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Review Article

# Methods of Enzyme Immobilization

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**ABSTRACT**
Enzyme immobilization is a technique specifically designed to restrict the freedom of movement of an enzyme. Immobilization of enzymes is a common practice, mainly in order to minimize enzyme costs on the process economics by making it possible to reuse the enzyme many times and also minimize the operation cost as the immobilization technique may be modify the enzyme behavior, thus reducing the enzyme and product costs significantly. Many techniques have been used previously for enzyme immobilization, as entrapment, adsorption, covalent binding, encapsulation, and cross linking. Here we compare and contrast the basic properties of all immobilization methods.

**Keywords**: Immobilization, Entrapment, Adsorption, Covalent, Encapsulation, Crosslinking.

## INTRODUCTION

*Immobilization of Enzymes*

The overall utilization of enzymes in industries (with the exception of in medicines) is about € 1.5 billion. In Nutrition and medicine industries, as enzymes cannot be easily isolated from the product, and they are lost after the first use, so it is not better to be utilized in a free liquid state, also, some of them are very expensive (Figure 1). The immobilization method would empower the reusability of enzymes (Fig. 1b) for many of times, therefor, the cost of enzymes and product will be reduced significantly[1]. So, Immobilized enzyme form is more favorable than free enzyme one since it offers the possibility of continuous flow processing and easy recovery of the immobilized enzyme and low cost operation can be done in industrial processing[2].

The immobilization of enzymes is a common practice, mainly in order to minimize the share of the enzyme costs on the process economics by making it possible to reuse the enzyme many times. This means that the enzyme is physically confined, often in a polymer matrix in the form of beads or membranes, in such a manner that it cannot loss into solution[3]. The use of an immobilized enzyme also generally facilitates the downstream processing because it can simply be removed by sieving, whereas a considerable effort and money would have to be invested in removing a soluble enzyme from a reactor stream. In addition, immobilized enzymes also tend to be more stable than the dissolved enzyme. Unfortunately, there are also disadvantages, e.g. a (partial) loss of activity, changed kinetics, and diffusion or mass transfer limitations[4].

*Choice of Support and Principal Method*

Over the previous decade, some synthetic routes for immobilized enzyme have been developed. To compare enzyme immobilization nowadays with the past, we searched database for studies reporting enzyme immobilization. Our search includes peer-reviewed English-language publications tending to at least one enzyme immobilization, and publication year between 1990 and 2015. Fig. 2 shows the outcomes of publication by year. As in figure, around 1426, 1628 and 1568 articles were published in 2013, 2014 and 2015 respectively; though in 1990 the corresponding figure was just 336, demonstrating expanding interest in enzyme immobilization over the late years.

In solution, soluble enzymes can disperse in the solution and also free in movement[5]. Enzyme immobilization is a technique particularly designed to limit the enzyme movement. Immobilization provides support for enzymes. For immobilization you have to decide the support material first, and then the immobilization method, taking into account the intended use and application. Some points required to be considered are listed in table 1.

Numerous methods have been utilized previously for immobilization of enzymes, for example: adsorption, covalent binding, entrapment, encapsulation, and cross linking[6].

*Principal methods for immobilization of enzyme.*

*Adsorption*

Using adsorption as immobilization method (Figure 3) is the easiest technique and includes reversible surface interaction between carrier and enzyme[7]. The forces

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formed are weak force, mostly electrostatic, for example Van der Waals forces, ionic bond and hydrogen bonding interactions, although hydrophobic bonding can be significant, but although these forces are very weak, but sufficiently large in number to enable reasonable binding.
This method done by mixing the enzyme(s) and a support material with each other in adsorption properties, at optimum pH, ionic strength, etc., for a time, after that collect immobilized enzyme and wash it to remove unbound enzymes.

*Advantages of this method are:*

- Little or no damage to enzyme /cells.
- Easy, cheap, and fast.
- No changes happened to carrier or enzyme/ cells.
- Reversible.

*Disadvantages are:*

- Leakage of enzyme/cells from the support
- Separation of product is not easy.
- Nonspecific binding.

The most signification disadvantage is the separation of enzyme from the support material, desorption may be happen under changes in pH, temperature, and also ionic strength.
Desorption may be happen as a result of physical factors, for example, flow rate, agitation, particle-particle Collisions.
Nonspecific binding may be lead to diffusion limitation and reaction kinetic problem, with consequent alteration in parameter V*max* and K*m*[8]. Further, binding of protons to the support with consequent shift in pH optimum (1-2 pH), which may be important enzymes with precise pH optimum requirement[9]. Unless carefully controlled, overloading the support can lead to low catalytic activity, and the presence of a suitable spacer between the enzyme molecule and the support can produce problems related to steric hindrance.

*Covalent binding*

Covalent binding immobilization method (Figure 4) consists of formation of a covalent bond, strong bond, between the enzyme/cell and a carrier[10,11]. This covalent bond formed between the functional groups present on the surface of carrier and the surface functional groups of the enzyme.
These functional groups on the surface of the enzyme such as amino groups ($NH_2$) of arginine or lysine, carboxylic group (COOH) of glutamic acid or aspartic acid, hydroxyl group (OH) of threonine or serine, and sulfhydryl group (SH) of cysteine[12].
Many factors affect the choice of specific carrier, and research work has demonstrated that hydrophilicity is one of the most important factors for keeping up enzyme activity[13]. Thus, hydrophilic carriers such as polysaccharide polymers are popular materials for enzyme immobilization. For example, cellulose, starch, dextran (sephadex), and agarose (sepharose). The sugar residues in these polymers contain ideal functional groups, hydroxyl groups, for covalent bond formation[14]. Also, hydroxyl groups can form hydrogen bonds with water and create an aqueous (hydrophilic) environment in the support. The supports are usually used in bead form[3].
Other popular supports for immobilization of enzymes are porous silica and porous glass. Porous silica contains small spherical particles of silica fused together having micro cavities and small channels. The carrier is normally sold in bead form, and is very strong and durable. Sintered borosilicate glass has a system of uniform channels. Porous glass is also durable and resistant to microbial disintegration or solvent distortion. However, these two supports are procedures for coupling an enzyme and a carrier is a covalent bond[15]. Most reactions may be one of the following categories:

- Isourea linkage Formation.
- Diazo linkage Formation.
- Peptide bond Formation.
- An alkylation reaction.

It is very important to choose a technique that no effect on the enzyme as it may be inactivate it by reacting with enzyme active site. Covalent binding consists of two steps. First one, activation of functional groups found on carrier surface by a specific reagent, and the second, adding enzyme to form covalent bond with activated surface of carrier. Normally the activation reaction is designed to make strong electrophilic (electron deficient) functional groups on the carrier. In the coupling reaction, these activated groups will react with strong electron donating nucleophiles, such as the amino group ($NH_2$), functional groups of certain amino acids on the surface of most enzymes, to form strong covalent bond[16].
Cyanogen bromide (CNBr) is usually used to activate the hydroxyl groups in polysaccharide materials. This method contains isourea linkage between enzyme and carrier. In the case of carbodiimide activation, the support materials should contain carboxyl group ($CO_2$ H) then enzyme and support are combined by peptide bond. If the support material contains an aromatic amino group, it can be diazotized utilizing nitrous acid, addition of enzyme leads to the formation of diazo linkage between the activated diazo group on the support and the ring structure of an aromatic amino acid, for example tyrosine. No technique of immobilization is confined to a specific type of carrier, and large numbers of Probabilities are possible between immobilization technique and support material. This is possible by chemical modifications on the support material to produce different functional groups. For example, the normal function group in cellulose is the hydroxyl group, and the chemical modification of this has produced a range of cellulose derivative, such as AE-cellulose (amino ethyl), carboxymethyl cellulose, and DEAE-cellulose. Thus, chemical modification increases the number of immobilization methods that can be utilized for each support material.

*Entrapment*

One of the easiest techniques of immobilization is entrapment. In recent years, calcium alginate has attraction as an immobilization support material. It has been utilized for immobilization of variety of cell types, sub-cellular organelles, multi-component systems, and enzymes. The physicochemical characteristics of this matrix in gel form have an important effect on the reactions of entrapped biologically active material in the gel. Critical parameter

in selecting a matrix is pore size[17]. The difference between entrapment technique and adsorption and covalent binding is that however the enzyme is restricted in movement by the structure of a gel lattice but it is free in solution[18]. The pore size of a gel lattice is controlled to ensure that the structure become tight enough to prevent loose of enzyme or cells, it also allow free movement of the substrate and product. The support acts as a barrier to mass transfer, and although this have serious reaction kinetics implications, but it can prevent interaction between harmful cell, proteins, and enzymes and immobilized biocatalyst[19,20].

There are several major methods of entrapment:

- Ionotropic gelation of macromolecules with multivalent cations (e.g. alginate).
- Temperature-induced gelation (e.g. agarose, gelatin).
- Organic polymerization reaction by chemical/photochemical (e.g.Polyacrylamide).
- Precipitation from an immiscible solvent (e.g. polystyrene).

Entrapment can be accomplished by cross linking the polyionic polymer material with multivalent cations in an ion-exchange reaction after mixing with enzyme to form a structure that traps the enzymes/cells (ionotropic gelation) (Figure 5). Change in temperature is a simple way of gelation by phase transition utilizing 1-4% solutions of gelation. κ-carrageen a polymers that can easy form gels by ionotropic gelation and by temperature-induced phase transition, which has form a greater degree of flexibility in gelation frameworks for immobilization[20].

On the other hand, it is possible to mix the enzyme with material that is then polymerized to frame a crosslinked polymeric system, trapping the enzyme in the internal spaces of the lattice. The last method is more widely utilized, and various acrylic materials are available for the formation of hydrophilic copolymers. For example, acrylamide monomer is polymerized to form polyacrylamide and methylacrylate is polymerized to form polymethacrylate. In addition to the monomer, a crosslinking agent is added during polymerization to form cross linkage between the polymer chains and help to create a three-dimensional network lattice. The formed polymer may be broken up into particles of a desired size, or polymerization can be arranged to form beads of defined size. Precipitation occurs by phase separation rather than by chemical reaction, but does bring the enzymes/cells into contact with a water-miscible organic solvent, and most enzymes/cells are not tolerant of such solvents. Thus, this method is limited to highly stable/previously stabilized enzymes or nonliving cells[21].

This method is depending on localization of an enzyme inside polymer network or membrane lattice. Entrapment has been advanced and broadly utilized for the immobilization of cells more than for enzymes. It is limited for enzymes immobilization as it may be lost during repeatedly using because of the small molecular size of enzyme compared to the cells. Diffusion limitations are also disadvantages for this method. This method may be classified into five categories: lattice, microcapsule, liposome, membrane, and reverse micelle[22]. The most widely one is the lattice method, in this type enzyme is entrapped in the lattice of the different natural or synthetic polymers. Alginate which is naturally occurring polysaccharide that has the ability to form gels by ionotropic gelation, is the most popular one[23]. Another type, microcapsule, involves entrapment to porous polymer. The preparation of micro capsules containing enzyme requires highly controlled conditions. Taqieddin and Amiji[24] developed a new method for encapsulation in which the alginate-chitosan core-shell microcapsules were prepared to immobilize β-galactosidase. The enzyme was confined and protected in the inner core, alginate, while the outer shell, chitosan, manages the transport properties. Utilizing $Ca^{2+}$ ions for alginate crosslinking, microcapsules with liquid core were developed with 60% loading efficiency. And using $Ba^{2+}$ ions, microcapsules with solid core were produced and 100% loading efficiency was obtained[25]. The entrapment in liposome is increasingly recognized as a technique of protecting biocatalysts from inactivation by proteolytic enzymes. Also, this enzyme, liposome, offers a noticeable increment in thermal protection. In the third type, reversed micelle, entrapping within the reversed micelles, it is formed by adding a surfactant with an organic solvent, such as aerosol OT/isooctane reverse micelles[26] and used to entrap β-galactosidase**.** While in the membrane type, the enzyme is isolated from the reaction solution microfiltration membrane or a hollow fiber[27].

*Encapsulation*

Encapsulation (Figure 6) of enzymes as well as cells can be accomplished by wrapping the biological components inside different forms of semi permeable membranes[28,29]. It is as entrapment in that the enzymes/cells are free in movements, however limited in space. Vast proteins or enzymes cannot out or inter capsule, however small substrates and products can go freely across the semi permeable membrane. Numerous materials have been utilized to form microcapsules are in range of 10-100 µm in diameter; such as, nylon and cellulose nitrate. Rupture of the membrane is a problem associated with diffusion may be result if products from a reaction accumulate rapidly.

Biological cells also may be used as capsules, and a famous example of this is the use of erythrocytes (red blood cells). The membrane of the erythrocytes is normally just permeable to small molecules. However, when erythrocytes are placed in hypotonic solution, they swell, expanding the cell membrane and substantially expanding the penetrability. In this condition, erythrocytes proteins go out of the cell and enzymes can inter into the cell. Returning these erythrocytes, swollen, to the isotonic solution enables the cell membrane to return to the normal state, and the enzymes inside the cell can't leak out. A distinct advantage of this technique is co immobilization. Cells and/or enzymes may be immobilized in any type of combination to be suitable for particular application[30].

*Cross linking*

This method of immobilization (Figure 7) depend only on enzyme and it is support- free as it done by joining the enzyme (or the cells) to each other to prepare a large, three-dimensional complex structure, and it can be done

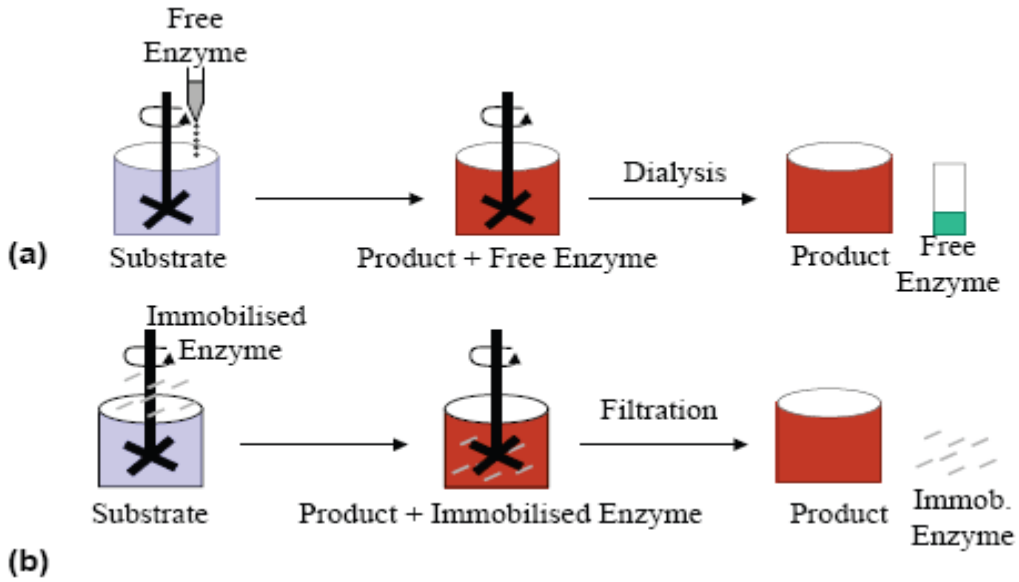


Figure 1: a&b Schematic diagram of free and immobilized enzyme reactions.
(a), Reaction of free enzyme with substrate and formation of product, which has to be separated via dialysis;(b), Reaction of immobilized enzyme with substrate and formation of product, which can be separated via filtration.

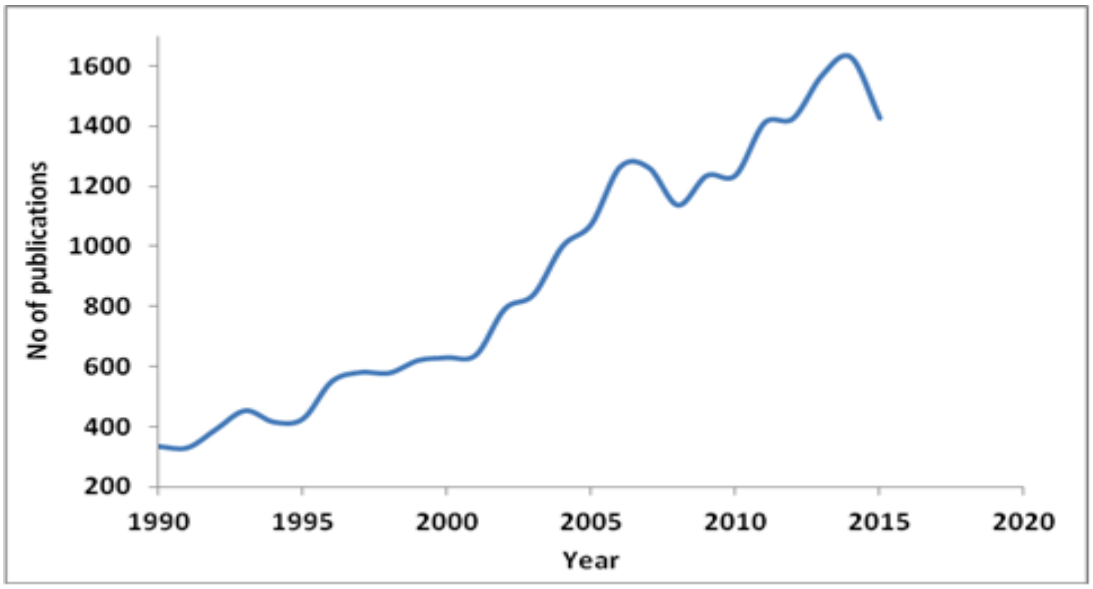


Figure 2: Annual number of articles on enzyme immobilization indexed in Scopus over the 1990–2015 period

chemically or physically[31]. Chemical type of crosslinking normally includes formation of covalent linkage between the cells by means of a bi- or multifunctional reagent, for example glutaraldehyde and toluene diisocyanate. However, limiting factors can be used in this method for living cells and many enzymes because of harmful materials. To minimize the close problems that can be found because of crosslinking of single enzyme, both albumin and gelatin have been used.

This technique uses a bi- or multifunctional compounds, which serve as the reagent for intermolecular cross-linking of the biocatalyst[22].

Covalent binding or crosslinking methods are done under relatively severe conditions in comparison with those of physical adsorption or encapsulation.

Hence, in the previous cases, conformational change of the enzyme structure and partial destruction of the active site may occur. Accordingly, unless covalent binding method is done under well controlled conditions, immobilized enzyme having high activity cannot be obtained. Also, the enzyme cannot easily be lost from carriers because of the strong binding forces between the enzyme and carrier.

## CONCLUSION

Enzymes can catalyze reactions in different forms: as individual free enzyme in solution, in aggregates with others, and as attached to carrier surfaces. The attached or "immobilized" form has been of particular interest[32].

Enzymes can be immobilized by using many methods. And every method has its advantages and disadvantages as shown in table 2.

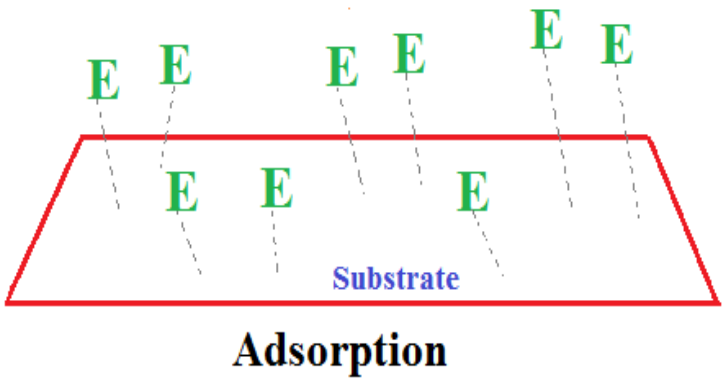


Figure 3: Immobilization by adsorption

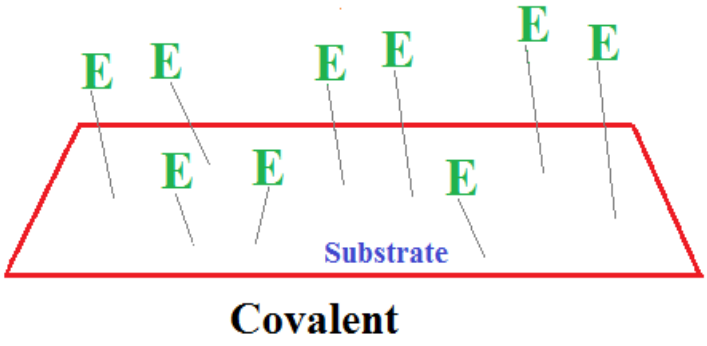


Figure 4: Immobilization by covalent

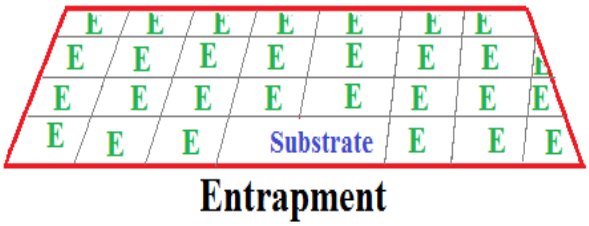


Figure 5: Immobilization by Entrapment

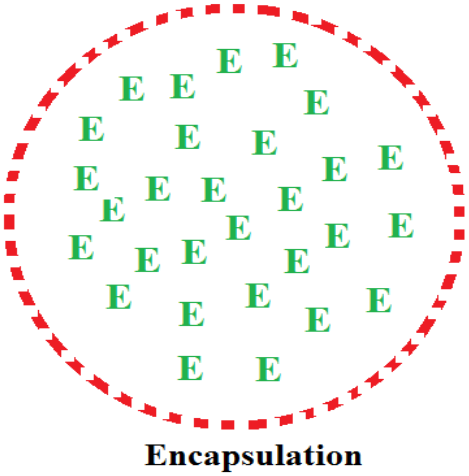


Figure 6: Immobilization by Encapsulation

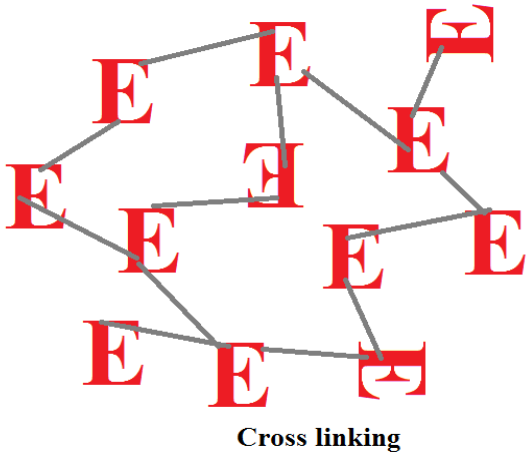


Figure 7: Immobilization by crosslinking

Table 1: Fundamental considerations in selecting a support and methods of immobilization

| Property | Points of Consideration |
|---|---|
| Physical | Strength, non-compression of particles, available surface area, Share/form (beads/sheets/fibers), degree of porosity, pore volume, permeability, Density, space for increased biomass, flow rate, and pressure drop. |
| Chemical | Hydrophilicity (water binding by the support), inertness toward enzyme/cell, available functional groups for modification, and regeneration/ reuse of support |
| Stability | Storage, residual enzyme activity, cell productivity, regeneration of enzyme activity, maintenance of cell availability, and mechanical stabilityof support material |
| Resistance | Bacterial/fungal attack, disruption by chemicals, pH, temperature, organic solvent, proteases, and cell defence mechanism (proteins/cells), Biocompatibility (invokes an immune response), toxicity of component |
| Safety | reagents, proteases, health and safety for process workers and end-product users, specification of immobilized preparation (GRAS list requirements for FDA approval) for food, pharmaceutical, and medical applications Availability and cost of support, mechanicals, special equipment, reagents, technical skill required, environmental impact, Industrial-scale chemical preparation, feasibility for |
| Economic | scale-up, continuous processing, effective working life, reusable support, and CRL or zero contamination (enzyme/cell-free product) Flow rate, enzyme/cell loading and catalytic productivity, reaction kinetics, side reactions, multiple enzyme and/or cell systems, batch, CSTR, PBR, FBR, |
| Reaction | ALR, and so on: diffusion limitations on mass transfer of cofactors, substrates, and products |

Table 2: Preparation and Characteristics of Immobilized Enzyme Carrier-binding method

| Characteristic | Physical adsorption | Encapsulation | Covalent binding | Cross-linking method | Entrapping method |
|---|---|---|---|---|---|
| Preparation | Easy | Easy | Difficult | Difficult | Difficult |
| Enzyme activity | Low | High | High | Moderate | High |
| Substrate specificity | Unchangeable | Unchangeable | Changeable | Changeable | Unchangeable |
| Binding force | Weak | Moderate | Strong | Strong | Strong |
| Regeneration | Possible | Possible | Impossible | Impossible | Impossible |
| General applicability | Low | Moderate | Moderate | Low | High |
| Cost of immobilization | Low | Low | High | Moderate | Low |

On the other hand, enzymes immobilized by encapsulation technique can be achieved simply under mild conditions but the binding forces between enzyme and support are very weak in comparison with those found in the covalent-binding methods. So, losing the enzyme from the carrier may happen after changes condition like ionic strength, pH of the substrate, or product solution.

Unlike encapsulation, covalent-binding, and cross linking methods, in the entrapping method, no bond formed between enzyme and carrier should occur in theory. Therefore, in many cases, preparations having high activity are acquired. However, in this strategy, recovery of activity losses is impossible, in comparison with covalent-binding method.

Table 3: Properties of immobilized enzymes
Technological properties of immobilized Enzyme system:

| Advantages | Disadvantages |
|---|---|
| Catalyst reuses | Loss or reduction in activity |
| Easier reactor operation | Diffusional limitation |
| Easier product separation | Additional cost |
| Wider choice of reactor | |

Table 4: products of immobilized enzymes.
Major products obtained using immobilized enzymes:

| Enzyme | Product |
|---|---|
| β- Galactosidase | Hydrolyzed lactose (whey) |
| Glucose isomerase | High- fructose corn syrup |
| Amino acid acylase | Amino acid production |
| Penicillin acylase | Semi-synthetic penicillins |
| Nitrile hydratase | Acrylamide |

But the significant disadvantage of immobilization by entrapping method is that it is limited to small molecular substrate and product; the entrapped enzyme has little or no activity toward macromolecular substrates.

Despite the fact that a number of immobilization techniques have been studied, there is no ideal general technique suitable for many enzymes have yet been developed. Every technique has specific advantages and disadvantages. Thus, in practice, it is important to find an appropriate method and optimum conditions for the immobilization of each enzyme in the light of planned application.

While immobilization procedures often decrease the enzyme activity and selectivity, it keeps the enzyme in their native state, and also can be easily separated from the products by a semi-permeable membrane. Micro filtration- and hollow-fiber- membrane utilizing is described in "Ullmann's Encyclopedia of Industrial Chemistry" (1989). Enzymes can be also cross-linked by using different bi-functional agents (e.g. glutardialdehyde) and thus converted to insoluble form[33,34,35]. Some of the discussed immobilization strategies are represented schematically in Figure 2.

In the recent years the dilemma concerning the choice between "carrier-bound or carrier-free enzyme" was subject of some researches. Kasche and Tischer[36] discussed possible advantages and disadvantages of enzyme in Free State compared to the immobilized one onto support materials. Table 3 demonstrates a few disadvantages of the carrier used in enzyme immobilization: decreased mobility of the biocatalyst; possible steric hindrances; diffusional limitations (depends on the material size and its pore size); development of pH gradients (can be noticed also by enzyme-crystals); fouling of the carrier-pores with substrate and/or product; extra costs for carrier activation.

The mathematical description of the diffusional and mass transfer effects of the enzyme kinetics is done by Kasche et al.[37]. The degree of mass transfer control is frequantly expressed by the stationary effectiveness factor η:

$$\eta = \frac{v_{imm}}{v_{free}}$$

Where $v_{imm.}$ and $v_{free}$ are the rates of the reaction catalyzed by immobilized and free enzyme at the same condition of the active enzyme[38]. The kinetic behavior of immobilized-enzyme systems depends principally on the carrier pore size and also its size, likewise on enzyme density, but it is independent on whether carrier-bound or carrier-free biocatalyst is used (table 4).

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