THE IMMOBILIZATION OF *CANDIDA RUGOSA* LIPASE ON THE CHITOSAN BEADS

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by

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Muliati Binti Mohd Romli

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LIST OF SYMBOLS

Symbol		Unit
A_{400}Blank	The absorbance of the sample at wavelength 400 nm	-
A_{400}Sample	The absorbance of the blank at wavelength 400 nm	-
BCA	Bicinchoninic Acid	-
DF	Dilution factor	-
E_f	Final enzyme protein in the soluble enzyme after immobilization	g/mL
E_i	Initial enzyme protein in the soluble enzyme before immobilization	g/mL
t	Time of assay per unit definition	minute
V_E	Volume of enzyme	mL
V_T	The total volume of assay	mL
IY	Immobilization of Yield	%
ε_{ext}	Micromolar extension coefficient of p-Nitrophenol at 400 nm	-

LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid Solution
CCD	Central Composite Design
DF	Degree of Freedom
DHA	Docosahexaenoic Acid
EDTA	Ethylenediaminetetraacetic Acid
EPA	Eicosapentaenoic Acid
H_2O	Water
GA	Glutaraldehyde
GC	Gas Chromatography
GG	Guar Gum
IU	International Unit
KH_2PO_4	Potassium Dihydrogen Phosphate
Na_2HPO_4	Sodium Hydrogen Phosphate
PNPB	p-Nitrophenyl Butyrate
RSM	Respond Surface Method
TGA	Triglycerides Acid
UV	Ultraviolet
2-D	Two Dimension
3-D	Three Dimension

CANDIDA RUGOSA LIPASE TERSEKAT GERAK PADA MANIK CHITOSAN

ABSTRAK

Enzim tersekat adalah pemangkin penting yang digunakan terutamanya untuk meningkatkan kadar tindak balas. Salah satu cara enzim untuk disekat gerak adalah dengan ikatan kovalen. Dalam kajian ini, *Candida rugos*a (*C. rugosa*) lipase berjaya disekat gerak oleh kaedah ikatan kovalen ke manik kitosan aktif. Sekat gerak lipase pada manik kitosan telah disahkan dengan menentukan aktiviti lipase semasa hidrolisis bahan piawai. Kesan kepekatan lipase dan glutaraldehyde (GA) terhadap hasil sekatan gerak dan hasil aktiviti disiasat. Secara umum, kepekatan GA dan lipase memberikan kesan yang signifikan terhadap hasil sekat gerak dan hasil aktiviti. Memandangkan peningkatan kepekatan GA, hasil aktiviti akan menurun, sementara semakin meningkat kepekatan lipase, hasil sekat gerak berkurang. Hasil sekat gerak tertinggi adalah 77.335 %, di mana dicapai pada 6 % daripada GA dan 0.001 g / mL daripada *C. rugosa* lipase. Selepas itu, aktiviti tertinggi ialah 50.3613 IU, di mana diperolehi pada 2 % daripada GA dan 0.010 g / mL daripada *C. rugos*a lipase.

Pengoptimuman parameter proses sekat gerak dijalankan melalui Kaedah Permukaan Respon (RSM). Telah didapati bahawa keadaan optimum untuk enzim disekat gerak adalah pada 4 % kepekatan gluatarldehyde dan 0.005 g / mL daripada *C. rugosa* lipase yang memberikan 65.14 % hasil imobilisasi dan 32.84 IU daripada aktiviti unit. Lipase tersekat digunakan berulang kali dalam tindak balas hidrolisis. Telah didapati bahawa enzim sekat gerak lebih stabil dan mempunyai keupayaan untuk digunakan beberapa kali. Oleh itu, lipase tersekat gerak pada sokongan chitosan diaktifkan boleh dilakukan dan boleh digunakan semula sebanyak 7 kali. Walau bagaimanapun, aktiviti ini berkurangan kepada 53.67 % selepas kitaran ke-7.

THE IMMOBILIZATION OF *CANDIDA RUGOSA* LIPASE ON THE CHITOSAN BEADS

ABSTRACT

The immobilized enzyme is an important catalyst which used mainly to increase the rate of reaction. One of the methods to immobilize the enzyme is by covalent bonding. In the present research, *Candida rugosa* (*C. rugosa)* lipase was successfully immobilized by the method of covalent bonding onto activated chitosan beads. The immobilization of lipase on the chitosan beads was confirmed by determining lipase activity during hydrolysis of a standard substance. The effect of lipase and glutaraldehyde (GA) concentrations on immobilization yield and activity yield were investigated. In general, the concentration of GA and lipase gave significant effect on immobilization yield and activity yield. As the GA concentration increase, the activity yield was decreased, while as the lipase concentration increase, the immobilization yield relatively decrease. The highest immobilization yield was 77.335 %, were achieved at 6 % of GA and 0.001 g/mL of *C. rugosa* lipase. After that, the highest activity was 50.3613 IU, where obtained at 2 % of GA and 0.010 g/mL of *C. rugosa* lipase.

The optimization of immobilization process parameters was carried out via Respond Surface Method (RSM). It has been found that the most optimal condition for immobilization was at 4 % glutaraldehyde concentration and 0.005 g/mL of C. *rugosa* lipase which gave 65.14 % of immobilization yield and 32.84 IU of unit activity. The immobilized lipase was used repeatedly in the hydrolysis reaction. It was found that the immobilized enzyme was more stable and has the ability to be used multiple times. Therefore, immobilization of lipase onto activated chitosan support was feasible and it can be reused for 7 times. However, the activities reduce to 53.67 % after the 7^{th} cycle.

CHAPTER ONE

INTRODUCTION

1.1 Research background

Enzyme is a type of protein that can lower the activation energy then increase the rate of reaction where it commonly found in plants, animal digestion system, and microbes. The application of enzyme as a catalyst for an industrial process will improve the productivity. Among all the enzymes, lipase has caught manufacturer interest as its useful for the production of various types of chemicals (Vassiliadi et al., 2018). Lipase is commonly used in the production of biofuel, lubricants, waxes, flavors, and fragrances. This is due to the versatility of lipase that capable to catalyze esterification, interesterification, and transesterification reactions in non-aqueous media (Houde et al., 2004).

Nowadays, many researchers and industries have switched to enzyme rather than chemical-based catalyst as it appears to be more green, sustainable and other advantages (Vassiliadi et al., 2018). Immobilized enzymes are preferable compared to free enzyme because the handling of the immobilized enzyme is easier for batch or even in a continuous process. In addition, the immobilized enzyme is reusable which is an added advantage to increase the productivity and reduces cost. There are several methods to immobilize the enzyme. These methods are including adsorption, covalent bonding, cell to cell cross-linking, encapsulation and entrapment.

From the previous study conducted by Knezevic et al., (2006), high activity retention was obtained when immobilizing enzyme in covalent bonding. The immobilization of enzyme by covalent bonding on a polymeric support is more durable

compared to the other methods since the enzyme is irreversibly bonded. The polymeric support of natural origin is more desirable for enzyme immobilization due to biodegradable properties and gives low environmental impact. For an instant, use a chitosan. Chitosan beads are highly porous and suitable for enzyme immobilization (Zhou et al., 2003). It provides a high surface area for the enzyme to bind. In 2003, a research had been conducted by Chiou and Wu (2004) using chitosan beads. From the research, it was found that the wet chitosan beads have highest enzyme activity compared to the dry chitosan.

Traditionally, esters are produced from plant sources using an extraction methode. But these methods are not viable nowadays due to their presence in fewer quantities. While Chemical production of flavor esters are not environmentally friendly and have toxic to the health. Nowadays biocatalytic synthesis had been more preferable used in researchs and industries (Garlapati and Banerjee, 2013). Using biocatalyst such as immobilized enzyme generally is a green process of producing the ester. Biocatalysts provide mild reaction conditions, high reaction selectivity and able to work in a solvent-free system (Neil W. B., 2013).

1.2 Problem statement

Free enzyme is not reusable, hence it less preferable to be used in industry as it increases the operational cost. As a solution, several methods have been developed to immobilize the enzyme. Immobilized enzyme offers more advantages compared to the free enzyme. Free enzymes also are not flexible to use in sensitive industries such as pharmaceutical. But the immobilized enzymes are more flexible and can be applied in various reactor configurations. Other than that, it's also such a simple catalyst that is easily reproduced, and provides a promising solution for catalytic reaction in the aqueous and non-aqueous system (Knezevic et al., 2006).

There are several techniques for the immobilization of enzymes such as covalent bonding, adsorption, cell to cell cross-linking, entrapment and encapsulation. Each technique will give a different result to an efficiency of the enzyme. In this research, we focus on the covalent bonding technique as it having an advantage of irreversible binding of the enzyme to the support matrix (Trevan, 1988). This method needs a support that formed a covalent bonding with the enzyme. Chitosan is an excellent supports derived from a natural resource for immobilization of lipase.

The main objective of this research is to investigate the immobilization *C. rugosa* lipase by using chitosan beads as a support. Hence, through this research, we will investigate the effect of immobilization process parameters on the performance of immobilized enzyme. The concentration of GA solution and concentration of lipase *C. rugosa* lipase solution are among the parameters that will be studied.

The optimum concentration of GA lipase for immobilization was determined. Subsequently, the reusability of the enzyme also was investigated. Theoretically, as the GA concentration increases, the activity yield was decreased. While, when the *C. rugosa* lipase increases, the immobilization yield was decreased.

1.3 Research objectives

The objectives of the study are:

i. To immobilize lipase from *C. rugosa* lipase onto the chitosan beads.
ii. To study the effect of lipase and GA concentration to immobilization and activity yield in the immobilization of *C. rugosa* lipase.
iii. To study the reusability of immobilized C. *rugosa* lipase.

1.4 Scope of study

The study was conducted according to the following scopes;

(i) The preparation of the support

Chitosan beads were used in this study due to its high porosity and can form a more covalent bond with the enzyme. Activation of the chitosan beads plays important roles in achieving optimum efficiency of the support. Different concentration of GA solution will affect the degree of activation of the support. In this research, 2-10 % (v/v) of GA was used during chitosan beads activation.

(ii) The immobilization of lipase

The immobilization process requires activation step of chitosan beads. Then the activated support was soaked in *C. rugosa* lipase solution. The concentration of lipase was varied between 0.001-0.002 g/mL (w/v) to study the effect of different concentration of enzyme solution to the immobilization of lipase. After immobilization process completed, protein assay and enzyme activity assay were conducted to measure the immobilization yield and activity yield, respectively.

(iii) The reusability of immobilized *C. rugosa* lipase.

The optimum condition of lipase immobilization was used to study the reusability of the immobilized lipase. The immobilized lipase was used repeatedly by using hydrolysis reaction of standard p-Nitrophenyl Butyrate (PNPB) and their activity was measured at each cycle. The reduction of activity was calculated by measuring the activity of enzyme before and after reaction. The reaction process was repeated until a constant number of enzyme activity was achieved.

CHAPTER TWO

LITERATURE REVIEW

2.1 Enzyme

Enzymes or biocatalyst is a protein that is able to catalyze chemical reactions which required for the survival and reproduction of animals, plants, and microorganism. Generally, the enzyme is acting as a catalyst to facilitate the rate of reaction and it is currently being used to assist the productivity in various industries.

The mechanism of the enzyme assisting the reaction process is a complex phenomenon. In general, it involves three steps. Firstly, the substrate is non-covalently bonded into the functional group in the binding subsites of the enzyme. Then, the bonds interaction will change the tertiary structure of the enzyme's surface. This will lead the substrates to correctly bind to the binding sites, and then the functional groups will activate the amino acid residue in the active site for catalysis purposes. Most important side in the enzyme is an active site, where the reaction occurs. In binding steps, the amino acid residue in these sites moved to allow better interaction to the substrate. Table 1 shows the classification of an enzyme that known and has been used in the bio-catalysis processes (Khlaus et al., 2005).

Table 2.1. The enzyme classification (Khlaus et al., 2005)

Enzyme Classes and sub classes(Function)	Remarks
1. Oxidoreductases	Cosubstrate is required
(Oxidation-reduction reaction)	Two-substrate reaction
i. At –CH-OH	
ii. At -C=O	
iii. At –C=C-	
2. Transferases	Two substrate reactions, one substrate must be activated
(Group transfer reaction)	
i. C1-groups	
ii. Aldehyde- or keto group	
iii. Acyl-group	
iv. Glycosyl-group	
3. Hydrolases	Two substrate reactions, one of these is H_2O
(strictly transferases that transfer groups to H_2O)	
i. Ester Bond	
ii. Glycoside bond	
iii. Ether bond	
iv. Peptide bond	
v. Amide bond	
4. Lyases	
(Non-hydrolytic bond-breaking reactions)	One-substrate reaction → bond breaking
i. C-C	Two-substrate reactions ← bond formation
ii. C-O	
5. Isomerase	
(Isomerization reactions)	
i. Racemizations	One-substrate reactions
ii. *Cis-trans*-isomerizations	
iii. Intramolecular oxidoreductases	
6.Ligases	
(Bond-formation reactions)	
i. C-O	Required ATP as a cosubstrate
ii. C-S	Two-substrate reactions
iii. C-N	
iv. C-C	

For each process, suitable enzyme needs to be determined to enhance the efficiency of process. In screening process, the sources of enzyme and it properties must be suited to the purpose of the reaction processes. Furthermore, the selectivity and stability of enzyme under specific reaction condition must be considered too.

2.2 Lipase in industries

Since the early 2010s, more than 200 lipases that have been used in the industries. Table 2.2 below shows the most common lipase used.

Table 2.2. Commercial lipase (Gaur and Khare, 2017)

Commercial	Sources	Supplier
Lipozyme RMIM	*Rhizomucor miehei*	Novozyme A/S, Bagsvaerd, Denmark
Lipase A "Amano"	*Aspergilus niger*	Amano Enzyme, Inc., Nagoya, Japan
Lipolase 100T	*A. niger*	Novozyme A/S, Bagsvaerd, Denmark
Lipomod	*Rhizopus oryzae*	Novozyme A/S, Bagsvaerd, Denmark
Lipase F-AP15	*R. oryzae*	Novozyme A/S, Bagsvaerd, Denmark
Lipase AYS "Amano"	*Candida rugosa*	Amano Enzyme, Inc., Nagoya, Japan
Lipase MY	*C. rugosa*	Meito Sangyo, Tokyo, Japan
Novozyme 435	*Candida antartica*	Novozyme A/S, Bagsvaerd, Denmark

Nowadays, industries have switched to the biocatalytic flavor synthesis. In the biocatalytic process, it used mild operating conditions, having fewer side reactions, and produce high purity flavor compounds without any expensive separation techniques

(Garlapati and Banerjee, 2013). Lipases are the most widely investigated among of all enzymes. Lipase-catalyzed reactions are able to prevent degradation of starting materials and reduce potential of side reactions (Awang et al., 2000). Owing to their greater stability at high temperatures, over a wide pH range, easy handling, and repeated use, immobilized lipases have been employed in a number of industrial reactions.

According to Lee et al., (2006) lipase also had been used in the modification of fats and oils and the synthesis of flavor. Lipase commonly found in foods and cosmetics industry. lipase used in manufacturers of food products such as ice cream, seasonings, and drinks to impart their flavor of choice. It's also used in the manufacture of cosmetics and detergents (Webmaster, 2017).

2.2.1 Lipase for esterification

Esterification is a process of forming an ester from a carboxylic acid. Figure 2.2 shows the general reaction of esterification reaction.

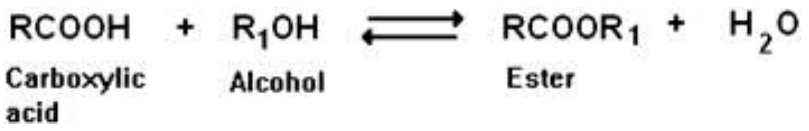

Figure 2.1. The general reaction of esterification reaction

In general, esterification carboxylic acid with alcohol catalyzed by immobilized enzyme will produce an ester. Ester is commonly used as solvents, plasticizers, flavors and fragrances, agrochemicals and other compounds (Carmo et al., 2009). Back then, esterification is a major chemical process for production of biodiesel (Suwannakarn et al., 2009). However, in esterification reaction is limited by chemical equilibrium, and separation product must be considered in favor to obtain attractive yields. Here,

conventional and reactive distillation technologies emerge as possible process alternatives to overcome equilibrium limitations and in order to evaluate their technical and economic flexibility (Leyva et al., 2013).

2.2.2 Lipase for transesterification

In contrast to esterification, transesterification is the process of converting a carboxylic acid ester into a different carboxylic acid ester. The biodiesel industry is an example of industries that using immobilized lipase in the transesterification reaction. biodiesel is derived from Triglycerides Acid (TGA) and it benefits as renewable sources, biodegradable, and also nontoxic fuel (Fukuda et al., 2001). Figure 2.3 below shows the general equation of transesterification of TGA with alcohol.

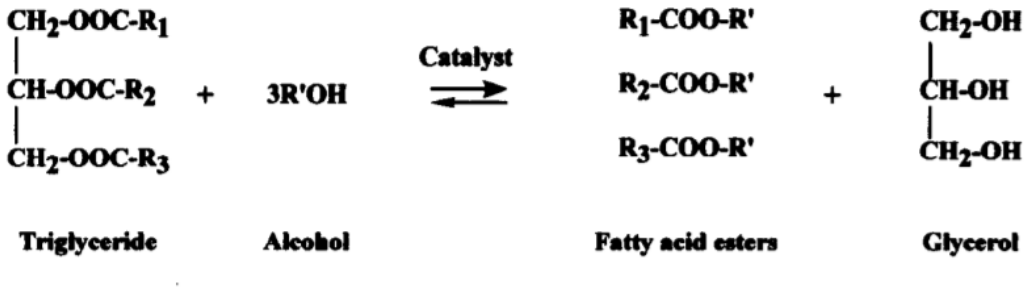

Figure 2.2. The general reaction of transesterification of TGA with alcohol

Bhangu et al., (2017) had study the transesterification of biodiesel where canola oil in the presence of lipase *C. rugosa* undergo transterifation process to produced biodiesel using the ultrasonic horns and plate transducer. From this study, it was found that the process is efficient at low ultrasound intensity.

2.2.3 Lipase for hydrolysis

Meanwhile, hydrolysis is a breaking bond process of an ester or amide molecule by using water. Enzyme-catalyzed by hydrolysis had been presented as an alternative process because it carried out under mild reaction and minimize the by-product production (Chiplunkar et al., 2018). One of the industries that using the immobilized enzyme in hydrolysis process is hydrolysis of fish oil. In this process, hydrolysis used to extract of omega-3 fatty acids, that mainly consist Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) from fish oil (Fernández-Lorente et al., 2011). DHA is important for the growing up kids neuronal function as it enhances the ability of learning and visual acuity while EPA good for prevention of cardiovascular diseases. So, by using the immobilized lipase on porous supports will enhance it hydrolysis process. On another side, there are some limitations on hydrolysis process. According to Chiplunkar et al., (2018), it limitation can be overcomed by the addition of solvent and ultrasound that will improve the mass transfer. As reported by others, enzyme-catalyzed hydrolysis has been presented as an alternative to the chemical route since it is carried out under mild reaction conditions, minimizing the formation of undesirable byproducts.

2.3 Immobilized enzyme

The free enzyme has a common problem such as lack of structural stability and cannot be reused (Izrael et al., 2015). Other than that, a high cost of free enzyme, make it economically unattractive for the industries (Lee et al., 2006). Due to this problem, the immobilized enzyme seems to be more common to be used.

The immobilized enzyme is an enzyme that attached to insoluble material after undergoing some processes. The main purpose of immobilization process is to increase

enzyme stability and to enable long-term operation (Ashly et al., 2011). Other than that, the immobilized enzyme can be used continuously and the enzyme can be reused. The reaction catalyzed by immobilized enzyme requiring mild reaction conditions, their high specificity and ease of processing make it more desirable to be used (Jeromin and Zoor, 2008). Convenient in handling and easy for separation process also made it desirable to be used in industrial applications (Ashly et al., 2011).

There are several immobilization methods of an enzyme that have been used in industries. Generally, immobilization can be classified into two groups, physical and chemical method. These methods are including adsorption, covalent bonding, cell to cell cross-linking, encapsulation, and entrapment. Enzyme immobilization onto solid support leads to increase in thermal and operational stability (Vassiliadi et al., 2018). Figure 1 shows the principles of enzyme immobilization (Khlaus et al., 2005).

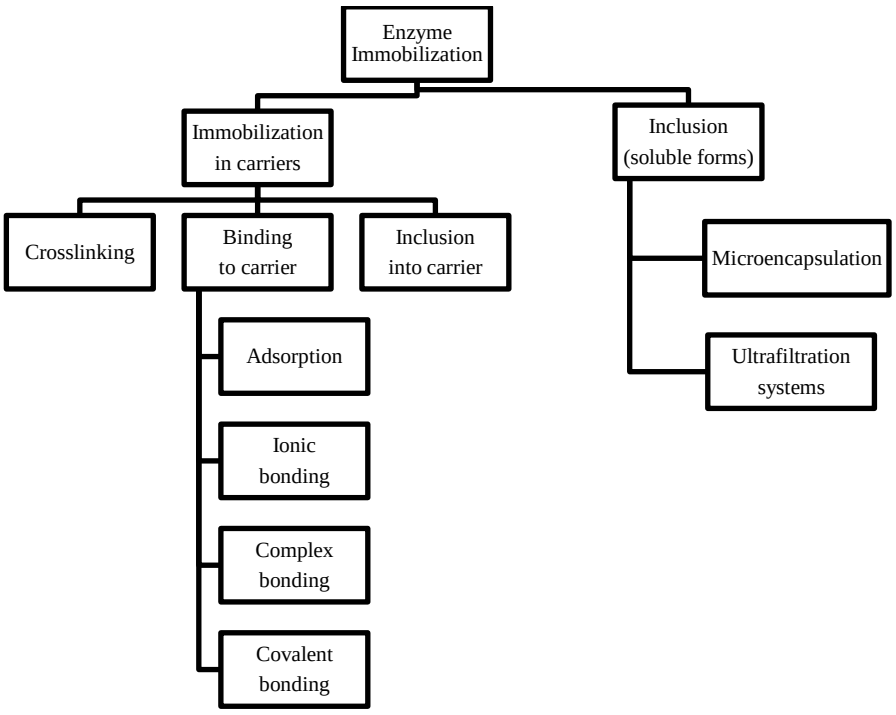

Figure 2.3. Principles of enzyme immobilization

Firstly, Adsorption is a common method of enzyme immobilization that forms a weak bond to the support or carrier. This method is preferable since it's simple and inexpensive so it reduces the operational cost of immobilization process. Other than that, by applying this method, the active site will avoid any conformational change or destruction. But the disadvantages of this method is it forms weak binding force between the enzyme and the carrier and the adsorbed enzyme leaks from the carrier during the reaction (Izrael et al., 2015). Wang et al., (2006) reported that adsorption method will make the immobilized lipase less sensitive to pH and higher optimum temperature.

Next, covalent bonding is an immobilization method that will form a covalent bond between enzyme and support or carrier**.** This method is the most frequently used in the industries. The covalent bond formed provides a permanent link between the enzyme and its support. The enzyme immobilized through covalent bonding can be reused more often than other available immobilization methods, such as adsorption and entrapment.

Moreover, cell to cell cross-linking or called copolymerization is a method which enzyme directly linked by a covalent bond between a various group of the enzyme without any matrix or support involved. The cross-linking offers high stability which preferable for industrial used but it also has a disadvantage as it can cause steric resistance by the substrate (Lee et al., 2006).

Others immobilization approach is by using an encapsulation method. This method immobilizes enzyme by enclosing enzyme in a semi-permeable membrane. By using this method, it can enhance its thermal stability, resistance to organic solvents and exhibits high enzymatic activity, while maintains the structure of the enzyme. These advantages made enzyme more stable and can be reused more often. But on another

side, entrapped enzyme did not have a suitable conformation to form the product (Hu et al., 2013). It makes the catalytic efficiency of the entrapped enzyme was low. This study had found that encapsulated lipase particularly exhibited high conversion. In the same research, it has been observed that excellent selectivity was obtained for the encapsulated lipase (Ozyilmaz et al., 2014).

Lastly, entrapment is a method that uses physical restriction of the enzyme within confined space. The advantages of this method are mild gelling properties and non-toxicity. However, a common problem with entrapment is leakage of the entrapped enzyme. It needs coating on the alginate gel surface, such as silica in order to overcome the leakage problem (Won et al., 2005). The coating will provide higher operational stability to the alginate beads. But with the addition of a coating, it will increase the cost and make the cost of immobilization of the enzyme higher compared to the others.

2.4 Supports for enzyme immobilization

The support for enzyme immobilization can be divided into three major group which are natural polymers, synthetic polymers (eg: polyanilines) (Ashly et al., 2011), and inorganic polymers. Natural polymers are a group of polymers that can form covalent bonds with the enzyme. Collagen and gelatin are the example of natural polymers. Next, synthetics polymers are polymers that insoluble and had a porous surface that can trap the enzyme.

2.4.1 Chitosan

Chitosan is a poly-*N*-acetylglucosamine support that has been used extensively in various applications field. Figure 2.4 shows the structure of chitin and chitosan (Pokhrel et al., 2016).

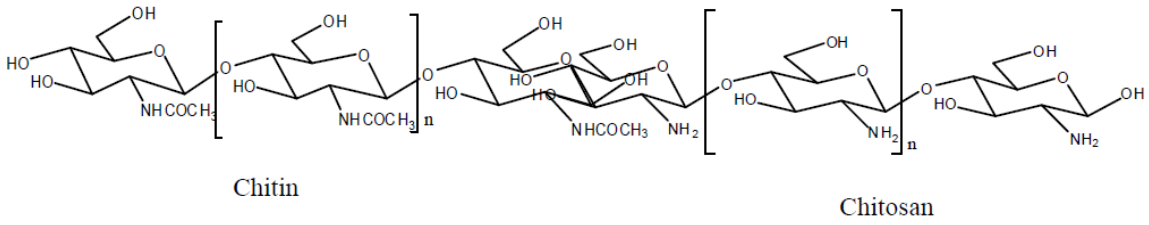

Figure 2.4. Structure of chitin & chitosan

Chitosan is a highly porous bead (Zhou et al., 2003). It can be derived from deacetylation of chitin and then forms chitosan. Chitin is biopolymer that mostly found in nature such as the prawn shell. Figure 2.5 below shows the conversion of chitin into chitosan. It's also a copolymer of N-acetyl-D-glucosamine (polymeric chain) units linked by alpha-(1-4) glycosidic bond (Pokhrel et al., 2016).

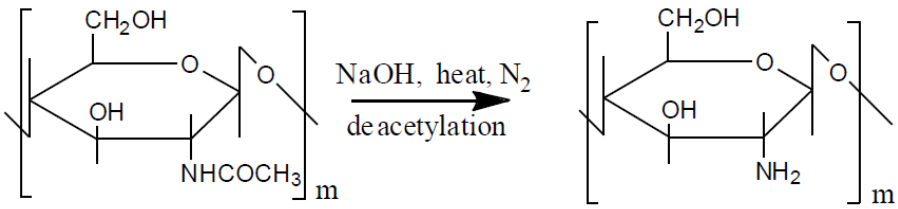

Figure 2.5. Conversion of chitin into chitosan

There are several advantages of using chitosan as a support, such as it excellent on its biodegradability, biocompatibility, strong hydrophilicity and also had a good antibacterial properties (Satar et al., 2017). It also non-toxic, able to absorb liquids and able to form films and beads (Vassiliadi et al., 2018). Although chitosan has several advantages as a support, limited studies that using chitosan as a support in immobilization of enzyme.

2.4.2 Alginate

Alginate is consist of 1,4-linked β-D-Mannuronic acid and α-L-Guluronic acid that arrange in different proportions and sequential arrangements (Ertesvåg and Valla, 1998). Their mild gelling properties and non-toxicity make it's favorable to be used in industry. Entrapment is one of the immobilization methods at insoluble beads. From the research that had been conducted on lipase immobilized in alginate gel by won and co-workers (Won et al., 2005). It was found that when the alginate concentration increases, its causes the loading efficiency increased. On the other hand, it made the immobilization yield decreased. The same research also found that loading efficiency and the immobilization yield of lipase does not affected by the weight of enzyme to alginate.

2.4.3 Guar Gum

Guar gum (GG) is a type ofpolysaccharides that derived from the endosperm of guar beans. Its characteristic is water-soluble, non-ionic, and high molecular weight. From a study conducted by Ragothaman et al., (2014), who has synthesized Poly (dialdehyde) guar gum from the selective oxidation of guar gum using sodium periodate. Immobilization of enzyme with guar gum will create a covalent bond. The temperature resistance, swelling and biodegradation properties of the hybrid scaffolds will be improved by present of this covalent bond (Ragothaman et al., 2014). Guar gum has been widely used in the pharmaceutical products because it has high biocompatibility, biodegradability and rheological properties (Gliko-Kabir et al., 2000).

2.5 Immobilization of *C. rugosa* lipase on the chitosan beads

Chitosan beads can be prepared via ionotropic gelatine in a basic coagulant both such as sodium tripolyphosphate. Chitosan beads are made from chitosan powder that dissolves in acetic acid. The droplet of chitosan dissolved in an acidic solution into coagulant bath of sodium triphosphate solution will result in the formation of the beads, the chitosan is positively charged due to the primary amino group, whereas, tripolyphosphate is negatively charged. The interaction between the charges in the mixture will stabilize the chitosan in beads form. This solution then will be extruded through a syringe needle into coagulant bath of sodium triphosphate solution under stirring condition. Then the spherical beads will form (Chiou and Wu, 2004). In preparing the chitosan beads, there are some parameters need to be considered such as chitosan concentration, acetic acid concentration and pH value of chitosan. From research conducted by Vassiliadi et al., (2018), it was found that chitosan can form a stable hydrogel with a mass fraction of polymer in the gels from 0.13 to 0.50. Within this range of concentrations, their structural integrity can be maintained for several days at temperature 25 ◦C. Moreover, the gels prepared with acidic water at pH 3 were more rigid and able to maintain their structural integrity in various solvents, while gels prepared with basic water at higher pH values could not retain their structure when introducing to a solvent (Vassiliadi et al., 2018).

GA has been used as a crosslinker for improving the immobilization of enzymes in which the amino groups on the structure are expected to form a Schiff base with the GA for better binding (Lee et al., 2006). Figure 2.6 below shows the chemical properties of GA.

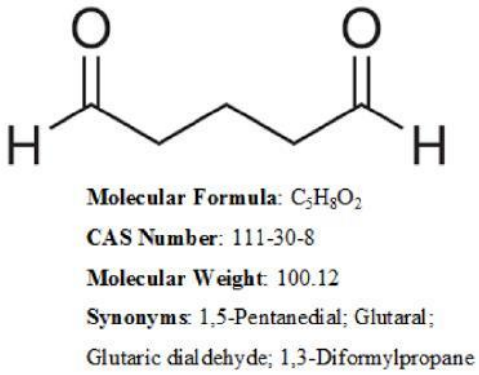

Figure 2.6. Chemical properties of GA

GA is commonly used as crosslinker due to its advantages in term of low cost and highly reactive. Satar et al. (2017) reported that GA is proven to be more efficient and effective as compared to other aldehydes in the formation of stable crosslink (Satar et al., 2017). Dislike of GA, carbodiimide also can be used as a coupling agent (Chiou and Wu, 2004). But in this study, it seems GA is being more suggestive to be used. Hence, the concentration of GA will give significant effect on the immobilization yield.

2.6 Reusability of immobilized enzyme

From the research conducted by Chiou and Wu, (2004), the specific activity of lipase immobilized to wet chitosan beads retained 100 % after 6 reused . On same research and after 10 reused, the residual activity of lipase immobilized on the wet chitosan beads were about 85 % and the residual specific activity was about 92 % (Chiou and Wu, 2004).

2.7 Summary

The enzyme is a very useful protein that has been used in oleo-chemical industry, detergent industry, production of the biodegradable polymer, food processing, flavor development, pharmaceutical, cosmetics and biodiesel (Choudhury and Bhunia, 2015). Since not reusable, the free enzyme has limited application in industries. The enzyme needs to undergo immobilization process to make it more desirable and maximize its potential.

The immobilization of enzyme can be carried out by adsorption, covalent bonding, cell to cell cross-linking, encapsulation, and entrapment. Each method has its own pros and cons. For this research, the enzyme will be immobilized by covalent bonding to prolog the reusability of the enzyme. Covalent bonding gives permanent attachment of enzyme, no leaching of the enzyme, which is an added advantage, compared to others method. The support can be used for immobilizing of the enzyme by covalent bonding. These include chitosan, guar gum, and alginate. Chitosan is promising support because chitosan has great biocompatibility (Zhou et al., 2003).

In this study, *C. rugosa* lipase will be immobilized. Through this research, we will figure out the effect of different concentration of the solution during the immobilization of lipase. The concentration of GA and of *C. rugosa* lipase solutions will be varied to study its effect on the immobilized *C. rugosa* lipase.

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Materials and chemicals

Table 3.1 shows a list of materials and chemicals that were used in this study.

Table 3.1. List of materials and chemicals used in this study

Materials	Purity	Brand
Candida rugosa	-	Sigma
Chitosan powder	-	Aldrich Chemistry
Glutaraldehyde Solution	25 % in water	Sigma-Aldrich
Acetonitrile	-	Merk KGaA
mSodium Hydrogen Phosphate (Na_2HPO_4)	-	Fisher Scientific
Potassium Dihydrogen Phosphate (KH_2PO_4)	-	Fisher Scientific
Sodium Triphosphate	-	Merk KGaA
Bicinchoninic Acid (BCA) Solution		Novagen
4 % Cupric Sulfate	4 %	Novagen
Triton™ X-114	10 %	Calbiochem
p-Nitrophenyl Butyrate (PNPB)	>98 %	Sigma

3.2 Types of equipments and analytical instruments

Table 3.2 shows the list of equipment and instruments that were used in this study.

Table 3.2. List of equipments and analytical instruments

Equipment	Brand	Model	Country of Origin
Water Bath	Protech	Model 830	Malaysia
Incubator Shaker	IKA	Ks 4000ic	United State
Spectrophotometer	Agilent	Cary 60 UV–Vis	United State
Ultrasonic Cleaning	Elma Schmidbauer	S 30	Germany
pH Meter	Mettler Toledo	Delta 320	Malaysia

3.3 Experimental procedure

3.3.1 Preparation of chitosan beads

Chitosan powder, 1.25 % (v/v) acetic acid solution, 1.5 % (v/v) sodium triphosphate solution and distilled water were used for the formation of chitosan beads. 1.50 g of chitosan powder was dissolved in 100 mL 1.25 % (v/v) acetic acid solution for preparing chitosan beads. Chitosan beads were formed by dropping chitosan solution into 100 mL of 1.5 % (v/v) sodium triphosphate solutions by using a hypodermic needle of 2.2 mm of diameter under stirring condition. The resulted mixture was allowed to standing for 3 hours, the spherical gels formed are known as wet chitosan beads. The mixture was left or rest overnight and then the beads were removed by filtration with filter paper. Afterward, the beads were washed with phosphate buffer solution at pH 6.86 until the residual solution reaches neutrality. Finally, the beads were restored in a phosphate buffer solution at pH 6.86 until further use.

3.3.2 Immobilization of lipase

Lipase was immobilized onto the chitosan beads by covalent bonding. On the surface of lipase, it had a substantial amount of amino group. These amino group can react with one of the aldehyde group of GA to form amide bond. Whereas, at other end of aldehyde group that belonging to GA, it allowed to react with amino group of chitosan beads to form covalently bonded immobilized lipase. The chitosan was soaked in a GA solution for 2 hours under a static condition at room temperature. Then, the chitosan beads were removed and washed with phosphate buffer solution at pH 6.86 until it reaches to neutrality. 0.20 g of beads were added to enzyme solution at varies concentration. Then, the resulted mixture was left for 40 hours at room temperature. Finally, the immobilized enzyme was recovered and weighed.

3.4 Enzyme immobilization yield

The immobilization yield (IY) was determined by the following Equation 3.1. Where E_i is the initial concentration of enzyme protein (g/mL), while E_f is the final concentration of the enzyme protein (g/mL) in the soluble enzyme before and after immobilization of lipase.

$$IY\ (\%) = \frac{E_i - E_f}{E_i} \times 100 \qquad (3.1)$$

3.5 Protein assay

A protein assay was determined by using BSA and 4 % cupric sulfate solution at ratio 20:1. The solution was prepared using 20:1 volume ratio of BSA and 4 % cupric sulfate. 0.05 mL of immobilized/free enzyme solution was added into the solution. The mixture was incubated for 30 mins at 37℃. After incubation, the resulted colour change was measured at 562 nm by a Cary 60 UV–Vis spectrophotometer (Agilent Technologies Inc., Santa Clara, CA, USA). A calibration curve as seen at Appendix B was plotted for references.

3.6 Enzyme assay

Enzyme assays are performed to identify and to determine the quantity of the enzyme in the sample (Bisswanger, 2014). Firstly, 100mM of phosphate buffer with 150mM sodium chloride and 0.5 % Triton X-100 was prepared in 2 mL tube, pH 7.2 at 37 ºC. Then, prepare 0.1 mL of 50Mm PNPB solution (prepare 1 mL in acetonitrile using PNPB) was added to the tube. Subsequently, 0.1 mL of the free or immobilized enzyme solution was added into the resulted mixture. The solution was prepared, in a water bath for 5 minutes at 37℃. After 5 minutes, the reaction was stopped by adding 1 mL of 0.5 M EDTA (Saifuddin and Raziah, 2008). The Unit activity (IU) was calculated by using Equation 3.2 below;

$$Activity\ Yield\ (IU) = \frac{(A_{400}Sample - A_{400}Blank)(V_T)(DF)}{(\varepsilon_{ext})(t)(V_E)} \quad (3.2)$$

Where A_{400}Sample and A_{400}Blank were absorbances of the sample and blank determined at 400 nm. Meanwhile, V_T is the total volume of the assay, V_E is the volume of the enzyme, DF is dilution factor, t is a time of assay, and ε_{ext} is micromolar extension coefficient of p-Nitrophenol at 400 nm which is 0.0148 $cm^2/\mu mol$.

3.7 Experimental design for immobilization of *C. rugosa*

Response Surface Methodology (RSM) using a design called a Central Composite Design (CCD) was used to study the parameter of immobilization of *C. rugosa* lipase. The range of and level of the parameter studied are given in Table 3.3 below.

Table 3.3. Independent factors and their coded levels for immobilization lipase

Variable	Code	Units	Coded variable levels		
			-1	0	1
Enzyme concentration	X_1	g/mL	0.005	0.01	0.02
GA concentration	X_2	%	4	6	10

A complete design matrix of the experiments employed as shown in Table 3.4. For two variables; 1 factorial point, 1 axial (star) point and 5 replicates at the center points were employed, indicating that total of 13 experiments as calculated from Equation 3.3:

$$N = 2^n + 2n + n_c \tag{3.3}$$

Where *N* is the total number of experiments required and n is the number of factors.