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An Overview Of Some Enzyme Stabilization Strategies: Advantages And Drawbacks

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Abstract

The use of enzymes as industrial biocatalysts had become a subject of interest. As they play an important role in different industries including food, detergents, textiles, leather, paper and pharmaceutical industries. However, the practical use of enzymes in industry may cause denaturation of enzymes by the working conditions such as high temperature, different reaction pH and the presence of some chemicals. Since the stabilized enzymes are strong and more resistant to environmental changes, a great attention was given to enzyme stabilization by different techniques. This review offers an overview on enzyme stabilization using different techniques such as immobilization, chemical modification and protein engineering. Each technique has its advantages and drawbacks. So, it is so important to find a suitable stabilization technique at the optimum conditions for each enzyme according to the targeted industrial application.

Keywords: Industrial enzymes; genetic engineering; immobilization; enzyme stabilization; chemical modification

1. Introduction

Enzymes are proteins found in plants, animals and microorganisms that accelerate many biochemical and chemical reactions, reduce the initial energy input by increasing the rate of the reaction **[1]**. Enzymes from microbial sources are preferable than enzymes from other sources due to their higher productivity, high catalytic activity and low production costs **[2-4]**. The requirement for enzymes in different industries is continuously increasing; however, the use of enzymes in the industry is still limited because they are unstable and easily inactivated by heat, chemicals and environmental factors **[5].** The practical use of enzymes in industry may cause denaturation of enzymes by the working conditions such as the high temperature, the aqueous-organic environment, different reaction pH and the presence of some chemicals. As a good industrial catalyst should be stable under the operating

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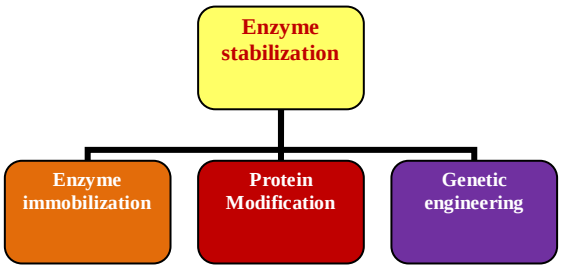
conditions for a long time, many strategies have been pursued recently to increase the enzyme stability **[5]**. Enzyme stability includes both thermodynamic and kinetic stabilities. Good knowledge of the enzyme stability is an important factor for its application in biotechnological processes, as it can provide information about the structure of the enzyme and facilitate its economical applications. Moreover, knowing more information about enzyme stability could help to improve the effectiveness of enzymatic processes **[6]**. Immobilization of enzymes on insoluble carriers has been considered an excellent method for enzyme stabilization. It also facilitates the recovery and reuse of the water-soluble catalysts **[7]**. The chemical modification of enzyme protein has been used to obtain stabilized enzymes in a soluble form. Also the use of genetic engineering is a very promising technique for enzyme stabilization **[5]**. In the present article we will discuss some important parameters to judge enzyme stability and the stabilization of enzymes by using various approaches such as immobilization, chemical modification and protein engineering.

Methods of enzyme stabilization:

Stabilization of enzymes is a very useful strategy that is used to protect industrially important enzymes against inactivation caused by heat, chemicals and other environmental factors. This can be achieved using different techniques including enzyme immobilization, protein modification and Genetic engineering.



1. Enzyme immobilization:

Immobilization is a process in which enzymes are fixed to solid supports which stabilize the structure of the enzymes, prevent structural denaturation and maintain their activities at the different reaction conditions. This Technique also can help in solving the problem of enzyme recovery and reuse **[8-10]**. As there are many complications associated with utilization of enzymes in industrial processes because enzymes are usually expensive and the cost of their isolation and purification is very high. They are also highly sensitive to various denaturing conditions such as temperature, pH and chemicals at trace levels. Most enzymes act in solutions which lead to product contamination and difficulty in their recovery from the reaction mixtures and reuse. Therefore, the ideal solution to overcome these problems is the use of immobilized enzymes **[11]**. The immobilized enzymes are more resistant to the environmental changes compared to free enzymes and can be separated easily from the reaction mixture, washed, and reused for multiple times.

Enzymes can be immobilized on various organic and inorganic materials or carriers. The organic materials include the synthetic polymeric beads (such as amberlite), water-insoluble polysaccharides and polymeric membranes **[12, 13]**. Inorganic materials such as silica, clay, magnetic particles, and inorganic oxides have been used as supports for enzyme immobilization **[14-17]**. Enzyme immobilization techniques include physical adsorption, covalent binding, entrapment, encapsulation and cross-linking **(Fig. 1)**.

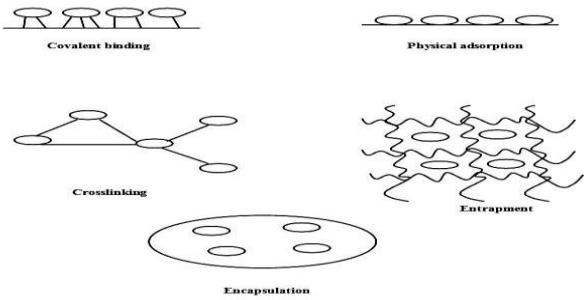


Fig.1: Methods of enzyme immobilization

1.1. Physical adsorption:

Adsorption is the oldest method used for enzyme immobilization. This method involves the attachment of enzyme to the external surface of an inert material. The bonds between the enzyme and support are weak, these including ionic interaction, hydrogen bonds and Vander Waal forces. The main advantage of this method is that there is no pore diffusion limitation since enzyme is immobilized on the outer surface of the carrier. Moreover, it is easy and costless method. However, there are many drawbacks as the active site of the enzyme may be blocked by the matrix resulting in reduction of the enzyme activity **[18]**. Also the physical adsorption is weak and fails to keep the enzyme fixed to the carrier under industrial conditions.

1.2. Covalent binding:

The enzyme is covalently bonded to a solid matrix through a chemical reaction of the functional groups on the protein surface either by direct binding of enzyme with the matrix or by using a cross linking reagent that binds to both the enzyme and the matrix (e.g. glutraldehyde). The aminated supports activated with glutaraldehyde are an example of supports that bearing many different characteristics **[19].** The multifunctional structure of support gave the amino-glutaraldehyde supports a very useful immobilization characters **[19]**. Covalent attachment is the most effective immobilization method which enhances enzyme stability and reusability, as the linkage between enzyme and support is very strong with no leakage problems. The drawbacks of this method involve mass transfer limitations due to steric hindrance after immobilization. However, the use of a spacer molecule helps to reduce steric hindrance and overcome this problem **[18]**.

Covalent immobilization of enzymes by using glutraldehyde as a spacer was investigated by many researchers for stabilization of several enzymes on different carriers (For example, immobilization of inulinase on *Luffa cylindrica* **[20]**, immobilization of inulinase on alginate–CMC beads **[21]**, immobilization of protease on Lewatit R258-K **[22]**, immobilization of naringinase enzyme on woodchips **[23]** and others).

1.3. Entrapment:

In this method the enzyme is physically trapped in insoluble beads or gels (e.g. calcium alginate beads). The enzymes is not directly attached to the surface of the matrix, but entrapped inside it. The bonds involved may be covalent or non-covalent. Entrapment is carried out by mixing the enzyme into a monomer solution, followed by polymerization initiated by a change in temperature or by a chemical reaction. Entrapment is easy to prepare, cheap and the loss of enzyme activity is minimized. However there are some disadvantages such as the enzyme leakage into the surrounding medium during repeatedly usage and mass transfer limitations of substrate and product **[24]**.

1.4. Encapsulation:

Encapsulation is a type of entrapment that involves the formation of a spherical particle in which the enzyme solution is enclosed within a semi permeable membrane from polymeric material. In encapsulation the enzymes cannot pass through the capsule,

however small substrates and products can diffuse through the semi permeable membrane. However, rupture of the membrane may occur if products accumulate rapidly from the reaction **[24]**.

1.5. Cross linking:

Cross-linking occurs by the formation of covalent bonds between the enzyme molecules to form a large, three dimensional complex structures. There is no matrix or support in this method. It can be achieved by physicochemical methods such as heat treatment, utilization of alkaline conditions, mechanical agitation, and the use of chemical multifunctional reagent with the enzyme **[25]**. Glutaraldehyde is the most powerful reagent used as cross-linker **[19]**, as it has the advantage of self-reaction. Cross-linking technique is very simple, cheap, and the enzyme is strongly bounded. However, the cross linking may cause some changes in the enzyme active site and it is not suitable with pure enzymes.

1.6. Immobilization on nanoparticles:

Nanoparticles are extensively used in medicine, cosmetics, and food industries. Therefore, the effect of enzyme immobilization on nanoparticles on both enzyme activity and stability has been essentially studied during the last few years. The most attractive advantage of nanomaterials is their big surface to volume ratio that facilitates better with increased loading of enzymes **[26]**. Enzymes can be immobilized on various nano-materials such as nanoporous materials, nano-fibers, metallic and magnetic nanoparticles using adsorption and covalent attachment. This offers a high surface area of nano-structured materials which results in improving the enzyme loading and increases the enzyme activity compared to the enzyme immobilized on conventional materials. The other advantages of nano-structured materials involve the controlled size (the pore size and the particle size in nanometer scale), similarity in size with enzyme molecules, conductivity and magnetism properties. As a consequence, the enzyme properties can be improved **[11]**. However, there's a limitation in the substrate diffusion through the nanomaterials due to the small pore size.

From the previously mentioned immobilization techniques it was clear that there is no ideal technique suitable for enzymes immobilization. So, for industrial enzymes it is important to select the immobilization methods which are suitable for their applications.

2. Protein modification:

Although the immobilization of enzymes has overcome the problem of stabilization, the use of immobilized enzymes is impossible in several applications. For example, water-insoluble enzymes cannot be used for catalytic processes when water-insoluble substrates are involved. As in this case, the access of the substrate to the active site of the enzyme is very difficult or impossible which reduce or inhibit the enzymatic process. Also in the medical applications it is necessary that the enzymes can circulate within the organic media for a long period of time without any immunological responses. In such cases modification of enzyme protein (chemical modification) can be used to obtain soluble stabilized enzyme preparations. As protein chemical modification has been evolved to get a more controlled and directed modified enzyme to attain further enzyme improvements **[27-29]**. Chemical modification of enzymes can be achieved by different methods: monofunctional substitutions of the amino-acid function groups on the enzyme surface, polyfunctional substitutions using polyfunctional agents (glutraldehyde) to achieve inter- or intramolecular linkages and attachment of the

enzyme molecules to some water soluble polymers (activated polysaccharides) **[5]**.

2.1. Monofunctional substitutions:

An example of the monofunctional substitutions of the amino acid groups is the alkylation or acylation of NH_2- residues with different reagents to increase the hydrophilicity of the surface area of the protein globules (transformation of protein NH_2-groups into -$NHCH_2COOH$ groups or other carboxyl derivatives). This technique is applied to decrease the non polar clusters on the enzyme surface whose contact with water molecules cause destabilization of the enzyme.

2.2. Polyfunctional substitutions:

This includes the use of polyfunctional agents such as glutaraldehyde which produce intra molecular cross-linkages in one or more regions of the enzyme molecule. These additional linkages increase the rigidity of the enzyme protein and enhance its stability **[5]**.

2.3. Attachment to soluble polymers:

It has been observed that glycoenzyme show higher stability compared to carbohydrate free enzymes so, many attempts have been made to enhance the stability of enzymes by covalently attaching to carbohydrate residues. The polysaccharide polymers (for example: cellulose, starch, dextran, sephadex, and agarose) are preferred for enzyme stabilization due to several reasons:

- The sugar residues in these polymers contain hydroxyl groups which can form hydrogen bonds with water and create a hydrophilic environment in the support to attain stabilized water soluble enzyme.
- The formation of additional inter- and intra-molecular bridges in the glycosylated enzyme molecule increases the enzyme rigidity and stability.
- Polysaccharides are more effective than mono- or disaccharide stabilizers because multipoint attachment of such poly-hydroxyls increases the rigidity as well as hydration of the enzyme molecule.

The activation of the carbohydrate residues by certain reagents such as cyanogens bromide and sodium periodate is designed to make strong electrophilic functional groups on the carrier molecule which in turn react with the amino groups located on the enzyme surface to form strong covalent bond. However, Sodium periodate is favored over cyanogen bromide as an oxidizing agent because cyanogen bromide may precipitate polysaccharides during activation and need high pH for coupling which might affect the enzyme structure **[24, 30-32]**. Chemical modification of enzymes using different polysaccharides was investigated by many researcher for stabilization of several enzymes (For example, chemical modification of alkaline protease using pectin **[33]**, chemical modification of alpha amylase with Carboxy methyl cellulose **[34]**, binding of invertase and cellulase to chitosan **[35-36]**). Also many attempts have been made to attach the enzyme to soluble polymers such as polyethylene glycol (PEGylation) which increase the enzyme rigidity against proteolytic enzymes, increase hydrophilicity and enhance its stability **[5, 37]**.

3. Genetic engineering:

Recently, genetic engineering technology has been developed to produce large quantities of natural proteins by cloning the desired genes into the selected microorganism **[5]**. Furthermore, using recombinant DNA technique can alter the structural and functional properties of natural proteins via site-directed mutagenesis to produce protein with different and novel characteristics compatible to the desired interest. However, the main drawback is the high cost of this strategy. Enzyme stabilization using genetic engineering can be achieved by several methods **[5, 38-39]**:

- Expression of a gene of a thermophilic enzyme into a mesophilic microorganism.

- Mutagenesis:

  -Random mutagenesis: by using physical mutagenesis (X-rays, γ-rays, UV) and chemical mutagenesis (ethyl methyl sulfonate, nitrous acid, methyl methane sulfonate, and ethidium bromide) to induce mutation in the producing microorganism.

  -Site directed mutagenesis using oligonucleotide primer to make specific changes in the DNA sequence of a gene to alter its properties and enhancing its stability.

- The introduction of internal or surface disulphide bridges.

- The substitution of some amino acid residues.

There are a great number of literatures reported the use of mutant strains for producing various industrial enzymes such as lipase, chitinase, amylase, cellulase and protease **[40-42]**.

Conclusion:

Enzyme stabilization is a very useful approach that is used to protect industrially important enzymes against inactivation caused by heat, chemicals and other environmental factors. Enzymes can be stabilized by using many techniques including enzyme immobilization, chemical modification and protein engineering. However, it is clear that there is no ideal technique for enzymes stabilization as each technique has its own advantages and disadvantages. So, for industrial applications it is important to find a suitable stabilization technique at the optimum conditions for each enzyme according to the targeted application.

Conflicts of interest

There are no conflicts to declare.

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