CHAPTER 2

LITERATURE REVIEW

2.1 Biocatalysis

Chemical transformations carried out by living organisms are enabled by thousands of protein (enzymes) which have catalytic activity to convert particular set of substrates into specific products. Biocatalysis is the general term for the transformation of natural and non-natural compounds by biological molecules or enzymes used as catalysts in specific chemical reactions (Gardossi and Molinari, nd).

Enzymes made of complex protein molecules are the key components in biocatalysis, where they are produced by living organisms to catalyze the biochemical reactions required for life. Although enzymes are formed within living cells, they can continue to function *in vitro* and their abilities to perform very specific chemical transformations are making them increasingly useful in industrial processes. To date, biocatalysis have exploited the abilities of enzymes to transform compounds which are not their natural substrates. Biocatalysis have therefore become an important tool for the production of fine chemicals especially in the field of pharmaceuticals and many others (Gardossi and Molinari, nd).

2.2 Enzymes as Biocatalysts

Apart from being proteins (enzymes), in some cases enzymes may be nucleic acids (e.g. ribozymes are enzymes made of ribonucleic acids, RNA).

The excellent properties of enzymes for rapid, selective and efficient catalysis have indeed made them important and necessary for the survival and reproduction in all living systems. These properties are valuable as they can also be used to catalyze chemical reactions on an industrial scale in a sustainable manner. Their applications cover the production of a wide range of desired products for almost all human needs (e.g. food, animal feed, pharmaceuticals, fine and bulk chemicals, fibers, hygiene, and environmental technology), as well as in a wide range of analytical purposes, especially in diagnostics (Ning et al., 2015).

For the past decades, the rapid increases in knowledge of enzymes together with their biosynthesis and molecular biology have allowed their rational use as biocatalysts in a variety of processes. In addition, their modifications and optimizations have allowed the emergence of new synthetic schemes and solutions for various analytical problems (Buchholz et al., 2005).

More than 2000 enzymes are currently known and a system of classification has been developed that takes into account both their reaction and substrate specificity. Each enzyme is entered in the enzyme catalog with a four-digit enzyme commission number (EC number). The first digit indicates membership of one of the six major classes. The next two indicate subclasses and sub-subclasses. The last digit indicates where the enzyme belongs in the sub-subclass. The enzymes thus have characteristics that make them vital molecules within the living world. Indeed, two closely related properties make powerful enzymes: Specificity of substrate binding associated with the optimal arrangement of their catalytic site (Walid et al., 2012).

2.3 Behaviours of Enzymes

Enzymes are solvent-soluble molecules that need to be separated from the reaction media in order to be re-used. This is especially important to ensure the cost effectiveness of a process and also to facilitate the control in a reactor (Oveimer et al., 2015). Enzymes, when used in suitable reactions systems are capable of catalysing the reactions with up to 1017-fold rate accelerations and with exquisite control of regiochemistry and stereochemistry, in the synthesis of complex and high value molecules. Such control makes the use of enzymes very attractive as an alternative to the traditional chemical catalysts which are sometimes difficult to implement. Over the years, enzymes have evolved to operate most effectively under physiological conditions, on a narrow range of natural substrates, and usually at concentrations in the low range (Dalby, 2007).

As catalysts, enzymes accept a wide array of complex molecules, including synthetic molecules with structures very different from the substrates found in nature. In general, the basis of the selectivity of all enzymes such as chemo-, regio-, and stereo-specificity lays in their structure. Amino acids forming an enzyme are responsible in providing the microenvironment for substrate binding and subsequent chemical transformation in the enzyme active site (Liese et al., 2006).

2.4 Enzyme Catalysis and Kinetics Reaction

Enzymes catalysis is important throughout the areas of biochemistry and physiology and allows various reactions to take place at ambient temperature, under mild condition, such as in living organisms. During catalysis, they provide a lower energy pathway for a reaction to take place. Enzymes frequently function as catalyst in very specific ways, which can be described as follows:

i. Absolute specificity; the enzyme can only catalyze a single reaction.

ii. Group specificity; a reaction of only single type of functional group is catalyzed by the enzyme.

iii. Linkage specificity; the enzyme makes specific type of bond labile.

iv. Stereochemical specificity; enzymes catalyze reactions of only one stereoisomer of a compound.

2.4.1 Enzyme Kinetics

Kinetics of reactions catalyzed by enzymes is a challenging topic to discuss. However, kinetics modelling is essential for process scale-up and reactor design because it provides solution in achieving optimal reaction conditions, the rate of product reaction and changes in experimental systems for proper design of bioreactors. A number of systems have been represented by rather simple kinetic models and their applications have been proven to be successful (Yadav & Manjula Devi, 2004).

In most cases, the kinetic studies of lipase-catalyzed esterification in organic solvent are remarkable rare and are mostly based on the Michaelis-Menten assumptions (Kuperkar et al., 2014). Although there are many reports discussing about the kinetics of biocatalysis, but not many have highlighted about the kinetics and modelling of reactions by immobilized lipases, as affected by different parameters.

2.4.2 Kinetic Modelling and Mechanistic Study

In order to optimize the use of a catalyst, identification of the optimal conditions to perform ester syntheses is indeed important. To do so, several theoretical models have been proposed to better explain lipase-catalyzed esterification by immobilized lipase in organic solvents (Paiva et al., 2000).

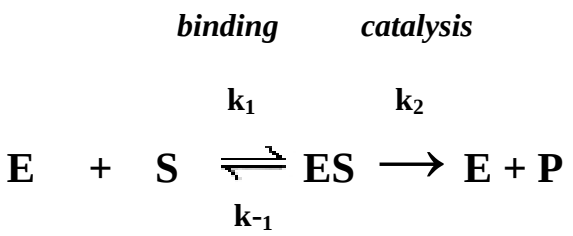
In general, enzymatic models that are based on the simple Michaelis–Menten equation are only valid for most simple enzymatic reactions, involving one substrate. Nevertheless, when a two-substrate, two-product reaction takes place, the general scheme, "bi-bi" has to be taken into account. This scheme includes two possible mechanisms: (i) Ping-Pong (non-sequential) and (ii) ternary complex (sequential). The latter type can be in ordered or random form (Lopresto et al., 2014).

Most lipases are said to follow and favour the Ping-Pong bi-bi mechanism. The bi-bi reaction model is like to occur because it takes into account the product formation following the formation of enzyme-substrate complex (Kuperkar et al., 2014). In a two-substrate reaction, however, the initial reactions rates obtained at various acid and alcohol concentrations fitted well to the Michaelis Menten kinetics with Ping-Pong bi-bi mechanism by a nonlinear regression method (Lopresto et al., 2014).

2.4.2.1 Michaelis-Menten Equation

A commonly used model for enzymatic reactions is the Michaelis–Menten equation, which approximates the original dynamics under the assumption that the concentration of the enzyme remains constant. The enzyme interacts with the substrate to form an enzyme–substrate complex, which leads to synthesis of the product and the release of the enzyme. The simplest model which accounts for tis behavior is shown in Scheme 2.1.

Scheme 2.1: Basic Model of Enzyme-Catalyzed Reaction



Whereby,

E = enzyme

S = substrate

ES = enzyme-substrate complex

P = product of the enzyme-catalyzed reaction

k_1 = rate constant of the forward reaction of E+S

k_{-1} = rate constant of the reverse reaction where the enzyme-substrate complex (ES) breakdown to E+S

k_2 = rate constant of the forward reaction of ES forming E+P

In this model, the catalytic reaction can be divided into two processes. The enzyme and substrate first bind to give an enzyme-substrate complex [ES] by physical forces. It is assumed that none of the product reacts with enzyme during the formation of the enzyme-substrate complex [ES]. This step is assumed to be rapid reversible with no chemical changes occur. The chemical processes the take place in a second step with a first order rate constant, k_{cat} (k_{-1} and k_2) is often called the turnover number of the enzyme because it represents the maximum number of substrate molecules converted to products per active site per unit time, or the number of enzyme 'turn over' per unit time. From scheme 2.1 above, the reaction velocity (V_o) can be expressed as $V_o = k_2[ES]$. Since ES is intermediate and its concentration unknown, the suitable expression of [ES] must be in terms of values as shown as below:

Rate of formation of [ES]:

$$\mathbf{V_{[ES]} = k_1\,[E][S]}$$

Rate of breakdown of [ES]:

$$\mathbf{V_{[ES]} = k_{-1} + k_2\,[ES]}$$

Under the steady-state approximation, the concentration of the intermediate [ES] stays a constant since the rate formation and breakdown of [ES] are equal. Hence, the expression is:

$$\mathbf{k1[E][S] = (k_{-1} + k_2)[ES]}$$

by rearranging the equation, the constant term was defined as Michaelis Menten constant (K_M):

$$\mathbf{[ES] = \frac{k_1[E][S]}{k_{-1} + k_2}}$$

$$\mathbf{K_M = \frac{k_{-1} + k_2}{k_1}}$$

Since in the most situations the enzyme concentration is very small ([E] << [S]), the concentration of the uncombined S is almost equal to the total concentration of S. The concentration of uncombined E is equal to the total enzyme concentration, [E_o] minus the concentration of enzyme-substrate complex [ES]. The maximum reactrion velocity (V_{max}) is reached when all enzyme sites are saturated with the substrate.

Therefore, the Michaelis-Menten equation for this system is:

$$\mathbf{v = \frac{V_{max}\,[S]}{K_M + [S]}}$$

Here, V_{max} represents the maximum velocity achieved by the system, at maximum (saturating) substrate concentrations. K_M (the Michaelis constant; sometimes

represented as K_S instead) is the substrate concentration at which the reaction velocity is 50% of the V_{max}. [S] is the concentration of the substrate S.

This is a plot of the Michaelis-Menten equation's predicted reaction velocity as a function of substrate concentration, with the significance of the kinetic parameters V_{max} and K_M graphically depicted as shown as in Figure 2.1.

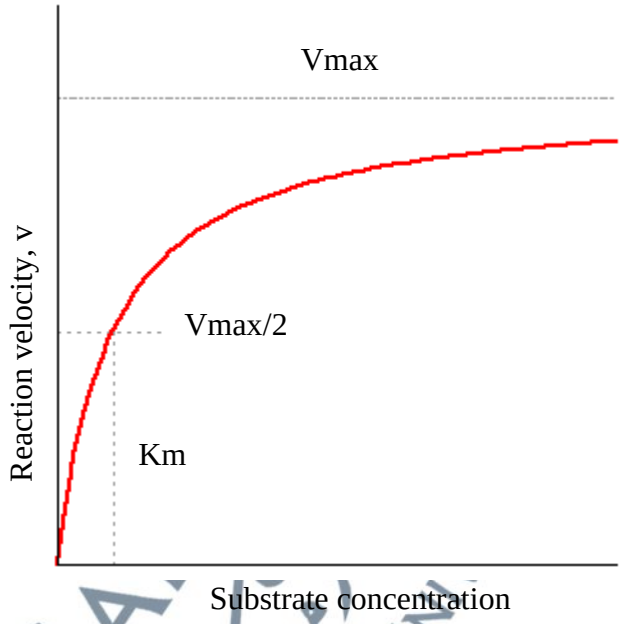


Figure 2.1: Reaction Velocity Plotted Against Substrate Concentration for a Reaction Obeying Michaelis Menten Kinetics.

Some of the principal advantages of integrated Michaelis-Menten equations are as follows (Rui et al., 2007):

(1) neglection of the need to differentiate data to obtain velocity;

(2) more information can be obtained from each experiment which will result in saving of both time and reagents;

(3) the possible accuracy to determine kinetic constants since they are based on products of the actual stereochemistry and of complete purity;

(4) the presence of end product inhibitor can be neglected;

(5) the possibility to predict initial substrate concentration and determine kinetics constants without the knowledge of exact substrate concentration

In general, the strength of the equation is in its ability to define relationship between initial velocities and substrate concentrations. Initial velocities are usually determined by linear regression of initial data points of the progress curves at different substrate concentrations. However, this determination method becomes theoretically incorrect when the product of reaction is an inhibitor. In this situation, the initial velocity obtained is inferior to the substrate concentrations and rate of reaction (Choi et al., 2017). Enzyme kinetics operates in a linear regime with respect to the substrate concentration when the latter is sufficiently low, before reaching a maximum value as the substrate concentration increases (Albert, 2013).

2.4.2.2 Transformation of Michaelis Menten Equation to Lineweaver Burk

Because straight-line plots are easier to evaluate than curves, it is convenient to reformulate Michaelis Menten equation to yield straight-line plots. The maximum initial rate of substrates consumption (V_{max}) and Michaelis Menten constant (K_M) were determined by linear regression using Lineweaver Burk approach. A plot of $1/V_0$ versus $1/[S]$, called a Lineweaver-Burk or double-reciprocal plot, yields a straight line with an intercept of $1/V_{max}$ and a slope of K_M/V_{max} as shown as in Figure 2.2.

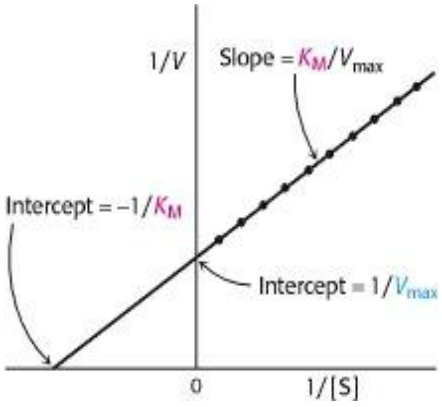


Figure 2.2: A double-reciprocal or Lineweaver-Burk plot

A double-reciprocal plot of enzyme kinetics is generated by plotting $1/V_0$ as a function 1/[S]. The slope is the K_M/V_{max}, the intercept on the vertical axis is $1/V_{max}$, and the intercept on the horizontal axis is $-1/K_M$. A straight line plot is much preffered over a curve, particularly when the data is slightly scattered due to experimental error. Slopes and intercepts are relatively easily obtained from a staright line graph, also known as Lineweaver Burk Plot (Berg et al., 2002).

The Lineweaver Burk plot is usually extrapolated to the negative x-axis. Although there is no data with negative x values, the negative intercept can be used to obtain KM or Ki if inhibition occurs (Chulalaksananukul et al., 1992). X –intercept is –y intercept/slope. When the y-axis is $1/V_o$, its intercept is $1/V_{max}$. Similarly, when the x-axis is 1/[S], then its intercept is $1/K_M$.

$$\frac{1}{V_o} = \frac{K_M + [S]}{V_{max}[S]}$$

$$\frac{1}{V_o} = \frac{K_M}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$$

2.4.2.3 Ping-Pong Bi-Bi Kinetic Model

In most esterification reactions, lipases were found to follow the Ping-Pong bi-bi model where it involves binding of acid with alcohol in successive steps, followed by

the release of water and the ester products in succession. This was proven in a study of the kinetic behaviour of *candida rugosa* lipase (CRL) in the esterification of tetrahydrofurfuryl alcohol with butyric acid, in which the binding of an acid leads to an acyl enzyme complex followed by the release of water molecules. Subsequently, binding of an alcohol leads to the transfer of the acyl group to the alcohol, which results in ester formation. Thereafter, an ester product is released (Somashekar et al., 2007).

In this type of reaction, bi-bi indicates the reaction involving two substrates and two products where the enzyme reacts with the first substrate (the acid) to give a covalently modified enzyme (acyl-enzyme intermediate) with a release of water. Ping-Pong, on the other hand indicates the second substrate (alcohol) with a release of an ester as the second product. The Ping-Pong bi-bi model therefore implies initial rate conditions with negligible product levels (Marie-Pierre et al., 2001).

As illustrated below, Ping-Pong bi-bi model describes a specialized bi-bi mechanism in which the binding of substrates and release of products is ordered. Whereas, in Ping-Pong mechanism, at any point of time only one substrate [A] is bound to the enzyme which leads to the formation of acyl-enzyme complex [E.A]. This is followed by product [P] detachment and formed as it fragmentates from the original substrate [A]. [F] indicates the enzymes intermediate of [P] and [B]. The rest of the substrate [B] is then covalently attracted to the covalent fragment of [A] still attached to the enzyme to form final product [Q] which is released and the enzyme is restored to its initial form [E]. A Ping-Pong mechanism with inhibition by both substrates is given in the following Scheme 2.2

Scheme 2.2: Ping-Pong mechanism with inhibition by both substrates, A and B

$$E+A \overset{K_A}{\Leftrightarrow} E.A \overset{K_{EA}}{\Leftrightarrow} F.P \overset{K_P}{\Leftrightarrow} F.B \overset{K_B}{\Leftrightarrow} F.B \overset{K_{FB}}{\Leftrightarrow} E.Q \overset{K_Q}{\Leftrightarrow} E+Q$$

According to the Ping-Pong bi-bi model, mechanism with inhibition by both substrates is given by the following equation:

$$v_0 = \frac{V_{max}[A][B]}{[A][B]+K_B[A]+(1+[A]/K_{iA})+K_A[B](1+[B]/K_{iB})} \quad (2.1)$$

Whereby,

v_0 - the initial rate of reaction;

V_{max} - the maximum velocity;

[A], [B] - initial concentrations of the two substrates;

K_A - the Michaelis-Menten constant for substrate [A];

K_B - the Michaelis-Menten constant for the substrate [B];

K_{iA} - the inhibition constants for [A] and

K_{iB} - the inhibition constant for [B]

The Ping-Pong bi-bi model (Figure 2.3) also implies initial rate conditions with negligible product levels. In the particular case of esterification reactions, water is one of the products and is usually present initially in the reaction medium since it is essential for enzyme activity. Since a negligible product level cannot be achieved in such a system, Janssen et al. (1999) recently proposed a revised model for esterification model for esterification reactions. They included an extra parameter

which is solid-gas catalysis substrate which takes into account the presence of water (Bousquet et al., 2001).

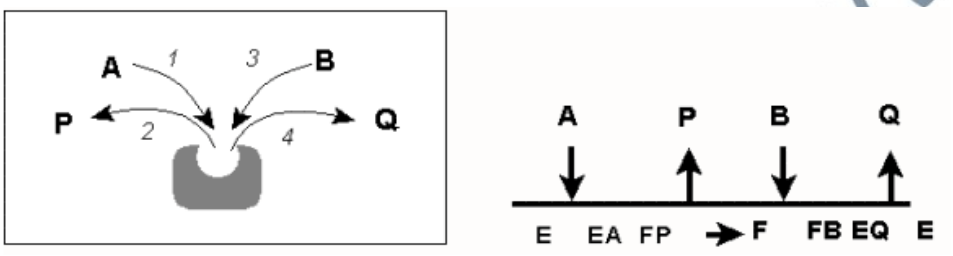


Figure 2.3: Ping-Pong bi-bi kinetic model with two substrates (A and B) and two products (P and Q)

Kinetic models for the entire systems of the reactions were developed based on ordered bi-bi mechanism of esterification. In another work, Wang et al. (2014) studied the kinetics of enzymatic esterification between palmitic acid and starch in a solvent-free system with a conclusion that the reaction followed Ping-Pong bi-bi mechanism. Similarly, Phuah et al. (2012) investigated the hydrolysis of palm oil by lipase from *R. miehei* in solvent-free system and reported that the reaction followed Ping-Pong bi-bi mechanism with competitive inhibition by water (Gofferje at al., 2014).

Ping Pong bi-bi model have also been widely applied to explain the kinetics of biodiesel production via enzymatic transesterification. Similarly, kinetic equations for enzyme-catalyzed reactions, which are based on Ping-Pong bi-bi model with competitive inhibition by both substrates and products, have been developed and used at starting point for the enzymatic kinetic studies. Kinetic studies of enzymatic biodiesel productions are however, often based on the initial rate of the reactions involved and only accounts for substrate inhibition, where glycerol is not present in the system initially (Meunier et al., 2014).

2.5 Lipases

Lipases or triacylglycerol acylhydrolases, (E.C. 3.1.1.3), are water-soluble enzymes that catalyze the hydrolysis of triacylglycerols with the formation of diacylglycerides, monoacylglyceride, glycerol and fatty acids at the interface between the aqueous and organic phases, with great potential for biotechnology. In lipases, since the water insoluble lipid are present with the water soluble lipase, digestion of these triglycerides takes place at the water-oil interface. Therefore, lipase can be used to catalyze the reverse reaction to form ester bonds in ester synthesis as well as transesterification in the reactions containing low water concentrations, opening up the possibility to synthesize polyester (Rahman et al., 2006).

This family of enzymes is also widely used in organic synthesis owing to the commercial availability, structural stability, solvent tolerance, broad substrate scope and good catalytic activity. In organic solvents, the enzymes generally catalyze different acylation reactions to provide esters, thiolesters and amides. In addition to intermolecular acylations, some lipases are furthermore able to catalyze promiscuous, intramolecular reactions (Zhang et al., 2015).

Since lipases are ubiquitous enzymes of considerable physiological significance and are able to perform important roles in the digestion, transport and processing of dietary lipids in most of living organisms, they can be easily found in diverse sources, such as plants, animals, and microorganisms. More abundantly they can be found in bacteria, fungi and yeasts (Miletic et al., 2012).

2.5.1 *Candida rugosa* Lipase

The previous sections have highlighted the remarkable abilities of lipases. These include catalyzing a plethora of reactions such as hydrolysis, esterification, and

transesterification reactions, placing them among the most sought biocatalysts for industrial applications. Among others, *Candida rugosa lipase* (CRL) has been widely studied for its selectivity either with respect to specific functional groups or to stereo- and regio-selectivity (Iole et al., 2015).

CRL is widely used in organic synthesis due to the high selectivity observed. Lipase produced by *C. rugosa* is becoming one of the most industrially used enzymes because of its uses that embrace a variety of processes due to its high activity and enantioselectivity, both in hydrolysis as well as synthesis, especially organic synthesis (Casa et al., 2005).

Among the commercially available lipases from different sources, CRL has been used in a wide range of catalytic reactions in both aqueous and water-restricted environments which include non-specific and stereo-specific hydrolysis. As far as biocatalysis is concern, no lipase is commercially available with a broad range of specificity as attributed to CRL (Calleri et al., 2003).

2.6 Immobilization of Enzymes

The term "immobilized enzymes" refers to enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, which enable enzymes to be used repeatedly and continuously (Brena and Batista-Viera, 2007). Immobilization of enzymes is a key to expand the applications of these natural catalysts by enabling easy separation and purification of products from reaction mixtures and efficient recovery of enzyme (Wang & Hsieh, 2008). The major components of an immobilized enzyme system are the enzyme, the carrier and the mode of attachment (binding) of the enzyme to the carrier (Figure 2.4). The terms solid-phase support, carrier and matrix are used synonymously (Juan et al., 2016).

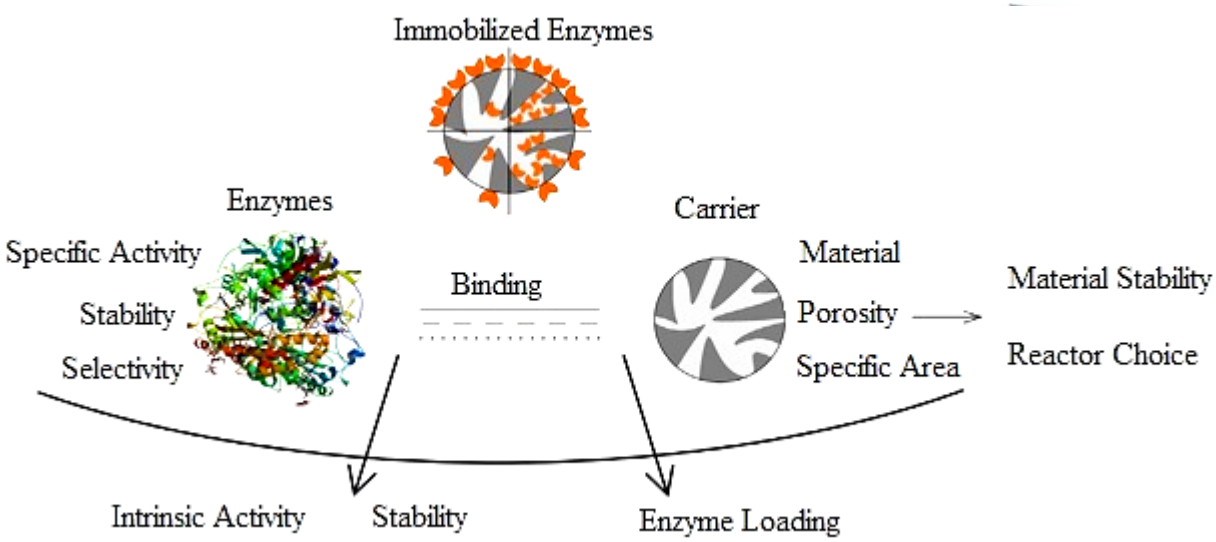


Figure 2.4: Enzymes immobilization in porous carriers and the parameters that were related to the support, the enzyme and the mode of enzyme

Although enzymes have excellent catalytic functions to obtain a wide range of products under mild and environmental friendly conditions, improvements in enzyme stability, activity and recovery are necessary (Miletic et al., 2012). Furthermore, they need to be stable enough to survive under industrially relevant conditions, such as presence of polar organic solvents, high temperatures to avoid contamination, etc. Also enzymes in the free form may not have high enough activities, selectivities or specificities towards the target industrial substrates. Moreover, some free enzymes available are produced in conjunction with many other similar proteins, where some of them possess undesired catalytic activities versus the substrates, or even the products. When this kind of enzymes is immobilized, decrease of the final volumetric activity may occur because some surfaces of the support will be occupied by other inactive proteins. The activity of the minority proteins/enzymes with opposite catalytic activities may also decrease the enantio or regio-selectivity or specificity of

the whole “biocatalyst” if these contaminant enzymes are present (Oveimer et al., 2015).

Some of the initial attempts to immobilize enzymes were done by adsorbing enzyme onto charcoal. However the effort was proved to be very unstable. During 1950s, several groups of researchers began to immobilize enzymes onto other supports. The first industrial application of immobilized enzymes was in the production of optically pure amino acids and the hydrolysis of penicillin G (Miletic et al., 2012).

Successful immobilization of enzymes simplifies enzyme applications and supports a reliable and efficient reaction technology. Improved activity and reusability has been achieved by immobilizing the lipases/enzymes onto various supports by the techniques of physical adsorption, covalent binding and entrapment or cross-linking enzyme aggregates (Kumar et al., 2015). As the properties of immobilized enzyme preparations are governed by both, the properties of enzyme and the carrier material, specific interaction between the two provides an immobilized enzyme with distinct chemical, biochemical, mechanical and kinetic properties. By an appropriate choice of the immobilization process, operational costs for enzyme industrial processes can be reduced by the selection of an appropriate immobilization method (Nigam et al, 2014).

2.6.1 Methods of Enzyme Immobilization

In general, a classification of immobilization methods is according to different chemical and physical principles. When selecting an immobilization strategy, the important parameters which must be considered for efficient enzyme immobilization

include optimal enzyme catalytic activity, effective catalyst utilization, minimal enzyme deactivation and the cost-effective operations (Muhammad et al., 2016).

Likewise, the immobilization procedure should be conducted in a way that allows the enzyme to maintain its active conformation and necessary catalytic flexibility. Basically, there are numerous methods of enzyme immobilization; each involves a different degree of complexity and efficiency. The various methods are adsorption, covalent binding or attachment to different matrices, cross-linking, entrapment, and encapsulation, as shown in Figure 2.5 (Calleri et al., 2003; Murty et al., 2002).

The selection of an immobilization strategy should be based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of lipase/enzyme utilization, deactivation and regeneration characteristics, cost of the immobilization procedure, toxicity of immobilization reagents and the final properties of the immobilized lipase (Murty et al., 2002).

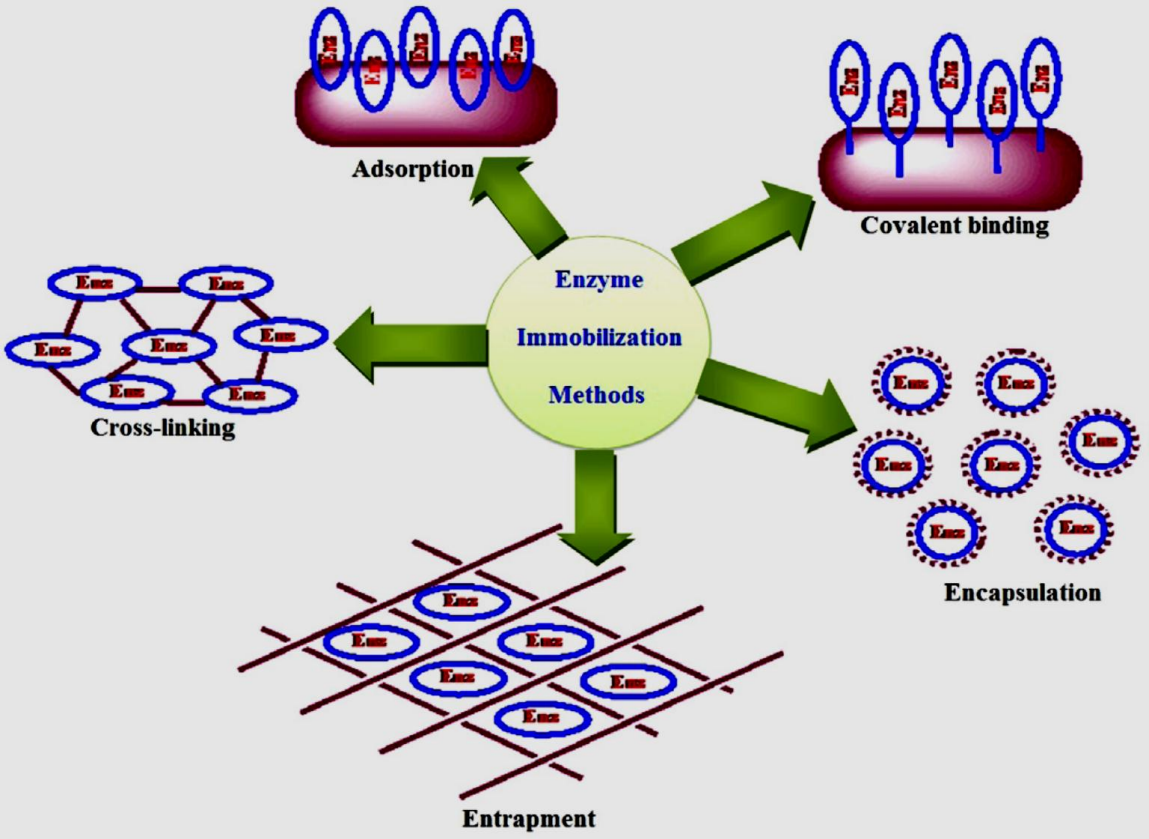


Figure 2.5: Enzyme immobilization methods

2.6.1.1 Immobilization through Adsorption

This is the simplest method and involves reversible surface interactions between enzymes and support material. It is mainly based on physical adsorption or ionic binding. In physical adsorption, the enzymes are attached to the matrix through hydrogen bonding, van der Waals forces, or hydrophobic interactions; whereas in ionic bonding the enzymes are bound through salt linkages. The forces involved in noncovalent immobilization can be reversed by changing the conditions that influence the strength of the interaction (e.g., pH, ionic strength, temperature, or polarity of the solvent).

Among the advantages of adsorption immobilization are; it is mild, cheap, fast and simple process where no chemical changes to support or enzymes are necessary. It

is also an easy to perform process, which usually preserves the catalytic activity of the enzyme. Such method is therefore economically attractive, but may suffer from problems such as enzyme leakage from matrix when the interactions are relatively weak. The possible steric hindrance by the support and nonspecific binding may also occur. Inorganic (activated carbon, silica, etc.) or organic (natural or synthetic polymers), insoluble matrices can be used as supports (Murty et al., 2002).

2.6.1.2 Immobilization through Covalent Binding

Covalent binding is the most widely used method of enzyme immobilization, in which the enzyme is covalently bound to the support (Miletic et al., 2012). This method is based on the formation of covalent bonds between a support material and some functional groups of the amino acid residues on the surface of the enzyme. Usually, the support has to be first activated by a specific reagent, to make its functional groups strongly electrophilic. These groups are then allowed to react with strong nucleophilic groups of the enzyme. The advantage of this method lies in the strength of the bond and the consequent stability of immobilization between enzyme and the matrix, where the enzyme is not easily released into the solution upon use.

However, in order to achieve high levels of activity, the amino acid residues essential for catalytic activity must not be involved in the covalent linkage with the support. This may be a difficult requirement to fulfill in some cases while the disadvantages are the higher costs and the lower yields, as the enzyme conformation and activity will be strongly influenced by the covalent binding (Murty et al., 2002).

2.6.1.3 Immobilization through Cross-Linking

This type of immobilization is support-free and involves joining the enzymes to each other to form a three-dimensional structure. The bond is formed by means of a bi-or multifunctional reagent such as glutaraldehyde. The disadvantages are the very low immobilization yields, the absence of mechanical properties and the poor stability (Murty et al., 2002).

2.6.1.4 Immobilization through Entrapment

The entrapment method is based on the occlusion of an enzyme within a polymeric network and constraining structure tight enough to prevent the protein from diffusing into surrounding medium while allowing the substrate and products to be in contact with the enzyme. The advantages of entrapment include the simplicity of the process, the potential for the successful protein loading and the possibilities of incorporating more than one type of enzyme along with other compounds. Furthermore, the constraining structures may protect the enzyme from denaturing factors such as pH, temperature and organic solvents, thus potentially enhancing the stability of the enzymes (Wang & Hsieh, 2008).

Using this method, the enzyme will be free in the reaction solution and the enzyme is not bound to a matrix or membrane but restricted in movement by the lattice structure of a gel network. There are different approaches of immobilizing enzyme following the entrapment, such as using gel or fiber, temperature-induced gelation, polymerization by chemical/photochemical reaction, ionotropic gelation of macromolecules with multivalent cations, etc. (Miletic et al., 2012).

Entrapment is mainly used for immobilization of whole cells, but has the inevitable disadvantage of low amount of enzymes/whole cells loaded and the support

will act as a barrier to mass transfer (Murty et al., 2002). Membranes or gels are the cause of these limitations. This method also does not completely prevent leaking, but it considerably decreases it with regards to physical adsorption present (Miletic et al., 2012).

2.6.1.5 Immobilization through Encapsulation

Encapsulation of enzymes or cells can be achieved by enveloping the biological catalysts within various forms of semi-permeable membranes, usually microcapsules varying from 10-100 µm in diameter. Large proteins cannot pass in/out of the capsule, but small substrates and products can pass freely across the semi-permeable membranes.

A disadvantage of the method would be the acute diffusion problem; while an advantage would be the co-immobilization of different enzymes/cells to suit particular applications.

2.6.2 Supports for Enzyme Immobilization

The carrier (or the support) is an entity larger in size than the enzyme molecule, to which the enzyme is directly bound or which the enzyme is confined within. This support is however absent in the case of cross-linking and precipitation of enzyme in organic solvent. The support may be a liquid, as in reversed micelles, or a solid, as in most of the commonly employed immobilization protocols (Balcao et al., 1996). Since most of the immobilization methods require a support, and the properties of the immobilized enzymes are absolutely determined by support used, therefore immobilization support should be selected wisely (Miletic et al., 2012).

Ideal support properties include physical resistance to compression, hydrophilicity, inertness toward enzymes, ease of derivation, biocompatibility,

resistance to microbial attack and availability at low cost. A suitable support for enzyme immobilization is the one which provide improved activity and stability of the immobilized enzyme without permitting leakage or hindering the diffusion of substrate molecules and reaction products (Sato et al., 2014).

To date, enzymes have been successfully immobilized on various water-insoluble supports, such as inorganic materials, synthetic polymeric gels, and natural macromolecules, through physical adsorption, covalent bonding, microencapsulation, or matrix entrapment methods (Calleri et al., 2003; Murty et al., 2002).

It has been recognized that the physical properties of enzyme carriers, such as surface functionality, porosity, and large surface area, strongly influence the stability and catalytic activity of an enzyme immobilized. On the other hands, proper linkage of the enzyme to the carrier increases enzyme activity by favorable conformational changes (Liu et al., 2015).

Supports can be classified as inorganic and organic according to their chemical composition. In general, inorganic supports often provide greater stability than organic supports, due to their higher inertness towards reaction conditions like high pressure and temperature. The organic supports, on the other hands, can be further divided into two categories; natural and synthetic, usually polymers (Miletic et al., 2012).

The physical characteristics of the matrices such as mean particle diameter, swelling behaviour, mechanical strength and compression behaviour, will be a major factor and importance for the performance of the immobilized system which will later determine the type of reactor to be used under technical conditions. In particular, pore parameters and particle size determine the total surface area and thus critically affect the capacity for binding of enzymes. Nonporous supports show diffusional limitations and have a low loading capacity. Therefore, porous supports are generally preferred

because the high surface area allows higher enzyme loading and ensures the immobilized enzymes receive greater protection from the environment (Brena and Batista-Viera, 2007).

An evident of the influence of the support particle size on the distribution of enzyme was reported by Chen et al. (2008). In their study, *Candida antarctica* lipase B (CALB) was immobilized on methyl methacrylate resins with identical average pore diameter (250 Å) and surface area (500 m^2/g) but with varied particle size (35 to 560 - 710 µm). The results showed that a non-uniform distribution with most enzymes present in the outer layer of the support particles found for resins with particle sizes 560-710 and 120 µm. In contrast, CALB appears evenly distributed throughout 35 µm resins.

Porous supports should have a controlled pore distribution in order to optimize capacity of immobilized enzymes bound. In spite of many advantages of inorganic supports such as high stability against physical, chemical and microbial degradation, most of the industrial applications are performed with organic matrices. The hydrophilic character of organic supports is one of the most important reasons determining the level of activity of an immobilized enzyme (Miletic et al., 2012).

2.6.2.1 Organic Supports

Natural or Biopolymer Organic Supports

Biopolymers are increasingly being used and studied for many applications in which synthetic polymers have traditionally been the materials of choice. The reason of high demanding in the new technology is the need to increase the use of biodegradable and recyclable materials to limit the number of materials sent to landfills, and the desire to

use renewable raw materials sources instead of non-renewable petroleum sources (Segura et al., 2003).

Natural organic polymers such as polysaccharides (cellulose, dextrans, agar, agarose, chitin and alginate), structural proteins (keratin, collagen), globular proteins (albumin) or carbohydrates are cheap starting materials for the production of support materials and are available in large quantities. From this group, carbohydrates are the special interest because they do are not affected by the biological safety aspects like protein matrices isolated from animal sources and because they are highly hydrophilic which provides a desirable microenvironment for many enzymes (Miletic et al., 2012).

On the other hands, organic support from a natural source, mostly polysaccharides, have the advantage of a great compatibility with the enzymes. Due to their hydrophilicity, they undergo only weak interactions with the enzyme, leading to a minimal inactivation, but unfortunately it also leads to poor binding and therefore the materials often have to be functionalized. Also, the mechanical stability of these materials is rather weak which can be increased by cross-linking. Agarose, cellulose derivatives and cross-linked dextrans are commonly used supports from natural source (Miletic et al, 2012).

In 2010, Luo and Zhang reported the successful immobilization of penicillin G acylase (PGA) in the magnetic cellulose porous microspheres. Results have shown that the spherical magnetic γ-Fe_2O_3 nanoparticles were uniformly dispersed and embedded in the cellulose substrate and that the structure and nature of γ-Fe_2O_3 were perfectly conserved. The immobilized PGA exhibited a highly effective catalytic activity, thermal stability and enhanced tolerance to pH variations. Furthermore, the cellulose microspheres loaded with PGA could be removed and recovered easily by introducing a magnetic field, leading to an acceptable reusability.

Cellulose

Cellulose is one of the most abundant naturally occurring biopolymer and it is commonly found in the cell walls of plants and certain algae (Ibrahim et al., 2011). Cellulose is a fibrous, insoluble and crystalline polysaccharide composed of β-D-glucopyranosyl units joined by 1,4-glycosidic bonds (Paljevac et al., 2007). It is biocompatible and has excellent thermal and mechanical properties (Kim et al., 2015).

Cellulose is an acceptable support which can be easily activated. However, the binding capacity of cellulose for enzymes is generally lower as compared to agarose but it is inexpensive and commercially available in fibrous and granular forms. Some drawbacks of this support is the small particle sizes, which impairs its use in rapid, high-pressure applications. Cellulose are also susceptible to microbial cellulases.

Cellulose having abundant hydroxyl groups can be used to prepare membranes and hydrogels easily with fascinating structures and properties. Cellulose membranes and hydrogels can be prepared from a cellulose solution through physical crosslinking and this is because has many hydroxyl groups which can easily form from networks via hydrogen bonds. The use of cellulose membrane for the immobilization of *Candida Rugosa Lipase*, where the lipase was later used as catalyst in the enzymatic esterification of butyloleate was reported by Hilal et al (2006).

Cellulose derivatives including methyl cellulose (MC), hydroxylpropyl cellulose (HPC), hydroxylpropyl methyl cellulose (HPMC), and carboxymethyl cellulose (CMC) have been used to fabricate cellulose-based hydrogels through physical cross-linking and chemical cross-linking (Ibrahim et al., 2013).

CMC (Figure 2.6) is one of the most important water-soluble derivatives of cellulose that is formed by its reaction with sodium hydroxide and chloroacetic acid. Among all the polysaccharides, CMC is easily available and it is widely used in many

industrial sectors including food, textiles, paper, adhesives, paints, pharmaceutics, cosmetics, detergents, mineral processing and oil well drilling operation (Paljevac et al., 2007). CMC is water soluble cellulose derivatives. Due to ability of gel formation, the presence of CMC in gel products provides excellent water retention property (Mai et al., 2013).

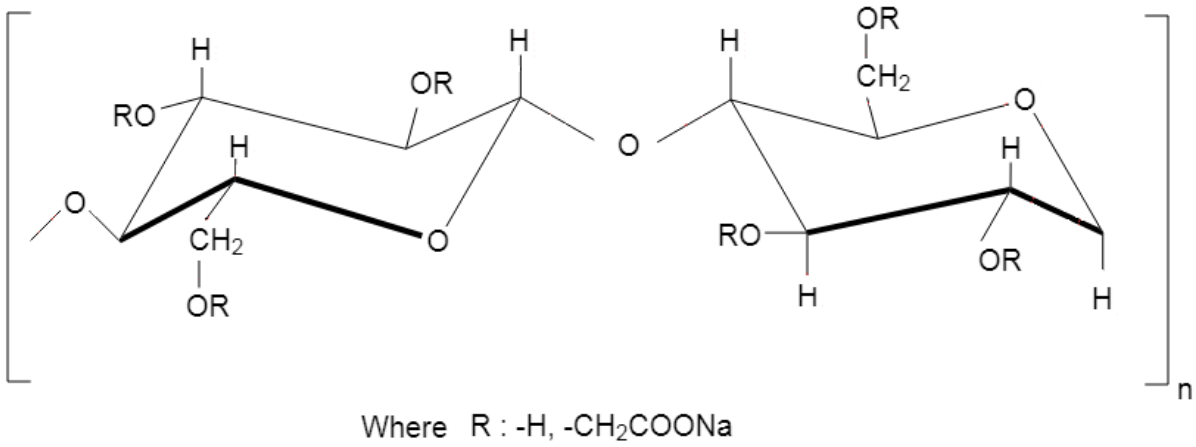


Figure 2.6: Chemical structure of CMC

Synthetic Organic Supports

Organic synthetic supports, mostly synthetic polymers such as polystyrene, polyacrylate, polyvinyl, polyamide, polypropylene and polypeptide structures, have been widely used as immobilization supports because they display the greatest variability with regard to physical and chemical characteristics. In principle, they can be adapted to the requirements of nearly any enzymatic process because they are inert to microbial attack. Such eco-friendly polymers have gained special importance not only for enzyme immobilization, but also for drug delivery system and tissue engineering due to their easy recovery and reusability from the reaction mixture (Wu et al., 2011).

They are commercially available either as purely adsorptive resins, ion exchangers with a variety of different basic and acidic groups, or as pre-activated supports carrying for instance epoxide or azlactone groups.

Among those synthetic organic polymers, polyvinyl alcohol (PVA), Figure 2.7, is one of the most promising type of synthetic polymer used since early 1930s in a wide range of industrial, commercial, medical and food applications including in the fabrication of resins, lacquers and surgical thread.

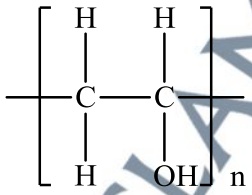


Figure 2.7: Chemical structure of PVA

It is a polymer of great interest because of its excellent compatibility for the preparation of miscible blend. PVA also has impressive features like excellent film formation ability, high interfacial adhesion, flexibility, emulsification, high tensile strength, non-toxicity, biodegradability and essentially resistant to organic solvents which make them more ideal for lipase and microbial immobilization (Badgujar et al., 2013).

PVA is also a good candidate for the preparation of hydrogels, where it can be cross-linked by using several chemical agents such as benzyol peroxide as well as methods including the freeze-thaw cycles, electron beam and γ-irradiation. The use of PVA in the fabrication of hydrogel for controlled drug release had been extensively studied by Bourke et al. (2003) while its use as membrane material for chemical

separations, barrier membrane for different applications and as biomaterials have been reported by Ibrahim et al. (2013).

In a study by Dhake et al. (2011) a blend of hydroxylpropyl methylcellulose with PVA was successfully used to immobilize *Rhizopus oryzae* lipase for acetate synthesis. Badgujar et al. (2013) also reported the success of using biodegradable polymeric film prepared using ternary blend of polylactic acid, PVA and chitosan for lipase immobilization where the film showed better biocompatibility and stability as compared to film prepared using binary blend without the presence of PVA.

2.6.2.2 Inorganic Supports

Inorganic carriers (e.g. glass, silica gel, alumina, bentonite, hydroxyapatite, nickel/nickel oxide, titania, zirconia) often show good mechanical properties, thermal stability and resistance against microbial attack and organic solvents. On the other hand, non-porous materials like metal and metal oxides only have small binding surfaces (Tisher & Wedekind, 2006).

Among inorganic supports, several silica-based and other oxide-based materials are accounted. These are widely considered the materials of choice for enzyme insolubilization since they are endowed with all the features mentioned above. For instance, the thermal and mechanical resistance of inorganic supports are generally higher (Hartmann & Kostrov, 2013).Furthermore, two main features distinguishing inorganic supports are rigidity and porosity. Organic materials can also be obtained with strictly controlled porosity, but they are usually very sensitive to pressure or pH, or in many cases to both. On the contrary, the typical stiffness of the inorganic supports ensures the invariance of pore diameter/pore volume, which

guarantees constant volume and shape to the support itself. Inorganic carriers showing various pore diameters are commercially available (Tran et al., 2011).

2.6.3 Advantages of Immobilization

There are a number of advantages of attaching enzymes to a solid support. The major advantages are:

1) Multiple or repetitive use of a single batch of enzyme.
2) The ability to stop the reaction rapidly by removing the enzyme from the reaction solution or vice versa.
3) Immobilization has a significant effect on stabilizing the enzyme activity against the denaturing effects.
4) The product is not contaminated with the enzyme which is especially useful in the food and pharmaceutical industries.
5) Helping in easy recovery of the enzymes from the reaction solutions and separation of the enzymes from substrates and products (Ghiaci et al., 2009).

In addition, immobilized enzymes have many benefits over free and soluble enzymes in laboratorial and industrial applications. In large-scale, commercial enzymatic processes, the use of immobilized lipases in batch operations were compared to operations employing soluble lipases for the production of food flavours. The result obtained was that, a high efficiency per unit volume of reactor corresponding to the high rate of return of capital costs was achieved using immobilized lipases (Sarmah et al., 2018).

In terms of promoting easy separation of immobilized enzymes from the product, the use of insoluble immobilized lipases leads to decrease in possibilities for contamination of the product via residual lipases, thus avoiding the need for extra cost

for downstream treatments. The reusability of the isolated immobilized enzymes also leads to multiple cost reduction in enzyme-catalyzed processes (Pospiskova & Safarik, 2013). For example, the costs of using immobilized enzymes in continuous processes are more than 20 times lower than using free enzymes, arising primarily from the cost of the relatively large amount of non-reusable enzyme required by the latter process (Murty et al., 2002).

2.6.4 Properties of immobilized enzymes

In general, the properties of immobilized enzymes are governed by the properties of both the enzymes and the support materials. The interactions between them provide immobilized enzymes with specific biochemical and kinetic properties. The biochemical properties of the enzyme, such as its molecular mass, functional groups present on the surface and its purity are some of the important factors for immobilization (Muhamad et al., 2015).

The functional groups on the surface of the enzyme for instance, provide information on the kind of interactions that take place between the enzyme and the support. The purity of the enzyme on the other hand, determines if interference with the substrates would take place. Other properties of the immobilized enzymes that determine their parameters are the reaction type and the kinetics of the reaction catalyzed by the enzymes. Temperature, pH, solvents and impurities are some of the many conditions that may cause activation or inhibition, which would then affect stability of the immobilized enzymes. Most often, immobilized or modified enzymes demonstrate better activity, selectivity and stability (Abdul Rahman et al., 2012).

The ways enzymes are distributed on supports play important role in determining their properties. If enzymes are attached on supports with smaller pore

sizes, structural rearrangement of the enzymes and their subsequent activity will occur, besides diffusion limitation of the substrates and/or products. However, for supports with very large pore sizes, a large amount of enzymes can accommodate the pores of the support causing enzymes to cluster together and lose activity due to saturation.

The diffusion limitation and mechanical properties of the immobilized enzymes (due to the support material they are attached to) is a crucial criteria for consideration in their applications. For example, when immobilized enzyme is to be applied in a stirred tank reactor, it has to be resistant to abrasion. Meanwhile, when immobilized enzyme is to be applied in a column or fed-bed reactor, it has to be resistant to flow (Miletic et al., 2012).

When the characteristics of the enzymes and support are combined in immobilization, other conditions will also influence the properties of the immobilized enzyme. This depends on the immobilization method applied and optimized conditions during immobilization, such as pH, temperature and duration of immobilization, which need to be studied to understand the properties of immobilized enzymes (Zhang et al., 2013).

For example, the rate of the enzyme-catalyzed reaction is greatly influenced by mass-transfer effects, where mass transfer to, from and inside the immobilized enzyme create micro and nano-environments that are sensitive towards changes in pH and concentration. This effect arises from the fact that an immobilized enzyme is bound to the support and has a deliberate restricted mobility. This will affect the mobility of the solutes as well, where the solutes can be adsorbed to the support, resulting in a reduced diffusion of the solutes, which in turn causes a decreased reaction rate compared to the use of soluble enzymes (Buchholz et al., 2005).

2.7 Industrial Applications of Lipases

The industrial applications of biocatalysts depend on the development of effective and stable immobilized enzymes (Asgher et al., 2014). Lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Lipases are also valued biocatalysts in industry because they are able to act under mild conditions, highly stable in extreme conditions and various organic solvents, and help reduce waste production.

Lipases, especially those of microbial origin, have great potential in commercial applications, such as in the production of food additives (for flavour modification), fine chemicals (synthesis of ester), detergents (hydrolysis of fats), waste water treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceutical (digestion of oil and fats in foods), leather (removal of lipids from animals skins) and medical products (blood triglyceride assay). For the control of pollution, lipases can be used to accelerate the degradation of fatty wastes and polyurethane (Andualema & Gessesse, 2012). The following section describes in detail the major industrial applications of lipase.

2.7.1 Detergents

One of the most significant industrial applications of lipases have been mainly found in detergent sector (Houde et al., 2004). Their use in detergents was reported to be almost 32% of the total lipase sales in 1994. Thermostability and ability to remain active in the alkaline environment are necessary for lipases to be use in detergents formulation.

Lipases are added to detergents such as household and industrial laundry and in household dishwashers, where their function is in the removal of fatty residues and cleaning clogged drains (Abhijit, 2012). Other enzymes such as proteases, amylases, cellulases are added together with lipases, to the detergents to improve their efficiency The greater consumption of lipases and other enzymes in detergent arises from the increasing preference for greener detergents arising from the concerns over persistence of detergent chemicals in the environment and its possible contamination of ground water, other fresh sources and their subsequent health related issues (Hasan et al., 2010).

In the development of commercial enzymes used in detergent formulations, Novo Nordisk, a Danish multinational company is responsible in introducing the first commercial recombinant lipase, Lipolase, in 1994, which originated from the fungus *Thermomyces lanuginosus* expressed in *Aspergillus oryzae*. In the following year, two more bacterial lipases were introduced by researchers from Genencor International, USA. The lipases were Lumafast, from *Pseudomonas mendocina* and Lipomax from *Pseudomonas alcaligenes*. An alkaline lipase produced by *P. alcaligenes* M-1, which well suited in removing fatty strain under conditions of modern machine wash was then reported by Gerritse *et al.* in 1998. Extensive and continued research is still going on to develop lipases, which will work under various suitable conditions as fat removers (Hasan et al., 2010).

2.7.2 Food industry

Esters play important roles in the food industry as flavour and aroma constituents. In esterification, the position of the fatty acid in the glycerol backbone, the chain length of the fatty acid, and its degree of unsaturation are important in determining the

physical properties of a triglyceride with desired nutritional and sensory values (Rahman et al., 2006).

In food industry involving palm oil, lipases are used in upgrading of initially less desirable and relatively inexpensive fats to cocoa butter substitutes through transesterification reaction that transforms palmitic acid in palm oil to stearic acid (Pinyaphong P & Phutrakul S, 2009).

In dairy and confectionery processing, lipases are used to hydrolyze milk fat to dairy products, particularly cheeses with desired specific flavours as a result of different carbon chain length of the resulting free fatty acids. In addition, lipases are also used for development of particular flavours in the beverages and bakery products (Jooyandeh et al., 2009).

2.7.3 Pulp and Paper Industry

In pulp and paper industry, treatment of the pulp with lipase leads to a considerable improvement in productivity and a sustained high paper quality with less frequent clearing of the drying cylinders. Lipases are useful in removing the pitch from the pulp during paper making (Fischer & Messner, 1992). Pitch control is important in paper and pulp manufacturing, where triglycerides cause deposits in softwood mechanical pulping. For this reason, both microbial based on colorless strains of sapstain fungi and enzymatic products from improved lipases, including thermostable variants from directed evolution have been commercialized to be applied on wood and pulp, respectively. These enzymes are among the additives of choice in pulping of high-resin-content softwoods (Gutiérrez et al., 2009).

However, lipases are not useful when pitch originates from other lipids, such as steroids and terpenes, where the sapstain inocula are only partially effective. In

order to degrade recalcitrant lipids, the inocula of white-rot fungi and their enzymes were tested while wood treatment was controlled to avoid cellulose degradation. The efficiency and selectivity of the laccase-mediator system was found to permit its integration with lipase, where a double benefit can be obtained from these treatments. As a result, pitch is controlled at the same time where residual lignin is removed facilitating the implementation of totally chlorine free pulp bleaching (Gutiérrez et al., 2009).

2.7.4 Biodiesel Production

Biodiesel (fatty acid methyl/ethyl esters), which is derived from triglycerides by transesterification with methanol/ethanol, has attracted considerable attention during the past decade as a renewable, biodegradable and nontoxic fuel. Enzymatic transesterification using lipase has become more attractive for biodiesel fuel production, since the glycerol produced as a by-product can be easily recovered and the purification of fatty methyl esters is simple to accomplish by the use of whole cell or biocatalysts immobilized within biomass support particles (Fukuda et al., 2001).

In a study by Arumugam & Ponnusami (2017), waste sardine oil was employed as a low cost feedstock for biodiesel production using lipase from *Aspergillus niger* immobilized on activated carbon as the catalyst for the transesterification reaction with metahnol. Variable Compression Engine performance fuelled by the produced biodiesel was studied and the results confirm that waste sardine oil is a potential alternate and low-cost feedstock for biodiesel production.

In 2015, Kimtun et al. extracted and characterized lipase from post harvested oil palm for application as catalyst in biodiesel production. The lipase in purified form was utilized in the transesterification reaction between vegetable oil and methanol; and the partial properties of the biodiesel were compared with commercial biodiesel. Interestingly, the acid value and free fatty acid content of the biodiesel from lipase were not significantly different from commercial biodiesel and it also passed Thailand's fuel standards.

2.7.5 Crude Oil Refining Processes

Oil degumming process plays a critical role in the physical refining of edible oil. Traditional degumming processes, including water degumming, super degumming, total degumming, ultrafiltration process, acid treatment, etc., cannot guarantee the achievement of low phosphorus contents required for physical refining, and are not always optimally suited for all oil qualities (Yang et al., 2006). The yield loss, the apparatus requirement and the energy expenditure of these processes are also great (Gibon & Tirtiaux, 2000).

Enzymatic oil degumming is a more suitable process for the physical refining, in which a phospholipase is used to convert the nonhydratable phosphatides into hydratable form. Apart from the reduction in the amount of acid, alkali and wastewater during the refining process, an enhancement in product yield and a reduction in operating costs can also be observed (Klaus, 1998). In crude oil refining processes, lipases from thermophiles are expected to play increasingly important roles as they are thermostable and resistant to chemical denaturation. Inherently thermostable biocatalysts are well appreciated and hence systematic screening on potential new organisms is necessary. Alternatively, molecular cloning incorporated

with site-directed mutagenesis is being performed to produce recombinant enzymes with desired characteristics (Salihu & Alam, 2014).

In a study by Yang et al. (2006), Lecitase® Ultra, a new microbial lipase developed by Novozymes was applied in degumming of the rapeseed and soybean oil. Lecitase® Ultra is a protein-engineered carboxylic ester hydrolase from *Thermomyces lanuginosus*/*Fusarium oxysporum* produced by submerged fermentation of a genetically modified *Aspergillus oryzae*. Therefore, it takes part in the activity towards both phospholipid and triglyceride structures. Interestingly, when the temperature is over 40 °C, the phospholipase activity predominates, and the lipase activity is partly suppressed. It its application for the degumming of vegetable oil, and the phosphatides in the oil were found to be easily converted by enzymatic catalyzed hydrolysis to less than 10 mg/kg within 5 hours at 50 °C.

In another study, the phospholipase A_2 (secreted by the archae *Pyrococcus horikoshii*) had been used in crude oil refining. The enzyme reacted optimally at 95 ^{o}C and pH 7.0 was found to be a better degummer for oil refineries, reducing the wastewater problems and running cost.

2.7.6 Other Applications of Lipases

Lipases are endowed with a substrate specificity that surpasses any other known enzymes. Consequently, the industrial usage of lipases is expected to increase rapidly in the future (Momsia & Momsia, 2013).

Some examples of lipase applications include the synthesis wax esters from long chain carboxylic acid and alcohol, which have wide applications as lubricants and additives in cosmetics formulation (Khan & Rathod, 2015). Lipases have also been widely used to obtain fatty acids and glycerol for soap production (Cherif et al.,

2014). All these are due to the ability of lipase to hydrolyze lipids (Zimmermann et al., 2004).

For environmental sustainability, lipases are used in the processing of hides and skins, where removal of residual fats and protein debris associated with the hide and the skin/hair are being carried out by enzymatic process instead of chemical process. This is due to the inefficiency and environmental problems caused by the latter process. Likewise, in leather industry, lipases help the removal of subcutaneous fat for easily processing of leather. In textile industry, their applications include the digestion of non-cellulosic impurities from raw cotton before further processing with dyes into finished products take place. Besides, lipases are also utilized in sludge and other aerobic waste processing for the removal of fats from the surface of aerated tanks to permit oxygen transport.

In green synthesis of fine chemical, lipases are being used to produce glucoside esters that are difficult to obtain using conventional chemical means. The glucoside esters are safer to consumers and environment, and are more acceptable due to their rapid biodegradation and low toxicity. They also exhibit surfactant properties and offer attractive alternative to the existing non-environmental surfactants in various areas, ranging from cosmetics, to household detergents, to industrial de-greasing of metals, electronics and leather processing.

In the fields of pharmaceuticals, cosmetics, agrochemicals and medical diagnostics, lipases are employed due to their enantioselectivity. In the synthesis of pharmaceutical products for example, the chirality of a compound produced determines the inhibition effects against tumor and bacterial agents. Likewise, lipases have been employed for efficient production of enantiopure (S)-indanofan, a novel herbicide used against grass weeds in the paddy fields.

The remarkable application of immobilized lipases is in biosensors, where lipases serve as recognition and signalling elements for the detection of specific molecular analyte of interest. Interestingly, the performance of biosensors depend on the extent of enzymatic activity after surface immobilization governed by the binding procedure and interactions between enzymes (detector) to substrates (molecular analyte of interest) (Abel & Aslan, 2014).

2.8 Enzymatic Synthesis of Esters

Synthetic esters of carboxylic acids and alcohols have great commercial importance because of their wide applications in industries such as food and beverages, cosmetics, emulsifiers, flavours and fragrances (Varma & Madras, 2010). In particular, short-chain aliphatic esters are mainly used as flavours and fragrance compounds in the food, cosmetic and pharmaceutical industries. Their fruity notes make them suitable as additives in fruit juices, cheeses, baked goods, candies, beverages and ice creams. Likewise, esters produced from long-chain fatty acids and short-chain alcohols are used in the food, cosmetic, pharmaceutical and detergent industries; as well as plasticizers for PVC, solvents, diesel additives, water-resisting agents, lubricants and in hydraulic fluids (Lopresto et al., 2014).

Interest in lipase-catalyzed preparation of liquid wax ester has also grown in the last few years as a result of the possibility to obtain a wide variety of high-quality products under mild conditions by utilizing the substrate selectivity of such biocatalysts. Liquid wax esters have been obtained in high yields by esterification of long-chain fatty acids and long chain fatty alcohols and in moderate to good yields by interesterification (alcoholysis) of triacylglycerols or natural fats and oils with long-chain alcohols.

A range of fatty acid esters are being produced using immobilized lipase in non-aqueous solvents. They are esters produced from long chain fatty acids (12-20 carbon atoms) as well as short-chain fatty chains (2-8 carbons atoms) with various applications in industries (Kraai et al., 2008). Recently, fatty acid esters with surfactant and combustible properties, have also received widespread attention from scientists due to their similar wide applications in various industries, including chemical industry. This include the biodiesels, the mono-alkyl esters of long chain fatty acids, which is receiving increasing attention from researchers due to the global shortage of fossil fuels, the increasing price of crude oil and environmental pollution concerns (Shah et al., 2018).

The production of biodiesel using base-catalyst in the transesterification of edible oils with methanol have several drawbacks such as the complication of the procedure, more methanol needed for the process, products are hard to reclaim, and environmental pollution. Lipase-catalyzed synthesis has therefore become a promising method to synthesize biodiesels owing to the advantages of the biocatalysis (Ghamgui et al., 2004).

2.8.1 Butyl Oleate Esters

The esterification of oleic acid and alcohols, catalyzed by lipases, leads to the production of a long-chain oleate esters with properties suitable for use in cosmetics, pharmaceuticals, lubricants, candles, polishers and protective coating agents. In addition, butyl oleate, an ester produced from the combination of oleic acid and butanol (short chain alcohol) is useful as a diesel additive, a polyvinyl chloride plasticizer, as water resistant agent and as hydraulic fluids. The syntheses of oleate

esters via enzymatic processes have been reported by many researchers, over the years.

Among the various forms of enzymatic oleates syntheses reported include canola phytosterols oleate as cholesterol lowering agent by plant lipases from Carica papaya, Ricinus communis (castor bean lipase) and microbial lipases from *Rhizomucor miehei*, *Candida antarctica* lipase B and Candida rugosa (Villeneuve et al., 2005), fructose oleate by Candida antarctica lipase B (Novozym 435) (Šabeder et al., 2006), ascorbyl oleate by Candida antarctica lipase B (Adamczak & Bornscheuer, 2009), propyl oleate by Candida antarctica lipase B (Novozym 435) and immboilized *Aspergillus oryzae* (Lipozyme TL IM and Lipozyme TL 100L) (Wang et al., 2007) and butyl oleate by Lipozyme® (Kwon et al., 1995).