CHAPTER - 1

GENERAL INTRODUCTION

CONTENTS

1.1 Enzymes

Nature is extremely diverse in terms of the large number and many types of organic molecules required for life. Enzymes are organic biocatalyst which govern, initiate and control biological reactions important for life processes. They are produced by living cells but can act independently of the cell if appropriate environmental conditions are created. Enzymes are large, three-dimensional protein molecules with an active site at a defined location on the folded surface. This part of the surface can be envisioned as a pocket that will permit entry only to a specific substrate for a reaction to occur. Their molecular weights range from about 10,000 to more than 10,00,000.

Specially in enzymes the active site is known to represent only a small portion of the large protein molecule called enzyme.[1] The active site may lie at or near the surface, but it may also be buried in an active site groove or crevice that limits access of all but the desired substrate. Clearly, the total surface area of the protein is significantly larger than that of the active site. In many enzymatic systems, the metal also plays an important role at the active site.

The temporary bonds between the enzyme and substrate that form the enzyme-substrate complex will loosen the bonds that hold the substrate together. Thus, the energy barrier for cleaving is lowered and the reaction can proceed and reach equilibrium at room temperature. Products are formed from the substrate and the enzyme is liberated again ready to catalyze the next reaction.

1.1.1 Sources of enzymes

Enzymes are isolated from microbial fermentation's and animal and plant by-products. Microbial enzymes from bacteria, fungi and yeast's are by far the most common and account for about 80% of the total industrial enzyme production.[2] Enzymes from animals are highly dependent on the availability of by-products from livestock slaughter.[3] Similarly, plant enzymes are obtained as by-products

from the fruit and vegetable industries. Often the animal and plant enzymes are available only as unpurified powders or suspensions, and this can limit their use.

Microbial enzymes do not suffer the same limitations as animal or plant enzymes. They can be produced in large fermentation processes, and the scope of isolatable enzymes is tremendous. Most commercial enzymes are produced extracellularly.

Enzymes that are used as commodity products need to be prepared economically. The fermentation processes are inexpensive and fairly simple raw materials such as starches, whey and cereal can be used as energy soures.[4] On the other hand, speciality enzymes that are employed as diagnostics or for highly selective biotransformations often must be prepared in a high-purity manner.

1.1.2 Enzymatic vs. chemical catalysis

Enzymatic catalysis is inherently more selective than conventional chemical catalysis.[5] This selectivity is often positional (i.e., regioselectivity) or chiral (i.e., stereoselectivity). While a great deal of work has been carried out to make organic catalysts highly selective[6] enzymes have evolved over billions of years to their high degree of perfection. High selectivity reduces side reactions, improves product separations, and shows fewer negative environmental effects. In addition to the great selectivity of enzymes, other factors making these catalysts potentially useful for commercial applications, include wide variety of reactions catalyzed, the ability to operate optimally under mild reaction conditions (i.e., ambient temperature and pressure), and high turnover numbers found in many enzyme-catalyzed reactions which are often higher than those for conventional chemical catalysts, which typically operate at much higher temperatures.[7]

1.1.3 Classification of enzymes

At present approximately 3000 enzymes have been characterized to some degree, whereas only about 300 are available commercially from enzyme suppliers.[8] Most enzymes are broadly classified according to the substrates they act upon. The classification proposed by the International Union of Biochemistry and its commission of enzymes is the scientifically accepted method of nomenclature. This categorizes enzymes into six classes[9] depending on the reactions that are catalyzed.

CLASSIFICATION OF ENZYMES

CLASS	*REACTION CATALYZED*	*EXAMPLE*
OXIDOREDUCTASE	**Oxidation-Reduction reactions between two substrates**	**Peroxidase, Alcohol Dehydrogenase**
TRANSFERASES	**Functional group transfer from donor molecule to acceptor molecule**	**Fructosyl-Transferases**
HYDROLASES	**Hydrolysis of ester, ether, peptide linkages**	**Lipase, Trypsin, Chymotrypsin**
LYASES	**Cleavage of bonds like C-N, C-C, C-O by elimination or addition of group to produce double bonds**	**Aspartase, Fumarase**
ISOMERASES	**Isomerization, catalyzing interconversion of optical, geometrical, positional, Cis-trans**	**Glucose-Isomerase**
LIGASES	**Catalyses the linking of two or more molecules**	**Glutathione-Synthetase**

In addition to the formal classification of enzymes, one may classify enzymes in terms of the synthetic chemistry involved. For example hydrolytic enzymes are capable not only of breaking down esters, amides, lactones, glycosides and anhydride linkages but often are stereo - and regioselective,[10,11] and also catalyse reverse reactions such as ester and peptide synthesis in organic media,[12,13] and can be classified accordingly.

1.2 Immobilization of enzymes

1.2.1 Why immobilize enzyme?

Enzymes have been utilized by human beings since ancient times, well before their nature was understood. Enzymes are produced by organisms for their own requirements, and although enzymes are efficient and effective catalysts, they are not always ideal for practical applications.

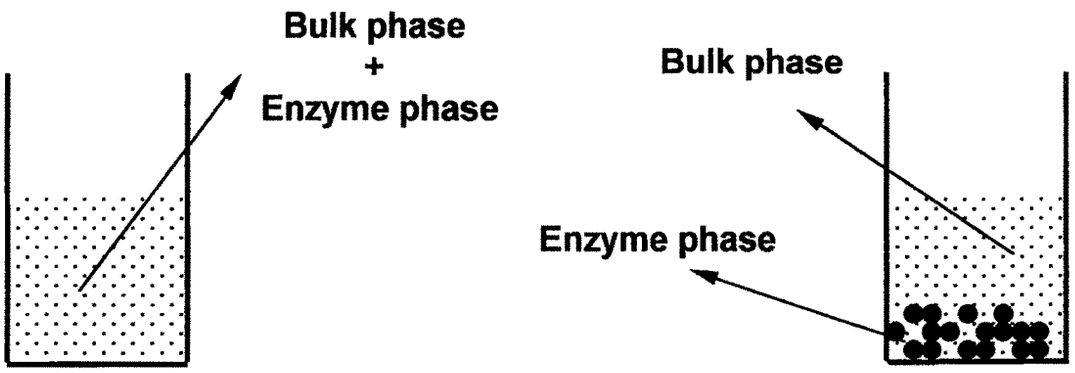
There are several factors for which enzymes cannot be used more effectively.

- Limited sources
- Inconvenient handling
- Altered reaction conditions
- Separation from product
- Reuse (cost)
- Reactor study

In order to overcome above disadvantages modification of an enzyme is required, which can be achieved to some extent through immobilization.

1.2.2 What is immobilization?

Immobilization of an enzyme has been defined as "Enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously." The enzyme is localized by various means in a water-insoluble high molecular weight polymer.



Free Enzyme **Immobilized Enzyme**

1.2.3 How to immobilize?

A specific conformation and an active center interacting with the substrate are regarded as essential features for the catalytic activity of enzymes. The active center consists of two sites having different functions. One is the reactive or catalytic site participating in the catalytic action, and the other is the specific or binding site controlling the substrate specificity of the enzyme. These sites are usually composed of several amino acid residues held in a specific spatial

relationship. The three-dimensional conformation of the entire enzyme protein also has an important effect on the catalytic activity. Consequently, to retain the catalytic activity of the enzyme in immobilized state, it is necessary to retain the native structure of an enzyme as far as possible.

Immobilization of enzymes can be generally be described as a process leading to their restricted mobility. Methods used for the immobilization of enzymes fall into two main categories - chemical and physical.

Chemical methods of immobilization involve the formation of at least one covalent (or partially covalent) bond between residues of an enzyme and a functionalized water-insoluble polymer or between two or more enzyme molecules. In reality, more than one covalent bonds between the reacting components are normally formed. Chemical methods are usually irreversible and the original enzyme cannot be regenerated or recovered. However, irreversibility is not an inherent feature of chemical methods, it is only a commonly found feature due to the nature of the chemical reactions employed for immobilization.

Physical methods involve localizing an enzyme in any manner which is not dependent on covalent bond formation. In this class, the immobilization of enzymes is dependent on the operation of certain physical forces (e.g. electrostatic interaction, formation of ionic bonds, protein-protein interaction etc.), the entrapment within microcompartments, or the containment in special membrane-dependent devices. In principle, physical methods of immobilization are completely reversible. However, many specific examples of the individual methods do not follow idealized behavior and considerable irreversible bond formation occurs.

Table 1.1
Techniques employed for the immobilization of enzymes

• **Physical**
- Physical adsorption
- Ionic binding
- Entrapment
- Microencapsulation
• **Chemical**
- Covalent binding
- Cross-linking

Over the last few years the number of methods available for enzyme immobilization have increased dramatically. In the following sections the general methods are described within the classification system described above.

1.2.3.a Physical adsorption

Physical adsorption is historically the earliest artificial method of enzyme immobilization. This is the simplest way of preparing immobilized enzyme. The method relies on non-specific physical interaction between the enzyme protein and the surface of the matrix, brought about by mixing a concentrated solution of enzyme with the solid, either with a stirrer or continuously agitated in a shaking water bath. The process results in a uniform loading of enzyme.

A major advantage of adsorption as a general method of insolubilizing enzymes is that usually no reagents and no activation steps are required. As a result, adsorption is inexpensive, easily carried out and tends to be less disruptive to the enzymic protein than chemical means of attachment. The binding in adsorption is mainly through hydrogen bonds, multiple salt linkages, and Van der Waal's forces.

Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Nelson and Griffin[14] reported for the first time in 1916 that invertase adsorpbed on activated carbon retained its activity towards saccharose.

1.2.3.b Ionic binding

Immobilization via ionic binding is based, mainly on ionic binding of the enzyme molecule to solid supports containing ion-exchange residues. The main difference between ionic binding and physical adsorption is the strength of the enzyme to carrier linkage which is much stronger in ionic binding although less strong than in covalent binding. The preparation of immobilized enzymes through ionic binding can be done by following the procedures described for physical adsorption.

Owing to ionic nature of the binding forces, leakage of enzyme from the carrier may occur in some situations, as with physical adsorption, when high substrate concentrations, high ionic strength solutions, or pH variations are involved. However, the mild conditions used for immobilization do mean that conformational changes only occur to a small extent. The most common ion exchangers used include DEAE-derivatives,[15] TEAE-derivatives[16] as anion and

CM-derivatives[17] as cation exchangers. Immobilization of an enzyme by this method was first reported by Mitz[18] in 1956.

1.2.3.c Entrapment

Confining enzymes within the lattices of polymerized gels is another method of immobilization. This allows the free diffusion of low molecular weight substrates 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 the desired particle size.

As there is no bond formation between the enzyme 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. Another disadvantage is that only low molecular weight substrates can diffuse rapidly to the enzyme, thus making the method unsuitable for enzymes that act on macromolecular substrates, such as ribonuclease, trypsin, dextranase, etc.

Cross-linked polyacrylamide gel was first employed for the entrapment of trypsin, papain and β-amylase by Bernfeld and Wan[19] in 1963.

The major disadvantages of entrapment technique are :

- × the occasional inactivation of the enzyme during entrapment
- × the requirement of high enzyme concentration
- × leakage of the entrapped enzyme

The major advantages of entrapment include :

- ✓ the extremely large surface area for the contact of substrate and enzyme within a relatively small volume

- ✓ the real possibility of simultaneous immobilization of many enzymes in a single step

1.2.3.d Covalent binding

The most intensely studied immobilization techniques is the method involving formation of covalent bonds between the enzyme and the support matrix. The choice of the selection of the method for immobilization is limited by the fact that the binding reaction must be performed under conditions that do not cause loss of enzymic activity, and the active site of the enzyme must be unaffected by the reagents used.

The selection of conditions for immobilization by covalent binding is more difficult than in the case of other carrier binding methods, but since covalent bonds are being formed, stable immobilized enzyme preparations which do not leach enzyme into solution in the presence of substrate or high ionic concentration solutions are achieved.

The immobilization of an enzyme by covalent attachment to a support must involve only functional groups of the enzyme that are not essential for catalytic action, and those, if reacted, would not alter detrimentally any chemical or physical properties of the enzyme. Thus, the 'active center' residues and functional groups needed for purposes such as allosteric control or subunit interaction should, if possible, be avoided. Amino acid residues of enzymes that have been either implicated or proven to be involved in the covalent bond

formation with supports are lysine, arginine, histidine, tryptophan, tyrosine, cysteine, aspartic and glutamic acids and serine.

A host of widely different water-insoluble supports are employed for the covalent attachment of enzymes, and **Table 1.2** gives a partial list of them.

Table 1.2
Commonly employed water-insoluble supports for the covalent attachment of enzymes

• **Synthetic**
- Acrylamide-based polymers
- Maleic anhydride-based polymers
- Methacrylic acid-based polymers
- Polypeptides
- Styrene-based polymers
• **Natural**
- Agarose (Sepharose)
- Cellulose
- Chitin
- Chitosan
- Dextran (Sephadex)
- Glass
- Starch

In principle, two steps are involved in the covalent binding of an enzyme to a polymer :

1) activation of the polymer through functional groups using bi or multifunctional reagent and

2) attachment of an enzyme molecule through their binding site to the activated polymer.

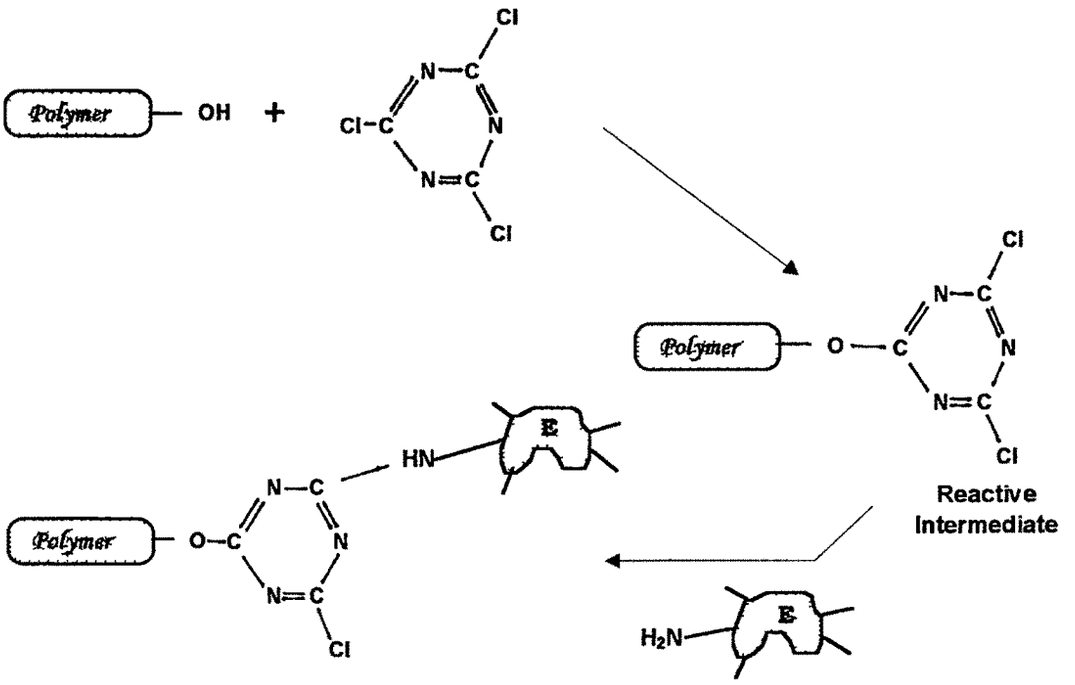
Selection of bi or multifunctional reagent used for activation depends upon the nature of functional group present in the polymer. Polymer containing hydroxyl groups can be activated by cyanuric chloride, p-tolyl sulphonyle chloride (PTS), cyanogen halide and p-benzoquinone. Polymer with carboxyl groups can be activated by water soluble carbodiimides, while those containing amino groups can be activated by glutaraldehyde.

a) activation of polymers containing hydroxyl groups

The hydroxyl groups of the polymer can be activated with cyanogen halide, cynuric chloride, p-tolyl sulphonyle chloride or with p-benzoquinone.

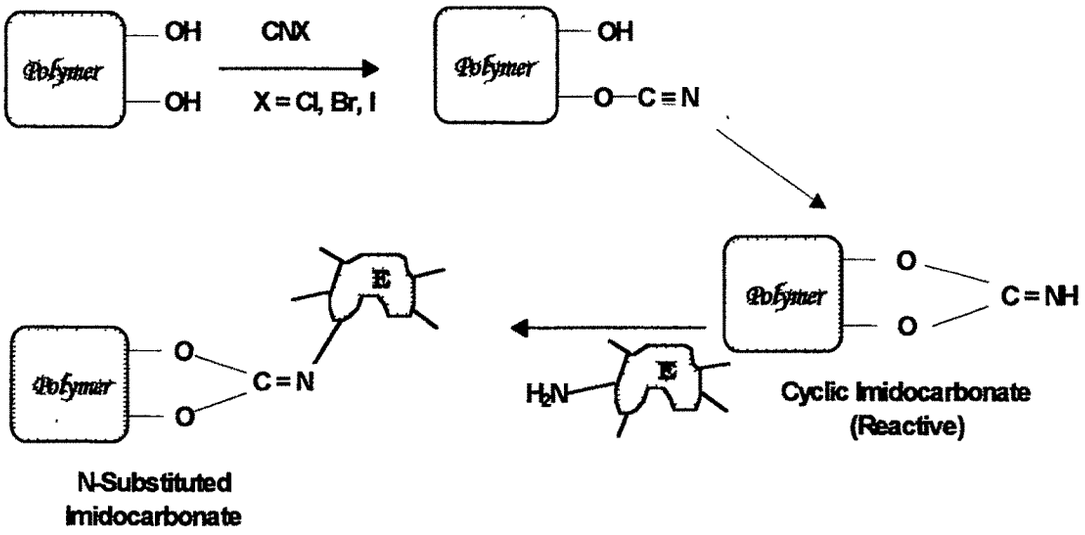
- **activation through cynuric chloride (2,4,6 trichloro s-triazine)**

As shown in the following reaction, carrier containing hydroxyl group reacts with trichlorotriazine and the active intermediate is formed. This then reacts with enzyme to give immobilized enzyme.



- **activation through cyanogen halide**

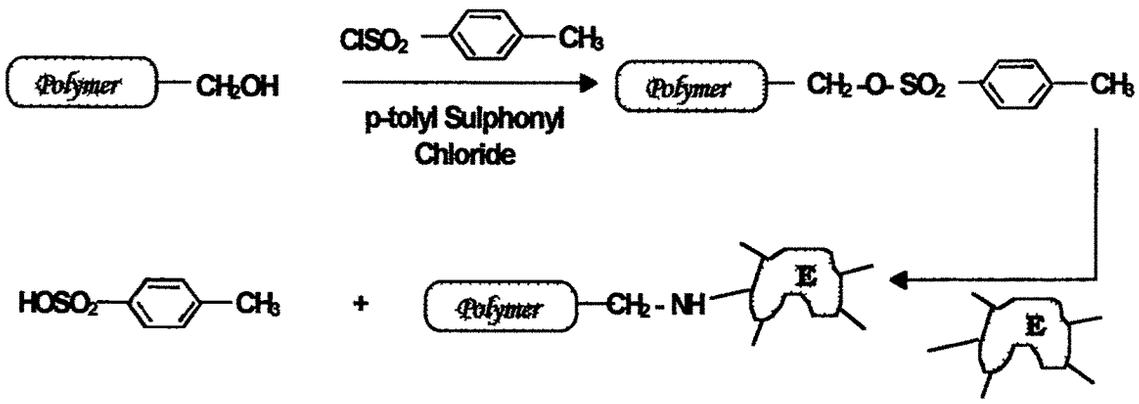
The method involves the activation of polymer with cyanogen bromide (or chloride or iodide) to give an inert carbamate and reactive imidocarbonate through the intermediate cyano derivative. The reactive imidocarbonate subsequently reacts with enzyme as follows :



This method was first reported by Axen et. al.[20] in 1967 for the activation of sephadex to immobilize chymotrypsin, trypsin and papain

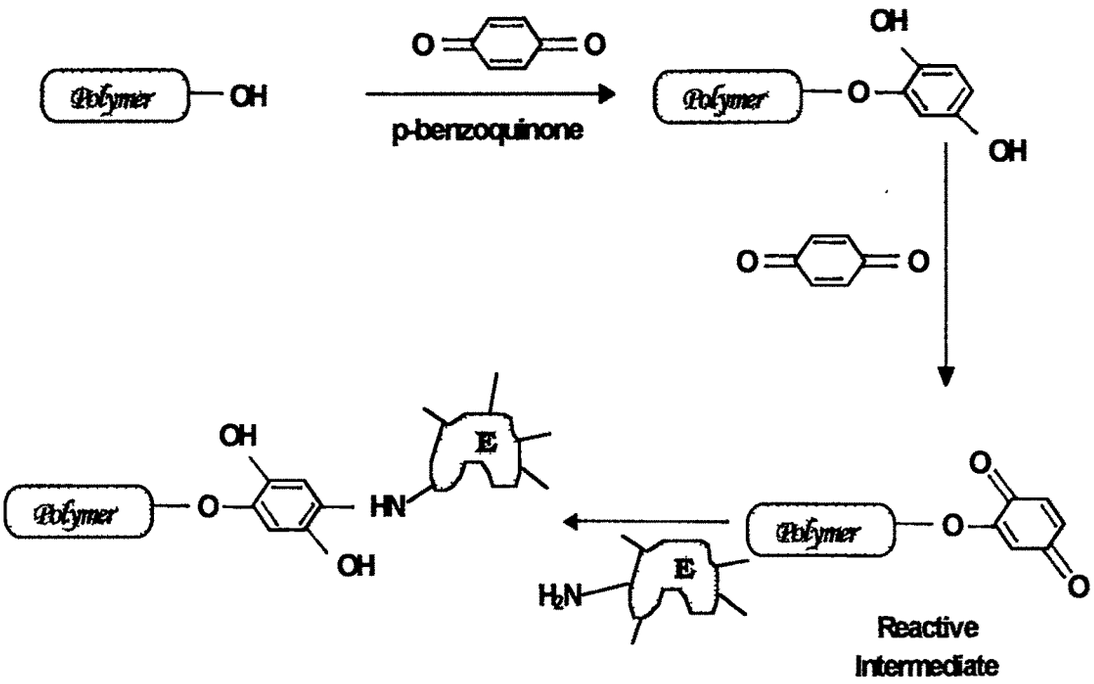
- **activation through p-tolyl sulphonyl chloride**

In 1981 Nilsson et. al.[21] used p-tolyl sulphonyl chloride for the first time for the activation of hydroxyl groups of agarose. This resulted in the formation of p-tolyl sulphonic ester in which tosyl group has excellent leaving properties in reactions with nucleophiles. They used this activation procedure to immobilize trypsin, peroxidase and alcohol dehydrogenase.



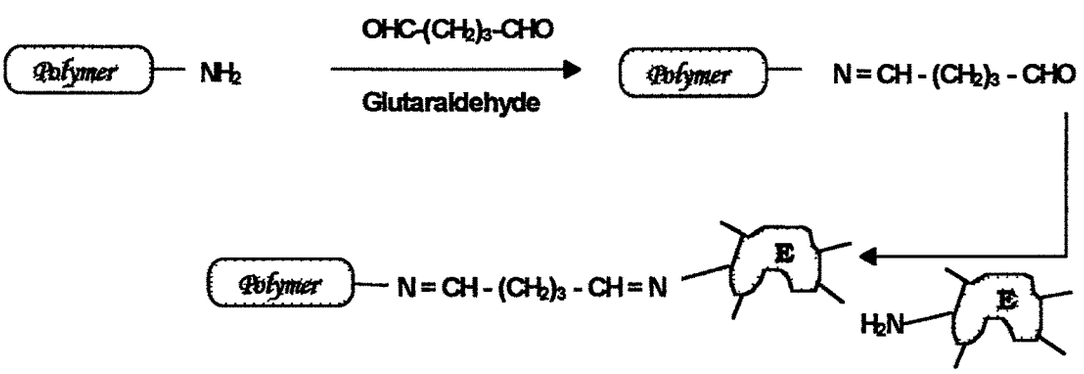
- **activation through p-benzoquinone**

This method was first used in 1975 for enzyme immobilization on polysaccharides such as sepharose and sephadex by Brandt et. al.[22]



b) activation of polymers containing amino group

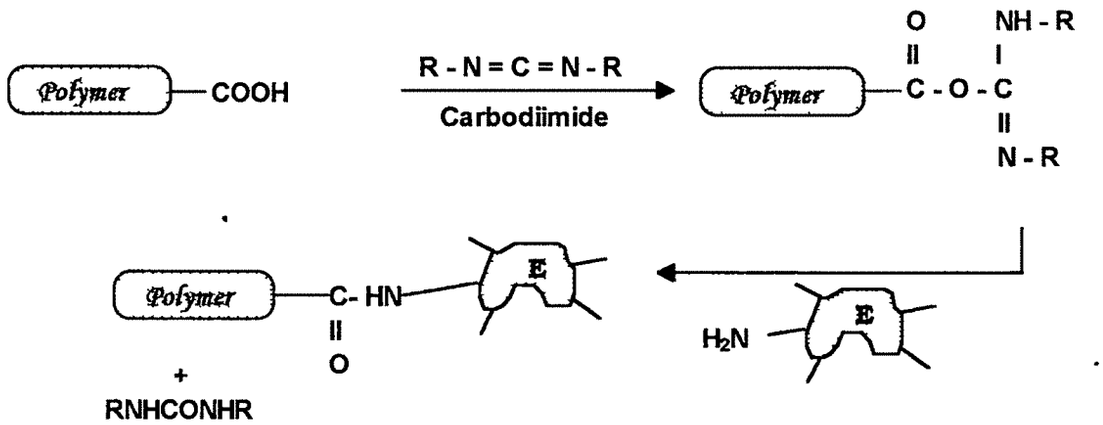
Glutaraldehyde is most commonly employed as the functional reagent, and many enzymes[23-28] have been immobilized by the formation of Schiff bases between the amino groups of carriers and of enzyme protein.



Carriers possessing amino groups, such as amino ethyl cellulose, DEAE-cellulose, amino derivatives of sepharose, partially deacylated chitin, chitosan, aminosilane derivatives of porous glass, etc., have been used for enzyme immobilization.

c) activation of polymers containing carboxyl group

Variety of methods are available for the activation of carboxyl groups of the polymers These supports can be activated with N-ethyl 5-phenyl isoxazolium-3'-sulphonate,[29] with N-ethoxy carbonyl-2-ethoxy-1-2-dihydroquinoline,[30] with thionyl chloride[31] and with carbodiimides.[20] The representative reaction between carbodiimide and carboxyl groups is given below.



1.2.3.e Cross-linking

This method is based on the formation of covalent bonds between enzyme molecules, by means of bi or multi-functional reagents, leading to three-dimensional cross-linked aggregates which are completely insoluble in water but which do not require the use of water insoluble carriers. This method involves

the addition of the appropriate amount of cross-linking agent to an enzyme solution under conditions which give rise to the formation of multiple covalent bonds. Optimum conditions for obtaining maximum insolubility whilst retaining high enzymatic activity must unfortunately be determined for each system by trial and error until sufficient is known about the enzyme's primary, secondary, and tertiary structures to allow prediction of the best conditions which will exhibit minimal distortion of the enzyme upon its reaction to give a cross-linked immobilized enzyme.

The reagents required for cross-linking possess two identical functional groups or two or more different functional groups, the latter being more common in binding enzymes to insoluble carriers than in intermolecular crosslinking reactions. Cross-linking reagents like glutaraldehyde,[32] isocyanate derivatives,[33] bisdiazobenzidine[34] and N,N'-ethylene bismaleimide[35] have been reported.

The first report of an immobilization reaction by this method involved the cross-linking of carboxypeptidase A with glutaraldehyde[36] which provides an immobilized enzyme with intermolecular linkages which are irreversible and can survive extremes of pH and temperature. However, the major disadvantages of the method, which severely limit the application of the method are :

- the difficulties in controlling the reaction.
- the need for large quantities of enzyme, much of which looses its activity by involvement of the active site in bond formation or by being at the centre of the cross-linked aggregate and out of contact with the substrate.
- the gelatinous nature of the final product.

1.2.4 Choice of immobilization method

Although many methods of immobilization have been developed and applied to many enzymes. It is now well recognized that no method can be regarded as the universal method for all applications or for all enzymes. This is because of the widely different chemical characteristics and compositions of enzymes, different properties of substrates and products, and the different uses to which the product can be applied. Therefore, for each application of an immobilized enzyme it is necessary to find a procedure which is simple and inexpensive to perform and which gives a product with good retention of activity and high operational stability.

When immobilization is accompanied by a chemical reaction, as in the cross-linking and covalent binding methods, conformational changes in the protein molecule must be kept to a minimum to avoid partial deactivation due to involvement of the active sites in the immobilization reaction. This requires the use of the mildest conditions possible to effect immobilization. However, once an enzyme is successfully immobilized by chemical means, the operational stability of the product is high, owing to the strength of the bonds between enzyme molecules or between enzyme and carrier, and the reluctance of these bonds to disruption by substrate or salt solutions. Cross-linking is generally not a suitable method for large-scale industrial applications, because of the lack of mechanical stability of the final product, whilst covalently bound enzymes which utilize organic matrices can rarely be regenerated and are again unattractive for large-scale industrial use on this account.

Physical adsorption and ionic binding are attractive methods for enzyme immobilization owing to mild conditions involved in the binding reactions. However, because the binding forces are generally weaker than for chemical binding methods, operational stabilities are lower through loss of enzyme from

the matrix as a result of changes in ionic concentration, pH, substrate concentration, or temperature of the reaction medium. To offset this disadvantage the possibility of regeneration does mean that industrial applications using these techniques are possible.

With the entrapment methods of immobilization, high retention of activity is possible as a result of there being no binding between enzyme and carrier, but limitation of enzyme activity can occur owing to diffusion effects of large molecular weight substrates and products. Therefore the use of entrapment methods must be limited to reactions involving small molecular weight substrate and product molecules.

1.3 Immobilized enzymes

1.3.1 Immobilization onto inorganic carriers

Inorganic carriers - such as glass, silica gel, alumina, bentonite, hydroxyapatite, titania, zirconia etc., often show good mechanical properties, thermal stability, and resistance against microbial attack and organic solvents.[37] On the other hand, non-porous materials like metal and metal oxides only have small binding surfaces. Minerals usually display a broad distribution of pore size. Besides these, inorganic supports have advantages, like no swelling and porosity changes with pH.

Silica gels are available under the trade names Promaxon, Spherosil or Aerosil. Silica compounds can be prepared with defined pore sizes and binding surfaces, but they suffer from high production costs and show limited stability under alkaline conditions. Furthermore, silica carriers are chemically inert and need activation and modification. Usually, they are treated with aminoalkyl triethoxysilanes to introduce amino groups, which can subsequently be activated

for enzyme coupling reactions by a variety of different methods.[38] The most common procedure is the reaction with dialdehydes to transform amino groups into reactive unsaturated aldehyde residues. In this way, penicillin G amidase was first coupled to dextran.[39] Inorganic oxides such as silica gel have received great attention due to the ability of immobilizing organic functional groups onto its surface by using some silane type coupling agents, which react with the silanols on the solid surface.[40]

Organopolysiloxanes constitute macroporous inorganic synthetic materials carrying organic functional groups and are commercially available. These supports were used to immobilize glutaryl-7-ACA-acylase to split glutaryl-7-aminocephalosporanic acid to 7-aminocephalosporanic acid, a key intermediate for semi-synthetic cephalosporing antibiotics.[41]

Porous glass has been widely used as support because of the normalization of particle and pore size, the availability of coupling procedures, the chemical resistance of the base material, and for its suitable mechanical properties.[42] L-Aminoacylase was recently covalently immobilized on glass using two different chemical procedures and three different pore sizes by Gomez et. al.[43]

Magnetic carriers attracted the attention of researchers, as magnetic particles with immobilized biocatalysts could be easily removed in the presence of the magnetic field providing another simple method of separation.[44-47] Magnetic particles for the enzyme immobilization may be prepared by various methods such as silanization of magnetic material,[48] encapsulation of magnetic particles with a proper polymer, or adsorption followed by cross-linking on magnetic particles.[49]

Magnetic poly(styrene) particles including active groups were prepared for the enzyme immobilization by the solvent evaporation method.[50,51] Glucoamylase,

was immobilized onto magnetic poly(styrene) particles without any activation process.[52]

Covalent attachment of acid phosphatase on the surface of amorphous $AlPO_4$ was carried out by Bautista et. al ,[53] whereas dextransucrase was immobilized in calcium alginate in the presence of organic solvents.[54]

1.3.2 Immobilization onto naturally occurring organic carriers

Natural organic polymers - such as structural proteins (ceratin, collagen), globular proteins (albumin) or carbohydrates - are cheap starting materials for the production of support materials and are available in large quantities. From this group, carbohydrates are of special interest, because they do not suffer from biological safety aspects like protein matrices isolated from animal sources suffer and also because they are highly hydrophilic they provide a desirable microenvironment for many enzymes. Alginate,[55] carrageenan,[56] chitin or chitosan (prepared from chitin by deacetylation) have been particularly reported to be useful for encapsulation of microorganisms by ionotropic or by acidic gelation.[57] Enzymes have been as well linked to carbohydrates simply by adsorption followed by cross-linking.[58]

Chitosan as a carrier is of importance because of its primary amino groups that are susceptible for coupling reactions. Furthermore, porous spherical chitosan particles are commercially available allowing non-covalent or covalent attachment of enzymes.[59] This support matrix can be easily prepared[60] and various activation methods useful have been summarized by Scouten.[61] Treatment with polyethyleneimine or with hexamethylenediamine and glutardialdehyde has been reported to improve the mechanical characteristics[62] of the biocatalyst, which is poor otherwise. However, this is often accompanied by some activity loss or increase of diffusional limitations.

Dextran and agarose need to be cross-linked, for instance with epichlorohydrin, to improve their mechanical characteristics and compressibility. Covalent linking of enzymes to commercially available bead forms of sephadex and sepharose is summarized in.[63] The most widespread activation method is the cyanogen bromide method,[64] yielding isourea and imidocarbonate functions which react with amine groups of enzymes to form N-substituted carbamates. Cyanogen bromide was substituted by 1-cyano-4-dimethylaminopyridinium tetrafluoroborate.[65]

Cellulose is also an acceptable support and can be activated in a similar way. The binding capacity for enzymes is reported to be generally lower as compared to agarose but it is inexpensive and commercially available in fibrous and granular forms. However the low particle size impairs the use in rapid high-pressure applications, and also its susceptibility to microbial cellulase. A good account of engineering aspects of immobilization has been given by Gemeiner et. al.[66]

1.3.3 Immobilization onto synthetic organic carriers

Synthetic organic polymers display the greatest variability with regard to physical and chemical characteristics. In principle, they can be adapted to the requirements of nearly any enzymatic process. Furthermore, they are inert to microbial attack. They are commercially available as purely adsorptive resins, as ion exchangers with a variety of different basic and acidic groups, or as preactivated supports. The commonly used synthetic polymers are polystyrene, polyacrylates, polyvinyls, polyamides, polypropylene and copolymers based on maleic anhydride and ethylene or styrene, polyaldehyde, and polypeptide structures.

Polystyrene was the first synthetic polymer used for enzyme immobilization by Grubhofer and Schleith[67] in 1963. Binding of enzyme was mainly by adsorptive forces. Usually, binding of the enzyme is favoured at low salt conditions. However, the hydrophobicity of the matrix often leads to partial enzyme denaturation during the binding process and therefore to low activity yields. By combining adsorption and cross-linking, penicillin G amidase was covalently coupled to a fairly hydrophilic polyacrylic ester, which had been pre-coated with glutaraldehyde at alkaline pH.[68] The same enzyme was adsorbed on macroporous polymethacrylate, which was optimized with regard to monomer-comonomer composition, and cross-linked by glutardialdehyde treatment.[69]

Ionic binding on ion-exchange matrices of mixed monomers (e.g. weakly basic styrene-divinylbenzene resin) often leads to higher immobilization yields as compared to adsorption on hydrophobic adsorber resins. Immobilization can indeed be improved by the above mentioned cross-linking reactions. This is of particular importance if the biocatalyst is used in aqueous systems with a high salt content or in the case of processes which liberate charged compounds, thereby increasing the ionic strength. However, most non-covalent immobilization processes on organic supports are of interest for non-aqueous applications. Lipases and, to a lesser extent, protease are the enzymes mainly used in organic solvents. Their immobilization and application technologies have been recently reviewed.[70-72] The hydrophobic polypropylene matrix is one of the most often used supports for lipases.[73,74]

Activation of synthetic polymers can be achieved by a variety of methods. Basically, reactive groups can be introduced during polymerization by selection of suitable monomers or can be generated through modification of the polymer backbone.

Epoxyactivated organic carriers can be prepared by copolymerization of glycidylmethacrylate with ethylenedimethacrylate or methacrylamide with N,N'-methylenebis(acrylamide) and allylglycidyl ether.[75] A different method of synthesis uses vinyl acetate and divinylethylene urea to build up the polymer backbone which is then surface modified with oxirane groups after hydrolysis of the acetate groups. Particle size, porosity, and the density of the functional groups can be adjusted by appropriate monomer mixtures and polymerization conditions. Generally, polymethacrylates are characterized by high mechanical strength and chemical stability.

They can be used directly for enzyme coupling primarily via protein amino groups in buffered aqueous solution, or can be modified by variety of different reagents, for example, introducing spacers with hydrophilic or hydrophobic groups. By this method a suitable distance of enzyme to carrier backbone can be adjusted to allow optimal enzymatic flexibility. Furthermore, the microenvironment of a bound enzyme can be influenced selectively.

1.4 Proposed Work

Immobilized enzymes exhibit better stability over broad pH range, temperature and organic solvents. Due to possibility of repeated use, they can be successfully used in the development of continuos processes.

Various types of enzymes from hydrolase, oxidoreductase, and esterase groups have been immobilized on organic and inorganic, synthetic and natural polymeric materials. Some of the immobilized systems have been used in fluidised bed, packed bed and other types of reactors. We have undertaken enzyme immobilization work with following objectives:

Objectives of the work

- Optimization of various immobilization conditions such as concentration of cross-linking reagent, crosslinking time, pH of the coupling medium, enzyme concentration, and immobilization time for α-chymotrypsin, yeast alcohol dehydrogenase and horse radish peroxidase.

- Modification of entrapment technique to get maximum entrapped enzyme activity.

- Comparison of properties of free and immobilized enzymes e.g. pH, thermal and storage stability, resistance to organic solvents.

- Evaluation of kinetic parameters for free and immobilized enzymes.

- Application of covalently immobilized enzyme for continuos operation in packed bed reactor.

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