IMMOBILIZATION OF LIPASE FROM *CANDIDA RUGOSA* USING ALGINATE BEADS

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2018

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by

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Thesis submitted in partial fulfilment of the requirement for degree of Bachelor of Chemical Engineering

June 2018

ACKNOWLEDGMENT

First and foremost, I would like to convey my sincere gratitude to my supervisor, Dr Fadzil Noor Gonawan for his precious encouragement, guidance and generous support throughout this work.

Apart from that, I would also like to thank all SCE staffs for their kindness cooperation and helping hands. Indeed their willingness in sharing ideas, knowledge and skills are deeply appreciated. I would like to express my deepest gratitude to my beloved parents Rohana Binti Othman and Azeman bin Mohamed for their continuous love and support.

Once again, I would like to thank to my best friends Nurul Syazliza binti Mohd, Nur Allia binti Asri and Noor Nadhirah binti Harith and all the people, including those whom I might have missed out and my friends who have helped me to the accomplishment of this project. Thank you very much.

Nursyarinie Nabihah binti Azeman

June 2018

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LIST OF SYMBOL

Symbol		Unit
C	Lipase Concentration	g/mL
t	Time	min
T	Temperature	^{o}C
Y_1	Immobilization Yield	%
Y_2	Unit Activity	IU/mg
V	Solution volume	L
λ	Wavelength	nm

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
Ba	Barium
CCD	Central composite design
Co	Cobalt
Cd	Cadmium
Cu	Copper
Ca	Calcium
$CaCl_2$	Calcium Chloride
Ca^{2+}	Calcium ion
Pb	Lead
Mn	Manganese
NaOH	Sodium Hydroxide
PNPB	p-Nitrophenyl Butyrate
RSM	Response Surface Method
Sr	Stronium
UV	Ultraviolet
Zn	Zinc

IMMOBILISASI LIPASE DARI *CANDIDA RUGOSA* KEDALAM BUTIRAN ALGINAT HIDROGEL

ABSTRAK

Aplikasi enzim di dalam industri sentiasa meningkat dari semasa ke semasa. Dalam usaha untuk meningkatkan aplikasi enzim, terdapat banyak kajian telah diperkenalkan. Lipase dari *Candida Rugosa* telah diimobilisasi di dalam butiran alginat hidrogel untuk aplikasi yang sesuai. Enzim yang terperangkap semasa titikan campuran algid an enzim di dalam cecair pengeras, kalsium ion. Kesan kepekatan alginat enzim lipase, suhu di keadaan optimum dan tahap boleh guna semula telah di kaji. Semakin meningkat kepekatan alginat, semakin meningkat unit aktiviti. Hasil immobilisasi (%) tertinggi adalah 99.17% dan unit aktiviti tertinggi adalah 2.365 IU/mg. Di bawah keadaan optimum dari analisis Design of Experiment (DoE), 0.03 g/ml kepekatan lipase dan 2% kepekatan alginat dan tahap guna semula enzim telah menyumbang kepada unit aktiviti 70.28%. Manakala, unit aktiviti untuk enzim teleh meningkat apabila suhu semasa rendam menaik. Suhu optimum untuk enzim adalah 37^{o}C yang telah menyumbang kepada unit aktiviti yg tinggi. (0.331 IU/mg). Namun begitu, unit aktiviti bagi enzim telah menurun apabila suhu mencecah kepada 40^{0}C hingga 55^{o}C. Keadaan ini berkemungkinan bahawa enzim telah pecah semasa suhu tinggi yang menyumbang kepada unit aktiviti yang rendah (0.037 IU/mg).

IMMOBILIZATION OF *CANDIDA RUGOSA* LIPASE INTO ALGINATE HYDROGEL BEADS

ABSTRACT

Applications of enzyme in industry are becoming more relevant these days. In an effort to increase the application of enzyme, several techniques have been introduced in the research. In the present work, lipase from *Candida rugosa* was immobilized on alginate beads for possible applications. The enzyme was entrapped by drop-wise addition of an aqueous mixture of sodium alginate and the biocatalyst to the hardening solution of calcium ion. Effect of alginate concentration, lipase concentration, temperature at optimum conditions and reusability of the enzyme were investigated. An increased in alginate concentration give higher unit activity of the enzyme. This techniques provided gives higher immobilization yield (%) and activity yield at 99.17% and 2.365 IU/mg respectively. Under optimal conditions generate by ANOVA analysis using Design of Experiment (DoE) software (0.03 g/ml of lipase concentration and 2% of alginate concentration), the lipase beads retained enzyme activity equivalent to 70.28%. The optimum temperature suitable for immobilized enzyme also studied. Enzyme activity increases when the temperature was increased to 37^{o}C, then decreases at higher temperature. The optimum temperature was determined at 37^{o}C with unit activity of 0.331 IU/mg. At temperature 55^{o}C, the activity declined to 0.037 IU/mg which suggested due to denaturation of lipase structure.

CHAPTER ONE

INTRODUCTION

1.1 Immobilization of Lipase from *Candida Rugosa*

Enzyme are biocatalyst that increasingly influence all area of daily life. The biocatalyst regulates and enhance the reaction rates of various biological and chemical processes (Madhavan *et al.,* 2017). The development of the biocatalyst include in medicine, pharmacy, nutritional products, analytics, environmental technology, biosensors in bioengineering and catalyst for chemicals and biochemical reaction (Betigeri *et al.,* 2002; Peter Grunwald, 2009). According to Madhavan *et al.* (2017), the major source of biocatalyst is microorganisms due to the advantage of easy laboratory culturing, natural abundance and rich diversity. The advantages of enzyme application in industrial processes are due to their high specificity, good rate of the reaction, non-toxicity, and water solubility (Betigeri *et al.*, 2002).

The operational stability of the enzyme used in industrial processes being advance steadily over the years. The desirable features in heterogeneous catalyst onto biological catalyst is the most commonly use in enzyme immobilization (Won *et al.*, 2005). Immobilization of enzyme also exhibit to the development of continuous processes and flexible to the variety of configurations and specific processes (Won *et al.*, 2005). Recently, there is high condition for dominant biocatalyst and are considered eco-friendly alternatives for high value chemical synthesis (Madhavan *et al.*, 2017).

According to Sharma *et al.* (2001), lipases in natural environment catalyze the hydrolysis of esters usually formed from the glycerol and long chain fatty acids. Nonetheless, the enzyme are very active biocatalyst for the esterification of fatty acids, alcoholysis and transesterification reaction.(Balcão *et al.,* 1996) The activity, selectively and operational stability of lipase are modified by immobilization in order to able to use in multiple application in food and flavor making, pharmaceuticals, synthesis of carbohydrates ester, amines and amide bio-detergents and recently cosmetics and perfumery (Hung *et al.*, 2003).

1.2 Problem Statement

The use of enzyme and other proteins has been limited and the number and diversity in application of enzyme remain modest (Betigeri *et al.,* 2002; Won *et al.*, 2005). Due to unstable nature and requirement of rigid conditions, such as particular pH and temperature, the operational stability of the enzyme use in industrial applications are improve by immobilization, genetic engineering or process alterations (Betigeri *et al.,* 2002; Won *et al.*, 2005). The immobilization of the enzyme means its transformation from the soluble to an insoluble state by attaching it to an insoluble carrier material or by encapsulating the catalyst within a corresponding matrix (Peter Grunwald, 2009). Immobilization of enzyme are importance in industrial for separation and purification of the products (Zhang *et al.*, 2014). Moreover, the purified free enzymes are rather costly and not economical because the enzyme will be discarded after used. Free enzyme also have disadvantage for separation of reactants and products to recycle. To overcome the problem, the immobilization has been studied to reduce the cost by enabling efficient separation, recycling and reuse the expensive enzyme in catalytic reaction. The

benefit of the immobilization enzyme also is the greater stability and enzyme activity over a broader range of pH and temperature (Bayramoglu *et al.*, 2011).

1.3 Research Objectives

The main objectives of this study are:

1. To study the immobilization yield and unit activity of immobilized lipase at different concentration of lipase in *Candida Rugosa* and sodium alginate.
2. To study the optimum immobilized enzyme preparation conditions including the concentration of lipase and sodium alginate using response surface methodology (RSM).
3. To compare the unit activity of immobilized enzyme using different incubation temperature.
4. To investigate the reusability of immobilized enzyme by repeating the reaction.

1.4 Scope of Study

In this work, lipase from *Candida Rugosa* in calcium alginate is immobilized in calcium alginate. The lipase is prepared by dissolving in distilled water before mix with sodium alginate solution. The mixture was shaken by vibration bed to make sure of the mixture dissolve completely. The lipase solution was mixed with sodium alginate solution at different concentration at room temperature. The solutions were incubated at same temperature 37℃ and time during the experiment. The best operating parameters were then used for experiment on effects of incubation temperature and reusability.

CHAPTER TWO

LITERATURE REVIEW

2.1 Type of Immobilization Enzyme

An enzyme that is insoluble has many benefits towards heterogeneous catalysis. Enzyme may be immobilized by transformation into insoluble form. The rate and yield of immobilization depend on the parameter involved, type of carrier, method of immobilization, concentration, pH, temperature and reaction time (Buchholz *et al.,* 2005).

` The standard method that can be used in laboratory and industry is by binding the enzyme on to the insoluble porous carrier. The major role player during the binding to the carrier is the properties of the external protein surface and the available functional groups. Besides that, the adsorption is dependent upon the hydrophilic and hydrophobic characteristics of the surface regions. The utilization of several functional group for covalent binding when the protein binding is accessible.

Interaction of the carrier surface with the protein must be relevant to prevent the unwanted side effects or the reaction may occur. The accessible surface within the pores must be large enough in order to take in the amount of enzyme required to provide high activity of the biocatalyst (Buchholz *et al.*,2005).

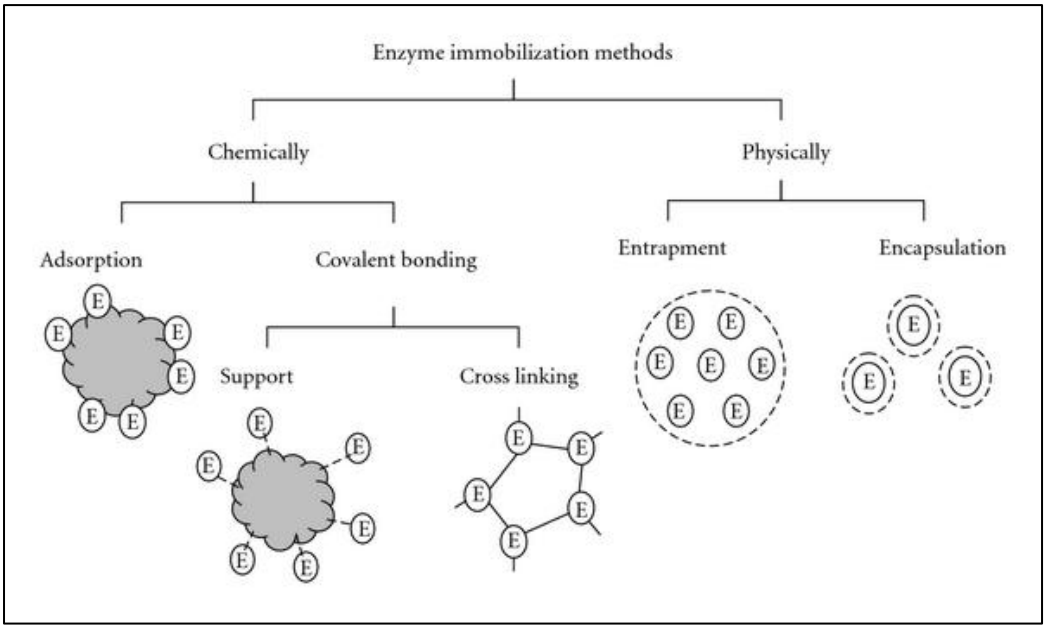


Figure 2.1: Enzyme immobilization methods (Al-marzouqi et al., 2014)

From Figure 2.1, the immobilization techniques are sorted into chemical and physical methods. The physical method does not involve in covalent bonding with the enzyme meanwhile, the chemical methods involving the formation of the covalent bond between functional group on the enzyme and functional group on the support material. The methods for immobilization can be divided into two main categories, binding and entrapment.

The most obvious reason for immobilization of enzyme is the need to reuse the enzyme to prevent the effect of economic towards the industrial processes. From Table 2.1, show the benefits and shortcoming of continuous processing . Plenty of investigations have been reported on the stabilization of enzyme by immobilization on soluble carrier.

Table 2. 1: Reasons and limitations for enzyme immobilization (Buchholz *et al.*,2005)

Reasons	Limitations
Reuse of enzyme, reducing cost	*Cost of carriers and immobilization*
Continuous processing - Facilitated process control - Low residence time (high volumetric activity) - Optimization of product yield	Mass transfer limitations Problems with cofactors and regeneration. Problems with multienzyme systems
Easy products separation and recovery	Changes in properties (selectivity)
Stabilization by immobilization	Activity loss during immobilization

2.1.1 Entrapment of Immobilized Enzyme

Enzyme entrapment is contain the enzyme into a semi-permeable supports such as alginate, which prevent the enzyme from leaving while allowing the substrate to and the product to pass through for organic reaction (Zhang *et al.*, 2013a). The entrapment technique has been developed by using various polysaccharides. As stated by Betigeri *et al.,* (2002), the efficiency and the retention of enzymatic activity hugely function of the technique in immobilization, the reagents used and the process variable. The microencapsulation has to be a successful immobilization technique.

Polymeric network or gels that formed from cross-linking of water-soluble polyelectrolyte with either polycations or polyanions. Alginate, pectin and carrageenan are the example of polysaccharides with carboxyl- or sulfonyl groups that are specifically suited for this. Moreover, this materials available at low and reasonable cost and non-toxic (Buchholz *et al.,* 2005).

The advantages from this technique, the entrapment can be easily separate from the reaction medium by filtration, immobilized enzyme have potential of being used in several reaction batches (Betigeri *et al.*, 2002).

However, the limitation of this technique are, the protein entrap are usually low efficiency, volatile organic solvents are typically required which causes to the environmental hazards and the structure and the function of protein may alter because of organic solvent (Betigeri *et al.*, 2002). Furthermore, even the immobilization of the enzyme by entrapment in calcium alginate well-known and effectives, the entrapment of enzyme leak out during the course of the time as result of the large pores of the matrix (Won *et al.*, 2005).

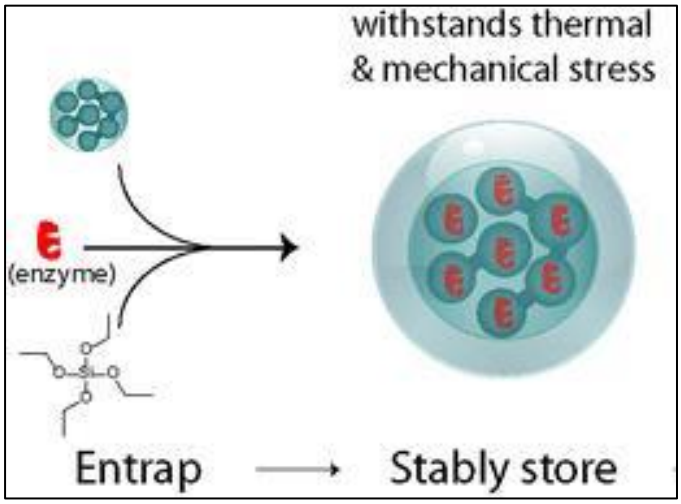


Figure2.2:Schematic diagram of encapsulation of enzyme (Zlateski *et al.*,2015)

2.2 Lipase from *Candida Rugosa*

Lipase is the enzyme that most frequently applied in organic synthesis. A large different number of lipase's origin with different catalytic properties is commercially available. Lipase have famous characteristics and positions amongst other enzyme because the wide spectrum that use in biotechnological applications

including their function in hydrolysis and transesterification of triglycerides that is natural fats and oils. The main significances of lipase application in organic chemistry are hugely active in wide range of non-aqueous solvents, display excellent stereo selectivity and accept nucleophiles other than water (Buchholz *et al.*,. However, from the number lipase available, only a few been shown to widely applicable to have stability. These are *Porcine pancreatic lipase* (PPL), *Bulkholderia cepacia* (BCL), *Candida rugosa* lipase (CRL) and *Rhizomucor miehei* (RML) (Buchholz *et al.,* 2005). In this study, the enzyme present is *Candida Rugosa* lipase. The functional lipase from C. rugosa can work in non-aqueous media as well as in water (Won *et al.,* 2001).

2.3 Sodium Alginate

Alginate are commercially available as water-soluble sodium alginate that have been used for more than 65 years in the food and pharmaceutical industries as thickening, emulsifying, film-forming and gelling agents (Knezevic *et al.*, 2002). In entrapment technologies and immobilization, alginate is the most broadly used polymer because of their thermo stability, and can be stored in room temperature (Zhang *et al.*, 2013a). Sodium alginate is alginate that are available as sodium salt of polyuronic acid that containing varying proportions of 1→4 linked β-D- mannuronic acid (M) and α- L- guluronic acid (G) residue arranged randomly along the chain (Betigeri and Neau, 2002; Zhang *et al.*, 2013a). From Figure 2.3, the residue occurs in varying proportions depending on the source, and are arranged in block patterns comprised of homopolymeric regions (MM blocks and GG blocks) infused with alternating regions of heteropolymeric regions (MG blocks) (Peter Grunwald, 2009).

The most abundant acidic groups in the alginate polymer are generally carboxylate (-COO-), ether (-C-O-C-) and hydroxyl (-OH-) (Zhang *et al.*, 2013b) .

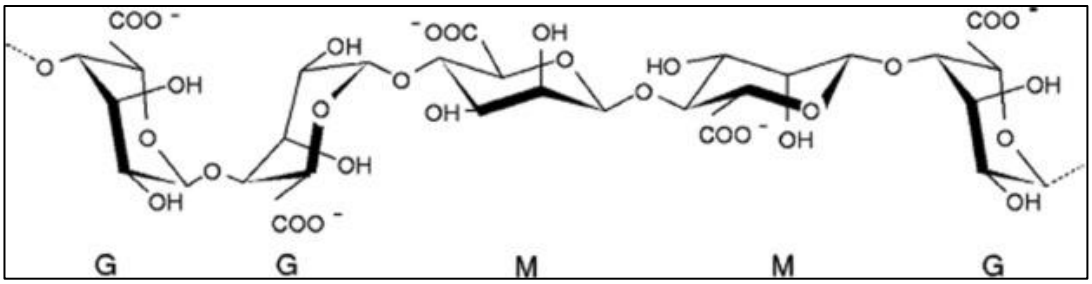


Figure 2. 3:Alginates with D-mannuronic-,L-guluronic-,and mixed structure elements (Al-marzouqi *et al.*,2014)

2.4 Calcium Alginate entrapment

Alginate are polysaccharides that are extracted naturally from the brown seaweeds found in the shoal waters of the temperate zones. Alginate does not have regular repeating unit and they are unbranched binary copolymer (Buchholz *et al.*, 2005) . Calcium alginate are the material commonly used for the enzyme encapsulation due to the natural biocompatibility, ease of formation and the mild physiological gelation condition (Zhang *et al.*, 2013a). The alginate bead are produced by pumping alginate biocatalyst solution through a needle and the solidification of the alginate droplets in the hardening solution via counter ion exchange (Knezevic *et al.*, 2002). From the Figure 2.4 ,the calcium chloride use as hardening solution and the cation, Ca^{2+} acts as cross linking agents towards the alginate. According to Smidsrod (1973), Ca^{2+} is the most frequently employed ions because of its low toxic. The rigidity of ionic alginate gels increases with affinity according to the order. Mn> Co> Zn>Cd> Ni> Cu> Pb> Ca> Sr> Ba. However, most of these ions cannot used for immobilization.

The droplet of the alginate and the biocatalyst entrapped as a bead during the droplet precipitate (Won *et al.*, 2005). At the room temperature, the bead with the porous structure are forming by metal-alginate suspension cross-linked in the presence of the calcium cation. The cross-linking between the carboxyl group of the α- L- guluronic acid with the solution of the cationic cross linker made the alginate support during the immobilization process (Zhang *et al.*, 2013b).

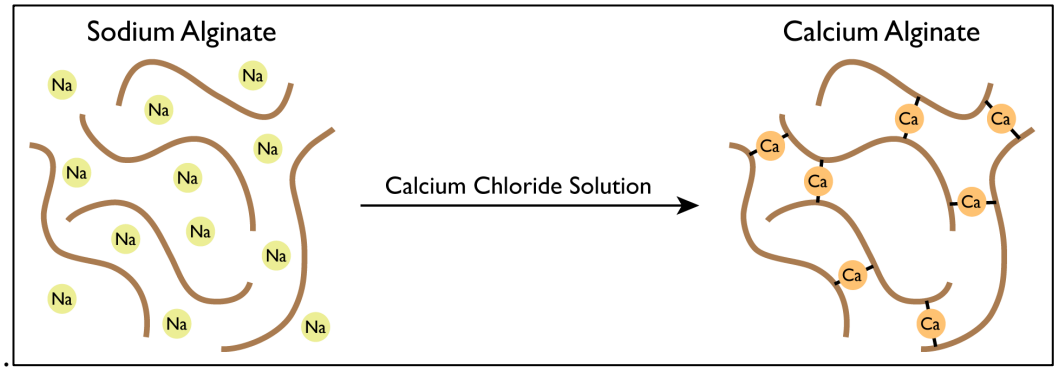


Figure 2.4: Schematic diagram of calcium chloride and sodium alginate during reaction (Phan *et al.*,2013)

2.5 Enzymatic assays

The purpose to perform the enzymatic assays are to identify a special enzyme, to prove its existence or absence in the specimen like organism or a tissue. For enzyme assays, it need to acknowledge the enzyme reaction depends on more factors than pH, temperature, and ionic strength. Commonly to all type of enzyme-catalyzed reaction is the substrate becomes converted into a product and thus the aim of any assay to identify the time-dependent formation of the product.

In the assay mixture, the activity of enzyme are depends on the pH. The different effects are responsible for this action are the state of protonation of functional groups of amino acid and cofactors involved in catalytic reaction and the

three-dimensional protein structure of the enzyme, the irreversible damaging of the protein structure. Moreover, as stated by Bisswanger (2014), the enzyme stable at is own optimum pH and suitable for testing and stored. This significance for the performance of enzyme assays, since addition of an aliquot of the enzyme stock solution to the assay mixture will not affect the assay pH.

CHAPTER THREE

METHODOLOGY

3.1 Materials and Chemicals

In this study, lipase was used as raw material for the immobilization of enzyme. the calcium alginate was used as a carrier to form gel beads that entrapped the lipase. During the reaction, calcium chloride acts as hardening agents for sodium-lipase to form beads. Table 3.1 shows the list of chemicals used throughout the experiment including the protein and enzymatic assays.

Table 3. 1: List of chemicals used during immobilization of enzyme experiment

Chemicals	**Supplier**
Lipase	Sigma Chemical Company
Sodium Alginate	BDH Laboratory Supplies
Calcium Chloride	Sigma Chemical Company
BCA solution	Novagen
4% Cupric Sulfate	Novagen
Sodium Phosphate	Fisher Scientifics
Sodium Chloride	Fisher Scientifics
Triton X-100	Sigma Chemical Company
Acetonitrile	Sigma Chemical Company
p-Nitrophenyl Butyrate	Sigma Chemical Company
Ethanol	VWR Chemicals

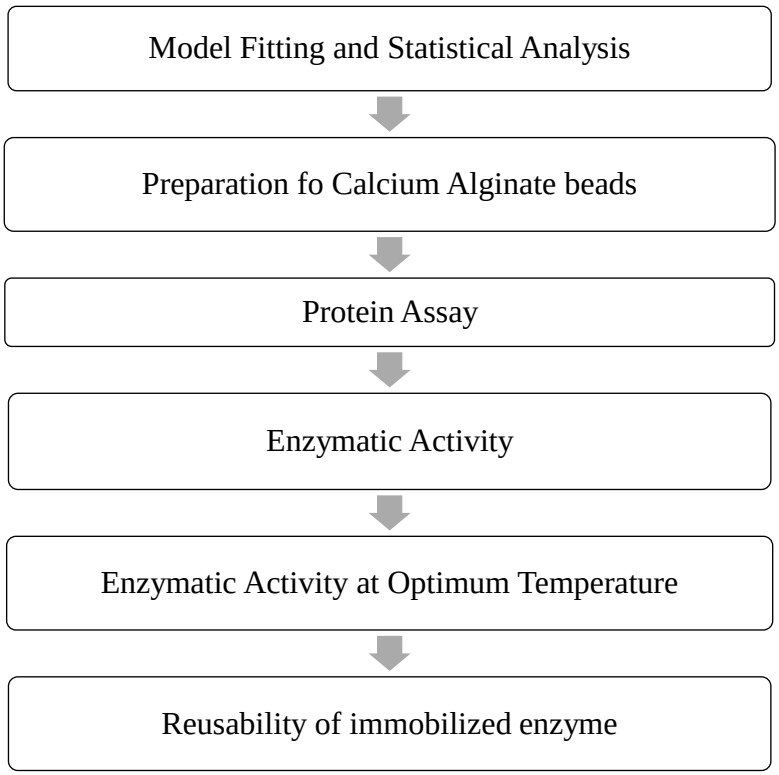
3.2 Equipments

Magnetic stirrers will be used to stir the sodium alginate solution until completely dissolve. The water bath will be used for incubation during protein and enzymatic assays. Finally, the Spectrophotometer (ModelCarry 60 Agilent Technologies) is used after performing protein and enzymatic assay to measure the absorbance. The list of the equipments and its general used is tabulated in Table 3.2 below.

Table 2.2: List of equipments and general uses

Equipment	**Function**
Magnetic stirrer	To mix the sodium alginate with distilled water
Water bath	to incubate the sample in desired temperature
Spectorphotometer	to measure the absorbance

3.3 Project Plan / Methodology



3.4 Experimental Procedure

3.4.1 Experimental Design for Immobilization of Lipase from *Candida Rugosa*

The standard response surface methodology (RSM) design called a central design (CCD) was used to study the parameter of preparing immobilization enzyme from lipase and sodium alginate concentration.

Table 3. 3: Independent factors for both response

Variables	Factor	Units	Coded variable levels		
			-1	0	1
Lipase Concentration	X_1	g/mL	0.01	0.025	0.04
Alginate Concentration	X_2	%	1	2	3

The range and the levels of the variable investigated in the study given in Table 3.3. For 2 variables indicating that total 12 experiments as calculated from Equation 3.1

$$N = 2^n + 2n + n_c = 2^2 + 2*(2) + 2 + 4 = 12 \quad (3.1)$$

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

The centre point are used to determined error and the reproducibility of the data. The axial points located at (±α, 0, 0), (0, ±α, 0) and (0, 0, ±α) where α is the distance of the axial point from center and makes the design rotatable. The experimental sequence was randomized in order to minimize the effects of the uncontrolled factors. Both response were immobilization yield (Y_1) and unit activity (Y_2). Each response was used to develop an empirical model which correlated that response to the both variables.

Table 3.4: Experimental matrix of prepared immobilized enzyme

Run No.	Factors	
	X_1: Lipase Concentration (g/mL)	X_2: Alginate Concentration (%)
1	0.01	1
2	0.02	1
3	0.03	1
4	0.04	1
5	0.01	2
6	0.02	2
7	0.03	2
8	0.04	2
9	0.01	3
10	0.02	3
11	0.03	3
12	0.04	3

3.4.2 Preparation of calcium alginate beads

Sodium alginate were dissolve at different concentration (1-3 w/v %) in distilled water. the solution were mixed until the alginate fully dissolve at room temperature. 2ml of lipase solution concentration (0.02 g solid/ ml) was added to 2 ml of sodium alginate solution and the mixture were shaken in vibration bed to ensure complete mixing. Then, the enzyme-alginate suspension was added dropwise to 15ml of calcium chloride (10wt%) aqueous solution and it formed colorless transparent beads. After they were embedded for 60 min, these beads were repeatedly wash with deionized water by funnel filtration process. Then, these beads were transfer to a clear glass dish, which sealed with film and can be stored in refrigerator and used within a week.

3.4.3 Protein assay

Firstly, pipette 50µL of sample protein (free lipase) into labeled test tubes. Then, add 1.0 ml of BCA working reagent and mixed in gently. The solution in the test tube then incubated at 37℃ for 10 minutes. During the incubation, the solution in the test tube turned from blue to purple color. After 10 minutes, 1ml of distilled water were added to a clean cuvette and the absorbance were adjusted to 562 nm to zero. Then, the reaction solution were transferred into the clean cuvette. All reactions were measured and recorder within 10 minutes. To obtain the corrected absorbance, all the reactions absorbance were subtracted to the absorbance of blank standard. The standard curve were plotted by corrected absorbance versus the known mass of the BCA standards. By using the calibration curve, the recorded absorbance reading for samples assayed were interpolated which fall within the linear range of the calibration curve.

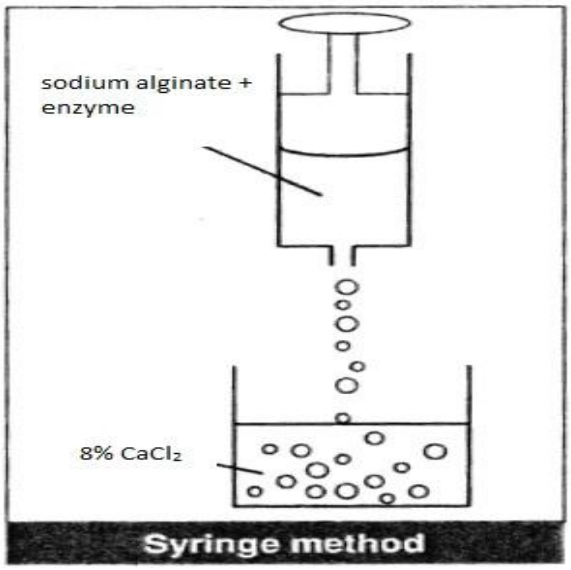


Figure 3. 1: Schematic diagram on how the calcium alginate beading process. (Buchholz *et al.*,2005)

3.4.4 Enzymatic assay

3.4.4.1 Preparation of solution

To prepare the phosphate buffer solution, by using sodium phosphate, monobasic, anhydrous, Sodium Chloride and Triton X-100 in 100ml deionized water and adjust to pH 7.2 at 37℃ with 1M NaOH. Then, prepared 50mM of PNPB in 1 ml Acetonitrile

3.4.3.2 Enzymatic assay of lipase

0.06 g of enzyme beads (immobilize enzyme) was placed in labeled test tube and 1 ml of 0.025M phosphate buffer solution (pH 7.2) was added also. Then, 10µL p-nitro-phenol butyrate (50mM) added into the test tube for reaction. Then the test tube was placed in water bath to incubate at 37℃ for 5 minutes. After 5 minutes, 1 ml of ethanol was added to stop the enzymatic reaction.

The solution was tranfered into clean cuvette. The analysis of the solution were conducted by UV-vis spectroscopy at wavelength 400nm.

The calculation for enzymatic assay as below:

$$\frac{\text{units}}{\text{ml}}\ \text{enzyme}=\frac{(\Delta A_{400\text{nm}}/\ \text{min}\ \ \text{Test-}\Delta A_{400\text{nm}}/\ \text{min Blank})(1.01)(\text{df})}{(0.0148)(0.1)}$$

The calculation for unit activity of immobilized enzyme (IU mg^{-1}) :

$$\frac{\text{unit}}{\text{mg}}\ \text{protein}=\frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

1.01 = Volume (in millimeters) of assay

df = Dilution factor

0.0148 mM = Micromolar extinction coefficient of p-Nitrophenol at 400nm

0.1 = Volume (in millimeter) of enzyme used.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Preliminary Studies

The concentration of lipase and calcium alginate used will affect the amount of lipase immobilized and its activity. Thus, the investigation was initiated by varying the concentration of these parameters. The optimum condition was also determined before further investigation on the performance of the immobilized lipase.

4.1.1 Experimental Design

The experimental data were analyzed using statistical software Design Expert software (STAT-EASE Inc.,Minneapolis, USA) for regression analysis to fit the appropriate emperical equation and also the evaluation of the statistical significance of the equation proposed. The two variables being studied were lipase concentration (g/mL) and alginate concentration (%). The responses being considered in this study were X_1 immobilization yield (%) and X_2 unit activity (IU/mg). From the experimental works, the complete design matrix obtained by preparing the solution together.

The empirical model were suggested for both response. The cubic and quadratic models have been suggested to represent the effect of immobilization process parameters on immobilization yield X_1 and unit activity X_2 respectively.. The final empirical formula models for the immobilization yield (X_1) and unit

activity (X_2) in terms of coded factors are represented by Equation 4.1 and 4.2 respectively.

$$Y_1 = 82.34 + 7.59X_1 + 5.24X_2 + 6X_1X_2 + 1.08X_2^2 - 1.74X_2^2 + 1.37\ X_1^2X_2$$

$$-20.26X_1X_2^2 + 1.45\ X_1^3 \quad (4.1)$$

$$Y_2 = 1.99 + 0.24X_1 + 0.73X_2 - 6.975^{-0.003}\ X_1X_2 - 0.1\ X_1^2 - 0.5X_2^2 \quad (4.2)$$

Table 4. 1: Experimental factors and response values for different experimental conditions.

Run No.	Factors		Responses	
	X_1: Lipase Concentration (g/mL)	X_2: Alginate Concentration (%)	Immobilization Yield (%)	Unit Activity (IU/mg)
1	0.01	1	92.9	0.279
2	0.02	1	83.62	1.049
3	0.03	1	63.07	0.732
4	0.04	1	61.18	0.744
5	0.01	2	73.82	1.485
6	0.02	2	73.65	1.86
7	0.03	2	99.17	2.086
8	0.04	2	85.1	2.292
9	0.01	3	93.46	2.168
10	0.02	3	92.37	1.877
11	0.03	3	75.88	2.271
12	0.04	3	87.05	2.365

For the responses, the model coefficient was estimated using multiple regression analysis technique included in RSM. The quality of the model developed was evaluated based on the correlation coefficient value. The models that developed the have highest R^2 value which were closer to unity as it will predict the response closer to the actual value. In this experiment, the R^2 for equation 4.1 and 4.2 were respectively 0.7322 and 0.9306. This indicated that 73.22% and 93.1% of the total variation in immobilization yield and unit activity were attributed to the experimental variable studies. The R^2 value of 0.9306 for equation 4.2 was considered relatively high, indicating the value for unit activity would be more accurate and closer to its actual value. The R^2 for equation 4.1 was considered lower to validate the fit, which might lead to larger variation in immobilization yield predicted from the model.

The significance and adequacy of the models were further justified through analysis of variance (ANOVA). In the ANOVA, the mean squares were obtained by dividing the sum of the square of each variation sources, the model and the error variance, by the respective degree of freedom. If the value of Prob > F less than 0.05, the model terms were considered as significant. The ANOVA for the models for unit activity was listed in Table 4.2 and Table 4.3. From Table 4.3, the response give interact between X_1 and X_2 to generate the response. However, the response between for immobilization yield from Table 4.2 does not give any interaction and not significant since the p-value were larger than 0.05.

Table 4. 2: Analysis of variance (ANOVA) for response surface cubic model for Immobilization Yield.

Source	Sum of Square	Degree of Freedom,df	Mean Square	F value	p-value >F	Comment
Model	1212.116	8	151.514	1.025	0.5492	not significant
X_1	26.152	1	26.152	0.177	0.7023	
X_2	85.650	1	85.650	0.580	0.5019	
X_1X_2	159.960	1	159.960	1.082	0.3747	
X_1^2	2.755	1	2.755	0.019	0.9000	
X_2^2	8.108	1	8.108	0.055	0.8299	
$X_1^2X_2$	2.965	1	2.965	0.020	0.8963	
$X_1X_2^2$	608.175	1	608.175	4.115	0.1355	
X_1^3	0.991	1	0.991	0.007	0.9399	

Table 4. 3: Analysis of variance (ANOVA) for response surface quadratic model for Unit Activity (IU/mg)

Source	Sum of square	Degree of Freedom, df	Mean Square	F-value	Prob > F	Comment
Model	5.366	5	1.073	16.099	0.0020	significant
X_1	0.370	1	0.370	5.547	0.0567	
X_2	4.317	1	4.317	64.769	0.0002	
X_1X_2	0.000	1	0.000	0.003	0.9564	
X_1^2	0.024	1	0.024	0.367	0.5667	
X_2^2	0.654	1	0.654	9.807	0.0203	

4.1.2 Optimization of Operating Parameters

One of the main aims of this study was to find the condition of the immobilization process parameters to obtain high immobilization yield and unit activity. In this optimization analysis, the target criteria was set at range for both values of immobilization yield and unit activity from the experimental and both of parameter are being studied. The experimental conditions with highest desirability were selected and then was verified at 0.03 g/ml of lipase concentration and 2% of alginate concentration. From Table 4.4, the experimental conditions were applied to prepare the optimum immobilized enzyme. From Figure 4.1, from the experimental values obtained were in good agreement with the values predicted from the models. Relatively small errors between the predicted and the actual values were observed, this indicated that the models were suitable and sufficient to predict the responses from the operating variables fixed.

Table 4. 4: Optimum conditions for Immobilization of lipase.

Alginate Concentration (%)	Lipase Concentration (g/mL)	Immobilization Yield (%)	Activity of alginate-immobilized lipase (IU/mg)
1	0.03	63.07	0.732
2	0.03	99.17	2.086
3	0.03	75.88	2.271

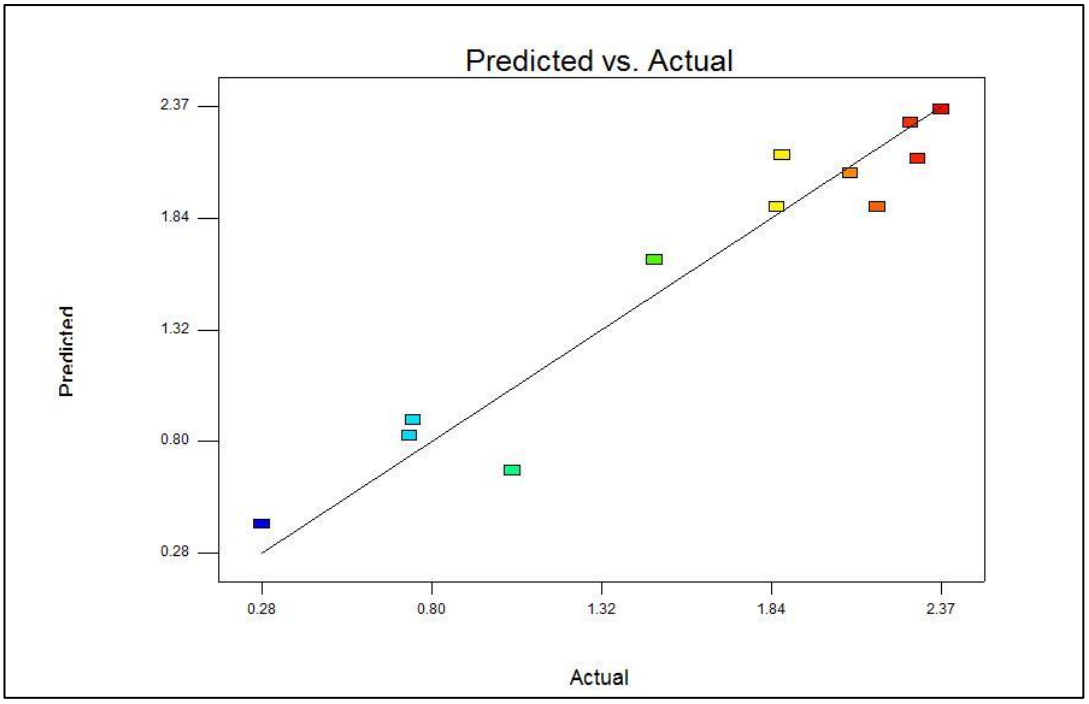


Figure 4. 1: Result of predicted versus Actual from the response

4.2 Effect of Alginate concentration

4.2.1 Unit Activity of Immobilized Lipase

The investigation was carried out by varying the sodium alginate concentration while keeping the lipase from *Candida Rugosa* at 0.01 g/ml, 0.02g/ml, 0.01g/ml and 0/04 g/ml. Alginate concentration was increased from 1% to 3% and $CaCl_2$ concentration was fixed at 10 w/v %. It was found that the unit activity of alginate-lipase increased when the alginate concentration increased. The immobilized lipase with 3% alginate yielded the maximum unit activity, 2.271 IU/mg while the minimum unit activity is 0.279 IU/mg which achieved when 1% of alginate was used. From Figure 4.2, the data indicates that the unit activity have interaction between lipase concentration, X_1 and alginate concentration, X_2. A lowest unit activity was obtained at lipase concentration of 0.01g/mL , whereas at 0.03 g/mL gives highest unit activity of immobilized lipase. From the Figure 4.2