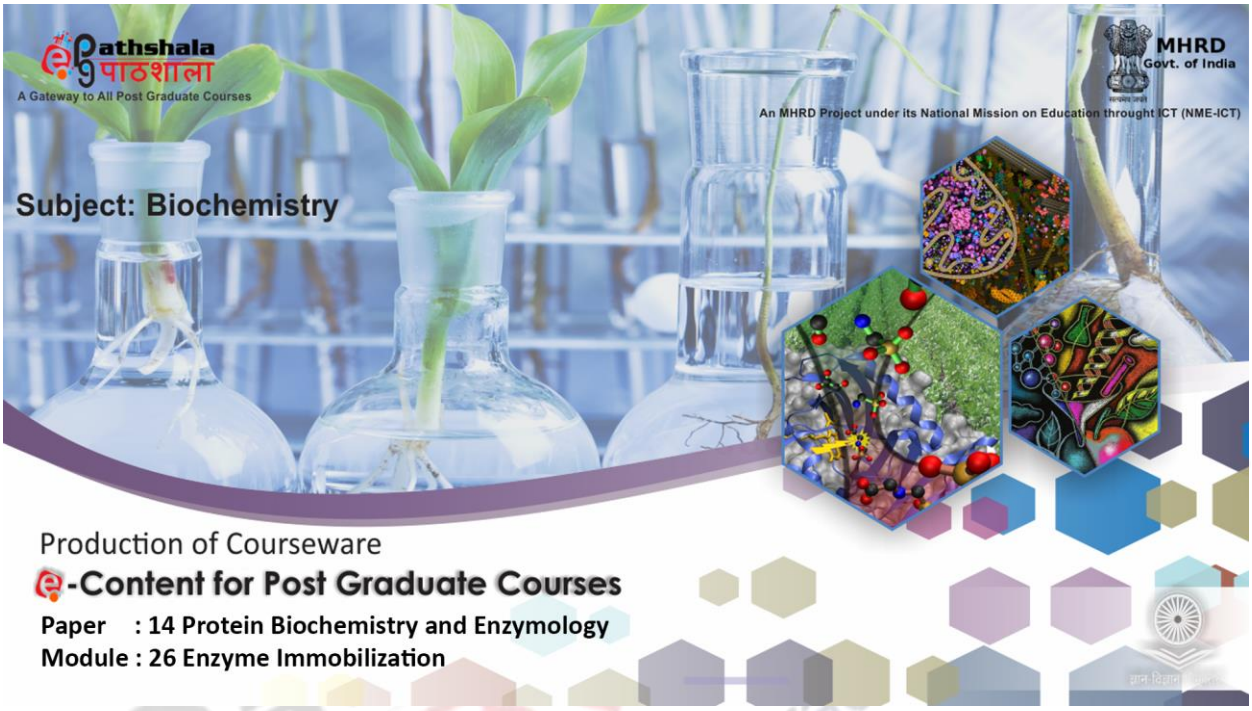

Production of Courseware

e-Content for Post Graduate Courses

Paper : 14 Protein Biochemistry and Enzymology

Module : 26 Enzyme Immobilization

athshala पाठशाला

MHRD Govt. of India

Development Team

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Description of Module	
Subject Name	Biochemistry
Paper Name	
Module Name/Title	**Enzyme immobilization**

1. Objectives

1. What is an immobilized enzyme
2. Merits and Demerits of Immobilization
3. Methods of immobilization
4. Matrix or Solid support used in immobilization
5. Properties of immobilized enzymes
6. Applications of enzyme immobilization

2. Enzyme Immobilization

Enzymes are biocatalyst that carries out all the essential biochemical reactions inside the body of an organism. Their unique feature is that they remain unaltered after the reaction is completed. Therefore, they can be used again and again. But the limitation of soluble enzymes is their isolation from the product and the substrate. Most of the Enzymes in the living organism are attached to the cell membrane or entrapped within the cells. This observation led to the concept that pure isolated enzymes may actually perform better when they are immobilized on a solid support. The term immobilized enzyme is used to denote "enzymes physically confined or localized in a defined region of space with retention of their catalytic activities and which can be used repeatedly and continuously". Immobilization is beneficial because it facilitates work up product isolation. Some of the potential advantages and disadvantages of immobilization are highlighted below.

Soluble Enzyme + Substrate---------- Product (single time usage of enzyme)

Immobilized Enzyme + Substrate---------Product (Repeated usage of enzyme)

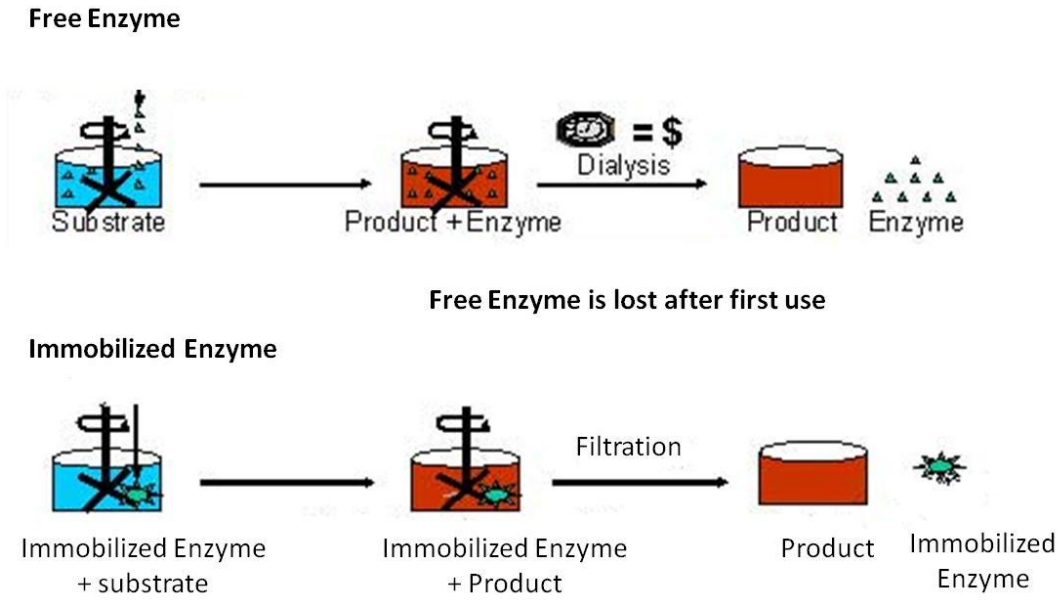


Figure 1: Enzyme Immobilization

2.1. Advantages:

1. Easy recovery of the product
2. Product is free from enzyme, so there is no cost of purification of enzyme.
3. Enzyme can be used repeatedly
4. The enzyme generally get stabilized after adsorption

2.2. Disadvantages:

1. Loss of catalytic properties for some enzymes
2. Some enzymes become unstable
3. Additional cost of immobilization
4. Differential limitations.

Intensive study in the area of enzyme immobilization started in mid 1950 and has since continued. The first industrial use of immobilized enzymes was reported in 1967 by Chibata and co-workers who immobilized *Aspergillus oryzae* amino acylase for the resolution of synthetic , DL amino acids into the corresponding optically active enantiomers. At present, the use of immobilized enzyme is well established in various industries.

Number of important points should be kept in mind while immobilizing an enzyme

1. The biological activity of the enzyme should be retained
2. The enzyme should be more stable as compared its soluble counterpart.
3. The cost of immobilization should not be too high
4. It should be used repeatedly.

3. Enzyme immobilization Techniques

The various methods of enzyme immobilization are broadly classified as

1. Reversible immobilization
   a. Adsorption
2. Irreversible immobilization
   a. Covalent coupling
   b. Entrapment and microencapsulation
   c. Crosslinking

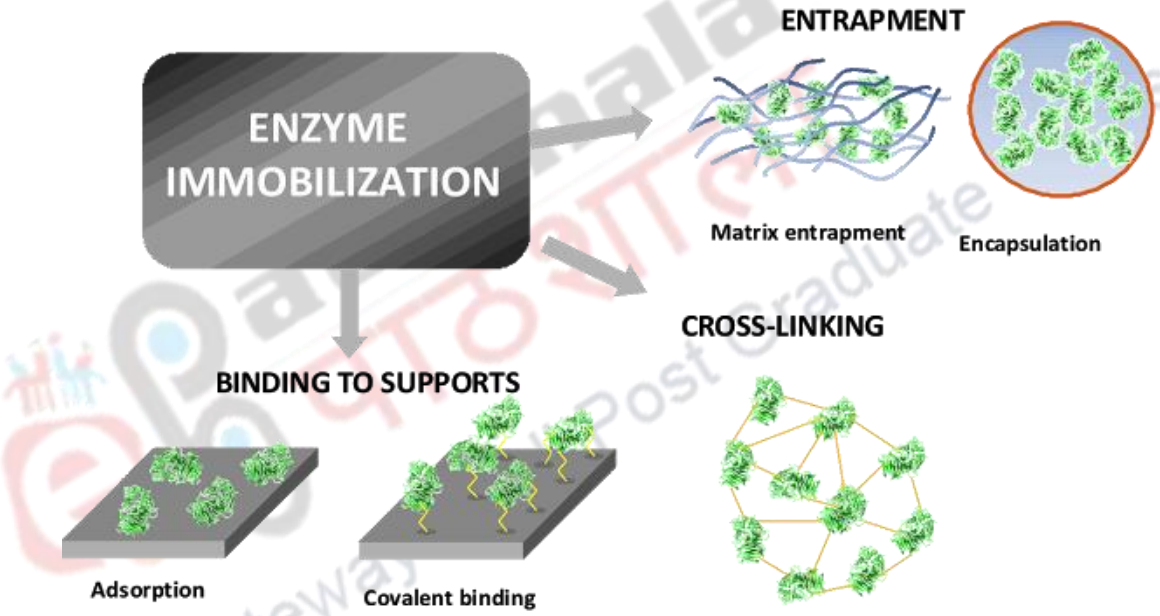


Figure 2: Diagrammatic representation of the various methods of immobilization

The advantage of reversible immobilization over irreversible systems can be summarized as follows:

1. No chemical modification of the enzyme is required
2. If the enzyme gets activated during use, it can be replaced in reversible immobilization.
3. The immobilization of enzyme by adsorption of bioaffinity can be accomplished rapidly

3.1. Reversible immobilization

3.1.1. Adsorption

Immobilization by adsorption is the easiest and fastest method. The adsorption is dependent on the experimental variables such as pH, nature of solvent, ionic strength, quantity of enzyme and adsorbent, the time and temperature. A close control of these variables is required owing to the relatively weak binding forces between protein and adsorbent (hydrogen bonds, van der Waals forces, hydrophobic interactions, etc.). Enzymes can be immobilized by simply mixing the enzymes with the matrix, under appropriate conditions of pH and ionic strength. Adsorption process is based on vander Waal forces, ionic and hydrogen bonding as well as hydrophobic interactions, which are very weak forces, but in large number, impart sufficient binding strength. Adsorbed enzymes can be protected from agglomeration, proteolysis and interaction with hydrophobic interfaces. In order to prevent chemical modification and damage to enzyme, the existing surface properties of enzymes and support are need to be considered. The adsorption through physical method generally involves multipoint protein adsorption between a single protein molecule and a number of binding sites on the immobilization surface. The main disadvantage of this method is that the enzyme is easily desorbed by factors like pH, temperature fluctuations, changes in substrate and ionic concentrations. Few advantages of adsorption methods are

- Easy to carry out
- No reagent are required
- Minimum activation step involved
- Comparatively cheap method
- Less disruptive to protein than chemical methods

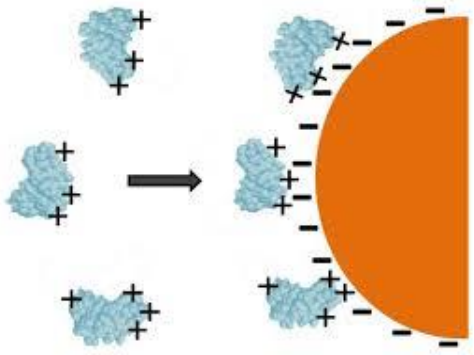


Figure 3: Adsorption

3.2. Irreversible immobilization

3.2.1. Covalent coupling

Covalent coupling is the most frequently used approach of immobilization in which covalent bonds are formed between surface amino acids of the enzyme and the matrix. Hydrophilic amino acids which are likely to be present on the protein surface, can be exploited for this purpose. ε-amino group of lysine residue, cysteine (via SH), tyrosine, histidine, aspartic and glutamic acids, tryptophan and arginine mostly takes part in bond formation. A number of chemical reagents and protocols are available for covalently linking an enzyme to the matrix.

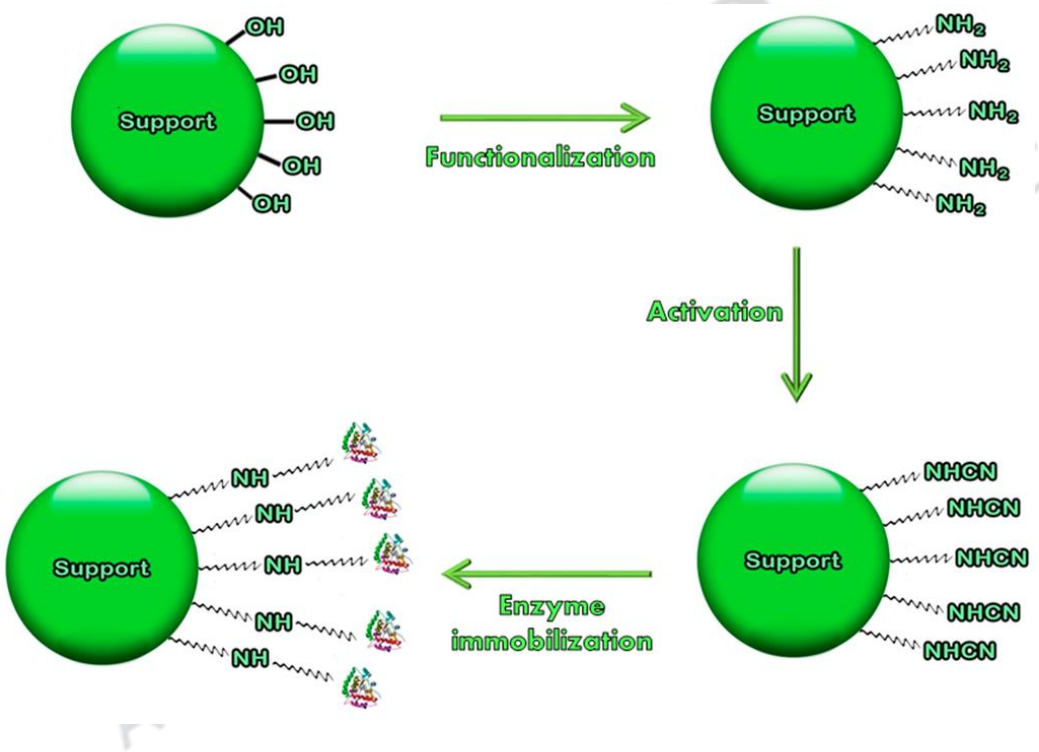


Figure 4: Covalent binding

Most commenly used methods of covalent bonding are:

1. **Diazoation:** It is based on the diazo linkage between protein and aryldiazonium electrophilic groups of the matrix.
2. **Formation of Peptide bond**: bond formation between amino/carboxyl groups of support and amino or carboxyl group of the enzyme
3. **Poly functional reagents:** use of bi-functional or multifunctional reagent (glutaraldehyde) which forms bonding between the amino group of the support and amino group of the enzyme

Figure 5: Glutaraldehyde

4. **Amidination reaction**: The matrix containing amido ester functional groups can be used for immobilization of protein.
5. **Thiol–disulphide interchanged reaction:** This methods is used for protein bonding via thiol groups of both carrier and protein.
6. **Akylation and arylation**: This methods is based on alkylation of amino, phenolic and thiol groups of protein with reactive matrix containing halides, vinyle, sulphonile etc.

Advantage of Covalent bonding method:

- Strong linkage of enzyme to the support
- No leakage or desorption problem
- Comparatively simple method
- A variety of support with different functional group available
- Wide applicability

Disadvantage of Covalent bonding method:

- Chemical modification of enzyme leading to functional conformational loss
- Enzyme inactivation by change in the conformational when undergoes reactions at active site
- This can be overcome through immobilization in the presence of enzyme substrate or a competitive inhibitor

3.2.2. Entrapment

Entrapment and encapsulation is based on the occlusion of an enzyme within a constraining structure, but tight enough to release an enzyme while allowing penetration of a substrate. However, due to diffusion limitations, such methods are often unsuitable for the immobilization of enzymes hydrolyzing macromolecular substrates.

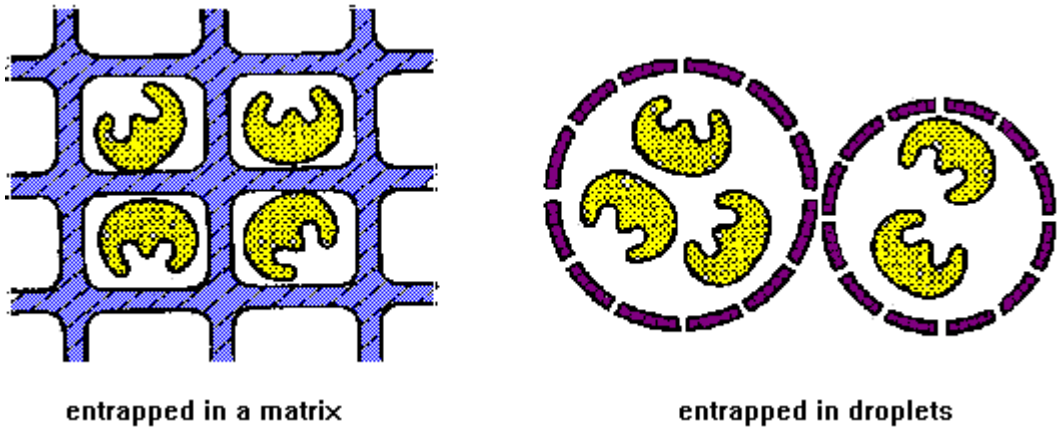


Figure 6

Methods of Entrapment

- **Inclusion in the gels:** enzymes trapped in gels
- **Inclusion in fibers:** enzymes supported on fiber formate
- **Inclusion in microcapsules:** enzymes entrapped in microcapsules formed by monomer mixtures such as polyamine, calcium alginate

Advantage of Entrapment method:

- Fast
- Cheap (low cost matrix available)
- Mild conditions are required
- Less chance of conformational change in the enzyme

Disadvantage of Entrapment method:

- Leakage of enzyme
- Pore diffusion limitation
- Chance of microbial contamination

3.2.3. Cross linking

This method involves attachment of biocatalysts to each other by bi- or multifunctional reagents or ligands. In this way, very high molecular weight typically insoluble aggregates are formed. Cross-linking is a relatively simple process. It is not a preferred method of immobilization as it does not use any support matrix. So they are usually gelatinous and not particularly firm. Since it involves a bond of the covalent kind, biocatalyst immobilized in this way frequently undergoes changes in conformation with a resultant loss of activity. Still it finds good use in combination with other support dependent immobilization technologies, namely to minimize leakage of enzymes already immobilized by adsorption.

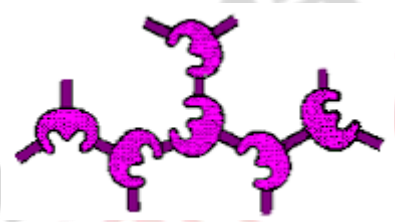


Figure 7: Cross-linking

Recent advancement to crosslinking method is the formation of CLEC, CLEA and Spherezyme

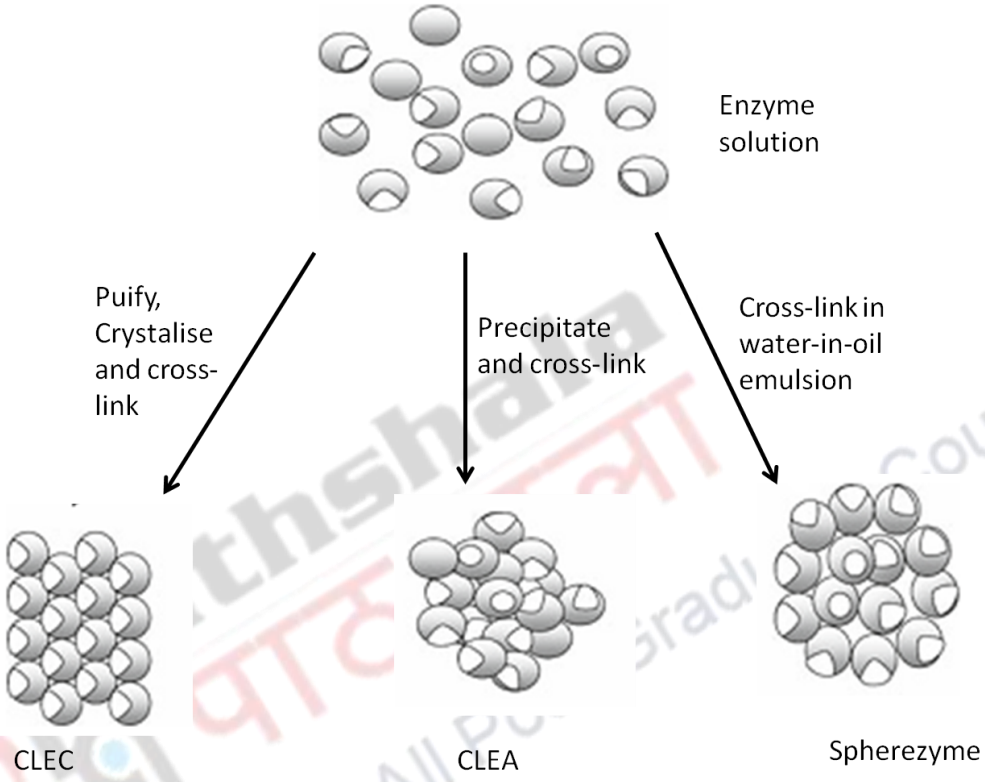


Figure 8: Formation of CLEC, CLEA and Spherezyme by crosslinking

Advantage of Cross linking method:

- It is used mostly as a means of stabalizing adsorbed enzyme and also for preventing leakage

Disadvantage of Cross linking method:

- Cross linking may cause significant change in the active site of enzyme which may lead to loss of activity

4. Charcteristic for a Solid support

one of the major component of immobilized enzyme is the support materials. Ideal support material should posses following characteristic:

1. Large suface area
2. Permeability
3. Hydrophilic character
4. Insolubility
5. Chemical mechanical and thermal stability
6. High rigidity
7. Soutable shape and particle size
8. Resistance to microbial attack
9. Regeneratibilty

4.1. Support material are classified into two categories based on their morphology

1. Non porus support: eg glass
2. Porus support: eg silica

Porus support have high surface area as compare to non porus support.

4.2. Support material are classified into two categories based on their chemical nature

1. Organic
2. Inorganic

4.2.1. Organic support material are further classified as:

1. Natural
2. Synthetic

Natural organic supports include Polysacchrides and proteins.

Some of the commonly used polysaccharides are

- **Alginate :** are polymers of n-acetyl glucouronic acid, having hydroxyl group which can be derivatized easily

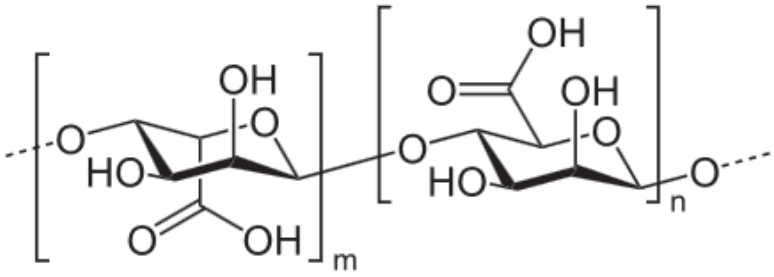
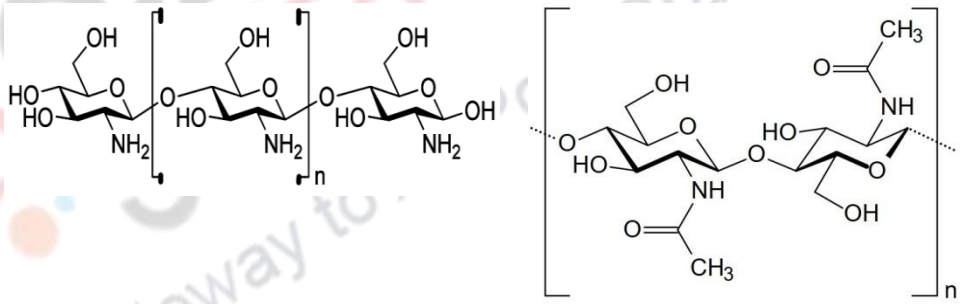


Figure 9: Alginic acid

- **Chitosan and chitin:** chitosan is deacetylated chitin



(a) (b)

Figure 10

- **Carrageenan**: Carrageenan, a linear sulfated polysaccharide.

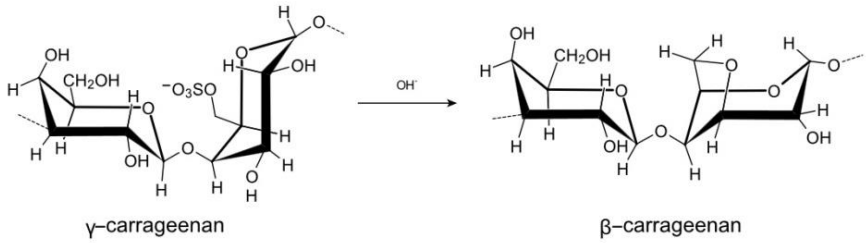


Figure 11: The molecular structures of carrageenan

- **Cellulose**: a linear polymer of D-glucose units (two are shown) linked by β(1→4)-glycosidic bonds

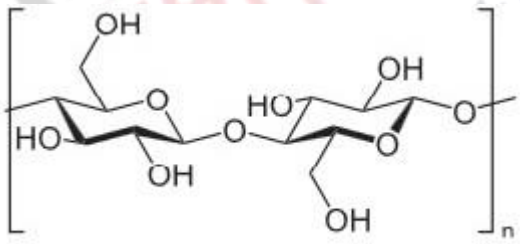


Figure 12

- **Starch:** linear polymer of glucose
- **Pectin:** A heteropolysaccharide made of galacturonic acid present in plant cell wall.

Some of the commonly used Proteins are:

- **Collagen**
- **Gelatin**

Synthetic support are the largest number of support materials avalaible for protein immobilization due to their physical and chemical characteristic.

- DEAE cellulose
- polyvinyl chloride (PVC)
- UV-activated polyethylene glycol (PEG)
- glutaraldehyde-activated nylon
- cyclodextrin glucosyltransferase

4.2.2. Inorganic materials as supports: the surface of most inorganic support is mainly composed of oxide and hydoxil group, such as silanol group in glass, provide mild reactive surface for activation and protein binding.

- **Zeolites:** Zeolites are microporous crystalline solids with well-defined structures.

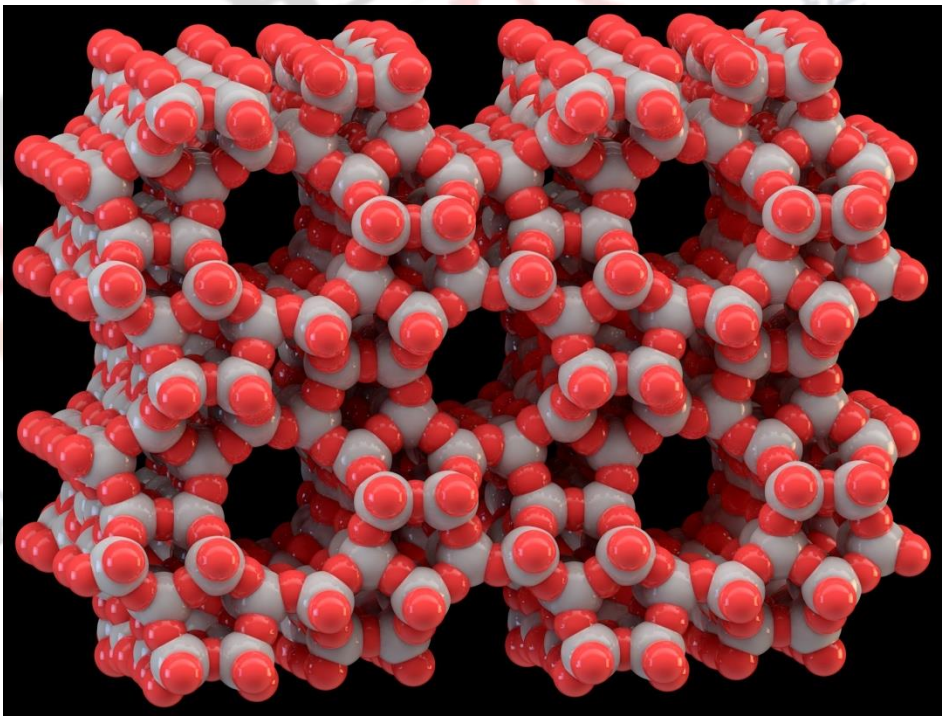
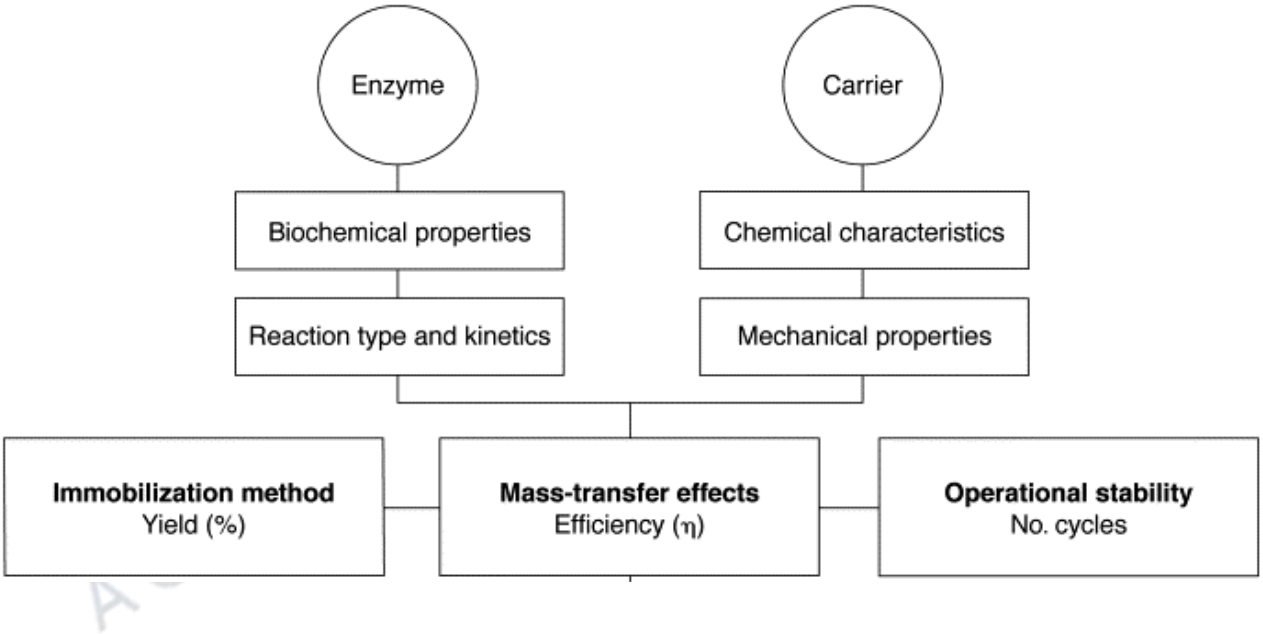


Figure 13: The microporous molecular structure of a zeolite, ZSM-5

- **Ceramics:** inorganic , non metal
- **Celite:** Celite is highly porous diatomaceous material.
- **Silica**: oxide of silicon
- **Glass:** Glass is a highly viscous liquid
- **Activated carbon:** Activated carbon is produced from carbonaceous source materials, such as coal, coconuts, nutshells, peat, wood, and lignite.
- **Charcoal**: allotrope of carbon, has excellent adsorption capacity

5. Biochemical Properties of the Immobilized Enzymes

The biochemical properties of the immobilized enzyme are different when compared to the free enzyme, this is due to the change in environment of the immobilized enzyme



- the physical and chemical properties of the support matrix and interactions of the matrix with substrates or products changes the kinetics of the immobilized enzyme.
- Generally there is a decrease in the rate of enzyme catalyzed reaction because the matrix restric the diffusion of the substrate towards the enzyme.

- The Km of the enzyme also changes after immobilization because of diffusion limitations. If the matrix is positively charged and substrate is also positively charges due to electrostatic repulsion the substrate will not come in the viccinity of the enzyme hence Km is altered.
- Sometimes the 3D structure of the enzyme is also changed which also result in altering the kinetic properties of enzyme.
- The performance of the immobilized enzyme can be improved further by studying the structural changes of the immobilized enzyme

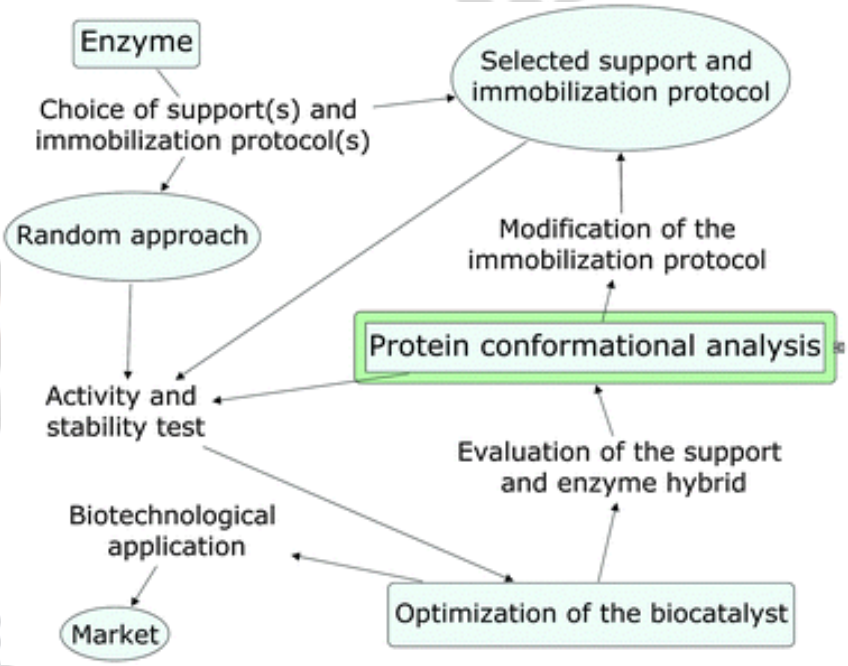


Figure 14: Structural studies improves the performance of the immobilized enzyme

6. Applications of enzyme immobilization

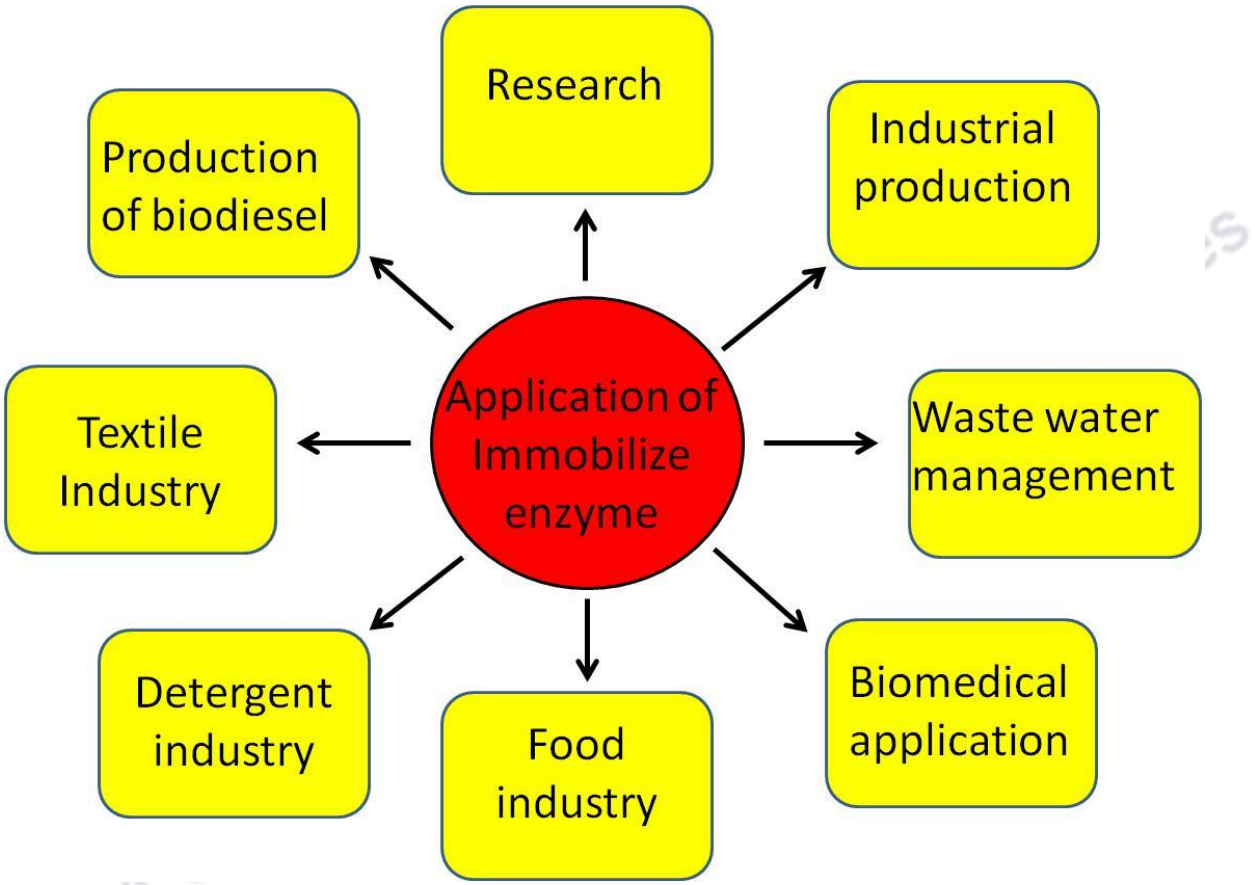


Figure 15: Applications of immobilized enzyme.

6.1. Biomedical Application

Biosensor: Biosensor are electronic monitoring devices that make use of an enzyme's specificity and the technique of enzyme immobilization

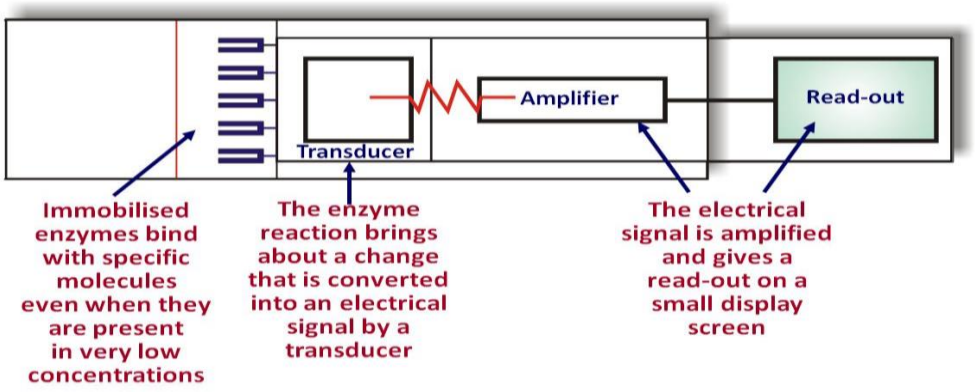


Figure 16: Biosenser

A biosensor has been developed for detecting glucose in the blood of diabetics.

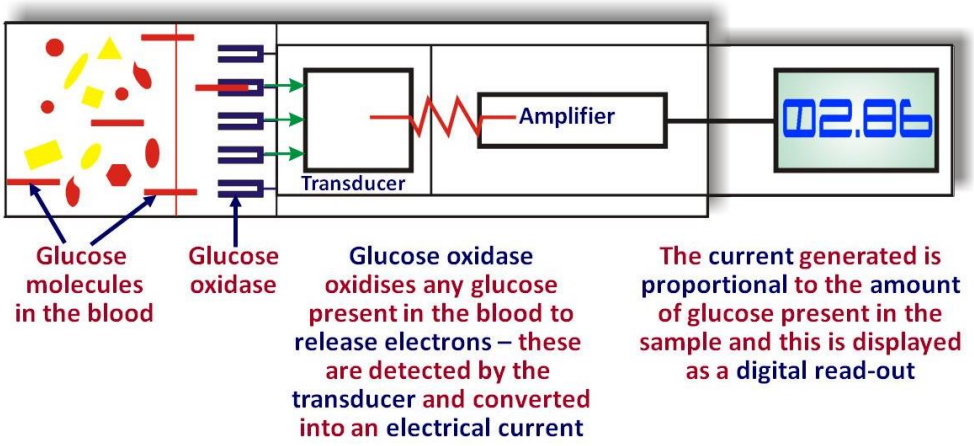


Figure 17: Detecting glucose in blood

Table 1: Immobilized enzymes used as biosensors

Enzymes	Inhibitor	Immobilization matrix	Samples
Biosensors for the determination of pesticides			
Acetylcholinesterase	Paraoxon	Mutiwell carbon nanotubes	Real water sample
Acetylcholinesterase	Paraoxon	Entrapment in PVA-SbQ	Spiked
Variants	Carbofuran	polymer	River water sample
Catalase	Azide	Gelatinwith GA	Fruit juice
Biosensors for the determination of heavy metals			
Urease	Hg2+, Cu Cd	Entrapment in sol-gel matrix	Tap and river water
Glucose oxidase	Hg2+	Cross-linking with GA and BSA	Spiked water
Biosensors for the determination of other chemical components			
Butyrylcholinesterase	A-chaconine, α-solanine	Cross-linking with BSA and GA	Agriculture
Acetylcholinesterase	Anatoxine-a	Entrapment in PVA-SbQ	Fresh water

6.2. Industrial applications of immobilized enzymes

Table 2 shows some of the immobilized enzymes used for the synthesis of various antibiotics.

Enzyme	Immobilization support	Antibiotic produced
Penicillin acylase from *E. coli*	Polyacrylamide gel	Cephalexin
Penicillin G acylase	Nylon hydrolon membrane	Cephalexin
Penicillin G acylase from *E. coli*	Silica gel	6-APA
Penicillin G acylase	Eupergit	6-APA
Acetyl xylan estrase	CKEAs using glutaraldehyd	Desacetyl β-lactam
Penicillin acylase	Poly-N-isopropylacrylamide	Cephalexin

6.3. Immobilized enzymes in food industry

Immobilized enzymes are used in the processing of food samples and its analysis.

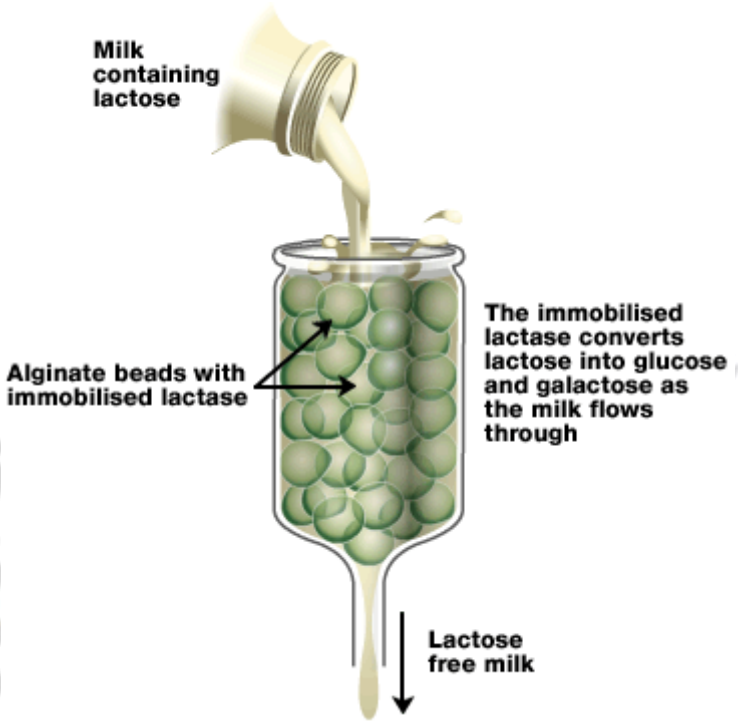


Figure 18: Removal of lactose from milk by Immobilized enzyme

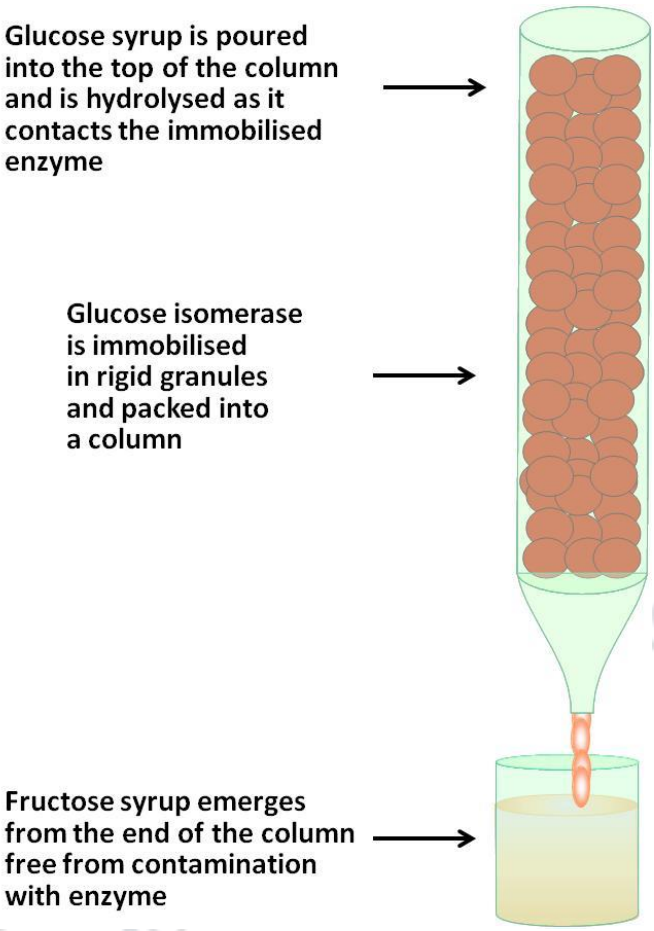


Figure 19: Conversion of glucose syrup into fructose corn syrups

Table 3 shows the processing of various food substrates using respective immobilized enzymes.

Table 3: Immobilized enzymes used in food industry

Enzyme	Immobilization support	Food substrate
β-galactosidase and amyloglucosidas	Bone powder	Lactose, Whey, Whey permeates, skimmed milk
Pectinase	Amino exchange resin	Pectin
Laccase	Silica gel	Ine, fruit juice and beer processing
Trypsin	cellulose	B-lactoglobulin
Tyrosinase	Polyacrylic acid carbon nanotubes	Phenolic in red wine
Pectinase	Amino exchange resin	Pectin solution

6.4. Biodiesel production

Biodiesel has gained importance for its ability to replace fossil fuels which are likely to run out within a century.

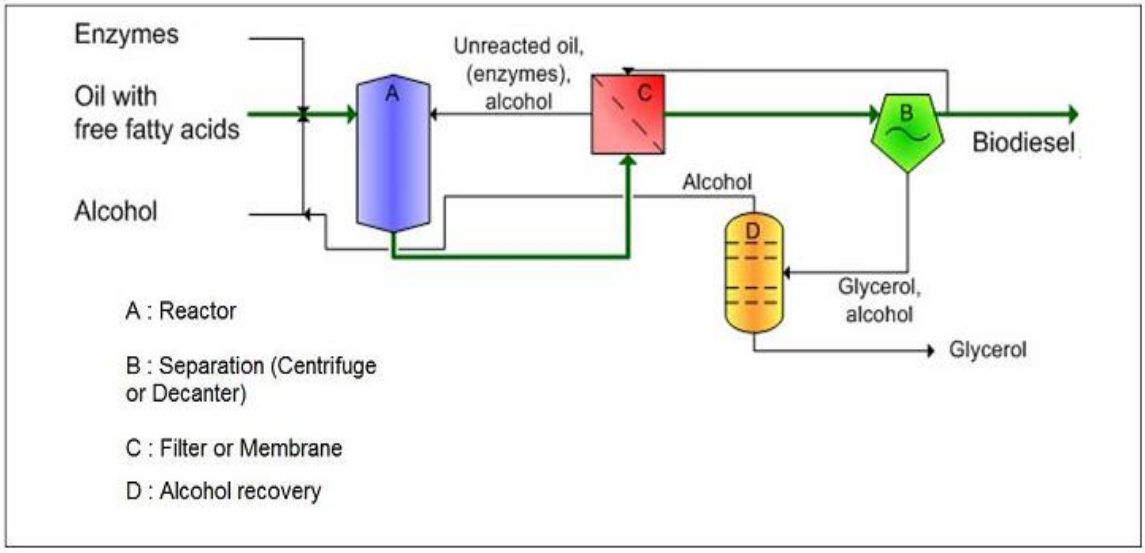


Figure 20: Enzymatic Biodiesel Production

Table 4: Immobilized lipases used biodiesel production

Source of lipase	Immobilization support	substrate
T. lanuginous	Polyurethane foam	Canola oil and methanol
C. antarcrica	Ceramic beads	Waste cooking oil
P. fluoescens	Porous kaolonite	Safflower oil
P. expansum	Silica gel (resin D4020)	Waste oil
T. lanuginous	Microporous polymeric matrix	Sunflower, soyabean
C. nigosa	chitosan	Rapeseed oil
Rhozopus oryzae	Biomass support particles	Jatropha oil

6.5. Immobilized enzymes for bioremediation

Bioremediation is a technique that involves the use of enzyme and biological organism to remove pollutants from a contaminated site.

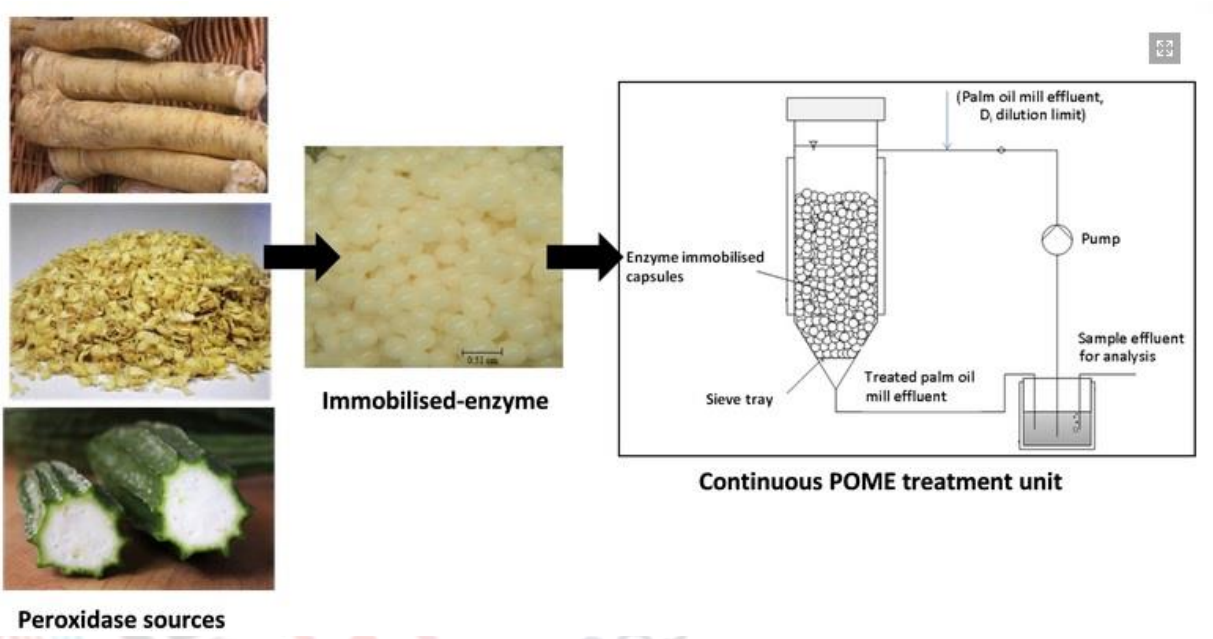


Figure 21: Peroxidase immobilized on support and used for continuous palm oil mill effluent (POME) treatment

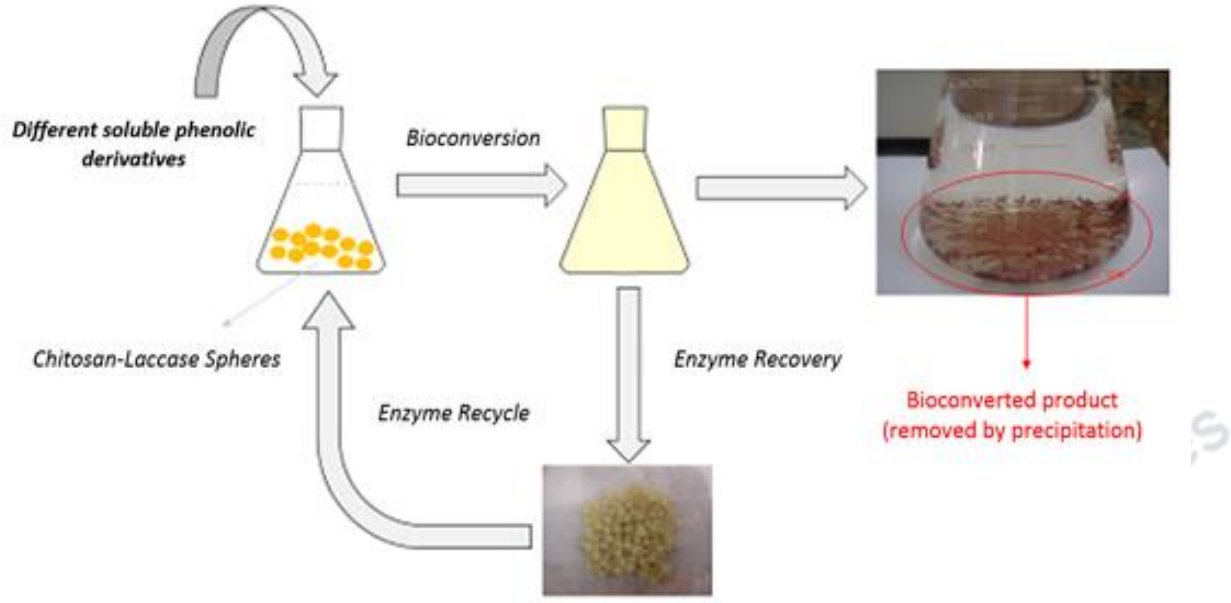


Figure 22: Removal of phenolic derivative by immobilized enzyme.

Table 5 lists some recent research about the use of enzymes in different immobilized forms for dye and phenolic compounds removal.

Table 5: Immobilized Enzymes in bioremediation

Enzyme	Immobilization support	substrate
Lipase	Polypropylene membrane	Dimethylphthalate
Laccase	Silica	Reactive dye
Polyphenol oxidase	Chitosen coated polysulphone membrane	Industrial phenolic effluent
Polyphenol oxidase	Celite-545	Textile and non textile dyes
peroxidase	Con A-sephadex	Textile dye
laccase	Epoxy activated carriers	Synthethic reactive dye
Fungal laccase	Porous glass beads	Anthraquinone and indigoid dyes

Summary

- Immobilization means restricted movement of the enzyme.
- Immobilization can be carried out by attaching the enzyme to a solid support.
- The enzyme is linked by noncovalent interaction or by covalent linkage to the solid support
- The immobilized enzyme is more stable in terms of thermal stability, pH etc as compared to free enzyme
- The major advantage of immobilization is repeated use of the enzyme.
- Nowadays immobilized enzymes are used in all the sectors like medicine, food industry, pharmaceutical industry and for bioremediation etc.