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Treball Final de Grau

Comprehensive review of enzyme immobilization in industry: applications, advantages, and challenges

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To know how to wonder and question is the first step of the mind toward discovery

Louis Pasteur

I want to thank my parents for always trusting me in everything.

And I want to thank my FDP tutor, Dr. David Faulon Marruecos for guiding me in the process of learning about the world of enzymes.

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SUMMARY

Enzymes bring lots of energetical, environmental and economic advantages that are obfuscating the vision of classical chemistry. Since enzymes are susceptible to destabilizing under industrial harsh conditions, industrial feasibility of enzyme use relies on making enzymes stronger and more active under such environments. Thus, catalytic chemistry has taken the path of conjugating enzymes with other molecules or surfaces that give new characteristics to render them more stable and, sometimes, even more active with respect to the original form.

The mechanisms underlying enzyme conformational changes and their correlation with activity have yet to be unveiled, but recent research has shed some light on how intermolecular and intramolecular interactions combined with immobilization techniques govern enzyme thermodynamics and kinetics. Thus, harnessing such knowledge can potentially give us a roadmap to ensure enzyme effectiveness in ubiquitous industrial sectors, such as food and pharmaceutics.

In short, enzyme immobilization can be the cornerstone that might impulse a new generation of biochemical synthesis in industry which is the greenest and most efficient ever utilized up until now.

Keywords: Enzyme immobilization, protein conformation, surface interactions, novel supports, bioconjugation, polymer brushes, enzyme stability, biomaterials

RESUM

Els enzims aporten molts avantatges energètics, ambientals i econòmiques que estan ofuscant la visió de la química clàssica. Atès que els enzims són susceptibles de desestabilitzar-se en condicions industrials adverses, la viabilitat industrial de l'ús d'enzims es basa a fer-les més forts i actives en aquests entorns. Així, la química catalítica ha pres el camí de conjugar els enzims amb altres molècules o superfícies que els confereixen noves característiques per a fer-les més estables i, a vegades, fins i tot més actives respecte a la forma original.

Els mecanismes que subjuguen als canvis de conformació dels enzims i la seva correlació amb l'activitat encara no s'han revelat, però recerques recents han llançat una mica de llum sobre com les interaccions intermoleculars i intramoleculars combinades amb les tècniques d'immobilització regeixen la termodinàmica i la cinètica dels enzims. Així, l'aprofitament d'aquests coneixements pot proporcionar-nos un full de ruta per a garantir l'eficàcia dels enzims en sectors industrials omnipresents, com l'alimentari i el farmacèutic.

En resum, la immobilització d'enzims pot ser la pedra angular que impulsi una nova generació de síntesis bioquímiques en la indústria que sigui la més ecològica i eficient utilitzada fins ara.

Paraules clau: Immobilització enzimàtica, conformació de proteïnes, interaccions superficials, nous suports, bioconjugació, raspalls de polímers, estabilitat enzimàtica, biomaterials

ODS

Aquest treball es pot classificar segons les ODS de l'ONU en direcció al creixement econòmic, a la innovació de la industria amb visió de protegir al medi ambient amb noves tecnologies.

El fet d'actualitzar l'industria a treballar amb enzims immobilitzats millorarà l'ús dels recursos del planeta, que estem esgotant a una velocitat alarmant a la vegada que embrutim el mon. Els enzims redueixen l'energia necessària per du a terme una reacció i la immobilització ens permet reciclar aquest valuosos enzims a la vegada que redueix el recursos necessaris per processar els productes per separar-los dels enzims. A més, amb l'ús d'enzims ja que els costos de procés baixen, també augmenta el valor econòmic de les industries.

Es a dir que, en general va en direcció de la prosperitat de nosaltres com societat moderna i la protecció del planeta, ja que van agafades de la ma, des de el meu punt de vista.

1. INTRODUCTION

The constant interest of designing and synthesizing novel molecules to improve the ones that are already in use or making completely new structures with desired properties has given the chance for enzymes to shine in various industries, such as the pharmaceutical, agricultural and food industry. Improvement is a challenge that must be faced with an enhanced synthetic chemistry toolbox that will allow new chemical structures to be made. Enzymes are the main player in the making of specific molecules because of their nature since they can be synthesized or obtained from microorganisms to do highly specific tasks in biochemical reactions. The revolution in molecular biology enabled Biocatalysis to take the lead in applied technologies, becoming relevant to a big range of chemical transformations, for example the use of Rhizopus oryzae (rROL) lipase for biodiesel production or B-galactosidase for the making of glucose (Federsel, Moody and Taylor, 2021).

The main drawback of free enzyme use is that, at the end of the process, enzymes are lost since they become part of the product. In other words, through free enzyme use, it is hard to recover the enzyme. Also, there is a high probability of rendering the enzyme irreversibly inactive via denaturation. The need to recover the enzyme is principally an economical and environmental problem, thus directly affecting the sustainability of biocatalytic processes, which needed to be solved.

One way to address the stability problems of free enzymes is Protein Engineering, which studies the modification of enzymes via mutagenesis in order to obtain more stable ones that can survive harsh conditions and have a longer shelf life. However, this does not solve the issues with the reusability of the enzyme. Thus, enzyme immobilization provides a framework for recovering the enzyme and operate continuously as any other non-biological catalyst in industrial processes. Immobilization lowers the environmental and economic impact of enzyme catalyzed reactions, by allowing the recovery of the enzyme and in addition offering operational flexibility (Imam, Marr and Marr, 2021).

Specifically, enzyme immobilization is a process through which an enzyme is placed on a surface (e.g., though chemisorption or physisorption) in such way that it becomes insoluble and reusable, while also enhancing its activity and stability under industrial harsh conditions. In other words, it is a way of obtaining improved biocatalysts that can bear the stress that industrial processes cause (Bernal, Rodríguez and Martínez, 2018).

The study of enzyme immobilization paved the way for further improvements in the arena. Its popularity stems from the performance advantages of enzymes that translate to cost reduction and higher product quality, since the specificity of enzymes evades a big amount of waste production and immobilizing them greatly reduces the loss of the expensive catalyst in the process. By combining selectivity, stability and kinetic of an enzyme and the physical and chemical properties of the carrier in a certain way, it is possible to maximize the enzymatic and physical stability of the biocatalyst. In industrial processes, the interest of using immobilized enzyme is the number of cycles the enzyme can be reused as an indirect measure of total productivity on a kg product per kg biocatalyst basis (Federsel, Moody and Taylor, 2021).

The use of immobilized enzyme biocatalysts requires a good understanding of both technical and economic factors.

The main challenge of using enzymes is that they are unstable under many conditions and environments, such as pH, temperature, and pressure or even zones with positive or negative charges or hydrophobic and hydrophilic interactions with the surrounding medium. These instabilities cause the leaching or denaturalization of enzymes, which lose their characteristics. These instabilities are magnified at big scales; meaning that the use of this technology out of the lab must have been previously studied under controlled conditions to be feasible and productive (Mohamad *et al.*, 2015).

The scaling up of immobilized enzymes is evaluated by the associated costs implied in their use. Since the process may be very expensive, the main factors to keep in mind while designing the process are (Mohamad *et al.*, 2015; Basso and Serban, 2019):

- Native enzyme and its initial stability
- Resin cost for enzyme immobilization
- Downstream processing
- Reactors that will be used that have to be directed for immobilization

- Disposal of immobilized enzyme after use
- Regeneration of carriers

It is essential to preserve the catalytically active tertiary structure of the enzyme to keep the stability and reactivity of the enzyme in its immobilized state, therefore, three factors must be taken into consideration: supports, conditions and methods of immobilization. For every immobilization method there are a series of changes of the physical and chemical properties of the enzyme. All changes in the microenvironment that surrounds the enzyme can alter the stability and kinetics of it; that englobes the supporting matrix and the resulting products. Thus, the proper selection of the support and the method of immobilization that enhances both the activity and conformational stability of the enzyme by attaching the right residues is the priority for the productivity of immobilized enzymes (Mohamad *et al.*, 2015).

Some of the decisive factors that should be considered for the application of the immobilization technology in industry are enzyme deactivation and regeneration characteristics, cost of immobilization procedure, toxicity of immobilization reagents and the desired final properties of the immobilized enzymes (Chapman, Ismail and Dinu, 2018).

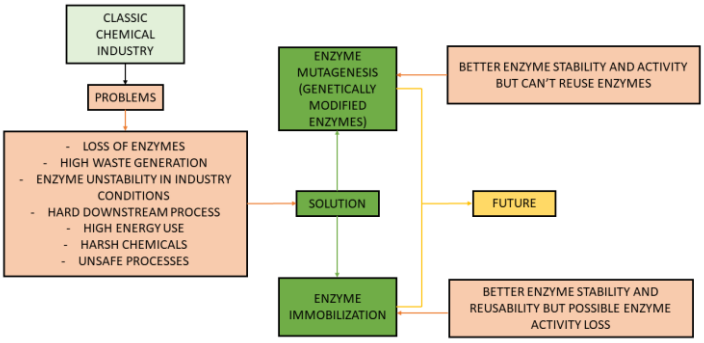

Figure 1. Future of industrial processes

2. OBJECTIVES

The objective of this review is to create a guide where the principal criteria, factors, and mechanisms that regulate enzyme stability and activity are summarized and categorized. Also, this work provides a comprehensive and extensive snapshot of the current state of the art of enzyme immobilization, especially at industrial level, as well as prospections of future directions of its applications.

Furthermore, we highlight here the main molecular mechanisms underlying enzyme stability and activity at a molecular level, taking into account the main inter and intramolecular forces that govern such processes. Understanding the molecular basis of enzyme stability, we can adjust and engineer both the enzyme, support, and immobilization technique in order to increase industrial productivity and reusability.

While a wide variety of immobilization techniques exist, we will focus on the most ubiquitous in industry, such as adsorption, covalent bonding, entrapment, and crosslinking.

Also, the structures that are used for immobilization such as polymers, metal organic frameworks (MOFs), and porous supports will be examined since the comprehension of chemical and physical properties of those is crucial for a proper immobilization.

Immobilization can be a very complex problem since there are many kinds of interactions between the enzyme, support, and the environment that might make an enzyme change its tertiary structure, which certainly will stop it from being active or may significantly reduce its activity. That is why knowing the equations that govern such interactions comes in handy when designing the support and the technique of immobilization for any given enzyme.

Lastly, we will focus on the applications of enzyme immobilization to improve the efficiency of industrial processes and waste reduction. This review will also focus on the economic and industrial feasibility of immobilization to give a grasp in what kind of industry will be more efficient to introduce it.

Shortly, the specific objectives of this work are the following:

- Make a guide of enzymes stability and activity
- Understand folding kinetics
- Understand enzyme-substrate kinetics
- Comprehend intermolecular and intramolecular interactions
- Make a guide on immobilization techniques
- Show the state of current enzyme industry
- Understand why enzyme-based industry is better than tradition chemistry

3. PROTEIN AND ENZYME STRUCTURE

Proteins are organized in four consecutive structures: primary, secondary, tertiary and quaternary. The primary structure of proteins is related to the sequence of amino acids that form the peptide chain and does not give any kind of information about its spatial arrangement. The secondary structure is defined by the spatial arrangement of the proteins since proteins can be folded in α-helix or β-sheet conformations. The tertiary structure is determined by side chain interactions in the protein such as ionic or hydrophobic interactions, thus it gives an understanding of the 3-dimensional arrangement of the protein, the interaction between tertiary structures from different peptide chains gives the quaternary structure as result (Koshland, 2020).

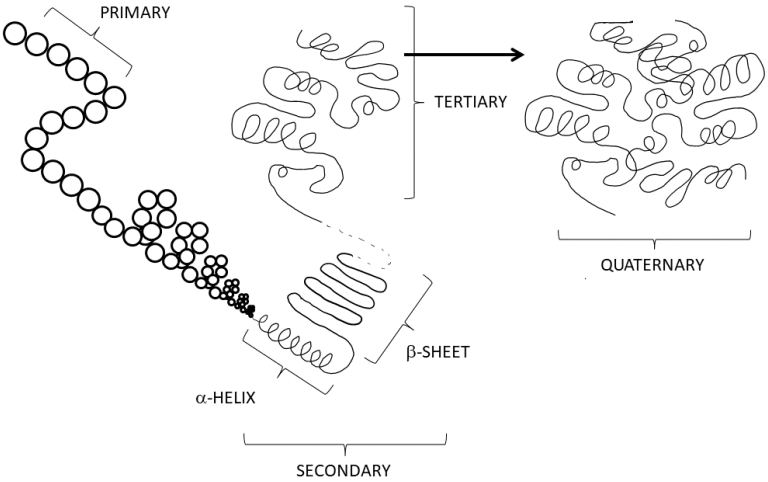

Figure 2. Primary, secondary, tertiary, and quaternary structures of a protein

Surface activity is related to the primary structure of the protein, which is determined by the sequence of amino acids that conforms the protein. The size of the molecules usually conditions the interaction with the surface, since a larger molecule can contact at more sites than a smaller one. However, there are exceptions like hemoglobin molecule compared to a fibrinogen molecule, fibrinogen being much larger has a lower surface interaction (*Protein-Surface Interactions*, no date).

Hydrophobicity is also a factor to consider in surface interactions, because of it charged amino acids are located on the surface of proteins which influences protein absorption. Like molecular size, charge is not the only determining factor in protein-surface interactions, for example, proteins show greater activity near their isoelectric point.

This phenomenon can be explained by the complexity of charge distribution on a mixture that contains huge amounts of charged molecules that might interact with each other, when the mixture gets to the isoelectric point the reduced electrostatic repulsion allows more protein to bind. Other explanation is related to changes on amino acid charges that might distort protein conformation, exposing unwanted amino acids to bind to the surface and causing protein unfolding (*Protein-Surface Interactions*, no date).

The isoelectric point, (pI) is a parameter that shows the pH point where the molecule is electrically neutral. Thus, changing the pH that surrounds a molecule may change its net charge.

All charges inside a protein when considered as a whole shows as a neutral charged molecule, if the pH is lower that the pI then the protein brings a positive charge, when the pH is above a negative charge. The pI is calculated as the mean of pKa of the molecule.

The pKa is a measure of strength of an acid, a constant of the equilibrium of dissociation in the scene of acid-base reactions.

To give an example of how the pI is calculated for an amino acid that only contains the amine and carboxyl group:

$$pI = \frac{pK_{a1} + pK_{a2}}{2} \quad (1)$$

Proteins with less intramolecular cross-linking are likely to unfold more or faster. The amphipathic nature of proteins, their polar, nonpolar, and charged amino acids, also contributes to surface activity. Hydrophilic polar and charged amino acids are generally on the surface and the hydrophobic ones are in the interior, there are some hydrophobic amino acids that can be on the surface which cause interactions with substrates. Additionally, unfolding can generate more interactions with the surface since it exposes hydrophobic amino acids (*Protein-Surface Interactions*, no date).

It must be pointed out that what is more important in an enzyme is its active site, all interaction that can cause an enzyme to change its conformation must be in a way that does not break the active site's functionality. The active site of an enzyme is what gives it the power to accelerate reactions. The substrate binds to the active site where it transforms into the product. The enzyme will also change a bit its conformation to perfectly fit the substrate. Sometimes, a cofactor or a coenzyme will be needed to proceed with the catalysis. A cofactor is usually a metal ion and coenzymes are organic molecules, both enter the active site of the enzyme and change the conformation for the catalysis of a given enzyme. Furthermore, the conditions of the environment such as the pH and the temperature can also change the enzyme conformation and can denature the enzyme.

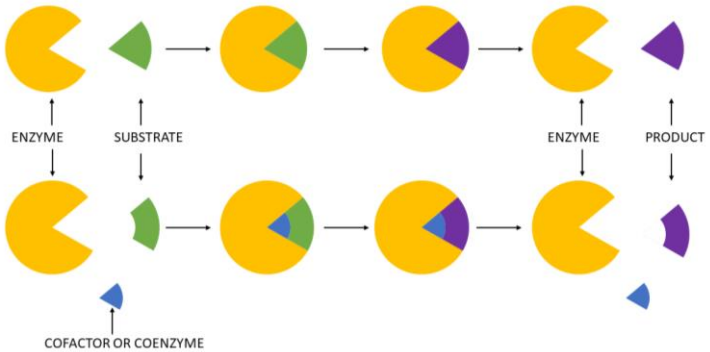

Figure 3. Enzyme-substrate transformation with and without additional cofactor or coenzyme

To sum up, there are many different forces that rule the interactions that conform an enzyme, and which will give its properties to interact with other molecules or surfaces, such interactions will be described below.

PROTEIN PROPERTIES	INTERACTION	SURFACE PROPERTIES
SIZE		TOPOGRAPHY
CHARGE		COMPOSITION
ACTIVITY		HYDROPHOBICITY
STABILITY		HETEROGENEITY (DOMAINS)
UNFOLDING RATE		ELECTRIC POTENTIAL

Figure 4. Protein and surface properties that affect interactions

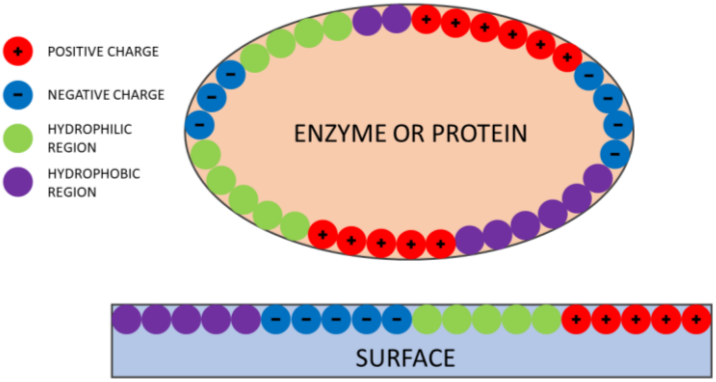

Figure 5. Protein and surface domains

3.1. INTRAMOLECULAR FORCES

Intramolecular forces are the ones that fold and maintain the stability of enzymes. Five main kind of interactions can be found inside a molecule: hydrophobic and hydrophilic interactions, disulfide and hydrogen bonds, and ionic bonds.

3.1.1. Hydrophobic interactions

Water molecules tend to form hydrogen bonds with each other (Section 3.1.3) but when a nonpolar molecule (e.g. alkanes, hydrocarbons, fluorocarbons, and inert atoms) or a vapor cavity comes into contact with water a hydrophobic effect is taken place. In this situation, no matter how the water molecule is rotated one of its tetrahedral charges is faced to the molecule or the cavity, what causes hydrogen bonds to break or to not form. If the molecule is small, in such situation the water molecules pack around without the necessity of losing hydrogen bond sites (Israelachvili, 2011).

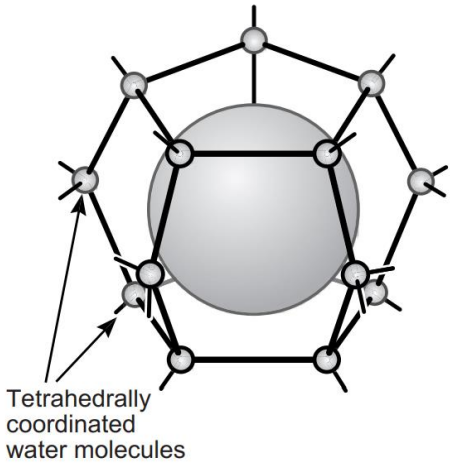

Figure 6. Water structure around a small hydrophobic molecule or vapor bubble (Israelachvili, 2011)

On the other hand, if the molecule is big enough or the water is faced to a vapor interface, the hydrogen bonds between water molecules bend, reorienting themselves in such a way that maximizes the number of hydrogen bonds (3 are the maximum hydrogen bonds that can be formed in this situation). Since hydrophobic surfaces are considered inert, the charge faced to the surface can be both positive or negative (Israelachvili, 2011).

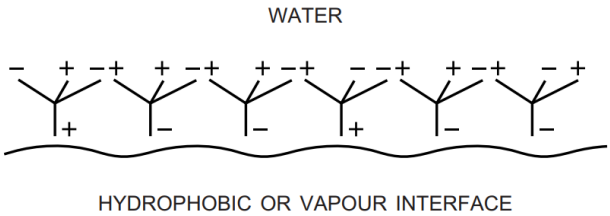

Figure 7. Water structure when faced to a hydrophobic or vapor interface (Israelachvili, 2011)

The above-mentioned phenomena are called hydrophobic solvation or hydrophobic hydration. To sum up, water molecules that are in contact with hydrophobic surfaces must rearrange their structure. The nature of this immiscibility is dependent on the corresponding surface free energy, which is related to the entropy of the system (Israelachvili, 2011).

Thus, hydrophobic interactions come from an entropic phenomenon, which comes from the reordering of hydrogen bonds between two hydrophobic species that come together, and not from any “hydrophobic bond”. Such interactions are of longer range than typical bonds. The hydrophobic pair-potential between two hydrophobic surfaces can be described by an exponential equation, proportional to the diameter of the hydrophobic molecules or group of molecules (Israelachvili, 2011).

Considering σ as the molecule or molecules diameter (in nm) and D_H as the characteristic hydrophobic decay length (around 1.0 nm) the energy equation is described as:

$$\omega_H(r) \approx -20 \cdot \sigma \cdot e^{-\frac{(r-\sigma)}{D_H}} \quad [kJ \cdot mol^{-1}] \qquad (2)$$

Thus, to obtain the free energy of dimerization for a given molecule with diameter σ in nm

$$r = \sigma \tag{3}$$

$$\Delta G_{dimer} = \omega_H(\sigma) \approx -20 \cdot \sigma \quad [kJ \cdot mol^{-1}] \tag{4}$$

For instance, the hydrophobic force that is necessary to separate two molecules that are in contact is given by

$$F_H(\sigma) = -\left(\frac{d\omega_H}{dr}\right)_{r=\sigma} \approx -3 \cdot 10^{-11} \cdot \sigma \quad [N] \; at \; 298 \; K \tag{5}$$

The range of interaction described by these equations is important to understand for example the dynamics of micelles and bilayers, hydrophobic aggregation, biological membranes, and more important for the purpose of this section protein folding (Israelachvili, 2011).

3.1.2. Hydrophilic interactions

Hydrophilic molecules and groups of molecules are water-soluble and repel each other in water medium. Often this kind of molecules are hygroscopic, which means that they retain moisture from the environment that they are in, that is why some polymers can form hydrogels when they get swollen by the water of the ambient. Hydrophilic molecules are not only polar or charged like hydrated ions and zwitterions (i.e., molecules that have formal charges but overall are neutral), respectively, since nonpolar and uncharged molecules can be hydrophilic if they can form hydrogen bonds with water and with the adequate geometry (e.g., alcohols and N atoms in amines). Some solid surfaces such as gold and chromium have hydrophilic characteristics. Other molecules like urea, even having nonionic character, are called chaotropic agents i.e., this kind of molecules tend to unfold proteins when they are in dissolved in water.(Israelachvili, 2011).

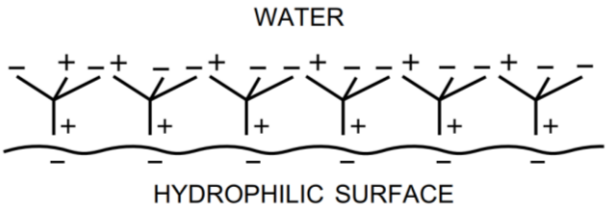

Figure 8. Water structure when faced to a hydrophilic surface must note how the charges are faced against contrary charges (2 negatives from the oxygen and 1 positive for every hydrogen) (Israelachvili, 2011)

In this case the surface and water are strongly and directionally bound, which was not the case in hydrophobic interactions, so the arrangement in one direction does not permit easy charge transportation. The bond that is formed adds a steric barrier to the molecule which causes an augmentation on the repulsion caused by the surface or molecule (Israelachvili, 2011).

The ordering of water molecules at surfaces may affect interaction forces, these parameters define the order of water molecules: positional ordering into layers, orientational ordering and mean density variations near the surface (Israelachvili, 2011).

To sum up, unlike hydrophobic interactions, hydrophilic molecules and surfaces interact with water forming bonds and are repulsive to another hydrophilic molecule or surface, this repulsion is further potentiated by the steric barrier that is formed by the layer of water.

3.1.3. Hydrogen and disulfide bonds

Hydrogen bonds are interactions between a hydrogen atom and electronegative atoms such as oxygen, nitrogen, and fluorine when the hydrogen atom is covalently bound to another atom that is similarly electronegative. This kind of bonds can occur intermolecularly and intramolecularly and its strength is both directional and distance dependent. Since hydrogen atoms are positively polarized and small, they can effectively bond two electronegative atoms, this interaction is accepted to be an electrostatic or a fairly strong dipole-dipole interaction. By

this means the hydrogen atom is shared in a way that it remains closer to the covalent bond. The directionality of this kind of bond permits the formation of weak three-dimensional structures in solids and in liquids longer range bonds are involved (Israelachvili, 2011).

The strength of most hydrogen bonds is around 5 to 10 times stronger than van der Waals bonds but around 10 to 20 times weaker than covalent or ionic bonds, so for 298 K hydrogen bonds strength per bond is 5-10 kT (where k is the Boltzmann constant and T is temperature). The strength of the bond tends to follow the $1/r^2$ distance dependence and is given by the dipole-dipole interaction equation:

$$\omega(r) = -Q_{H^+} \cdot u \cdot \frac{\cos(\theta)}{4 \cdot \pi \cdot \varepsilon_0 \cdot \varepsilon \cdot r^2} \; [J] \tag{6}$$

For example, for a water molecule model, the calculated strength of the water-water hydrogen bond is 4.9 kcal/mole that is equivalent to the stablished 4.5 kcal/mole from literature (Israelachvili, 2011).

Disulfide bridges work with similar mechanics as hydrogen bonds. The difference is denoted by the atoms that are involved in the bond. In the case of disulfide bridges the interaction is between two sulfur atoms.

Proteins usually use two sidechains (residue amino acids) cysteines to form disulfide bonds for the stabilization of the tertiary structure of the protein or to form intermolecular bonds, for example in immunoglobulins (Vanduijn *et al.*, 2022). This bond can be reduced using electrochemistry (Vanduijn *et al.*, 2022) or reducing agents can be used like dithiothreitol (DTT) which is a thiol-based agent or non-thiol-based agents like tris(2-carboxyethyl)phosphine (TCEP) to brake disulfide bonds, it has been observed that reduction of proteins such as lysosome and BSA can affect the aggregate formation depending on disulfide scrambling (Yang, Dutta and Tiwari, 2015)

Disulfide bonds from, for example cysteine, can also be reduced and re-bridged, process called disulfide stapling, next-generation stapling involves 2 in 1 reagent (e.g. dithioaryl(TCEP)pyridazinedione) that can reduce and re-bridge in one step (Lee *et al.*, 2016).

3.1.4. Ionic bonds

Ionic bonds are a kind of electrostatic interactions that are defined by the Coulomb laws that will be more detailed in section 5.1.1 Electrostatic forces. In general, is a force generated by interaction between charges that is dependent on $1/r^2$, permittivity of the media and ionic valency of interacting ions.

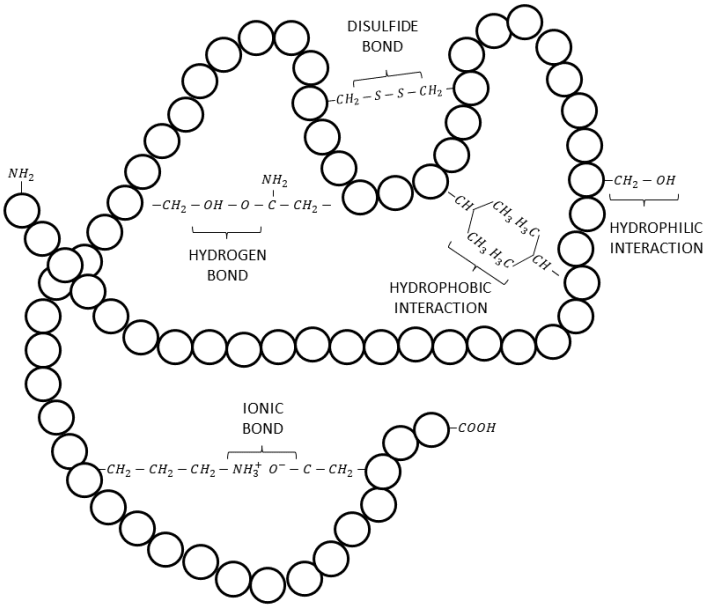

Figure 9. Different intramolecular interactions of an amino acid

4. PROTEIN THERMODYNAMICS AND KINETICS

Enzymes are powerful tools, the way that they transform substrate to product is very efficient, but how are enzymes formed is not understood in the sense that it is a very complex system with hundreds of interactions happening at the same time, thus is very important to investigate its kinetics and thermodynamics to achieve better use of enzymes.

4.1. KINETICS

Enzyme folding kinetics is not fully understood by researchers since the static ground state structures are not enough to determine the enzyme energetic trajectory that will define its final conformation, in other words, how enzymes are structured comes from a free energy landscape that researchers have not determined fully yet. Reactions are catalyzed by steps which are defined by the energy landscape (Villali and Kern, 2010). Static ground states are stationary states of lowest energy.

A kinetic model developed by (Rollins and Dill, 2014) dictates how the development of the native tertiary structure comes from the folding route of secondary structures. Meaning that enzymes are formed by little steps from the unfolded state until they achieve the native conformation. Predicts that the free energy landscape is only downhill for the native state step (i.e., also referred as folding funnel) and that folding is described as two-state kinetic model. Two-state kinetic refers to a simple reversible reaction between the unfolded and native conformation of an enzyme.

The folding funnel concept is introduced to explain the speed at which enzymes go from unfolded globular conformation to native globular conformation, since a plane like energy landscape would imply that jumps from step to step were random since the free energy change would be 0, meaning that the time that would take for an enzyme to adapt its native conformation wouldn't be as fast as the experimental time which is nanoseconds. Immobilization prevents the enzyme to directly contact the surface, thus evading its denaturation.

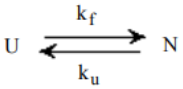

Figure 10. Two-state kinetic representation

$$\frac{d[U]}{dt} = -k_f[U] + k_u[N] \tag{7}$$

$$\frac{d[N]}{dt} = k_f[U] - k_u[N] \tag{8}$$

Equations 7 and 8 represent the rate of formation of each state (change of concentration over time). For equation 7 the rate is referred to the unfolded state and for the equation 8 is referred to the native state. The constants of the reaction in both directions are represented in figure 10.

Enzyme-substrate kinetics are defined by a series of steps that starts when the substrate enters the active site of the enzyme and ends when the product dissociates from the active site. It is important to understand that immobilization must not affect the active site by deforming it or blocking it, or the substrate might not be able to enter and react. Such reactions can be described by the Michaelis Menten equations.

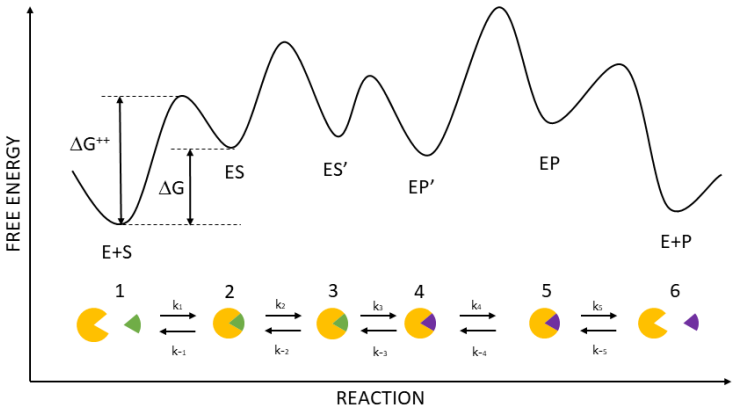

Figure 11. Representation of the steps involved in the substrate transformation to product. Step 1 the enzyme gets to the active site, step 2 there are conformational changes of the enzyme to fully adapt to the substrate, step 3 the chemical step, step 4 conformational changes of the enzyme, step 5 product dissociation. ΔG^{++} refers to the activation energy there is one for every transition but there is only one represented

4.2. THERMODYNAMICS

Thermodynamics explain the energy landscape of the enzyme folding process through the free energy changes of the process. Free energy is calculated by enthalpy “H” and entropy “S” and is dependent on the temperature “T”. Enthalpy decreases during protein folding since the internal energy decreases and entropy decreases too since the system is evolving to a more “ordered” state compared to the unfolded state.

$$\Delta G_{folding} = \left(H_{unfolded} - H_{folded}\right) - T\left(S_{unfolded} - S_{folded}\right) \quad (9)$$

When proteins fall in kinetic traps or local minima they might stop folding temporarily or permanently, thus misfolding can occur.

Enzyme stability is often defined by a parameter called Delta Delta G which is calculated as the difference in energy change of folded to unfolded states and the energy change when the enzyme suffers a modification or mutation.

$$\Delta\Delta G = \Delta G_{folding} - \Delta G_{folding(mutated)} \quad (10)$$

Mutations correspond to changes in the primary structure of a protein. For example, a point mutation indicates that one aminoacid in a given position within the protein is substituted by another one. Often, mutations trigger conformational changesor add/remove certein intramolecular interactions that alter protein stability/activity. For example, by introducing a cys group in the chain the molecule will be able to form disulfide bridges that before could not happen, disulfide bridges can be added for example to strengthen enzyme stability (they act as staples to increase rigibity within the protein). Thus, enzyme stability is also dependent on the functional groups that are attached to the enzyme, since those will change its conformation too. Multipoint covalent attachment has been studied as very interesting method of locking the enzyme conformation onto a surface to secure the enzymes stability thus reducing unfolding or leaching (Rodrigues *et al.*, 2021). The multipoint covalent attachment can be applied to a mutated enzyme with functional groups that the investigator added to it, so the attachment does not affect the active site of the enzyme.

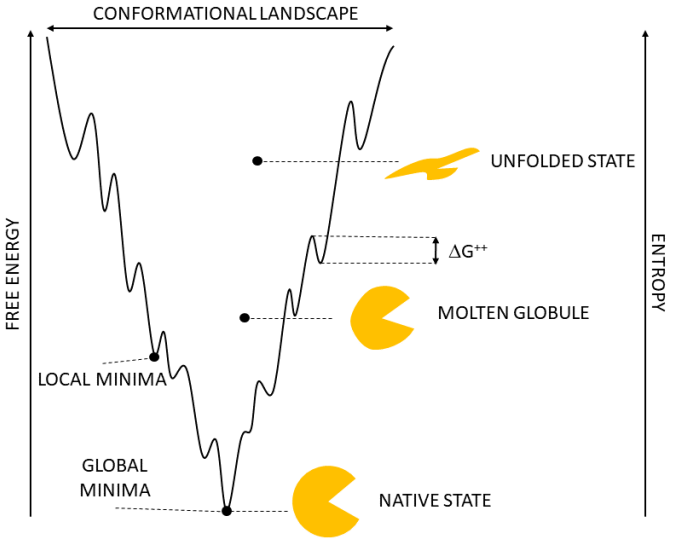

Figure 12. Representation of the energy landscape or folding funnel. There can be observed multiple local minima where if the enzyme falls in must overcome $\Delta G^{++}$to continue folding. Both free energy and entropy decay when diving into the folding funnel. Every enzyme has its conformational landscape, such as mutated enzymes have different landscape compared to the unmutated enzyme, the $\Delta\Delta G$ is calculated by the difference of the global minima of those two (enzyme and mutated enzyme)

5. IMMOBILIZATION

5.1. INTERMOLECULAR FORCES

Intermolecular forces are the ones that describe how a molecule will behave in reference to another molecule or surface.

5.1.1. Electrostatic forces

The force between two charged atoms or ions is much stronger than lots of chemical binding forces. Since electrostatic forces form physical bonds they are great holding molecules together with relatively long-range interactions when compared to chemical bonds. Electrostatic forces are basically defined by Coulomb forces, so the Coulomb laws are applied to these bonds (Israelachvili, 2011). The force that is applied on a charge Q_2 at a distance r by the field E generated from a charge Q_1 called Coulomb force or law is described by the next equation

$$F(r) = Q_2 \cdot E_1 = \frac{Q_1 \cdot Q_2}{4 \cdot \pi \cdot \varepsilon_0 \cdot \varepsilon \cdot \mathrm{r}^2} \quad [N] \tag{11}$$

By integrating the force between r and r = ∞ the free energy for the charge-charge interaction is given by

$$\omega(r) = \int_{\infty}^{r} \frac{Q_1 \cdot Q_2}{4 \cdot \pi \cdot \varepsilon_0 \cdot \varepsilon \cdot \mathrm{r}^2} = \frac{Q_1 \cdot Q_2}{4 \cdot \pi \cdot \varepsilon_0 \cdot \varepsilon \cdot \mathrm{r}} = \frac{z_1 \cdot z_2 \cdot e^2}{4 \cdot \pi \cdot \varepsilon_0 \cdot \varepsilon \cdot \mathrm{r}} \quad [J] \tag{12}$$

Both equations are r dependent the distance between ions center to the other. From these equations must be noted that the bigger the ionic valency of the molecules interacting the stronger the force at which they will interact. Also, the sign of the ions will determine if the force

is repulsive or attractive, a negative force or energy means attraction and a positive one means repulsion. For example, $z_1 = +1$ for Na^+ and $z_2 = -1$ for Cl^- from such interaction the resultant ω or F will be negative, thus an attractive interaction will occur. The media will also affect the Coulomb interaction since an increase in ε will cause a decrease in the force of interaction (Israelachvili, 2011).

Going back to the range of interaction may be interesting a comparison with the gravitational forces since those are r dependent too. Coulomb forces are by a factor of approximately 10^{43} stronger its strength starts to decay at about 56 nm range. This kind of interaction can be very complex to analyze when all electrostatic interactions are taken in consideration in a solution full of ions due to phenomenon's called field screening by moving charges in the media (Israelachvili, 2011).

When two charged surfaces with the same charge (positive or negative) are close an electric double-layer interaction occurs. This kind of interaction force decays exponentially with distance. Is somehow, like a fight between attraction and repulsion that is dependent on the distance that the two layers are. Three zones can be observed, when the distance is close to 0 the winner is the attraction force, as the layers are separated a maximum appears where the layers are repulsed, and when the distance is still growing the attraction force appears and tends asymptotically to a value. The interaction also depends on the geometry of the bodies that are interacting (Israelachvili, 2011).

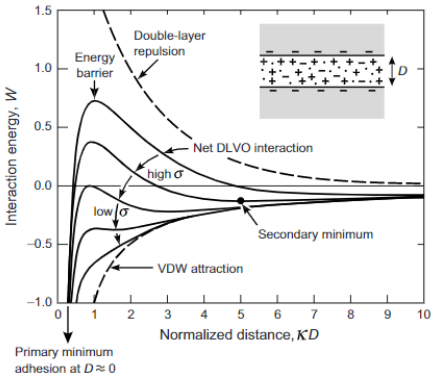

Figure 13. Representation of the different forces that repel or attract the two layers depending on the distance and the molecule radius (Israelachvili, 2011)

Geometry of bodies with surfaces D apart ($D \ll R$)		Electric 'Double-layer' Interaction	
		Energy, W	Force, $F = -dW/dD$
Two ions or small charged molecules	TWO IONS IN WATER	$\frac{+z_1 z_2 e^2}{4\pi\varepsilon_0\varepsilon r} \frac{e^{-\kappa(r-\sigma)}}{(1+\kappa\sigma)}$	$\frac{+z_1 z_2 e^2}{4\pi\varepsilon_0\varepsilon r^2} \frac{(1+\kappa r)}{(1+\kappa\sigma)} e^{-\kappa(r-\sigma)}$
Two flat surfaces (per unit area)	TWO FLAT SURFACES	$W_{Flat} = (\kappa / 2\pi) Z e^{-\kappa D}$	$(\kappa^2 / 2\pi) Z e^{-\kappa D}$
Two spheres or macromolecules of radii R_1 and R_2	TWO SPHERES	$\left(\frac{R_1 R_2}{R_1 + R_2}\right) Z e^{-\kappa D}$	$\kappa \left(\frac{R_1 R_2}{R_1 + R_2}\right) Z e^{-\kappa D}$ Also $F = 2\pi \left(\frac{R_1 R_2}{R_1 + R_2}\right) W_{Flat}$
Sphere or macro-molecule of radius R near a flat surface	SPHERE ON FLAT	$RZe^{-\kappa D}$	$\kappa R Z e^{-\kappa D}$ Also $F = 2\pi R W_{Flat}$
Two parallel cylinders or rods of radii R_1 and R_2 (per unit length)	TWO PARALLEL CYLINDERS	$\frac{\kappa^{-1/2}}{\sqrt{2\pi}} \left(\frac{R_1 R_2}{R_1 + R_2}\right)^{1/2} Z e^{-\kappa D}$	$\frac{\kappa^{3/2}}{\sqrt{2\pi}} \left(\frac{R_1 R_2}{R_1 + R_2}\right)^{1/2} Z e^{-\kappa D}$
Cylinder of radius R near a flat surface (per unit length)	CYLINDER ON FLAT	$\kappa^{-1/2} \sqrt{\frac{R}{2\pi}} Z e^{-\kappa D}$	$\kappa^{3/2} \sqrt{\frac{R}{2\pi}} Z e^{-\kappa D}$
Two cylinders or filaments of radii R_1 and R_2 crossed at 90°	CROSSED CYLINDERS	$\sqrt{R_1 R_2} Z e^{-\kappa D}$	$\kappa \sqrt{R_1 R_2} Z e^{-\kappa D}$ Also $F = 2\pi \sqrt{R_1 R_2} W_{Flat}$

Figure 14. Equations for different geometries of surfaces of the double-layer interaction (Israelachvili, 2011)

5.1.2. Covalent bonds

Covalent bond is a kind of chemical reaction that is characterized by the sharing of electrons between atoms. Covalent bonds are directional and may have rotational freedom depending on single, double, or triple bonds are formed, the first being the only one that is not fixed in a specific position and angle. The rotation has relevant effects on the molecular and macromolecular stability. The range of covalent forces is of the order 0.1 – 0.2 nm and the strength can vary from 200 – 800 kJ/mol (Israelachvili, 2011). Covalent bonds are the strongest and longest-lived bonds, ofter with an irreversible character.

5.1.3. Van Der Waals forces

Van der Waals forces are always present independently of the properties of any given molecule. Such is the importance of VDW forces that they play roles in phenomena like adhesion, surface tension, physical adsorption, protein and polymeric structures and more areas. Their features can be summarized as: long-range forces (10 nm or greater), repulsive or attractive that do not follow a power law, tendency to orient or align molecules and not additive which means that other close bodies effect con the VDW forces (Israelachvili, 2011).

McLachan's equation for the van der Waals free energy between two molecules is given as:

$$\omega(r) = \frac{C_{VDW}}{r^6} = \frac{6kT}{(4\pi\varepsilon_0)^2 r^6} \sum_{n}^{\infty} \frac{\alpha_1(i\nu_n)\alpha_2(i\nu_n)}{\varepsilon_3^2(i\nu_n)} \qquad (13)$$

Where $\alpha_n i\nu_n$ are the polarizabilities of the molecules that are interacting and $\varepsilon_3^2(i\nu_n)$ is the dielectric permittivity of the medium that the molecules are in, and $(i\nu_n)$ the imaginary frequency.

5.1.4. Repulsive forces

Repulsive forces arise when two molecules are getting close together. Three potentials are described commonly the hard sphere, the inverse power-law and the exponential potentials (Israelachvili, 2011). Steric and overlap forces are also repulsive forces that appear when two

surfaces covered with polymers or one of them covered get close to each other and start overlapping.

5.1.4.1. Steric and overlap forces

As said before steric or overlap forces appear when two surfaces get close enough, this interaction is closely related to the osmotic pressure. When the chains that are between the surfaces start compressing the unfavorable entropy associated with the system makes them to interact repulsively. This interaction can be favorable to evade for example the formation of colloids in a medium by adding polymer additives (Israelachvili, 2011).

The repulsive energy for two surfaces at distance D, where Γ is the number of grafted chains per unit area and Rg the radius of gyration:

$$W(D) = 36\Gamma kTe^{-\frac{D}{Rg}} \left[\frac{J}{m^2}\right] \tag{14}$$

$$W(D) = 36kTe^{-\frac{D}{Rg}} \left[J\ per\ molecule\right] \tag{15}$$

The pressure's equation:

$$P(D) = 36\left(\frac{\Gamma kT}{Rg}\right)e^{-\frac{D}{Rg}} \left[\frac{N}{m^2}\right] \tag{16}$$

The radius of gyration can be of a protein or a polymer and indicates the distance from the axis of rotation where all the mass can be assumed to be concentrated.

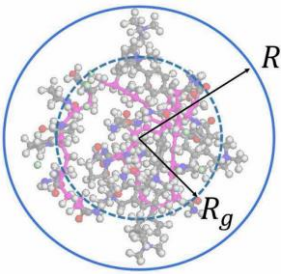

Figure 15. Radius of gyration (Vega-Paz *et al.*, 2019)

5.2. IMMOBILIZATION TECHNIQUES

The most used methods are based on physical immobilization (carrier-bound attachment and encapsulation or physical entrapment) and chemical immobilization (covalent binding and cross linking)(Chapman, Ismail and Dinu, 2018).

Immobilization onto a material with desired properties has been shown to give biocatalysts higher stability under a broad range of operating conditions. The additional functionality of the enzymes comes from both the method of immobilization as well as the properties of the material used to immobilize them (Chapman, Ismail and Dinu, 2018). The benefit of immobilizing enzymes resides on techniques that allow the recovery of enzymes and the additional stability and functionality without affecting the activity and selectivity (Mohamad *et al.*, 2015).

To characterize the immobilization effects two kinetic parameters are used the Michaelis constant and the maximal reaction velocity (Km and Vmax respectively). Km gives the rate of substrate to enzyme binding respective to dissociation, smaller Km values suggest higher enzyme-substrate affinity. On the other hand, Vmax measures the rate at which and enzyme converts substrate to product, it is a way to quantify the catalytic activity(Chapman, Ismail and Dinu, 2018).

5.2.1. Carrier-bound enzyme immobilization through both physical and chemical binding

Carrier-bound enzyme immobilization is defined by the attachment of the biocatalyst onto a solid material. The immobilization method is selected based on optimizing catalytic performance. The two common methods of carrier-bound enzyme immobilization are physisorption and chemisorption (Chapman, Ismail and Dinu, 2018).

5.2.1.1. Physical adsorption (physisorption)

Physical adsorption offers a facile non-site-specific bonding the opposing party of covalent bonding. The bonds that are formed with the support are weak, those can be van der Waals, hydrophobic interactions, and hydrogen bonds. This kind of bonding is reversible which is very useful for the recovery of the support when the enzymes activity has been decayed and for it to be attached to new fresh enzymes (Chapman, Ismail and Dinu, 2018).

There are two ways of immobilizing an enzyme by this method, on one hand, the enzyme can be soaked into the support and be immobilized after a period of incubation, and on the other hand is allowing a solution of enzymes to try on the electrode surfaces and then clean away the enzymes that hasn't been attached to it (Mohamad *et al.*, 2015).

There are a wide variety of organic and inorganic supports such as ceramics, metal oxides, nanomaterials, and polymers but the investigation of such immobilization method has concluded to limitations in enzyme stability which means enzyme leaching and a decrease in catalytic efficiency. The stability is also dependent on the pH of the solution since the isoelectric point might bear changes by modeling systems the charge distribution can be defined (Chapman, Ismail and Dinu, 2018).

This kind of immobilization can be exploited by the principle of affinity binding. Control of the orientation of the immobilized enzyme and minimal changes in its tertiary structure are key for high retention of the enzyme and catalytic activity enhancement. Either is achieved by the support pre-coupled to an affinity ligand or the enzyme is conjugated to an entity that has affinity to the support, for example, the binding between polyhistidine tag and metal ions, lectins and free saccharidic chains or glycosylated macromolecules, avidin and biotin, etc. (Mohamad *et al.*, 2015).

Entropically driven hydrophobic interactions are also used for binding enzymes to supports. When an enzyme displaces many water molecules from the surface both the support and its own, results in entropy gain that produces hydrophobic interactions between them (Mohamad *et al.*, 2015).

The strength of the hydrophobic interactions depends on hydrophobicity of the adsorbent and the enzyme, this interaction is regulated by both size of the ligand and the degree of substitution of the support. These interactions can be adjusted by modulating pH, temperature, and concentration of the system during immobilization (Mohamad *et al.*, 2015).

5.2.1.2. Covalent bonding (chemisorption)

Covalent bonding is widely used for irreversible enzyme immobilization. Immobilization via covalent attachment was shown to offer a stronger chemical bonding that significantly reduces enzyme leaching and further preservation of enzyme active sites (Mohamad *et al.*, 2015).

The functional group that takes part in the binding usually involves side chains of cysteine's thiol group, lysine's e-amino group, and aspartic and glutamic acids carboxylic, imidazole and phenolic groups. Since those are not essential for the catalytic activity of the enzyme (Mohamad *et al.*, 2015).

Covalent binding methods are however more intensive and chemically aggressive than physical adsorption, often requiring activation steps capable of denaturing enzymes (Chapman, Ismail and Dinu, 2018).

The selection must be in a way that ensures the enzyme to keep its catalytic activity, by not affecting on the active site amino acids, and if possible, to ensure an increase of efficiency (Mohamad *et al.*, 2015).

There are two ways of coupling the enzyme to the support, by modifying the support matrix to generate activated groups or they can be added without the use of biotechnological equipment, in both cases the electrophilic groups on the group that are generated will react with strong nucleophiles on the enzyme (Mohamad *et al.*, 2015).

5.2.2. Enzyme entrapment

Entrapment consists of immobilizing a biocatalyst into carriers with a particular porosity and permeability, it is an irreversible method of immobilization. The stability obtained by immobilizing via entrapment is higher due to better control of the microenvironment that surrounds enzymes, also the enzyme does not chemically react with the support, so denaturation is usually avoided in that sense. The control of the microenvironment is dedicated to optimizing the pH, polarity or amphilicity (Mohamad *et al.*, 2015; Chapman, Ismail and Dinu, 2018).

Also, this kind of immobilization technique shows a better catalytic activity on enzymes at higher temperatures in organic solvents, and easier separation of the substrate-product mixture. The enzymes are entrapped in a support or inside of fibers(Mohamad *et al.*, 2015). There are a variety of carriers such as, nanomaterials, hydrogels, polymers, and many others. They have been researched for novel biosensing systems and organic compounds synthesis. Entrapped enzymes have been reported to be able to be locked in more catalytically active conformations which directly affect the enzyme efficiency (Mohamad *et al.*, 2015; Chapman, Ismail and Dinu, 2018).

5.2.3. Cross-linked enzyme aggregates (CLEA)

Enzyme immobilization via the formation of CLEAs is a new class of irreversible immobilization technique that has been strongly researched for industrial biotransformation of fine chemicals and pharmaceuticals, the method is also called carrier-free immobilization since the enzyme is its own carrier. This way it's a virtually pure enzyme without the disadvantages and advantages that are associated with carriers (Mohamad *et al.*, 2015; Chapman, Ismail and Dinu, 2018) .

The preparation of CLEAs via the aggregation of a soluble enzyme using a precipitating reagent like acetone, ethanol or tert-butanol followed by the copolymerization of enzyme aggregates with a cross-linking agent like glutaraldehyde. Each precipitating and cross-linking agents must be carefully optimized for every biocatalyst, since aggregate cross-linking is not a universal technique, the immobilization must be in a way that does not effect on the enzyme activity (Chapman, Ismail and Dinu, 2018).

If CLEA technique is prepared correctly it has been shown that the immobilized catalyst's operational life and stability is increased, so the leaching of the enzyme is reduced. CLEAs are

shown to hyperactivate enzymes since enzymes are aggregated in a preorganized tertiary structure that renders them in a permanent insolubility form (Chapman, Ismail and Dinu, 2018).

CLEA technology must be better defined for continuous processes separation and improve its mechanical properties, but in general is an inexpensive immobilization method with high catalytic productivity (Chapman, Ismail and Dinu, 2018).

Table 1. Immobilization techniques and examples of enzyme-substrate attachment

Substrate/Support	Enzyme	Immobilization technique	Reference
Silica nanoparticles	Lipase	Covalent bonding	(Ali *et al.*, 2016)
Nanoporous rice husk	Oxylipin	Covalent bonding	(Le *et al.*, 2018)
COF-coated magnetic graphene	Trypsin	Covalent bonding	(Wang *et al.*, 2017)
Titania membranes	Laccase	Covalent bonding	(Abejón *et al.*, 2015)
Zirconia ceramic capillary membranes	Protease	Covalent bonding	(Tajiri and Al-Qureshi, 2020)
Halloysite	Alkaline phosphatase	Adsorption	(Atyaksheva *et al.*, 2021)
Silica and zirconia	Lipase from Candida rugosa	Adsorption	(Izrael Živković *et al.*, 2015)
Hematite	b-glucosidase	Adsorption	(Zang *et al.*, 2020)
Wet ground corncob residues	Cellulase	Adsorption	(Liu *et al.*, 2020)
Myristyl-	Peroxidase	Entrapment	(Falcone *et al.*, 2019)

phenylalanine hydrogel			
Myristyl-phenylalanine hydrogel	a-amylase	Entrapment	(Falcone *et al.*, 2019)
Latex	b-glucosidase	Entrapment	(Javed *et al.*, 2016)
Agar-agar	Protease	Entrapment	(Sattar, Aman and Qader, 2018)
Lipase	Lipase	CLEA	(Guajardo, Ahumada and Domínguez de María, 2021)
Laccase	Laccase	CLEA	(Nguyen, Seow and Yang, 2017)
Glucoamylase	Glucoamylase	CLEA	(Nadar and Rathod, 2016)
Xylanase	Xylanase	CLEA	(Abdul Wahab *et al.*, 2019)

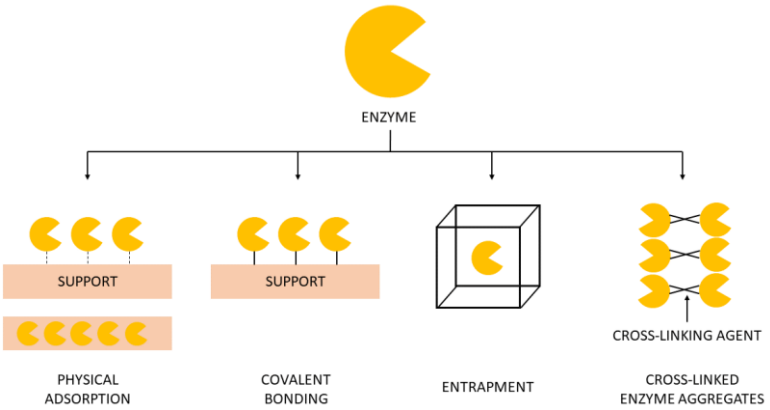

Figure 16. Graphic visualization of the general immobilization techniques

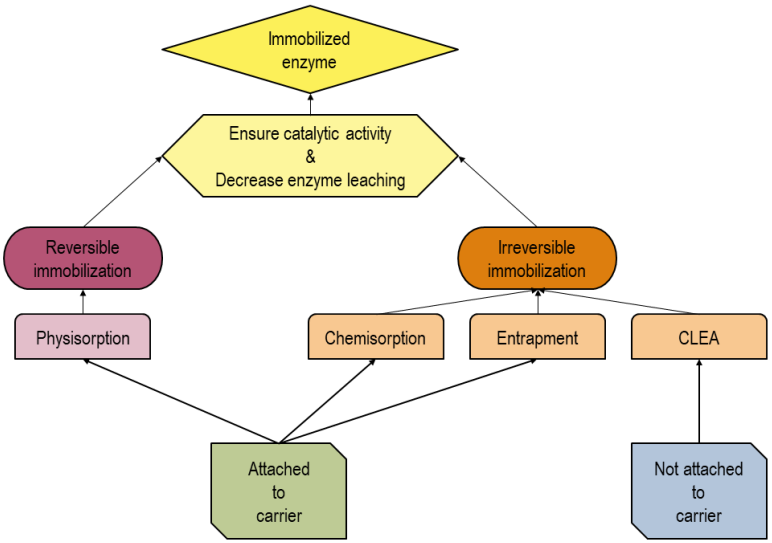

Figure 17. Diagram of immobilization techniques reversibility differentiation

6. IMMOBILIZATION SURFACES

The support that is selected to immobilize an enzyme is key for good results in this practice. Since the properties of the surface both chemical and physical will cause interactions with the protein that will be attached to it (Mohamad *et al.*, 2015). (Faulón Marruecos, Schwartz and Kaar, 2018) studied how there is a correlation between surface properties and enzyme unfolding, also how environmental changes effect on enzyme stability. Thus, the selection of an ideal carrier is a complex activity. Some supports are designed to offer better results of the catalytical activity of an enzyme, as happens with immobilization technologies (Mohamad *et al.*, 2015).

As (Mohamad *et al.*, 2015) indicates, there are some general properties that are important for the selection of the carrier, those are hydrophobic characteristics, inertness (i.e., they must not interact with the functional groups that are important in the catalytical activities), biocompatibility, resistance to microbial attack, resistance towards compression, high surface area or more contact sites between support-enzyme (e.g., mesoporous structures), and economically accessible. Therefore in (*Protein-Surface Interactions*, no date),suggests that the properties to take in consideration when selecting an immobilization surface are the geometric, chemical, and electrical properties.

Surface geometry is referred to more surface area which means more interactions with proteins, for example a surface with pores, grooves or machine marks will add more area to a surface. The chemical composition of a surface will determine which functional species are available for interaction with biomolecules. Functional species, such as amino, carbonyl, carboxyl, and aromatic groups, can be present on polymeric biomaterials or passivated metallic surfaces that expose metal and oxygen ions add more interaction possibilities. On a microscopic scale, patches, or domains, of different functionality can exist on a surface which can affect positively or negatively depending on what enzyme is going to be attached to them and what kind of interaction can be determinant during the immobilization. The surface potential influences the structure and composition of the solution adjacent to its which consecutively

changes the interaction forces that bond both surface and enzyme, those forces can be both enhanced or hindered depending on the distribution of potential (*Protein-Surface Interactions*, no date).

6.1. SURFACES

6.1.1. Polysaccharides

They are low cost and widely available. Prominent materials are alginates, chitosan, cellulose, agarose, guar gum, agar, carrageenan, gelatin, dextran, xanthan, and pectins. In the study of polysaccharides as enzymes raised the potential for the development of super-biocatalysts. This can be used to make a bioprocess in one step, or it can be used for a sequence of chain reactions. That is all because multiples enzymes are loaded onto a single support (Federsel, Moody and Taylor, 2021).

6.1.1.1. Agarose

Agar or agar-agar is extracted from Rhodophyceae class red seaweeds and is generally used in the food industry as an additive and in microbial cultures, its two main components are agarose and agaropectin. Generally, agarose must be de-esterified before it's used, to free every hydroxyl group, four per agarobiose (i.e., the repeating disaccharide units from the agarose structure are called agarobiose). In some cases, the hydrogen bonds that the hydroxyl groups can form are not strong enough for high temperature procedures, so cross-linking might be necessary. Agarose's hydrophilic nature, structural invariance after solvent changes, chemical stability, biological strength, possibility of co-immobilization and low cost makes them versatile immobilization surfaces (Zucca, Fernandez-Lafuente and Sanjust, 2016).

6.1.1.2. Chitosan

Chitosan is a biopolymer derived from waste crustacean shells or mushrooms and other fungi. Since it is a byproduct implies that its available in a large scale and its low cost. Chitosan has biodegradable, non-toxic, bio-adhesive. Entraps bioactive biomolecules through chemical cross-linking, ionic cross-linking, and ionic complexation. Electrospinning is now being used to

make thin mats of chitosan with good mechanical stability, the advantages of such mats include high surface-to-volume ratio, high porosity, and high mass transfer. Usually, its crosslinked with glutaraldehyde functionalized with glucose oxidase. It also forms mats that exhibit antimicrobial properties by generating hydrogen peroxide so it can be used for wound-care applications (Federsel, Moody and Taylor, 2021).

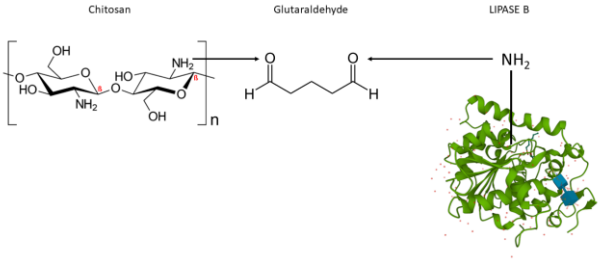

Figure 18. Chitosan and Lipase B attached via Glutaraldehyde as a cross-linking agent

6.1.2. DNA

DNA a promising substrate for enzyme immobilization. The advantages of this support are the formation of multiple enzyme-DNA conjugates and a high spatial precision, and the phenomena such as substrate channeling that allows the product of an enzyme reaction to be efficiently delivered to the active site of the next enzyme in sequence (Federsel, Moody and Taylor, 2021).

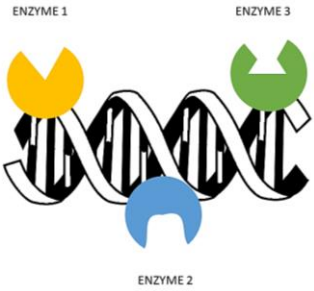

Figure 19. General representation of how different enzymes can be attached via DNA to catalyze a substrate in a chain reaction

6.1.3. Metal-organic frameworks

Metal–organic frameworks (MOFs) are made of metal ions or clusters linked by organic ligands what makes them highly crystalline porous material with tunable porosity and high surface area also with high thermal stability. The functionality of MOFs is designable which means they can be used in lots of manners. The adjustable properties of MOFs, make it feasible to optimize them for specific enzymes. MOF structure can tightly confine enzymes to give them an extremely high stability (Federsel, Moody and Taylor, 2021).

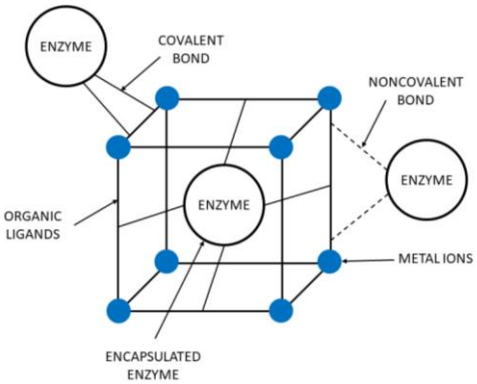

Figure 20. MOF representation and different ways enzymes can interact with it

6.1.4. Controlled pore glass

Controlled pore glass is well known for enzyme immobilization. EziG is an immobilization technology that can be used for a vast enzyme type. It is a material based on controlled pore glass, coated with an organic polymer, and chelated with a metal ion. The most common ions used are Fe(III), Ni(II) or Co(II), nowadays it's known that Fe(III) gives a stronger bond that results in no leaching. The enzyme is attached via His-tag binding (oligomeric his6-homopeptide) which is a residue structure of enzymes. Because of interconnecting pores and selective and non-destructive binding by His-tags, a high enzyme mass loading can be archived effectively with diffusion limitation and deactivation which is translated to gain of activity (Federsel, Moody and Taylor, 2021).

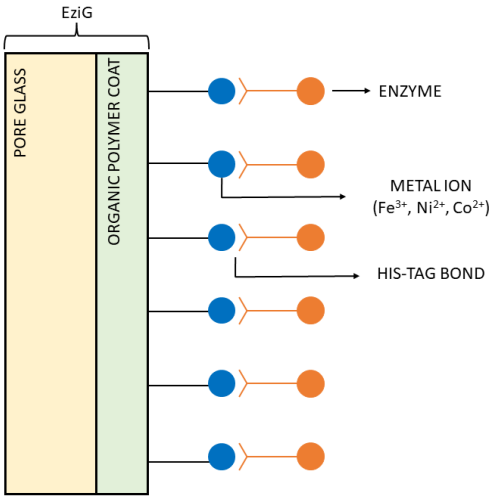

Figure 21. Controlled pore glass with EziG technology

6.1.5. Magnetic nanoparticles

The large surface area, surface to volume ratio and high mass transfer capacity, makes magnetic nanoparticles (MNP) as an interesting surface for enzyme immobilization. The most common type of MNP is iron oxide due to high biocompatibility, nontoxicity, and binding capabilities to enzymes. One of the best characteristics of MNPs as substrate is the ease with

which it can be recovered from the media by applying magnetic fields (Federsel, Moody and Taylor, 2021).

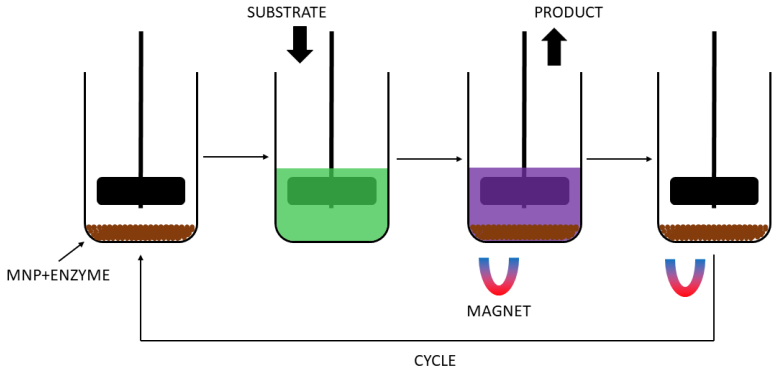

Figure 22. Cycle of a MNP + Enzyme system in a stirred-tank reactor, using a magnet to recover the enzyme-MNP

6.1.6. Polymer brushes

A recent type of immobilization surfaces is a kind of polymeric coating that is applied onto a substrate in a way that the polymer chains have one end bonded to the surface and the rest of the chain is forced to point away from the surface forming a brush conformation. Since the polymers have lots of functional groups, they can be ideal for enzyme immobilization. Enzymes' isoelectric point gives the opportunity of changing the media pH to produce charges on the enzyme surface to endure electrostatic adsorption mechanisms onto polyelectrolyte brushes (Koenig *et al.*, 2016).

There are two grafting techniques studied for attaching the polymers to the surface, "grafting from", and "grafting to". Grafting from method, is characterized by an initiator monomer being attached on the surface and through polymerization methods such as ATRP, NMP, and RAFT, more monomers are attached until the polymer is formed, thus cuprous catalysts or harsh

chemicals are needed on the process which can be dangerous when used for example in food industries. On the other hand, the grafting to method directly covalently attaches polymers to a surface forming the brush, but the drawback is that through this method the patterns of polymers are not as dense and controlled as the brushes formed through the grafting from method (Chen *et al.*, 2017).

The stress generated by the stretching of the polymer chains of the brush might cause mechanochemical effects that can affect the enzyme addition to the brush (Chen *et al.*, 2017).

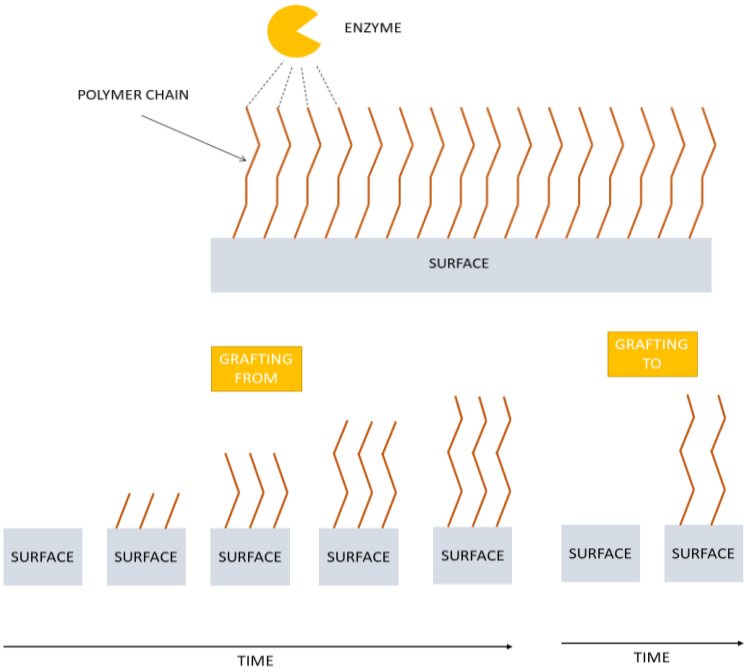

Figure 23. First general view of a polymer brush, second the two grafting methods side by side (grafting from left and grafting to right)

6.1.7. Renewables

There is a large availability of renewable materials that come from waste of other processes, and because of that condition they are economically favorable to be used. These materials may contain different chemical groups that can be used for enzyme binding or high surface area and porosity which also can give the possibility of immobilizing enzymes onto them. Such materials have to be further researched, here are some examples of substrates that can be used: coconut fiber, rice husk ash, chicken eggshells, lignocellulosic waste and biochar (Federsel, Moody and Taylor, 2021).

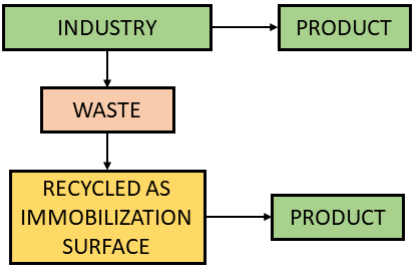

Figure 24. The waste of a process can be recycled as surfaces for immobilization

7. BENEFITS OF ENZYME IMMOBILIZATION

Enzymes as seen are very effective catalysts in mild conditions, which are selective and specific so can act in a wide variety of reactions. Enzymatic large-scale processes have demonstrated lower energy demands which translates to cost reductions and because of the catalytic characteristics they are less time consuming (Zucca, Fernandez-Lafuente and Sanjust, 2016). The implementation of enzyme immobilization comes from the idea of recovering the catalyst after the reaction (Zucca, Fernandez-Lafuente and Sanjust, 2016), the application of such technology brings additional costs and profitability seem to be the main factor when designing an industrial processes (Chapman, Ismail and Dinu, 2018). To give an example the production of HFCS (high-fructose corn syrup) has been observed to be economically more efficient to be obtained by the use of immobilized enzymes than by conventional chemical methods, putting HFCS production as the largest industrial process to apply immobilized enzymes producing about 10 million tons per year (Chapman, Ismail and Dinu, 2018).

The use of immobilization technology besides being economically driven effects on other parameters of the process that must be aware of. A drawback of immobilization is the reduction of mass transfer since the support material can affect the amount of reactive that can interact with the enzyme which does not happen with homogeneous enzyme in the reaction media. For its use, techniques and novel support materials must be developed as seen before that might change the enzyme kinetics so new models to understand those have to be developed too (Eş, Vieira and Amaral, 2015).

To study the environmental and economic impact of the implementation of immobilized catalysis on an industry two parameters must be analyzed, LCAs and TEAs.

LCAs meaning life-cycle assessment, is used to identify process materials and energy requirements, as well as waste and emissions. Usually, enzyme use is considered as a clean and energy saving method to draw upon. Since less waste is produced is more beneficial for the environment. On the other hand, TEAs meaning techno-economic analyses gives an understanding of the economic viability of a process and the process economics, which LCAs

does not take in account. Thus, by combining LCAs and TEAs the scale-up of immobilization enzyme technology applied on a process can be diagnosed by both economic and environmental point of view (Eş, Vieira and Amaral, 2015).

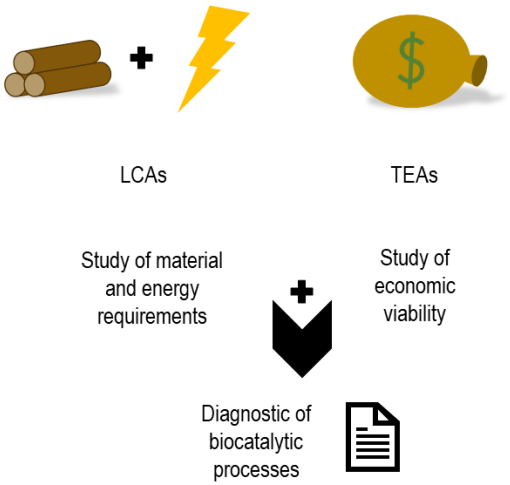

Figure 25. material and economical diagnostic of a biocatalytic process through LCA and TEA parameters

Table 2. Advantaged and disadvantages of biocatalytic processes

ADVANTAGES	DISADVANTAGES
Higher stability of the enzyme	Additional costs for designing the process
Easy downstream processing	Limitations on mass transfer
Catalyst can be recovered and reused	Possibility of enzyme activity loss
Better application of enzymes in continuous processes	The immobilization process can be time consuming and complex
Minimizes product contamination	
The use of the catalyst improves	

productivity

Overall cost reduction

Environmentally friendly processes

Reduction in energy use

Table 3. Advantages and disadvantages of the immobilization techniques

Immobilization technique	Advantages	Disadvantages
Physical adsorption	Simple and cheap technology to obtain high catalytic activity, without the need of changing the conformation of the enzyme.	The weak bonds that are formed might cause desorption and can generate stability problems to the enzyme.
Covalent Binding	Very strong binding that provides high stability to the enzyme, positions the enzyme in desired conformations for better contact with the substrate.	Mobility limitations might cause the decrease of the enzyme's activity since the bond is irreversible the support material cannot be renewed.
Entrapment/ Encapsulation	The enzyme is protected by a structure and can be easily applied on a continuous regime process since facilitates downstream processing.	The technology is limited by the mass transfer of substrate and a maximum loading of enzymes in structures.
CLEA	Provides strong bonds that benefit enzyme stability and a decrease in desorption.	Theres the probability of altering the active site and losing enzyme activity, also can generate diffusion limitations of the substrate.

To sum up, enzyme immobilization is in general a good technology to apply in modern processes, almost all the advantages are related to cost reduction because of the reuse of the enzymes or because the energetic need of an enzyme-based transformation is lower. Also, the recovery of enzyme and product separately is much easier when the enzyme is insoluble, which benefits the environment since less resources will be needed in the downstream processes and less harsh products will be used in the production step. However, it must be investigated for further application in bigger scales without the drawbacks of designing costs and the time consumption of immobilization.

8. INDUSTRIAL APPLICATIONS OF ENZYMES

As seen, enzyme immobilization can be very advantageous to use but it is not in its final form, since more investigation must be done. In the industrial sector, immobilization is getting attention because of the environmental and economic benefits that it provides.

8.1. ENZYMATIC REACTORS

Before talking about enzyme immobilization application in different industrial sectors, it can be helpful to see what kind of reactors are the most used with enzyme immobilization technology. The factors to keep in mind while selecting the enzyme reactor for the process are: the enzyme state (immobilized or free), the properties of the substrate since it can be soluble or insoluble, reaction requirements such as pH, temperature, gas production, and enzyme stability; and the economic cost determined by the full process cost (Zhang and Xing, 2019).

Table 4. Typical reactors in biocatalytic processes and examples

Reactor	Operating principle	Examples
Stirred-tank reactor (STR)	Enzymes are introduced in a tank which has stirring, pH and temperature control. Can be operated in two ways, in batch operation (BSTR) the produced amount is small, but the reactor is simple, in continuous (CSTR) operation the production is more efficient but more complex than batch operation. Usually followed by a filter to recover the enzyme, it is recommended the use of MNPs for easier recovery of the immobilized enzyme through magnetic fields (Zhang and Xing, 2019).	Cyclodextrin production: CGTase entrapped in alginate-gelatin gel beads (CSTR) (Rakmai and Cheirsilp, 2016). Ester synthesis: lipase from Burkholderia 47 epacian covalently bond to a magnetic-polymer composite

		(BSTR) (Mijone *et al.*, 2020)
Packed-bed reactor (PBR)	PBR is based on a tube filled with immobilized enzymes, through which the substrate enters from one side and is extracted as a product from the other side of the tube. The continuous PBR is preferred since it offers stable and low-cost operation conditions (Zhang and Xing, 2019).	Biodiesel synthesis: lipase from Pseudomonas cepacian adsorbed on sodium alginate (Kumar *et al.*, 2020) Juice production: pectinase entrapped in alginate beads (de Oliveira *et al.*, 2018)
Fluidized-bed reactor (FBR)	In this kind of reactor, the substrate is introduced with enough velocity to lift the immobilized enzyme particles thus fluidizing the bed, ending up with a reactor that combines the performance of CSTR and PBR. The feature that stands out of FBR is the excellent control of heat and mass transfer (Zhang and Xing, 2019).	Biolubricant production: lipase from Candida rugosa covalently bonded to MNPs (Hajar and Vahabzadeh, 2016) Juice production: Novozym 33095 crosslinked to chitosan beads with glutaraldehyde (Dal Magro *et al.*, 2021)

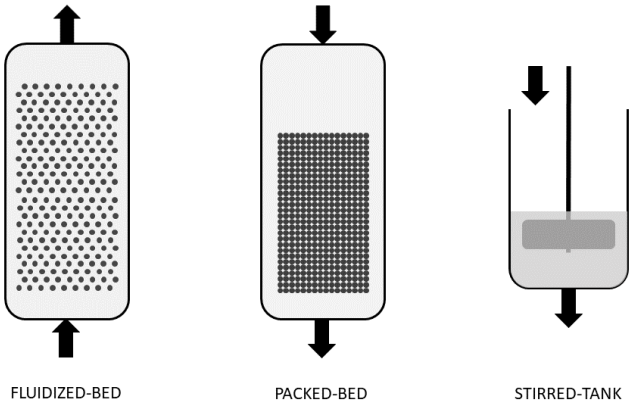

Figure 26. FBR, PBR, and STR from left to right

8.2. INDUSTRIAL SECTORS

The sustainability, low waste production and milder work conditions are some of the advantages and benefits that immobilized enzymes provide to the industrial processes (Basso and Serban, 2019), but first catalytic efficiency and stability to high temperature and organic solvent environments, that are usual in industrial processes, must be investigated to make it feasible (Choi, Han and Kim, 2015). Here below, some sectors that apply industrial catalysis will be described with some examples of biocatalysts that are being used.

8.2.1. Pharmaceutical industry

The production of pharmaceuticals often requires the introduction of some groups and the removal of undesired groups from the active center, the compounds are regio-, enantio-, and chemoselective which can be produced using immobilized enzymes since by traditional chemistry, it would require much more effort to obtain the same yield (Basso and Serban, 2019). Using enzymes' selective properties that can be solved and in addition to that the production steps do not require unsafe and harsh chemicals or high-temperature conditions (Chapman,

Ismail and Dinu, 2018). Also, the use of enzymes makes the production much greener and low-cost reducing steps in the process (Choi, Han and Kim, 2015).

8.2.2. Food industry

For instance, chemical routes usually produce side toxic products, consequently its use in the food industry is much more unsafe, biocatalyst offer a solution to that since they produce much cleaner products and does not need harsh chemicals, also the use of immobilized enzymes can even simplify the process by reducing the number of steps needed (Chapman, Ismail and Dinu, 2018). In the case of food production, the immobilized enzyme must be cheaper than in the pharmaceuticals production, and the production scale is much larger thus, the production usually is configured in a continuous model (Basso and Serban, 2019). Usually, enzymes in food production were dedicated to improving solubility, debranching and clarification, nowadays, there is an interest in the nutritional aspect of food, thus the industry is also using enzymes to produce prebiotics (i.e., food with properties such as improving mineral absorption inside the human intestine) and low-calorie sweeteners (Choi, Han and Kim, 2015).

8.2.3. Bulk chemistry industry

It has been proven how the use of biocatalyst improved the production time, the number of reaction steps and reduced the amount of waste in the production of a variety of chemical and biological substances (Choi, Han and Kim, 2015). Since chemical products are produced in tons like happens in the food industry, recyclability of the enzymes is key, consequently the industry is evolving towards immobilization technology (Basso and Serban, 2019).

8.2.4. Fuel and natural gas industry

Since biofuels popularity has been growing as a sustainable and low environmental impact product (Kumar *et al.*, 2020), enzyme immobilization has given the chance to change the traditional way of making them by using sodium methoxide which contaminated the product, to a much cleaner and safer process. The interest of enzymes in the natural gas industry comes from the need to increase the value of the methane extracted from the natural gas reserve, one way is converting methane to methanol, but the inconvenience is that through classic chemistry very high temperatures, high pressures, and noble metal catalysts are needed in the process

and the selectivity is relatively low. Using biocatalysts, such as MMO (methane monooxygenase), the selectivity turns out to be 100%, reducing the steps needed to obtain methanol, but more research is needed to make the scale-up feasible (Chapman, Ismail and Dinu, 2018; Srivastava *et al.*, 2022).

8.2.5. Flavors, aromas, and detergents industry

Using biocatalysts higher efficiency can be achieved by reducing contaminant production while reducing the cost and time of the process. Since flavors and aromas are chemo-, stereo-, or regioselective substances, enzyme use is much more lucrative than the conventional chemical processes that have been dominating the sector up to now. The same happens with detergents production but enzymes are not used as a process catalyst but as a product, some detergent mixtures can be prepared with enzymes such as lipase or amylase to remove fats and oils for example, which are less environmentally damaging or harsh to the surfaces and fabrics (Chapman, Ismail and Dinu, 2018).

8.2.6. Cosmetic, paper and textile industry

The need of products that are less harsh for the environment is a need nowadays, thus by changing the classic ways of producing cosmetics and fabrics through enzyme catalyzed processes is a big step into a greener future. For cosmetics production, enzymes augmented the yield for obtention of chemicals such as emollient esters which are widely used in cosmetic products. In fabric production the use of this kind of technology evades the use of high amounts of water, and energy (Choi, Han and Kim, 2015). With paper is the same story, energy and harsh chemical use can be reduced by enzyme-based processes (Kumar, Tazeb and Ram, 2021).

Table 5. Products obtained through enzymes in industrial sectors

Industrial sector	Product	Enzymes	Reference
Pharmaceutical	Sofosbuvir	Lipase CalB	(Chandra *et al.*, 2020)
Pharmaceutical	Sitagliptin	R-selective transaminase	(Desai, 2011)
Pharmaceutical	Atrovastatin	Ketoreductase and Halohydrin dehalogenase	(Rosenthal and Lütz, 2018)
Pharmaceutical	Pregabalin	Lipolase	(Rosenthal and Lütz, 2018)
Food	High fructose corn syrup (HFCS)	D-glucose/xylose isomerase	(Jin *et al.*, 2017)
Food	Allulose	Epimerase	(Li *et al.*, 2021)
Food	di-D-fructofuranose 1,2':2,3' dianhydride (DFA III)	Inulin fructotransferase	(Pudjirahart i *et al.*, 2011)
Food	Lactose-free products	b-galactosidase	(Saqib *et al.*, 2017)
Fuel	Biofuel	Pectinase	(Abraham and Puri, 2020)
Fuel	Biofuel	Cellulase	(Abraham and Puri, 2020)
Natural gas	Methanol	Methane monooxygenase	(Srivastava *et al.*, 2022)
Bulk chemistry	Acrylamide	Nitrile hydratase	(Jiao *et al.*, 2020)
Bulk chemistry	hydroxymethylfurfural	Glucose isomerase	(Gimbernat

	(HMF)		*et al.*, 2018)
Bulk chemistry	Cyclodextrins	Cyclodextrin glucanotransferase	(Larsen and Beeren, 2021)

8.3. ECONOMICS

As seen, there are lots of industries that are taking profit of the benefits of immobilization of enzymes technologies, thus the global enzyme market is growing year by year.

According to (Chapman, Ismail and Dinu, 2018) the industrial enzymes global market should grow from the 5.01 billion US dollars in 2016 to 6.32 billion US dollars in 2021, those projections were motivated by the development in enzymatic biofuel production, also by the growth of textile, leather, and paper industries.

Nowadays, according to Global Industry Analysts, Inc. USA, amid the COVID-19 the global market for industrial enzymes in 2020 was estimated to reach 6.1 billon US dollars and is projected to reach the 8.4 billion by 2027. The global biofuel enzyme market is projected to reach the 1 billion in 2022, and 1.3 billion by 2026. The food market is estimated to grow to 3.4 billion in 2027 from the 2.3 billion in 2020. All values in US dollar to make it clear.

From this it is understandable how profitable the catalytic industrial processes are. The market has been growing since 2016 and is projected to still growing until 2027, evidencing the need of a cleaner and more efficient alternative to the classic chemical processes of production that have been dominating the market.

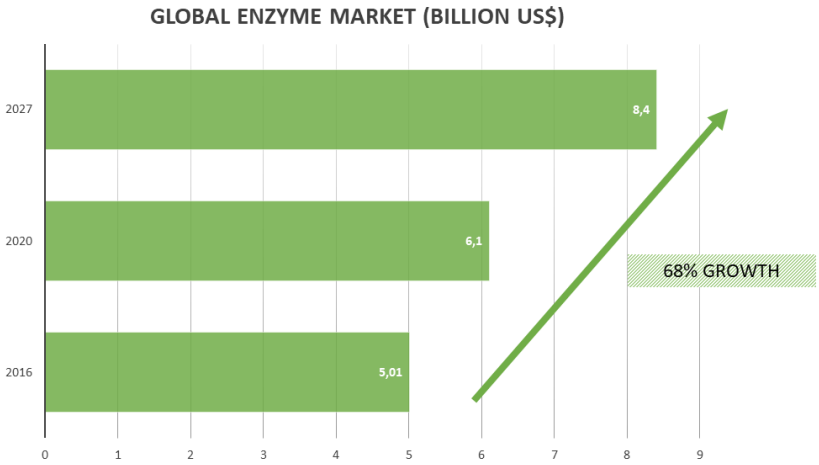

Figure 27. Economic growth of the Global Enzyme Market

The economic benefit of processes is also increased when the energy needed to process is reduced. Nowadays, the environmental crisis is alarming, thus industries must proceed optimizing the energy use. High energetic benefits have been observed in catalytic processes that involve enzyme use. For example, in paper production the pulp refining process is the most energy requiring step taking up to 50% of the paper making process in between of 150 and 500 kWh per ton of paper. Enzyme assisted paper making not only reduces the energy needed by 50% to process the pulp but the pulp obtained by this method has better physical properties than the traditional way. (Kumar, Tazeb and Ram, 2021).

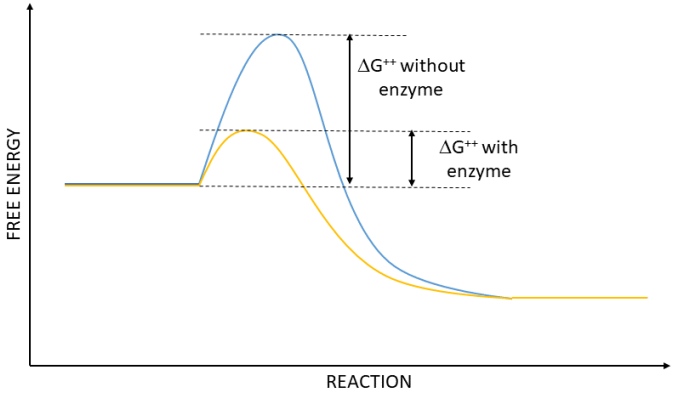

Figure 28. Activation energy is lower for enzyme based reactions

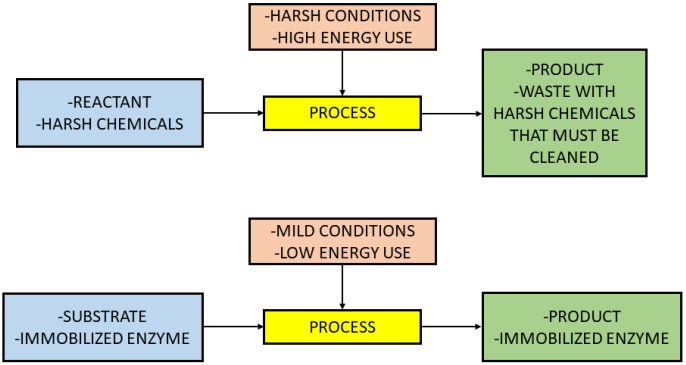

Figure 29. Waste is eradicated by enzymes' selectivity to the substrate and the conditions are milder

9. CONCLUSIONS

The need to reuse enzymes in industrial processes has brought to life the immobilization technology, making it shine in the key aspects that the industry needed. By immobilization enzymes gain stability to survive unnatural conditions, high temperatures, high pressures, and making it possible for enzymes to word in a wider spectrum of pH.

From a good analysis and selection of a carrier and an immobilization technique for an enzyme, those can provide higher product yields that translate to economic growth by boosting the catalytic activity of enzymes in some cases.

Another particularly important topic nowadays is the environmental crisis, the catalytic industries have the chance to change the world by producing without all the waste that traditional industries have been producing, because other feasible technologies were not available until now. Also, the need of energy for production is reduced by enzymes, which also benefits the environment and allows it to heal.

It is clear that this technology might need more investigation, but the future projections of the enzyme market give hope for the development of enzyme immobilization. Probably in the future with better understanding of enzymes mutagenesis and enzyme immobilization technologies, can be combined to achieve a new era in production and prosperity of all kinds of industries from pharmaceutical and food to textile and fuel industries.

To sum up, enzyme-based industries are the future because of the environmental and economic boost and efficiency that provide for production processes.

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ACRONYMS

ATRP - atom transfer radical polymerization

BSA – bovine serum albumin

BSTR – batch stirred tank reactor

CLEA – cross-linked enzyme aggregates

COF – covalent organic frameworks

CSTR – continuous stirred tank reactor

DTT – dithiothreitol

FBR – fluidized bed reactor

HFCS – high fructose corn syrup

LCA – life-cycle assessment

MMO – methane monooxygenase

MNP – magnetic nanoparticles

MOF – metal organic framework

NMP - Nitroxide-mediated radical polymerization

PBR – packed bed reactor

RAFT - Reversible addition−fragmentation chain-transfer polymerization

rROL – rhyzopus oryzae

STR – stirred tank reactor

TCE – techno-economic

TCEP – tris(2-carboxyethyl)phosphine

VDW – Van Der Waals