CHAPTER 1

INTRODUCTION

1.1 The Study's Background

Enzymes are biological and environmentally friendly catalysts with high effectiveness and efficiencies for use in a wide variety of processes. Interests on their use as catalysts are mainly due to their high selectivity and activity, usually under mild operating conditions and in aqueous environment (Ferreira-Leitão et al., 2017; Francolini et al., 2020). For these reasons, enzymes have become extremely important catalysts, with great potentials for many practical and industrial applications ranging from food to pharmaceuticals (Wohlgemuth, 2010; Zdarta et al., 2018).

Among the enzymes known, lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) are the most popular due to their abilities to catalyse the hydrolysis of triglycerides to glycerol and free fatty acids and the reverse reactions (Zaidan et al., 2010). The demand for lipase in industry is due to their abilities to catalyse several reactions such as esterification, transesterification and aminolysis. They are also endowed for their high specificities and catalytic activities against a wide range of substrates (Dong et al., 2012), low in price, available from a number of natural sources and are stable in organic substances and aqueous media (Reshmi & Sugunan, 2013; Kumar et al., 2013).

Microbial lipase from the *Candida rugosa* species is a remarkably significant enzyme, highly favoured for its broad substrate specificity and high activity. It has been widely used in various industries such as cosmetics, detergents, pharmaceutical, biodiesel productions and food processing (Kartal et al., 2007; Zheng et al., 2013;

Reshmi & Sugunan, 2013; Zhao et al., 2018). It has also been used in many esterification reactions operating in organic medium, under mild temperature and pH conditions, and requires lower energy consumptions as compared to chemical catalysts (Sri Kaja et al., 2018).

However, lipases in the free form are not reusable which limits their industrial applications with regards to cost effectiveness. Lipases in the free form are flexible protein molecules, which can be easily denatured and inactivated under extreme pH, temperature and polarities of organic mediums. This form of lipase is also highly soluble in aqueous solutions, which makes their recovery rather difficult, causing depletion of long-term stability during operation (Idris & Bukhari, 2012).

In order to solve the problems of instability and to improve the catalytic activity and reusability of free enzymes, several techniques including immobilization, structural modification, and use of additives have been investigated (Dong et al., 2012). Among these attempts, the immobilisation technique is the most widely utilised method for imparting the desirable characteristics of heterogeneous catalysts in enzymes. (Won et al., 2005).

Immobilization technique offers several advantages, which include reducing the operational costs involving enzyme in industrial processes and improve enzyme stability and activity. Hence, increase the opportunities for higher productivity and possibility for applications in various designed reactors, ease separation of enzymes from products and reduce cost for downstream processing (Idris & Bukhari, 2012). To date, lipases have been immobilized onto a variety of supports through physical adsorption, covalent binding, ionic interactions or entrapment. Among these methods, enzyme immobilized via physical adsorption is the most straightforward and extensively used method.

The physical adsorption method of immobilization is also low-cost and imposes lower destructive effects on the enzymes structures compared to the chemical means of immobilization and modifications (Šekuljica et al., 2016; Ismail and Baek, 2020). Hydrogen bonding, van der Waals forces, and hydrophobic interactions are among the forces that exist between supports and enzymes in this approach (Babaei et al., 2014).

In general, the chemical, biological, mechanical, and kinetic properties of the enzyme are all influenced by the interactions between the enzyme and the support material (Srivastava et al., 2013). Over the years, immobilization of enzymes and whole microbial cells has been carried out using a variety of solid supports (Ramos, 2014). This includes immobilization onto octyl-glyoxyl agarose beads (Rueda et al., 2015), sol-gel hybrid coating films (Yuce-dursun et al., 2016), nanocomposite membranes (Aghababaie et al., 2018), Celite 545, Sephadex G-25, and chitosan (Kaja et al.,2018), corn husk (Nuraliyah et al., 2018), montmorillonite K-10 (Ozdemir et al., 2020) and vermiculite (Duman et al., 2020).

Among the various supports for immobilization, natural clays can be readily found in abundant in nature, inexpensive, environment friendly, possesses high surface area, and can be modified to suite a variety of applications (Duarte-Silva et al., 2014). In addition, they possess appreciable chemical and mechanical stabilities, making them excellent supports for immobilization of biocatalysts. Due to the presence of silanol groups in clays that act as attachment sites for bioactive species, immobilization of enzymes on clays have been of much interest among researchers (Reshim & Sugunan, 2013).

In this study, kaolin which is a kaolinite mineral clay with empirical formula of $Al_2Si_2O_5(OH)_4$ and theoretical chemical compositions of 46.54% SiO_2, 39.50% Al_2O_3 and 13.96% H_2O (Mgbemena et al., 2013) have been used as support for the

immobilization of lipase from *Candida rugosa*. The mineral clay particles are usually between 0.2-2.0 μm in size and the kaolin 1:1 layer is made out of a single silica tetrahedral sheet and a single alumina octahedral sheet joined. (Li et al., 2012).

Two-thirds of the oxygen atoms in the layer common to the octahedral and tetrahedral sheets are shared by silicon and aluminium, where they exist as O^{2-} rather than OH^-. It is the silica and alumina content of kaolin which serve as immobilization sites for various enzymes (Ajayi et al., 2012) and this contributes to the excellence in the performances of immobilized enzymes (Šekuljica et al., 2016; Tanaskovic et al., 2017).

The physico-chemical characteristics of clay, such as pore structure, cation exchange capacity, particle size, adsorptive property, catalytic activity, and mineralogical property are greatly affected by thermal treatments (Zivica et al., 2011). Thermal treatments at temperatures lower than 450 ºC produces glass phase together with the crystallization of other phases such as mullite (Gamelas et al., 2014; Erasmus, 2016). Interestingly, calcination of kaolin clay at temperatures in the range of 450-750 ℃ leads to transformation of kaolin into metakaolin ($Al_2O_3 \cdot 2SiO_2$). These temperatures are high enough to cause loss of hydroxyl group in the kaolin and cause the crystal structure to collapse, hence amorphous structure to be produced. Metakaolin contains exclusively 50-55% SiO_2 and 40-45% Al_2O_3 when determined by weight (Poon, 2001). Other oxides that can be found in trace concentrations in metakaolin include Fe_2O_3, TiO_2, CaO, and MgO. Besides kaolin, metakaolin also offers good potentials for enzyme immobilization due to the presence of silica and alumina, the required substances for effective immobilization of enzymes (Ajayi et al., 2012; Tanaskovic et al., 2017).

1.2 Problem Statement

Currently, most ester productions involved in the perfumery, flavor and fragrance industries are still produced by traditional methods, which include extraction of essences from natural sources followed by the syntheses of their ester derivatives via chemical routes (Annapurna Devi & Bhanu Radhika, 2018). The use of chemical methods of syntheses causes several problems such as formation of undesired and toxic by products, hazardous to the environment, formation of low-quality products with impurities and difficulty in product recovery which requires extensive purification steps to separate products from the reaction mixture. On the other hand, extraction of esters from natural sources fails to meet its demand which in turn leads to increase in cost of the compound.

In turn, the use of free enzymes as catalysts is limited in extreme conditions (e.g. temperatures, solvents, pH). Free enzymes are also non-reusable, which defeat their use in robust industrial settings that sometimes require them to be used in specially designed reactors. Although the use of immobilized enzymes is a great solution to these problems, some enzyme immobilization supports do not serve as a 'good' support for free enzymes. They are either expensive, possess low adsorption capacities and/or not susceptible for further modifications.

In spite of the fact that natural kaolin clay possesses the important criteria needed for enzyme immobilization, further improvements on their adsorption capacity are still needed to ensure successful enzyme immobilization takes place. For this purpose, conversion of kaolin to metakaolin via thermal treatment alone may not be enough. Attempts to increase the basal spacing of the natural clay via chemical modification have been extensively studied with the use of organic cationic surfactant, benzyltriethylammonium chloride.

However, lack of information on the cation exchange capacity of clay may lead to unsuccessful chemical modifications, which in turn will affect interactions between enzyme and the support materials (Dong et al., 2012, Zdarta et al., 2018). Therefore, in this study the cation exchange capacity of the kaolin and metakaolin were identified prior to chemical modification, to ensure successful modification and performance of the immobilized enzymes.

1.3 Objectives of the Study

This study was conducted with the main aim of improving the catalytic performance of *Candida rugosa* lipase via immobilization onto natural kaolin and its derivatives. To achieve this, the following objectives are outlined:

1. To produce natural kaolin derivatives, which are metakaolin and organo-modified kaolin/metakaolin, through thermal treatment and organo-modification using benzyltriethylammonium chloride, respectively.
2. To characterize the physico-chemical properties of the natural kaolin, metakaolin and their derivatives using X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM) and nitrogen adsorption-desorption isotherms employing the Brunauer-Emmett-Teller (BET) and Barrett-Joyner-Halenda methods for the determination of their suitability as supports for enzyme immobilization.
3. To immobilize *Candida rugosa* lipase onto the natural kaolin, metakaolin and their derivatives via physical adsorption method.
4. To determine the catalytic activity, stability and kinetic behaviours of free and selected immobilized lipases in the esterification reaction between hexanoic acid and nonanol.

1.4 Research Scope

This study began with the derivatization of natural kaolin with the aim to increase its adsorption capacity and suitability for use as support for enzyme immobilization. Conversion of kaolin into metakaolin was done via thermal treatment (calcination) at 650 ℃. This was followed by modification of the natural kaolin and metakaolin using benzyltriethylammonium chloride (BTEACl) at amounts relative to their cationic exchange capacities (CEC). The natural kaolin, metakaolin and their derivatives were then characterized to determine the important physico-chemical properties of the supports. In this process, XRD was used to determine the d-spacing of the supports, FTIR spectroscopy was used to identify the functional groups in the supports, SEM was used to investigate the surface morphologies of the supports, while nitrogen adsorption technique was used to determine the surface areas and pore sizes of the supports. Successful derivatization of natural kaolin was distinguished through analysis using these instruments where differences between the outcomes from the analyses revealed whether natural kaolin have been successfully converted to metakaolin and modified to organo-kaolin clay. The natural kaolin and their derivatives were then used as supports for the immobilization of *Candida rugosa* lipase following simple immobilization technique of physical adsorption method. Although success of the immobilization technique depends on the hydrophobic interactions between the surfaces of the supports and the lipases, physico-chemical characterizations using XRD, FTIR, SEM and nitrogen adsorption-desorption isotherms were done to determine the success of the enzyme immobilization. Finally, the catalytic activities and stabilities of the free and immobilized *Candida rugosa* lipase were determined through their abilities in catalyzing the synthesis of nonyl hexanoate. The parameters involved were enzyme thermostabilities for free and immobilized lipases, and operational stability tested on

immobilized lipases. Kinetic behaviours of free and selected immobilized lipases with highest catalytic performances were determined via time course study conducted at various substrate concentrations. The kinetic parameters, maximal rate, V_{max}; Michaelis-Menten constant, K_m; and the nonlinear least squares regression were used to generate a model equation using SigmaPlot version 12.5 scientific data analysis and graphing software. The limiting substrates for each of the tested enzymes were then distinguished by the Ping Pong Bi Bi kinetic model.