INTRODUCTION

CHAPTER 1

CONTENTS

Thousands of years ago man learnt about the basic necessities like shelter, food and clothes. The ability to provide these basic necessities of everyday life did not of course imply an understanding of the biological processes involved. Understanding of cellular metabolism and the catalytic properties of enzymes is indeed a relatively recent process. However, acquisition of this knowledge has in turn led to a desire to exploit more fully the potential of cells and enzymes for domestic, industrial and medicinal purposes. Hence, the increasing use of enzyme-catalyzed processes in industry, chemical analyses and medicine has made enzymology more of a "main stream" chemistry topic rather than a biologically oriented one.

1.1 Enzymes

Enzymes are biocatalysts involved in various chemical reactions in the living systems. They mainly consist of globular proteins and are produced by living cells. Because of their wider acceptance in industry, chemical laboratory, dairy farms and other areas they have gained increased importance recently. Moreover, as they are found to be more efficient and versatile, the conventional inorganic catalysts are being replaced by these in phases. The most desirable character observed in enzymes is that unlike other catalysts in aqueous solution they can catalyze a reaction in mild conditions at normal temperature and pressure with very high reaction specificity, bond specificity and optical specificity. Based on the specificity character enzymes are divided into six major classes.

1.2 Classification of Enzymes

No	Class	Reaction Catalyzed
1	Oxido reductases	Oxidation-reduction reactions between two substrates A and B
2	Transferases	Group transfer from one molecule to another molecule
3	Hydrolases	Hydrolysis of ester, ether, peptide, glycosyl, acid anhydride by the addition of water
4	Lyases	Addition of group to double bond
5	Isomerases	Isomerization, catalyzing interconversion of optical, geometrical and positional
6	Ligases (synthetase)	Joining of two molecules coupled with cleavage of pyrophosphate bond of ATP or similar triphosphate

Even though enzymes are produced by living cells the activity of an enzyme varies with the source from which it is produced. Normally enzymatic reactions are highly efficient. The turnover number, a measure of efficiency is relatively higher for the enzymes. The optimum temperature for enzyme activity is 25°C to 40°C. Moreover, they can act effectively at neutral and close to neutral pH values. They are very specific in the nature of the reactions catalyzed and the substrate used.

However, extraction and purification of enzymes is not easy task and hence their uses in various reactions are stringently governed by their necessity in the reactions. Besides, enzyme activity is very low or lost completely at elevated temperatures and in organic solvents. Above all, the high cost, the less stability and durability have restricted their uses in the areas where they are quite unavoidable.

Conventionally enzymatic reactions have been carried out in batch processes by incubating a mixture of substrate and soluble enzymes. However, it is impossible to recover the active enzyme from the mixture and hence it is lost. It was found that this problem can be solved by using immobilized enzymes.

1.3 What is Immobilization of Enzymes?

Immobilization means physical confinement or localization of enzyme molecules. This can be brought through:

a) physical adsorption of enzymes onto water insoluble organic or inorganic supports,
b) the entrapment of enzyme within gel matrices or semipermeable microcapsules,
c) the covalent attachment of enzyme to the water insoluble supports.

1.4 Why Immobilize Enzymes?

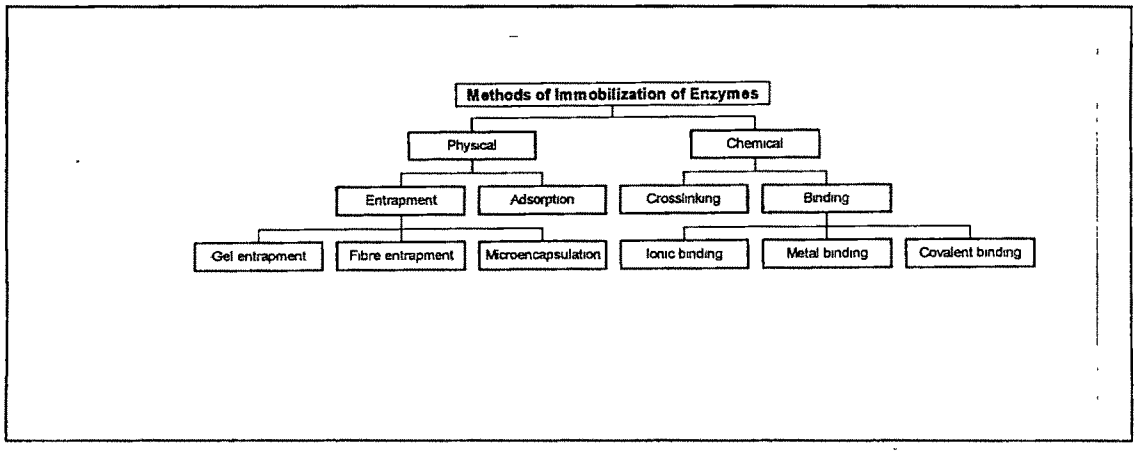
There are three major reasons for immobilizing enzymes. Because they:

a) offer a considerable operational advantage over freely mobile enzymes, such as reusability, applicability in repeated continuous operations, rapid termination of reaction and controlled product formation,
b) alter chemical or physical property, and
c) serve as model enzymes or systems for natural, in vivo, membrane-bound enzymes.

In addition, they are highly stable and resistant to shear stress and contamination. The rate of reaction is very fast due to high catalyst concentration and easy separation of biocatalyst from termination medium is possible. Moreover repeated use of biocatalyst is also possible. Immobilized enzymes have also certain demerits. The existance of mass transfer resistance and cumbersome immobilization processes are some aspects which can be highlighted against the immobilization of enzymes. However, an excellent general introduction to this topic is given by Zaborsky [1] and Chibata [2].

1.5 Methods of Enzyme Immobilization

There are several ways of classifying the various types of immobilization processes. The classification is based on the nature of interaction responsible for immobilization. Over the last few decades number of methods have been attempted for enzyme immobilization and new methods are being tried for improving the previous ones. The immobilization processes hence are classified as:

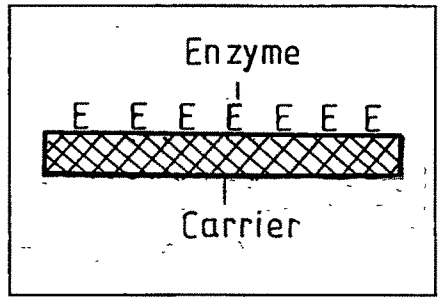


In all enzyme immobilization techniques, the binding reaction is carried out under mild conditions so that the protein does not denature. In the covalent binding the amino, carboxyl, sulphydryl, hydroxyl, or phenolic groups of the amino acids of the residual side chains of the proteins are used to couple with the reactive groups of the polymer : A few selected examples of various modes of immobilization are discussed here.

1.5.1 Physical Adsorption

The immobilization of enzymes by physical adsorption involves little or no conformational change in the protein. However, this method has the disadvantage that the adsorbed

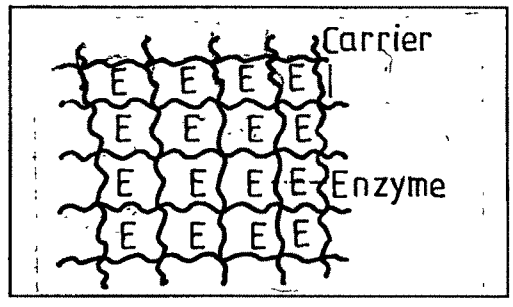
enzyme may leak from the carrier during use, as binding forces between the enzyme protein and the carrier are weak. Another disadvantage is that substrate diffusion problems can hamper the performance of the enzyme. A major factor influencing the quantity of enzyme adsorbed onto a solid support is enzyme concentration available to the unit surface of the carrier during immobilization process. The adsorption of an enzyme onto a water insoluble material is dependent on experimental variables such as pH, nature of the solvent, ionic strength, concentration of protein and adsorbent, time and temperature. A very wide range of systems has been used for this purpose. Both inorganic and organic supports have been used as carriers. Inorganic supports include alumina, clay, silica gel, titania, calcium phosphate gel, porous glass, zeolite, zirconium oxide, etc. Organic supports such as activated carbon, starch, chitosan, derivative of cellulose, gluten etc, have also been used for the immobilization of enzymes by adsorption techniques [1,2].



1.5.2 Entrapment

1.5.2.a Gel Entrapment

This method involves the formation of a highly crosslinked polymer network in the presence of protein where protein molecules are physically entrapped within the polymer matrix. As the enzyme molecules are trapped inside the matrix the leak out from the matrix could be prevented to a certain extent. However, such systems suffer from problems of diffusion. If the protein is located deep inside the solid matrix the diffusion of the reactants or products is difficult. Also the retained reaction products can inhibit the enzyme activity. Polyacrylamide is one of the most commonly used crosslinked polymer for protein entrapment. This technique was first used and reported by Bernfeld and Wan in 1963 [3] who entrapped trypsin, papain, amylase and ribonuclease in a gel matrices or lattices of polyacrylamide.



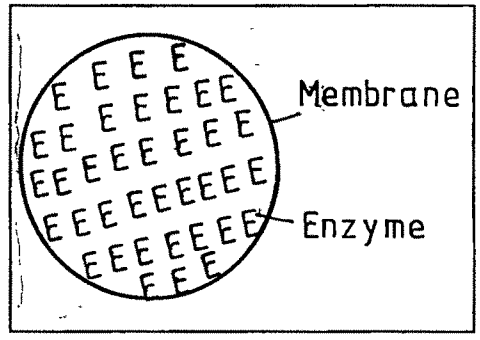
When a gel is formed from a naturally occuring polymers such as gelatin, alginate, agar etc., the polymer is dissolved in aqueous media and mixed into the enzyme solution. This enzyme-polymer mixture is then gelled by mixing with a solvent which is immiscible with water. A wide range of gel entrapment systems are reported in the literature [1,2].

1.5.2.b Fibre Entrapment

A method of immobilization of enzymes by entrapment within the microcavities of synthetic fibres has been developed by Dinelli and coworkers[4]. In this method enzyme molecules can be entraped in fibres. This method has several advantages over gel entrapment. High surface area for enzyme binding can be obtained by using very fine fibres. The fibres are resistant to weak acids and alkalis and some organic solvents. Depending on the polymer used they can show good resistance to microbial attack. Cellulose acetate is one of the most commonly used polymer for fibre entrapment due to low cost and good biological as well as chemical resistance.

1.5.2.c Microencapsulation

In this method, the proteins are immobilized in microcapsules, that have either permanent or non-permanent semipermeable character.



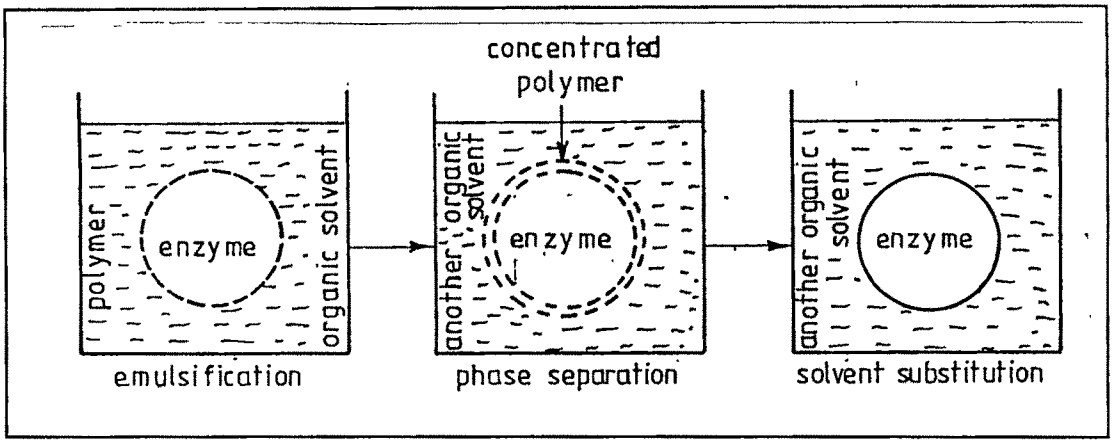
The immobilization of enzymes by entrapping the molecules within permanent semipermeable microcapsules was first reported by Chang in the mid sixties[5] . Since then, various enzymes have been immobilized in microcapsules of different chemical compositions. Permanent membranes are formed by interfacial polymerization or phase separation method and the non-permanent membranes are formed by the use of appropriate surfactants, additives and hydrocarbons in the membrane formation.

Technically immobilization of enzymes by encapsulation has certain advantages over other proposed methods. Among other things, conformation of an enzyme molecule is retained after the immobilization because enzyme molecules are in the form of microcapsules instead of being chemically bound to the supporting materials. Encapsulation may lead to the highly selective catalysis due to the difference in permeability of substrate molecule. Microencapsulation is achieved through [2,6]

(i) phase separation,
(ii) interfacial polymerization,
(iii) liquid drying and
(iv) liquid surfactant membrane methods.

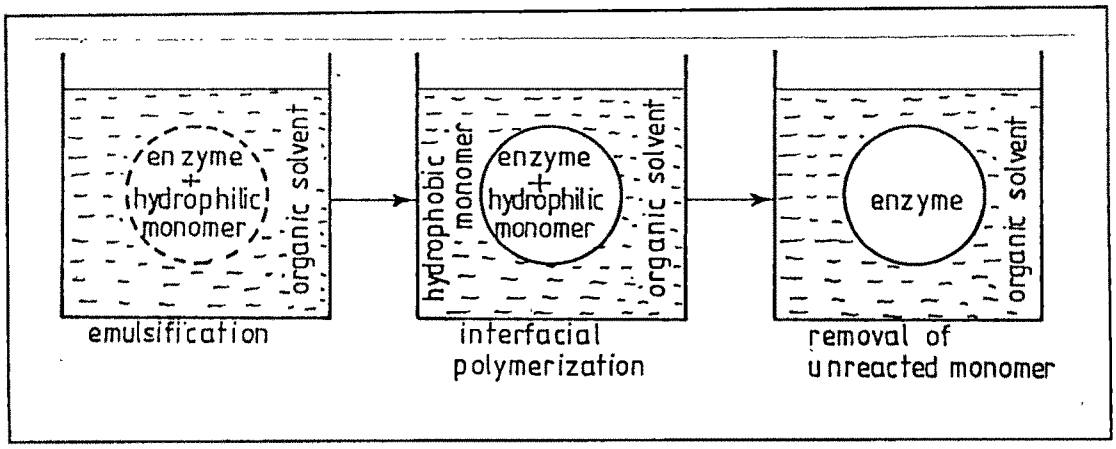
(i) Phase separation

This is the most common method of enzyme microencapsulation and is based on a physical phenomenon. In this process an aqueous solution of the enzyme is emulsified in a water immiscible organic solvent containing the polymer. This polymer-enzyme emulsion is then vigorously stirred and another water immiscible organic solvent in which the polymer is insoluble is added. The polymer is concentrated and membranes are formed around the microdroplets of aqueous enzyme solution, as shown diagramatically below:



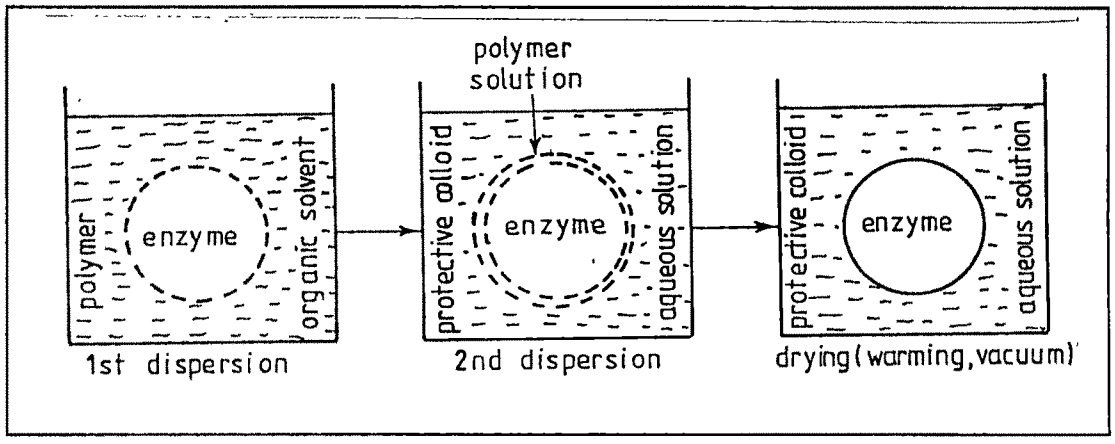
(ii) Interfacial polymerization

This procedure is based on enclosing enzymes in semipermeable membranes of polymers by polymerizing hydrophobic and hydrophilic monomers at the interface. In this technique, an aqueous solution of the enzyme and a hydrophilic monomer is emulsified in a water immiscible organic solvent. To the emulsion, hydrophobic monomer solution dissolved in the same organic solvent is added with stirring. Polymerization of both monomers occurs at the interface between the aqueous and organic solvent phases in the emulsion. By this method, enzyme in the aqueous phase is enclosed by a membrane of polymer as shown in Fig



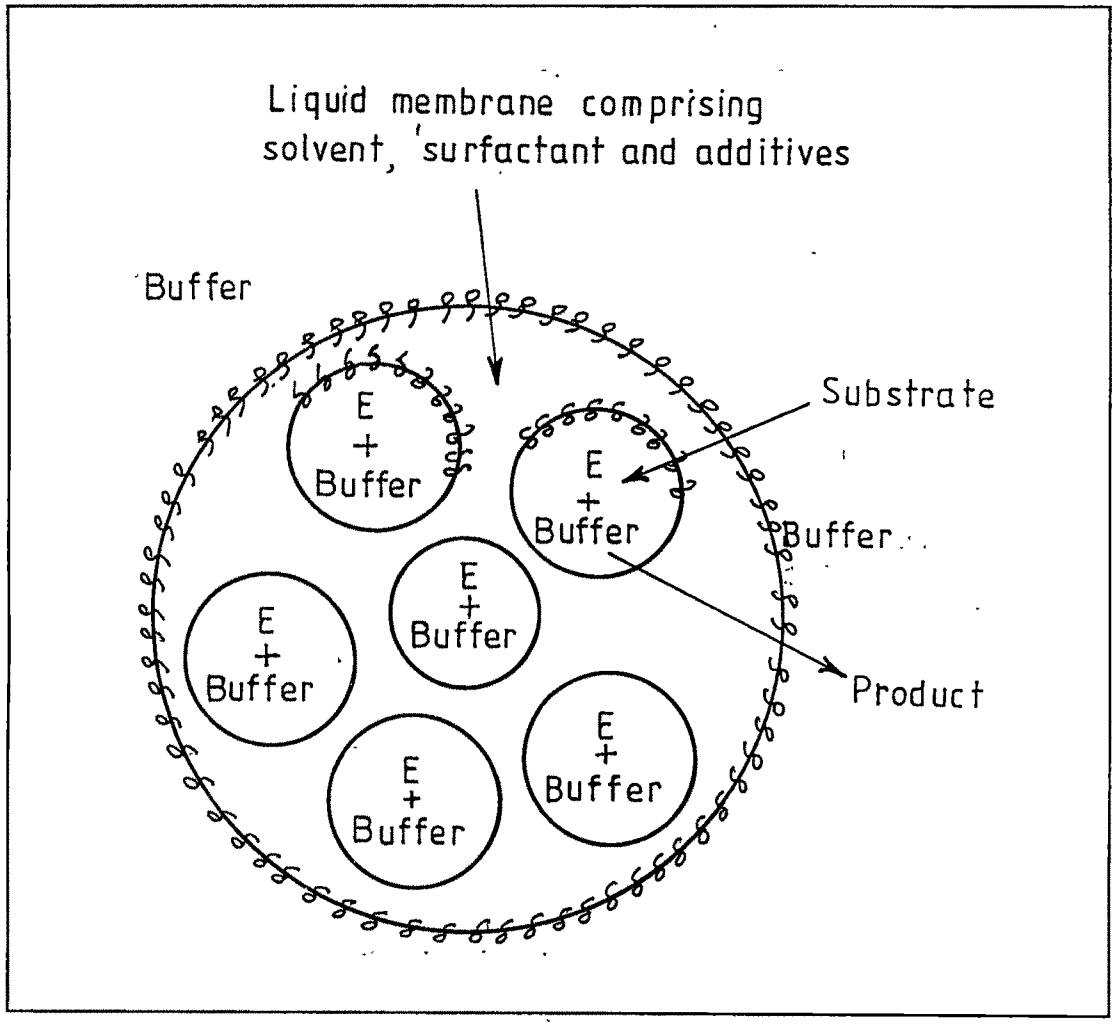
(iii) Liquid drying

In this technique, a polymer is dissolved in a water immiscible organic solvent which has a boiling point lower than that of water. An aqueous solution of enzyme is dispersed in the organic phase to form a first emulsion of water-in-oil type. In this case, the use of oil-soluble surfactants as emulsifying agents is effective. The first emulsion containing aqueous microdroplets is then dispersed in an aqueous phase containing protective colloidal substances such as gelatin, poly(vinyl alcohol) and surfactants. Thus a secondary emulsion is prepared with continued stirring the organic solvent which is removed by warming in vacuo. By this process, a polymeric membrane is produced to give enzyme microcapsules as shown diagramatically.



(iv) Liquid surfactant membrane

This method which provides immobilized enzymes within a non permanent microcapsule is based on the liquid surfactant membrane concept developed by Li[7]. Enzyme immobilization is brought about by emulsifying an aqueous enzyme solution with a surfactant to form the liquid membrane encapsulated enzymes. The major advantage of this procedure is the non-chemical nature of the method and reversibility of the immobilization process as shown diagramatically.



A large number of microencapsulated systems have been reported [1]. Recently Iso *et al* [8] reported immobilization and application of lipase by microencapsulation. In this study, microencapsulation was carried out using (w/o)/w two phase emulsion techniques. Polystyrene and Styrene-Butadiene-Rubber (SBR) were used as wall materials either separately or in mixture. Performance of the encapsulated lipase enzyme was evaluated

by employing the hydrolysis of triacetin as a model substrate for the enzyme catalysis. A mathematical model was developed to simulate the behaviour of hydrolysis.

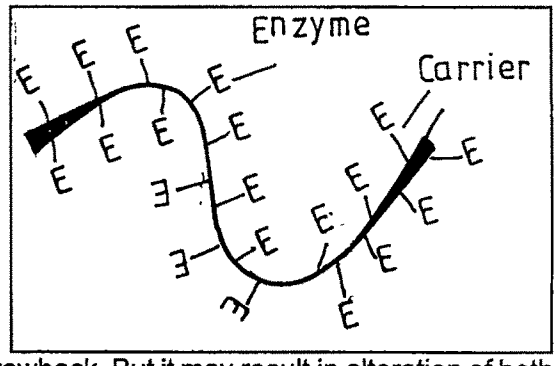
1.5.3 Chemical binding

1.5.3.a Ionic binding

Immobilization via ionic binding is based, mainly on ionic binding of the enzyme molecules to solid supports containing ion exchange residues. In some cases, not only ionic binding but also physical adsorption may take place simultaneously. The main difference between ionic binding and physical adsorption is the strength of the enzyme to carrier linkages which are much stronger for ionic binding although less strong than covalent binding. Polysaccharides and synthetic polymers having ion exchange residues are used as carriers for ionic binding. The disadvantage of this method is that a change in pH, electrolytic concentration or temperature results in the desorption of enzymes. Immobilization of an enzyme by this method was first reported by Mitz in 1956[9]. In this work catalase was immobilized on DEAE-cellulose. Since then wide variety of carriers for ionic binding have been used and the details are discussed in the Handbook of Enzyme Biotechnology by Wiseman[6].

1.5.3.b Covalent binding

In this immobilization process atleast one covalent or partially covalent bond between the amino acid residue of the protein and a water insoluble polymer exists. The physical immobilization techniques suffer the disadvantage of protein being slowly leaked out from the bulk of the material. The covalent attachment of the enzyme overcomes this drawback. But it may result in alteration of both the conformation and the active centres of protein. As a result a considerable reduction in biological activity or change in the substrate specificity is observed.



The enzymes can also be covalently bound to the carrier in the presence of competitive inhibitor or substrate to prevent the coupling at active sites of enzymes. Binding is generally carried out using any of the following reactions :

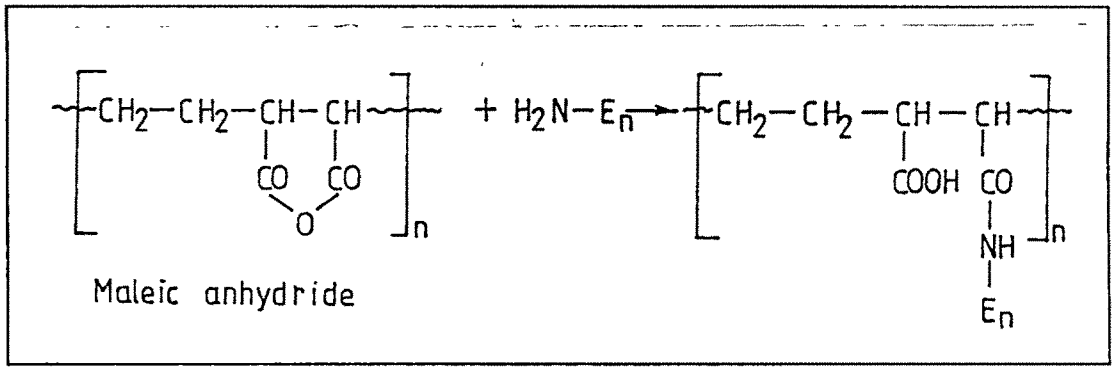
- Peptide bond formation,
- alkylation and arylation,

- ► diazolinkage,
- ► isourea linkage and
- ► other reactions.

The majority of polymeric supports have free hydroxyl, amine, amide and carboxyl groups which can be activated before use. Coupling reaction must be carried out at well controlled conditions, so as to avoid the denaturing of the enzyme-protein. Outline of some of the selected and relevant methods is given below.

(i) Immobilization of enzymes without activation of supports

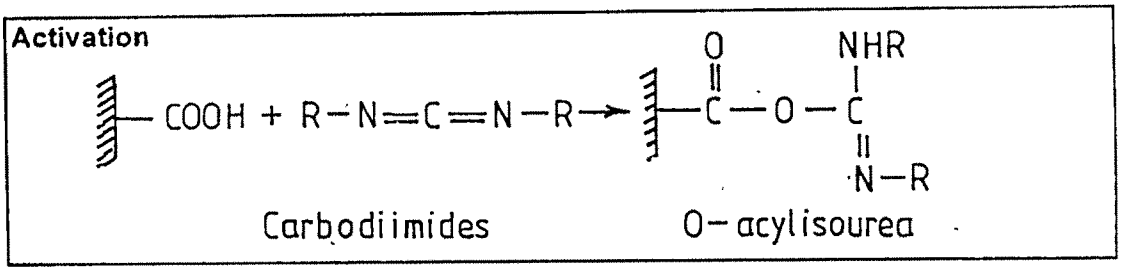
Some of the polymeric materials do not require activation step before the coupling of the enzyme. Manecke *et al* [10-12] prepared a polymer with flourinated styrene and used it for immobilization of invertase and other enzymes without activation of the support. Abdel-Hay *et al* [13] grafted m- and p- fluoro styrene onto nylon. After the nitration of polystyrene and further reduction to the amino compounds, BSA and other enzymes were coupled to the support. The coupling yield was reported to be good but the retention of enzyme activity was considerably low. Levin *et al* [14] coupled trypsin to a number of maleic anhydride/ethylene block copolymers and obtained good coupling yields (46-700 mg/g) with ~ 50% retention of enzyme activity without prior activation of the support. Similarly Manecke and Korenze [15] used maleic anhydride/N-vinyl pyrrolidine block copolymers to immobilize 348-442 mg papain/g copolymer. The retention of enzyme activity was observed to be 8-12%. Maleic anhydride copolymers are an example of the kind of polymers that do not require activation before immobilization. In this case, the reaction is as follows :

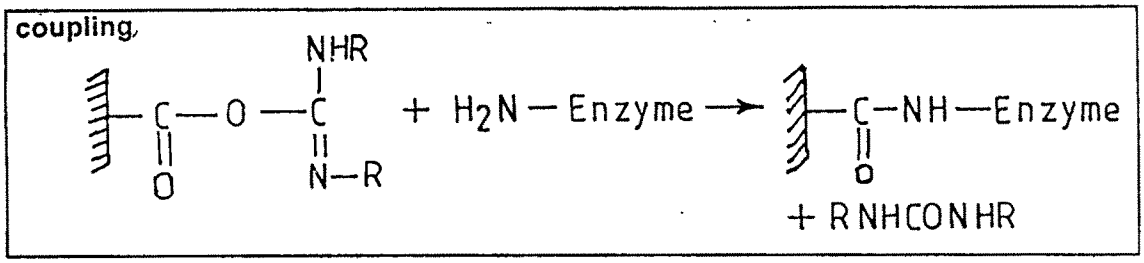


(ii) Activation of the polymers containing carboxyl groups

A 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 (Woodward's Reagent K) [16], with N-ethoxy carbonyl 2-ethoxy-1-2 dihydroquinoline [17], with thionyl chloride [18] and with carbodiimides [1]. The representative reaction between carbodiimides and carboxyl groups is given here.



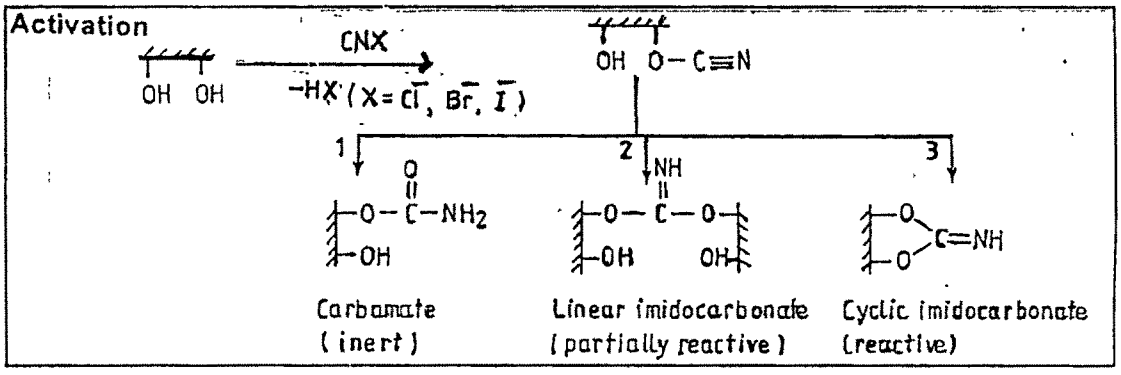


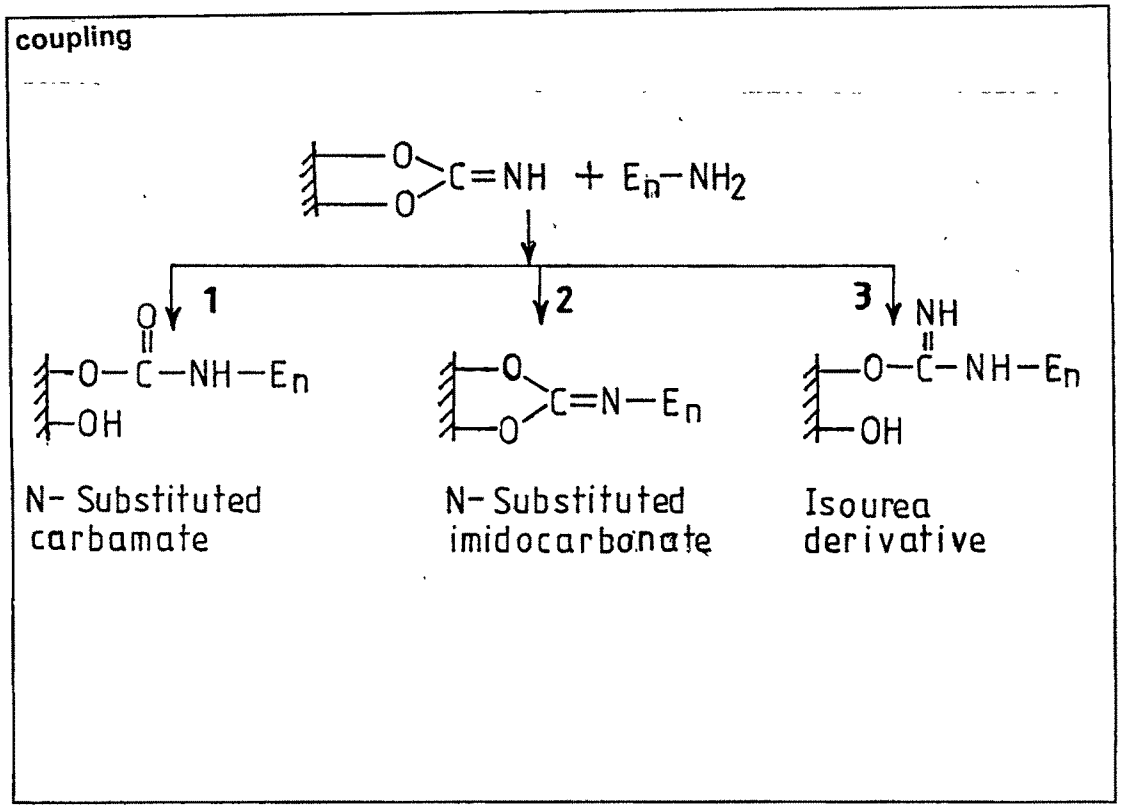
(iii) Activation of polymers containing hydroxyl groups

A wide range of methods are available for activation of polymers containing hydroxyl groups.

► **Activation with cynogen halide (chloride, bromide or iodide)**

Here the hydroxyl groups of polymeric materials are activated with cynogen bromide/chloride/iodide to form an imido carbonate intermediate which is then coupled with the enzyme as follows:





Axen *et al*[19] used this method for the immobilization of chymotrypsin, trypsin and papain on sephadex using cyanogenbromide.

► **Activation with cynuric chloride (2,4,6 trichloro s-triazine)**

A commonly employed method for the immobilization of enzymes on various types of celluloses is through the activation of the poly saccharides with cynuric chloride or some of its dichloro derivatives[20]. The coupling reaction is an alkylation reaction involving the primary amino groups of the enzyme and the activated support. In our study we have used cynuric chloride for the activation of hydroxy polymeric supports. The reactions involving hydroxy polymers with cynuric chloride activation are discussed in Section 2.4.2.c.

► **Activation with p-benzoquinone**

This method was first used in enzyme immobilization with modified poly saccharides such as sepharose and sephadex by Brandt *et al*[21]. Later on it was used by Chun *et al*[22] for the immobilization of ß-galactosidase.

The double bonds of a quinone ring structure are activated by conjugation with the carbonyl groups and react with hydroxyl groups of polymers [18]. The second conjugated pair of double bonds are left free to undergo a similar reaction with nucleophiles on the proteins. As this method is used in our study the details are discussed in Section 2.4.2.a.

► **Activation with p-tolyl sulphonyl chloride**

In 1981 Nilsson *et al*[23] used p-tolyl sulphonyl chloride 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. Since water rapidly hydrolyses tosylate this method is preferable in non-aqueous medium. This activation procedure to immobilize trypsin, peroxidase and alcohol dehydrogenase on agarose was used. They could achieve coupling of 70mg of peroxidase, and 100 mg of alcohol dehydrogenase g^{-1} of copolymer with 25% of the retention of their activity. The mechanism of p-tolylsulphonyl chloride activation is discussed in Section 2.4.2.b as we have used this method for the immobilization of enzymes.

(iv) Activation of polymers with amine groups

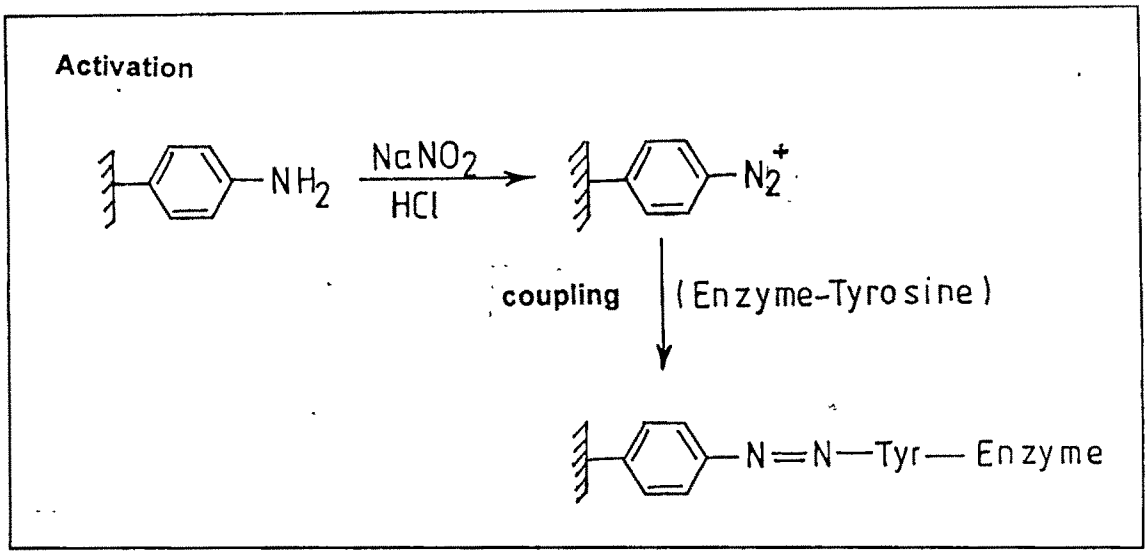
► **Activation through glutaraldehyde**

Supports containing amino groups can be activated by variety of methods such as diazotization and use of glutaraldehyde. The chemical nature of the reaction between glutaraldehyde and proteins is not clearly understood. But it is known that glutaraldehyde forms a schiff's base with amino groups. The effect of glutaraldehyde on protein immobilization has been well described by Olson and Stanley [24].

Glutaraldehyde can also be used for the crosslinking of proteins or whole cells. Griffith's and Muir [25] immobilized whole cells of *Bacillus stearothermophillus* on DEAE-cellulose using glutaraldehyde activation process. They observed 2-4 mg of dry mass of cells coupled g^{-1} of DEAE cellulose with an activity of 22 unit/g support. They also observed that the activity of the ß-galactosidase was much higher in cells covalently bound to the support than those adsorbed. They observed that the immobilized whole cells were active over a wide range of pH values and temperature. In our study we have used glutaraldehyde crosslinking for protein immobilization and the reactions involved in glutaraldehyde treatment are discussed in Section 2.4.2 d.

► **Diazotization**

Generally this method is based on the formation of a diazolinkage between enzyme protein and aryldiazonium electrophilic groups on the insoluble support. Supports containing aromatic amino groups are treated with sodium nitrite in acidic solutions and the resultant activated support reacts with aromatic residues such as the phenolic residue of L-tyrosine or imidazole residue of L-histidine to form azo derivative as shown below:



1.5.3.c Metal binding

Activation of support materials with transition metal compounds, usually titanium (IV) chloride, was originally thought to give supports containing active transition metal chelates which were able to bind enzyme molecules by replacement of metal-chloride bonds with groups present in the enzyme molecule. Immobilization of enzymes through metal binding is discussed in the Handbook of Enzyme Biotechnology by Wiseman[6].

1.5.4 Cross linking

This immobilization method is based on the formation of chemical bonds, as in the covalent binding method, but water-insoluble carriers are not used in this method. The immobilization of enzymes is performed by the formation of intermolecular crosslinkages between the enzyme molecules by means of bi or multifunctional reagents.

The cross linking reactions are carried out under relatively severe conditions as in the case of covalent binding methods. Thus, in some cases, the conformation of active centres of the enzyme may be affected by the reaction, leading to significant loss of

enzyme activity.

A cross-linking reagents glutaraldehyde (schiff's base), isocyanate derivatives (peptide bond), bisdiazo benzidine (diazo coupling), N,N'-polymethylene bis iodo acetamide (alkylation) and N,N'-ethylene bis-maleimide (peptide bond) have been used for this purpose.

Some of the selected and relevant immobilization methods are already discussed. However, it is not possible and desirable to give complete list of the references to this area because very wide range of enzymes and supports have been used for the enzyme immobilization using different techniques.

1.6 Proposed work

In the present work it is proposed to study the immobilization of hydrolases enzymes such as ß-galactosidase, urease, pepsin, trypsin and chymotrypsin on some synthetic and natural polymeric supports.

The main objectives of the work are :

(i) Establishment of conditions for the immobilization of ß-galactosidase, urease, pepsin, trypsin and chymotrypsin on the polymeric supports namely polysaccharide chitosan, crosslinked poly(vinyl alcohol), salicylic acid-resorcinol-formaldehyde resin and Amberlite-IRA-400.

(ii) The conditions to be optimized include type and concentration of crosslinking agent, pH of the coupling medium, enzyme concentration and reaction time.

(iii) Comparison of the stability of the free and immobilized enzymes towards pH, temperature, solvents, kinetics, storage and number of cycles.

(iv) Reactor studies for the exploration of potential of immobilized enzymes at industrial scales.

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