Methods for immobilizing enzymes

Introduction :

In living cells, enzymes are immobilized in well-defined compartments (plasma membrane, nucleus, mitochondria, cytosol, lysosomes, endoplasmic reticulum, etc.), either associated with membranes or attached to cell walls in plants.

Their behavior differs from that of enzymes in solution. It is no longer homogeneous catalysis, but heterogeneous catalysis.

For more than 30 years (since the 1980s), various enzymes have been artificially immobilized on a solid support for use in biotechnology and as models for enzymes immobilized in vivo in order to determine the kinetic laws that govern their functioning.

History :

In 1916, Nelson and Griffon discovered that invertase “showed the same activity when observed on a solid (charcoal or aluminum hydroxide).” This discovery was the first of several enzyme immobilization techniques currently available. To date, more than 5,000 publications and patents have been issued on enzyme immobilization techniques.

1. Definitions :

Homogeneous catalysis :

Catalysis is said to be homogeneous when the catalyst is in the same phase as the reactants; therefore, the reaction mixture is homogeneous.

Heterogeneous catalysis :

Catalysis is said to be heterogeneous when the catalyst, reactants, and products are not in the same phase. These reactions occur at the interface and involve concepts of surface chemistry.

Supported catalysis :

In supported catalysis processes, homogeneous catalysts are bound to insoluble solid supports in order to take advantage of the selectivity, efficiency, simplicity of post-reaction treatment, and recyclability of homogeneous catalysts.

Immobilized enzyme :

Is an enzyme attached to an inert, insoluble material by physical-chemical bonding. In this case, the enzymes are held in place throughout the reaction, as they are easily separated from the products and can be reused.

Enzyme immobilization :

Is a set of chemical or physical methods by which some enzymes are attached to supports in order to increase the stability of these enzymes and prolong their existence by immobilizing them, in order to preserve their activity.

2. Interest in immobilizing enzymes :

Why immobilize enzymes ?

- Obtaining free enzymes (extraction and purification) is costly ;
- Enzymes are unstable in solution ;
- Free enzymes are difficult to recycle after catalysis (separation of the reaction product) ;
- Catalysis is discontinuous.

So immobilization allows :

- Recovery and reuse of enzymes ;
- Stabilization (against thermal denaturation, extreme pH, improved preservation) ;
- The enzyme's reaction mechanism is not modified ;
- The products obtained are of greater purity and easy to recover ;
- Reduction or even elimination of inhibition processes ;
- Sometimes, the enzyme is little or not purified ;
- Continuous catalysis over time, thus greatly increasing productivity.

3. Differences between free enzymes and immobilized enzymes :

Free enzyme	Immobilized enzyme
~ Isolated from the cellular environment ; ~ All molecules are identical ; ~ The environment is continuous ; ~ Homogeneous catalysis (Michaelis kinetics different from in situ)	~ Fixed or confined ; ~ Not all molecules are identical ; ~ The environment is discontinuous ; ~ Heterogeneous catalysis (close to in situ)

4. Methods for immobilizing enzymes :

Enzymes can be immobilized by physical retention or chemical binding. These two methods can be combined to ensure better enzyme fixation :

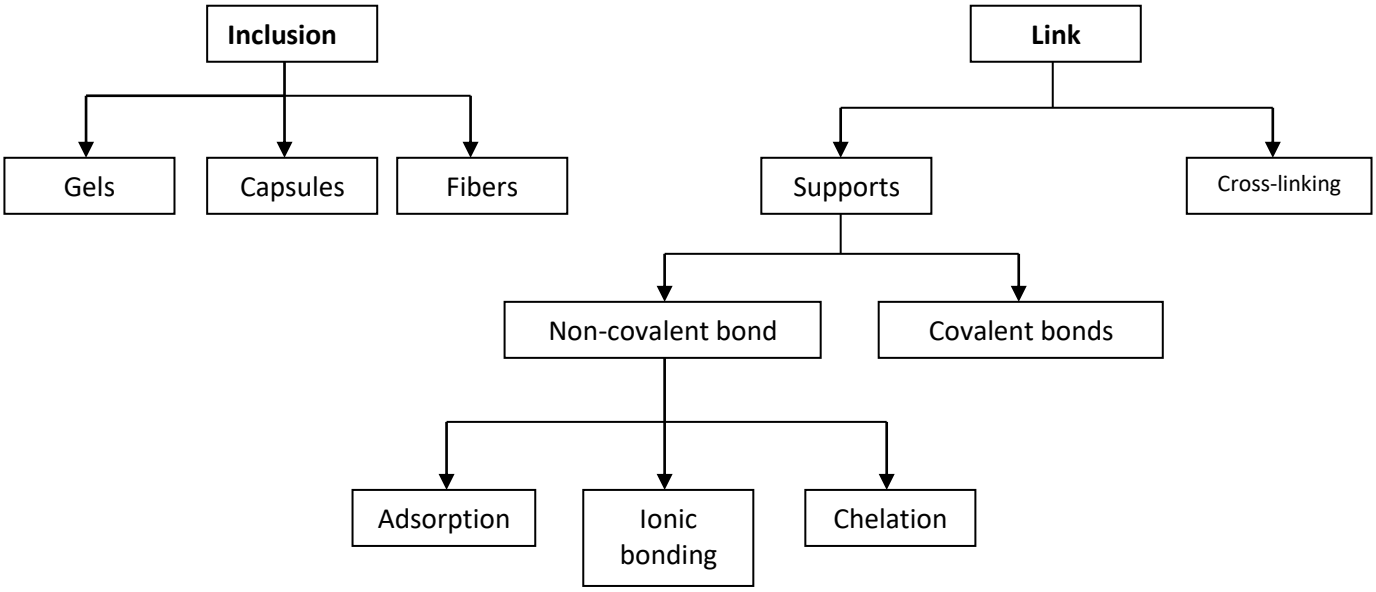
- **The chemical bond :**

This bond is formed between the enzyme and the support. Chemical immobilization often has the advantage of stabilizing the protein and allowing prolonged use.

- **Physical retention :**

It exploits the large difference in size between the enzyme and the substrate, hence the idea of creating a semi-permeable barrier to retain the enzyme. This can be a network, a capsule, or a membrane.

In fact, there are many methods cited in the literature. However, there are four main methods commonly used to immobilize enzymes :



4.1. Inclusion (confinement) :

In this method, enzymes are retained in a porous matrix. The enzyme is dispersed in a homogeneous solution of monomers or emulsion. Polymerization of the monomer leads to the formation of a network in which the enzyme is trapped in a purely physical manner. The polymerization reaction must be carried out under conditions that are as non-denaturing as possible.

The polymer can be : polyacrylamide, polyethylene glycol, polyvinyl pyrrolidone, starch. The matrix can be inorganic, such as clay.

4.1.1. Inclusion in a matrix :

- *Polyacrylamide gels :* acrylamide is the starting monomer and N,N-methylenebisacrylamide is the cross-linking agent.
- *Starch gels.*
- *Dextran gels (Sephadex).*
- *Polyvinyl alcohol gels :* vinyl alcohol is polymerized in the presence of enzymes by exposure to gamma rays or accelerated electron beams.
- *Other gels* : silica gels, siliceous resins or polyvinylpyrrolidone, alginates, carrageenans.

4.1.2. Inclusion in a microcapsule :

This technique can be used to prepare both collodion and nylon membranes (polystyrene, silicone and ethylcellulose derivatives, polyurea). The size of the capsules obtained varies from a few microns to a few hundred microns.

4.1.3. Advantages and disadvantages of inclusion :

Advantages **:**

- ✓ This technique is inexpensive;
- ✓ The entire enzyme mass is immobilized;
- ✓ Polymerization reactions are generally well known, their application can be well controlled, and they take place in a single step;
- ✓ The molecular integrity of the enzyme is preserved during immobilization;
- ✓ The technique can be used to immobilize any enzyme;
- ✓ Inclusion appears to be favorable for the immobilization of oligomeric enzymes and multienzyme systems.;

Disadvantages :

- ✓ The conditions for obtaining the polymer can be more or less denaturing (solvents, pH);
- ✓ The distribution of pore diameters is difficult to control (continuous loss of enzyme);
- ✓ The location of the enzyme inside the polymer implies diffusion limitations and steric encumbrance problems ;
- ✓ The mechanical properties of the gels do not allow for use in large reactors.

4.2. Adsorption :

In this method, enzymes are retained on the surface of an insoluble mineral or organic support by establishing non-covalent interactions between the functional groups of the enzyme and the support. This is achieved by placing the support and enzymes in contact for a defined period of time.

Reactive chemical groups on the enzyme surface used for immobilization :

widely used (++) for immobilization	NH_2 (Lys)
Frequently used (+)	NH_2 (Terminal N) COOH (Asp, Glu, terminal C)
Used occasionally (±)	OH (Ser, carbohydrate)
Not used (-)	Trp, Tyr, Mrt, Cys, Arg

4.2.1. The types of bonds involved in adsorption :

- *Van der Waals interactions* : these are electrostatic interactions between atoms or molecules resulting from variations in electron distribution on orbitals, leading to the appearance of a dipole. These are weak interactions that depend on the intermolecular distance.
- *Hydrophobic interactions* : this is the tendency of two or more nonpolar groups to aggregate by expelling water molecules from their immediate environment.
- *Hydrogen bonding* : this is an electrostatic bond resulting from the polarization of bonds between a hydrogen atom and another more electronegative atom.
- *Charge transfe r*: these interactions occur through the more or less marked transfer of electron doublets from one molecule to another (between nucleophilic substances and other electrophiles).
- *Ligand exchange :* this mechanism results in the substitution of one or more ligand molecules of the adsorbent by the adsorbed molecule. The adsorbed molecule (enzyme) must be a more powerful chelating agent than the displaced ligand.
- *Ion exchange* : this is due to the ionic charges of the acidic and basic amino acid residues carried by enzymes. These interactions are highly dependent on the pH of the medium due to the amphoteric nature of amino acids.
- *Chemisorption :* this involves the formation of a chemical bond between the adsorbate and the adsorbent. It is an exothermic process.

4.2.2. Parameters influencing adsorption :

- *Protein concentration* : generally, the mass bound to the support increases with the concentration of the adsorbed species until the adsorbent is saturated.
- *Contact time* : the adsorption rate is determined by the physicochemical characteristics of the adsorbent and the adsorbed protein (dimensions of the molecule and support particles, presence and nature of electrical charges, etc.). Under normal adsorption conditions, the time required to immobilize an enzyme preparation is short and does not exceed a few hours.
- *pH* : changes in pH mainly interact with ionic bonds. They cause a change in the nature of the charges carried by the support and by the enzyme depending on the pK value of the ionizable groups. It has been observed that adsorption is highest near the isoelectric point of the adsorbed protein.
- *Composition of the medium* : this may or may not favor adsorption. The main factors are the influence of organic solvents and salts.
- *Temperature* : in general, the mass of proteins adsorbed onto a support increases with temperature (irreversible denaturation must be avoided).
- *Amount of adsorbent* : increasing the amount of adsorbent allows the entire mass of enzyme contained in a solution to be immobilized.

4.2.3. The supports :

- *Clay mineral supports (aluminosilicate*) : this is a very abundant and inexpensive raw material. The mechanisms involved in the adsorption of enzymes on these supports include ionic bonds and weaker bonds.
- *Porous glass, quartz (silicon dioxide*) : in this case, there is an optimum depending on the size of the enzyme molecule and the diameter of the pores ; the mass of enzyme adsorbed increases with the specific surface area up to a limit corresponding to pore diameters that are too small to allow the enzyme to enter. The bonds involved in protein retention are mainly ionic bonds. Hydrogen bonds may also be involved. There is also porous glass carrying ion exchange groups.
- *Ion exchangers* : these are mainly resins, cellulose derivatives and dextrans, agarose, and amberlite.
- *Collagen.*
- *Other supports* : these include glutenin, collodion, activated carbon, aluminum hydroxide, porous titanium oxides, a sepharose-concanavalin A complex, concanavalin A, a polyacrylamide gel (biogel), silica gels, polyurethane, starch, calcium phosphate gel (hydroxyapatite), chitin-cellulose complex.

4.2.4. Advantages and disadvantages of adsorption :

Advantages :

- ✓ The complexes are very easy to prepare;
- ✓ Despite their nature, the bonds formed between the enzyme molecules and the support are strong enough that changes in conditions (pH, ionic strength) do not cause the enzyme to leach out;
- ✓ The support is easy to regenerate (reversible).;

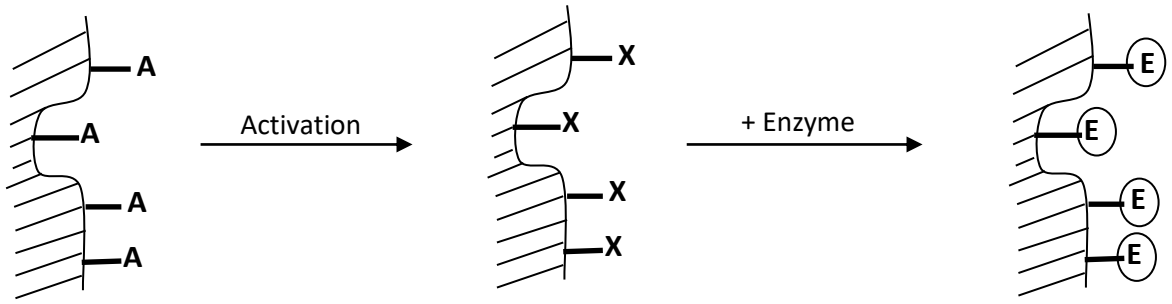
Disadvantages :

- ✓ Enzyme desorption is relatively easy by modifying pH and ionic strength conditions;
- ✓ Some adsorbents cause clogging problems (in reactors);
- ✓ Steric hindrance problems;
- ✓ Poor enzyme orientation, leading to loss of activity.

4.3. Covalent bond :

This immobilization is achieved through irreversible, covalent bonds between the functional groups of the enzyme and the reactive groups of the support (carboxyl COOH, primary amine NH_2, less frequently hydroxyl OH or thiol SH).

In this method, it is necessary to activate either the enzyme groups or the support groups. Since activation of the enzyme causes a loss of activity, the support must therefore be activated :

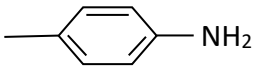


Functional groups of enzymes	
α- and ε-amino functions (-NH_2)	Terminal amino acids, lysine
α, β, and γ-carboxylic functions (-COOH)	Aspartic acid and glutamic acid
Thiol functions (-SH)	Cysteine
Phenolic ring	Tyrosine
Hydroxyl functions (-OH)	Serine, threonine
Imidazole ring	Histidine

In order to bind the enzyme to the support via covalent bonds, reactive chemical functions are introduced onto the support.

These can be created by direct reaction (cellulose + cyanogen bromide) or by grafting a bifunctional compound (spacer arm) that allows the enzyme to be fixed at a certain distance from the support and provides better accessibility of the enzyme to its substrate.

4.3.1. The main activation methods :

Functional groups of the support	Activation methods	Functional groups of the enzyme
-COOH	Carbodiimide / Acid chloride / azoture	-NH_2
-OH	Chlorotriazine / Cyanogen halides / halogenation / silanes	-NH_2
-NH_2	Glutaraldehyde / other multifunctional reagents	-NH_2
-NH_2	Carbodiimide / Isothiocyanate	-COOH
	Isocyanate / Isothiocyanate / Diazonium salts	-tyrosine
-SH	Disulfide bridges	-SH
-SH	3,maleimidopropionic acid / N, hydroxysuccinimide	-NH_2

4.3.2. The supports :

The supports generally used for immobilization can be classified into two main groups :

1. ***Organic supports :*** This type of support is chemically activated using various techniques, which is one of their main advantages as it allows enzymes to be attached in different ways.

2. ***Inorganic supports :*** These are generally more stable (resistant to wear, chemicals, and bacteria). However, covalent bonding to these supports is difficult due to their low reactivity.

Types of support				Reactive groups
Organic substances	Polyosides	Cellulose		OH
		Carboxymethylcellulose		COOH
		Diethylaminocellulose		OH
		Para-aminobenzylcellulose		φ-NH_2
		Other cellulose derivatives		φ-NH_2, OH, CHO
		Dextrans and agarose		OH
		Dialdehyde starch		CHO
		Starch		OH
		Polygalacturonic acid		COOH
	proteins	Collagen		NH_2, COOH
	Synthetic polymers	Polyamino acids		COOH, φ-NH_2
		Polyvinyl (ethylene/maleic anhydride)		Anhydride
		Polyacryliques	Polyacrylamide	NH_2
			Acrylamide copolymer	Variable
			Polyacrylates	COOH
			Polymer and copolymer of methacrylic acid	COOH
			Polystyrene and derivatives	φ-X
			Polyamide (nylon)	NH_2
Mineral substances	Porous glass			OH
	Aluminosilicates			OH
	Metal oxides			OH
Mixed supports	Magnetic supports			Variable
	Organo-mineral supports			Variable

4.3.3. Advantages and disadvantages of covalent bonding methods :

Advantages **:**

- ✓ Covalent immobilization is characterized by the permanence of the enzyme-support bond (expensive enzymes or enzymes with excluded release).
- ✓ Acquisition of new properties in terms of enzyme resistance.
- ✓ The quantities of immobilized enzymes are lower, which does not necessarily imply lower specific activity.
- ✓ There is a wide variety of supports.
- ✓ The stiffening of the three-dimensional structure of the supports allows for greater stability.

Disadvantages :

- ✓ Complexity of methods (introduction of reactive groups);
- ✓ In situ regeneration possibilities are very limited;
- ✓ Modification of the enzyme structure, resulting in loss of activity;
- ✓ Impossible to predict grafting yield;
- ✓ Organic supports are susceptible to attack by microorganisms and their mechanical properties are unsatisfactory.

4.4. Cross-linking :

It is a chemical process that immobilizes enzymes in the absence of a support by forming covalent intermolecular bonds between the enzyme and a bi- or multifunctional agent (cross-linking agent).

This coupling is based on the reaction of the functional groups of the cross-linking agent with the amine groups of the enzymes to form covalent bridges, in order to obtain high molecular weight structures that may be insoluble. There are two modes of cross-linking :

- **Direct cross-linking :**

Results in stable immobilized enzymes, but with a loss of some of their activity due to the rigidification of their three-dimensional structure.

- **Indirect cross-linking (co-cross-linking) :**

To prevent loss of activity, the enzyme is cross-linked with an inactive protein such as bovine serum albumin (BSA).

Characteristics :

- ✓ There are several reagents (cross-linking agents) for this immobilization method : glutaraldehyde, diazobenzidine, hexamethylene, diisocyanate, and toluene diisothiocyanate.
- ✓ Glutaraldehyde is the most commonly used cross-linking agent.
- ✓ Immobilization by cross-linking is a simple but costly technique.
- ✓ The disadvantage of this method is the risk of enzyme denaturation by polyfunctional reagents.

5. Comparison between methods :

Characteristics	Adsorption	Covalent bond	Cross-linking	Inclusion
Preparation	Simple	Difficult	Difficult	Simple
Cost	Low	High	High	Moderate
Bond strength	Variable	Strong	Strong	Low
Enzyme loss	Yes	No	No	Yes
Applicability	wide	Selective	wide	Very wide
Known issues	High	Low	High	High
Matrix effect	Yes	Yes	Yes	No
Wide diffusion barriers	No	No	Yes	Yes
Microbial protection	No	No	Yes	Yes

6. Choice of a technique :

- The choice of immobilization technique depends on the final application of the enzyme.
- Covalent binding of the enzyme to a matrix is generally the most stable immobilization technique.
- The capacity of the matrix, as well as its mechanical and chemical stability, cost, and the difficulty of activating the support are also important factors in the development of an immobilized enzyme system.
- It should be noted that there is no “best” technique.