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Review Article

Enzyme Instability between Problem and Solution

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Abstract

One of the main problems obstructed to the use of enzymes in industrial biotechnology is their improper stability under processing conditions. In the last decades, a lot of research has focused on the improvement of enzymes stability in the conditions in which they were to be used, and especially on thermal stability improvement. Long time ago different methods were suggested to enhance the enzyme stability. This review trying to explain the enzyme structure and the factors affecting its stability. also, tried to highlight and summarized most of these methods such as chemical modification, immobilization, and addition of various compounds. Also, give an overview of the recent progress in these methods.

Keywords: Enzyme; Chemicals; *Bacillus*; *Aspergillus*

Introduction

Industrial manufacturing processes often require the use of harsh conditions, specifically toxic chemicals, high temperatures, or extremes in pH. A large portion of biotechnology research is focused on replacing these processes with those mediated by enzymes that can tolerate these extremes and reduce the use of chemicals, or perform the same reactions under more moderate conditions. The production of heat-resistant enzymes could be allow enzymatic reactions to work at high temperatures, and therefore, conversion rates and substrates solubility increase accordingly, reducing the risk of microbial growth and the viscosity of the reaction medium. Success in these areas of research might mean reduced harm to the environment, lower costs in terms of energy used, less dependence on non-renewable resources and less risk to production employees. Several strategies are at hand to increase operational stability the use of stabilizing additives, chemical modification of enzyme structure, derivatization, immobilization, crystallization and medium engineering.

What are enzymes?

Enzymes are proteins that are produced naturally by plants, animals, bacteria, fungi and all other living things, and are absolutely necessary for life. They are catalysts that accelerate the rate of chemical reactions without changing themselves. Commercially available enzymes are produced from bacteria and fungi like *Bacillus, Aspergillus and Trichoderma* species [1].

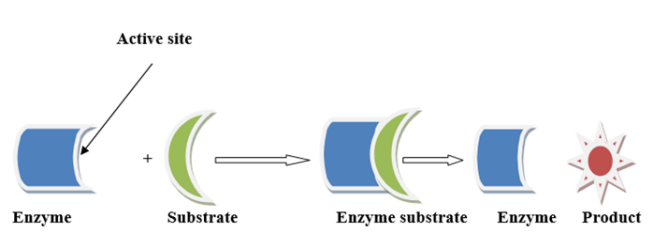

Figure 1: Simple diagrams explain the enzyme mode action.

Cofactors

Some enzymes do not require any additional components to get full activity. While, others need non-protein molecules called cofactors to be bound for activity [1]. Cofactors can be either inorganic (e.g., metal ions and iron-sulfur clusters) or organic compounds, (e.g. flavin and heme) (Figure 2). Organic cofactors could be either prosthetic groups, which are tightly bound to an enzyme, or coenzymes, which are released from the enzyme's active site during the reaction. Coenzymes include Nicotinamide adenine dinucleotide H^+ (NADH), NADPH and adenosine triphosphate. These molecules work to transfer chemical groups among the enzymes.

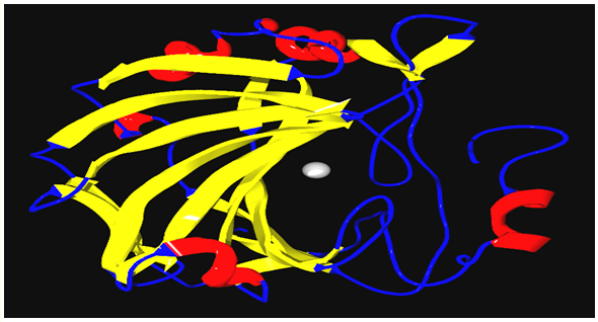

Figure 2: Zinc cofactor of carbonic anhydrase.

An example of an enzyme included a cofactor is carbonic anhydrase which, is shown in the ribbon diagram (Figure 2 and 3) with a zinc cofactor bound as part of its active site [2]. These tightly bound molecules are usually present in the molecule active site and are embedded in catalysis. For example, flavin and heme cofactors are often involved in redox reactions.

Enzymes that the cofactor is essential for their activities but do not have one bound are called *apoenzymes* or *apoproteins*. An apoenzyme together with its cofactor (s) is known as a *holoenzyme* (this is the active form). Most cofactors are not covalently attached to an enzyme but are very tightly bound. However, organic prosthetic groups could be covalently bound (e.g., thiamine pyrophosphate in the enzyme pyruvate dehydrogenase).

Coenzymes

Coenzymes (Figure 3) are small organic molecules that transport chemical groups from one enzyme to another. Examples of these chemicals are riboflavin, thiamine and folic acid as vitamins. When these compounds cannot be made in the body and must be acquired from the diet. The chemical groups carried include the hydride ion (H^-) carried by Nicotinamide adenine dinucleotide (NAD) or (Nicotinamide adenine dinucleotide phosphate).

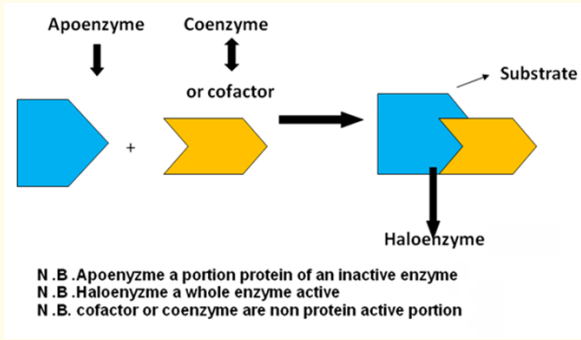

Figure 3: Showed the coenzyme or cofactor mode of action.

$NADP^+$, the acetyl group carried by coenzyme A, formyl, methenyl or methyl groups carried by folic acid and the methyl group carried by S-adenosylmethionine.

Since coenzymes are chemically converted according to the enzyme action. It is useful to consider the coenzymes to be a special class of substrates, or second substrates, which are common to many different enzymes. For example, about 700 enzymes are known to use the coenzyme NADH [3].

Coenzymes are usually regenerated and their concentrations kept at a steady level inside the cell: for example, NADPH is regenerated through the pentose phosphate pathway and *S*-adenosylmethionine by methionine adenosyltransferase.

How Do Enzymes Work?

Enzymes work through breaking apart the large complex compounds (substrates) into smaller, more easily absorbed nutrients that the bacteria could be use. Enzymes from different sources have a specific temperature and pH range at they are optimally active. This is considering an important factor when selecting an enzyme product. Enzymes are classified according to their substrate they work on. For example, proteases work on proteins, breaking them down into amino acids and peptides. Cellulases break down cellulose, the major indigestible component of plant cell walls, into simple sugars. Only very small quantities of enzymes are required for converting very large quantities of substrate: typically, enzyme to substrate ratios can range from 1:1,000 to 1:1,000,000.

Factors affecting enzyme inactivation

Many factors affected the enzyme structure and led to complete enzyme denaturation (Figure 4). The following sections tried to highlight most of these reasons:

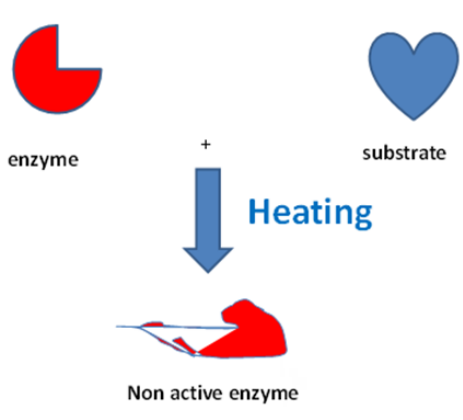

Figure 4: A simple diagram explains the heat effect in complete enzyme destruction.

Microbial and proteolytic degradation: Enzymes degradation by the microorganisms' action and exogenous proteases is a process responsible for their operational and storage inactivation [4].

Autolysis and aggregation: Proteases assembled one of the three largest groups of industrial enzymes. It represented about 59% of total worldwide enzyme sale [5]. The proteolytic enzymes have been studied widely and in depth, are easily available, and are very important from a practical viewpoint. Thermal, operational, and storage inactivation of free proteases occur mainly owning to intermolecular self digestion (autolysis).

Unfolding due to heating, denaturing agent, ETC: It has been already mentioned that general mechanisms of irreversible enzyme inactivation have not been clarified. It is quite certain, however, that inactivation of the enzymes under the action of, for example heating, pH shifts, or denaturing agents, involves considerable conformational changes in the protein molecules, for example, unfolding [6].

Effect of dissociation on oligomeric enzymes

Many oligomeric enzymes dissociate and as a result, are inactivated under the action of different reagents such as urea, guanidine hydrochloride, and high salt concentrations [7].

Inactivation of enzyme due to unfavorable pH

Inactivation of the enzymes due to unfavorable pH of the medium is frequently encountered. Such inactivation may be a result of change in the extent of ionization of enzyme catalytically essential inorganic group (s), conformational changes, dissociation of oligomeric enzymes, autolysis (in the case of proteases), aggregation, etc. In certain cases, it is desirable to perform an enzymatic reaction in solutions containing an organic co- solvent.

Inactivation of enzyme in organic solvents

In certain cases, it is desirable to perform an enzymatic reaction in solutions containing organic solvents. There are two main reasons (a) poor solubility in water of substrates or other low molecular weight components of the enzymatic process and (b) an unfavorable equilibrium position for reactions involved in pure water. Research contains into the properties and applications of enzymes in organic solvents. In an apparent paradox, water miscible solvents are, in many cases, more problematic than hydrophilic solvents as regards protein stability. This is because hydrophilic solvents, unlike their hydrorophobic counterparts, can remove the essentially bound water from the surface of the enzyme molecules [8].

Inactivation of enzyme by hydrogen peroxide

Many enzymes undergone irreversible inactivation under the action of hydrogen peroxide (owing to the oxidation of catalytically essential groups in the protein). This inactivation assumes

significance from the viewpoint of applied enzymology when such enzymes are required for the technological process and hydrogen peroxide is the product of the enzymatic reaction. Glucoamylase and glucose oxidase are good examples [9].

Inactivation of enzymes by oxygen

Some enzymes are readily inactivated by oxygen. Among these are the hydrogenase, nitrogenase, ferrdoxin, succinate dehydrogenase, dioxygenase, aconitate dehydrogenase, amidophosphosphoribosyl transferase, and the sulfhydryl proteases papin, bromelain, and ficin. The inactivation can be both reversible and irreversible [10].

Enzyme stabilization

Enzyme stabilization is one of the most important fields in basic and applied enzymology. In basic enzymology, it is of relevance to understand enzyme stabilization principles first elucidating how and why the enzymes lose their biological activity and then deriving structure-stability relationships existing in enzymatic molecules. In applied enzymology, the most important goal is to achieve useful compounds by biocatalysts'. Enzymes are good catalysts had high catalytic and specific activity with capability to work under mild conditions. However, they are not always ideal catalysts for practical applications because they are generally unstable, and they inactivate rapidly through several mechanisms. To increase the enzyme stability, many strategies have been dominated in the recent years.

Strategies for enzyme stabilization

Chemical modification

The active site of cross-linked enzyme crystals could be quick modified by chemical means and yielded a new type of enzyme chemically engineered. Previously, the crosslinked protein crystals characteristics as microporous materials have been studied and act as stationary phases in chromatographic separations [11]. The chemical modification of polypeptide R-groups remains benefit for enzyme stabilization, despite having been over shadowed in recent years by genetic strategies. Chemical and genetic techniques could be complementary as appear by the further increased in thermostability of an engineered consensus phytase following chemical crosslinking of its glycan chains to form phytase oligomers [12]. Some stabilizing chemical modifications methods are described below.

Crosslinked enzyme crystals

An artificial crosslink could be formed between a polypeptide chain (an intermolecular crosslinked). Such an internal crosslink could stabilize the protein by inhibit the unfolding under sever conditions. It is also possible to link tow polypeptides via an intermolecular crosslink. A form +of this included the crosslinking of small (1 - 200 µM) enzyme crystals with the bifunctional reagent such as glutaraldehyde. These crystals crosslinked enzyme (CLECs) clearly increased the storage and operational stability (at high temperature, in solvents and under agitation) compared with their untreated counterparts. Govardhan (1999) [13] has written enzyme stabilization without decreasing and diluting their activity is critical for their use as industrial catalysts. One practically had proved approach involves the enzyme (size 1 - 200 µm) crystallization followed by subsequent crosslinking. In many cases, the resulting crosslinked enzyme crystals appeared increased in storage stability as well as stability in applications. The technology is complementary to protein engineering methods that aim at boosting the inherent enzyme stability.

On contrary, Visuri., *et al*. (1999) [14] prepared crosslinked crystals of *Streptomces rubiginosis* GI to compare this fraction with the native soluble GI in buffer and in 45% glucose fructose solutions. Unexpectedly, native GI was more in a buffer at 80 °C. Inactivation of native enzyme in buffer did not obey first-order kinetics but proceeded with a rapid first phase followed by a stable phase. The stabilization process is discussed to be a result of a conformational change in the protein structure. Inactivation of the native enzyme in buffer was directly related to protein precipitation. In the presence of high substrate concentration, the inactivation was related to browning reactions between the enzyme and the reactive sugar, resulting in soluble sugar-protein complexes.

Covalent attachment to polymers

An alternative approach to crosslinking involves protein attachment of interest to multiple sites of a soluble polymer. To utilize this strategy, Bieniarz., *et al.* [15] reported the synthesis of polymeric cross-linking agents, poly (glutamic acid) poly (phosphorothioates), and their use in the cross-linking and stabilization of proteins upon treatment with alkaline phosphatase. It was shown that poly (phosphorothioates) are excellent substrates of alkaline phosphatase, yielding thiolated polymers which react covalently with electrophilic groups introduced into the proteins. Three proteins of different structure and function were cross-linked using this method: calf intestinal alkaline phosphatase, glucose oxidase (*Aspergillus niger*), and (*R*)-phycoerythrin. The cross-linking of alkaline phosphatase is self-catalyzed since this enzyme catalyzes the hydrolysis of phosphates, unmasking thiolates which react with the maleimide PR derivatized alkaline phosphatase. The incubation of native alkaline phosphatase in buffered solutions at 45^0C (7-14 days) caused a 35% loss of enzymatic activity compared to that of cross-linked enzyme. The effect of cross-linking glucose oxidase is

even more pronounce, ranging from 800% stabilization at 37^{0}C and pH 9.0 to 3000% at 37^{0}C and pH 7.4 [15]. (*R*)-Phycoerythrin cross-linked with 1 - 3 equiv of poly (phosphonothioates') and incubated at 45^{0}C for 45 days was 20% more fluorescent than the native (*R*)-phycoerythrin subjected to the same conditions. The stabilizing effect of cross-linking was confirmed by comparing the rate of loss of quaternary structure of the cross-linked (*R*)-phycoerythrin with that of the native protein. Mostafa and Abd El Aty [16] reported in alginate- polyethyleneimine gel beads was modified by using 0.3 M Na+. It was used for covalent immobilization of *Aspergillus flavus* xylanase and this led to a great improved in xylanases thermal stability and its tolerance to different pHs. In this finding, Clark., *et al.* [17] reported *in* myoglobin (cardiac enzyme) treated with an aliphatic diamine to produce a high degree of positive charge on its surface (Figure 5). The 'cationized' protein formed a complex with an anionic polymer surfactant and an aqueous solution of protein–polymer nanoconjugates was produced. The aqueous solution was lyophilized, followed by thermally induced melting at an ambient temperature and pressure. This step produced a free-flowing translucent syrup with myoglobin as the central ingredient. Spectroscopic analyses appeared a near- myoglobin native structure. The binding studies with dioxygen and other gaseous substrates proved the biological function.

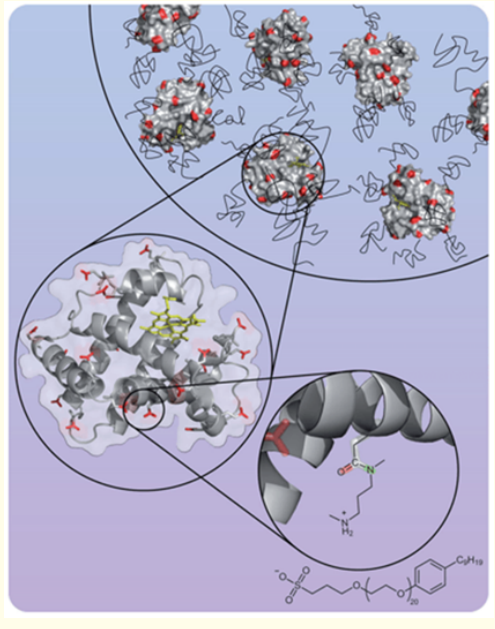

Figure 5: Chemical modification of myoglobin. Carboxylate groups on the side chains of myoglobin are reacted with a diamine to introduce positive charges on to its surface.

Clark (2010) [17].

Surface modification

It means chemical modification of the amino acid residue on the surface of the enzyme molecule. This alter the nature of the enzyme surface charge and its hydrophobicity/hydrphilocity. This include enzyme acetylation, succnilyation and alkylation. Also, the enzyme can be stabilizing even where crosslinking does not occur. Khajeh., *et al*. [18] reacted citraconic anhydride with the lysine side chains of tow bacterial α-amylases one from mesophlic *Bacillus amyloliquefaciens* (BAA) the other from thermophilic *Bacillus licheniformis* (BLA) The modification replaces the positive charge of the hydrcine residue with a negatively charged carboxyl group. It was estimated that 13 lysines were modified in (BAA) and 10 in (BLA). Modified BAA retained much more activity than native at 70, 80^{0}C. BAA undergoes deamidation of its asparagine residues at high temperatures but this occurred much less with modified form. BAA contains Lys-Asn dipeptide and it was suggested that modification of this particular lysine might prevent or delay deamidation. In this finding, Schiavon., *et al*. [19] reported in Bovine pancreatic ribonuclease A (RNase) was modified at different extent at the lysine residues using monomethoxypoly (ethylene glycol) (MPEG) which activated as an active ester. For pharmacokinetic experiments, a radioactive adduct was also done with tritiated amino acid as spacer between polymer and protein. The modification process reduced slightly the RNase catalytic activity and Km towards the substrate cytidine-2',3'-cyclic monophosphate. On the other hand, extensively modified MPEG-RNase samples, appeared significant decrease in activity towards ribonucleic acid. The polymer modification did not alter the pH activity profile, increased the stability for proteolytic digestion, while its behavior towards denaturants and heat was not modified. The native and MPEG-RNase showed IV, IM and SC to rats, administrative impressive variations in pharmacokinetics: the half-life of the modified enzyme, evaluated in blood by radioactivity, increased about 40-50 folds with respect to the native form.

Stabilizing additives

A range of low-molecular weight additives exert stabilizing effects by inducing preferential hydration of proteins, i.e. the additive tends to be excluded from the vicinity of the protein molecule. Interaction between the protecting additive and the peptide backbone is unfavorable. At least in the case of polyols, the preferential hydration arises from an increase in the surface tension of the solvent water. Loss of the protein's compact, properly folded structure (denaturation) will increase the protein–solvent interface. This in turn will tend to increase the degree of thermodynamically unfavorable interaction between the additive and the protein molecule.

The result is that the protein molecule is stabilized by the additive. Naturally- occurring stabilizing additives increase a protein's *T*m but do not affect the protein's denaturation [20]. This means that the intrinsic conformational stability of the protein molecule itself is not increased but its unfolding is greatly disfavored by the presence of stabilizing additives in the medium. The disaccharide trehalose (d-glucopyranosyl, d-glucopyranoside) effectively prevents protein denaturation at high temperatures. Singer and Lindquist [21] showed that trehalose also acts *in vivo* to suppress the aggregation of denatured proteins. Molecular chaperones can act on these partially folded denatured proteins to reactivate them but the continued presence of trehalose interferes with the refolding process. This may explain why trehalose levels *in vivo* decline swiftly following heat shock. Additionally, it was found that sucrose and maltose suppressed aggregation of GdCl-denatured rhodanese as effectively as did trehalose (each used at 0.5M concentration). Baptista., *et al*. [22] observed the effects of 0.5M trehalose on the unfolding of cutinase, a lipolytic enzyme, at alkaline pH. Although prone to aggregation at pH values below 9.2, the fungal cutinase was more stable in the lower alkaline pH range: as pH increased from 9.2 to 10.9, *T*m decreased by 14ºC. Trehalose increased *T*m by 4ºC at pH 9.2 but only by 2.6ºC at pH 10.5. The lower pH values favored reversibility of the unfolding process but trehalose slightly decreased the extent of reversibility. Arrhenius plots of the intrinsic rate constant of inactivation (as opposed to the apparent rate constant, unadjusted for the equilibrium between folded and reversibly unfolded cutinase) indicated that trehalose lowered the activation energy of the inactivation process from 30 to 16 kcal/mol. Among some representative low-molecular weight polyols studied with ribonuclease A-chymotrypsinogen, lysozyme, cytochrome *c*, and trypsin inhibitor, inositol was the most stabilizing on a molar basis, mannitol and sorbitol were intermediate, and xylitol and adonitol were the least stabilizing [23].

Catalase is of industrial interest for the hydrogen peroxide degradation which used to bleach cellulose fabrics. The effects of various additives on the stability of a *Bacillus* catalase. Glycerol and polyethylene glycol increased storage stability at 4 and 30 ºC (along with other substances) and were the only additives to improve tolerance of short-term exposure to pH 10 or 11 at 70ºC. Glycerol was the best-performing stabilizer overall. Polymers also can stabilize proteins. Polyethyleneimine (PEI) is a cationic polymer with numerous uses, including protein stabilization [24]. Both high- and low-molecular weight fractions of PEI, when added at 0.01–1% (w/v) concentrations. It was greatly increased the shelf lives of dehydrogenases and hydrolases stored at 36 ºC. The effect seems to be kinetic rather than thermodynamic, as the *T*m of lactate dehydrogenase was unaffected by PEI. Curiously, however, lactate dehydrogenase activity levels at pH 5 decreased with increasing PEI concentrations. On contrary, the polymer stimulated the activity at pH 7.2 and 9. PEI at 1% (w/v) concentration also protected the sulfhydryl groups of lactate dehydrogenase against oxidation and inhibited the consequential aggregation of the protein during 1-month storage at 36°C, even in the presence of Cu^{2+} ions. This protecting effect was ascribed to metal chelation by the PEI [24], as metal ions promote oxidation reactions. Surfactants have an efficient effect on both the stabilities and activities of enzymes. The cationic surfactant benzalkonium chloride (0.01 or 0.1%) maintained the activity of bovine lactoperoxidase stored at 37°C, pH 7 for much longer than that of a control sample. Significant activity remained up to 240 h at either concentration, while the surfactant-free control had no activity at 96 h. The lower concentration had an activating effect on the enzyme. CD indicated that the benzalkonium chloride stabilized the native protein's secondary structure. Curiously, however, this stabilizing effect was not observed at pH 6 [25]. Shimizu., *et al.* [26] reported in the effect of enzyme-stabilizing additives in sixteen proteins during freeze-thawing and freeze-drying. Five enzymes, each with different instabilities against freezing and dehydration, were choosing as the protein to be stabilized. Proteinaceous additives caused greater enzyme stabilization during freeze-thawing than sugars. The degree of stabilization effect for the basic lysozyme and protamine were inferior to that of neutral and acidic proteins. The presence of a proteinaceous reduced freeze-drying-induced inactivation of enzyme. Their extent was lower than that for a sugar. In both freeze thawing and freeze drying, the enzymes stabilization by the influence of proteinaceous additive increased with the increase of additive concentration. The enzyme inactivation increase caused by the pH alteration was also reduced in the presence of proteinaceous additives. The combination of a sugar such as sucrose and dextran led to enhance the stabilizing effect of the proteinaceous additive.

Immobilized-enzyme processes

Immobilization to solid carriers is perhaps the most used strategy for improving the operational stability of biocatalysts, other benefits being obtained as well, like better control of operation, flexibility of reactor design, and facilitated product recovery with-

out catalyst contamination. Thermal stability upon immobilization is the result of molecular rigidity and the creation of a protected microenvironment. Among immobilization methods available, multipoint covalent attachment is the most effective in terms of thermal stabilization, although thermal stabilization has also been reported for gel-entrapped enzymes. Dramatic increase in thermal stability by immobilizing different enzymes to glutaraldehyde-activated chitin matrices, where multiple Schiff-base linkages are established between free amino groups in the protein and the aldehyde group in the glutaraldehyde linker [27]. Despite its great technological potential, few large-scale processes utilize immobilized enzymes. Severe restrictions may arise because of additional costs, activity losses and diffusional restrictions. In the last few years, improvement in carrier and immobilization techniques is opening new options for process development. In general, immobilized biocatalysts will compete advantageously when the cost of the catalyst is a major component of the processing cost (which is not always the case) and substrates and products are readily soluble and of low molecular weight.

Enzyme immobilization include many strategies

Immobilization of enzymes enables their efficient and continuous use. The rationale behind immobilisation is the easy separation of product from the biocatalyst. Immobilization of enzymes may have a considerable effect on their kinetics. This may be due to structural changes to the enzyme and the creation of a distinct microenvironment around the enzyme [28]. The thermodynamic parameters represented a key in enzyme stability judgment. Within this context, Karam., *et al*. [29] recorded the effect of immobilization process of the immobilized *Aspergillus awamori* amylase on a novel carrier Ca^{+2} alginate (Alg) starch (St)/polyethyleneimine (PEI)/glutaraldehyde (GA), in the improvement of the thermodynamic parameters. Where, the immobilized form appeared the highest optimum temperature, activation energy (Ea) and deactivation rate constants (kd). Also, $t_{1/2}$, D-values (decimal reduction time), change in enthalpy (ΔH^0kJ mol^{-1}), and Gibbs free energy (ΔG^0) increased and was higher than the native enzyme within 50–80^{0}C. The activity of an immobilized enzyme is governed by the physical conditions within this microenvironment not those prevalent in the bulk phase. The method of immobilization and the used matrix affects the partition of material between the product phase and the enzyme phase and imposes restrictions on the rate of diffusion of material [30]. Figure 6 deduce that the immobilized form could be reuse many times while the free enzyme use one time only.

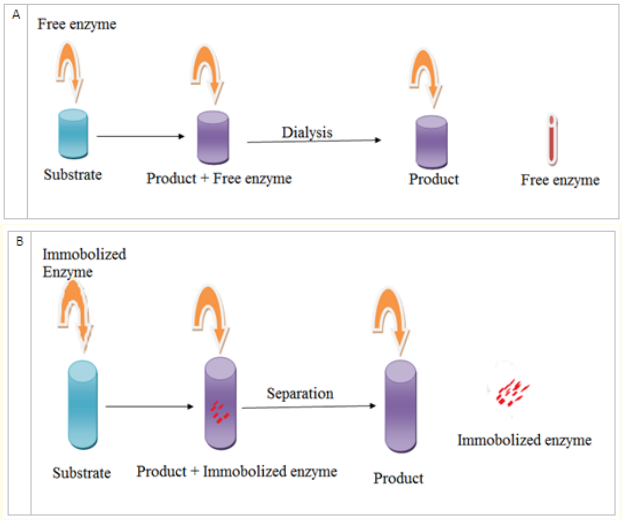

Figure 6: Explain the difference between the free and the immobilized form Previously, numerous methods have been utilized for enzyme immobilization, for example: adsorption, covalent binding, entrapment, encapsulation, and cross-linking [31].

Adsorption technique

Adsorption is another immobilization technique which considered one of the easiest techniques included a reversible surface interaction between the carrier and the enzyme [32] (Figure 7). Abdel el naby., *et al*. [32] reported in *B. subtilis* œ-amylase immobilization using the adsorption method in different carriers One gram of each carrier including AS-alumina, chitin, chitosan, hydroxyapatite, tannin-chitosan ortannin-Sepharose) was incubated with the enzyme solution (1177.5 U) in two ml of 0.05 M citrate-phosphate, pH 6.0) at 4°C for 12h.The unbound enzyme was separated from the carriers by washing with 0.05 M citrate-phosphate buffer (pH 6.0) until no activity or soluble protein was recorded. The enzyme physically adsorbed on AS-alumina showed the highest immobilization yield (24.6%).

Few years ago, the enzyme stabilization by adsorption in nanoparticles was mentioned. The idea depending on the enhancing the protein stability by interfacing them with nanomaterials such as C60 fullerenes. The adsorption of soybean peroxidase into fullerenes at 95 degrees C for 117 min. recorded 13-fold increase in compared to that of the native enzyme Asuri., *et al*. [33].

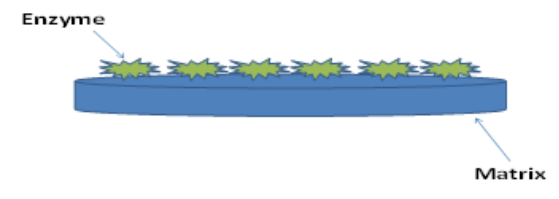

Figure 7: Simple diagram represented the adsorption process.

While adsorption technique has some disadvantages includes:

- Leakage out of the enzyme from the support
- The separation of product is not easy.
- Nonspecific binding.

Entrapment

Entrapment is one of the most famous and easiest immobilization techniques. Several years ago, calcium alginates were the most popular support in immobilization process. It has been implemented for immobilization of different cell types, sub-cellular organelles, multi-component systems, and enzymes. The physicochemical characteristics of this matrix in gel form have an important effect on facilitating the entrapment process into the gel. Entrapment is generally applicable but may cause diffusional problems. Previously, many authors reported in different entrapment methods [34]. The difference between entrapment, adsorption and covalent binding techniques is that the enzyme is restricted in movement by the structure of a gel lattice (Figure 8), but it is free in solution [35]. Esawy., *et al*. [34] reported in immobilization of pectinase in polyvinyl alcohol (PVA) sponge by entrapment. The polyvinyl alcohol (PVA) lattice structure sponge distinguished by very dense porosity and the specific pore volume are very high. These properties recommended PVA sponge strongly to be used for the enzyme entrapment. Polyvinyl alcohol sponge (PVA) was washed several times by distilled water, squeezed to get rid of water, then was cut into small pieces (5 mm × 5 mm × 2 mm) using scissors. The pieces of synesthetic sponge were inserted into the 50% ethanol enzyme fraction dissolved in 2.0 ml of 0.2 M acetate buffer, pH 4.0 at 4^0C for 24 h (1037 U/g carrier). The results recorded 66% immobilization yield with the enzyme dose 12.2 mg/g carrier.is method of immobilization improved the enzyme thermal stability to great extent where, the free enzyme lost its activity completely a 70 ºC while, the immobilized form keep 52% of is activity at the same condition. Also, the immobilized form showed more tolerance to acidic and alkaline pH.

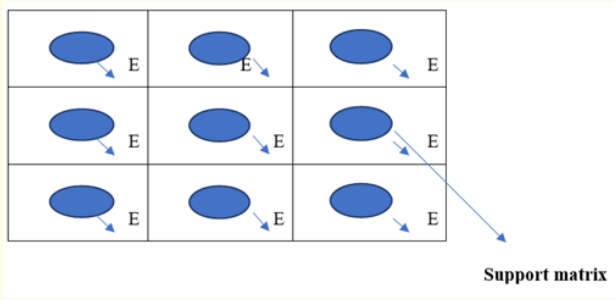

Figure 8: Simple diagrams discuss immobilization by Entrapment.

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 could be occurred by cross linking the polyionic polymer material accompanied by multivalent cations in an ion-exchange reaction. This step happened after mixing with the enzyme to form a structure supported the enzymes / cells entrapment (ionotropic gelation). Change in temperature is a simple way of gelation by phase transition utilizing 1-4% solutions of gelation. κ-carrageen a polymer that can easily form gels by ionotropic gelation and by the temperature-induced phase transition, which has formed a greater degree of flexibility in gelation frameworks for immobilization [36].

Enzyme encapsulation

Encapsulation of the enzymes (Figure 9) was achieved by wrapping enzymes inside different semi permeable membranes forms. This technique of immobilization was very similar to the entrapment technique in which the enzymes are in free movements and fixed in space. The vast proteins or enzymes cannot out or inter

the capsule. While small substrates and products could go freely across the semi permeable membrane. Numerous materials have been used for forming microcapsules in range of 10-100 µm in diameter, like nylon and cellulose nitrate. The membrane rupture is a fundamental problem accompanied with diffusion of the products from a reaction. This may be happened if the product rapidly accumulated. Moreover, cells or enzymes could be immobilized in any desired combination to suit the applications.

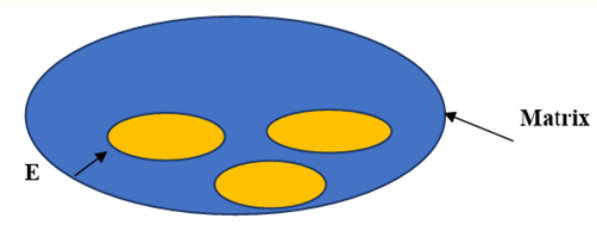

Figure 9: A simple diagram showing wrapping enzymes inside the capsule.

Wang and Frank [37] mentioned the combination of using porous particles for enzyme immobilization. Also, their subsequent coating with nanocomposite shells to effect encapsulation of the enzymes, thus overcoming the problem of enzyme desorption often encountered with the direct immobilization of enzymes on mesoporous silicas. Encapsulating enzymes in mesoporous silica spheres *via* immobilization, subsequent by assembling of an organic/inorganic nanocomposite shell on the particle surface resulted in high loadings, high enzymatic activity and stability, and protection from proteolysis.

Covalent binding

The covalent binding is one of the most famous and efficient methods for immobilization. The covalent bond (Figure 10) is a strong bond happened between the enzyme/cell and a carrier. This bond was formed between the functional groups present on the carrier surface and the enzyme surface. The enzyme functional groups are 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 [38].

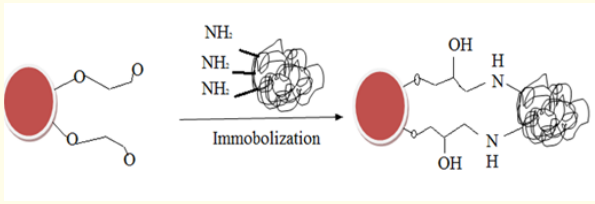

Figure 10: Immobilization by covalent binding.

Many factors controlled the selection of the specific carrier; a lot of research work showed that hydrophilicity is one of the most important factors for remaining the enzyme activity. Thus, hydrophilic carriers like the polysaccharide polymers are the famous materials for enzyme immobilization. For instance, cellulose, starch, dextran (sephadex), and agarose (sepharose). The sugar residue in these polymers contained an ideal functional group, hydroxyl groups, for the formation of covalent bonds [39]. As well as the hydroxyl groups formed hydrogen bonds with water and created an aqueous (hydrophilic) environment for the support. The supports are usually is bead form. The other popular supports for enzyme immobilization are porous silica or porous glass. Porous silica contained small spherical particles of silica fused together to form microcavities and small channels. The carrier is usually solid in bead form; it is very strong and durable. Sintered borosilicate glass has a uniform channel system. Also, the porous glass is durable and resistant to microbial disintegration and solvent distortion. However, the two supports are procedures for coupling an enzyme and a carrier by a covalent bond. A novel carrier for dextransucrase immobilization was invented. This enzyme produced by bacterial honey isolate (*Enterococcus faecalis*). The carrier invented by improving the mechanical properties of alginate and addition of carboxymethylcellulose (CMC) which raised the active groups on the surface of the polymer and entered a new functional group on the polymer surface. This was reached by the treatment of the carrier by polyethyleneimine then glutaraldehyde which made a very strong covalent bonds between the enzyme and the polymer and helped to use the polymer several times for dextransucrase immobilization, which reduce the total cost of the products produced by the enzyme loaded and also dissolved the major problem faced by many scientists before (loading this enzyme because dextran blocked the

active site of the enzyme), this invention eliminated this problem and the results showed the high activity of the enzyme and make it more applicable in medicine and industry [40].

Covalent immobilization has no leakage of enzyme. The enzyme could be easily contacted to the substrate and increasing the thermal stability of the enzyme. But unfortunately, this technique of immobilization has a high cost because the good supports are very expensive.

Cross linking

Cross-linking method (Figure 11) is another irreversible immobilization technique which depends only on enzyme and it supports. It done by joining the enzyme or the cells to each other to prepare a large, three dimensional complex formations by using polyfuncational reagent. There are several reagents that could be used in cross linking normally included the formation of a covalent linkage between the cells by means of a bi- or multifunctional reagent, as glutaraldehyde and toluene diisocyanate. However, limiting factors could be used in this method for living cells and many enzymes because of harmful materials. To minimize the close problems that could be found due to cross-linking of a 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 [41].

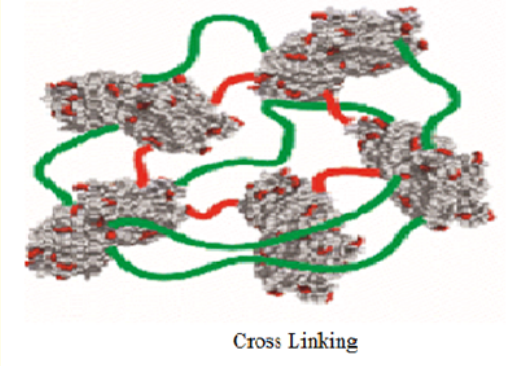

Figure 11: Immobilization by cross-linking.

Previously, immobilization in *A. flavus* NFCCI fructotransferase by the cross-linking method using the chitosan or by entrapment in alginate beads 0.3% (w/v), sodium alginate with 0.1% (w/v) of $CaCl_2$ solution was mentioned. The free and immobilized fructotransferase characteristics were studied, and the results recorded that the optimum pH and temperature for fructotransferase activities were changed by the immobilization. Moreover, Aguiar-Oliveira E, and Fernandes [42] reported in immobilization of *Rhodotorula* sp fructotransferase by adsorption on particles of niobium graphite alloy.

Some reports introduced immobilization techniques to levansucrase using different immobilization matrices including ceramic support glutaraldehyde activation of amino silica and hydroxyapatite [31]. *Z. mobilis levansucrase* was immobilized on the surface of hydroxy apatite and optimized conditions for the formation of levan. The immobilized enzyme has a storage stability as well as high efficiency in reusability for several times. The immobilized molecule means that its movement in space was completely restricted or to a limited region through solid structure attachment [41].

Enzyme stabilization in organic solvents

The organic solvent systems use as an alternative way of aqueous media for enzymatic reactions introduced a lot of advantages, including improved the solubility of hydrophobic substrates or suppression of water-dependent side reactions. Research continues into the properties and applications of enzymes in organic solvents. In an apparent paradox, water-miscible solvents are, in many cases, more problematic than hydrophobic solvents as regards protein stability. This is because hydrophilic solvents, unlike their hydrophobic counterparts, can remove the essentially bound water from the surface of the enzyme molecule [43]. Santucci., *et al*. [44] investigated HRP' stability to tolerate the common solvent DMSO. Their study was prompted by the widespread use of DMSO to dissolve polymers employed for HRP entrapment in electrochemical sensor configurations. HRP's structure was monitored by UV-Vis spectrophotometry and electron paramagnetic resonance (EPR) while its activity was measured by cyclic voltammetry and a colorimetric assay. The enzyme retained its aqueous levels of activity in the colorimetric assay up to 70% (v/v) DMSO. Cyclic voltammograms (CVs) at or below 60% DMSO closely resembled those in aqueous buffer. Minor alterations to EPR spectra occurred in the range 5 - 50% solvent, where the high-spin ferric heme persisted, and no low-spin peak was detected. The critical DMSO value appeared to be 80% (v/v), as all three structural probe techniques indicated radical changes to HRP's structure, with loss of the heme and an absence of catalytic activity, at this concentration. Activity was retained at DMSO concentrations of 70% or less and structural

alterations, although detectable, were minor. In thermal unfolding experiments, HRP was much less stable in 70% DMSO than in aqueous buffer but in 20 - 60% DMSO, its thermal profile closely resembled that in buffer. Sears., *et al*. [45] studied the stability of lyophilized subtilisin BPN in DMF mixtures. Retention of activity at very high DMF concentrations improved with increasing concentration of counterion in the aqueous solution from which the enzyme had been lyophilized. Multivalent counterions poorly soluble in organic solvents were much more stabilizing than monovalent, soluble ions. Sodium citrate was the most stabilizing of the salts tested. The percentage of water in the DMF mixtures also influenced subtilisin stability, as did the manner of stirring the enzyme suspension.

Klibanov [43] discovered the rules that enable enzymes to vigorously act as catalysts in organic solvents containing little or no water. When placed in this unnatural milieu, enzymes acquire some remarkable novel properties, such as greatly enhanced thermostability and strikingly different specificity, including stereoselectivity. Our goal is to obtain a mechanistic understanding of enzymatic catalysis in nonaqueous media. This knowledge will enable us to control predictably the behavior of enzymes by altering the solvent, rather than the protein molecule itself (as in protein engineering). Enzymes in organic solvents are also used as catalysts of synthetically interesting and challenging processes, such as asymmetric oxidoreductions.

Effect of salts in enzyme stabilization

Lozano [46] reported in the influence of different alkali halides (LiF, LiCl. LiBr, NaF, NaCl, NaBr, KF, KCl and KBr) on α-chymotrypsin-catalysed plastein synthesis has been studied in aqueous medium at different substrate concentrations. The results appeared an enhancing effect on the plastein synthesis enzyme action by the presence of salts which proportional to the salt concentration and was decreased when the substrate concentration was increased. Additionally, these facts allowed the ions to be classified as a function of their activation power ($F^- > Cl^- > Br^-$: $K^+ > Na^+ > Li^+$). Baptista., *et al*. 2000 [22] showed that the thermal unfolding induced by increasing temperature was analyzed in the absence and in the presence of trehalose according to a two-state model (which assumes that only the folded and unfolded states of cutinase were present). Trehalose delays the reversible unfolding.

Effect of polysaccharides in enzyme stabilization

The polysaccharide-β-glucosidase complex was extremely resistant to proteinases and far more stable against urea and temperature as compared with polysaccharide-free β-glucosidase. The β-glucosidase-polysaccharide complex was 18-, 36-, 40-, and 82-fold more stable against chymotrypsin, 3 mol/L urea, total thermal denaturation, and irreversible thermal denaturation, respectively, as compared with polysaccharide-free β-glucosidase. The starch conjugated *A. tenuissima* KM651985 laccase was active over a wide range of temperatures and pHs with the highest activity at 4 and 60°C, respectively. The conjugated *A. tenuissima* KM651985 laccase thermal stability was proved by, high $T_{1/2}$ values (half life) 1076.16, 382.42 and 191.23 min at 50, 60 and 70 °C, respectively. The low K_d (denaturation rate constant) recorded 6.44×10^{-4}, 18.13×10^{-4} and 36.25×10^{-4} min^{-1} at the same temperatures, while the high D-values (decimal reduction time) were 3575.56, 1270.61 and 635.38 min at the same temperatures [28].

Methods for stabilizing enzymes in liquid compositions,

Including those liquid compositions have a high-water content and those stabilized enzyme liquid compositions formed thereby. The method included forming the enzyme, so that it is in an insoluble form thereof and then adding thereto an agent for maintaining the enzyme in the insoluble form thereof. Examples of such insoluble forms are crystal forms of the enzyme. Enzymes which are stabilized in this case are useful for combining with liquid compositions, including liquid compositions having high water content. The method is particularly useful for the preparation of stable enzyme-containing liquid detergent compositions.

Protein engineering techniques

Many new techniques for progress the industrial effectiveness of pre-existing enzymes have been done possible since the PCR discovery. These involved DNA sequencing, site-directed mutagenesis and DNA shuffling. Other advances in biotech have led to the methods development that mange for screening large numbers of new recombinant or mutant microorganisms which express the modified enzymes and choose the best one suitable for a specific application. Large throughput capacity of screening facilitated significant advances in enzyme technology in recent years since It was difficult to test individual recombinant microorganisms, or their purified enzyme products, one at a time, for improved the potentiality of a particular process Bryan [47] has reviewed protein engineering of the detergent proteinase subtilisin, including stability aspects. Advances in the protein engineering of glucose isomerase (GI) have also been explained, so these enzymes do not receive detailed treatment here. The following paragraphs describe protein

stabilization examples by directed evolution, polypeptide chain extension, manipulation of chimeric proteins and rational design.

Directed evolution

Directed evolution techniques had a dramatic impact on protein engineering since their introduction. No knowledge or modeling of the target protein's molecular structure is required. Briefly, directed evolution involves the recombination of beneficial point mutations with the selection for further-improved properties.

This process could be repeat by successive cycles, leading to noteworthy alterations/improvements to the properties of the baseline protein. Typically, a number of DNA sequences coding for the protein of interest, each containing one or more beneficial point mutations, are mixed and then randomly fragmented with, e.g. DNase. Many methods for DNA sequence variegation have been mentioned since the directed evolution was first conceptualized. The majority of these methods fall into the following categories: error-prone PCR, DNA shuffling or chimeragenesis, site saturation mutagenesis, and random mutagenesis using chemical agents, physical agents, or hypermutator strains [48].

CiP peroxidase

A notable example of directed evolution concerns peroxidase (CiP) from the ink cap mushroom *Coprinus cinereus* [49]. CiP is potentially important in detergents: it can bleach dyes leached from colored clothing during the wash cycle, inhibiting the dye transfer to white clothes if white and colored garments are accidentally washed together. CiP is readily available but is not stable under wash conditions.

Site-specific mutagenesis

Site-specific mutagenesis continues to introduce a great scope for protein stability increasing where the target protein structure (or of a closely related protein) is known. This is especially so in situations since the screening of a large population of mutants is not possible or feasible [50].

Conclusion

Enzyme instability considered one of the main problems facing the enzyme application in industrial field. This review highlighted different factors affecting the enzyme stability and introduced different strategies which were used to improve the enzyme stability from different points of view.

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