CHAPTER 1

INTRODUCTION

1.1 General background

Enzymes are natural catalysts of biological molecules that accelerate the rate of chemical reactions several times in magnitude by providing an alternative reaction pathway, which require less activation energy compared to uncatalysed reaction. Enzymes consist of protein molecules with wide range of compositions and structural complexities precisely arranged to promote the transformation of chemicals in living systems. They are also versatile biocatalysts, capable of catalysing diverse and unique reactions that are highly specific in their catalytic mechanisms, enabling formation of structurally specific product and making them highly desirable for targeted reactions (Perez et al., 2007). Furthermore, enzymes have the ability to catalyse reactions under mild conditions with a very high degree of substrate specificity, thus decreasing the formation of by-products. Among the reactions catalysed are a number of very complex chemical transformations between biological macromolecules, which are not accessible to ordinary methods of organic chemistry. This makes them very interesting for biotechnological use. Among major applications of immobilized enzymes are in the industrial production of sugars, amino acids and pharmaceutical compounds (Brena& Batista-Viera, 2007).

In general, enzymes can catalyse reactions in different states, either as individual molecules in solution, in aggregates with other entities and as attached to

surfaces. The attached or "immobilized" state has been of particular interest to those wishing to exploit enzymes for technical purposes. The term "immobilized" refers to physically confinement or localization in a certain defined region of space. Interestingly, enzymes immobilization is often accompanied by changes in enzymatic activity, optimum temperature and pH and affinity to substrates. When immobilization takes place, enzymes catalytic activities are retained, where they can be used repeatedly and continuously. Enzymes immobilized onto water insoluble support also often possess improved storage and operational stabilities. In some industrial processes, whole microbial cells containing the desired enzymes are immobilized and used as catalysts (Brena& Batista-Viera, 2007).

Among the lipases from various sources, *Candida rugosa lipase* (CRL) received much attention due to its high activity and broad specificity (Yong *et al*., 2008). Within the hydrolase-based biocatalysis, lipases from *Candida rugosa* were firstly described as early as in the sixties, by isolating the yeast from natural soils due to its powerful lipase production capacity (Dominguez de Maria *et al*., 2006). *Candida rugosa* lipase (CRL) is a stable mesophilic lipase that has high activity and broad specificity in reaction medium (Wu et al., 2012). Furthermore, due to its low specificity with respect to the glycerol position, *Candida rugosa* lipase is very attractive enzyme for the industrial production of fatty acids, because it is able to hydrolyze all the ester bonds of triacylglycerols (Montero et al., 1993).

For the purpose of immobilizing enzymes, both chemical and physical methods have been successfully developed. For many years, researchers have been absorbed enzymes onto a variety of solid supports including inert solids (Homaei et al., 2013), ion-exchange resins (Gandhi et al., 1996), or physically entrapped/encapsulated in solids (Fan et al., 2003), such as crosslinked gels (Gregg &

Heller, 1990), microcapsules (Taqieddin & Amiji, 2004) and hollow fibers (Jurado et al., 2006). Enzymes can also be covalently bonded to solids via cross linking method using multi-functional reagents, or by creating surface reactive functional groups (Mateo et al., 2007).

The characteristics of solid supports that are desirable for biomolecular attachment include large surface area, good chemical, mechanical and thermal stability, hydrophilicity and insolubility (Perez et al., 2007). Among the various solid supports, nonporous materials possess no diffusion constraint, but have very low surface areas for protein binding. On the other hand, the high surface area, porous materials provide higher protein loading capacity. However, in cases where most of the surfaces are located internally, inefficient diffusion of solution can present major drawbacks during immobilization. It is therefore possible to engineer pore structures of porous solid for efficient diffusion of solution (Shang *et al*., 2015).

Polymers for example, are excellent candidates as supports for enzyme immobilization. This is due to their versatile chemical compositions, physical properties and widely available structural forms which can be naturally obtained, synthesized or altered. Natural polymers commonly used in the study of enzyme immobilization include polysaccharides of cellulose (Kim et al., 2015), cellulose derivatives (Murtinho et al., 1998), agarose (Prakash & Jaiswal, 2011) and chitosan (Shi et al., 2011) while synthetic polymers utilized in the study of immobilization include polystyrene (Wang et al., 2015), polyvinyl alcohol (Bai et al., 2014), polyurethane (Donato et al., 2014) and many others (Perez et al., 2007).

In this work, the use of carboxymethyl cellulose, CMC, poly (vinyl alcohol), PVA, and a blend of both were evaluated for their abilities in enhancing *Candida*

rugosa lipase performance in the esterification of butyl oleate was evaluated. CMC is a water soluble cellulose derivative formed through copolymeration of ß-D-glucose and ß-D-glucopyranose 2-O-(carboxymethyl)-monosodium salt which are connected via b-1,4-glycosidic bonds. It has several advantageous properties for use as thickener, binder, stabilizer, protective colloid, suspending agent and rheology, or flow control agent in various industries including, food, pharmaceuticals, as well as non-foods (e.g. cements, adhesives, textiles, pesticides, detergents, paper and ceramics) (Rachtanapun & Rattanapone.,2012).

Poly (vinyl alcohol) or PVA, on the other hand is a hydrophilic semicrystalline polymer produced by polymerization of vinyl acetate to produce poly (vinyl acetate) (PVAc), where subsequent hydrolysis of PVAc then produces PVA. PVA is a widely used thermoplastic polymer that is benign to living tissues, harmless, and nontoxic. This polymer is widely investigated because of its use in cross-linked products and nanofillers. PVA is a biodegradable polymer, and its degradability is enhanced through hydrolysis because of the presence of hydroxyl groups on the carbon atoms. Moreover, it is water-soluble and has a hydrophilic nature (Gaaz et al, 2015).

In biotechnological processes, it is of high importance to efficiently utilize enzymes. Identifying the optimal conditions to perform an enzymatic synthesis is therefore crucial because it helps to evaluate the reaction kinetics and derive the constants describing the kinetic behaviours of the enzymes (Lopresto et al., 2014). Kinetic studies of biocatalysts have only become important in the last few years. Although there are a great number of studies about lipase-catalyzed reactions, less information is available about their kinetic models and the parameters of control. Some of the most common model proposed for organic synthesis is the Michaelis-Menten model, but is only valid for the simplest enzymatic reaction. Therefore, a

better approach has been proposed, which is the "Ping Pong Bi Bi" model which seems to provide acceptable results in terms of reproducibility in experimental findings (Romero et al., 2007). Chulalaksananukul et al. (1990) was the first to propose the model used to explain the kinetics of immobilized *Rhizomucor miehei* lipase in the esterification between oleic acid and ethanol. This model is able to explain the kinetics of the reaction involving two substrates and two products (denoted as the "Bi Bi" reaction). The enzyme was explained to react with the first substrate (the acid) to form an acyl-enzyme intermediate before releasing water (the first product). This occurred before the enzyme reacts with the second substrate (the alcohol), to form the second product (denoted as the "Ping Pong" mechanism) (Bousquet-Dubouch et al., 2001). Since early development of the mentioned kinetic model, studies have been focused on the enzymatic esterifications involving short chain alcohols with long chain fatty acids, observing only alcohol inhibition, described by the Ping Pong mechanism (Hari Krishna &Karanth, 2001).

This study therefore aims to evaluate the viability of the thin film for lipase entrapment through characterization of lipase performance in the esterification reactions and derivation of the kinetic constants using the Ping Pong Bi Bi model. In the present work, oleic acid was reacted with n-butanol to produce butyl oleate, a compound with wide applications and uses in industries, as a diesel additive, a polyvinyl chloride plastisizer, a water-resisting agent and hydraulic fluids (Ghamgui *et al*., 2004). Although there are a number of researches used of immobilized *Candida rugosa* lipase for the synthesis of butyl oleate, the information on the kinetic behaviour of the enzyme immobilized within carboxymethyl cellulose (CMC) and polyvinyl alcohol (PVA),in the esterification reaction system for future industrial scale up continues to be relatively limited.

1.2 Research Scope

In this study, the extensive exploration on the abilities of carboxymethyl cellulose (CMC) and polyvinyl alcohol (PVA) as supports for *Candida rugosa lipase* immobilization were done due to the availability and possibility in alteration of pore sizes and characteristics of the materials suitable for enzyme immobilization.

To ensure successful immobilization and enhancement of lipase activity take place, the suitable compositions of the polymers and enzyme blends in the formation of thin film were extensively studied, together with the factors influencing immobilization, enzyme activity, stability, reusability and kinetics. In this study, the polymer supports and enzymes were characterized using SEM, EDX, XRD, FTIR and DSC to determine their suitabilities as support for enzyme immobilization. The data obtained from the instrumentational analysis were useful to explain the performance and effectiveness of the materials as supports. In all cases, comparison between the native and immobilized CRL was done in the esterification of butyl oleate. The enzymes performances as affected by time and substrates concentrations were subsequently used in the determination of the enzyme kinetic behaviours with the assistance from using SigmaPlot and GraphPad (Prism 5) softwares.

1.3 Research Objectives

In general, the present study was developed to address drawbacks encountered in reactions involving enzymes. The objectives of the study are stated as below:

1. To prepare the thin films containing CMC, PVA and/or CRL with suitable enzyme-polymer compositions with consideration of their mechanical strength and catalytic abilities.

2. To characterize the prepared thin films using SEM, EDX, XRD and FTIR

3. To compare the activity, stability, reusability and kinetic behaviour between the immobilized and native CRL on the enzymatic systems of esterification process.