Chapter 1

General Introduction

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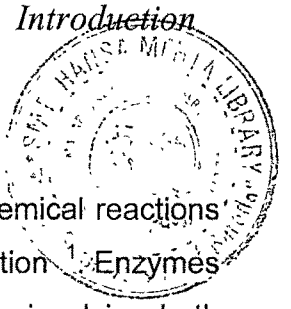


1.1 Enzymes

Enzymes are biological catalysts that increase the rate of chemical reactions taking place within living cells by lowering the energy of activation [1]. Enzymes catalyze most biological reactions in vivo [2,3] and reactions involving both natural and synthetic substrates in vitro [4,5,6,7] . The word enzyme was coined by Friedrich Wilhelm Kunhe to designate these biological catalysts in 1878.

An activated enzyme can transform a substrate selectively at an impressive rate and be deactivated until needed again . For many years the size , complexity and instability of enzyme molecules precluded an accurate understanding of their composition and structure. It is now well known that enzymes are proteins with molecular weight ranging from 10,000 to 5,00,000 .

Enzyme has traveled a long distance from the simple use as a catalyst in the cell to their use in reactions taking place in aqueous medium and organic medium since last two decades. More over it also finds use in pharmaceuticals [8,9] , agrochemicals and detergents [10,11] , biodesulphurisation [12,13] and also in wastewater treatment [14,15] .Thus enzyme has entered in every phase of human life with the passage of time.

1.1.1 Classification of Enzymes :

Enzymes have been classified into six major groups by the International Union of Biochemistry [16] according to the types of reactions they catalyze.

(i) Oxidoreductase :

These enzymes catalyze redox reactions resulting in oxygenation or overall removal or addition of hydrogen atom equivalent. They act on aldehyde and ketone groups. The enzymes of this class include horseradish peroxidase , alcohol dehydrogenase , cytochrome oxidase, aldehyde oxidase , tryptophan

oxygenase , D-lactase dehydrogenase, Dihydrofolate reductase , difluromethylene phosphonate.

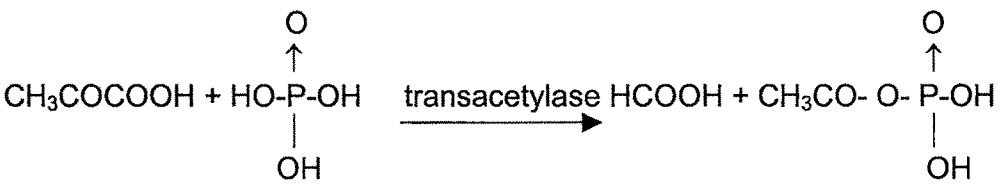
Alcohol dehydrogenase brings about the dehydrogenation of alcohol to aldehyde by transfer of two hydrogen atoms to the coenzyme .

$$CH_3CH_2OH + Co\text{-}I \xrightarrow{\text{Alcohol Dehydrogenase}} CH_3CHO + H_2Co\text{-}I$$

(ii) Transferase :

Those enzymes which catalyze the transfer of a group other than hydrogen between a pair of substrate are included in this class. i.e the enzyme catalyses the transfer of one carbon group , aldehydic or ketonic residue or acyl , glycosyl , alkyl , phosphorus or sulphur containing groups. Fructosyl-transferase , acyl transferase, hexokinase , transaminase , transacetylases are included in this class.

A transacetylase catalyses the transfer of an acetyl group from pyruvic acid to inorganic phosphate



(iii) Hydrolase :

The hydrolysis of ester , ether , peptide , glycoside , halide, thioether and acid anhydride groups is carried out by this class of enzymes by adding water and then breaking the bond. This class includes lipase , trypsin, pepsin ,β-galactosidase, chymotrypsin , urease , guanine deaminase , acetyl cholineesterate .

Urease catalyses the hydrolysis of urea to carbon dioxide and ammonia.

$H_2NCONH_2 + H_2O \xrightarrow{\text{Urease}} CO_2 + 2NH_3$

(iv) Lyases :

Lyases enzyme cleaves bonds without the addition of water , but by elimination reaction to form double bonds or rings. They break bonds like carbon-carbon , carbon-oxygen , carbon-nitrogen , carbon-sulphur and carbon-halide . Aspartase , Fumarase , Aldolase , Histidase , dehydratases are included in this class.

Fumarase catalyzes the splitting of malic acid into fumaric acid and water .

$HOOCCH_2CHOHCOOH \xrightarrow{\text{Fumarase}} CHCOOH + H_2O$

(v) Isomerase :

These catalyze interconversion of optical, geometrical or positional isomers by intramolecular rearrangement of atoms or groups. This class includes glucose –isomerase , alanine racemase, glucosephosphate isomerase .

L-Alanine $\xrightarrow{\text{Alanine racemase}}$ D-Alanine

(vi) Ligases :

These enzyme take part in formation of bonds like C-O , C-S, C-N, C-C . Ligases are also known as synthetases , they couple the hydrolysis of a pyrophosphate in ATP or other nucleoside triphosphate to a second reaction in which tow molecules are joined . Glutathione-synthetase , acetyl –co-A synthetase, glutamine synthetase are included in this class.

ATP + L-glutamine + NH_3 $\xrightarrow[\text{synthetase}]{\text{glutamine}}$ ADP + orthophosphate + L-glutamine

1.2 Immobilization :

1.2.1 What is enzyme immobilization ?

Enzyme immobilization is defined as physical confinement or localization of enzyme in certain defined region of space with retention of its catalytic activity in such a way that it can be used repeatedly and continuously [17]. The first report by Nelson and Griffin of enzyme immobilization appeared in 1916 where invertase was adsorbed onto aluminium hydroxide which retained its catalytic activity[18] .

1.2.2 Why to Immobilize ?

Free enzymes has certain drawbacks such as high cost and frequent deactivation under the desired reaction conditions. Moreover all enzymes are soluble in aqueous medium , which makes it more difficult and expensive to recover the enzyme once the process is over. Hence to overcome all these disadvantages and to obtain the enzyme in the form which can work more effectively, it needs to be immobilized. However, there are certain advantages and disadvantages of immobilization and they are listed below.

Advantages	***Disadvantages***
Immobilization increases the stability of enzymes.	The cost of immobilization is added to enzyme cost.
Immobilized enzyme offers an advantage of control of reaction.	During immobilization the activity of the enzyme may be lost.
The cost of enzyme reduces and the separation of the product becomes easy after immobilization	Great effort needs to be taken to select a suitable support for immobilization.

The activity of the immobilized enzyme increases and can be used in continuous operations.	Moreover , diffusional limitation of substrate is also observed.
Multistep reactions can be carried out without contamination of the product.	The catalyst volume will be increased due to immobilization.

1.2.3 Methods of Immobilization

Method of immobilization plays an important role during immobilization of enzymes. Such a method must be selected which causes minimum loss of enzyme activity during immobilization. For this , one should have sufficient knowledge of the active sites of the enzyme.

The other factor on which the method of immobilization depends is the support used for the immobilization. The support used for the immobilization of enzyme must possess certain characteristics such as mechanical strength , resistance against microorganisms , thermal stability , chemical stability , chemical functionality , low cost , hydrophobicity , regenerability and high immobilizing capacity of enzyme.

The immobilization of enzyme can be divided into two major classes .

- Immobilization through Physical Methods
- Immobilization through Chemical methods.

1.2.3.a. Physical Methods :

The physical methods of immobilization are further divided into three classes depending on the way of interaction of the enzyme with the support .

Adsorption :

Adsorption is one of the simplest method of enzyme immobilization. In this method the support is mixed with a solution of the enzyme where enzyme is allowed to get adsorbed on the support . The possible interaction between enzyme and supports are Vanderwaals interation and ionic-interaction . Commonly used adsorbents are alumina, activated charcoal, diatomaceous earth and glass . Several ion exchange materials have also been used to immobilize enzymes by ionic interactions. Adsorption has certain advantages and disadvantages which must be taken into consideration.

Immobilization of an enzyme by adsorption is simple , economical and less disruptive to the enzyme protein. Moreover the major advantage of immobilizing the enzyme by adsorption is that no activation step or reagent is required. However, leakage of the enzyme may occur , especially if the conditions such as pH, ionic strength etc in the reaction mixture are drastically different from those used during adsorption.

Ionic Bonding:

Immobilization via ionic binding is based, mainly on ionic binding of the enzyme molecule to solid supports containing ion-exchange residues. The binding of an enzyme to the carrier is easily carried out, and the conditions are much milder than those needed for the covalent binding method. Hence, the ionic binding method causes little changes in the conformation and the active site of the enzyme.

The most common ion-exchangers used are DEAE-derivatives [19],TEAE derivatives [20] as anion and CM-Derivatives [21] as cation exchangers.

The conditions of ionic binding are mild on the other hand ionic binding being a surface phenomena leakage of the enzyme is observed

Entrapment:

Entrapment means confining enzymes within the lattices of polymerized gels. This allows the free diffusion of low molecular weight substance and reaction products. The usual method is to polymerize the hydrophilic matrix in an aqueous solution of the enzyme and break up the polymeric mass to desired particle size. There is no bond formation between the enzymes and the polymer matrix . Entrapment provides a method that, in theory, involves no disruption of the protein molecules. However, free radicals generated during the course of polymerization may affect the activity of entrapped enzymes. Entrapment can be carried out in gels, or by microencapsulation . In case of entrapment in gels, enzyme can be immobilized by polymerizing a suitable monomer in presence of enzyme where enzyme gets entrapped in three-dimensional network of the polymer for entrapment . Polyacrylamide is the most commonly used polymer for entrapment . Different polymers such as polyethyleneglycol dimethacrylate [22],polyurethane [23] have also been used for this purpose.

Microencapsulation of enzyme can be carried out by using a semipermeable polymer membrane . The membrane may be prepared from nylon , cellulose nitrate or 1,6-diaminohexane .

Entrapment provides large surface area for the contact of substrate and enzyme in relatively small volume and can be used for simultaneous immobilization of many enzymes. In addition there is lesser possibility of conformational change during immobilization. However, occasional inactivation of enzyme , diffusional limitation for substrate with high molecular weight and leakage of enzyme cannot be over ruled.

1.2.3.b. Chemical Method

Covalent coupling

The most widely studied method of the immobilization is covalent coupling of enzyme to the support. However, care must be taken so that the method of coupling does not lead to loss of enzymatic activity and active site of the enzyme remains unaffected by the reagents used.

Many methods of covalent coupling of enzymes to solid materials have been developed. Enzymes are proteins and the amino side chain contains several functional groups suitable for coupling reactions .In most methods , amino groups of lysine residues are the principal reactive groups. However, in many cases coupling occurs also with sulfhydryl groups of cysteines ,phenolic hydroxyl groups of tyrosines , carboxyl groups of aspartic and glutamic acid [24].

Many solid materials have been used as supports in enzyme immobilization. The main requirement for the support material is that the material must have suitable properties for its use in the reactor and is stable under operational conditions, both chemically and mechanically. Further support should contain functional groups suitable for binding the enzyme. Many natural supports such as agarose, dextran, starch , cellulose [25,26,27] and synthetic supports such as polyacrylates, glass etc are used.

Covalent Coupling takes place in two steps ;

- Activation of the support through functional groups using bi or multifunctional reagents.
- Attachment of an enzyme molecule through their binding site to the activated polymer.

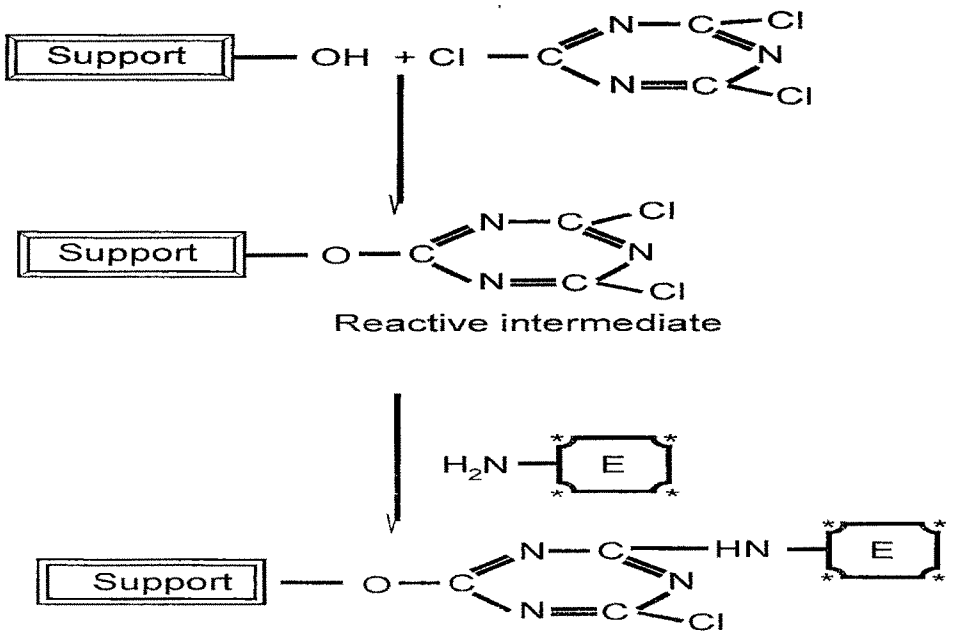
Different bi or multi functional activating agents are used depending on the type of functional groups present on the support.

Functional Group	***Activating agent***
-OH	p-Benzoquinone , cyanogen halide , cynuric chloride, p-tolyl sulphonyl chloride.
$-NH_2$	Glutaraldehyde
-COOH	N-ethyl 5-phenyl isoxazolium-3'-sulphnate, N-ethoxycarbonyl-2-ethoxy-1-2-dihydroquinone, Thionyl chloride, carbodiimide.

Activation of Hydroxyl Group :

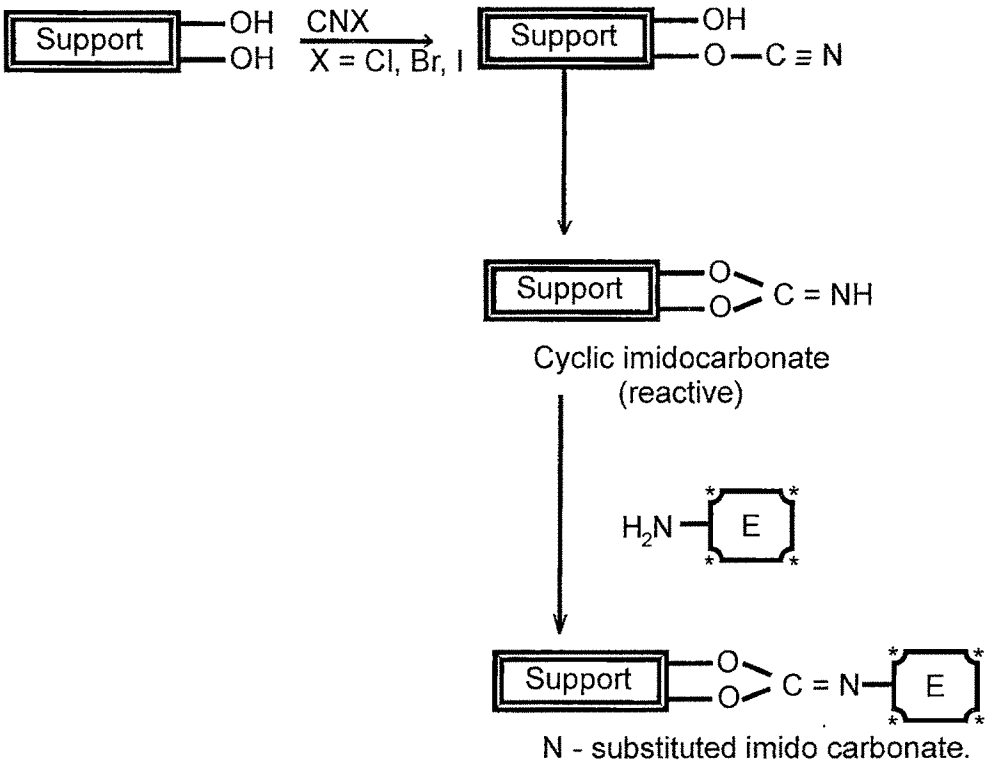
Activation by cynuric chloride :

In this case trichlorotriazine reacts with the hydroxyl groups of support and forms a reactive intermediate which is further coupled with NH_2 group of the enzyme resulting in immobilized enzyme system. This method has been used for immobilization of enzymes on various types of celluloses through the activation of the polysaccharides by cyanuric chloride or its dichloro derivatives[28].



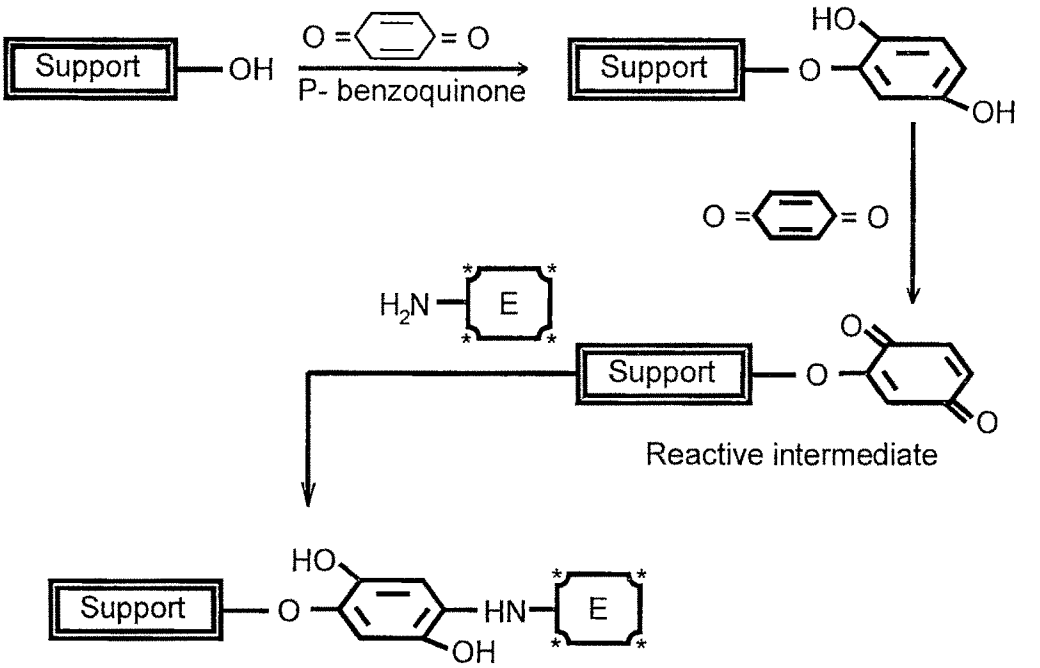
Activation with cynogen halide:

In this method the support is activated with cynogen halides to give intermediate cyano derivative which couples with enzyme to give immobilized enzyme system. Axen used this method for immoblization of chymotrypsin , trypsin and papain in 1967 [29].



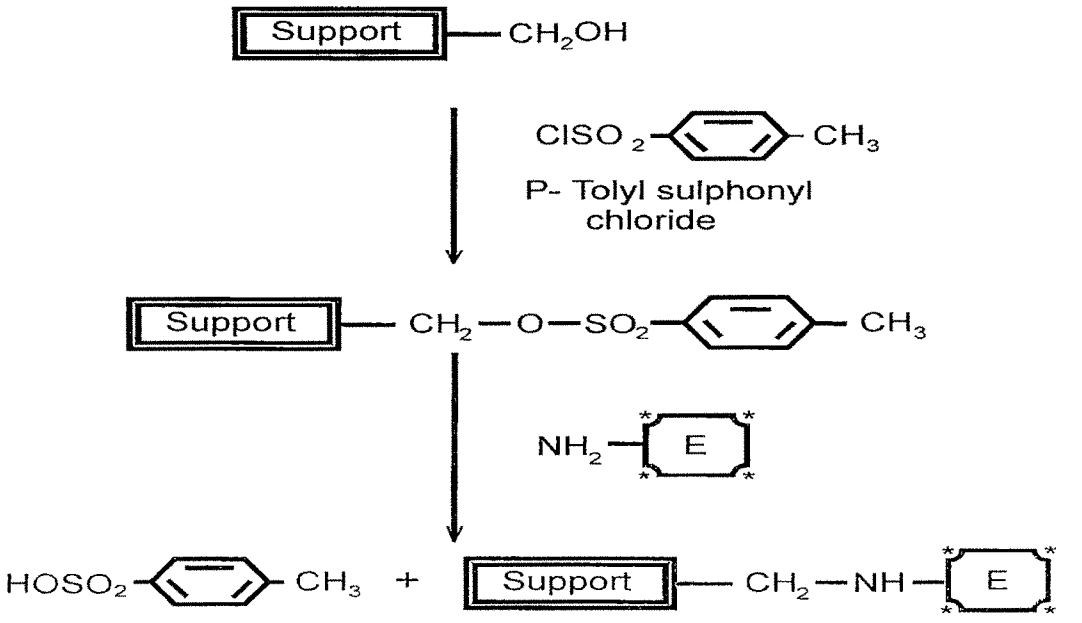
Activation by p-Benzoquinone :

In this method the hydroxyl groups of support are activated with p-benzooquinone. Reactive intermediate further couples with enzyme giving immobilized enzyme system. Brandt et al.[30] used this method in 1975 for immobilization of enzyme on polysaccharide like sepharose and sephadex.



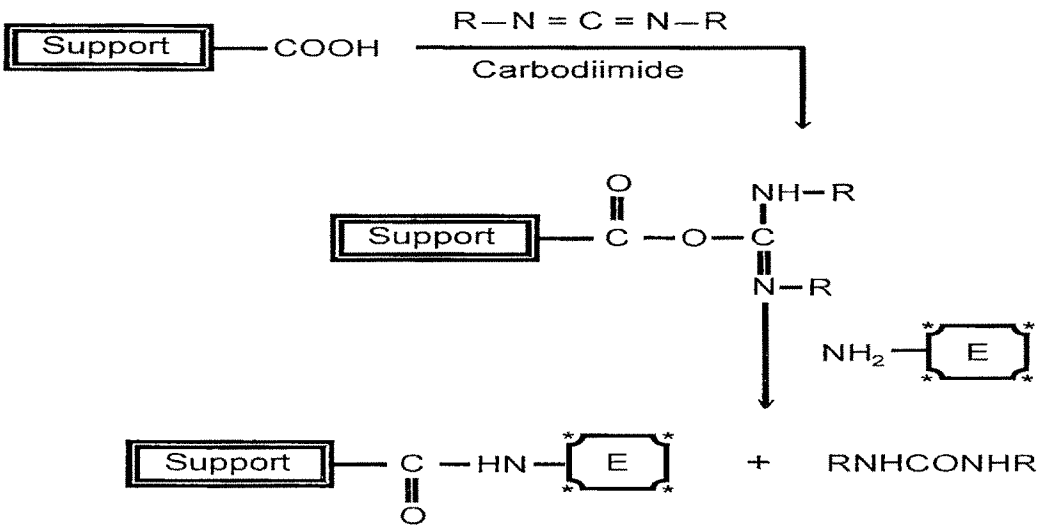
Activation by p-tolyl sulphonyl chloride

This method was first used by Nilsson in 1981[31] for activation of the hydroxy groups of agarose. This resulted in the formation of p-tolyl sulphonic ester in which tosyl group has excellent leaving properties in reaction with nucleophiles. They used this activation procedure to immobilize trypsin, peroxidase and alcohol dehydrogenase.



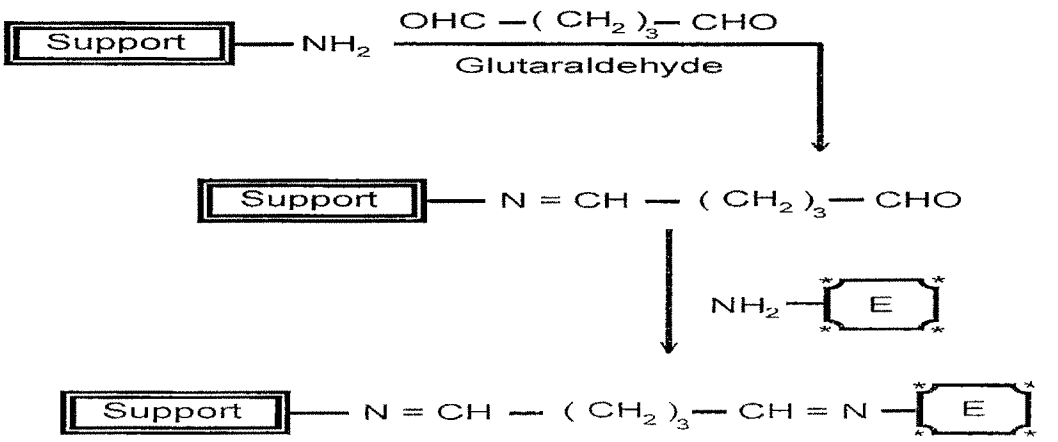
Activation of Carboxyl Group :

Many activating agents are used for activation of COOH groups of the support such as N-ethyl 5-phenyl isoxazolium –3'-sulphonate[32] N-ethoxy carbonyl –2-ethoxy-1-2-dihydroquinone[33], thionyl chloride[34], and carbodiimdes [29] .The reaction of carbodiimdes and carboxyl groups is given below.



Activation of Polymer Having amino Group:

Glutaraldehyde has been successfully used for activation of supports containing NH_2 group . The most commonly used support is chitosan . The immobilization takes place by formation of Schiff base between enzyme and support [35-40] .



Crosslinking :

Immobilization of enzymes by crosslinking has been achieved by intermolecular cross-linking of the protein, either to other protein or to functional groups on an insoluble support matrix. Cross-linking an enzyme to itself is both expensive and ineffecient, as some of the protein material will inevitably be acting mainly as a support , resulting in relatively low enzymatic activity . Generally, cross lining is best used in conjunction with one of the other methods.

By crosslinking the enzyme molecule large aggregates are formed that eventually become so large that they are no longer soluble .In this way , the enzyme functions both as a catalyst and as the support material. Glutaraldehyde , tannic acid , dimethyl adipimidate , diazobenzidine are some of the crosslinking agents .

1.3. Properties of immobilized enzyme:

It is important to understand the changes in physical and chemical properties which an enzyme would be expected to undergo upon solubilization if the best use is to be made of the various insolubilization techniques available. Changes have been observed in the pH , stability , kinetic properties because of the microenvironment imposed upon them by the supporting matrix and by the product of their own action.

1.3.1. Effect of pH.

As enzymes consist of proteins the catalytic activity is markedly affected by environmental conditions especially pH of aqueous medium. Thus, information on change in pH –activity behavior caused by immobilization of enzymes is useful for an understanding of the structure function relationship of enzyme proteins. Cases are known of change and no change in the

optimum pH of an enzymatic reaction and in the pH –activity profile upon immobilization

The change in optimum pH and nature of pH activity profile depends on the charge on the enzyme protein and / or on the water –insoluble carrier. There are many cases showing shifts in optimum pH but not showing any change in pH activity curve on immobilization, i.e , a parallel shift of the pH activity curve occurs without changing the pH –activity profile.

Tosa et al. have reported shift in the optimum pH of aminoacylase immobilized by ionic binding with DEAE-cellulose or DEAE-sephadex by 0.5 pH units to the acid side compared to the native enzyme[41-42]. Similar shifts in optimum pH was observed for invertase[43] and ATPdeaminase[44] immobilzed by ionic binding with DEAE cellulose, and NADH dehydrogenase[45] immobilized by crosslinking using glutaraldehyde.

These shifts towards the acid side are explained as follows. When an enzyme is bound to a polycationic carrier, positive charge on the enzyme protein increases and the pH of the region in viscinity of immobilized enzyme 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 towards acidic side. On the other hand , the optimum pH's of trypsin , chymotrypsin[46] immobilized by peptide binding with CM-cellulose azide shifted 0.5 pH unit to the alkaline side may be because of an increase in negative charge due to the carboxyl groups of CM-cellulose as the microenvironment of the immobilized enzyme becomes more acidic than that of the external solution.

Some immobilized enzymes show no shift in the optimum pH but do exhibit changes in pH activity profile . For example in β-fructofuranosidase [47] , immobilized by diazo binding with polyamino polystyrene and asparaginase [48] microencapsulated in nylon or polyurea the optimum pH did not shift but the pH –activity profile showed narrower pH region.

1.3.2. Temperature Dependence of enzyme activity :

The catalytic activity of enzyme is dependent on temperature and at temperatures above certain limit enzyme activity is lost due to the denaturation of the enzyme protein. Therefore, temperature dependence of enzyme activity studies are important for their use.

The optimum temperature of immobilized enzyme was reported to be 5 – 15 °C higher than that of native enzymes in case of trypsin [46] and chymotrypsin [48] immobilized on CM-cellulose azide . However , asparaginase microencapsulated in nylon or polymer membrane showed decrease in optimum temperature on immobilization and glucose isomerase [49] and leucine aminopeptidase [50] immobilized by diazo coupling to a porous glass did not show any change in optimum temperature . These observations show how diverse obsevations are achieved in immobilization pertaining to the type of enzyme used and method and support used for immobilization.

1.3.3.Effect of Kinetic Parameters :

The conformational changes of the enzyme protein may occur on immobilization and the affinity between enzyme and substrate may be changed . Hence the investigation of kinetic constants for free and immobilized enzymes is very important.

The variation in these values are reported in literature. The K_m value for ficin [51] immobilization by peptide binding with CM-cellulose azide was reported to be 1/10 th of that of the native enzyme, when benzoyl-L-arginine ethyl ester (BAEE) was used as substrate. This was reported to be due to the increase in electrostatic attractive forces between the polyanionic carrier and positively charged substrate. While in the case of chymotrypsin immobilized on CNBr-activated sephadex , K_m for acetly-L-tyrosine ethyl ester (ATEE) was reported to be 10 times higher than that for the native enzyme [52].

1.3.4.Storage Stability :

The stability of enzymes might be expected to either increase or decrease on insolubilization, depending on whether the carrier provides a microenvironment capable of denaturing the enzymatic protein or of stabilizing it. As a result of the immobilization of enzymes increase in stability has been observed in many cases. The enhancement in stability on immobilization is advantageous for the industrial applications of the enzyme and it is thus important in determining the feasibility of immobilized enzyme for a particular application.

An increase in storage stability on immobilization of enzyme was observed with lactate dehydrogenase immobilized on porous glass[53] using glutaraldehyde and trypsin [54] immobilization on amino acid containing copolymer.Goldstein et al. [55] have reported 50 % retention of enzyme activity on immobilization of naringinase on styrene – maleic anhydride copolymer after one week at 4°C whereas native enzyme did not show any loss in activity.

1.4.Applications of immobilized enzymes:

Immobilized enzymes are used in various chemical processes, analysis, medical treatment, food processing and affinity chromatography.Enzyme reactors used in these applications are classified by the mode of operations and the flow characteristics of substrate and product. Following types of reactors are commonly used .

Stirred tank reactor :

Stirred tank reactor is the simplest of all the types of reactors. It contains the reactor and a stirrer. This type of reactor is useful for substrate solution of high viscosity and for immobilized enzymes having low activity.

Packed bed reactor :

Packed bed reactors are most widely used reactors for immobilized enzymes . The advantage of this type of reactor is that it is easy to operate , reduces the labor cost , stabilizes the operating conditions and facilitates easy control of products.

Fluidized bed reactor :

This type of reactor is used when substrate solution of high viscosity and a gaseous substrate or product are used in continuous reaction system . While using this reactor care must be taken to avoid destruction and decomposition of immobilized enzymes.

1.5. Proposed Work :

In the present work we have proposed to study immobilization of Horseradish peroxidase , Pepsin and β-galactosidase on natural and synthetic supports such as K-carrageenan and copolymer of Acrylamide-2-hydroxyethyl methaacrylate (AAm-HEMA) respectively and examine their potential for specific applications.

The main objectives of the work are:

- Optimization of various immobilization conditions for entrapment of these enzymes on K - carrageenan such as type and concentration of hardening agent and enzyme loading.
- Optimization of various covalent coupling conditions for copolymer of AAm-HEMA for immobilization of Horseradish peroxidase , Pepsin , β-galactosidase such as concentration of activating agent , activation time , enzyme concentration , enzyme coupling time , effect of enzyme coupling pH and enzyme loading.
- Comparison of properties of free and immobilized enzymes such as pH , thermal stability , storage stability and reusability .
- Determination of Kinetic parameters for free and immobilized enzymes.
- Application of both entrapped and covalently bound enzyme in batch , fixed bed reactor and fluidized bed reactor.

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