CHAPTER 2

LITERATURE REVIEW

2.1 Enzymes in Nature

Enzymes are one of the most significant and interesting substances found in nature. Enzymes have three features that make them useful as biological catalysts. First, an enzyme's primary role is to speed up a reaction by giving an alternate pathway for a reaction with minimum energy; without enzymes, most biological reactions might be too slow to complete. Second, because of the active site, a specific portion of the enzyme that has a specific geometric form that is similar to the geometric shape of a substrate molecule, enzymes specifically react with only one or a few related chemicals (substrate) to make products (Figure 2.1). The third feature is that enzymes are characterised from a low to a high activity level and vice versa.

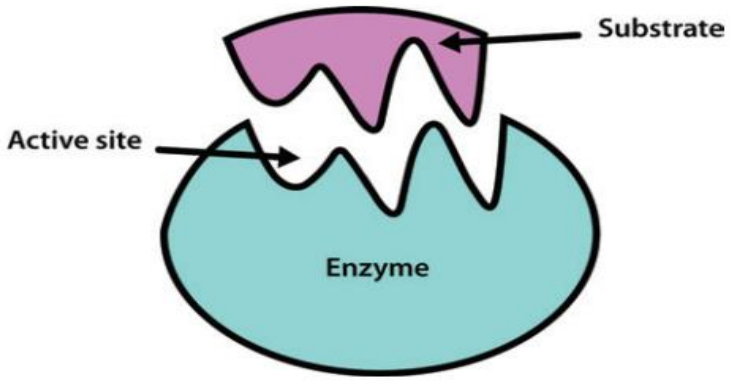
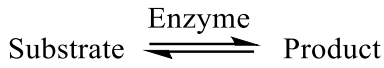


Figure 2.1: The Binding of a Substrate with the Active Site of an Enzyme Molecule (Robinson, 2015)

Less than ten of the enzyme's component amino acids are involved in the active site. The active site's shape and charge qualities enable it to attach to form of substrate molecule, causing differences in the levels of electrons in the substrate's chemical

bonds, which in turn triggers the reaction and generates the product (Robinson, 2015). Enzymes are able to catalyse the conversion of substrate molecules into product molecules in the following way (Scheme 2.1):



Scheme 2.1: Enzymatic Reaction

2.2 Lipase

Lipase (EC 3.1.1.3, triacylglycerol acyl hydrolase) is a pervasive enzyme, which accelerate the breakdown of triglycerides to glycerol and free fatty acids. Furthermore, lipase causes the breakdown and transesterification esters at the same time, synthesis of esters also take place. Lipases are group of enzymes that have a wide range of uses in biotechnology. as additives in detergents, food, pharmaceutical, leather, cosmetic, medical, diagnostics, dairy, beverage, fatty acid, and paper industries (Sarmah et al., 2018; Joshi & Kuila, 2018).

Lipases have a variety of features, including different substrate specificity, selectivity, and high stability, which is often paired with strong enantioselectivity. They can also execute their duty in a wide range of reaction media. As a result, lipases have a broad range of applications in commercial biocatalysis. They also have a unique method of action known as "interfacial activation," which allows lipases to bind to the hydrophobic surface (Arana-Pea et al., 2018; Ortiz et al., 2019).

The main source of lipases from animal includes porcine, pancreas of human, goats, sheeps, and calves. Seeds are the primary source of the enzyme in the plant. Santos et al. (2013) evaluated the application of lipases from the castor bean (*Ricinus communis*), maize (*Zea mays*), sunflower (*Helianthus annuus*), and passion fruit (*Passiflora edulis*) in the hydrolysis of oils for the production of concentrated fatty

acids. Fungi such as *Rhizopus spp.*, *Mucor spp.*, and *Aspergillus spp*, yeast such as *Candida rugosa, Candida tropicalis, Candida antarctica,* and *Candida cylindracea* and bacteria like *Staphylococcus spp., Pseudomonas spp., Chromobacterium spp., Achromobacter spp., Alcaligenes spp.* are the most common lipase sources that produce the lipase (Thakur, 2012; Filho et al., 2019).

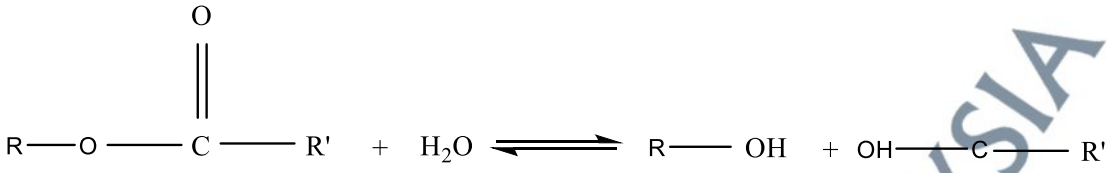
2.2.1 Lipase-Catalyzed Reactions

As shown in Scheme 2.2, lipases catalyse a number of reactions that are mostly determined by the presence of water. When the water activity in an aqueous solution is low, they catalyse hydrolysis and favour the production of esters from alcohols and long chain fatty acids.

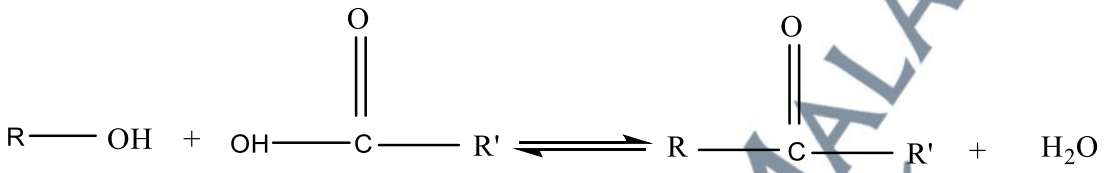
The four subclasses of transesterification are determined by the chemical species that react with the ester. The reaction between an alcohol and an ester is known as alcoholysis, whereas the reaction between an ester and an acid is known as acidolysis. Interesterification is a process that occurs when two distinct esters combine together. In aminolysis, an ester is reacted with an amine, and the resulting is an amide and an alcohol (Sharma et al., 2016).

However, there are differences between the chemical and lipase-catalyzed reactions such as lipases are a good option of catalyst in many sectors since they target a wide range of substrates and also the lipase-catalyzed reactions can proceed under milder conditions than the chemical reactions, thus undesired side reactions such as heat degradation of the substrates can be avoided. On the other hand, lipases as well as other enzymes employed in industry have limited reusability cycles due to their lack of long-term stability under operational conditions. (Yvergnaux, 2017; Musa et al., 2018).

(1) Hydrolysis

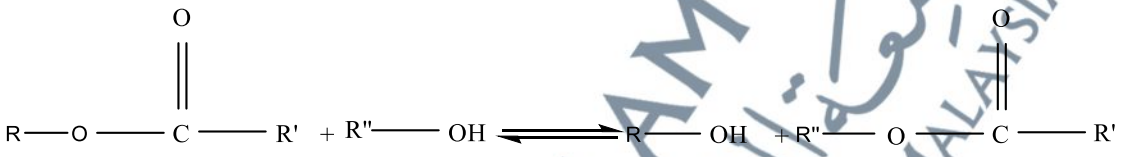


(2) Ester synthesis

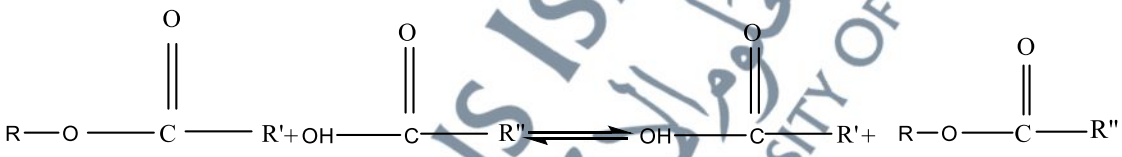


(3) Transesterification

(3,1) Alcoholysis



(3,2) Acidolysis



(3,3) Interesterification

(3,4) Aminolysis

Scheme 2.2: The Reactions Catalyzed by Lipase

2.2.2 Applications of Lipases

Lipases are the most notable because they have high catalytic activity and substrate specificity for some substrates, and they can catalyse a wide range of processes. Table 2.1 summarises the most common uses of lipases. Furthermore, lipases

can be employed in a variety of industries, including pulp and paper production, animal feed, textiles, fatty waste degradation, detergents, medicines, waste treatment, biofuel production, and cosmetics production (Filho et al., 2019). As seen in Table 2.2, the majority of commercial microbial lipases come from fungus and bacteria.

Table 2.1: Industrial Applications of Microbial Lipases

Industry	Action	Product / Application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy products	Hydrolysis of milk fat, cheese ripening	Development of flavoring agent in milk cheese and butter
Bakery	Flavor improvement	Shelf-life prolongation.
Beverages	Transesterification	Alcoholic beverages
Nutrition		Health foods, Improved aroma
Meat and fish	Flavor development	Meat and fish product fat removal
Laundry	Reducing biodegradable strains	Cleaning cloths
Cosmetics	Esterification	Skin and sun-tan creams, bath oil, etc.
Agrochemical	Esterification	Herbicides such as phenoxy propionate
Pharmaceutical	Breakdown of polyester alcohols	Produce various intermediates used in manufacture of medicine
Fuel	Transesterification	Biodiesel production
Paper	Hydrolysis	Paper with improved quality
Leather	Hydrolysis	Leather products

(Verma et al., 2012)

Table 2.2: Some Commercially Microbial Lipases Available by Different Companies

Source	Application	Producing Company
Alcaligenes sp.	Modification of oils and fats	Meito Sangyo. Co.
Burkholderia cepacian	Chiral synthesis	Amano
Aspergillus niger	Dietary supplement	Amano
Aspergillus oryzae	Cheese flavor enhancement	Chr. Hansen A/S
Rhizopus oryzae	Dough strengthening	Novozyme
Candida rugosa	Laboratory chemicals Flavor production from milk fat	Sigma-Aldrich Meito Sangyo. Co.
Candida antarctica	Laboratory chemicals Oil-based specialties	Sigma-Aldrich Novozyme
Rhizomucor miehei	Cheese flavor enhancement	Novozyme

(Source: Lam et al., 2015)

2.3 *Candida rugosa* Lipase

The easily produced of lipase enzyme on large scale by growing microbial sources such as fungi or bacteria in a bioreactor. *Candida rugosa (*also known as *Candida cylindracea)* makes at least five lipases that are strongly related (Kurtovic et al., 2020). Lipase from *Candida rugosa* (CRL) is a very attractive enzyme for the industrial applications due to its structure, broad substrate specificity, high activity under both, aqueous and nonaqueous conditions, and it has been successfully used in a variety of hydrolysis and esterification reactions, especially enantiomer resolution (Huang et al., 2015; Sharma et al., 2019).

Abundant literature is available on hydrolytic and synthetic reactions driven by CRL including biodiesel production (Sharma et al., 2019). It's also a great model for studying lipase immobilisation because it's been widely characterised in both free and immobilised forms (Kurtovic et al., 2020).

Lipase from *Candida rugosa* and other organisms can take two forms. One of them is known as the closed form (inactive), in which the active centre of lipase is separated from the reaction media by a polypeptide chain known as the "lid." The protein's surface becomes substantially more hydrophilic. The open form (active) is the other type, which has the lid removed and the active centre uncovered to the reaction media (Figure 2.2). The surface around the active site depression in the open conformation is much more hydrophobic than other parts of the protein surface (Zaidan et al., 2010).

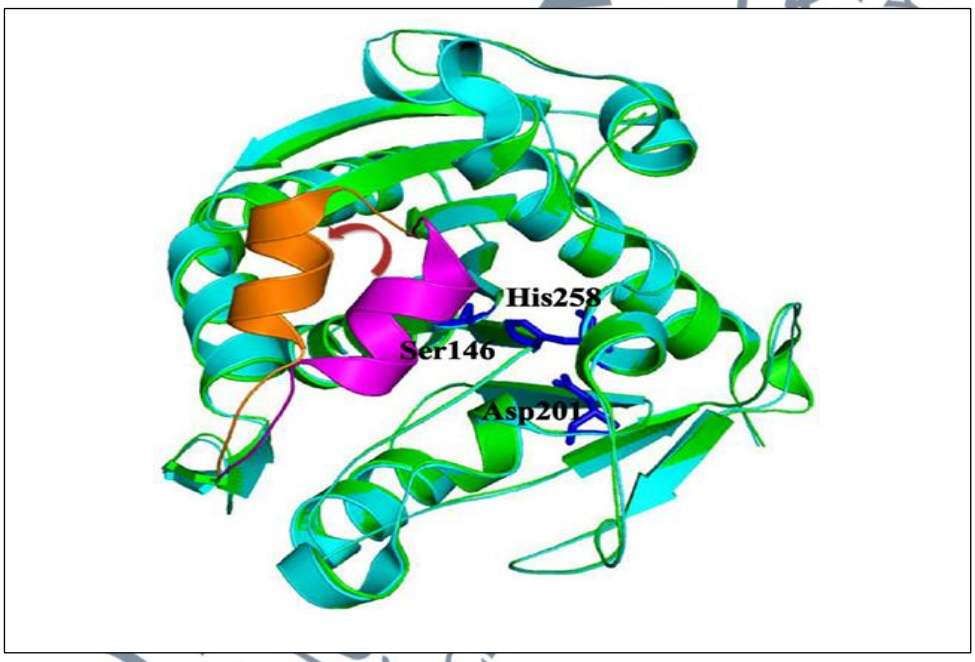


Figure 2.2: The Close Lid, Open Lid, and Catalytic Triads were Highlighted by Magenta, Orange, and Blue Colors, Respectively (Khan et al., 2017)

CRL exhibits interfacial activation in the presence of water-insoluble substrates. The conformational shift (the lid moves to reveal the active site) that lipases experience as a result of adsorption to an interface, as well as the subsequent increase in activity against an insoluble substrate, is known as interfacial activation. Several hydrophobic structures, such as insoluble substrates or hydrophobic supports, could increase

interfacial activation. When the lipase is immobilised on hydrophobic substrates, however, it experiences interfacial activation/conformational modification (Kurtovic et al., 2020).

2.4 Immobilization of Enzyme

Lipases usually solubilize in the reaction medium and this makes their recovery difficult. During the process, unwanted changes in some parameters of the medium's reaction, such as temperature and pH, may occur, causing the enzyme to denature and lose its function (Filho et al., 2019). Many researchers have argued that immobilizing enzymes onto solid materials could be a viable solution to these issues (An et al., 2015). The immobilization can be described as "enzymes physically confined at or localized in a certain region of space with retention of their catalytic activity and which can be used repeatedly and continuously".

Immobilized enzymes, which have various advantages over bulk or free enzymes, are employed in a variety of industries, including food and pharmaceuticals. Enzyme immobilization has many advantages such as more efficient, continuous processing and high storage stability. In addition, immobilized enzymes have improved catalytic activity, resistance to chemical condition and reusability of enzymes (Pradubsang et al., 2018; Asmat & Husain, 2019).

Unfortunately, due to changes in the enzyme secondary structure or a restriction on reactant-catalyst transport, the immobilisation process frequently results in activity loss. Furthermore, lipases' inclination to congregate in water may make the immobilisation process more difficult. High surface areas and hydrophobic supports have been shown to enhance lipase immobilisation in both monomeric and open conformation in this regard. In the presence of hydrophobic surfaces, the open form of

lipases typically occurs with the movement of the lid, boosting the enzyme activity (Pradubsang et al., 2018; Asmat et al., 2019; Francolini et al., 2020).

2.4.1 Techniques for Enzymes Immobilization

There are different methods for enzyme immobilization as shown in Figure 2.3, and the choice of the methods would certainly vary depending on the enzyme involved and its application.

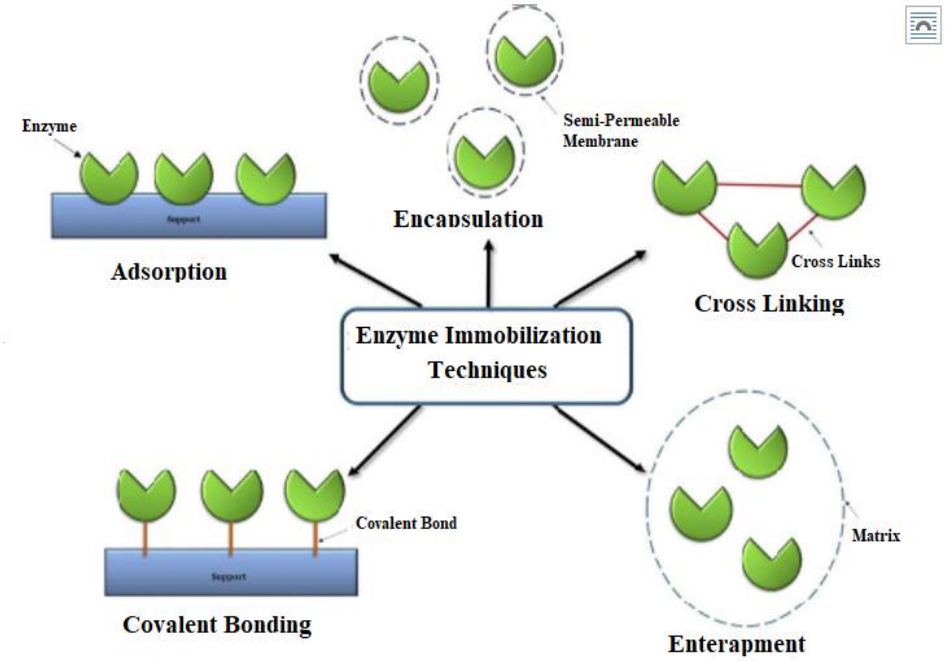


Figure 2.3: Techniques of Enzyme Immobilization (Jun et al., 2019)

2.4.1.1 Covalent Binding

The traditional method for immobilization is covalent binding, which involves physically attaching the enzyme to the substance via a covalent connection. This approach relies on the development of covalent connections between support materials and functional groups of amino acid residues on the enzyme surfaces.

Side chains of amino acids like histidine, arginine, and aspartic acid are where the covalent link between the enzyme and the matrix is created. Reactivity is caused by the presence of functional groups such as carboxyl, phenolic, amino, indole, thiol, and hydroxyl. To attain the highest level of enzyme activity, it will be necessary to avoid the active center of the amino acid from becoming involved in the binding process. To activate the support and make its functional groups substantially electrophilic, a particular reagent is usually utilized. The electrophilic groups are then permitted to react with the e's nucleophilic groups (Sirisha et al., 2016).

Glutaraldehyde is one of the successive cross-linking agents as it has the ability to connect with both the support and the amino group of the enzyme, forming a covalent interaction. The usage of microporous silica and chitosan in covalent binding with enzymes resulted in higher in the enzyme's thermal stability and half-life. The strength of the linkages gives stability for immobilization, and the enzyme cannot be easily leached from the surfaces of the supports with this approach. Unfortunately, this technique can change the enzyme's active site, rendering it inactive (Filho et al., 2019).

2.4.1.2 Adsorption

Adsorption method involves the physical binding of enzymes on the surface of an inert support (Hanefeld et al., 2009). Stoytchevs et al. (2011) stated that lipase has high adaptation towards support using physical adsorption method compared to other methods. The lipase and the support also could have direct contact to each other during immobilization by using physical adsorption method. In this method, the enzyme molecules adhere to the surface of the support by a combination of hydrophobic interactions and the formation of various salt linkages per molecule of enzyme (Sirisha et al., 2016).

The physical binding of enzymes on the surface of an inert support is used in the adsorption method (Hanefeld et al., 2009). Lipase has a strong adaptation to support when employing the physical adsorption approach, according to Stoytchevs et al. (2011). Using the physical adsorption approach, the lipase and the support could potentially be in direct touch during immobilisation. The enzyme molecules stick to the support surface using a mix of hydrophobic interactions and the creation of different salt connections per enzyme molecule in this approach (Sirisha et al., 2016).

Activated carbon and silica are inorganic support examples for attaching enzymes by the absorption approach. Kaolin is ecofriendly matrix upon acetylation, showed high enzyme retainability (Sirisha et al., 2016). Karagulyan et al. (2007) stated that the physical adsorption on kaolin granules are the most efficient methods of immobilization. It was reported that n lipase from *Candida rugosa* adsorbed onto poly(3-hydroxybutyrate-co-hydroxyvalerate), expressed 94% activity even after treating for 4 h at 50°C and reusing till 12 cycles (Cabrera-Padilla et al., 2012). When *Yarrowia lipolytica* lipase was immobilized on octyl-agarose and octadecyl-sepabeads supports by physical adsorption, resulted in greater stability, higher yields, better process control, and quite economical as compared to free lipase. These studies revealed that an environmentally acceptable support for enzyme immobilization via physical adsorption was shown to be cost-effective, biodegradable, long-lasting, and effective (Sirisha et al., 2016).

2.4.1.3 Ionic Binding

Electrostatic interactions, such as ionic or hydrogen bonding between differently charged ionic groups of the matrix and enzymes, provide the basis for ionic binding

(Datta et al., 2013). This form of immobilization is non-covalent, and it can be reversed by changing the ionic strength, polarity, and temperature.

Support materials for this type of method include polysaccharides and synthetic polymers with ion exchange centers. This approach has stronger connections between enzymes and support materials than adsorption but is less effective than covalent binding. The advantages and disadvantages of ionic binding are similar to those of adsorption, except they are achieved by ionic binding. This approach has the advantage of being simpler and using milder settings than covalent binding. This type of interaction changed the enzyme's shape and active site, resulting in increased catalytic activity in most cases. It's possible that the enzymes from the carrier will leak out (Dwevedi, 2016).

2.4.1.4 Cross Linking

Although the cross-linking approach is effective, it is costly and may have an impact on enzyme activity. It also causes enzyme instability and relatively low immobilization yields. This method is also known as carrier-free immobilization since it does not require a carrier and simply uses a cross-linking agent such as glutaraldehyde as a cross-linking agent. During immobilization, the agent is required to maintain the enzyme's functional and structural characteristics (Datta et al., 2013).

2.4.1.5 Entrapment

Entrapment of enzymes in gels or fibers is a handy approach that is only applicable for substrates or products with a low molecular weight. This approach enables substrates and products to move through, but the enzyme is kept in the cage by a covalent or non-covalent bond within a polymeric network. Entrapment can take the

form of simple physical confinement or covalent binding. Entrapment is commonly employed to immobilize cells, but its drawbacks include the support itself acting as a barrier to mass transfer and the fact that it is irreversible because the enzyme cannot be withdrawn once immobilized (Sirisha et al., 2016).

2.4.2 Clay as Immobilization Support

The appropriate support must be selected to guarantee that the enzyme's efficiency is maintained and that the carriers do not interfere with the enzyme's action. The carrier that is employed to immobilise the enzyme has an impact on its performance. As a result, it's critical to comprehend the supporting materials' qualities and properties (Fang et al., 2011). Musa et al. (2018) reported that dramatic differences exist in the activity of lipases, which immobilized on different supports. In addition, the interaction between the support and enzyme will provide an immobilized lipase some specific chemical, biochemical, mechanical, and kinetic specifications (Amin, 2013).

The support can influence on the substrates, products, and water in the reaction mixture, so it can have effects on the catalytic properties of the enzyme. For example, enzyme immobilization prefers support hydrophobicity, which facilitates attraction of the organic phase (substrate), and when the active center is completely visible. As a result, many studies have used this concept to selectively immobilize a variety of lipases on a range of hydrophobic supports using their open forms. (Asmat et al., 2019).

Different solid materials are used as supports for immobilization process. However, it is only separated into two types: organic (such as polysaccharides and polyacrylic) and inorganic (such as clay, sand and stone). These materials are best regarded as effective enzyme immobilisation carriers (Zucca, 2014).

To obtain successful immobilization, the support must fulfil all requirements as shown in Figure 2.4. One of them is that the support's chemical stability must be high in order to prevent the carrier from interfering with the reaction. The microbiological stability is a key consideration when selecting a carrier, and it must be strong to avoid rapid digestion. Furthermore, the support should be insoluble in all sorts of solvents so that it can be reused in subsequent reactions.

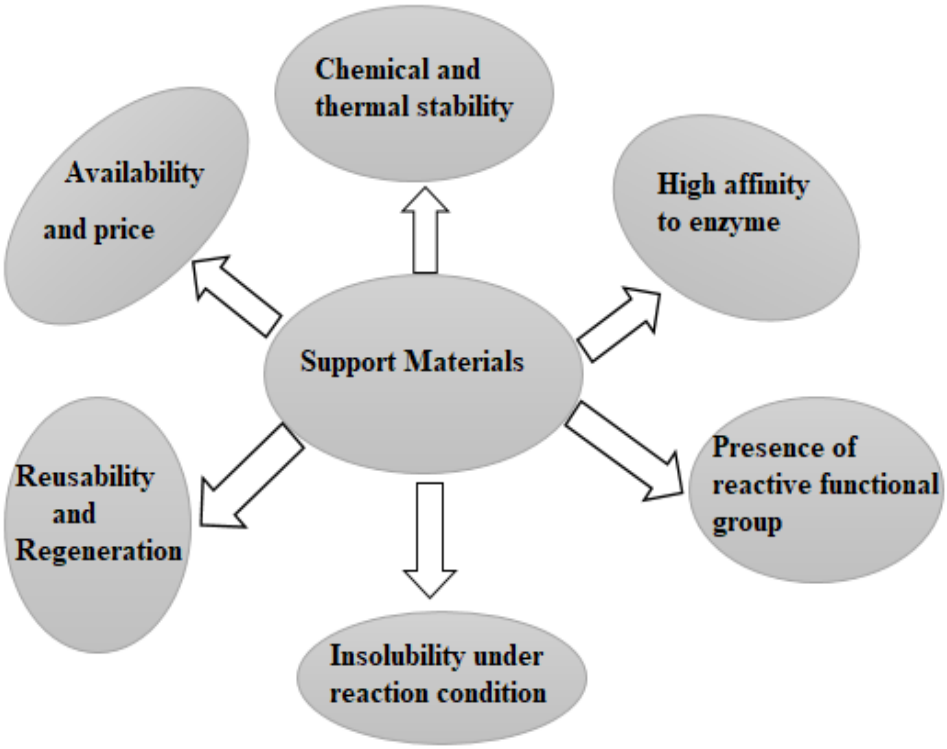


Figure 2.4: Main Features of Support Materials Used for Enzyme Immobilization (Zdarta et al., 2018)

In order to ensure good enzyme activity throughout the process, the morphological structure of the support should also be examined. The accessible surface inside the support must be large enough to allow an adequate amount of enzyme to be coupled to it (Stergiou et al., 2013).

Clay has become a popular choice for enzyme immobilisation in recent years, owing to its inexpensive cost, chemical inertia, thermal stability, well-defined layered structure, and ion-exchange capacity (Cacciotti et al., 2019).

The clay minerals are generally classified into two classes; cationic and anionic (Ghadiri et al., 2015). To balance the charge, cationic clays use negatively charged aluminosilicate layers with tiny cations in the interlayer region, whereas anionic clays use positively charged (metal hydroxide) layers with anions and water molecules to balance the charge. The presence of hydroxyl group (-OH) on the surface of clay minerals make it as a hydrophilic material, which can link very easily to the water molecules. Clay minerals can be divided into various groups as in Table 2.3 depending on the layer type (Bergaya et al., 2013).

Clay minerals are phyllosilicates of hydrous aluminium or magnesium that are prevalent in nature. On a nanometer scale, clay minerals have a two-dimensional layered structure. Every layer of montmorillonite (MMT), for example, is made up of one tetrahedral silicate (Si-O) sheet. Clay minerals with this structure feature unusual physicochemical properties, such as chemical inertia, thermal stability, a well-defined layered structure, and ion-exchange capacity. (Cacciotti et al., 2019).

The presence of silanol groups on tetrahedral silicate (Si-O) sheets can help clay minerals and organic enzyme molecules interact through hydrophobicity. Furthermore, hydrogen bonding between the enzyme and hydroxyl groups can develop at the broken edges of tetrahedral and octahedral clay sheets. A cation exchange process can also be used to replace exchangeable cations in the interlayer space, such as Na^{+} or Ca^{2+}, with positively charged enzyme molecules. Clay minerals are one of the best supports for immobilizing enzymes because of their qualities (An et al., 2015).

Table 2.3: The Important Groups of Clay Minerals (Bergaya & Lagaly, 2013)

Group of Clay Minerals	Ratio of Tetrahedral to Octahedral Sheets	Composition
Kaolinite	1:1	$Al_2Si_4O_{10}(OH)_8$
Smectite (MMT)	2: 1	$M_x(Al_2xMg_x)(Si_4)O_{10}(OH)_2.nH_2O$
Pyrophyllic	2:1	$Mg_3Si_4O_{10}(OH)_2$
Talc	2:1	$Al_2Si_4O_{10}(OH)_2$
Mica	2:1	$X_2Y_4Si_6Al_2O_{20}(OH)_4$ (X=K and/or Na, Y=Al and/or Fe and/or Mg)
Chlorite	2:2	$(Mg_{6-y-x}Fe_yAl_xSi_{4-x})O_{10}(OH)_8$
Serpentine	1:1	$Mg_6Si_4O_{10}(OH)_8$

On the other hand, 1:1 type of clay minerals have little isomorphous substitution and no exchangeable cations in the interlayer space. It has also hydrogen bondings between H and OH groups of their adjacent layers. Thus, it is difficult to introduce the enzyme molecules into their interlayer space. Nevertheless, it is possible to immobilize the enzyme on this type of clay, which can occur at their edges and on their external surface (An et al., 2015).

Furthermore, the compositional and structural features of layered clay minerals allow for a wide range of organic, polymetric, and organic particle alteration of clay minerals. These clay minerals, which have been modified, have an additional functional group and adhesion area. As a result, modified clay minerals have improved features for enzyme immobilisation techniques, such as increasing the loading amount, pH toughness, thermal stability, and biocompatibility of immobilised proteins (Zhou, 2011; Zhou & Keeling, 2013).

2.5 Modification of Clays

Clay minerals can be modified in various way to enlarge its surface area, therefore increasing the adsorption capacities, and to obtain specific chemical and structural properties for different applications (Boukhemkhem & Rida, 2017). In this case, lipases are hydrophobic enzymes so they prefer strong hydrophobic or electrostatic interaction with support in an immobilization process. It's possible that the enzyme has a slightly negative charge overall, resulting in limited interactions with the clay mineral surface. The layer charge, adsorption capacity, morphology and cation exchange capacity are factors which play an important role in the modification of the mineral clays (Uddin, 2017). After surface modification, many researchers have observed considerable improvements in the surfaces of numerous materials typically employed as adsorbents (Abegunde et al., 2020).

2.5.1 Methods of Modification of Clay Minerals

2.5.1.1 Pillared Clays

In addition to the improved pore structure, specific inorganic moieties (guest species) were intercalated into the clay mineral, resulting in thermally stable "pillared" clay materials. The pillaring agent is an organic or inorganic substance. The use of inorganic pillaring agents is more common than the use of organic pillaring agents. The pillared clay's nomenclature is determined by the host clay mineral and the pillaring agent. The host is MMT, while the pillaring agent is polyhydroxyaluminum in pillared polyhydroxyaluminum MMT, for example (Sarkar et al., 2019).

2.5.1.2 Polymer Modified Clays

Polymers can be added to the interlayer gaps of clays to improve their characteristics. Physical adsorption, chemical grafting, or ion exchange with surfactants

are the most common methods for modification. During the polymerization process, the structure of clay minerals is not destroyed. The polymer-clay nanocomposite, on the other hand, can increase the physical and chemical properties of the polymer and clay components individually (Mukhopadhyay et al. 2020; Barakan & Aghazadeh, 2021).

2.5.1.3 Organoclays

The quaternary ammonium salts of the form $[(CH_3)_3NR]^+$ or $[(CH_3)_2NRR']^+$ are commonly used as the cationic surfactants for the formation of organoclays (Aroke & El-Nafaty, 2014). As it is well known, the clay minerals usually have a net negative charge due to the isomorphous substitutions in the aluminosilicate layers of clay, which is balanced by metal cations such as K^+, Na^+ and Ca^{2+}. The substitution of these inorganic cations by quaternary ammonium cations at the exchangeable sites of natural clays results in organoclay derivatives (Aroke & El-Nafaty, 2014).

Bentonite have widely been used for the preparation of organomodified clay due to its high cation exchange capacity (CEC), and swelling capacity. Thus, the organic molecules able to intercalate in its interlayer structure and lead to increase *d*-value. Whereas kaolin would adsorb the organic molecules as an outer surface (Sarkar et al., 2019). The presence of many hydroxyl groups on the surface of kaolin favors its functionalization with modifier (de Souza Lima et al., 2019).

Cation-exchange reactions, on the other hand, have long been used to effectively substitute inorganic ions with organic cationic surfactant molecules that intercalate into the clay gallery, leading to an increase in the basal spacing by expanding the interlayer spacing. During the interaction of quaternary ammonium compounds with clay minerals, these organic cations are exchanged with hydrated cations available in the interlayer region of clay, resulting in hydrophobic organoclays (Sarkar et al., 2019).

In addition, the hydrophobic surface of support can induce the conformational change on lipases necessary to allow free access of substrates to their active centres. In other words, it has a 'pulling effect' on the lipase's a-helical 'lid' away from the active site, resulting in the lipase's 'open' conformation. Lipases contain certain secondary structural "lid" parts covering their active areas in the absence of this mechanism that make the active sites unavailable to the substrates. Thus, this mechanism is a key guidance to produce high activity lipase derivatives (Badgujar & Bhanage, 2014; Elias et al., 2019; Asmat et al., 2020).

Organo-modified supports should present an apparent positive charge on their surface due to the ammonium salts (Golbaha et al., 2016; Öztürk et al., 2016) which could interact favorably with the globally negatively charged enzyme. Kaolin treated with organically cationic surfactants could be an excellent hydrophobic support for enzyme immobilisation (Dong et al., 2012). As a result, the effects of the chemical change on the structure of the supports, as well as lipase immobilised supports, must be investigated. To the best of our knowledge, few studies on immobilization process of lipase on kaolin and metakaolin, and the effect of hydrophobicity of both on the activity of lipase have been reported.

It is crucial to pick the right cationic organic modifier (Singla et al., 2012). The use of organomodifiers such as octyl amine, octadecyl amine, dodecyl amine, dimethyl distearyl ammonium bromide, dimethyl benzyl hydrogenated tallow quaternary ammonium, dimethyl dehydrogenated tallow quaternary ammonium, and methyl tallow bis-2-hydroxy ethyl quaternary ammonium has been published (Mgbemena et al., 2013).

Benzyltriethylammonium ($BTEA^+$) are quaternary amine monovalent cations that can be effective in modifying the surface of the clay. Ramos et al. (2014) used a

benzyltriethylammonium chloride as organic modifier to immobilize of *Candida rugosa* lipase and they found it to be a suitable material for enzyme immobilisation; it is cost-effective and offers the ideal conditions for enzyme stability and efficient hydrolysis reaction performance. Aroke and El-Nafaty (2014) studied about characterization of kaolinite clay which was treated with cationic surfactant hexadecyltrimethylammonium bromide, they provided that the clay materials modified can achieve desired surface properties for best immobilization performance.

2.5.1.4 Thermal Activation

This type of modification is a physical treatment which includes the calcination of clay at high temperature (Nwuzor et al., 2018). Thermal activation breaks the hydrogen bonds between the dioctahedral layers (Cheng et al., 2019). Clay minerals are generally calcined prior to use to remove the adsorbed and hydrated water and impurities. Thermal treatment can bring about the modification of structure, physiochemical properties and composition of the clay minerals. These alterations differ depending on the clay mineral group you are dealing with, as well as the particle size and heating regime. The continuous of heating correspond to the dihydroxylation. This causes the bonds within the clay structure to break down, resulting in the collapse of the structure and a reduction in cation exchange capacity. (CEC) (Nwuzor et al., 2018).

2.5.1.5 Acid Activation

The modification by acid activation involves treatment the clay with inorganic acids such as, hydrochloric and sulphuric, at intended acid strength. The acid activation changes the physical characterization of the clay such as, the capacity of the acid to digest and eliminate related impurities such as calcite is largely responsible for

improved surface qualities such as increased porosity and surface area. Temperature, reaction duration, and acid concentration are reaction factors that determine the final acid activated clay mineral products during acid activation of clay minerals. It is critical to guarantee that the modified clay mineral's surface acidity is not caused by the presence of free acid on the clay's surface. This is avoided by thoroughly washing the modified clay until no free acid remains on the surface (Sarkar et al., 2019).

2.6 Kaolin Clay

Kaolin is either white, greyish-white, or slightly coloured solid which consists mainly of the mineral kaolinite and other minerals in small amounts such as quartz and feldspar. Kaolin is used in different of industrial applications such as ceramic, rubber industries, plastics, and it is utilized to improve color, opacity, and printability of paper. Natural kaolin is inexpensive and shows good potential as support material. Some studies have been conducted using kaolin clay for enzyme immobilization, as exhibited in Table 2.4.

Table 2.4: Recent Studies using Kaolin Clay from Different Sources for Enzyme Immobilization

Origin	Immobilization Method	Author
Imerys, Pará, Brazil	Covalent bond	de Souza Lima et al., 2019
Sinopharm Chemical Reagent Shanghai, China	Adsorption	Wen et al., 2019
Carlo Erba, Cornaredo, Italy	Adsorption	Tanaskovic et al., 2017
Sungai Sayong, Perak, Malaysia	Encapsulation	Rahim et al., 2013
Kankara Village, Nigeria	Adsorption	Ajayi et al., 2012
Kuala Kangsar, Perak, Malaysia	Adsorption	Rahman et al., 2005

The silica and alumina content of kaolin when upgraded, can serve as immobilization sites for various enzymes (Ajayi et al., 2012). Immobilized lipase on natural kaolin was found to be stable in hexane at room temperature for up to 12 days by Rahman and coworkers (2005), and also demonstrated superior stability than native lipase in a storage investigation.

2.6.1 Structural Features of Kaolinite Clay

Kaolinite is the main component of kaolin with chemical formula $Al_2Si_2O_5(OH)_4$ which can also be written in terms of oxides as Al_2O_3-$2SiO_2$-$2H_2O$, and whose theoretical composition of 46.54% SiO_2, 39.5% Al_2O_3 and 13.96% H_2O. The crystalline structure of kaolinite is made through the arrangement of two layers, which sandwich a common plane of oxygen atoms. The tetrahedral layer (T) contains silicates (SiO_4), and the octahedral layer (O) contains aluminate group, $Al(OH)_4$, as seen in Figure 2.5.

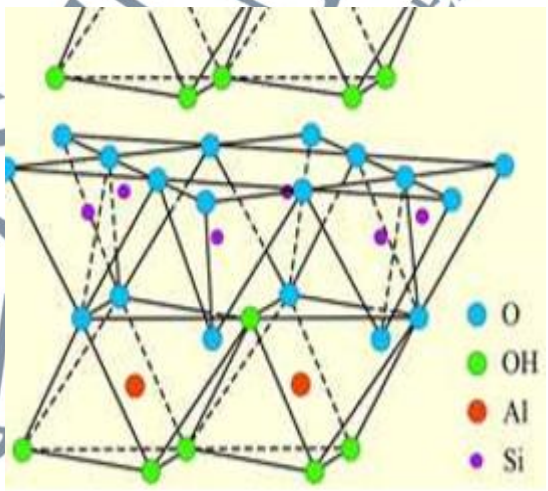


Figure 2.5: The Arrangement of Layers to Form Structure of Kaolin (Von-Kiti, 2017)

The adjacent layers are combined by hydrogen bonding involving aluminol (Al–OH) and siloxane (Si–O) groups (Gebremariam, 2015). Kaolinite is a layered silicate

mineral having one tetrahedral sheet connected to one alumina octahedral sheet by oxygen atoms (1:1 layer). Kaolinite's exchange sites are on the surface, and it has no interlayer exchange sites (Yao et al., 2014).

Between the layers of kaolinite clay, there is a high binding energy resulted from three different components which are Van der Waals' forces, hydrogen bonding between hydroxyls and basal oxygen groups and electrostatic interactions, which are caused by kaolinite's dipolar nature.

Because there is no basal inner-surface hydroxyl, no hydrogen bonds are formed between the layers of kaolinite clay, and only weak Van-der-Waals forces hold the layers together. Organic modifiers have a low adsorption capacity due to a tiny specific surface area induced by layer cohesion and a very low cation exchange capacity of kaolinite.

2.6.2 Cation Exchange Capacity

Cation-exchange capacity (CEC) is referring to the total capacity of a clay to hold exchangeable cations in its structure. These adsorbed cations will balance the negatively charged sites. In other words, the CEC is defined as the amount of exchangeable cations required to compensate for the charge shortfall on the clay particle surface. Higher CEC indicates more surface activity and, as a result, increased water absorption capability (Forouzan, 2016).

As illustrated in Figure 2.6; there are two parts of clay surface, external surface and internal surface. The external exchange capacity is determined by the number of cation bonding sites on exterior surfaces. The internal exchange capacity can be used to determine the overall charge imbalance on the layer structure and clay absorption

capacity (Forouzan, 2016). The cation exchange capacity of a clay may be caused by isomorphous substitution on the flat external surfaces of layers or by broken bonds.

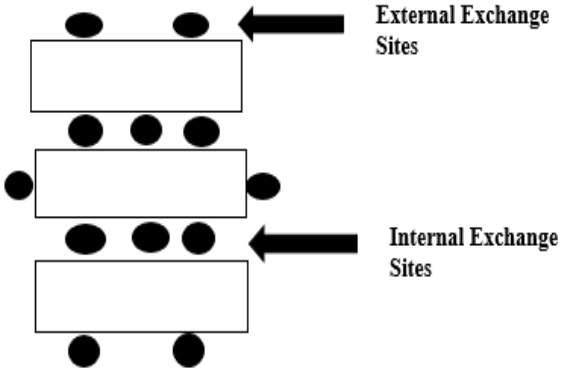


Figure 2.6: Different Types of Exchange Sites on Clay Particles, Surface and Absorbed Ion Interlayer Sites

Because of the prevalence of Al^{3+} in the octahedral sheet and the lack of substitutions by other cations such as Fe^{3+} or Mg^{2+}, the CEC value of kaolinite is quite low compared to other forms of clay (Czarnecka, 2013). The cation exchange capacity of kaolin (1:1 type clay) comes from the broken edges of the layers, in comparison; smectite (2:1 type clay) is attributed to that ionic substitution within the central octahedral sheet of the molecular layer. The CEC of kaolinite minerals range from about 2 - 10 meq/100 g, depending on the particle size (Grim, 1968), The low values are most likely pure kaolinite, whereas the increase may be due to contaminants. Olaremu (2015) reported that some researchers suggested the value of pure kaolin clay is about 1 - 10 meq/100. Table 2.5 shows the different value of the CEC corresponding to types of the clay minerals.

Table 2.5: Cation Exchange Capacity with Respect to Clay Minerals (Lambe & Whitman, 1969)

Clay mineral	CEC (meq/100 g)
Kaolinite	3 – 15
Illite	10 – 40
Montmorillonite	80 – 150

Some researchers determined the cation exchange capacities of clay minerals by adsorption of methylene blue (MBT) method from aqueous solution (Boxill, 2011; Ikhtiyarova et al., 2012; Anggraini et al., 2014; Forouzan, 2016; Acevedo et al., 2017; Marques et al., 2018; Rosário et al., 2019). Aprile & Lorandi (2012) found that using MBT to determine CEC is a simpler and more realistic method with lower operating expenses and a faster processing time. This approach has become the industry standard for determining the cation exchange capacity of clays in a variety of applications (Abayazeed & El-Hinnawi, 2011).

The methylene blue adsorption method is conducted through a laboratory procedure in which a solution of methylene blue made with de-ionized water is titrated in 1mL increments into an aqueous solution of the sample. The positive methylene blue ion installs Na^{+}, Ca^{2+}, K^{+}, and Mg^{2+} cations on the surfaces of clay particles when a methylene blue solution is introduced to a watery clay mixture (Scheme 2.3) allowing estimation of the cation exchange capacity and specific surface area of clay minerals present (Boxill, 2011).

Ca-Na Clay + MB hydrochloride ⟶ MB Clay + Ca-Na Chloride

Scheme 2.3: Adsorption of Methylene Blue (MB) onto Clay

Titration with methylene blue $C_{16}H_{18}N_3S$-Cl can also be used to produce a relative estimate of cation exchange capacity (CEC) of a clay soil because molecules are

preferentially adsorbed onto the negatively charged site that might otherwise attract cations (Erguler & Ulusay, 2003). CEC is calculated using the volume of methylene blue added to the clay dispersion until the visual saturation point is reached (marked by the formation of a halo of constant diameter around a drop taken from the mixture).

2.6.3 Surface Modification Reactions and Intercalation of Kaolinite

The functionalization the surface of immobilization support is a way to solve leaching problem and inactive enzyme aggregates. Table 2.6 shows some organic modifiers used to the synthesis of the organo-modified kaolin in previous researches.

Table 2.6: Some Surface Modifiers used to the synthesis of the organo-modified Kaolin

Surface Modifier	Author
Hexadecyltrimethylammonium bromide	Aroke & El-Nafaty, 2014
Tetraethylamonium iodide	Hashemian&Yaldaparsaei, 2015
Chlorhydric acid and sodium hydroxide	Boukhemkhem & Rida, 2017
Rubber seed oils (*Hevea brasiliensis*) and tea seed oils (*Camellia sinensis*)	Mgbemena et al., 2013
Tetrabutyldimethyl chlorosilane	Duarte-Silva et al., 2014
3-aminopropyltriethoxysilane and glutaraldehyde	de Souza Lima et al., 2019
Hexadecyltrimethylamonium bromide (HDTMA-Br) cationic surfactant	Mudzielwana et al., 2019

There are two general categories of the chemical modification of clay minerals: (i) the alteration that takes place on the particle's exterior surfaces, and (ii) the change that takes place on the interlayer surfaces. The first group includes modifications with polyatomic cations that generate ionic exchange processes between the clay surface and

the cation. Aluminol groups, which are found at the layers' edges and basal surfaces, have a lot of potential as functional groups for reacting with other chemical species and forming covalent connections between the particle surface and the reacting molecules (Shahverdi-Shahraki, 2014).

Although the modifying of kaolinite by intercalation or grafting of tiny molecules is significantly more difficult than that of smectites, some molecules are capable of intercalating the structure and modifying the kaolinite. According to Shahverdi-Shahraki (2014), these molecules can be divided into four categories; molecules such as urea, formamide, hydrazine, and others that have donor and acceptor groups for hydrogen bonding at distinct atoms and are capable of forming strong hydrogen bonds; polar compounds with a high dipole moment, such as dimethyl sulfoxide and pyridine N-oxide, for example; low molecular weight carboxylic acid alkali and ammonium salts, such as ammonium acetate and potassium propionate.

These groups provide different interactions between the surfaces of the enzyme molecules and support. Organics and polymers that can't be intercalated directly into the layers can be added via substitution processes or active molecules. Microwave radiations were utilised by Feriancová et al. (2021) to speed up the intercalation of potassium acetate solution and dimethyl sulphoxide (DMSO). Letaief et al. (2006) utilized urea to prepare ethyl pyridinium chloride-kaolinite. The urea acted as a starting material for achieving the final nanostructure.

Previous studies have been reported on enzyme adsorption onto modified clay. Dong et al. (2012) reported that the catalytic activity of lipase was improved after organo-modification and organo-bentonite creates a highly dispersed surface as a result of interfacial activation by a hydrophobic microenvironment. Öztürk et al. (2016) reported that the highest hydrolytic activities were obtained for lipase immobilized on

organo-modified clay minerals, highlighting the beneficial effect of organo-modification. An et al. (2015) also reported that organic modification of clay minerals is made to enhance the loading, activity and stability of enzymes.

The orientation and arrangement of the alkyl chain was determined by wide angle X-ray diffraction. Depending on the length of alkyl chain, packing density, temperature; the chains are known to form monolayer, bilayer, or paraffin complexes as shown in Figure 2.7. Furthermore, the longer the modifier alkyl chain means the more hydrophobic partition medium, and thus the higher adsorption capacity. In addition, a more paraffinic structure was observed for highly charged clay layer.

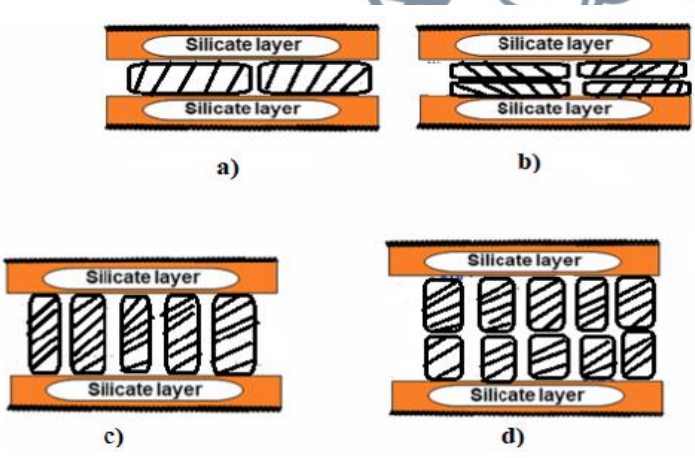


Figure 2.7: Surfactants Arrangements within the Clays, (a) Lateral Monolayer; (b) Lateral Bilayer; (c) Paraffin-Type Monolayer; (d) Paraffin- Type Bilayer (Award et al., 2019)

2.6.4 Calcination of Kaolinite

Thermal decomposition of pure kaolin clay yields amorphous structured material called metakaolin ($Al_2Si_2O_7$). Metakaolin is an artificial material used in advanced ceramics, as filler in papermaking, as a pozzolanic material in mortars, concrete and cement-wood composites, as extender in plastics and natural rubber (Gamelas et al., 2014).

According to prior study, the optimal temperature is between 550 and 800 °C and still different from one researcher to another (Astutiningsih et al., 2018). The heating period also is still exactly undetermined (Rashad, 2013). Metakaolin typically contain 50-55% SiO_2 and 40 - 45% Al_2O_3 (Poon et al., 2001). Other oxides that are found in minor levels are: Fe_2O_3, MgO, CaO, and TiO_2. Depending on the increase in the heating temperature, the removal stages of H_2O^- and H_2O^+/OH^- are the following (Phung-Thi, 2013):

(a) 100 - 120 °C: Release of moisture and absorbed water (H_2O^-);

(b) 120 - 400 ^oC: Mass loss (H_2O^-, H_2O^+/OH^-) correlated with a pre-dehydration process which takes place as a result of the reorganisation in the octahedral layer and first occurring at the OH^- group at the surface;

(c) 400 - 650 ^oC: De-hydroxylation of kaolin and formation of metakaolin according to the following reaction (scheme 2.4):

$$Al_2Si_2O_5(OH)_4 \rightarrow Al_2Si_2O_5(OH)_xO_{2-x} + (2-x/2)H_2O$$

Scheme 2.4: The Formation of Metakaolin

This occurs with a low value of x (about 10% of residual hydroxyl groups in metakaolin);

(d) 900 - 1000 ^oC: Metakaolinite decomposes and then crystallizes to form mullite or cristobalite, or both.

During thermal treatment of kaolin, the principal response that took place was the disintegration of the kaolin structure. The heating reaction separates the mineral's -OH chain, collapses the kaolinite structure, and yields metakaolin in the structure of an amorphous aluminosilicate ($Al_2O_3.2SiO_2$) (Astutiningsih et al., 2018). Metakaolin has a limited percentage of residual hydroxyl groups, and when heated for a long time, it

releases water (Guatame-Garcia et al., 2018). Calcination removes the hexagonal layer of kaolinite and causes atomic rearrangement, resulting in the conversion of hexa-coordinated Al ions in kaolinite to penta- and tetra-coordinated Al ions (Abdullah et al., 2018). Metakaolin contains high content of Si and Al and is obtained by heating raw material (kaolin) (Gamelas et al., 2014).

Metakaolin as a support has a lot of potential for enzyme immobilisation since it has strong mechanical and thermal properties and is easy to obtain from naturally available raw kaolin. It also has a low cost, is non-toxic, and has a low chemical reactivity (Tanaskovic et al., 2016). Tanaskovic and colleagues (2016) employed metakaolin as a substrate for immobilising *Candida antarctica* lipase B. The biocatalyst, which was obtained under ideal conditions, was then successfully used in the production of lipophilic antioxidants, with conversion yields of up to 100%.

2.7 Esterification Reactions

The esterification reaction happens when hydroxyl molecules such as alcohols or phenols react with acids to generate an ester. In most cases, an ester is created when the hydrogen of a carboxylic acid (COOH) is displaced by a hydrocarbon group from alcohol, with H_2O as a byproduct. Ester is a significant chemical group because it has numerous applications in various fields, including solvents, plasticizers, and ethyl acetate, which has been widely utilised in the production of paint, confectionery, perfumes, and fruit (Turhanen et al., 2019).

The esterification reaction necessitates the use of a mineral acid such as sulphuric acid, which has a number of drawbacks like harsh reaction conditions, difficult to separate, the corrosion of the reactor as a result of the use of a strong acid catalyst, heat sensitivity, and poor reaction selectivity leading to undesirable side reactions (Elias et

al., 2019). To solve these problems, enzymatic catalysts are receiving more attention to develop a method that is an eco-friendly technique to produce the esters.

As one of the most important and adaptable components of natural flavours and perfumes, short chain ester is in great demand and widely utilised in cosmetics, food beverages, and pharmaceutical industries. Despite the fact that flavour ester is manufactured by chemical synthesis, natural flavours are increasingly preferred over chemically synthesised flavours. This is because individuals are more aware of environmental issues and are concerned about their own health. As a result, they seek natural or natural-like flavours in their food and beverages. However, the restricted availability of such sources fails to match demand, resulting in high product costs (Monteiro et al., 2019).

2.7.1 Ester for Flavour and Fragrance

As one of the most significant and customizable components of natural flavours and perfumes, short chain ester is in great demand and widely employed in the food beverage, pharmaceutical, and cosmetic industries (Brault et al., 2014). Flavor is made up of two types of molecules: volatile and non-volatile, which have different chemical and physiochemical properties. The flavour is mostly influenced by non-volatile components, whereas volatile components influence both fragrance and taste.

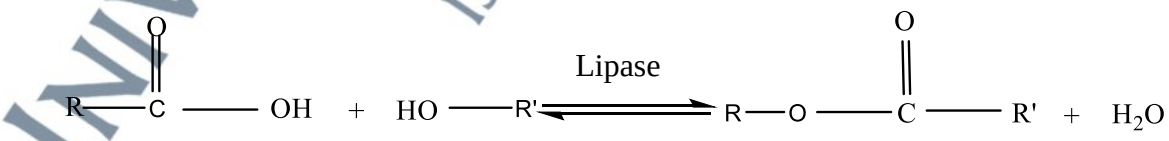
The flavour and fragrance components are extracted from the natural sources such as plants, fruits, and flowers or are produced by chemical synthesis. Natural extracts' commercial application, on the other hand, is hampered by a lack of supplies and expensive production costs (Musa et al., 2019).

Although flavour esters are now manufactured via chemical synthesis, there is a rising preference for natural flavours over chemically synthesised ones (Garlapati &

Banerjee, 2013). The odours of flowers and the flavours of fresh fruits were obtained by a complex mixture of chemical compounds, which is why they are employed in products such as beverages, jellies, jams, sweets, and a variety of other products that impart fruit flavour. The flavour ester created by the biocatalyst technique can be considered close to "natural," and it may be able to meet customer demands (Brault et al., 2014).

Chemically synthesised flavours are still a popular choice among manufacturers for mass-producing this ester. Chemical synthesis takes place at high temperatures, has negative environmental consequences such as toxicity from impurities or byproducts produced during the process, and necessitates post-treatment. Because of improved selectivity and quality of product, gentle reaction conditions, and the ability to identify the product as "natural," the use of natural enzymes to generate flavour esters has recently gotten a lot of interest (Musa et al., 2018; Elias et al., 2019).

Enzyme-catalyzed synthesis can produce various products depending on the specificity or stereospecificity of the lipase used. The esterification reaction is shown in Scheme 2.5. The past few decades have seen immobilized enzymes being used as biocatalysts for improving the application of enzymes in the industry (Musa et al., 2018; Elias et al., 2019). Table 2.7 shows optimized parameters in the synthesis of flavour esters using microbial enzymes.



Scheme 2.5: Esterification Reaction of Fatty Acid and Alcohol to Produce Ester

Table 2.7: Optimized Parameters in the Synthesis of Flavour Esters using Microbial Enzymes

Microbial Enzymes	Substrates	Optimized Parameter	Author
***Candida antarctica* Lipase**	Butyric acid with methanol and ethanol	Time: 8 h Temp.: 25 ℃ Continuous shaking at 150 rpm. Substrates Molar Ratio: 1:1 molar ratio acid-to-alcohol	De Souza et al., (2017)
***Pseudomonas* AMS8 Lipase**	Hexanoic acid Ethanol	Time: 2 h Temperature: 20 ℃ Continuous shaking at 200 rpm. Amount of Enzyme: 5% Substrate Molar Ratio: 1:1 molar ratio acid-to-alcohol	Musa et al., (2018)
***Candida rugosa* lipase**	Butyric acid Butanol	Time: 3 h Temperature: 50 ℃ Continuous shaking at 200 rpm. Substrate Molar Ratio: 1:2 M ratio of acid/alcohol	Elias et al., (2019)
***Candida rugosa* lipase**	Valeric acid Ethanol	Time: 24 h Temperature: 45 °C Continuous shaking at 150 rpm. Substrate Molar Ratio: 4:1 molar ratio of alcohol to acid	Asmat et al., (2020)

The esterification of nonanol and hexanoic acid is carried out in this work to form the ester nonyl hexanoate. The production of an acyl-enzyme intermediate between a fatty acid and an acyl donor, as well as lipase. Because this structure is unstable and thermodynamically unstable, it formed ester by reacting with alcohol as the nucleophile.

Lipase will break away from the product and reattach to another acyl donor. Lipase is not reactive, rather it is the catalyst that starts the reaction (Muhamad, 2012).

Nonyl alcohol, also known as 1-nonanol, is a primary structured organic molecule with the structural formula $C_5H_{12}O$, as shown in Scheme 2.3. It has a molar mass of 144.2545 g/mol, a boiling point of 213.3 °C, and a melting point of -5 °C. It presents as a colourless liquid with an aromatic or fruity odour and a molar mass of 144.2545 g/mol. Nonanol's main application is in the production of fake lemon oil; its refractive index ranges from 1.4324 to 11.4344, and its density is 0.827 g/cm^3.

Nonyl alcohol, often known as 1-nonanol, is a primary structured organic molecule with the structural formula $C_5H_{12}O$. It has a molar mass of 144.2545 g/mol, a boiling point of 213.3 °C, and a melting point of -5 °C. It displays as a colourless liquid with an aromatic or fruity odour. Nonanol's refractive index ranges from 1.4324 to 11.4344, and it has a density of 0.827 g/cm^3.

Other than that, the carboxylic acid which is hexanoic acid is acid derived from hexane and noun as caproic acid with chemical formula $C_5H_{11}COOH$. It has an undesirable fatty, cheesy, waxy odour similar to that of goats or other barnyard animals, and it is water insoluble to slightly soluble and less dense. It can be found in a variety of plant and animal fats and oils in nature. Hexanoic acid is primarily used in the production of hexanoic acid esters for artificial flavours and hexyl derivatives such as hexylphenols. It has a molar mass of 116.15828 g/mol, a melting point of -3.4 °C, and a boiling point of 205.8 °C. It presents as a colourless oily liquid with a molar mass of 116.15828 g/mol, a melting temperature of -3.4 °C, and a boiling point of 205.8 °C.

Hexanoic acid has a refractive index of 1.4161 and a density of 0.92 g/cm3.The product of esterification of hexanoic acid and nonanol is nonyl hexanoate, with the

structural formula $C_{15}H_{30}O_2$ and it is produced by combining hexanoic acid and nonanol. The other name for nonyl hexanoate is caproic acid nonyl ester. It has molar mass 242.3975 g/mol and its boiling point is 290.3 ℃ with the density 0.866 g/cm^3. The refractive index of this ester is 1.437.

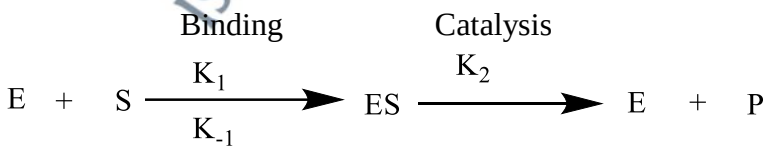
2.8 Enzyme Kinetics

Enzyme kinetics is the study of the chemical reactions that are catalysed by enzymes and It gives reliable data regarding the rate of product formation and changes in experimental systems, which may be used to build suitable reactors and then scale up to industrial size. At its most basic level, enzyme kinetics allows for the clarification of the mechanism of enzymatic action by continuously adding an inhibitor to alter the rate of a given enzyme-catalyzed process (Hess et al., 2015). In addition, knowledge the reaction kinetics and the constants that describe the kinetic behaviour of the reaction is useful to identify the optimal conditions for ester synthesis (Kuo et al., 2020).

2.8.1 Measurement of Enzyme Kinetic Properties

2.8.1.1 The Michaelis-Menten Equation

The kinetic properties of enzyme can be determined by measuring reaction rates of the enzyme-catalyzed reactions for different concentrations of substrate. The basic model which accounts for this behaviour is shown in Scheme 2.6.



Scheme 2.6: Basic Model of Enzyme Catalyzed Reaction

Whereby: E is enzyme, S is substrate, ES is enzyme-substrate complex, P is product, k_1 is rate constant of the forward reaction of E+P, k_{-1} is rate constant of the reverse reaction where enzyme-substrate complex [ES] breakdown to E+S, k_2 is rate constant of the forward reaction of [ES] forming E+P.

It can be seen that there are two processes in this model of the catalytic reaction. The enzyme binds with the substrate to give an enzyme-substrate complex ES, by physical forces. This process is thought to be quick and reversible, with no chemical alterations, it means none of the product produce in this step. In the second step, 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.

It can be also seen in the following series of reactions (2.1 - 2.8) that the reaction velocity (V_0) can be determined from [ES]. Since ES in an intermediate and its concentration unknown the suitable of [ES] must be in terms of known values as shown in equations below:

Rate of formation of [ES]:

$$V_{[ES]} = k_1 [E] [S] \quad (2.1)$$

Rate of breakdown of [ES]:

$$V[ES] = (k_{-1} + k_2) [ES] \quad (2.2)$$

Because the rate of formation and breakdown of [ES] are equal, so the concentration of intermediate [ES] stays a constant. Hence, the expression is:

$$k_1 [E] [S] = (k_{-1} + k_2) [ES] \quad (2.3)$$

By rearranging the equation, the constant term was defined as Michaelis-Menten constant (K_m):

$$\left[ES \right] = \frac{K_1 \left[E\right] \left[S\right]}{K_{-1} + K_2} \quad (2.4)$$

$$K_m = \frac{K_{-1} + K_2}{K_1} \quad (2.5)$$

The enzyme concentration is very small compared to the substrate ([E] << [S]). Hence, the concentration of uncombined S is almost equal to the total concentration of S. The concentration of uncombined E is equal to the total enzyme concentration. It can be reached to the maximum reaction velocity (V_{max}) when all enzyme sites are companied with the substrate. So, V_{max} can be expressed as:

$$V_0 = \frac{V_{max} \left[S\right]}{K_m + \left[S\right]} \quad (2.6)$$

Whereby, V_0 is reaction rate, V_{max} is maximum rate, [S] is substrate concentration, K_m is Michaelis-Menten constant.

Figure 2.8 shows the determination of initial rates from slopes of Michaelis Menten- plot, whereas each rate measured should be an initial velocity, by taking the slope of a progress curve ([S] plotted versus time) at t = 0. The reaction should be demonstrated to be linear over time interval and these slopes are the initial rates, V_0.

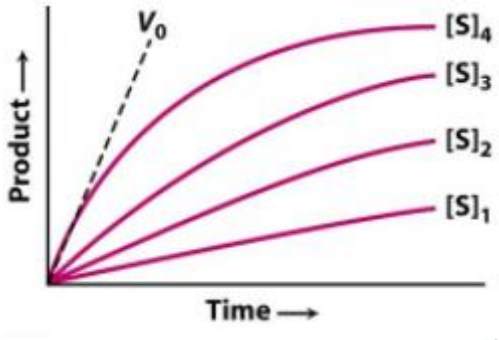


Figure 2.8: The Determination of Initial Initial Rates from Slopes of Michaelis Menten Plot

When [S] = K_m, then $V_0 = V_{max}/2$. Thus, K_m is equal to the substrate concentration at which the reaction rate is half its maximal value. Higher V_{max} means faster reaction and better catalysis. K_m indicates the affinity of the substrate for the enzyme whereby, low K_m means high affinity, and that means the enzyme binds the substrate strongly. The reaction will have a different K_m for each substrate as shown in Figure 2.9.

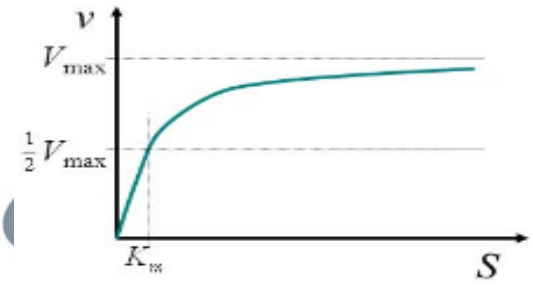


Figure 2.9: Reaction Rate Plotted Agains Against Substrate Concentration for a Reaction Obeying Michaelis-Menten Kinetics

2.8.1.2 Transformation of Michaelis Menten Equation to Lineweaver-Burk

When plotting experimental data, employing a straight line is more easier. The important terms in enzyme kinetic such as K_m and V_{max} were determined by linear regression using the Lineweaver-Burk approach. This is known as a linear transform Figure 2.10.

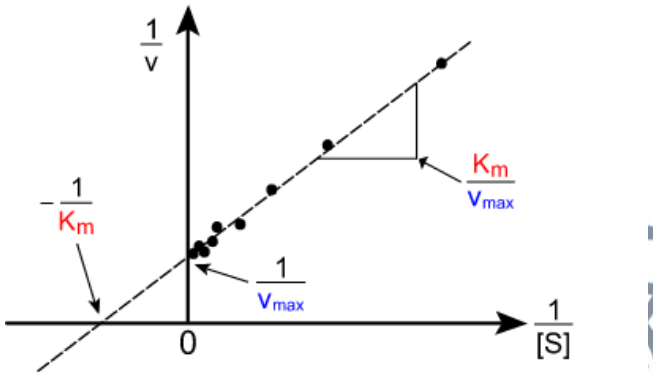


Figure 2.10: Linearization of Michaelis Menten to Lineweaver-Burk Plot

If we use y instead of $1/V_0$ and x for 1/[S], this is now the standard equation for a straight line, with slope = K_m/V_{max}, and y intercept=$1/V_{max}$. Slopes and intercepts are relatively easily obtained from a linear graph, also known as the Lineweaver-Burk Plot. Although there is no data with negative x values, the negative x-intercept can be used to obtain K_m or K_i if inhibition occurs (Romero et al., 2007). X-intercept is –y-intercept/slope. When the y-axis is $1/V_0$, its intercept is $1/ V_{max}$. Similarly, when the x-axis is 1/[S], then the intercept is $-1/K_m$.

$$1/V_0 = \frac{K_m + [S]}{V_{max}[S]} \tag{2.7}$$

$$1/V_0 = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{2.8}$$

2.8.2.3 The Ping Pong Bi-Bi Kinetic Model

The Ping Pong Bi-Bi Kinetic Model has been accepted method and it is the most used kinetic models for explaining lipase catalysed reaction kinetics (Kuo et al., 2020). Enzyme inhibition by one or more substrates is a very common phenomenon when

using enzymatic systems. The steps of the proposed mechanism considered are: First, the enzyme binds to fatty acid forming the acid–lipase complex. This complex then isomerizes to form the acyl–enzyme intermediate and a molecule of water is displaced. After that, the alcohol binds to the acyl-enzyme complex and the ester is formed and free lipase. Alongside this, inhibition substrate also combines with the free lipase and forms dead-end complex that would not interfere with the formation of the product (Cavallaro et al., 2019).

Some examples of studies implementing the mentioned model include the work by Elias et al. (2019) in the synthesis of butyl butyrate by esterification of butyric acid with butanol by using *Candida rugosa* lipase immobilized on chitosan-nanocellulose hybrid composite (CRL/CS-NC). The kinetic was found to obey the Ping Pong Bi-Bi mechanism with butanol substrate inhibition. Ping Pong Bi-Bi kinetics with inhibition of ethyl caprate and butyric acid were also observed in esterification of ethyl butyrate with lipase from *Candida rugosa* (Devi et al., 2017). In the work reported by Bezbradica et al. (2013), the kinetics of L-ascorbyl oleate synthesis catalysed by immobilized lipase from *Candida antarctica* in acetone was investigated and experimental data were successfully fitted with a ping pong bi–bi kinetic model with substrate inhibition.