADH

Various coupling conditions were optimized for the maximum retention of enzyme activity and stability of immobilized YADH using 200 mg of semidry chitosan beads.

Effects of glutaraldehyde concentration (0.0 to 0.2%, v/v), cross-linking time (30 to 150 min), coupling medium (50 mM phosphate buffer of range 4 to 10), enzyme to carrier ratio (0.05 to 0.5) and coupling time (1 to 14 h) on the extent of enzyme immobilization and on the retention of enzyme activity were studied through appropriate experiments as described for α-chymotrypsin in **section 2.3.6**. The immobilized protein content was estimated by determining the protein from the supernant liquid by Lowrys' method.

3.4.4 Comparative account of free and immobilized YADH

Yeast alcohol dehydrogenase was immobilized by entrapping into the network of poly(AAm-co-HEMA) copolymer and was also covalently bound onto porous chitosan beads activated through glutaraldehyde. The results obtained were compared for optimum pH and thermal, storage and operational stability of entrapped and covalently bound YADH and free YADH. The immobilized enzymes were also characterized for reusability and Michaelis constants through Lineweaver-Burk plots.

(a) pH activity profile

As enzymes consist of protein, the catalytic activity is markedly affected by environmental conditions, especially the pH of aqueous medium. Thus, information on changes in pH-activity behaviour caused by immobilization of enzymes is useful for an understanding of the structure-function relationship of enzyme protein.

Hence the activity of the free and immobilized YADH was measured by incubating free and immobilized enzyme at 27 °C for 30 min in the 50 mM phosphate buffers of different pH ranging from 4 to 10 and using ethanol as a substrate. The absorbance of the reaction mixture was measured at 340 nm and correlated to the concentration of enzyme. From the calibration plot activity of enzyme was determined.

(b) Thermal stability

As a result of the immobilization of enzymes if the heat stability is enhanced, it is advantageous for the industrial application of immobilized enzymes, and is thus important in determining the feasibility of immobilized enzymes for a particular

application. Therefore, the thermal stability of free and immobilized enzymes was investigated.

Free and immobilized enzymes were placed in the optimum pH buffer and incubated at different temperatures (40 to 70 °C) for different time intervals. The activity of the enzyme was then determined as described earlier. 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 and 'A_t' is the activity after heat treatment for 't' minutes.

(c) Storage stability

The residual activities of the free and immobilized enzymes stored at room temperature (35 °C) were determined and the activities were expressed as percentage retention of their residual activities at different times.

(d) Reusability of immobilized YADH

The reusability of immobilized enzymes is one of the most important factors affecting the success of industrialization of an immobilized system. To evaluate reusability of the immobilized YADH it was washed with water and buffer after each use and then suspended again in a fresh reaction mixture to measure the enzymatic activity. This procedure was repeated for ten cycles. Reusability of immobilized YADH was examined by using ethanol as substrate. Leakage of the enzyme, if any was determined by measuring the enzyme activity in the washings.

(e) Determination of kinetic constants

The Michaelis constant (K_m) and maximum reaction velocity constant (V_{max}) for the free and immobilized YADH were determined by measuring the velocity of the reaction varying ethanol concentrations from 50 to 500 mM and varying NAD concentrations from 2 to 10 mM. Free and immobilized enzymes in optimum pH buffer were incubated with substrates for 30 min at 27 °C. From the activity of the enzymes, K_m and V_{max} were calculated using the Lineweaver-Burk plot of 1/s vs. 1/v.

(f) Effect of urea

It is known that chemical agents like urea, bicarbonate, mercaptoethanol etc. have denaturing effect on enzyme stability. Hence we have carried out study on the stability of free and immobilized YADH in the presence of 0 to 4 M urea concentration. Free and immobilized enzymes were incubated with varying concentrations of urea for 30 min at 30 °C.

3.4.5 Oxidation of C_2 to C_5 alcohols using packed bed reactor

Immobilized enzymes are used in various fields, such as chemical processes, analysis, medical treatment, food processing, chromatography, and so on. A continuous column system employing an immobilized enzyme is suitable in cases where the cost of the enzyme is high. In the column system the enzymatic reaction can be easily controlled and hence automation of the process is easy, and the running cost will be relatively low. By employing an immobilized enzyme, a product of higher purity can be obtained in higher yield.

Continuous oxidation of various primary alcohols such as ethanol, propanol, butanol and pentanol is rarely investigated. Hence we have carried out oxidation

of these alcohols under optimum conditions through column of 1.2 x 20 cm dimension containing 2 g of each of chitosan and poly(AAm-co-HEMA) immobilized YADH. The column was maintained at 27 °C and aliquots of 25 mL of various alcohols were passed through the reactor at 1 to 4 mL min^{-1} using peristaltic pump. Efficiency of the reactor containing covalently bound YADH was determined by operating the reactor continuously and measuring the absorbance at 340 nm at fixed time intervals, for different concentrations of alcohols (100 to 400 mM) for 100 min operation.

3.5 Results and Discussion

3.5.1 *Insitu* entrapment of YADH

Insitu entrapment of YADH was done in the copolymer of AAm and HEMA with 1:1(w/w) ratio and 2% cross-linker bis-AAm. To the polymerizing solution 10 mL of glycine buffer solution of pH 9.2 containing 900 U of YADH was added after 5 min of initiation time. Reaction was allowed to complete for 1 h. The copolymer containing entrapped YADH was washed with cold water followed by buffer solution and meshed to 400 - 250 μ. Dried copolymer containing entrapped enzyme was stored at 4 °C till further use.

Activity of entrapped YADH was measured as per the method reported using ethanol as substrate. Percentage activity was calculated from the total initial activity of YADH before polymerization and the total activity after entrapment. Approximately 90% enzyme activity was observed to be retained after entrapment.

3.5.2 Optimization of conditions for covalent coupling

Covalent coupling of YADH to chitosan was optimized by varying experiment conditions as described below.

- The effect of glutaraldehyde concentration on immobilization significantly influences the amount of coupled enzyme and hence its activity. **Fig. 3.1** shows that at 0.05% (v/v) glutaraldehyde concentration, the immobilized enzymes shows the highest activity of 4.5 U g^{-1} support. Further increase in glutaraldehyde concentration decreases the coupled enzyme activity as well as protein bound.

- The effect of cross-linking time was studied by varying cross-linking time for 30 min to 180 min. Results are given in **Fig. 3.2**. From the results it is observed that maximum 20% retention of activity is achieved when the cross-linking time was 90 min.

- The effect of pH of the coupling medium on the extent of immobilization of YADH was studied over the pH range 4 to 10 using 50 mM potassium phosphate buffer. It was observed from the **Fig. 3.3** that maximum 9.9 U g^{-1} protein was bound and 24% of its enzyme activity was retained between pH 7.0 and 8.0.

- The results obtained by varying ratio of enzyme to carrier are given in **Fig. 3.4**. When the ratio of enzyme/carrier was increased, the activity of the immobilized enzyme was observed to increase. However, at and above 1 : 5 ratio active enzyme bound remained almost constant (9.9 U g^{-1}), but the % retention activity decreased. It can be attributed to the limitation of the functional groups on the carrier available for enzyme immobilization.

- The effect of coupling time on the extent of immobilization of YADH was studied at various time intervals of 1 to 15 h. From the **Fig. 3.5**, it was observed that within 10 h time quantitative coupling of YADH (9.9 U g^{-1} of wet chitosan beads) takes place with 24% retention of enzyme activity.

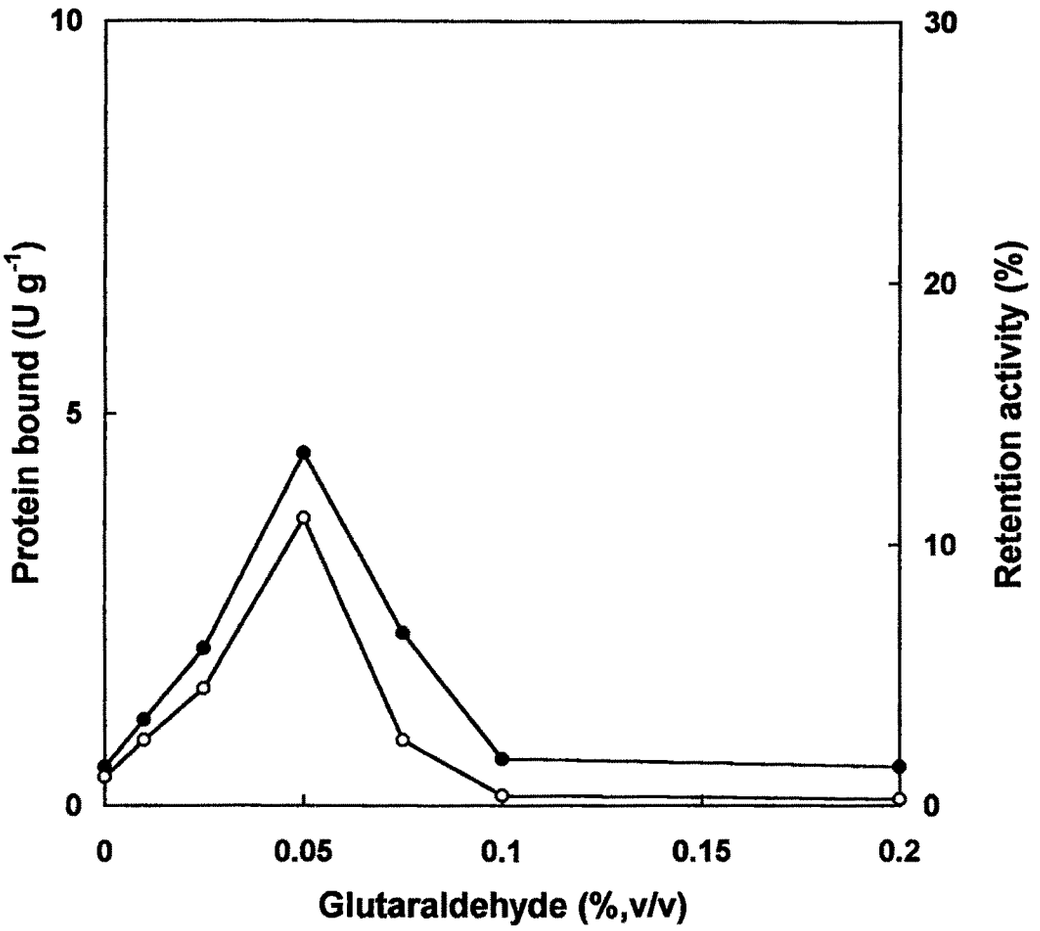

Fig. 3.1 Effect of glutaraldehyde concentration on immobilization of YADH :

Support 200 mg, Cross-linking time 120 mins,
Enzyme concentration 45 U, pH 7, Coupling time 14 h

Protein bound (●), % Retention activity (O)

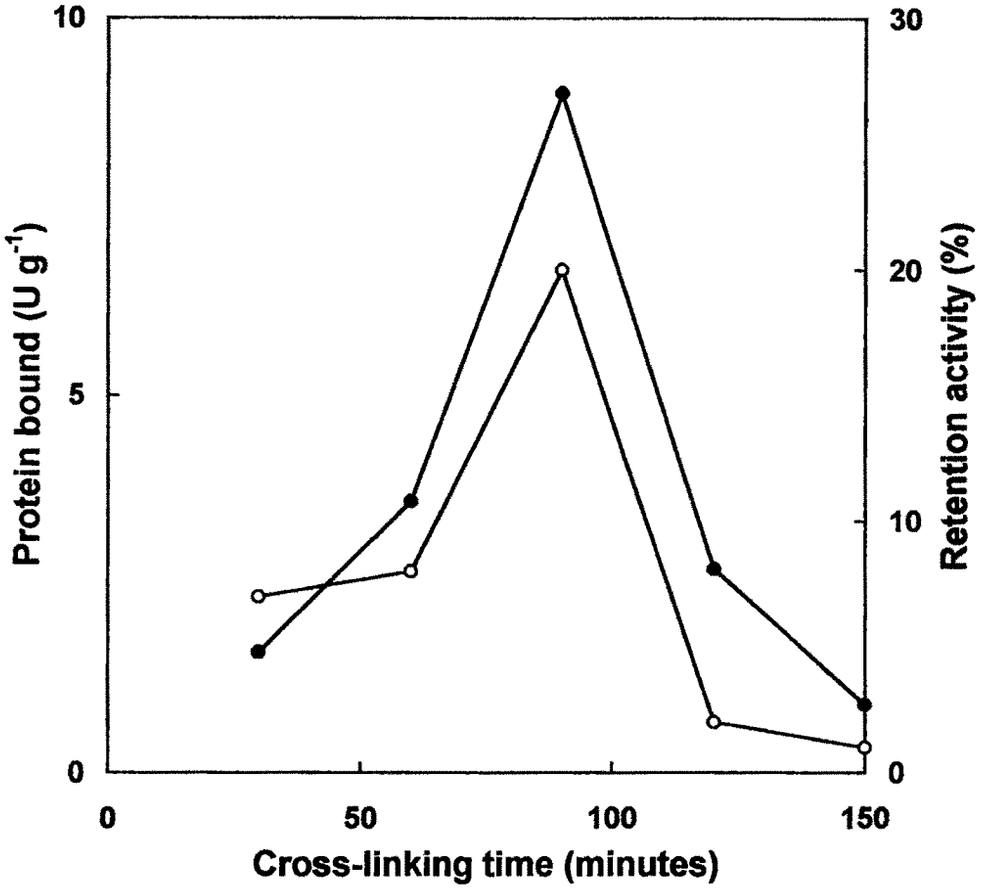

Fig. 3.2 Effect of cross-linking time on immobilization of YADH :

Support 200 mg, Glutaraldehyde conentration 0.05%, Enzyme concentration 45 U, pH 7, Coupling time 14 h

Protein bound (●), % Retention activity (O)

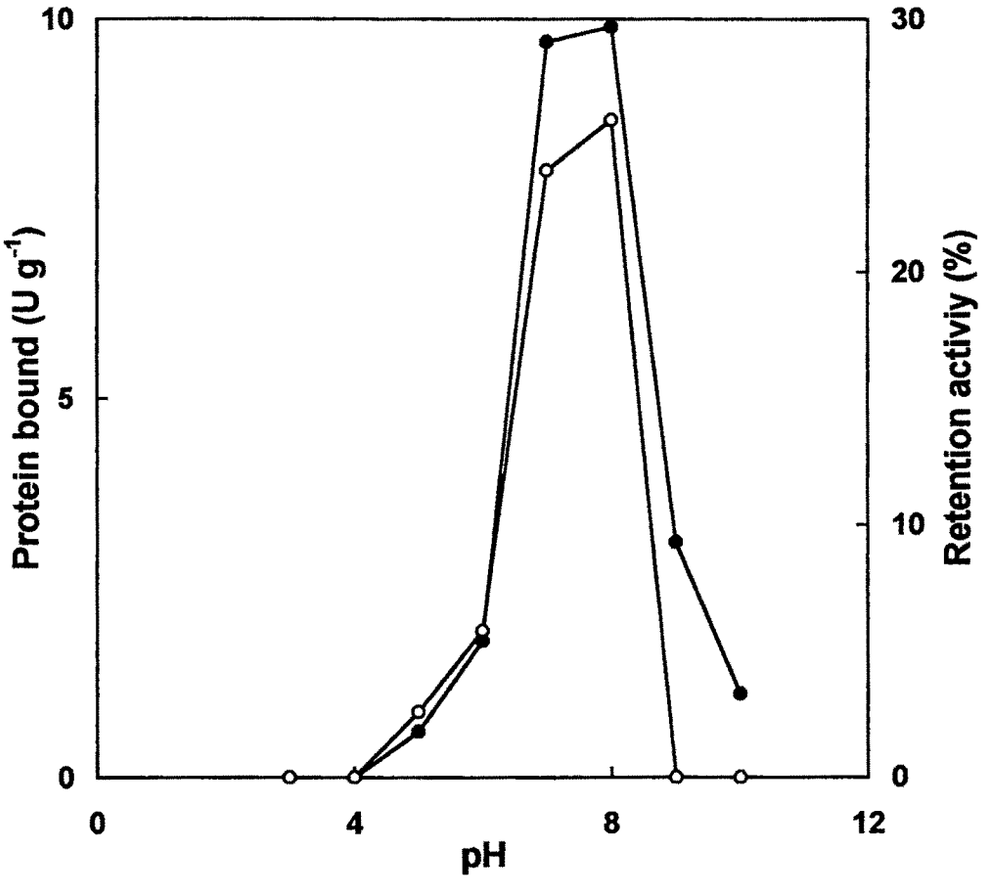

Fig. 3.3 Effect of pH of the medium on immobilization of YADH :

Support 200 mg, Glutaraldehyde concentration 0.05%, Cross-linking time 90 min, Enzyme concentration 45 U, Coupling time 14 h

Protein bound (●), % Retention activity (O)

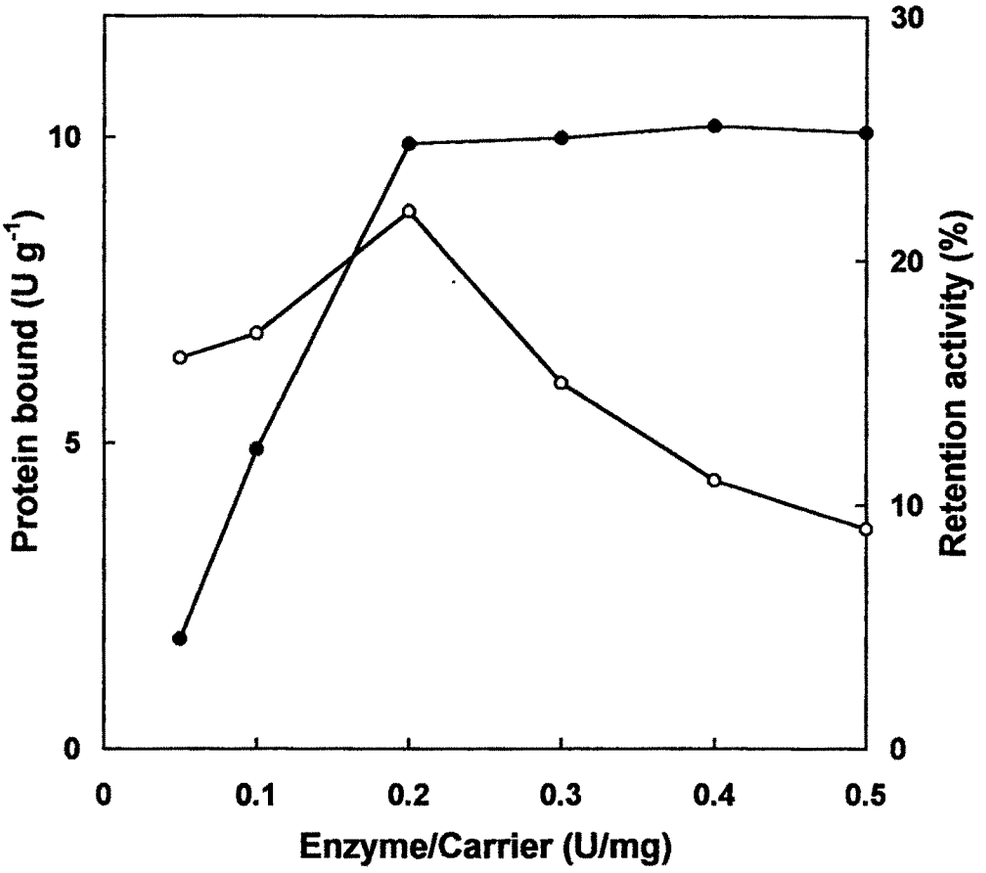

Fig. 3.4 Effect of enzyme/carrier ratio on immobilization of YADH :

Glutaraldehyde concentration 0.05%,
Cross-linking time 90 min, pH 8, Coupling time 14 h

Protein bound (●), % Retention activity (O)

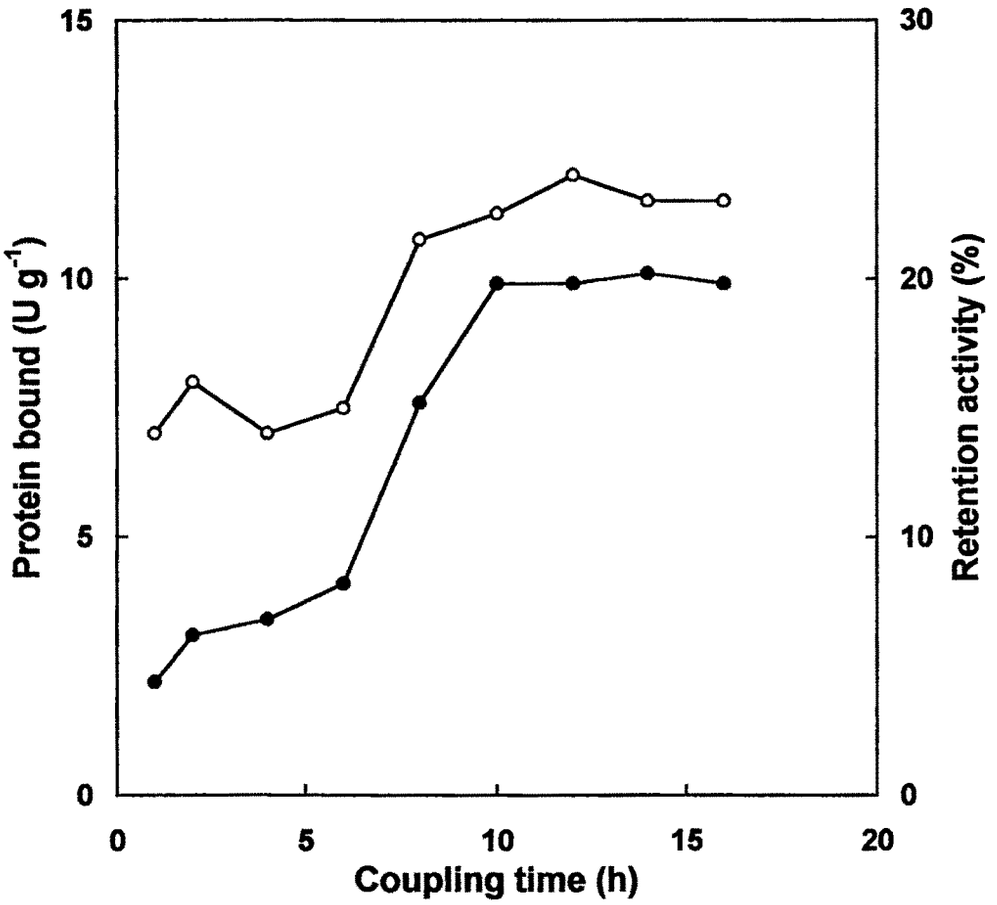

Fig. 3.5 Effect of coupling time on immobilization of YADH :

Glutaraldehyde concentration 0.05%,
Cross-linking time 90 min, pH 8, Enzyme/Carrier ratio 0.2

Protein bound (●), % Retention activity (O)

Thus, quantitative coupling of YADH is achieved at 0.05% concentration of glutaraldehyde in 50 mM phosphate buffer of pH 7 - 8 at 4 °C for 10 h coupling time. Though the protein coupled to the support was observed to be 9.9 U g^{-1} of wet polymer only 24% of the enzyme activity was retained. Optimized conditions for covalently immobilized YADH onto porous chitosan beads activated with glutarldehyde are summarized in **Table 3.1**.

3.5.3 Comparative account of free and immobilized YADH

(a) pH activity profile

Fig. 3.6 illustrates the effect of immobilization on the optimum pH for the enzyme activity. The free enzyme shows maximum activity at pH 9, whereas CB-YADH enzyme shows it at pH 8, indicating that polymer matrix behaves as a polycation. When an enzyme is bound to polycation carrier, positive charge on the enzyme increases and the pH of the immobilized enzyme region becomes more alkaline than that of the external solution. Accordingly the enzyme reaction effectively proceeds on the alkaline side of the external buffer pH, and the optimum pH apparently shifts to the acidic side. However, ENT-YADH shows optimum pH between 8 - 9.

(b) Thermal stability

Knowledge of thermal stability of immobilized enzyme is very useful in the investigation of potential applications of enzymes. **Fig. 3.7** shows the comparison of relative activity for free and immobilized YADH at 70 °C. It was observed that immobilized enzyme has higher thermal stability than free enzyme at all intervals of time. Free enzyme loses its 90% activity whereas ENT-YADH retained 30% and CB-YADH retained 40% activity over 30 min incubation at 70 °C. From the study the thermodeactivation constants (K_d) calculated as

Table - 3.1

Optimized conditions for covalently immobilized YADH on chitosan

Substrate	Ethanol
Glutaraldehyde concentration (mg g^{-1})	20
Cross-linking time (min)	90
Coupling pH	7.0
Coupling time (h)	10
Active enzyme bound (U g^{-1})	9.9
Retention of activity (%)	24

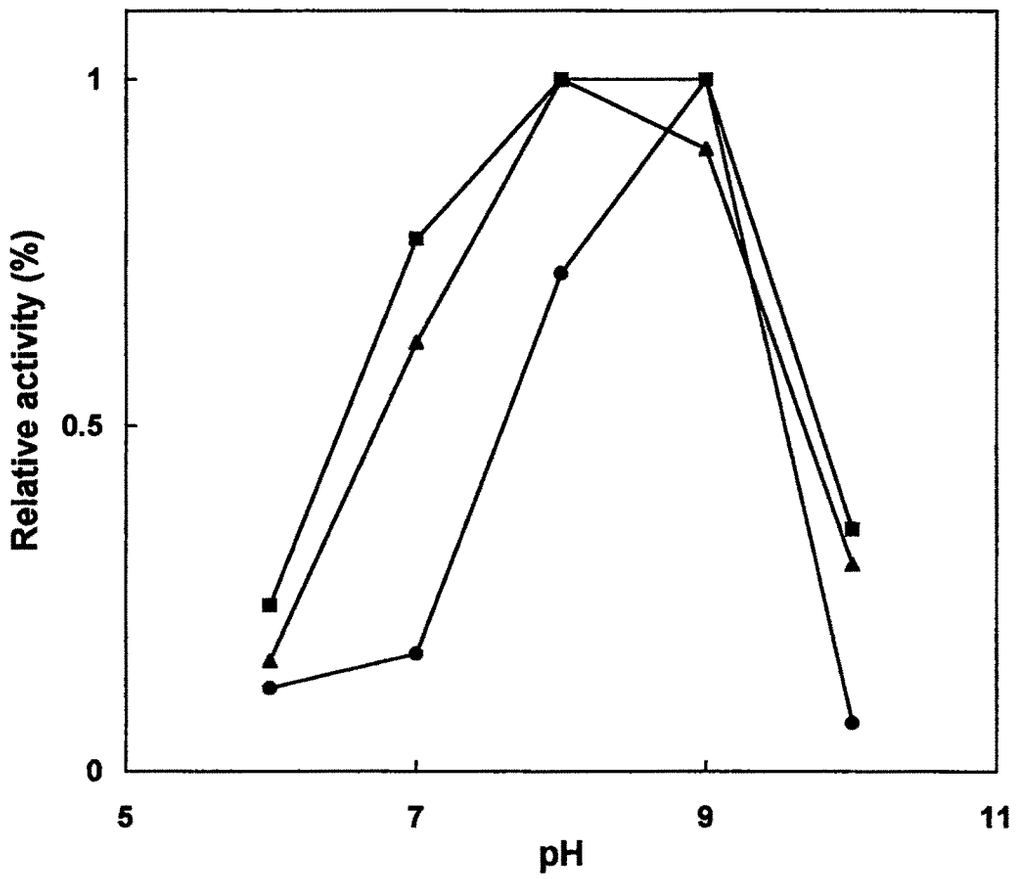

Fig. 3.6 pH activity profile at 27 °C using ethanol as substrate

Free YADH (●), CB-YADH (▲) and ENT-YADH (■)

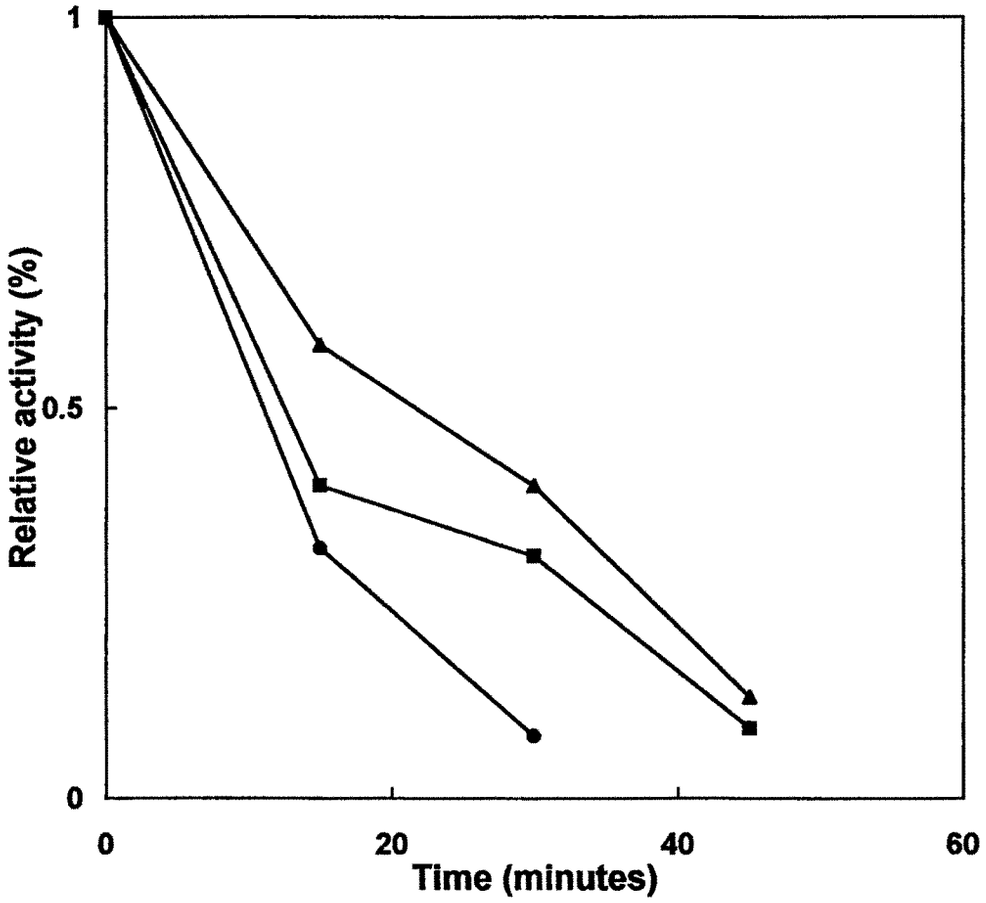

Fig. 3.7 Thermal deactivation of enzyme at 70 °C

Free YADH (●), CB-YADH (▲) and ENT-YADH (■)

discussed earlier are given in **Table 3.2**. From the data it is observed that rate of deactivation increases with temperature for both free and immobilized YADH. However, it can be seen that rate of deactivation is higher for the ENT-YADH system in comparison with CB-YADH indicating strong bond formation between the chitosan and YADH.

(c) Storage stability

Destabilization is considered to be caused by autolysis or microbial growth on the enzyme. Immobilization reduces autolysis and/or prevents microbial growth. The storage stability of free and immobilized YADH has been investigated and results are given in **Fig. 3.8**. At room temperature (35 °C) free enzyme loses its activity completely after 5 days whereas ENT-YADH and CB-YADH retain 50% of their activity after 60 and 30 days respectively. The stabilization on immobilization is attributed to multipoint attachment of the enzyme to the support and/or it's role as semipermeable membrane creating more rigid enzyme molecule as stated by Glassmayer and Ogle.[70] Hence disruption of the active centre becomes less likely to occur. Similar type of results were also observed for YADH immobilized on cyanogen bromide activated sepharose system by Li et. al.[71] However, Millis and Wingard[32] observed retention of only 10% activity of YADH immobilized in albumin matrix cross-linked with glutaraldehyde on storage at pH 8.8 at 30 °C after 2 days storage.

(d) Reusability

Free enzymes suffer from a major drawback of nonreusability. This is an advantage for immobilized enzymes. The activity of the immobilized systems after successive uses is given in **Fig. 3.9**. ENT-YADH and CB-YADH retained 50% of its initial activity after five and eight cycles respectively. However, ENT-YADH loses its activity after nine cycles completely, whereas CB-YADH

Table - 3.2

Effect of temperature on the deactivation of YADH

Temperature (°C)	Deactivation rate constant ($K_d \times 10^{-2}$)		
	Free YADH	CB-YADH	ENT-YADH
40	0.08	0.05	0.05
50	0.31	0.22	0.24
60	1.46	1.11	1.39
70	7.65	3.70	5.20

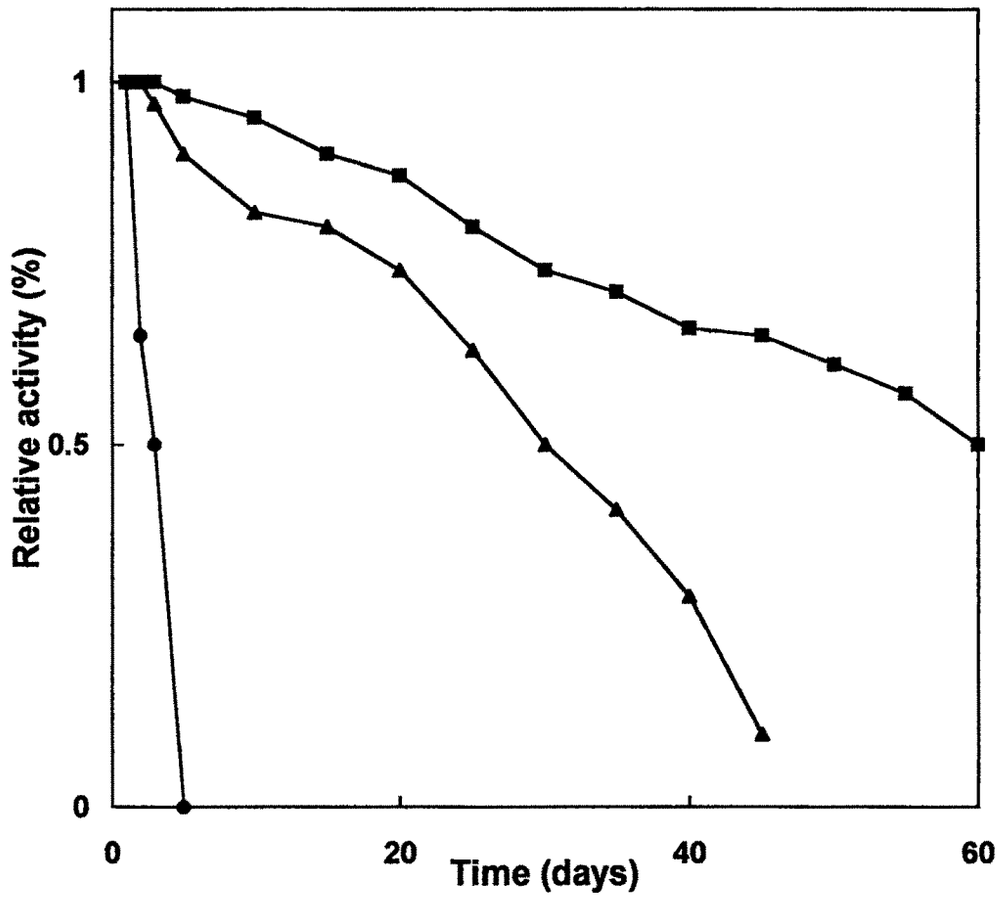

Fig. 3.8 Storage stability of enzymes at room temperature (35 °C)

Free YADH (●), CB-YADH (▲) and ENT-YADH (■)

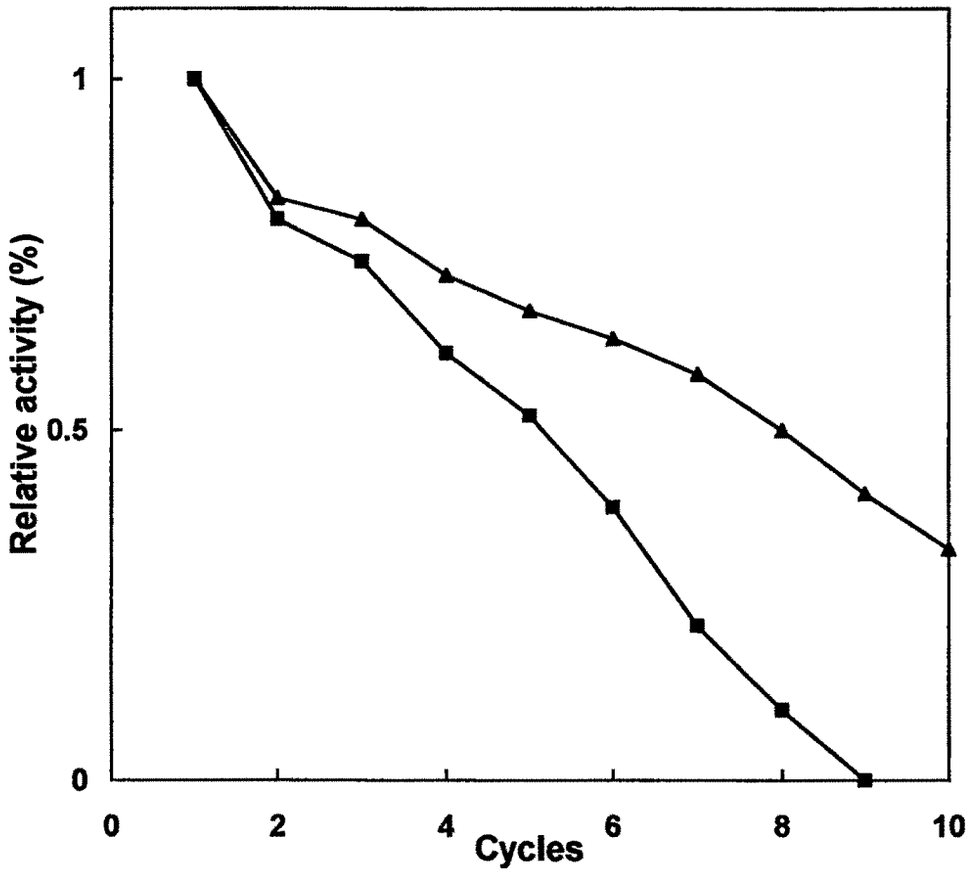

Fig. 3.9 Reusability of immobilized enzymes at 27 °C and pH 9.2

CB-YADH (▲) and ENT-YADH (■)

retains 30% of its original activity after ten cycles for ethanol oxidation. Higher reusability of covalently bound YADH further confirms strong chemical bond formation between enzyme and support.

(e) Determination of kinetic constant

The effect of substrate concentration on the reaction rate catalyzed by free and immobilized YADH was studied using ethanol substrate. The Lineweaver-Burk plots were used for calculation of the Michaelis constant (K_m) and maximum reaction velocity (V_{max}) of the free and immobilized YADH, which are presented in **Table 3.3**. The values of K_m and V_{max} of the free YADH were found to be 8.3×10^{-2} mM and 9.1 mM min^{-1} respectively.

When YADH immobilized by covalent binding onto chitosan and entrapped into poly(AAm-co-HEMA), K_m values were observed to decrease about 1.2 and 5.2 fold respectively. The lower values of K_m for immobilized enzyme may be due to strong electrostatic attractions between the polymeric support and the protein chains as well as protein substrate interaction. V_{max} values of CB-YADH and ENT-YADH were 1 7 and 2.5 fold smaller, than that of the free YADH. This may be due to the greater rigidity of the gel which limits the substrate diffusion in the matrix toward enzyme reaction sites, and due to a possible deactivation of the enzyme which has been occurring during the gel formation.

Similarly the values for K_m and V_{max} of the free and immobilized enzymes were calculated by keeping the ethanol concentration constant (200 mM) and varying concentration of the coenzyme NAD. The results are given in **Table 3.4**. Very little variation in the K_m and V_{max} values was observed on variation of NAD or ethanol concentrations.

Table - 3.3

Kinetic parameters* for free and immobilized YADH

	Free YADH	CB-YADH	ENT-YADH
K_m (mM)	8.3×10^{-2}	6.7×10^{-2}	1.6×10^{-2}
V_{max} (mM min^{-1})	9.1	5.3	3.6

* using fixed 6 mM NAD concentration, at 30 °C, pH 9.2 for 30 minutes

Table - 3.4

Kinetic parameters** for free and immobilized YADH

	Free YADH	CB-YADH	ENT-YADH
K_m (mM)	7.1×10^{-2}	3.7×10^{-2}	2.2×10^{-2}
V_{max} (mM min^{-1})	8.2	4.9	4.6

** using fixed 200 mM Ethanol concentration, at 30 °C, pH 9.2 for 30 minutes

(e) Effect of urea

The effect of a denaturing agent urea on the free and immobilized YADH was examined. The YADH activity in the presence of different concentrations of urea is shown in **Table 3.5**. The denaturation caused by urea was found to increase with increase in strength of urea for both free and immobilized YADH. However, about 25% and 10% of the activity was retained in the immobilized preparations in the presence of 4 M urea, while free YADH deactivates completely. The enhanced stability of the immobilized YADH to urea may presumably due to its stabilization by binding to the support at multiple points.

3.5.4 Oxidation of C_2 to C_5 alcohols using packed bed reactor

Operational stability of covalently bound YADH was determined by operating the reactor continuously for 100 min using 25 mL of aliquots of different alcohols like ethanol, propanol, butanol and pentanol at constant temperature 27 °C.

The percentage conversion for the oxidation of alcohols of different strength 100 to 400 mM as a function of flow velocities (1 to 4 mL min^{-1}) are presented in **Figs. 3.10** to **3.13**.

From the results it is observed that as the flow rate increase from 1 mL min^{-1} to 4 mL min^{-1} percentage conversion decreases at all concentrations of alcohols. It is also observed that as the number of carbon atoms in alcohol increase the percentage conversion decreases. This is because YADH is very specific enzyme, which can accept only a hydrogen atom or a methyl group of the substrate. Therefore it is less active as the chain length of corresponding alcohol increases.[72] These results are promising for the application of the immobilized YADH in the oxidation processes.

Table - 3.5

Effect of Urea on YADH activity

Urea concentration (M)	Retention of activity (%)		
	Free YADH	CB-YADH	ENT-YADH
0	100	100	100
1	44	71	73
2	17	51	38
3	5	39	17
4	0	25	10

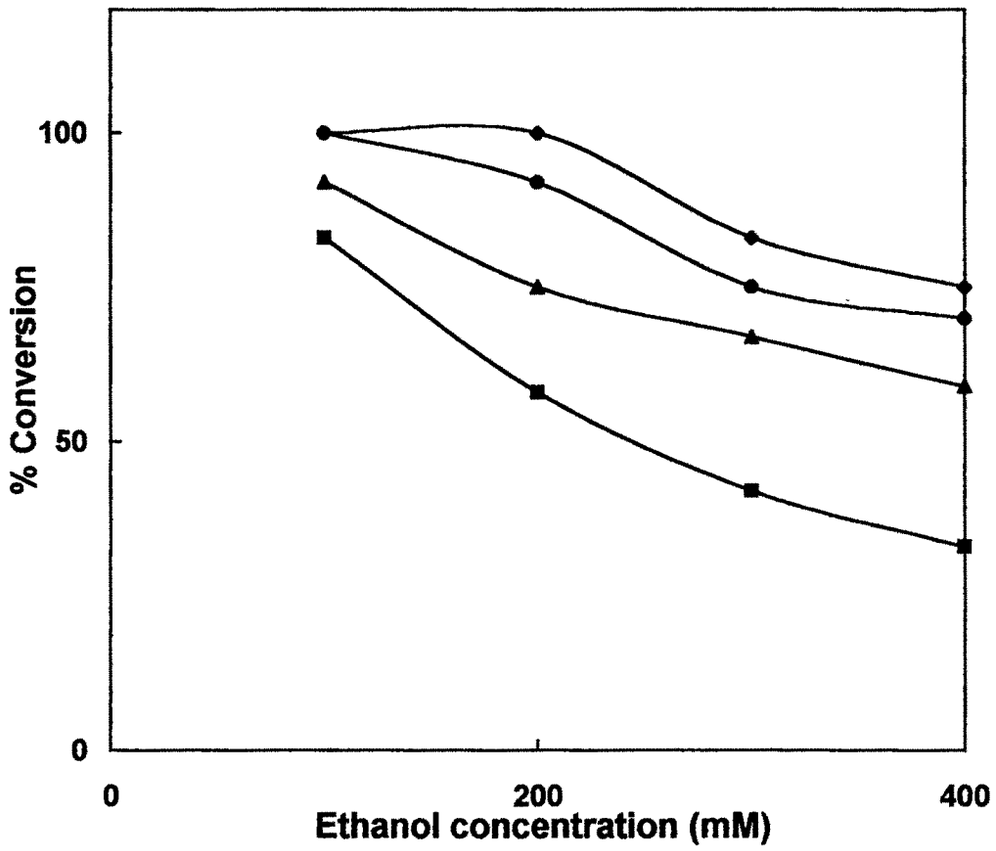

Fig. 3.10 Efficiency of column in oxidation of ethanol at 27 °C, pH 9.2 for 100 minutes

1 mL min^{-1} (◆), 2 mL min^{-1} (●), 3 mL min^{-1} (▲), 4 mL min^{-1} (■)

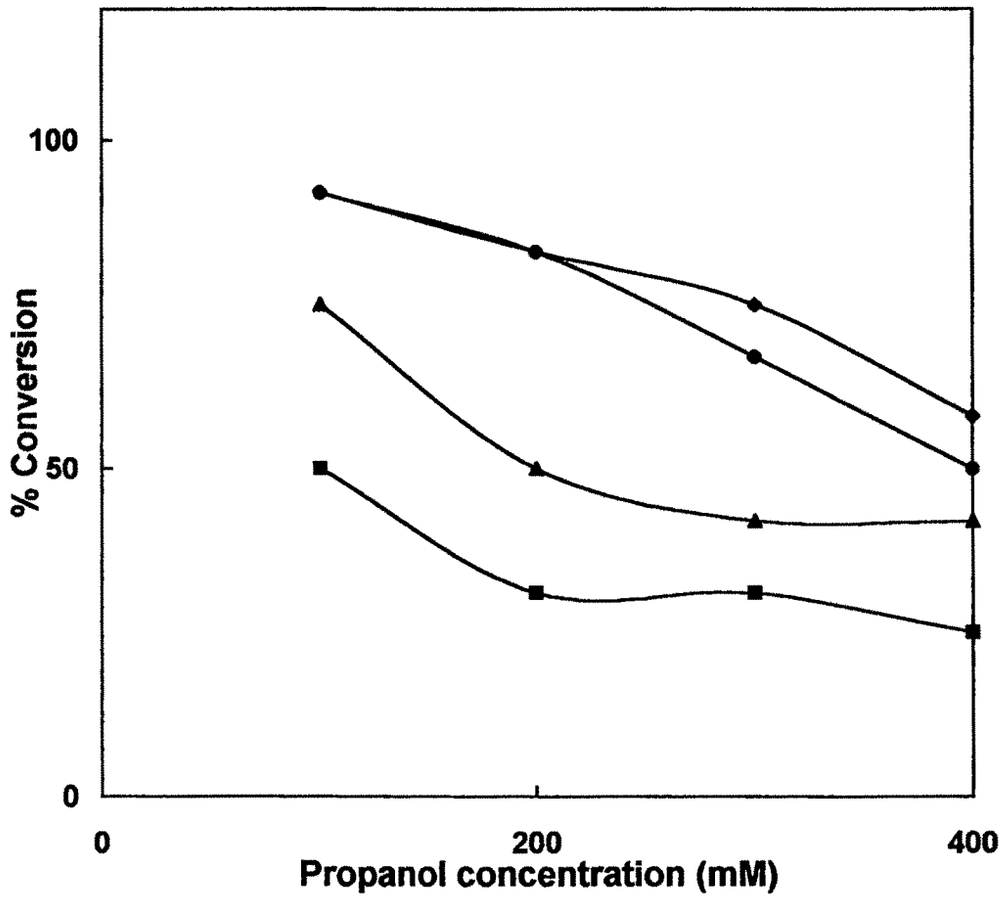

Fig. 3.11 Efficiency of column in oxidation of propanol at 27 °C, pH 9.2 for 100 minutes

1 mL min^{-1} (◆), 2 mL min^{-1} (●), 3 mL min^{-1} (▲), 4 mL min^{-1} (■)

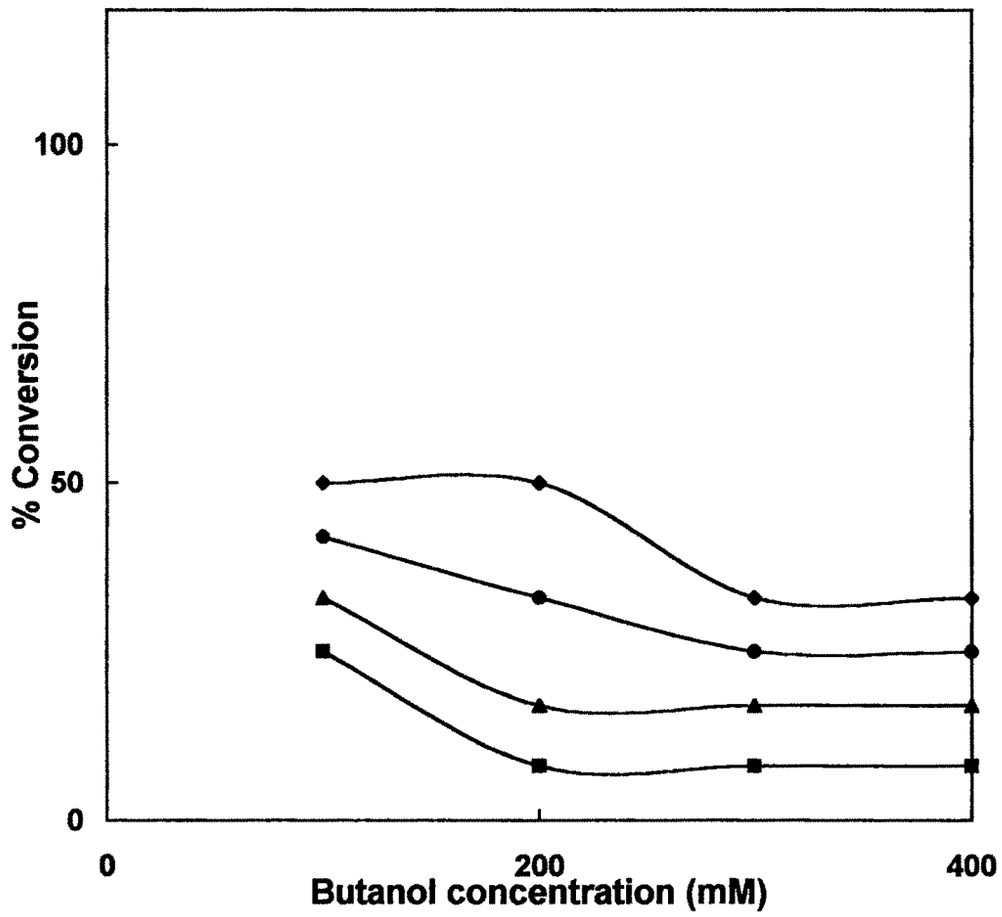

Fig. 3.12 Efficiency of column in oxidation of butanol at 27 °C, pH 9.2 for 100 minutes

1 mL min^{-1} (◆), 2 mL min^{-1} (●), 3 mL min^{-1} (▲), 4 mL min^{-1} (■)

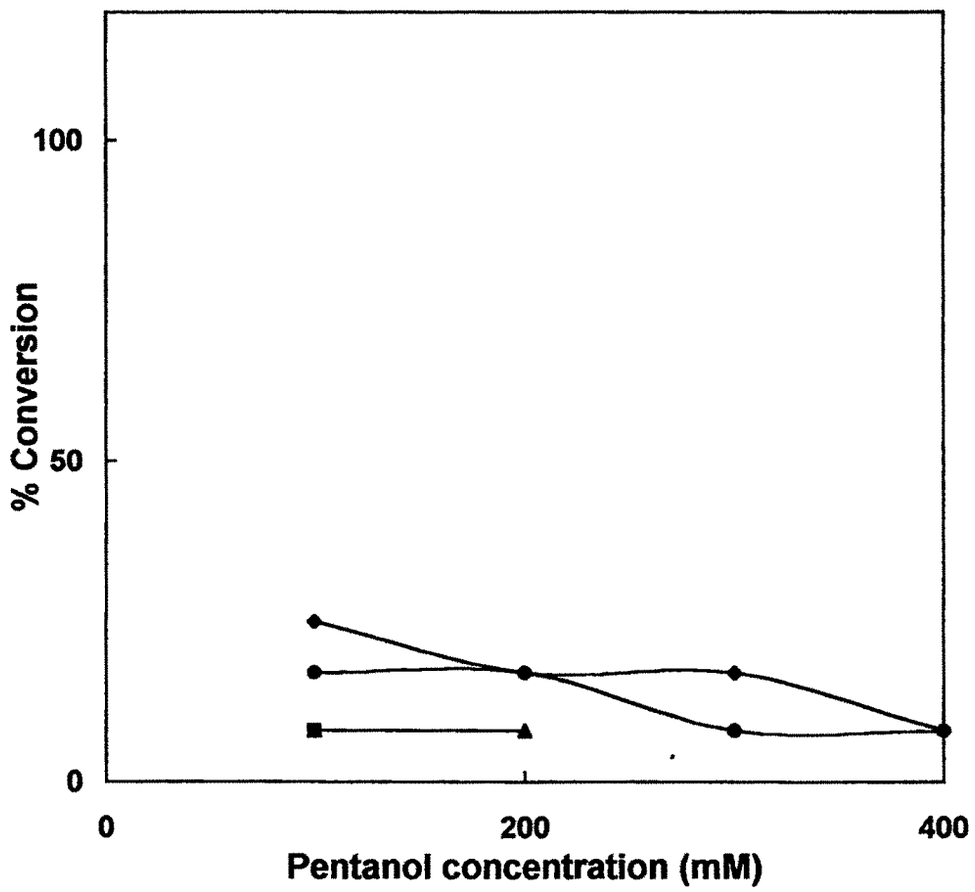

Fig. 3.13 Efficiency of column in oxidation of pentanol at 27 °C, pH 9.2 for 100 minutes

1 mL min^{-1} (◆), 2 mL min^{-1} (●), 3 mL min^{-1} (▲), 4 mL min^{-1} (■)

Similar results were obtained **(Table 3.6)** when ENT-YADH was used in the reactor. However, the values obtained are smaller than that of CB-YADH. This can be attributed to the difference in the nature and surface area of the polymeric supports used for the immobilization. This confirms that there is a strong bond formation between chitosan and YADH than entrapped YADH into poly(AAm-co-HEMA) in which the leakage of enzyme is likely to take place.

3.6 Summary and Conclusions

1. YADH which catalyzes oxidoreductions for a broad spectrum of primary alcohols was immobilized by entrapping into the network of poly(AAm-co-HEMA) copolymer and was also covalently bound onto porous chitosan beads activated through glutaraldehyde.

2. Maximum retention of YADH activity was 90% and 24% for entrapment and covalent binding technique respectively. Thermal stability of entrapped and covalently bound YADH was high and thermal deactivation constants were smaller when compared with free YADH. Fifty percent activity of free enzymes was retained for 3 days at 35 °C whereas CB-YADH and ENT-YADH could retain 50% activity upto 60 and 30 days at 35 °C storage. CB-YADH and ENT-YADH retained 50% activity after 8 and 6 cycles. Enzyme catalyzed oxidation of ethanol was observed to be diffusion controlled through Linweaver-Burk plots.

3. Immobilized YADH was successfully used in packed bed reactor for continuous oxidation of primary alcohols like ethanol, propanol, butanol and pentanol.

Table - 3.6

Effect of concentration and flow rate on the oxidation of ethanol using ENT-YADH

Concentration (mM)	% Conversion Flow rate (mL min^{-1})			
	1	2	3	4
100	100	92	50	25
200	90	83	42	17
300	83	70	33	11
400	75	58	23	8

4. Entrapped enzyme activity was observed to critically depend on enzyme addition time and concentration of cross-linking agent. While covalent attachment of enzyme was observed to depend on concentration of bi-functional reagent and also on enzyme/carrier ratio.

Thus, coupling of YADH to natural and synthetic organic polymeric supports resulted in an increase in the stability of enzyme enhancing their potential for continuous production of aldehydes from their respective primary alcohols at industrial scale. The various properties of free YADH and immobilized YADH are summarized in **Table 3.7**.

Table - 3.7

Comparison of properties of free and immobilized YADH

Properties	Free enzyme	Covalent binding	*Insitu* entrapment
Optimum pH	9.0	8	8 - 9
Optimum temperature (°C)	30	30	30
Storage stability at RT (days)	2	30	60
Turn over number (cycles)	-	11	9
Thermodeactivation constant (K_d) at 70 °C	0.077	0.037	0.045
Michaeli's constant, K_m (mM)	8.33×10^{-2}	6.66×10^{-2}	1.57×10^{-2}
Maximum velocity, V_m (mM min^{-1})	9.09	5.26	3.58
Effect of 4 M urea (%)	0	25	10

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