Immobilization of Enzymes

Enzymes are biological catalysts that accelerate the chemical transformations between biological macromolecules in living systems but are not themselves consumed in the reactions. Enzymes display high specificity and are not permanently modified by their participation in reactions and they may be used repeatedly for as long as they remain active. In most of the processes, enzymes are mixed with substrates in the reaction vessel and cannot be economically recovered after the reaction and are generally wasted. Enzymes can catalyze reactions in different states such as

1. individual molecules in solution
2. in aggregates with other entities, and
3. as attached to surfaces.

The attached—or "immobilized" - state has been of particular interest to the researchers to exploit enzymes for technical purposes. The term "immobilized enzymes" refers to "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". Thus it becomes imperative to use enzymes in an immobilized form so that they may be retained in a biochemical reactor for further catalysis. This is facilitated by enzyme immobilization which may be defined as the process whereby the movement of enzymes, cells and organelles in space is completely or severely restricted usually resulting in a water-insoluble form of the enzyme. Immobilized enzymes are also sometimes referred to as insolubilised, supported or matrix-linked enzymes.

Benefits of Immobilizing an Enzyme

There are a number of advantages in attaching enzymes to a solid support and a few of the major reasons are listed below:

- Multiple or repetitive use of a single batch of enzymes (catalyzing the same reaction many times)
- Immobilized enzymes are usually more stable and less likely to denature (lose their shape)
- The ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or vice versa)
- Product is not contaminated with the enzyme (especially useful in the food and pharmaceutical industries)
- In addition, there will be no enzyme left in the product at the end, therefore, purification is not necessary or we can say easy separation of the enzyme from the product
- Allows development of a multienzyme reaction system

Disadvantages of immobilized enzymes

Immobilization requires extra time, more infra structure facilities and is a highly laborious process. It invariably affects the stability and activity of enzymes. There may be a

reduction in reaction rates if enzymes cannot mix freely with the substrate; and immobilised enzymes cannot be used if one of the substrates is insoluble. Certain immobilization protocols offer serious problems with respect to the diffusion of the substrate to have an access to the enzyme.

Components of immobilization

The major components of an immobilized enzyme system are the enzyme, the matrix, and the mode of attachment of the enzyme to the matrix. The terms solid phase support, carrier, and matrix are used synonymously.

Choice of Supports

The characteristics of the matrix are of paramount importance in determining the performance of the immobilized enzyme system. In order to fully retain the biological activity, enzymes should be attached onto surfaces without affecting their conformational and functional properties. The choice of a suitable immobilization strategy is determined by the physico-chemical properties of both supporting surface and the enzyme of interest.

Ideal support properties include:

i. Physical resistance to compression,
ii. hydrophilicity,
iii. inertness toward enzymes,
iv. stability,
v. Regenerability after the useful lifetime of the immobilized enzyme,
vi. Enhancement of enzyme specificity,
vii. ease of derivatisation,
viii. biocompatibility,
ix. reduction in microbial contamination and nonspecific adsorption,
x. availability at low cost.

- The form, shape, density, porosity, pore size distribution, operational stability and particle size distribution of the supporting matrix will influence the reaction rate
- The support should be cheap, inert, physically strong and stable
- Ideally, it should:
  – increase the enzyme specificity (kcat/Km)
  – shift the pH optimum to the desired value for the process
  – discourage microbial growth and non-specific adsorption

Supports can be classified as inorganic and organic according to their chemical composition. In spite of the many advantages of inorganic carriers (e.g., high stability against physical, chemical, and microbial degradation), most of the industrial applications are performed with organic matrices.

The organic supports can be further subdivided into natural and synthetic polymers. The physical characteristics of the matrices (such as mean particle diameter, swelling behavior, mechanical strength, and compression behavior) will be of major importance for the performance of the immobilized systems and will determine the type of reactor used under technical conditions (i.e., stirred tank, fluidized, fixed beds).

In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show few diffusional limitations but have a low enzyme loading capacity. Therefore, porous supports are generally preferred because the high surface area allows for a higher enzyme loading and the immobilized enzyme receives greater protection from the environment. Porous supports should have a controlled pore distribution in order to optimize capacity and flow properties.

An excellent matrix that has been extensively used is agarose. In addition to its high porosity, which leads to a high capacity for proteins, some other advantages of using agarose as a matrix are hydrophilic character, ease of derivatization, However, an important limitation in the use of agarose and other porous supports is the high cost. This problem can be circumvented by employing reversible methods that allow matrix regeneration and re-use. The enzymes can be attached to the support via interactions ranging from reversible physical adsorption and ionic linkages to stable covalent bonds.

Enzyme immobilization methods

Enzyme immobilization methods have been classified into two types. They are

i. Irreversible enzyme immobilization and
ii. Reversible enzyme immobilization methods.

Irreversible enzyme immobilization includes covalent binding, cross-linking and Entrapment.

Reversible enzyme immobilization includes adsorption, ionic binding, affinity binding and metal binding.

Immobilization based on the formation of covalent bonds

Covalent binding is a conventional method for immobilization; it can be achieved by direct attachment with the enzyme and the material through the covalent linkage. The covalent linkage is strong and stable and the support material of enzymes includes polyacrylamide, porous glass, agarose and porous silica. An advantage of this method is that, because of the stable nature of the bonds formed between enzyme and matrix, the enzyme is not released into the solution upon use. Covalent methods for immobilization are employed when there is a strict requirement for the absence of the enzyme in the product.

The covalent reactions commonly employed give rise to enzymes linked to the support through either amide, ether, thio-ether, or carbamate bonds. Therefore, the enzyme is strongly bound to the matrix and, in many cases, it is also stabilized, however, because of the covalent

nature of the bond, the matrix has to be discarded together with the enzyme once the enzymatic activity decays.

Cross-Linking

This method is based on the formation of bonds between enzyme molecules, by means of bi-or multi-functional reagents, leading to three-dimensional crosslinked aggregates which are completely insoluble in water. The cross-linking may occur within the molecules itself, or between the other molecules and they are respectively known as intramolecular and intermolecular cross-linking. In such cases, the activity of immobilized enzymes depends on

1. Number of cross-links formed
2. Size of substrate molecule
3. Reagent used
4. Concentration of enzyme
5. Ionic strength of the solution medium and
6. Hydrogen ion concentration

Basically, there are two types of bifunctional cross-linkers

1. Homo-bifunctionals: having two or more identical functional groups, example – glutaraldehyde and 4-dinitrobenzene
2. Hetero-bifunctionals having two or more different functional groups, example – trichloro-S-triazine and 4-diisocyanate.

In cross-linking, each bifunctional reagent molecule binds to two enzyme molecules, thereby, a network of enzyme molecules linked together is produced. Glutaraldehyde is particularly useful for producing immobilized enzyme membranes for use in biosensors.

Entrapment

Enzymes are occluded in the synthetic or natural polymeric networks, it is a permeable membrane which allows the substrates and the products to pass, but it retains the enzyme inside the network. There are different approaches to entrap enzymes which involves

1. gel entrapment,
2. fibre entrapment and
3. microencapsulation.

The advantage of entrapment of enzyme immobilization is fast, cheap and only mild conditions are required for reaction process. The disadvantage is that limitation in mass transfer. The support matrix protects the enzymes from microbial contamination, proteins and enzymes in the micro environment.

In gel entrapment method, the enzyme molecules can be entrapped in the interstitial spaces of a gel lattice. It is done by polymerization of materials containing enzyme solution. Generally polyacrylamide gel is used which comprises acrylamide as monomer and N, N' Methylene –bis-acrylamide as crosslinking agent.

A method of immobilization of enzymes by entrapment within the microcavities of synthetic fibres has been developed. The advantage of this fibre entrapment method is high surface area for enzyme binding obtained using fine fibres.

Microencapsulation method in this, the enzyme molecules are capsulated within spherical semi-permeable membranes with a selective controlled permeability. Enzymes immobilized in this manner are physically contained within the membrane, whilst substrate and product molecules are free to diffuse across the membrane. This method provides the large surface area between polymeric material and the enzyme. The drawback of this method is inactivation of enzyme during encapsulation. An encapsulation technique which can be used for enzyme immobilization is the formation of liposomes, which are formed as lipid membranes, enclose the water droplets. Some of the enzyme will remain in the aqueous solution within the liposomes, while the rest will be incorporated into the membrane.

Methods of Reversible Immobilization

The use of reversible methods for enzyme immobilization is highly attractive, mostly for economic reasons because when the enzymatic activity decays, the support can be regenerated and re-loaded with fresh enzyme. The reversible immobilization of enzymes is particularly important for immobilizing labile enzymes and for applications in bioanalytical systems.

Adsorption (Noncovalent Interactions)

This is a simple method of preparing an immobilized enzyme where the enzyme molecules adhere to the surface of carrier matrix due to a combination of hydrophobic effects and the formation of several salt linkages per enzyme molecule. The interaction between the enzyme and the surface of the matrix is achieved through weak forces by salt linkage, hydrogen bonds, hydrophobic bonds, ionic bonds and van der waals forces. The materials used for adsorption are activated charcoal, alumina, ion exchange resins and this method is cheap and easy for use and the disadvantage is a weak binding force between the carrier and the enzyme.

The advantage of enzyme adsorption is minimum activation step and therefore no reagents required. Immobilization by adsorption is a mild, easy to perform process, and usually preserves the catalytic activity of the enzyme. Such methods are therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak.

Nonspecific Adsorption

The simplest immobilization method is nonspecific adsorption, which is mainly based on physical adsorption or ionic binding.

In physical adsorption, the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions; whereas in ionic bonding the enzymes are bound through salt linkages. This method is the easiest method of preparing immobilized

enzymes, being based on the physical adsorption of the enzyme molecules onto the surface of solid matrices.

Ionic binding

Ionic binding provides a slightly more specific way of attaching an enzyme to a carrier: many ion exchange resins, example DEAE- Sephadex and CM-cellulose have been used as support media. The enzyme will remain bound to the carrier provided pH and ionic strength are maintained at suitable values. The bonding involved between the enzyme and the support material is salt linkages. The nature of the forces involved in noncovalent immobilization results in a process can be reversed by changing the conditions that influence the strength of the interaction (e.g., pH, ionic strength, temperature, or polarity of the solvent).

Hydrophobic Adsorption

Another approach is the use of hydrophobic interactions. In this method, it is not the formation of chemical bonds but rather an entropically driven interaction that takes place. The strength of interaction relies on both the hydrophobicity of the adsorbent and the protein. The hydrophobicity of the adsorbent can be regulated by the degree of substitution of the support and by the size of the hydrophobic ligand molecule.

Affinity Binding

The principle of affinity between complementary biomolecules has been applied to enzyme immobilization. Two different methods are being followed in affinity immobilization. The first method is the activation of the support material which contains the coupled affinity ligand, so that the enzyme will be added. The advantage of this method is the enzyme is not exposed to any harsh chemicals conditions. In the second method, the enzyme modified to another molecule which has the ability to bind towards a matrix.

Chelation or Metal Binding

Transition metal salts or hydroxides deposited on the surface of organic carriers become bound by coordination with nucleophilic groups on the matrix. Mainly titanium and zirconium salts have been used and the method is known as “metal link immobilization”. The metal salt or hydroxide is precipitated onto the support (e.g., cellulose, chitin, alginic acid, and silica-based carriers) by heating or neutralization. Because of steric factors, it is impossible for the matrix to occupy all coordination positions of the metal; therefore some of the positions remain free to coordinate with groups from the enzymes. The method is quite simple, however, the operational stabilities achieved are highly variable and the results are not easily reproducible. The reason for this lack of reproducibility is probably related to the existence of nonuniform adsorption sites and to a significant metal ion leakage from the support. In order to improve the control of the formation of the adsorption sites, chelator ligands can be immobilized on the solid supports by means of stable covalent bonds. The metal ions are then bound by coordination and the stable

complexes formed can be used for the retention of proteins. Elution of the bound proteins can be easily achieved by competition with soluble ligands or by decreasing pH. The support is subsequently regenerated by washing with a strong chelator such as ethylene diamine tetraacetic acid (EDTA) when desired.

In metal linked enzyme immobilization, the metal salts are precipitated over the surface of the carriers and it has the potential to bind with the nucleophilic groups on the matrix. The precipitation of the metal ion on the carrier can be achieved by heating. The carrier and the enzyme can be separated by decreasing the pH, since it is a reversible process, the matrix and the enzyme can be regenerated.

Binding and entrapment methods are used for soluble enzymes. Derivatisation and without derivatisation methods are used for the enzymes that are insoluble in nature. Immobilization without enzyme derivatisation includes the use of membranes impermeable to enzyme molecules but permeable to product, and in some cases to substrate molecules and because no chemical modification occurs, the method allows the study of enzymes. Recently several reports have described chemical modification of enzyme without insolubilization, using low or high molecular weight compounds. For example, the acylation of enzymes with low molecular weight reagents can have a stabilizing effect.

Properties of Immobilized Enzymes

As a consequence of enzyme immobilization, some properties of the enzyme molecule, such as its catalytic activity or thermal stability, become altered with respect to those of its soluble counterpart. This modification of the properties may be caused either by changes in the intrinsic activity of the immobilized enzyme or by the fact that the interaction between the immobilized enzyme and the substrate takes place in a microenvironment that is different from the bulk solution. The observed changes in the catalytic properties upon immobilization may also result from changes in the three-dimensional conformation of the protein provoked by the binding of the enzyme to the matrix. These effects have been demonstrated and, to a lesser extent, exploited for a limited number of enzyme systems. Often when an enzyme is immobilized its operational stability is improved. The concept of stabilization has thus been an important driving force for immobilizing enzymes. In many cases, the observed operational stabilization is usually the result of loading an excess of enzyme, which in turn makes the process diffusion controlled. However, true stabilization at the molecular level has also been demonstrated, such as the case of proteins immobilized through multipoint covalent binding.

One of the main problems associated with the use of immobilized enzymes is the loss of catalytic activity, especially when the enzymes are acting on macromolecular substrates. This steric restriction may, in turn, change the characteristic pattern of products derived from the macromolecular substrate. There are several strategies to avoid these steric problems such as selection of supports composed by networks of isolated macromolecular chains, careful choice of the enzyme residues involved in the immobilization, and use of hydrophilic and inert, longer spacer arms.

Applications of immobilized enzymes

Immobilized enzymes have been widely used in the processing of variety of products. New strategies are continuously emerging for the formation of diverse immobilized enzymes having superior efficiency and usage.

Immobilized enzymes have immense potential in the analysis of clinical, industrial and environmental samples. Immobilized enzymes and proteins have been tremendously used in antibiotic production, drug metabolism, food industry, biodiesel production and bioremediation. The success of the vast usage of immobilized enzymes lies in the fact that they prove to be environmental friendly, cheaper and easy to use when compared to other parallel technologies. Biomedical Application: Immobilized enzymes are used in medicine from 1990 for diagnosis and treatment of diseases in the medical field. The inborn metabolic deficiency can be overcome by replacing the encapsulated enzymes (i.e, enzymes encapsulated by erythrocytes), the RBC acts as a carrier for the exogenous enzyme drugs and the enzymes are biocompatable in nature, hence there is no immune response. The enzyme encapsulation through the electroporation is an easiest way of immobilization in the biomedical field and it is a reversible process for which enzyme can be regenerated. The nanoparticles and nanospheres are often used as enzyme carriers for the delivery of therapeutic agents.

Food industry Application:

In food industry, the purified enzymes are used but during the purification process, the enzymes will denature. Hence the immobilization technique makes the enzymes stable. The immobilized enzymes are used for the production of syrups. Immobilized beta-galactosidase used for lactose hydrolysis in whey for the production of baker's yeast.