

Unit 4: Microbial enzymes of industrial interest and enzyme immobilization

Methods of immobilization of enzymes

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- This lecture is in continuation to what was covered in the class.
- This is being posted in pursuance of the notification on continued online learning during class suspension due to Covid-19.

Unit 4: Microbial enzymes of industrial interest and enzyme immobilization

- Microorganisms for industrial applications
- Hands –on screening for casein hydrolysis
- Hands-on screening for starch hydrolysis
- Hands-on screening for cellulose hydrolysis
- **Methods of immobilization**
- Advantages and applications of immobilization
- Large-scale applications of immobilized enzymes (glucose isomerase and penicillin acylase)

Introduction

- Humans have been using enzymes for millenia, even though the concept of enzymes was propounded much later.
- Ancient Egyptians are known to have used fermentation for producing beverages.

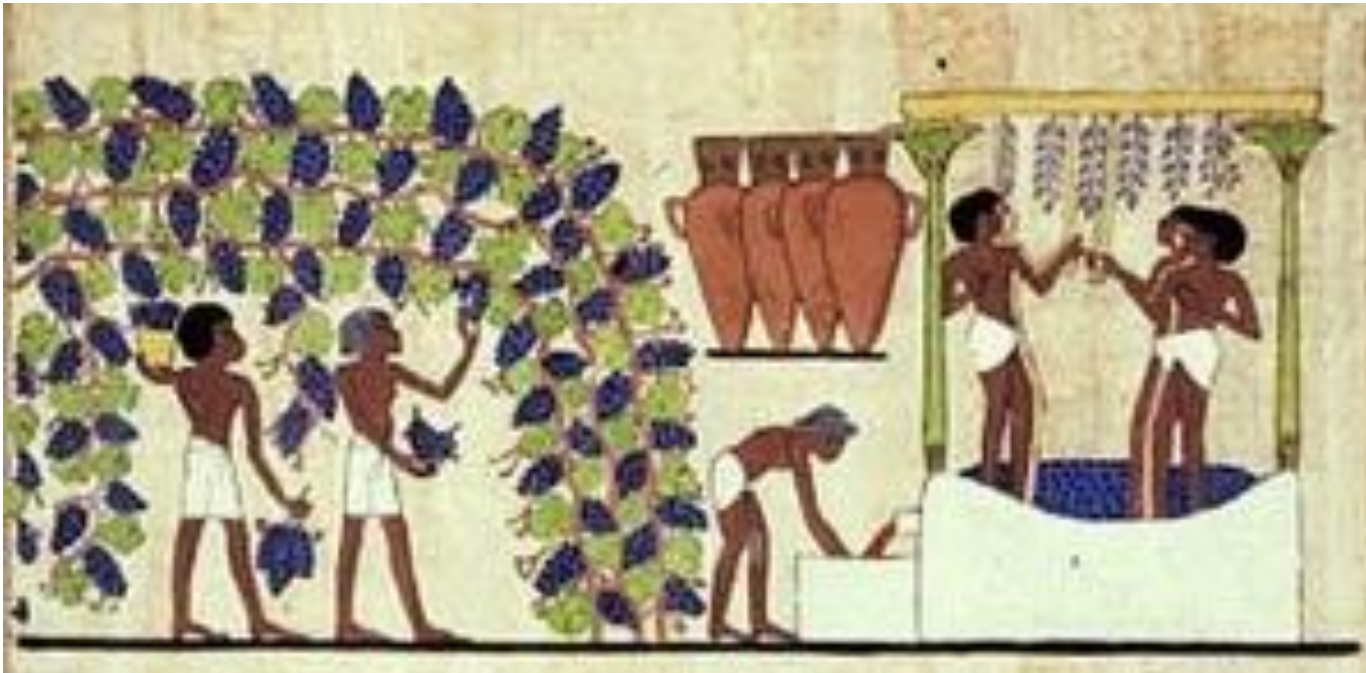


Image source:
<http://fermentedherbs.blogspot.com/2014/02/ancient-wines-thyme-and-artemesia-and.html>

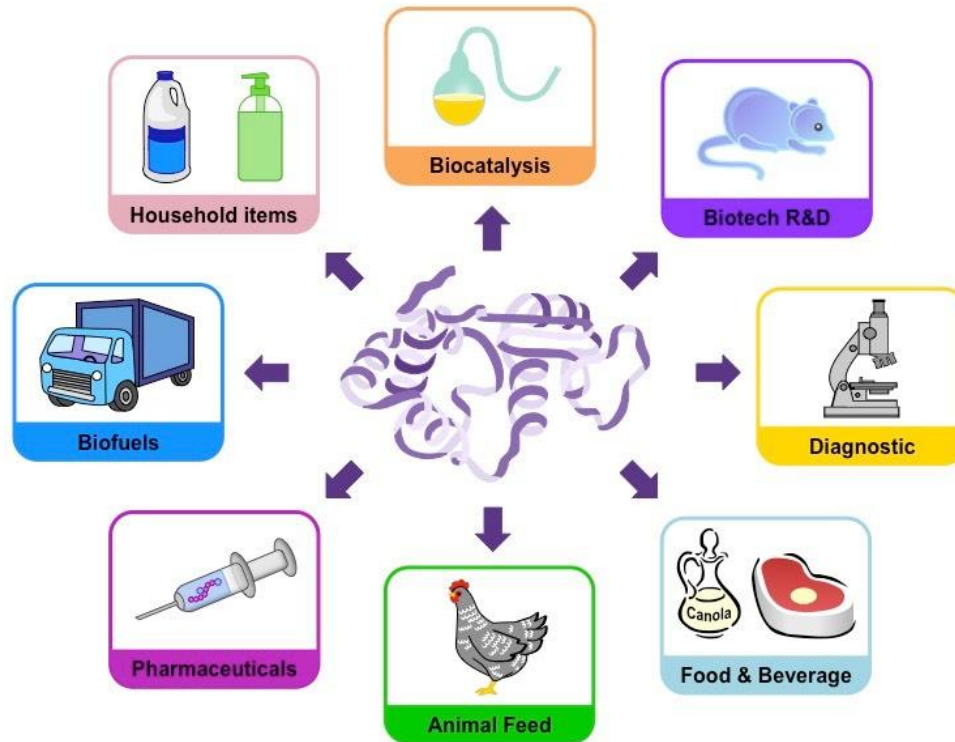


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- Enzymes are natural proteinaceous catalysts that accelerate many biochemical and chemical reactions. They are vital to living organisms and are found in all classes of organisms.
- They find their applications in myriad industries: food, pharmaceuticals, textiles, paper & pulp, biofuel, modern industries like production of biosensors, etc

Hurdles in the industrial usage of enzymes

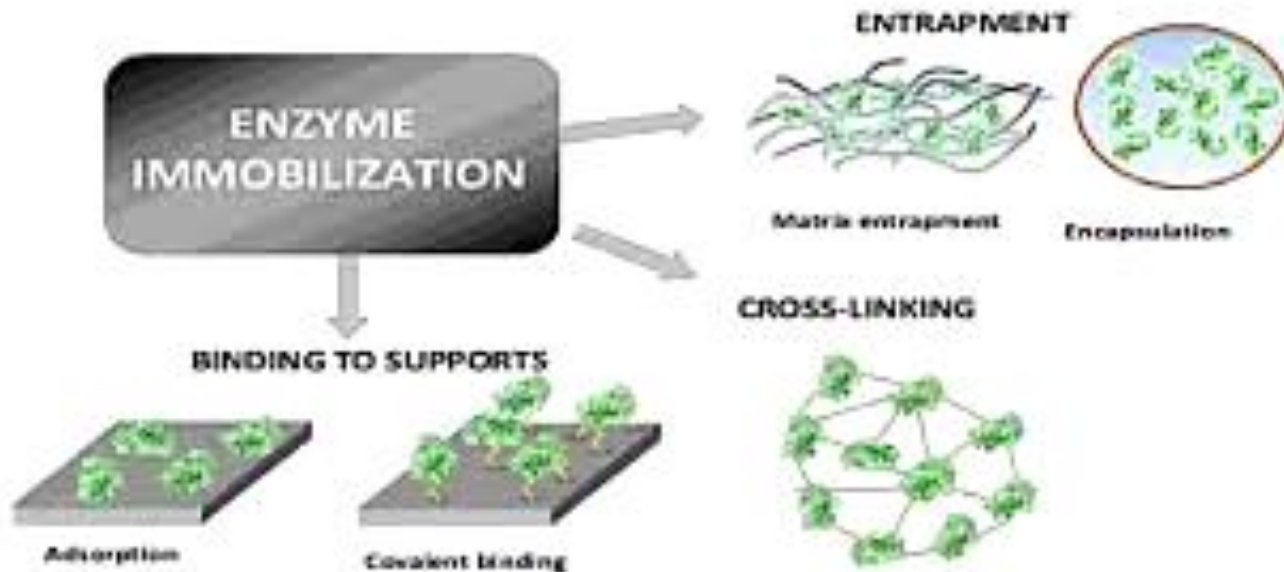
- Enzymes lack long-term operational stability and shelf-storage life
- They are generally expensive because the cost of their isolation and purification is many times higher than that of ordinary catalysts.
- Being proteinaceous, they are also highly sensitive to various denaturing conditions when isolated from their natural environments.
- They are also sensitivity to process conditions, such as temperature, pH, and substances at trace levels, can act as inhibitors which add to their costs.
- Their recovery and re-use are cumbersome. Unlike conventional heterogeneous chemical catalysts, most enzymes operate dissolved in water in homogeneous catalysis systems. This leads to product contamination, thus ruling out their recovery from most of the reaction mixtures for reuse in the active form.

A major challenge in industrial bio-catalysis is the development of stable, robust, and preferably insoluble biocatalysts.

These drawbacks can generally be overcome by immobilization of enzymes.

Enzyme immobilization

- It can be defined as the confinement of enzyme molecules onto/within a support/matrix physically or chemically or both, in such a way that it retains its full activity or most of its activity.
- An enzyme is fixed to a support (more recently nanostructures) while retaining its catalytic activity. Natural or artificial substrates can be used as efficient carriers.



Source: Int. J. Sci. Res. Sci. & Tec.

History

- In 1916, Nielsen and Griffin found that invertase, bound to artificial substrate of aluminium hydroxide [Al(OH)₃] and charcoal, had retained its catalytic activity similar to when it is uniformly distributed throughout the solution.
- However, the idea of exploiting these immobilized enzymes began only from 1950s, and by 1970s, the technology was quite developed.
- This discovery was later developed to the currently available enzyme immobilization techniques.

Function of an immobilized enzyme

Any immobilized enzyme contains **two essential functions**:

- (i) Non-Catalytic Functions (NCFs)** that are designed to aid the separation of enzyme from the reaction mixture and hence plays a role in its re-use
 - NCF relates to the physical properties of the immobilized enzyme, such as shape, size and length of the selected carrier.
- (ii) Catalytic Functions (CFs)** that are designed to convert the substrates into the products within the desired time and space.
 - CF is correlated with the biological activities of the enzyme such as substrate specificity, activity, ideal pH and temperature range.

Thus, it is mandatory to develop a 'robust' enzyme system which consists of an optimum NCF and a high CF to ensure purification and re-use, along with high product yield within a minimum time frame.

Methods of immobilization of enzymes

- Enzymes may be immobilized by a variety of methods, such as physical, where weak interactions between support and enzyme exist, and chemical, where covalent bonds are formed between the support and the enzyme.
- Here, we will discuss the methods under two broad classes:

(A) Reversible Enzyme Immobilization methods

(B) Irreversible Enzyme Immobilization methods

Methods of enzyme Immobilization

(A) Reversible Enzyme Immobilization methods

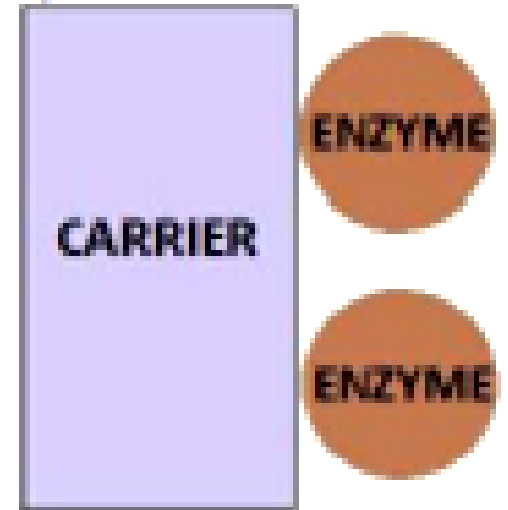
1. Adsorption
2. Chelation
3. Disulphide bonding
4. Ionic binding
5. Affinity binding

(B) Irreversible Enzyme Immobilization methods

1. Covalent Binding
2. Crosslinking:
 - a) Cross Linking Enzyme Crystals (CLEC);
 - b) Cross Linking Enzyme Aggregates (CLEA)
1. Entrapment
2. Encapsulation

(A)1: Adsorption

- It is the oldest and arguably the simplest method.
- The first industrial process that involved immobilized enzyme used an aminoacylase adsorbed to a DEAE-Sephadex for continuous resolution of amino acids.
- The first record of large scale industrial utilization of immobilized enzyme technology also involved adsorption of glucose isomerase to DEAE-Cellulose in the production of high fructose corn syrup by Clinton Corn Products.

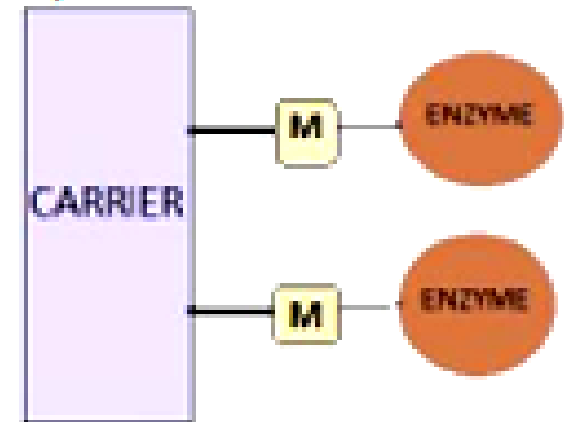


(A)1: Adsorption contd.

- **Principle:** It involves adhering of the enzyme to the surface of the carrier via several weak non-covalent interactions such as hydrogen bond, Van Der Waal's interactions and hydrophobic interaction.
- **Advantage:** It is cheap, easy to perform and allows easy recovery of the enzyme from the carrier, thus allowing re-use of both. It requires very little activation and no reagents. The forces being weak, enzyme is hardly distorted, retaining maximum enzyme activity.
- **Disadvantage:** There is significant enzyme loss as the binding forces are weak, reversible and susceptible to physical parameters such as pH and temperature. This may lead to the contamination the products with the enzyme and complicate the purification process
- **Carriers used:** activated charcoal, alumina, cellulose, Sephadex, agarose, collagen and starch & other new eco-friendly carriers like coconut fibre.

(A)2: Chelation

- Chelation or metal binding is another common type of reversible enzyme immobilization mostly used as a chromatographic method. Being reasonably expensive and involving safety issues, this method is less popular in industries.
- **Principle:** It is based on the ability of charged and polar amino acids (histidine, lysine, phenylalanine, cysteine and tyrosine) to bind to metal ions via coordinate bonds. The metal ions bound to the carrier surface have metal ligands weakly bound to them. Upon exposure of the enzyme to the carrier, the weak ligands are replaced by the enzyme molecules. The binding can be easily reversed either by the introduction of a ligand with greater affinity for the metal ion (ethylene diamine tetraacetate, EDTA) or by addition of excess of a competing ligand

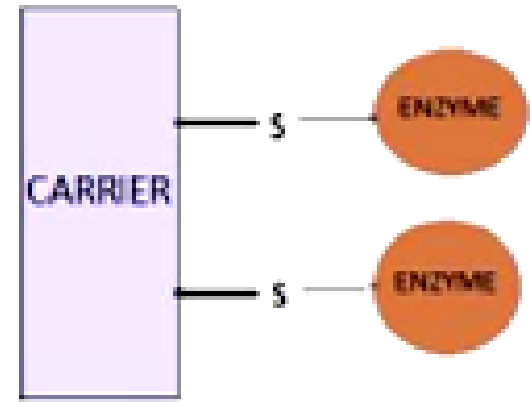


Chelation contd.

- **Advantage:** Enzyme leakage is restricted. The support can be easily regenerated. A variety of chelating anions can be used.
- **Disadvantage:** The reagents involved may not be safe for the production of food products and may cause health hazards. Enzyme activity may be reduced due to interaction with the metal ions at the active sites of the enzymes.
- **Carriers used:** The supports used are mostly organic materials, usually cellulose, chitin or silica-based carriers and also others like transition metal salts or hydroxides .

(A)3: Disulphide bonding

- It involves the formation of disulphide bonds between the enzyme and the matrix.



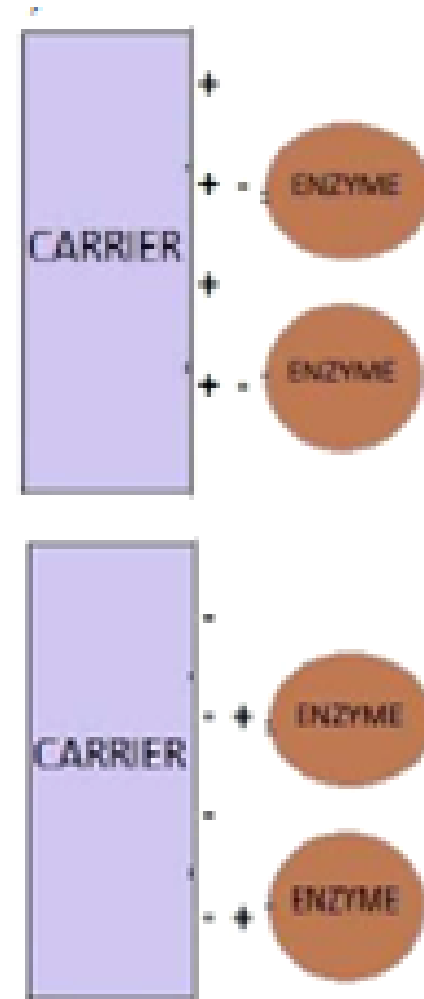
Though it is a covalent bonding, it is classified as a reversible technique because of the ease of reversal of the binding.

- **Principle:** Disulphide linkage is formed between the carrier and a free thiol group, usually on cysteine residues. The bond is stable under physiological conditions. But the binding can be reversed by the addition of reagents such as dithiothreitol (DTT) under mild conditions, or by altering the pH.
- **Advantage:** Under ambient conditions enzyme-leakage is restricted due to stable bonding. The activity of the thiol group can also be altered with pH.
- **Disadvantage:** As the reaction progresses, constantly changing pH and substrate concentration may alter enzyme binding, leading to enzyme loss.
- **Carriers used:** Inert substances like silica, which are chemically activated by agents such as iodoacetate or maleimide are used.

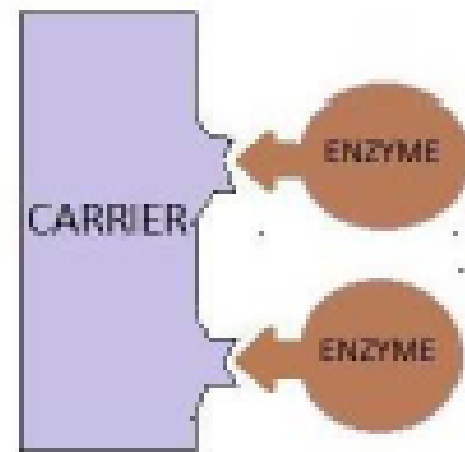
(A)4: Ionic binding

This is a simple reversible mode of immobilization of proteins, which involves ionic interaction between the enzyme and the support.

- **Principle:** The support used is generally charged, such that the protein to be bound has an opposite charge. The enzyme is therefore bound to the support via ionic interactions. It can be easily reversed by altering the pH or 'salting out' of the enzyme.
- **Advantage:** It is very easy, inexpensive and requires simple inputs for reversal of the binding. To maintain an optimum pH during the reaction tenure, easy manipulation of the acidity or alkalinity in the reaction mixture can be performed, as the matrix which immobilizes the enzyme is stably charged.
- **Disadvantage:** The presence of the charged support causes several problems like enzyme structure distortion and alterations in enzyme kinetics. High charge has the potential to disrupt the enzyme catalysis. As a result, maximum yield is hindered.

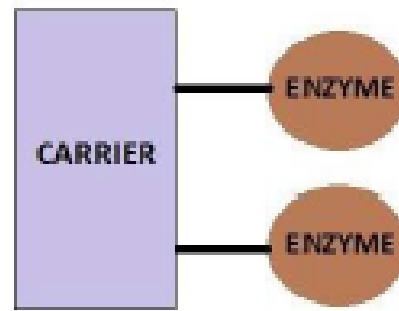


(A)5: Affinity binding



- **Principle:** This technique is based on high affinity interaction between biomolecules, like antigen-antibody interaction. The carrier matrix is synthesized specifically for a single type of enzyme and contains antibodies against specific epitopes on the antigen (enzyme).
- **Advantage:** The reaction is highly specific and no contaminants are present on the carrier. If the antibody on the support is highly specific for the enzyme, the step of enzyme purification can be bypassed. Enzymes from an impure solution can also specifically attach to the matrix. Maximum activity of the enzyme is also ensured if the antibody is targeted at an epitope away from the activity site.
- **Disadvantage:** The method involves use of specific antibodies, which are generally very expensive.

(B)1: Covalent binding

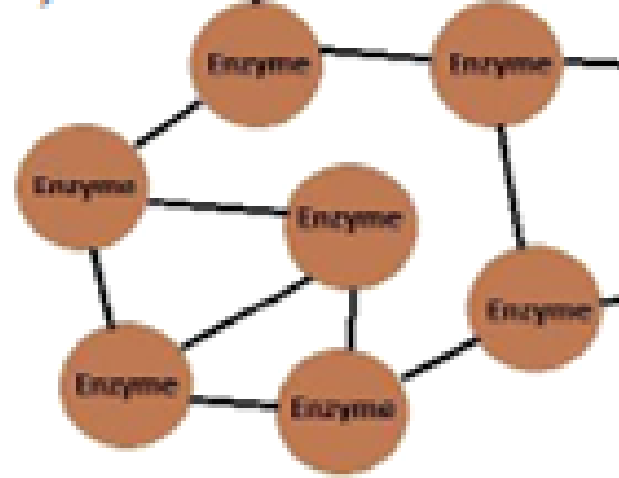


- It is a highly stable irreversible immobilization technique that has been used in industries since 1973 esp. in the synthesis of 6-aminopenicillanic acid from Penicillin G, which utilizes penicillin acylase covalently bound to Sephadex G-200.
- **Principle:**
- It covalent bond between the support and the side chains of the amino acids of the enzyme, most commonly lysine ($\hat{\mu}$ -amino group), cysteine (thiol group), aspartic acid and glutamic acid (carboxylic group), hydroxyl group, imidazole group, phenol groups, etc.
- These groups are nucleophiles and tend to bind to electrophilic groups of the support.
- A wide variety of reactions have been developed, depending on the functional groups available on the matrix. The support can be activated by (i) addition of a reactive group to the support polymer to activate it, or (ii) modification of the polymer backbone to produce an activated group. The activation processes are generally designed to generate electrophilic groups on the support. This allows the support to react with the strong nucleophiles on the proteins, allowing stable immobilization

Covalent binding contd.

- **Advantage:** The strong binding prevents leaking of the enzyme into the reaction mixture, thereby reducing contamination and the cost of purification of the product. It may also promote higher specific activity.
- **Disadvantage:** If the covalent bonding involves the amino acids of the active site of the enzyme, it may lead to significant loss in activity. Since the method is irreversible, the support cannot be recycled, as the enzymatic activity declines. The support and the bound enzyme has to be discarded.
- **Carriers used:** The common supports used are cyanogen bromide (CNBr)-activated Sephadex or CNBr-activated Sepharose. Other common carriers include activated forms of dextran, cellulose, agarose, etc. Artificial matrices include Polyvinyl chloride, ion exchange resins and porous glass.

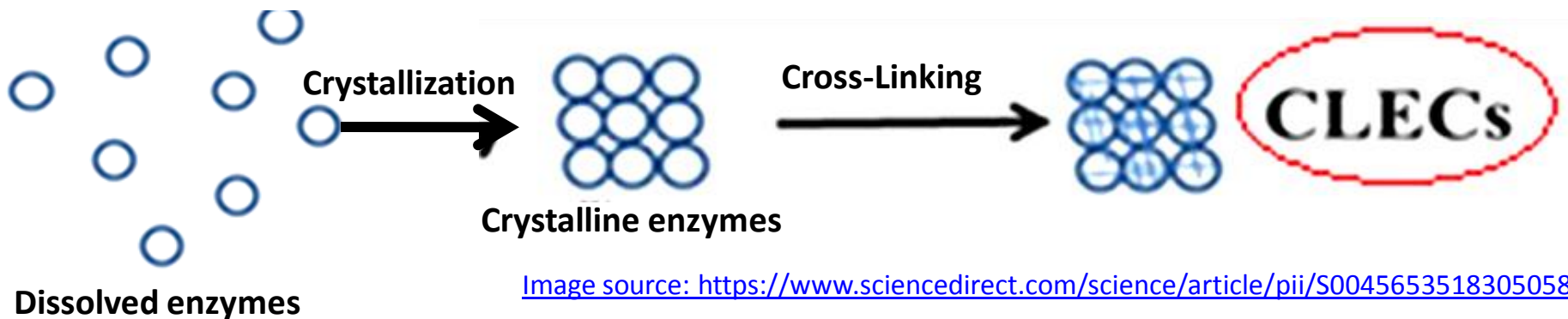
(B)2: Crosslinking



- It is an irreversible method of enzyme immobilization.
- Unlike other techniques, it does not require a support for the immobilization.
- There are two methods of cross linking in use, (i) Cross Linking Enzyme Aggregate (CLEA), and (ii) Cross Linking Enzyme Crystals (CLEC).
- Both CLEA and CLEC are modifications of an old method, where cross linking agents such as glutaraldehyde (which react with the amino group on the protein) were used.
- The CLEC or CLEA are added to the reaction mixture and can be later removed from the mixture during product purification. Hence, unlike the other systems of enzyme immobilization, the immobilized enzyme is not bound to any matrix, but is present in the reaction mixture, but in an immobilized form.

a) Cross Linking Enzyme Crystals (CLEC)

- **Principle:** In this method, cross-linking agent glutaraldehyde is used to crystallize the enzyme. The CLEC, upon addition to the reaction mixture, catalyzes the reaction with reasonably high efficiency.



CLEC contd.

- **Advantage:** The CLEC are very stable and are not easily denatured by heat or organic solvents. They are moderately resistant to proteolysis. They have a manageable size and stability in operating the bioreactor and can be recycled. They also give enhanced enantioselectivity to particular forms of their substrate.
- **Disadvantage:** Highly purified enzyme produced through a standard protocol of crystallization is required for the preparation of CLEC. These requirements make it time-consuming and expensive. Diffusion of substrate and product is limited with increase in size of the aggregate.

b) Cross Linking Enzyme Aggregates (CLEA)

- **Principle:** CLEA is an improved version of CLEC production aimed at nullifying the disadvantages of CLEC. While CLEC requires the formation of crystals, CLEA can work in aqueous solutions. Addition of salts, organic solvents or non-ionic polymers results in the formation of enzyme aggregates which retain their catalytic properties. These aggregates are called Cross Linked Enzyme Aggregates (CLEA).

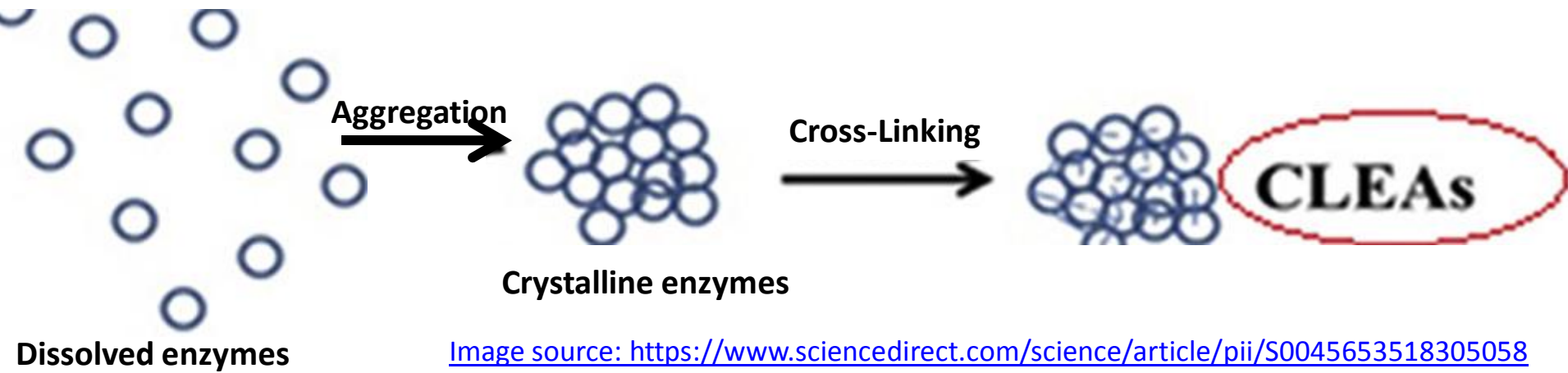


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CLEA contd.

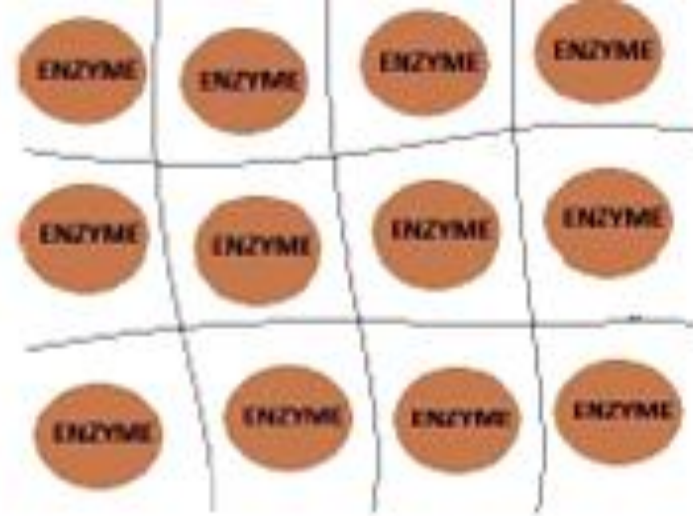
- **Advantage:** It is cheaper, easier to perform and has a wide range of applications. For multi-enzyme catalysis, it is possible to synthesize CLEA having more than one enzyme in the aggregate (called combi-CLEA).
- **Disadvantage:** The size of the derived aggregates is small and often similar to the size of the substrate or product, which may be inconvenient during product purification. Diffusion of substrate and product is limited with increase in size of the aggregate. The CLEAs are often fragile and tend to exhibit low stability in stirred tank fermenters or packed bed reactors

Crosslinking: Crosslinking agents used

- For most enzymes, the crosslinking agent used is glutaraldehyde, which is cheap, stable and easily available.
- For enzymes that are inactivated by glutaraldehyde, alternative cross-linking agents such as dextran polysaccharide, bis-isocyanate, bis-diazobenzidine, diazonium salts and functionally inert proteins, such as bovine serum albumin (BSA) are preferred.

(B)3: Entrapment

It is a technique of irreversible enzyme immobilization, where the enzyme is immobilized by entrapping it within a support matrix or within fibres.



Principle:

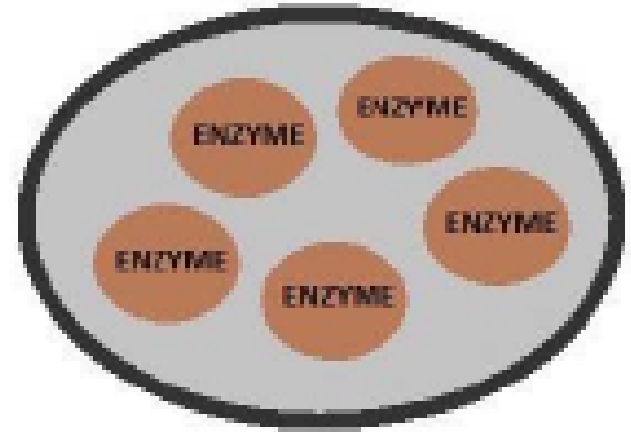
- Enzymes, being large macromolecules, tend to be larger than the substrates or products. Thus, the enzyme is immobilized within a matrix of appropriate pore size to allow only the substrates and products of a diameter smaller than the matrix pore size to diffuse in and out of the mesh respectively.
- The enzyme size-to-pore size of support is a deciding factor in selecting the support. Smaller the pores, lesser the enzyme entrapped, while larger the pores, more the leaking of the enzyme. Hence, accurate pore size selection of the support is crucial.

Entrapment contd.

- **Advantage:** The method is fast, cheap and easily carried out under mild or physiological conditions. As the enzyme remains confined within a matrix, it is protected from contamination by microbes, proteases or other enzymes.
- **Disadvantage:** The matrix cannot support a large volume of enzyme molecules as enzymes may be inactivated. Hence, it can be costly sometimes. The rate of diffusion of the substrate and product dictate the reaction rate. This is because unless the substrate molecules diffuse into the mesh, the reaction will not be initiated and according to Le Chatelier's principle, the reaction rate does not reach a peak unless the products sieve out.
- **Matrix used:** Common polymers used for enzyme entrapment include alginate, carrageenan, collagen, polyacrylamide, gelatin, silicon rubber and polyurethane.

(B)4: Encapsulation

It is a special type of entrapment where the enzyme is immobilized by entrapping it in a spherical semi-permeable membrane.



Principle:

- The basic underlying principle is the larger size of the enzyme as compared to substrates or products. Thus, when the enzyme is entrapped in a semi-permeable membrane, the substrate molecules diffuse in and the product molecules to diffuse out, while the enzyme remains within it.
- The enzyme, though restricted within the membrane, is free floating inside the capsule. The permeability of the membrane is controlled according to the enzyme being immobilized.
- Encapsulation is achieved by one of the two methods,
 - (i) Coacervation (allowing the polymer to separate out enzyme microdroplets in a water immiscible solvent), and
 - (ii) Interfacial polymerization (when a hydrophobic monomer is added to an aqueous solution of enzyme and another monomer which has been dispersed in a water immiscible solvent. This promotes polymerization at the interface of the two droplets and hence around the enzyme).

Encapsulation contd.

- **Advantage:** It maintains the enzyme structure in its native form and prevents leakage of the enzyme, protecting it from the harsh conditions of the medium. Multi-enzyme encapsulations can also be created by trapping more than one enzyme within a membrane .
- **Disadvantage:** The diffusion of substrate and product across the membrane controls the reaction rate. The pore size needs to be maintained accurately to prohibit enzyme leakage (if the pore is too large) or poor loading of the enzyme (if pores are very minute). This technique is not suitable for reactions involving substrate and enzyme molecules of similar sizes.
- **Biopolymers used:** Alginate, maltodextrin, cellulose, chitosan, etc. are used for encapsulation of an enzyme. These polymers form a single layered capsule around the enzyme. Double-layer microcapsules, built of two distinct types of polymers, are also popular. The most common ones are composed of chitosan, Poly-Llysine, Polyvinyl acetate, gelatin and boric acid. Lipid vesicles are also used for encapsulation (hence called liposomes).

Alternative strategy: Whole cell immobilization

- It is an alternative strategy to enzyme immobilization.
- Cells are living biocatalysts, with each one containing different enzymes to catalyze a plethora of reactions.
- Cells are generally not immobilized when used in bioreactors. Hence purification of the product is required.
- Surface-attached microbial communities consisting of multiple layers of cells embedded in hydrated matrices are known as **biofilms**.
- Whole cells maybe immobilized either in a viable or non-viable form.

Whole cell immobilization: Principle

- In nature, bacteria often grow attached to their substrate, especially in niches where the surrounding medium is not static or stable, e.g., on rocks under flowing rivers, on the walls of pipes, teeth, etc. It is often seen that bacteria bound to a surface grow and proliferate better. So, this principle was used in immobilizing cells for reactions. In this technology, the cells remain bound to a surface, while the nutrients (essential for cells to survive), substrates and products diffuse in and out of the cells (owing to the semi-permeable nature of the cell membrane).
- Two important points to consider for whole cell immobilization are as follows:
 1. Suitable support: the cells are immobilized under a porous support like polymeric gels, attached them to a micro carrier surface or entrapped them behind membranes (less favoured). The strategy used commonly is attaching them to a carrier; several motifs on the cell membrane or wall of the cell being immobilized are utilized. Targeting the characteristic epitopes on the membrane of commercially useful microorganisms can allow efficient binding of the cells to the carrier.
 2. Efficient diffusion the substrates and products within the cells: This is essential for economical industrial production. To achieve this, in case of enzymes that work in the cytoplasm, cells may be permeabilized physically (freezing & thawing), chemically (using organic solvents like toluene, chloroform, ethanol, etc. or mild detergents like N-Cetyl-N, N, N-trimethyl ammonium bromide, i.e., CTAB and digitonin). For other enzymes active in the periplasmic space such as catalase (in yeasts), urease, Penicillin G acylase (in bacteria), the cells can be utilized without permeabilization. Sometimes cells are genetically engineered to retain useful enzymes in the periplasmic space.

Whole cell immobilization contd.

Advantages:

- Enzymes function the best in the optimum conditions in the cells.
- Viable cells continue to divide and naturally replenish the enzymes inside a bioreactor, thereby maintaining the cell density easily during continuous fermentation processes. The cell density of the culture can be pre-determined as it is totally controllable.
- It is more economical than enzyme immobilization, because purified enzymes are expensive.

Disadvantages:

- The rate of catalysis is highly limited by the rates of diffusion of substrate and product across the membrane, as the enzymes remain entrapped within the cells.
- For aerobic cells, proper oxygenation is vital for survival and growth. The dissolved oxygen needs to be maintained at a high level, as the cells lie embedded on a solid surface (usually inside the media).
- The matrices may tear due to excessive proliferation of the cells.
- Immobilization can only be used for cells that release out the products to the exterior. Since the cells are immobilized, it is not possible to extract the cells and lyse them for releasing the products.
- Cells contain many different enzymes. So, there may be cross-reaction of the substrate with unwanted enzymes, or the product can be converted to some unwanted forms.

Conclusions

- Thus, immobilized form of enzymes mimic their natural mode in living cells, where most of them are attached to cellular cytoskeleton, membrane, and organelle structures.
- The solid support systems generally stabilize the structure of the enzymes and, as a consequence, maintain their activities.
- These alterations result from structural changes introduced into the enzyme molecule by the applied immobilization procedure and from the creation of a microenvironment in which the enzyme works, different from the bulk solution.

References

- Krishnamoorthi et al. Immobilized Enzyme Technology: Potentiality and Prospects. J Enzymol Metabol. 2015; 1(1): 104.
- Homaei et al. Enzyme immobilization: an update. J Chem Biol. 2013; 6:185–205.