

**Review Article**

# Maximizing Stability in Industrial Enzymes: Rational Design Approach – A Review

Abubakar Muhammad Nazif<sup>1, \*</sup>, Ayuba Yohanna Musa<sup>2</sup>, Muhammad Muawiya Alkali<sup>3</sup>, Ilesanmi Esther<sup>4</sup>

<sup>1</sup>Department of Biotechnology, Modibbo Adama University of Technology, Yola, Nigeria

<sup>2</sup>Department of Chemistry, Abubakar Tafawa Balewa University, Bauchi, Nigeria

<sup>3</sup>Department of Biochemistry, Modibbo Adama University of Technology, Yola, Nigeria

<sup>4</sup>Department of Chemistry, University of Benin, Benin, Nigeria

**Email address:**

nazeefba@gmail.com (A. M. Nazif)

\*Corresponding author

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**Abstract:** Indigenous enzymes found in nature have found wide application in industries ascribable to their ability to catalyze complex chemical processes under moderate experimental and environmental conditions. However, the use of indigenous enzymes is yet to achieve the needed industrial goal for, indigenous enzymes are readily unstable when subjected to harsh environmental conditions. Since the emergence of recombinant DNA technology and recent developments in protein engineering in recent years, there have been continuous reports regarding enzyme stability – most especially by the introduction of site-directed mutagenesis. With these new developments, scientists have been able to engineer enzymes using a variety of strategies in rational design such as the introduction of disulfide bridges and engineering hydrophobic residues. This review aims to highlight rational design methods and enzyme immobilization from various studies, which may be used to increase stability in industrial enzymes.

**Keywords:** Protein Engineering, Stability, Rational Design, Immobilization

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## 1. Introduction

The speed of sequencing of microbial genomes and metagenomes is providing an ever increasing resource for the identification of new robust biocatalysts with industrial applications for many different aspects of industrial biotechnology [1]. Enzymes are biological molecules that increase the rates of chemical reactions. Industries have been using enzymes since traditional or recorded history in cheese production, utilizing indirectly yeasts and bacteria in food manufacturing. Isolated enzymes were first used in detergents in 1914, their protein nature was proven in 1926 and their large scale microbial production began in the 1960s. Many hydrolase enzymes including proteases, lipases, esterases and

cellulases are already used commercially in industrial biocatalysis as documented in recent review chapters [2] [3]. The enzyme biocatalysts required for industrial processes need to withstand conditions that they are not usually exposed to within their natural environments (cited in [1]). However, enzymes found in nature have been exploited in industries due to their inherent catalytic properties in complex chemical processes, under mild experimental and environmental conditions [4].

Despite the fact that biochemists have characterized several thousand enzymes, only few could be considered of commercial importance [5]. Recent progress in genetic-manipulation techniques enables the large-scale supply of many enzymes for industrial purposes. However, identification of new biocatalysts (for example, by screening

of soil samples or strain collections by enrichment cultures) does not always yield suitable enzymes for a given synthetic problem [6]. The limiting factor in the industrial application of enzymes, however, is the high cost of isolating and purifying large amounts that would meet industrial requirements but, beyond cost there are other limitations associated with industrial enzymes especially, the conditions subjected to industrial processes differ with that of physiological pathways, and even the desired industrial application may differ significantly from physiological roles. To overcome these limitations, tailor-made biocatalysts can be created from wild-type enzymes by protein engineering using computer-aided molecular modeling and site-directed mutagenesis (cited in [6]). Most importantly, industrial applications require robust enzymes with long half-life under process conditions.

Generally, indigenous enzymes do not meet the requirements for industrial application, therefore, there is a need to modify, optimize or effect positively the properties of such enzymes especially, in terms of stability for, the conditions (such as temperature and pH) at which reactions take place in industries vary significantly to conditions in natural systems. The aim of protein engineering is to overcome the limitations of natural enzymes as biocatalysts and engineer process-specific biocatalysts. As a result of recent advances in recombinant DNA technology techniques, numerous examples of the optimization of certain enzyme traits (thermo-stability, tolerance towards organic solvents, and enantio-selectivity) have been reported. 21st century's advances in technology is making it possible to analyze the structure of enzymes and to study the direct correlation between enzyme structure and function using tools and graphical user interfaces, with which models can be drawn and stability can be maximized through applying necessary changes at specific positions that do not change an enzyme's original conformation and function.

Enzyme design presents a huge challenge, not only in the de novo design of catalysts for which no natural counterparts are known, but also in the design of multipurpose enzymes, which may have a wide range of biotechnological applications in fields, such as industrial organic synthesis and metabolic engineering [7]. In this review, methods for maximizing stability in industrial enzymes and enzyme immobilization, concentrating on methods published in the last two decades were discussed.

## 2. Protein Engineering

The introduction of Protein engineering in the early 1980s evolved from the knowledge of gene cloning, DNA sequencing and in-vitro DNA manipulations (cited in [4]). Majority of enzymes isolated from their natural source do not have the desired features for industrial application in one way or the other. For decades, scientists have been searching extensively for new enzyme variants in organisms that can grow in extreme conditions, but this has recorded a few successes. The increasing usage of enzymes in industrial

processes and household catalysis has spurred the development of protein engineering methodologies to produce novel enzymes with new or improved properties. The advances in recombinant DNA technology, high throughput in technology, genomics and proteomics have fueled the development of novel enzymes and biocatalytic processes (cited in [4]).

Protein engineering can be considered a sub discipline within the broader category of genetic engineering [8]. The main difference between protein engineering and genetic engineering is that the final product is a protein with a modified amino acid sequence, rather than a new or modified living organism. Generally, proteins do not reproduce, many of the concerns found in the broader field of genetic engineering, for instance, the current controversy surrounding genetically modified organisms are not an issue in protein engineering. In this regard, engineered proteins more closely resemble new chemical compounds from non-biological sources, for which concerns around safety and toxicity apply, but which by their very nature are readily biodegradable (cited in [8]). Table 1 below shows in summary, some of the reasons why industrial enzymes need to be engineered for improved stability.

*Table 1. Change in enzyme characteristics by Protein engineering.*

S/No.	Enzyme	Industry	Goal
1.	Proteinase	Detergent	Thermo-stability Oxidative stability Alkali stability
2.	Xylanase	Food	Temperature stability
3.	Glucoamylase	Starch	Optimum pH stability
4.	Glucose Isomerase	Fructose	Acid stability Thermo-stability

Several enzymes have already been engineered to function efficiently in industrial processes. Xylanase is a good example of an industrial enzyme that needs to be stable in high temperatures and active in physiological temperatures when used as feed additive and as bleach in food and paper industries respectively.

### 2.1. Rational Design

The creation of biocatalysts from scratch enables scientists and engineers to build synthetic enzyme for a series of different chemical reactions (cited in [4]). Rational design is a strategy in protein engineering where proteins with improved characteristics are created based on the available information obtained from the three-dimensional structure and the relationship between protein structure and its function, which scientists believe over the years play a crucial role in protein's function. Because of its roots in protein chemistry, rational design was the earliest approach to protein engineering and still is widely used, either as a stand-alone approach or combined with random mutagenesis or directed evolution [9].

The rational design strategy employed in protein engineering is a step wise process which involves x-ray crystallography or NMR spectroscopy to view the three-dimensional structure, which can be represented

graphically and mathematically on a computer. The computer model allows visualization of the protein/enzyme in all its spatial detail by zooming and rotation. The computer model then allows predictions to be made, particularly the effect of mutation on structure based properties.

## 2.2. Enzyme Stability

Enzyme stability is the net balance of forces which determines whether a protein would be in its native folded conformation or a denatured state. Enzyme stability normally refers to its thermodynamic stability, not chemical stability. Enzyme stabilization has notable importance due to the increasing number of enzyme application [10].

Stability is an important parameter which co-determines the economic feasibility of applying an enzyme in an industrial process [11]. High stability is generally considered an economic advantage because of reduced enzyme turn over. In addition, stability in enzymes permits the application of extreme conditions, which may have beneficial effect on rate of reaction and even eliminate the risk of contamination. In order to enhance the stability of enzymes, protein engineering employs the following methods;

1. Engineering disulphide bonds
2. Engineering hydrophobic residues
3. Engineering Asparagine and Glutamine residues
4. Engineering hydrogen bonds

### 2.2.1. Engineering Disulfide Bonds

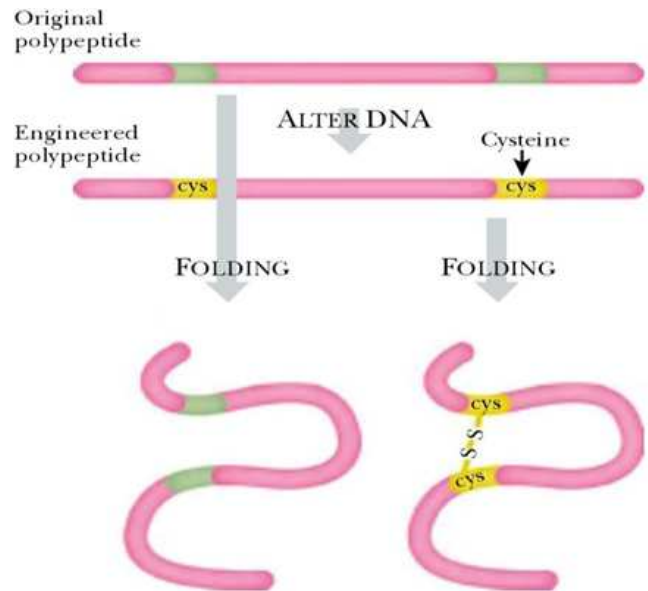
Disulphide bonds are very important in maintaining the 3D (three-dimensional) structure of proteins/enzymes. Most enzymes used in industries are exposed to oxidizing conditions and for this reason, disulphide bonds are relevant. Introduction of disulphide bonds is a straightforward and at the same time a cautious process of increasing stability in enzymes. After viewing and modeling of an enzyme's 3D structure using necessary computational tools, two cystein residues can be introduced to the polypeptide chain at appropriate positions so that when the polypeptide chain folds, the two cystein residues will come into contact and form a disulphide bond. Generally, the longer the loop of amino acids between the cystein residues, the greater the increase in stability. However, formation of disulphide linkage can create a strained conformation if the two cystein residues are not properly aligned [12], which may result in a decrease in stability of the enzyme.

[13] Have replaced isoleucine at position 3 with a cysteine (Ile 3 -Cys) in phage T4 lysozyme (a disulfide-free enzyme), and under mild oxidation they have generated a disulfide bond between the new Cys 3 and Cys 97, one of the two unpaired cysteines of the native lysozyme. The disulfide mutant protein retained full enzymatic activity and was more stable against thermal inactivation than the wild-type protein. The addition of new disulfides has not always led to increased stability. Based on the examination of four disulfide mutant T4 lysozymes, [14] have suggested that for the successful improvement of protein stability by disulfide bonds;

1. The introduction of the cysteine(s) should minimize the

disruption or loss of interactions stabilizing the native structure.

2. The size of the loop formed by the crosslink should be as large as possible.
3. The strain energy introduced by the disulfide bond should be kept as low as possible.



**Figure 1.** A disulfide bond can be added to a protein by changing two amino acids into cysteines by site-directed mutagenesis. When the engineered protein is put under oxidizing conditions, the two cysteines form a disulfide bond, holding the protein together at that site. Adapted from cited in [12].

### 2.2.2. Engineering Hydrophobic Residues

Hydrophobic residues tend to exclude water, these residues tend to cluster in the center of proteins and avoid the outer surface. The hydrophobic effect is considered an important driving force in protein folding and stability. The filling of cavities in the hydrophobic core is believed to increase stability as demonstrated by [15]. The filling of the cavities may be done by replacing small hydrophobic residues in the core with larger hydrophobic residues. For example, the substitution of alanine by valine or leucine by phenylalanine at suitable positions would result in achieving stability.

However, most proteins have hydrophobic cores that are already fairly stable and have few cavities. Furthermore, inserting larger hydrophobic amino acids to fill these cavities often causes twisting of their side chains into unfavorable conformations (cited in [12]), which cancels out any gains of stability from packing the hydrophobic core more completely.

### 2.2.3. Engineering Asparagine and Glutamine Residues

Generally, industrial conditions such as high temperature and pH do have adverse effects on amino acid residues that make up an enzyme. Asparagine and glutamine residues are relatively unstable for, if subjected to high temperatures and extreme pH, they are converted to their corresponding amides, aspartic acid and glutamic acid respectively. The replacement of these neutral amides by the negatively charged carboxyl may damage enzyme structure and activity. The de-amination

of Asparagine in Glutamine residues in peptides and proteins however depend upon both the identity of other nearby amino acid residues, some of which can catalyze the de-amination reaction [16]. However, this can be avoided by engineering polypeptide chains to replace asparagine and glutamine residues by an uncharged hydrophilic residue of comparable size like threonine (cited in [12]).

#### 2.2.4. Engineering Hydrogen Bonds

Hydrogen bonding is an omnipresent interaction contributing to protein stability. It was one of the first interactions to be analyzed consistently by protein engineering. The destruction of a hydrogen bond by a single amino acid substitution often reduces protein stability. Alber [17] have shown a strong correlation between a major reduction in stability and the replacement of Threonine 175 in phage T4 lysozyme with other amino acids unable to form a hydrogen bond with the amide of Asparagine 159. Although, the urea and thermal unfolding studies of twelve mutants of ribonuclease T1 have suggested that hydrogen bonding and the hydrophobic effect make comparable contributions to the conformational stability of the enzyme, in general hydrogen bonds seem to be quite variable in their contribution to protein stability.

### 3. Immobilization

Immobilization is the conversion of an enzyme from a water-soluble, mobile state to a water-insoluble, immobile

state. The techniques of enzyme immobilization may be divided into five groups. Since 1967, when the first immobilized enzyme, amino acylase, was used for the resolution of amino acids. Enzyme immobilization technology has attracted attention and considerable progress has been made over the past decade. There has been considerable interest in the development of enzyme immobilization techniques because, immobilized enzymes have enhanced stability compared to soluble enzymes, and can easily be separated from the reaction [19]. Stabilization due to immobilization can be ascribed to mutual spatial fixation of enzyme molecules (against aggregation or autolysis of proteases), an increase in their rigidity (against unfolding, i.e. conformational changes) and their protection from possible inactivators (pH, oxygen, hydrogen peroxide).

Approaches used for the design of immobilized enzymes have become increasingly more rational and are employed to generate improved catalysts for industrial applications [20]. There are a variety of methods used to immobilize enzymes, the three of the most common being -- adsorption, entrapment, and cross-linking or covalently binding to a support.

However, there is no doubt that the use of immobilized enzymes provides more advantages than demerits. The practical use of immobilized enzymes in industries is restricted when compared to the extent of research carried out. Moreover, avoiding the use of a polymer support has been suggested to be more useful in enzyme stabilization.

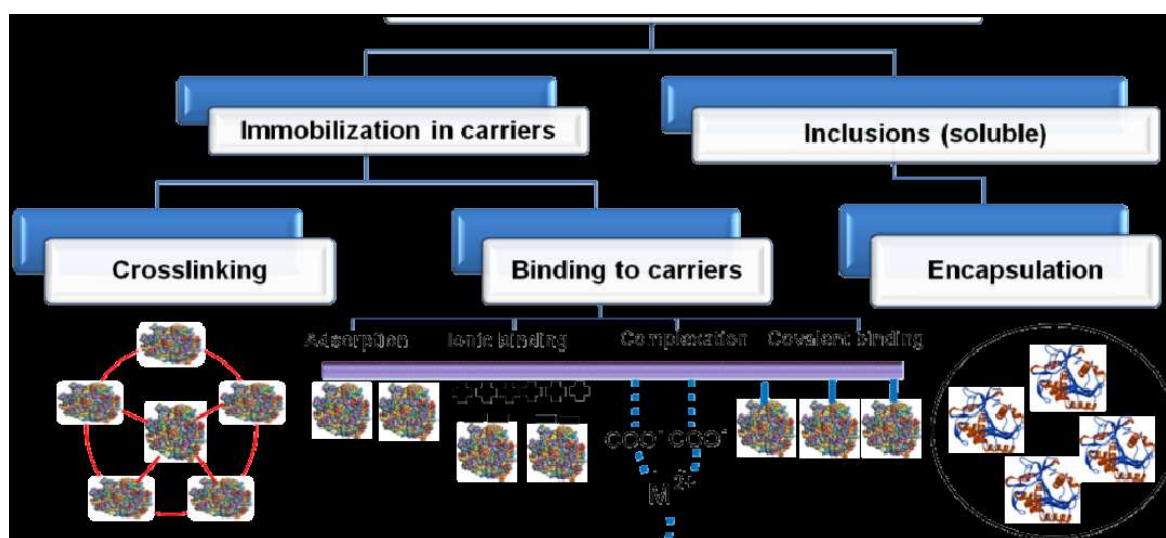


Figure 2. Enzyme immobilization via different routes. Adapted from (cited in [18]).

Table 2. Advantages and disadvantages of enzyme immobilization.

S/No	Advantages	Disadvantages
1.	High stability and resistance to shear stress and contamination.	Necessity of immobilization processes.
2.	Ease of developing continuous process.	Existence of mass transfer resistances.
3.	Easy separation of biocatalysts from fermentation medium.	High cost of immobilization reagents.
4.	Fast reaction rate.	
5.	Repetitive use of biocatalysts.	

Adapted from (cited in [19]).

### 4. Conclusion

Approaches to produce more stable enzymes are based on protein engineering, which can either use a rational design strategy or a direct evolution strategy. Nowadays, there are several methods of improving enzyme stability for industrial processes. Alas, there is no single preferred method for improving stability in enzymes for; each of the methods discussed in this review has its own advantage and disadvantage. The pace at which the biotechnology industry is

growing especially in the aspects of computational biology, protein engineering and immobilization of enzymes, coupled together, seems to be a powerful tool to greatly improve a number of industrial processes, and more effort may be expected in this regard.

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