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Immobilized enzymes

Definition:

Immobilized enzymes have been defined as enzymes that are physically confined or localized, with retention of their catalytic activity and which can be used repeatedly and continuously.

OR

It is the imprisonment of an enzyme in a distinct phase that allows exchange with, but is separated from the bulk phase in which the substrate, effector or inhibitor molecules are dispersed and monitored. Imprisonment refers to arresting the enzyme by certain means where polymer matrix is formed. The first commercial application of immobilized enzyme technology was realized in 1969 in Japan with the use of *Aspergillus oryzoe* amino acyclase for the industrial production of L-amino acids. Consequently, pilot plant processes were introduced for 6-amino penicillanic acid (6 APA) production from penicillin G.

In some cases, these biocatalysts are bound to or within insoluble supporting materials (carriers) by physical or chemical binding. In other cases, biocatalysts are free but confined to limited spaces of supporting materials (entrapment).

History:

In 1916, Nelson & Griffen observed that yeast invertase adsorbed on charcoal was able to catalyze the hydrolysis of sucrose. Later Bernfeld and WAN described the entrapment of trypsin, papain, amylase and ribonuclease in polyacrylamide gel in 1963. The first industrial application of immobilized enzyme microbial cells was performed in 1973.

The first immobilized enzymes to be scaleded up for industrial production are immoblized amino acid acylase, Penicillin G acylase and glucose isomerase. Some other industrially important enzymes produced by this technique are aspartase, estrase and nitrilase.

Advantage of Immobilized Enzymes:

The advantages of immoblized enzymes are as follow:

1. They can be reused.
2. The production is less labour intensive.
3. The contineous use is possible.
4. There are less chances of contamination with their use.
5. The reaction time required is minimized.
6. The saving in capital cost is possible.
7. High enzyme:substrate ration can be achieved.

8. The are more stable as compared to conventional means.
9. Improved process control is a reality.

Methods of Enzyme Preparation:

Different methods have been discovered for immobilization of enzymes.

All these methods can be categorized in two different techniques:

A. Immobilization in a Support
B. Immobilization on a Support

A. Immobilization in a support:

1) Entrapment
2) Microencapsulation

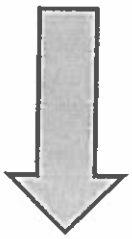
1) Entrapment:

In this technique, enzyme is entrapped within a cross-linked polymer matrix. Enzyme is dissolved in a solution of polymer and then polymerization initiated, enzyme is physically entrapped within the matrix and it cannot escape permeation. However, substrate molecules can diffuse in and can be enacted upon by the enzyme and the product molecules can diffuse out of the matrix.

Acrylamide + N-N methylene biacrylamide + enzyme

Pot. Persulphate (initiator)

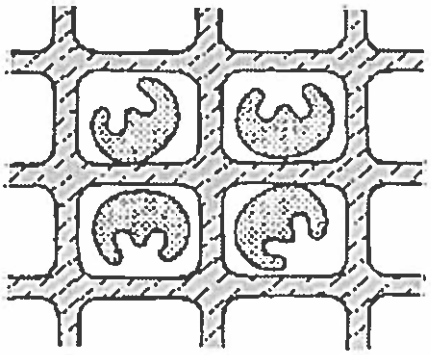
3- dimethyl aminopropionitrile (stimulant)



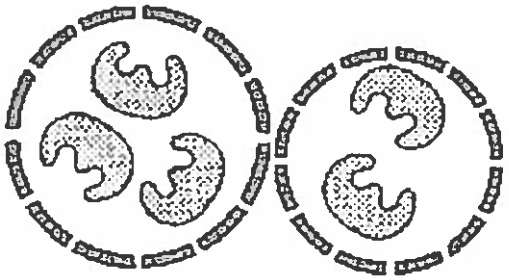
Polymer + enzyme

(Enzyme entrapped within polymer)

Others examples of polymers are starch, agar, alginate gel.



entrapped in a matrix



entrapped in droplets

Disadvantage:

There will be continuous leakage of enzymes. Some of the enzyme activity is lost due to free radicals produced during polymerization.

2) **Microencapsulation:**

In this method, enzyme is immobilized by enclosing enzymes in a semi permeable membrane capsule.

Once encapsulation is done, the enzyme cannot escape whereas low molecular weight substrate and products can diffuse through the membrane.

Composition of capsule :

Capsule may be made up of either permanent material like nylon or biodegradable material like polyacetic acid.

Methods of microencapsulation:

- Interfacial polymerization method
- Liquid drying
- Phase separation

Interfacial polymerization:

Enzymes are enclosed in semipermeable membrane of polymer. An aqueous mixture of the enzyme and hydrophilic monomer emulsified in a water immiscible organic solvent. Then same hydrophilic monomer is added to the organic solvent by stirring. Polymerization of monomer occurs at the interface between aqueous and organic solvent phases in the emulsion.

As a result, enzyme in the aqueous phase is enclosed in membrane of polymer.

Liquid drying:

An aqueous solution of enzyme is dispersed in organic phase to form first emulsion of w/o type.

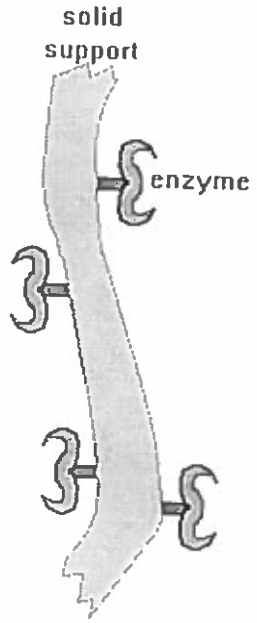
The first emulsion containing aqueous micro droplets is then dispersed in an aqueous phase containing protective colloidal substances i.e. gelatin. Secondary emulsion is prepared in microcapsules.

Phase separation:

Dissolve polymer in an organic solvent, re-precipitate it by adding another organic solvent which is miscible with first but which does not dissolve polymer.

B. Immobilization on a support: (also known as carrier binding)

The carrier binding method is the oldest immobilization technique for enzymes. In this method, the amount of enzyme bound to the carrier and the activity after immobilization depend on the nature of the carrier. The following picture shows how enzymes are bound to the carrier.



The selection of the carrier depends on the nature of the enzyme itself as well as

Particle size

Surface area

Molar ratio of hydrophilic to hydrophobic groups

Chemical composition:

In general, an increase in the ratio of the hydrophilic groups and in the concentration of bound enzymes results in a higher activity of the immobilized enzymes. Some of the most commonly used carriers for enzyme immobilization are polysaccharides derivatives such as cellulose, dextran, agarose and polyacrylamide gel.

According to the binding mode of the enzyme, the carrier-binding method can be further sub-classified into:

- Adsorption
- Ionic bonding
- Covalent bonding
- Cross-linking or copolymerization

- **Adsorption:**

This method for the immobilization of an enzyme is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. An enzyme may be immobilized by bonding to either external or internal surface of a carrier or support e.g. mineral support (aluminium oxide, clay), organic support (Starch), modified sapharose and ion-exchange resins.

Bonds of low energy are involved e.g. ionic interactions, hydrogen bonds, van der Waals forces etc.

The earliest example of enzyme immobilization using this method is the adsorption of beta-D-fructo-fruanosidase onto aluminium hydroxide.

If the enzymes is immoblized externally, the carrier particle size must be very small (500 angstrom to 1mm) in order to achieve the an apppreciable surface of bonding. In this technique of external immoblization, no pore diffusion limitations are encountered.

The enzymes immoblized on an internal surface is proteced from abrasion, inhibitory bulk solutions and microbial attack, and a more stable and active enzyme system can be achieved. The pore diameters of carriers may be optimized for internal surface immoblization.

The processes available for physical adsorption of enzymes are:

- **Static process:** The solution containing the enzyme is allowed to contact the carrier without stirring for immobilizing the enzyme.

- **Dynamic batch process:** In this process the carrier is placed into the enzyme solution and mixed by stirring or agitated contineously in a shaker.
- **Electro deposition process:** The carrier is placed proximal to on of the electrodes in an enzyme bath, and the current is put on. The enzyme migrates to the carrier, and it is deposited on the surface and immoblization takes place.
- **Reactor loading process:** The carrier is placed into the reactor, then the enzyme solution is transferred to the reactor and the carrier is loaded in a dynamic enviornment by agitation the carrier and the enzyme solution together.

- **Covalent Bonding:**

Covalent bond is formed between the chemical groups of enzyme and chemical groups on the surface of the carrier/ support. Covalent bonding is thus utilized under a broad range of pH, ionic strength and other variable conditions. Immobilization steps are:

1. Attachment of coupling process followed by an activation process
2. Attachment of functional group
3. Attachment of the enzyme

Types of Carrier:

Different types of carriers are used in immobilization such as carbohydrates, proteins, amine bearing carriers, inorganic carriers etc. Covalent attachment may be to specific group e.g. amine, hydroxyl, tyrosil etc. on the surface of the enzyme. Hydroxyl and amino groups are the main groups of the enzymes with which it forms bonds, whereas sulphydryl groups least involved.

When trying to select the type of reaction by which a given protein should be immobilized, the choice is limited by two characteristics; (1) the binding reaction must be performed under conditions that donot cause loss of enzymatic activity and(2) the active site of the enzyme must be unaffected by the reagents used.

Functional group added to enzymes:

The covalent binding method is based on the binding of enzyme and water insoluble carriers by covalent bonds. The functional groups that may take part in this binding are listed below:

- Amino group
- Hydroxyl group
- Thiol group
- Carboxyl group
- Imidazole group
- Tareonine group
- Sulfhydryl group

- Phenolic group
- Indole group

This method can be further classified into diazo peptide and alkylation methods according to the mode of linkage. The conditions for immobilization by covalent bonding are much more complicated and less mild than in cases of physical adsorption and ionic binding. Therefore the covalent binding may alter the conformational structure and active center of enzyme, resulting in major loss of activity and/or changes of the substrate. However, the binding force between enzyme and carrier is so strong that no leakage of the enzyme occurs, even in the presence of substrate or solution of high ionic strength.

Hence covalent bonding can be brought about by the following

- Diazotitation — Support N=N enzyme
- Amide bond formation — Support CO-NH enzyme
- Alkylation and arylation — Support CH_2-NH enzyme
- Schiff's base formation s — Support CH_2-S enzyme
- Amidation reaction — Supports CH=N enzyme
- Thiol-disulfide interchange — Supports S-S enzyme
- UGI reaction
- Mercury enzyme interchange
- Gamma irradiation induced coupling
- Carrier binding with bifunctional reagents — Support $O(CH_2)_2$ $N=CH(CH_2)_3$ CH=N-Enzyme

The active sit of the enzyme must not be hindered. There must be an ample space between the enzyme and the backbone. Enzymes can be covalently bound to polymer by many methods this can be done by two ways

1. Through reactive groups or the side chains of its amino acids such as lysine, arginine and tyrosine.
2. Through terminal amino and carboxyl group of polypeptide

Advantages:

1. Because of tight binding they don't leak or detach from the support during utilization
2. Immobilized enzyme easily come in contact with substrate because enzymes are localized o the surface of support.
3. Not affected by the pH.

Disadvantages:

1. Strong interaction between support and enzyme often hinders the free movement of enzyme resulting in decreased activity.
2. Supports are not renewable.

- **Ionic Binding**

The ionic binding method relies on the ionic binding of the enzyme protein to water insoluble carriers containing ion exchange residues.

Polysaccharides and synthetic polymers having ion exchange centers are usually used as carriers. 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 cause little changes in the conformation and the active site of the enzyme. Therefore this method yields immobilized enzymes with high activity in most cases.

The main difference between ionic binding and physical adsorption is that the enzyme to carrier linkages are much stronger for ionic binding, although weaker than covalent binding.

3) **Cross linking or copolymerization:**

Cross linking is characterized by covalent bonding between the various molecules of an enzyme via a polyfunctional reagent such as glutaraldehyde. diazonium salt, hexamethylene disocyanate and N-N ethylene bismaleimide. The demerits of using polyfunctional reagents are that they can denature the enzyme.

Three techniques are used for immobilization of enzymes by cross linking

1. Cross linking of enzyme with glutaraldehyde to form an insoluble aggregate.
2. Adsorption of enzyme onto a surface followed by cross-linking.
3. Introduction of enzyme in polymer matrix and then cross-linking the enzyme within the process.

The choice of method of immobilization varies with the enzyme and also with the application of immobilized enzyme.

Advantages:

1. It is an economical method.
2. It is a simple method.
3. It is widely used in commercial preparation.

Cell Immobilization:

This technique is now well developed and successfully used for industrial – scale production, in cell immobilization technology, the enzymes are active and stable for a long period of time.

The method of cell immobilization is same as described for enzyme immobilization, involving the processes of the adsorption, covalent bonding, cell to cell cross linking, encapsulation and entrapment in polymeric network. Since a long time, adsorption of cells to preformed carrier has been undertaken. The woodchips are used as carrier for *Acetobacter* in production of vinegar since 1823.

Whole-cell immobilization

Support Material	Cell	Reaction
Gelatin	Lactobacilli	Lactose
Glass	Saccharomyces	Glucose/ethanol
Cotton	Zymomonas mobilis	Glucose/ethanol
Cellulose	Nocardia erythropolis	Steroid Conversion
Carboxy methyl cellulose (CMC)	Bacillus subtilis	L- Histidine/ uronic acid
Diazotized diamines	Streeptomyces	Glucose/fructose
Glutaraldehyde	E. Coli	Fumaric acid/L-aspartic acid
Chitosan	Lactobacillus brevis	Glucose/ethanol
Aluminium alginate	Candida topicalis	Phenol degradation
Calcium alginate	Saccharomyces cerevisiae	Glucose/ethanol
Carrageenan	E. Coli	Fumaric acid/L-aspartic acid
Polyester	Streeptomyces sp.	Glucose/fructose
Alginate-polylysine	Hybridoma Cells	Monoclonal antibodies

Application of enzymes

Enzymes in general are limited in the number of reactions they have evolved to catalyze and also by their lack of stability in organic solvents and at high temperatures. Consequently protein engineering is an active area of research and involves attempts to create new enzymes with novel properties either through rational design or in vitro evolution.

The enzymes and cells have wide spectrum of applications that can be grouped into four broad categories

1. Therapeutic
2. Analytical
3. Manipulative

4. Industrial

Therapeutic Applications:

Some inborn errors of metabolism occurred due to missing of enzymes where specific genes are introduced to encode specific missing enzymes. Certain diseases are treated by administering the appropriate enzyme e.g. virilzation of a disease developed due to loss of hydroxylase enzyme from adrenal cortex and introduction of hydroxyl group (-OH) on 21-carbon in ring structure of steroid hormone. The missing enzyme synthesizes aldosterol in excess leading to masculinization of female baby and precocious sexual activity in males within 5-7 years. Similarly treatment of leukemia is possible by administering asparaginase of bacterial origin.

Analytical Applications:

The enzymes are used in kinetic analysis. End point analysis refers to total conversion of substrates into products in a few minutes in the presence of enzymes whereas kinetic analysis involves the rate of reaction in substrate product concentration. The analysis of anti bodies and immunoglobulins necessary for human use is also possible with the enzymes. The usable enzymes are alkaline phosphatase, β-galactosidase, lacatamase etc. another important utility of enzyme is in bio sensors. It is a device of biologically active material displaying characteristic specificity with chemical or electronic sensor to convert an electrode, oxygen electrode or a photocell may be used as a biosensor.

Manipulative Applications:

A variety of enzymes isolated from different sources are used in genetic engineering as one of the biological tool.

Industrial Application:

The industrial use of enzyme maybe broadly categorized as follows

1. **Detergent industry:** The stains on cloth can be easily removed by adding proteolytic enzyme to the detergent. The enzyme attacks on peptide bonds and therefore dissolves protein. The alkaline serine protease obtained from *B. licheniformis* is commonly used in manufacture of detergent in addition the serine protease of *Bacillus amyloliquefacians* that contains α-amylase is also extensively used.
2. **Starch industry:** Currently various enzymatic processes are successfully applied for different products. Glucose isomerase is an important enzyme used commercially in conversion of glucose to fructose via isomerization. Fructose is used for the fructose syrup preparation. The enzyme glucose isomerase is widely used in production of fructose syrup.

The reaction mixture at the end contains 42% fructose, 52% glucose and 6% dextrins. The mixture is sweeter than glucose and as sweet as sucrose. The technique has been developed to obtain 55% fructose concentration in syrup.

3. **Rubber industry:** Catalase is used to generate oxygen from peroxide to convert latex into foam rubber.
4. **Photographic industry:** Protease (ficin) dissolves gelatin of scrap film allowing recovery of its silver content.
5. **Dairy industry:** For a long time calf rennet has been used in dairy industry. In recent years calf rennets are replaced by microbial rennets (e.g. *Mueor michei*) that are acid aspartate proteases. Lactase produced by *Bacillus stearothermophlius* is used for hydrolysis of lactose in milk. The enzyme lipase is used for flavor development in cheeses.
6. **Brewing industry:** The enzymes commonly used in brewing industry are α-amylase, β-gluconase and protease that are required for malt in substitution of barley. The biological sources for these enzymes is *B. amyloliquefaciens.* The neutral protease helps In the inhibition of alkaline protease by an inhibitor β-Glucanase that takes care of filtration problems due to poor quality of malt.
7. **Wine industry:** The pectic enzymes are used in wine industry for high yields with improved quality. The peptic enzymes give good results when combined with other enzymes e.g protease glucoamylase.
8. **Pharmaceutical industry:** The enzyme penicillin ON acylase is widely used in the production of semisynthetic penicillins. All pencillins consists of an active β-lactam ring i.e. 6-aminopenicillinic acid (6-APA) group combined with different side chains (R group). The enzyme penicillin ON acylase removes ON group resulting in the separation of 6APA and R groups. Finally new synthetic side chains are coupled with 6-APA to synthesize new semi synthetic penicillins.

Reactions:

The *E. coli* strains are the most explored and exploited ones for the production of penicillins G acylase. The biosynthesis of penicillin acylase in *E. coli* is controlled by alterations in concentrations of nutrients and culture conditions. Sudhakaran and Berkar (1989) reported the effects of growth substrate, inducers and regulators on formation of enzyme. The strain *E. coli* NCJM-2400 produced penicillin G acylase intracellularly when grown in nutrient

Both the growth and enzyme biosynthesis whereas glucose, sorbitol, lactose, acetate and lactate (All 0.1%) catabolically repressed the enzyme formation.

Penicillin G acylase occurs in fungal and actinomycetes sources however its activity has also been reported in many bacteria such as *Bacillus sphaericus*, *Erwinia aroideae* and *Pseudomonas acidovorans*. Lowe et al.(1986) have identified a strain of *Fusarium oxysporum* that exhibited intracellular penicillin V acylase activity which was induced by phenoxyacetic acid in culture. The enzyme was partially purified and concentrated from disrupted cells (cells hydrolyzed with 5% penicillin V solution) by fractional precipitation with miscible solvents.

Paper industry: Amylases, xylanases, cellulases and lignilases, degrade starch to lower viscosity aiding sizing and coating paper. Xylanases reduces the bleach required for the decolourizing. Cellulases smoothens fibers, enhance water drainage and promote ink removal. Lipases reduce pitch and lignin degrading enzymes remove lignin to soften paper.

Biosensors

A biosensor is an analytical device consisting of an immobilized layer of biological mater in an intimate contact with a transducer i.e. sensor that analyzes the biological signals and converts them into an electrical signal (Gronow, 1984). A sensor can be a single carbon electrode, an ion sensitive electrode, a photocell, an oxygen electrode or a thermistor.

The principle of biosensor is simple. The biological material is immobilized on the immobilization support permeable membrane. The substance to be measured passes through the membrane and interacts with the immobilized material to yield the product. A product (i.e the product monitored substrate) may be gas (oxygen), heat, hydrogen ions, electrons or ammonium ions. The product passes through another membrane to transducers. The transducers converts product into electrical signals which is them amplified. The signal processing equipment converts the amplified signal into a display most commonly the electric signal which can be read out and recorded.

The glucose electrode is built up by immobilizing glucose oxidase in poly acrylamaide gel around a platinum oxygen electrode which is separated by a teflon membrane. Potassium chloride solution surrounds the platinum oxygen electrode. From the upper surface glucse oxidase is intimately covered by a cellulose acetate membrane when glucose solution is brought in contact with membrane glucose and oxygen pass through the membrane into the enzyme layer and as a result of oxidation reduction reaction convert into gluconic acid and hydrogen per oxide in presence of water, oxygen and glucose oxidase. Consequently concentration of oxygen in the gel around the electrode is lowered down. Hydrogen peroxide brings about a change in the current i.e measurable signal. The electrode records the rate of the reaction. The rate of diminution of oxygen concentration is proportional to glucose concentration of the sample. It responds linearly to glucose concentrations over a range of 10^{-1} to 10^{-5} mol/dm^3 with a