Immobilization of Enzymes and Biotechnological Perspectives

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Introduction

**Immobilized enzymes are the enzymes either covalently bound or adsorbed on to the surface of an insoluble support or incorporated in a gel matrix.** In other words, immobilized enzymes have restricted movement in space either completely or to a small limited region.

The first Enzymes Engineering Conference was held at Henniker, New Hampshire, USA in 1971. The main theme of this conference was immobilized enzymes. The definition and classification of immobilized enzymes were proposed at the conference. Before that time, various terms such as "water-insoluble enzyme", "trapped-enzyme", "fixed-enzyme" and "matrix-supported enzyme" had been used. In that conference immobilized enzymes were 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". 
WHY IMMOBILIZED ENZYMES ARE IMPORTANT?

Immobilized enzymes have several advantages over the soluble enzymes. The immobilized enzyme can be easily removed from the reaction making it easy to recycle (reuse) it in the reaction. Since, immobilized enzyme can be removed from the reaction mixture, therefore, reaction mixture does not get contaminated with the enzyme protein and contains only the solvent and reaction products. Besides, it has been found that immobilized enzymes typically have greater thermal and operational stability than the soluble form of the enzyme. A greater variety of engineering designs for a continuous process can also be made with immobilized enzymes and they also show greater efficiency in constructive multi-step reactions. Immobilized enzymes offer the opportunities to reduce the cost of biologically active catalysts (enzymes). Therefore, use of immobilized enzymes makes the reaction product(s) more economical.
If we talk of chemical synthesis of a compound, it is awesome and uneconomical. Besides, there are certain compounds, that can not be synthesized by chemical process. On the other hand, immobilized enzymes offer promising results in the production of many compounds at low cost and enzyme immobilization has become one of the vital branch of Biotechnology now-a days. Enzymes are biological catalyst consisting of proteins, that participate in many chemical reactions occurring in living things. Enzymes have been used by human beings since ancient times, well before their nature was understood. The use of enzymes has gradually been extended into variety of fields, such as brewing, food production, textiles, tanning and medicine. Further the recent development of biochemistry has resulted in the clarification of the mechanisms of enzyme reactions and the development of new enzyme sources, and, together with progress in applied microbiology, has greatly extended the range of application of enzymes. The enzymes are produced by the living organisms for their own requirements, and are efficient and effective catalysts. These are not always ideal for practical applications. Some of the advantages of the enzymes may even be disadvantages in practical use as catalysts. Enzymes are generally unstable and can not be used in organic solvents or at elevated temperature.
Conventionally, enzyme reactions have been carried out in batch processes by incubating a mixture of the substrate(s) and the soluble enzyme. It is technically very difficult to recover active enzyme from the reaction mixture after the reaction for reuse. Accordingly, the enzyme and other contamination proteins are generally removed by denaturation by changing pH or heat-treatment during procedures to isolate the product from the reaction mixture. This is uneconomical, as active enzyme is lost after each batch reaction.

To eliminate the disadvantages inherent in ordinary chemical catalysts and enzymes, and in order to obtain superior catalysts for applications, that is highly active and stable catalysts having appropriate specificity, two approaches have been investigated. One is the synthetic approach, using recently developed techniques of organic synthesis and polymer chemistry to synthesize catalysts having enzyme-like activities. These catalysts are sometimes called "synzymes". Another approach is the modification of the enzymes produced by organisms. Immobilization of the enzymes is a part of the latter approach.
If active and stable water-insoluble enzymes that is immobilized enzymes having appropriate substrate specificity are prepared, most of the above disadvantages are eliminated and it becomes possible to use enzymes conveniently in the same way as ordinary solid catalysts used in synthetic chemical reactions. In addition, since enzymes can catalyze specific reactions under mild conditions, (normally temperature and pressure), application of the immobilized enzymes in the synthetic chemical industry can reduce energy requirements.

Immobilized enzymes are considered to be the modifications of the enzymes and should also be useful in clarifying the relationship between protein structure and enzyme activity or reaction mechanism. Many enzymes are known to be bound to cell membranes or cellular particles in the cell. Immobilized enzymes are considered to be models of these bound enzymes and are becoming the subject of extensive academic interest. Immobilization of enzymes may also provide stable and active enzyme system by change in physical and chemical properties of the enzymes.
For the first time in 1916, Nelson and Griffin reported that invertase enzyme extracted from yeast was adsorbed on charcoal and showed the same activity as native enzyme. In 1948, Sumner found that urease from jack bean became water-insoluble on standing in 30% alcohol and sodium chloride for 1 to 2 days at room temperature and the water–insoluble urease was active. Thus it has been known for a long time that enzymes in water in water-insoluble form showed the catalytic activity. In 1953, for a particular application, Grubhofer and Schleith immobilized carboxypeptidase, diastase, pepsin and ribonuclease on diazotized polyaminopolystyrene resin. On the other hand, prior to this in 1949, immobilization of physiologically active protein was carried out by Micheel and Ewers. Further, in 1951, Campbell and his colleagues prepared immobilized antigen by binding albumin to diazotized p-aminobenzyl cellulose.

Israel and Japan have carried out extensive studies on immobilization of enzymes and their physical and chemical properties. For the first time in the world, immobilized aminoacylase was shown industrially important for the optical resolution of DL-amino acids.

In context of immobilization, enzymes have been categorized into two groups namely native enzymes and modified enzymes. The modified enzymes have been further categorized as entrapped enzymes and bound enzymes. Both entrapped and bound enzymes have been further classified into matrix entrapped and microencapsulated, and adsorbed and covalently bound, respectively. The following line diagram shows the above mentioned groupings of the enzymes.
Many techniques have been developed for the immobilization of enzymes depending upon the nature and purpose of the enzyme catalyzed reactions.
Immobilized enzymes have been divided into three groups namely carrier bound, cross linked and entrapped. Carrier bound immobilized enzymes have been further divided into physically adsorbed, ionic bound and covalent bound depending upon the nature of binding the enzyme on the solid support. The entrapped enzymes have been divided into lattice type and microencapsulated depending on the nature of entrapment.
For immobilization of an enzyme, it is necessary that functional groups in the active site should not be involved in the reaction leading to inactivation of the enzyme. Since tertiary structure of the enzyme protein is maintained by relatively weak binding forces like hydrogen, hydrophobic and ionic bonds, therefore, it is necessary to carry out the immobilization reaction under mild conditions. Reactions at high temperature and strong acid or alkali treatments must be avoided to preserve the structural integrity of enzymes. Even treatment with organic solvents or high salt concentrations may cause denaturation and loss of activity.
Methods of Immobilization of enzymes

Carrier binding method

It is the method of binding enzymes to water-insoluble carriers. In fact, this is the oldest immobilization technique. When enzymes are immobilized in this way, care is taken regarding the selection of carriers as well as binding techniques. Generally, the amount of the enzyme bound to the carrier and the activity after immobilization depends markedly on the nature of the enzyme itself and therefore some aspects must be considered. These are a) Particle size; b) surface area; c) molar ratio of hydrophilic to hydrophobic groups; and d) chemical composition. As carriers for enzyme immobilization, polysaccharide derivatives such as cellulose, dextran, agarose and polyacrylamide gel are most commonly used. Some are available commercially under trade names such as Sephadex, Sepharose, Bio-gel and porous glass.

The carrier-binding method can be further divided into physical adsorption, ionic binding and covalent binding methods depending upon the binding mode of the enzyme.
Physical absorption method

This method is based on the physical adsorption of enzyme protein on the surface of water-insoluble carriers. The method often causes little or no conformational change of the enzyme protein or destruction of its active center. Though this is very simple method, it has the disadvantage that the adsorbed enzyme may leak from the carrier during use because binding forces between the enzyme protein and carrier are weak.

In our laboratory, protocols have been designed and optimized for physical absorption method. Starch phosphorylase from sorghum leaves after partial purification using ammonium sulfate fractionation and subsequently desalting through gel filtration chromatography was immobilized on alumina. To 2 gm of alumina suspended in 12 ml of Buffer A (0.05M Tris-HCl buffer, pH 7.0), 2.5 ml of the enzyme preparation having 12 mg protein was added and kept on a shaker with 75 rpm at 5oC for one hour. After decantation, the residue was washed with Buffer A until no protein was detected in the washings. The residue was suspended in 10 ml of Buffer A. Upon immobilization on alumina, there was about 28% retention of the enzyme activity (Venkaiah and Kumar, 1994).
Our laboratory has also designed and optimized for physical absorption on egg shell using starch phosphorylase from cabbage leaves. Egg shells were broken into small pieces and kept in boiling water for 15 minutes. After decantation of water, washed several times with acetone and dried in an oven at 60°C. The pieces were then crushed to mesh 100. Two gm of prepared egg shell was added to 8 ml of the partially purified enzyme preparation (2.4 units, 108 mg protein) with stirring. The suspension was incubated for four hours with gentle stirring. The pellet obtained after centrifugation under the cold condition was washed with buffer A (0.01 M Tris-HCl, pH 7.5 containing 20 mM 2-mercaptoethanol and 0.05 M EDTA) until no protein was detected in the washings and then suspended. Upon immobilization on egg shell there was about 56% retention of the enzyme activity (Garg and Kumar, 2007). Earlier Chatterjee et al (1990) also described immobilization of goat liver catalase on egg shell where Anil Kumar was also a co-author.
Our laboratory has also designed and optimized for physical absorption on egg shell using pectinmethylesterase from germinating seeds of *Vigna sinensis*. Pieces of hen egg shell were boiled in water for 30 minutes, washed 4-5 times with acetone and dried in oven at 60°C. Thereafter, the pieces were crushed to mesh 100. Two gm of crushed egg shell were added to 10 ml of partially purified pectinmethylesterase preparation containing 46 mg protein in 10 mM Tris-HCl, pH 7.5 and stirred for 12 hours at 4°C. The residue obtained after centrifugation under cold conditions at 5000 x g for 10 minutes was washed with 10 mM Tris-HCl, pH 7.5 until no protein was detected in the washings and then suspended in 20 ml of 10 mM Tris-HCl, pH 7.5. Upon immobilization on egg shell there was about 24% retention of the enzyme activity. However, when immobilization was carried out at pH 7.0 instead of 7.5, there was about 56% retention (Nighojkar et al., 1995). Using the same procedure, we also immobilized endo-polygalacturonase from germinating *Vigna sinensis* on egg shell. With endo-polygalacturonase, crushed egg shell was suspended in 6 ml of 10 mM sodium acetate, pH 5.0 containing 0.02% sodium metabisulfite and 4 ml of the partially purified enzyme preparation having 45 mg protein was added. Upon immobilization on egg shell there was about 14% retention of the enzyme activity (Nighojkar et al., 1996).
**Ionic binding method**

This method is based on the ionic binding of enzyme protein to water-insoluble carriers containing ion-exchange residues. In some cases not only ionic binding but also physical adsorption may take part in the binding. As carriers for ionic binding, polysaccharides and synthetic polymers having ion-exchange residues are used. The binding of enzyme to the carrier is easily carried out and the conditions are mild compared to those necessary for the covalent binding method. Therefore, the ionic binding method causes little or no changes in conformation and also in the active site of the enzyme protein and yields immobilized enzymes having high activity in many cases. As the binding forces between enzyme protein and carriers are lesser strong than in covalent binding, leakage of the enzyme from the carrier may occur in substrate solutions of high ionic strength or upon variation in pH. In 1956, for the first time, catalase was immobilized on DEAE Cellulose.

Our laboratory has also designed and optimized for immobilization of starch phosphorylase from Bengal gram seeds on DEAE cellulose. DEAE cellulose (3gm) was regenerated and equilibrated in 0.02 M Tris-HCl, pH 7.6 and suspended in 30 ml of the same buffer. To it, 16 ml of the partially purified enzyme preparation (91 mg protein) was added and the suspension was slowly stirred for 2 hours in the cold condition. The residue obtained after centrifugation at 7000 x g for 10 minutes in cold condition was washed with 0.02 M Tris-HCl, pH 7.6 until no protein was detected in the washings and then the residue was suspended in 100 ml of 0.02 M Tris-HCl, pH 7.6. Upon immobilization on DEAE cellulose, there was about 79% retention of the enzyme activity (Upadhye and Kumar, 1996).
Covalent binding method

The covalent binding method is based on the binding of enzymes and water insoluble carriers by covalent bond. The functional groups that take part in the covalent binding of enzyme to carrier are a) $\alpha$-or $\varepsilon$-amino group; b) $\alpha,\beta,\gamma$-carboxyl group; c) sulfhydryl group; d) imidazole group; e) phenolic group; f) hydroxyl group; g) indole group. In coupling reactions, these functional groups react with carriers containing reactive groups such as diazonium, acid azide, isocyanate and halides. This method can be further classified into diazo, peptide and alkylation methods according to the mode of linkage. The selection of conditions for immobilization by covalent binding is more difficult than in the case of physical adsorption and ionic binding. The reaction conditions required for covalent binding are relatively complicated and not mild. Therefore, in some cases, covalent binding alters the conformational structure and active center of the enzyme. However, the binding force between the enzyme protein and carrier is very strong and leakage of the enzyme does not occur even in the presence of substrate or salt solutions of high ionic strength.
Covalent binding to a support matrix should involve only functional groups of the enzyme that are not essential for catalytic activity. Higher activity results from prevention of inactivation reactions with amino acid residues of the active sites. The following protective methods have been suggested:

- Covalent attachment of the enzyme in the presence of a competitive inhibitor or substrate.
- A reversible, covalently linked enzyme inhibitor complex.
- A chemically modified soluble enzyme whose covalent binding to the matrix is obtained by newly incorporated residues.
- A zymogen precursor.

The active site of the enzyme should not be blocked. There must be sufficient space between the enzyme and the backbone.

Mitra’s group (Sree Divya et al., 1998) is active in covalent enzyme immobilization onto glassy carbon matrix. They used a three step procedure for covalent immobilization of horse radish peroxidase. The steps involve heat treatment of carbon matrix, chemical pretreatment to introduce functional groups and enzyme coupling.
They heated glassy carbon (procured from Hochtemperatur Werke, Germany) in a hot air oven at 100-110°C for one to two hours. The treatment removes volatiles and other absorbates from the surface of the glassy carbon particles so that the full surface is available for the chemical pretreatment. Some people prefer to wash the matrix with organic solvents prior to heat treatment to remove the tar and other waxy (non-volatile) contaminants. For chemical pretreatment, they described three different independent procedures namely nitration followed by reduction of nitrated matrix, bromination followed by hydrolysis with alcoholic potassium hydroxide and peroxidation.

For nitration, they treated heat treated matrix with nitration mixture having nitric acid and sulfuric acid in the ratio of 1 to 3 at 100°C for 3 to 4 hours. Thereafter, they suspended it in sodium hydroxide to neutralize the acid prior to centrifugation. The matrix was washed with dilute sulfuric acid to remove excess alkali. Afterwards, nitro groups present on the matrix were reduced by addition of one ml of sodium borohydride having 300 to 500 mg sodium borohydride (for 250 mg matrix) and incubation overnight at the room temperature. The matrix was washed with water to remove excess reagent. This treatment introduces nitro groups on the matrix and the same are reduced to amino group by sodium borohydride.
For bromination, the heat treated matrix (250 mg) was suspended in 5 ml of bromine solution in 20% (v/v) carbon tetrachloride and incubated at the room temperature till all the bromine evaporated from the solution. It took nearly 3 days. After that, washing was done with water to remove bromine. Thereafter, the matrix was heated with alcoholic potassium hydroxide (20% in 90% ethanol) for 2 hours in a boiling water bath. Washed with water. Reaction with bromine introduces bromine atoms onto the matrix surface. Treatment with alcoholic KOH hydrolyzes the bromine moieties to hydroxyl groups.

For peroxidation, the heat treated matrix was suspended in 30% hydrogen peroxide for 48 hours at the room temperature followed by washing with water. It introduces hydroxyl groups onto the matrix.

The amino derivative of the matrix was incubated with 2% glutaraldehyde for 12 hours at 4oC followed by washing with water to remove excess glutaraldehyde. Thereafter, matrix was suspended in 1 to 2 ml of water and 3 mg of the enzyme protein (for 250 mg of matrix) in 1 ml of phosphate buffer and incubated for 12 hours at 4oC. The matrix after enzyme coupling was washed with distilled water and dried under vacuum. Glutaraldehyde attaches to the matrix via the amino group attached on the matrix and the other end remains free to couple the enzyme. Enzyme couples to free aldehyde group of glutaraldehyde. The mechanism of reaction of glutaraldehyde with amines is the subject of much speculation and may involve condensation and subsequent reaction between this polymer and the amino group rather than reaction with a simple aldehyde.
The matrix having hydroxyl groups was modified by carboxymethylation. For that, matrix was suspended in an equal volume of 0.1 M sodium chloride and was put in an ice bath. Sodium hydroxide (6 M) was added slowly with stirring till temperature reached 15°C. Thereafter, mixture was removed from the ice bath and 5 ml of 2% monochloroacetic acid was added and incubated for 70 minutes at 25°C with continuous stirring. After that, matrix was washed with 0.1 M sodium chloride 3 to 4 times to remove excess reagent. The resulting carboxymethylated (CM)-matrix was stored in 0.1 M sodium chloride containing 0.04% (w/v) sodium azide (for prevention of bacterial growth). In the presence of chloroacetic acid and alkali, hydroxyl groups are converted to carboxymethyl groups. For coupling of horse radish peroxidase, they took suspension of CM-matrix (roughly 250 mg) and to it 40 to 60 mg of dicyclohexyl carbodiimide (DCC) was added with stirring. Afterwards, one ml of the enzyme (3 mg protein) was added and incubation was done at 25°C for four hours. Thereafter, matrix was washed with 0.1 M sodium chloride and evaporated to dryness under vacuum. The pH was maintained at near 5 throughout during the entire reaction.
Glucose isomerase has been covalently linked by a disulfide group containing bridge on inorganic or organic support carrier. In this method, spent immobilized enzyme is readily regenerated by reduction under conditions of the disulfide bridge to provide the carrier with mercaptan groups and adding fresh enzyme to the carrier (http://www.wikipatents.com/4176006.html, US patent No. 4176006).

Xylanase has been covalently immobilized by radical grafting of acrylamide on cellulose acetate membranes. During the method, grafting of acrylamide on cellulose acetate powder and flat membranes was done followed by activation with glutaraldehyde to provide support for the enzyme immobilization. The grafting of acrylamide was confirmed by Fourier Transformation Infra Red (FTIR) study and by determination of nitrogen content of the cellulose acetate material. The grafting degrees of acrylamide on cellulose acetate flat membranes increased almost linearly with the initiator/membrane ratio, polymerization time and monomer/membrane ratio whereas the activation time had a more complex influence. The immobilization of xylanase was successfully carried on the acrylamide grafted cellulose acetate membranes. The immobilized enzyme showed enzyme activity in beechwood xylan solution after subsequent incubations at 50oC lasting 7 hours altogether. The immobilized enzyme was more resistant to inactivation by heat than the soluble enzyme (Sarbu et al., 2006).
Cross linking method

The cross linking is performed by the formation of intermolecular cross linkages between the enzyme molecules by means of bi- or multifunctional reagents. As cross linking reagents, glutaraldehyde (Schiff's base), isocyanate derivatives (peptide bond), bisdiazobenzene (diazocoupling), N,N'-polymethylene bisiodoacetamide (peptide) have been employed. The functional groups of enzyme proteins participating in the reactions include the α-amino group at the amino terminus, the ε-amino group of lysine, the phenolic group of tyrosine, the sulfhydryl group of cysteine and the imidazole group of histidine. 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 center of the enzyme may be affected by the reaction, leading to significant loss of activity.

Immobilization of enzymes has been carried out on siliceous supports. Siliceous support having surface hydroxyl groups such as silica gel is contacted with a solution of a polyaldehyde cross linking agent such as glutaraldehyde to produce a modified support having bound glutaraldehyde. The modified support has been contacted with the enzyme to bind to free aldehyde groups of glutaraldehyde. The resulting immobilized enzyme has been found to be more stable (http://www.freepatentsonline.com/5998183.html, US patent No. 5998183).
Similarly, a magnetic support matrix has been prepared comprised of a porous, refractory inorganic oxide containing ferromagnetic particles dispersed throughout its interior and a polyamine cross linked with an excess of a bifunctional reagent impregnated therein to furnish functional groups. The magnetic support did not substantially decrease loading of subsequently immobilized enzyme and also not altered the properties of the immobilized enzyme compared to non-magnetic support (http://www.freepatentsonline.com/4343901.html, US patent No. 4343901).

Our laboratory is engaged in immobilization of enzymes on egg shell using glutaraldehyde as cross linking reagent. After preparation of egg shell as described above under the physical adsorption method, 30 gm of ground egg shell was added to 70 ml of sorghum leaves partially purified starch phosphorylase preparation (336 mg protein) with stirring. Glutaraldehyde was added slowly to the suspension with shaking to a final concentration of 1.2% (v/v) and the mixture was incubated at 10°C for 12 hours. The residue obtained after centrifugation in the cold condition at 6000 x g for 15 minutes, was washed with buffer (0.05 M Tris-HCl buffer pH 7.0) until no protein was detected in the washings and suspended in 70 ml of buffer (0.05 M Tris-HCl buffer pH 7.0) (Venkaiah and Kumar, 1994). Using the similar type of protocol, starch phosphorylase from starch based industrial waste water (Venkaiah and Kumar, 1995) and pectinmethylesterase from Vigna sinensis (Nighojkar et al., 1995) have also been immobilized.
We also immobilized endo-galacturonase from germinating *Vigna sinensis* seeds on sand by cross linking using glutaraldehyde. Purified sand grains (2 gm) were constantly stirred with 10% glutaraldehyde in 0.2 M sodium bicarbonate buffer, pH 9.5 for one hour at 25°C and washed with 0.05 M sodium acetate buffer, pH 5. Thereafter, enzyme (2 ml; 22 mg protein in sodium acetate buffer was added to the sand grains and incubated for one hour at 30°C. The sand was washed with 0.01 M sodium acetate buffer, pH 5 and treated with sodium borohydride (50 mM final concentration) for 3 minutes at 0°C to stabilize the linkage of the enzyme to support and to reduce the unreacted carboxylic groups of the cross linking agent. The immobilized enzyme was washed with 0.05 M sodium acetate buffer, pH 5 and finally suspended in 10 ml of this buffer (Nighojkar et al., 1996).
ENTRAPPING METHOD

The entrapping method is based on confining enzymes in the lattice of a polymer matrix or enclosing enzymes in semi-permeable membranes, and can be classified into the lattice and microcapsule types. In this method the enzyme does not bind to the gel matrix or membranes. Thus this method may have wide applicability. However, if a chemical polymerization reaction is employed for entrapping, relatively severe conditions are required and loss of enzyme activity occurs in some cases. Therefore, it is necessary to select the most suitable conditions for the immobilization of various enzymes.

Our laboratory immobilized pectinmethylesterase from Vigna sinensis in gelatin (Nighojkar et al., 1995). For that, partially purified enzyme (13.8 mg protein) was added to 5 ml of 10% gelatin solution in 20 mM Tris-HCl buffer, pH 7.5 followed by 50 mg celite and 0.16 ml of 25% glutaraldehyde, and incubated at 4°C for three hours. The resulting gel was washed, centrifuged and suspended in 10 ml of buffer (10 mM Tris-HCl, pH 7.5).
Lattice type

The method involves entrapping enzymes within the interstitial space of a cross linked water-insoluble polymer, i.e., within the gel matrix. Various synthetic polymers such as polyacrylamide, polyvinylalcohol and other natural polymers such as starch and konjak powder have been used for the immobilization of enzymes.

We have immobilized sorghum leaves starch phosphorylase on polyacrylamide (Venkaiah and Kumar, 1994). Seventy mg of methylene bisacrylamide in 2 ml of 0.05 M Tris-HCl buffer, pH 7.0 (buffer A) was mixed with the following in sequence: 0.2 ml of TEMED, 5 ml of enzyme (26 mg protein) and 4 mg of ammonium persulfate. After mixing, the suspension was kept at 10ºC for one hour without any motion. The insoluble polymer thus formed was dispersed gently with a glass rod and centrifuged in the cold condition at 15000 x g for 30 minutes. The insoluble polymer was washed many times with cold distilled water to remove any adhering protein and the flocculent precipitate was suspended in 20 ml of buffer A.
**Micro-encapsulated type**

This method involves enclosing the enzymes in semi-permeable polymer membranes and used in many fields like medical, foods, cosmetics, dyes and fuel etc. For the preparation of enzyme microcapsules, extremely well-controlled conditions are required compared to micro-encapsulation of other chemical materials. The procedure for micro-encapsulation of enzymes may be classified into three categories, namely Interfacial polymerization; Liquid drying; and Phase separation.

The membranes are prepared by the process of phase separation, which is similar to homogenization of water in oil. One phase is not miscible with the other but forms a droplet or coacervate with the other phase, when mixed. The enzyme is entrapped within this droplet or coacervate.
A water insoluble membrane is prepared by chemical polymerization and enzyme is entrapped during this polymerization. Spheres made up of semipermeable nylon or collodion membranes are often used for microencapsulation of an enzyme. Enzymes are also entrapped inside fibers of cellulose triacetate. The choice of the method depends according to the enzyme involved and its application. Sufficient variation has been reported in the level of retention of enzyme activity on immobilization to different supports.

Urease enzyme has been immobilized in tetramethylorthosilicate derived sol-gel matrix on the sensing surface of glass pH electrode. No significant leaching of the enzyme has been shown from the microencapsulated enzyme film. It is used for the estimation of urea.
**Enzyme membranes**

Enzyme membranes can be prepared by attaching enzymes to membrane-type carriers by molding into membrane form after the enzymes have been enclosed within semi-permeable membranes of polymers by the entrapping method. Enzyme membranes have also been prepared by evaporating a mixture of an aqueous solution enzyme and a solution of nitrocellulose or polyvinylalcohol dissolved in a mixed solvent of diethylether, ethanol, acetone, amylalcohol and ethyleneglycol.

**Enzyme tubes**

Nylon, polyacrylamide and polystyrene tubes can be employed as enzyme carriers. The inside surface of a nylon tube is partially hydrolyzed with acid and newly liberated amino groups were destroyed by treatment with nitrous acid. The remaining carboxyl groups are converted to aminoaryl derivatives by reaction with benzidine in the presence of carbodiimide reagent. The derivative is further diazotized and enzyme is bound via diazo coupling to give an enzyme tube. The preparation of enzyme tubes by carrier cross-linking method has also been reported. Amino groups in the enzyme and amino groups liberated by partial hydrolysis of the inside surface of a nylon tube are cross-linked with glutaraldehyde.
Besides enzyme tubes, enzyme fibers and ultra-filtration membranes are being used for immobilization of many enzymes. Immobilization of enzymes often incurs an additional expense and is only undertaken if there is a sound economic or process advantage in the use. The most important benefit derived from immobilized enzyme is the easy separation of the enzyme from the products of the catalyzed reaction. This prevents the enzyme from contaminating the product minimizing downstream processing costs and possible effluent-handling problems particularly if the enzyme is noticeably toxic or antigenic. It allows continuous processes to be practical with a considerable decrease in enzyme labor and overhead costs. Immobilization often affects the stability and activity of the enzyme but conditions are usually available where these properties are little changed or even enhanced. The productivity of an enzyme so immobilized is greatly increased as it may be more fully used at higher substrate concentrations for longer periods than is the free enzyme.
The value of world enzyme market has rapidly increased recently from £110 M in 1960, £200 M in 1970, £270 M 1980, £500 M in 1985, £1000 M in 1990 and £2000 M in 1995. This increase has reflected the rise in the number of enzymes available on an industrial scale at relatively decreasing cost and the increasing wealth of knowledge concerning enzymes and their potential applications. As enzyme cost generally represents a small percentage at most, of the cost of the final product, it can be deduced that enzymes are currently involved in industrial processes with annual turnovers totaling many billions of sterling pounds. Several enzymes especially those used in starch processing, high-fructose syrup manufacture, textile de-sizing and detergent formulation, are now traded as commodity products in the world's markets. Although the cost of enzymes for use at the research scale is often very high, where there is a clear large-scale need for an enzyme, its relative cost gets reduced dramatically with increased production. Relatively few enzymes, notably those in detergents, meat tenderizers and garden composing agents, are sold directly to the public. Most are used by industries to produce improved or novel products, to bypass long and involved chemical synthetic pathways or for use in the separation and purification of isomeric mixtures.
## SOME IMPORTANT INDUSTRIAL USES OF IMMOBILIZED ENZYMES

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<tr>
<th>ENZYME</th>
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<tr>
<td>Starch phosphorylase</td>
<td>Glucose-1-phosphate</td>
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<tr>
<td>Amylases</td>
<td>Maltose</td>
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<tr>
<td>α-glucosidase</td>
<td>Glucose</td>
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<tr>
<td>Aminoacylase</td>
<td>L-amino acids</td>
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<tr>
<td>Aspartate ammonia-lyase</td>
<td>L-aspartic acid</td>
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<tr>
<td>Cyanidase</td>
<td>Formic acid from waste cyanide</td>
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<td>Glucoamylase</td>
<td>D-glucose</td>
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<td>Glucose isomerase</td>
<td>High fructose corn syrup</td>
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<td>Histidine ammonia-lyase</td>
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### Enzymes and Products

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<th>Enzyme</th>
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<tr>
<td>Invertase</td>
<td>Invert sugar</td>
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<td>Lactase</td>
<td>Lactose-free milk and whey</td>
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<tr>
<td>Pectinmethyl esterase</td>
<td>Low methoxyl pectin</td>
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<tr>
<td>Pectinase</td>
<td>Galacturonic acid</td>
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One of the triumphs of enzyme technology so far been the development of glucose isomerase. Glucose is normally isomerised to fructose during glycolysis but both sugars are phosphorylated. The use of this phosphohexose isomerase may be ruled out as a commercial enzyme because of the cost of the ATP needed to activate the glucose and because two other enzymes (hexokinase and fructose-6-phosphatase) would be needed to complete the conversion. The immobilized glucose isomerase greatly reduces the expenditure of production of fructose from glucose. Sucrose can be produced from starch by using a combination of the enzymes starch phosphorylase, glucose isomerase and sucrose phosphorylase.
The development of raffinase (\(\alpha\)-D-galactosidase) suitable for commercial use is another triumph of enzyme technology. The raffinase hydrolyzes raffinose that interferes in sucrose production from molasses and corn. The product, galactose formed does not cause any problem in purification of sucrose. This process results in a 3% increase in productivity and a significant reduction in the costs of the disposal of waste molasses. Immobilized raffinase may also be used to remove the raffinose and stachyose from soybean milk. These sugars are responsible for the flatulence that may be caused when soybean milk is used as a milk substitute in special diets.

Invertase was probably the first enzyme to be used on a large scale in an immobilized form. It is used in the production of invert sugar containing syrups. Enzymic inversion avoids the high-colour, high salt-ash, relatively low conversion and batch variability problems of acid hydrolysis. A productivity of 16 tones of inverted syrup (dry weight) may be achieved using one litter of the granular enzyme (immobilized enzyme).
Another early application of an immobilized enzyme was the use of the aminoacylase for resolving racemic mixtures of L-and N-acyl-DL-amino acids. There is one more important enzyme, hydantoinases of differing specificity for the production of D-amino acids which are essential constituents in antibiotics and insecticides. Immobilization of these enzymes play a major role in the production of pharmaceuticals. Lactase is one of relatively few enzymes that have been used both free and immobilized in large-scale processes. Immobilized lactases are mainly important in the treatment of whey, as the fats and proteins in the milk emulsion tend to coat the biocatalysts. These both reduces their apparent activity and increases the probability of microbial colonization.

Benzylpenicillins and phenoxyethyl penicillins are produced by fermentation and are the basic precursors of a wide range of semi-synthetic antibiotics. The amide link may be hydrolyzed conventionally but the conditions necessary for its specific hydrolysis, whilst causing no hydrolysis of the intrinsically more labile but pharmacologically essential \( \beta \)-lactam ring, are difficult to attain. Such specific hydrolysis may be simply achieved by the use of immobilized penicillin amidases. Acrylamide is another important monomer needed for the production of a range of economically useful polymeric materials. It is largely produced by the immobilized nitrile hydratase. Acrylamide production using this method is about 4000 tones per year. The closely related enzymes cyanidase and cyanide hydratase are used to remove cyanide from industrial waste and in the detoxification of feeds and food stuffs containing amygdalain.
Much work on immobilization of enzymes is being carried out in the author’s laboratory. Pectin is widely used in the preparation of jams, jellies and other food gels. The gels obtained from low methoxyl pectin are softer, more spreadable and relatively independent of pH compared to high methoxyl pectins and yield a low calorie gel, with or without sugar in the presence of bivalent cations such as calcium. The high methoxyl pectins require large amounts of sugar and low pH for gel formation.

Plant pectin methylesterase is used in the preparation of low methoxyl pectin and in the destabilization of clouds in fruit juices. Pectin methylesterase deestersifies pectin linearly creating blocks of free carboxyl groups and releasing methanol. After a desirable degree of esterification is achieved, pectin methylesterase is preferably denatured by heating or lowering the pH at low solid contents. Instead, the use of immobilized pectinmethylesterase ensures the controlled deesterification of pectin, easy removal, enhanced stability and reusability of the enzyme. The author’s laboratory isolated pectinmethylesterases from cow pea seeds and *cuscuta reflexa* filaments. Lab scale technologies have been developed to produce low methoxyl pectin. Pectin methylesterases have been immobilized on various solid supports including egg shell, gelatin as matrices.
Starch phosphorylase (EC. 2.4.1.1) catalyzing the reversible conversion of starch and inorganic phosphate into glucose-1-phosphate, plays an important role in starch metabolism in plants. It can be used for the production of glucose-1-phosphate, a cytostatic compound used in cardio-therapy. Starch phosphorylase may also be used to estimate inorganic phosphate in serum under the pathological conditions as well as to detect amount of inorganic phosphate pollution in the environment. Immobilized enzymes are in great demand in industries due to their reusability. In spite of its great importance, starch phosphorylase has been immobilized from few sources only. Kumar and Sanwal for the first time immobilized starch phosphorylase from mature banana leaf on methylene bis acrylamide and also characterized immobilized enzyme. Potato phosphorylase was subsequently immobilized by coupling to an insoluble support through diazonium salts and covalent bonding between the enzyme and Eupergit C. Author’s laboratory is engaged in studying starch phosphorylase from various sources and their immobilization on various solid supports namely egg shell, gelatin, polyacrylamide, DEAE cellulose, brick dust, alginate beads etc. Technologies at lab scale have been developed for production of glucose-1-phosphate.

Immobilized enzymes, therefore, can be exploited as biological machines in the service of man as now a days, biotechnology is emerging as powerful technology.
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Further reading:


Enzymes in Biomass Conversion (Leatham, G.F. and Himmel, M.E. eds.), American Chemical Society, Washington D.C.


Immobilized Enzymes: Research and Development (Chibata ed.) Tokyo Kodansha Ltd., Tokyo.