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JUDUL: **APPLICATION OF CALCINED KAOLIN AS SUPPORT FOR THE IMMOBILIZATION OF LIPASE FROM *Candida rugosa***

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APPLICATION OF CALCINED KAOLIN AS SUPPORT FOR THE IMMOBILIZATION OF LIPASE FROM *Candida rugosa*

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A thesis submitted in fulfillment of the
requirements for the award of the degree of
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MAY 2008

DECLARATION

I declare that this thesis entitled “Application of calcined kaolin as support for the immobilization of lipase from *Candida rugosa* ” is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Date : May, 2008

Special Dedication of This Grateful Feeling to My…

Beloved Mak and Ayah;

Supportive Lecturer
Mrs. Nina Suhaity binti Azmi

Special friend at Moscow Medical Academy
Zalikha

My life aspiration
Sya

"non scholae sed vitae discimus"

(we learn not for school, but for life)

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ABSTRACT

Lipase from *Candida rugosa* was immobilized onto calcined-kaolin by physical adsorption method. Lipase offers wide ranges of applications; where as the ability of lipase to act in both organic and inorganic solvents making it more valuable in term of application. However the major concern still will be the cost of lipase, the availability of lipase and reusability of lipase. In order to curb the problems, immobilization of lipase is becoming normal practices. Kaolin is natural substance that obtained from clay. With abilities to act as good as ceramic material; (no toxicity, ability to withstand extreme heat and pressure) it is believed that kaolin has tremendous potential to be utilized as solid support material in both laboratory scale and industrial scale. The study was focus on the preparation of treated kaolin to utilize natural resources instead of inorganic substances. The objectives of this study are to study, amount of lipase attach to kaolin as support material in form of immobilized lipase, to record performance of immobilized lipase with the effect of temperature and effect of pH. The last objective is to compare enzyme activity between free lipase and immobilized lipase. It is determined that percentage of protein loading is 68% (relative amount of lipase attached to solid support material)

ABSTRAK

Lipase daripada *Candida rugosa* telah dilekatkan kepada kaolin yang telah dibakar dengan suhu tinggi (1000^{o}C) dengan menggunakan kaedah lekatan fizikal. Lipase boleh digunakan dalam pelbagai bidang di mana kebolehannya untuk berfungsi di dalam pelarut organik dan inorganik menjadikan kegunaannya sangat penting. Bagaimanapun masalah utama masih lagi berkenaan kos lipase, jumlah lipase yang kurang dan juga kebolehgunaan semula lipase. Untuk mengatasi masalah-masalah ini, kaedah lekatan lipase telah menjadi amalan yang kerap dipraktikkan. Dengan kebolehan untuk berfungsi seperti mana bahan seramik, (tiada keracunan, kebolehan untuk berfungsi pada suhu dan tekanan ekstrem) kaolin dipercayai mempunyai potensi yang luas untuk digunakan sebagai bahan bantu-lekatan pada skala makmal dan juga skala industri. Matlamat kajian ini untuk mengkaji jumlah maksimum lipase yang akan terlekat pada kaolin yang berfungsi sebagai bahan bantu-lekatan, mengkaji prestasi lipase yang telah dilekatkan terhadap kesan suhu dan kesan pH. Akhir sekali adalah untuk membuat perbandingan aktiviti lipase bebas dan juga lipase yang telah dilekatkan. Jumlah relatif lipase yang dilekatkan pada bahan bantu lekatan adalah 68%.

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%	-	percent
°C	-	degree Celsius
µg/ml	-	microgram per milliliter
g	-	gram
g/l	-	gram per liter
h	-	hour
kg	-	kilogram
L	-	liter
L/h	-	liter per hour
min	-	minute
ml	-	mililiter
mm	-	milimeter
rpm	-	rotation per minute
v/v	-	volume per volume
wt %	-	weight percent
µg/ml	-	microgram per milliliter
kDa	-	kilo Dalton

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Enzymes are used on an industrial scale as catalysts for processing various crude materials. Enzyme displayed excellent properties (high activity, selectivity, and specificity) which mean enzyme can work in wide range of environments; that is perfect for industrial purposes as economic point of view is regarded as most crucial criteria. Despite the advantages above, current practice do not reflect enzymes as ideal industrial-scale catalyst because of several factors, mainly high cost of the enzymes, and enzymes instability. Enzymes are cost-effective only when it can be re-used many times. For recirculation the enzymes need to be separated from the process liquid. This is possible when the enzymes are attached to a carrier which can be filtrated and recovered. This is very important as some enzymes are soluble in aqueous media and it is not economic to recover them as enzymes are difficult and expensive to recover after reaction. Thus reaction only limited to batch operation.

Immobilization means associating biocatalysts (enzymes) with an insoluble matrix, so that enzymes will retain at it support as substrate flow through of enzymes in reactor packing. These eradicate problems with recovery of enzyme and purity of products which are significance to economical impact of operation.

Immobilization of lipases has proved to be a useful technique for improving enzyme activity in organic solvents. Several methods have been reported, such as deposition on solid supports covalent binding and entrapment within a hydrophobic sol–gel material or within a polymer matrix. The latter method has been more widely used to variety of lipases (Dave and Madamwar, 2005)

The availability of a large number support materials and methods of enzyme immobilization leave virtually no feasible route of immobilization. It is important that the choice of support material and immobilization method over the free bioactive agent should be well (Arica *et al.*, 2004

1.2 Problem Statement

In order to execute various biochemical reactions in an industrial field by using an organic catalyst, for example an enzyme catalyst, many researches and studies on an enzyme immobilization have been actively carried out in recent years. To immobilize enzyme, there are two major consideration; properties of the enzyme and support material (carrier). For the carrier, various materials may be used, for example high molecular organic materials such as cellulose, agarose, chitin, zeolite, kaolin carrageenan, poly acrylamide, and inorganic materials such as commonly used porous glass, ceramics, and so on. However, such organic materials are apt to have a poor mechanical strength, and mostly require a high temperature treatment in order to prevent the contamination of various germs in a reaction system employing the above described enzyme immobilizing carrier. (John *et al.,* 1990)

In fabrication of the immobilize lipase, there are many processes must be done; firstly is to decide the best solid support, preparation the support material and lipase preparation. The next will be selection of method of immobilization and eventually amount of enzymes which are immobilized to the support. The last consideration will be assessment of performance of immobilized enzyme against free enzyme in several parameters; temperature, effect of solvent, and time.

In this study kaolin mineral is added with a strong acid, and subjected to a hydrothermal treatment and a baking treatment. There are two major consideration in order to conduct this study; cheapest available solid support material and method of immobilization. After considering factors such as availability, cost and method of immobilization; it is decided usage of kaolin as solid support material and the method will be physical adsorption as these choices among the practical yet reliable options available.

1.3 Research Objectives

This research was conducted to achieve several objectives. One of the most important objectives is to determine amount of lipase that will attached to calcined-kaolin as solid support material. Then the next objective is to record performance of immobilized lipase to effect of temperature and effect of pH. The last objective will be comparison of enzyme activities between free lipase and immobilized lipase.

1.4 Research Scope

The scope for this study is:

- To determine optimum performance in term of activity of immobilized lipase in different parameters; namely enzyme properties (stability at various temperature, stability towards solvent), and effect of temperatures.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

One of basic principal of immobilization procedures is to fix maximum amount of enzyme and keeping maximum activity at lowest cost. More than 5000 publications, including patents, have been published on enzyme immobilization techniques. Several hundred enzymes have been immobilized in different forms and approximately a dozen immobilized enzymes, for example amino acylase, penicillin G acylase, many lipases, proteases, nitrilase, amylase, invertase,; have been increasingly used as indispensable catalysts in several industrial processes. Although the basic methods of enzyme immobilization can be categorized into a few different methods only, for example adsorption, covalent bonding, entrapment, encapsulation, and cross-linking, hundreds of variations, based on combinations of these original methods, have been developed. Correspondingly, many carriers of different physical and chemical nature or different occurrence have been designed for a variety of bioimmobilizations and bioseparations.

Rational combination of these enzyme-immobilization techniques with a great number of polymeric supports and feasible coupling chemistries leaves virtually no enzyme without a feasible immobilization route. It has recently been increasingly demonstrated that rational combination of methods can often solve a problem that cannot be solved by an individual method.

For instance, the physical entrapment of enzymes in a gel matrix often has drawbacks such as easy leakage, serious diffusion constraints, and lower stability than that for other immobilized enzymes. (Katzbauer *et al.*,1995). These drawbacks can, however, be easily solved by rational combination of different methods. For instance, higher stability can be achieved by means of the so-called pre-immobilization stabilization strategy or post-immobilization strategy.

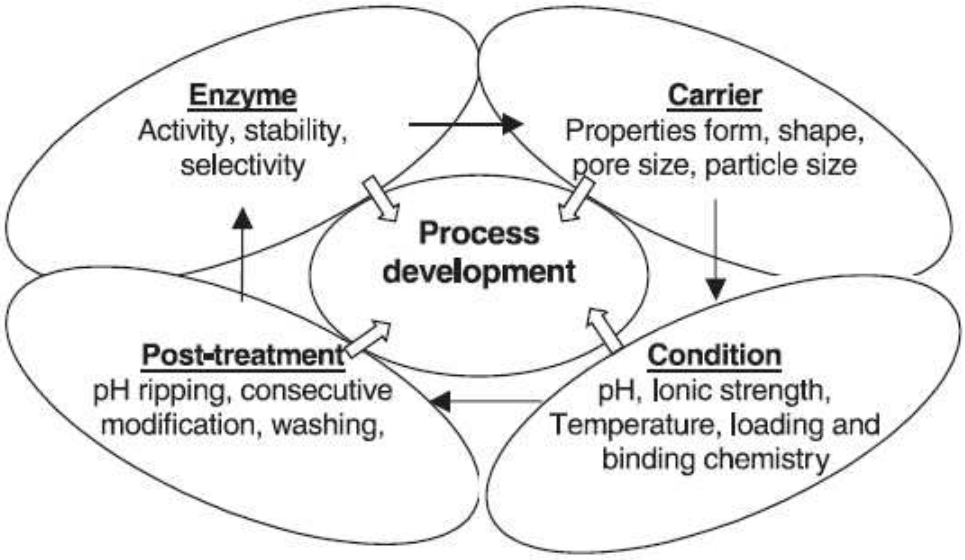

Figure 2.1 Illustration of general procedures for enzyme immobilization (Katzbauer *et al.*,1995)

2.2 General overview of lipase

Today, lipases stand amongst the most important biocatalysts carrying out novel reactions in both aqueous and no aqueous media. This is primarily due to their ability to utilize a wide spectrum of substrates, high stability towards extremes of temperature, pH and organic solvents, and chemo-, regio- and enantioselectivity.

More recently, the determination of their three-dimensional structure has thrown light into their unique structure–function relationship. Among lipases of plant, both animal and microbial origin, it is the microbial lipases that find immense application. This is because microbes can be easily cultivated and their lipases can

catalyze a wide variety of hydrolytic and synthetic reactions. Lipases find use in a variety of biotechnological fields such as food and dairy (cheese ripening, flavour development), detergent, pharmaceutical (naproxen, ibuprofen), agrochemical (insecticide, pesticide) and oleochemical (fat and oil hydrolysis, biosurfactant synthesis) industries. Lipases can be further exploited in many newer areas where they can serve as potential biocatalysts.

The demand for industrial enzymes, particularly of microbial origin, is ever increasing owing to their applications in a wide variety of processes. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods. Enzymes find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile, and cosmetic industries. In the above scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates. However, with the realization of the biocatalytic potential of microbial lipases in both aqueous and nonaqueous media in the last one and a half decades, industrial fronts have shifted towards utilizing this enzyme for a variety of reactions of immense importance.

Thus, lipases are today the enzymes of choice for organic chemists, pharmacists, biophysicists, biochemical and process engineers, biotechnologists, microbiologists and biochemists. Lipases (triacylglycerol acylhydrolases) belong to the class of serine hydrolases and therefore do not require any cofactor. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved.

Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The occurrence of the lipase reaction at an interface between the substrate and the aqueous phase causes difficulties in the assay and kinetic analysis of the reaction. The usual industrial

lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyze them initially into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids. (Godfredson *et. al.*,1990).

Lipases are not involved in any anabolic processes. Since this enzyme acts at the oil–water interface, it can be used as a catalyst for the preparation of industrially important compounds. Lipases catalyse the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and fatty acids, and under certain conditions the reverse reaction leads to esterification and formation of glycerides from glycerol and fatty acids. As lipases act on ester bonds, they have been used in fat splitting, as known as interesterification or transesterification. (Base on catalyst used)

2.2.1 Sources of lipase

2.2.1.1 Bacterial lipases

A relatively smaller number of bacterial lipases have been well studied compared to plant and fungal lipases. Bacterial lipases are glycoproteins, but some extracellular bacterial lipases are lipoproteins. (Winkler *et al*.,1979) reported that enzyme production in most of the bacteria is affected by certain polysaccharides.

Most of the bacterial lipases reported so far are constitutive and are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable among bacteria, *Achromobacter sp*., *Alcaligenes sp*., *Arthrobacter sp*., *Pseudomonas sp*., *Staphylococcus sp*., and *Chromobacterium sp*. have been exploited for the production of lipases. *Staphylococcal* lipases are lipoprotein in nature. Lipases purified from *S. aureus* and *S. hyicus* show molecular weights ranging between 34–46 kDa. The optimum pH varies between 7.5 and 9.0.

The lipase genes from *S. hyicus* and *S. aureus* have been cloned, sequenced, and compared with other lipases. This revealed two conserved domains separated by 100 amino acids which are likely to form active site. (Kotting *et al.*,1994).

2.2.1.2 Fungal lipases

Fungal lipases have been studied since 1950s, and (Lawrence *et al.*,1967) have presented comprehensive reviews. These lipases are being exploited due to their low cost of extraction, thermal and pH stability, substrate specificity, and activity in organic solvents. The chief producers of commercial lipases are *Aspergillus niger, Candida cylindracea, Humicola lanuginosa, Mucor miehei, Rhizopus arrhizus, R. delemar, R. japonicus, R. niveus* and *R. oryzae (*Godfredson.S.E, 1990)

2.2.2 Lipases in organic synthesis

For synthetic chemists, the application of lipases as catalysts in organic synthesis is of much advantage. These enzymes can show stereo- and regiospecificity, and tolerate organic solvents in the incubation mixture. Both the activity of the enzyme and the identity of the product depend upon the solvents used, which may vary from aqueous buffer systems through biphasic emulsions and microemulsions, to organic solvents. For synthetic purposes, crude enzyme preparations are often convenient. Because the catalyst is always an expensive factor in a chemical reaction, strategies for enzyme recycling are being developed. Synthetic strategies involving microbial lipases can be used to prepare molecules of high positional and configurational purity. Lipases can be used to create biologically active analogues of naturally occurring messenger molecules as antagonists or inhibitors in biological systems.

2.2.2.1 Bioconversions in organic media

The synthetic potential of lipases in organic solvents has been widely recognized and is well documented, particularly on the basis of activity of lipases in organic solvents containing low water content. The main application of lipases in organic chemistry is the resolution of enantiomeric compounds, making use of the enantioselectivity of these enzymes.

The use of organic solvents for lipase-catalysed resolutions has four main advantages in comparison with water as the solvent:

a) Racemic mixtures of alcohols or acids need not be esterified before resolution into enantiomers,
b) Lipase are more stable in organic solvents than in water,
c) Lipases used need not be immobilized for recovery, owing to their insolubility in organic solvents; they can be collected by filtration in their active state, and
d) Substrates and products may be unstable in aqueous solution. In this case, reaction in organic solvents is essential for formation and isolation of the products. The three main areas of lipase-catalyzed reactions in organic solvents are:

- Hydrolysis
- Transesterification reaction
- Ester Synthesis

2.2.3 Applications in industries

Table 2.1: Application of lipase in industries

Industry	Effect	Products
Bakery	Flavour improvement and shelf-life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Chemical	Enantioselectivity	Chiral building blocks and chemicals
Cleaning	Synthesis Hydrolysis	Chemicals Removing of cleaning agents like surfactants
Cosmetics	Synthesis	Emulsifiers, moisturising agents
Dairy	Hydrolysis of milk fat Cheese ripening Modification of butter fat	Flavours agents Cheese Butter
Fat and oils	Transesterification Hydrolysis	Fatty acids, glycerol, mono- and diglycerides
Food dressing	Quality improvement	Mayonnaise, dressing and whipping
Health food	Transesterification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavour development and fat removal	Meat and fish products
Paper	Hydrolysis	Paper products
Pharmaceuticals	Transesterification Hydrolysis	Speciality lipids Digestive aids

Source: (Kooting and Eibl 1994, Lawrence 1967, Godfrey *et al*.,1991, John and Abraham 1990)

2.2.3.1 Lipases in dairy industry

Lipases are used extensively in the dairy industry for the hydrolysis of milk fat. Current applications include flavour enhancement of cheese, acceleration of cheese ripening, manufacture of cheese-like products, and lipolysis of butter fat, and cream. While the addition of lipases primarily releases short-chain (C_4 and C_6) fatty acids that lead to the development of sharp, tangy flavour, the release of medium-chain (C_{12} and C_{14}) fatty acids tends to impart a soapy taste to the product.

More recently, a whole range of microbial lipase preparations have been developed for the cheese manufacturing industry, such as those of *Mucor miehei, Aspergillus niger* and *A. oryzae.* Ranges of cheese of good quality were produced by using individual microbial lipases or mixtures of several preparations. Lipases are widely used for imitation of cheese made from ewe's or goat's milk. (Lawrence *et al.,*1967) Addition of lipases to cow's milk generates a flavour rather similar to that of ewe's or goat's milk. (Godfrey, and Hawkins 1991) This is used for producing cheese or the so-called enzyme-modified cheese (EMC). EMC is a cheese that has been incubated in the presence of enzymes at elevated temperatures in order to produce a concentrated flavour for use as an ingredient in other products such as dips, sauces, soups, and snacks.

2.2.3.2 Lipases in detergents

The usage of enzymes in washing powders still remains the single biggest market for industrial enzymes. The world-wide trend towards lower laundering temperatures has led to much higher demand for household detergent formulations. Recent intensive screening programmes, followed by genetic manipulations, have resulted in the introduction of several suitable preparations, for example, Novo Nordisk's Lipolase (*Humicola lipase* expressed in *Aspergillus oryzae*).

2.2.3.3 Lipases in oleochemical industry

The scope for application of lipases in the oleochemical industry is enormous as it saves energy and minimizes thermal degradation during hydrolysis, glycerolysis, and alcoholysis. Miyoshi Oil and Fat Corperation, Japan, had reported commercial use of *Candida cylindracea* lipase in production of soap. The introduction of the new generation of cheap and very thermostable enzymes can change the economic balance in favour of lipase use.

The current trend in the oleochemical industry is a movement away from using organic solvents and emulsifiers the various reactions involving hydrolysis, alcoholysis, and glycerolysis have been carried out directly on mixed substrates, using a range of immobilized lipases. This has resulted in high productivity as well as in the continuous running of the processes. Enzymatic hydrolysis perhaps offers the greatest hope to successful fat splitting without substantial investment in expensive equipment as well as in expenditure of large amounts of thermal energy.

2.2.3.4 Lipases in synthesis of triglycerides

The commercial value of fats depends on the fatty acid composition within their structure. A typical example of a high-value asymmetric triglyceride mixture is cocoa butter. The potential of 1,3-regiospecific lipases for the manufacture of cocoa-butter substitutes was clearly recognized by Unilever and Fuji Oil. Comprehensive reviews on this technology, including the analysis of the product composition, are available. In principle, the same approach is applicable to the synthesis of many other structured triglycerides possessing valuable dietic or nutritional properties, for example, human milk fat. This triglyceride and functionally similar fats are readily obtained by acidolysis of palm oil fractions which are rich in 2-palmitoyl glyceride with unsaturated fatty acid(s).

Acidolysis, catalysed by 1,3-specific lipases, is used in the preparation of nutritionally important products which generally contain medium-chain fatty acids. Lipases are being investigated extensively with regard to the modification of oils rich in high-value polyunsaturated fatty acids such as arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acids.

2.2.3.5 Lipases in synthesis of surfactants

Polyglycerol and carbohydrate fatty acid esters are widely used as industrial detergents and as emulsifiers in a great variety of food formulations (low-fat spreads, sauces, ice-creams, mayonnaises.) Lipase from *A. terreus* synthesizes a biosurfactant by transesterification between natural oils and sugar alcohols. Lipases may also replace phospholipases in the production of lysophospholipids. *Mucor miehei* lipase has been used for the transesterification of phospholipid in a range of primary- and secondary alcohols. (Godfredson, 1990) Lipases may also be useful in the synthesis of a whole range of amphoteric bio-degradable surfactants, namely amino acid-based esters and amides.

2.2.3.6 Lipases in synthesis of ingredients for personal care products

Unichem International has recently launched the production of isopropyl myristate, isopropyl palmitate, and 2-ethylhexyl palmitate for use as emollient in personal care products like skin and sun-tan creams, and bath oils. Wax esters have similar application in personal care products and are being manufactured enzymatically, using *C. cylindracea* lipase, in a batch bioreactor. (Godfredson, 1990)

2.2.3.7 Lipases in pharmaceuticals and agrochemicals

The utility of lipases in the preparation of chiral synthons is well recognized and documented. The resolution of 2-halopropionic acids, the starting materials for the synthesis of phenoxypropionate herbicides, is a process based on the selective esterification of (S)-isomers with butanol, which is catalysed by porcine pancreatic lipase in anhydrous hexane. Another impressive example of the commercial application of lipases in the resolution of racemic mixtures is the hydrolysis of epoxyester alcohols. The reaction products, (R)-glycidyl esters and (R)-glycidol are readily converted to (R)- and (S)-glycidyltosylates which are attractive intermediates for the preparation of optically active ß-blockers and a wide range of other products. A similar technology has been commercialized to produce 2(R),3(S)-methylmethoxyphenyl glycidate, the key intermediate in the manufacture of the optically pure cardiovascular drug Diltiazem.

Lipases have applications as industrial catalysts for the resolution of racemic alcohols in the preparation of some prostaglandins, steroids, and carbocyclic nucleoside analogues. Regioselective modification of polyfunctional organic compounds is yet another rapidly expanding area of lipase application, particularly in the field of AIDS treatment. Lipases from *A. carneus* and *A. terreus* show chemo- and regiospecificity in the hydrolysis of peracetates of pharmaceutically important polyphenolic compounds. Lipases are also useful in the synthesis of the artificial sweetner sucralose by regioselective hydrolysis of octa-acetylsucrose.

2.2.3.8 Lipases in polymer synthesis

The stereoselectivity of lipase is useful for synthesis of optically active polymers. These polymers are asymmetric reagents, and are used as absorbents. In the field of liquid crystals, suitable monomers can be prepared by lipase-catalysed transesterification of alcohols, which with racemic alcohols may be accompanied by resolution. Lipases have been employed successfully in the food industry as well as in high technology production of fine chemicals and pharmaceuticals. Furthermore,

this enzyme has potentials in newer fields, for example lipases have successfully been used in paper manufacturing, the treatment of pulp with lipase leads to a higher quality product and reduced cleaning requirement. Similarly, the enzyme has also been used in association with a microbial cocktail for the treatment of fat-rich effluents from an ice-cream plant. This could also be utilized in waste processing of many food industries.

2.3 Immobilization of lipase

The preparation of fatty acid esters by enzyme catalysis has stimulated the optimisation of reactions under nonaqueous media for the production of compounds broadly used in both, the chemical and pharmaceutical industries The increase in the number of patents and publications in this research area can illustrate the commercial importance of this enzymatic approach. In biotransformation, the lipases stand out for their versatility to carry out hydrolysis, esterification and interesterification reactions with extreme process simplicity, superior quality of the end product and high yield.

These characteristics give the lipases a comparable biotechnology potential with proteases and amylases; enzymes used in large-scale level; stimulating research on the optimisation of the lipase production, immobilization and industrial applications. With immobilized lipases, improved stability, re-use, continuous operation, the possibility of better control of reactions and, hence, more favourable economical factors can be expected.

Lipase has been immobilized by several methods, namely adsorption, cross-linking, adsorption followed by cross-linking, covalent attachment and physical entrapment using many inorganic and organic materials as carriers, including activated charcoal, aluminium oxide, diatomaceous earth (Celite), controlled pore glass and synthetic resins. However, the activity and operational stability of the immobilized lipases depend on several parameters, such as the lipase source, the type

of support and the immobilization protocol. Among the immobilization techniques, the adsorption may have a higher commercial potential than other methods as they lend themselves a minimal resistance in the reaction mixtures, and possible supports are mechanically durable and re-usable. (Oliviera *et al*., 1999)

2.3.1 Techniques and supports for immobilization

A large number of techniques and supports are now available for the immobilization of enzymes or cells on a variety of natural and synthetic supports. The choice of the support as well as the technique depends on the nature of the enzyme, nature of the substrate and its ultimate application. Therefore, it will not be possible to suggest any universal means of immobilization. It can only be said that the search must continue for matrices which provide facile, secure immobilization with good interaction with substrates, and which conform in shape, size, density and so on to the use for which they are intended.

Care has to be taken to select the support materials as well as the reagents used for immobilization, which very safe (fail-proof and no contaminant), particularly when their ultimate applications are in the food processing and pharmaceutical industries. (Mitz, 1956)

Macromolecular, colloidal, viscous, sticky, dense or particulate food constituents or waste streams also limit the choice of reactor and support geometries commercial success has been achieved when support materials have been chosen for their flow properties, low cost, nontoxicity, maximum biocatalysts loading while retaining desirable flow characteristics, operational durability, ease of availability, and ease of immobilization (Mitz, 1956)

Techniques for immobilization have been broadly classified into four categories, namely entrapment, covalent binding, cross-linking and adsorption. A combination of one or more of these techniques has also been investigated. It must be emphasized that in terms of economy of a process, both the activity and the operational stability of the biocatalysts are important. They determine its productivity, which is the activity integrated over the operational time.

2.3.1.1 Entrapment

Entrapment has been extensively used for the immobilization of cells, but not for enzymes. The major limitation of this technique for the immobilization of enzymes is the possible slow leakage during continuous use in view of the small molecular size compared to the cells

Notable among them are the photo-crosslinkable resins, polyurethane prepolymers, and acrylic polymers like polyacrylamide. Among these, the most widespread matrix made from monomeric precursors is the polyacrylamide gel. Polyacrylamide may not be a useful support for use in food industry in view of its toxicity, but can have potentials in the treatment of waste and in the fabrication of analytical devices containing biocatalysts.

One of the major limitations of entrapment technique is the diffusional limitation as well as the stearic hindrance, especially when the macromolecular substrates like starch and proteins are used. Diffusional problems can be minimized by entrapment in fine fibres of cellulose acetate or other synthetic materials or by using an open pore matrix.

Recently, the developments of so-called hydrogels and thermoreactive water-soluble polymers, like the albumin-poly (ethylene glycol) hydrogel, have attracted attention in the field of biotechnology. In the area of health care, they offer new avenues for enzyme immobilization. Such gels with a water content of about 96%

provide a microenvironment for the immobilized enzyme close to that of the soluble enzyme with minimal diffusional restrictions.

2.3.1.2 Covalent binding

Covalent binding is an extensively used technique for the immobilization of enzymes, though it is not a good technique for the immobilization of cells. The functional groups extensively investigated are the amino, carboxyl, and the phenolic group of tyrosine. Enzymes are covalently linked to the support through the functional groups in the enzymes, which are not essential for the catalytic activity. It is often advisable to carry out the immobilization in the presence of its substrate or a competitive inhibitor so as to protect the active site. The covalent binding should also be optimized so as not to alter its conformational flexibility.

Covalent binding has been extensively investigated using inorganic supports. Enzymes covalently bound to inorganic supports have been used in the industry. Enzymes have also been bound to synthetic membranes, thus integrating biconversion and downstream processing. Large-scale processes using such an approach have been demonstrated for the preparation of invert sugar using invertase.

2.3.1.3 Cross-linking

Biocatalysts can also be immobilized through chemical cross-linking using homo- as well as heterobifunctional cross-linking agents. Among these, glutaraldehyde which interacts with the amino groups through a base reaction has been extensively used due to its, low cost, high efficiency, and stability.

The enzymes or the cells have been normally cross-linked in the presence of an inert protein like gelatine, albumin, and collagen. Adsorption followed by cross-linking has also been used for the immobilization of enzymes. The technique of

cross-linking in the presence of an inert protein can be applied to either enzymes or cells

2.3.1.4 Adsorption

This is perhaps the simplest of all the techniques and one which does not grossly alter the activity of the bound enzyme. In case of enzymes immobilized through ionic interactions, adsorption and desorption of the enzyme depends on the basicity of the ion exchanger. Moreover, a dynamic equilibrium is normally observed between the adsorbed enzyme and the support which is often affected by pH as well as the ionic strength of the surrounding medium. This property of reversibility of binding has often been used for the economic recovery of the support.

This has been successfully adapted in industry for the resolution of racemic mixtures of amino acids, using amino acid acylase. A variety of commercially available ion exchangers have been investigated for this purpose. One of the techniques, which have gained importance more recently, is the use of polyethylenimine for imparting polycationic characteristics to many of the neutral supports based on cellulose or inorganic materials. Enzymes with low pI, like invertase, urease, glucose, oxidase, catalase, and other enzymes have been bound through adsorption followed by cross-linking on polyethylenimine-coated supports.

Physical adsorption mode is the common method for the immobilization of enzyme on the surface of water-insoluble carriers. Hence, the method causes little or no conformational change of the enzyme or destruction of its active centre. If a suitable carrier is found, this method can be both simple and cheap.

However, it has the disadvantage that the adsorbed enzyme may leak from the carrier during use due to a weak binding force between the enzyme and the carrier. The earliest example of enzyme immobilization using this method is the adsorption of beta-D-fructo-furanosidase onto aluminum hydroxide. The processes available for physical adsorption of enzymes are:

- Static Procedure
- Electro-deposition
- Reactor Loading Process
- Mixing or Shaking Bath Loading

Among the four techniques, the most frequently used in the lab is Mixing-Bath Loading. For commercial purposes the preferred method is Reactor Loading.

A major advantage of adsorption as a general method of immobilizing enzymes is that usually no reagents and only a minimum of activation steps are required. Adsorption tends to be less disruptive to the enzymatic protein than chemical means of attachment because the binding is mainly by hydrogen bonds, multiple salt linkages, and Van der Waal's forces. In this respect, the method bears the greatest similarity to the situation found in natural biological membranes and has been used to model such systems.

Because of the weak bonds involved, desorption of the protein resulting from changes in temperature, pH, ionic strength or even the mere presence of substrate, is often observed. Another disadvantage is non-specific, further adsorption of other proteins or other substances as the immobilized enzyme is used. This may alter the properties of the immobilized enzyme or, if the substance adsorbed is a substrate for the enzyme, the rate will probably decrease depending on the surface mobility of enzyme and substrate.

2.4 Solid support material

Supports used for immobilizing enzyme should possess mechanical strength, microbial resistance, thermostability, chemical durability, chemical functionality, low cost, hydrophylicity, regenerability and a high capacity of enzyme (Kilara, 1981). The various immobilization protocols used with enzymes have been extensively studied (Kennedy and Cabral, 1983). Immobilization of enzyme consist four different methods: adsorption of the enzyme on a carrier, entrapment of the enzyme within an insoluble gel matrix, containment of enzyme within porous hollow fibers or microcapsules, binding of the enzyme to dried mycelia and ion exchange between the enzyme and support (Malcata *et al*.,1990). Immobilization of enzyme through physical method is still the most commonly used because it is the easiest to perform and the least expensive. In this method, the forces between a support and the enzymes include hydrogen bonding, Van der Waals forces and hydrophobic interactions (Burns, 1986).

2.4.1 Calcined Kaolin

In this study, support that used is calcined-kaolin (Aluminium silicate hydroxide) $Al_2Si_2O_5OH)_4$. The kaolin is made from India (Himafil) courtesy of AT Chemical Sdn Bhd. It is a layered silicate mineral, with one tetrahedral sheet linked through oxygen atoms to one octahedral sheet of alumina octahedral. Kaolin has a low shrink-swell capacity and a low cation exchange capacity (1-15 meq/100g.)

Calcination is the process of heating the small particles of Kaolin to about 1800 degrees Fahrenheit for about 45 minutes. In other word, calcined kaolin is also produced by heating spray dried fine particle kaolin to temperatures in the range of 1,000 C. The kaolinite becomes anhydrous and transformed to mullite (Al_2 Si_2 O_5) and SiO_2. This process 'pops' the structure of the Kaolin molecule and increases the surface area. The brightness goes up and the reflective characteristics are increased.

Rotary calcining kiln removes chemical water from clay crystal and forms porous aggregate structures. These thermally structured calcined clays are used to replace and substitute of TiO_2 - 15 to 25% in many applications. (Sabir.*et al*.,2001)

For this research calcined kaolin that used is HIMAFIL (brand name) obtained from English Indian Clays Limited courtesy of AT Chemical Sdn Bhd.

CHAPTER 3

MATERIALS & METHOD

3.1 Introduction

This chapter will discuss about processes to prepare immobilization lipase and analysis based on several parameters. Free lipase and immobilized lipase was reacted at different temperature and different pH. Amount of lipase attached was determined using Bradford method. Lipase activity was determined by chemical method as known as soap colorimetry method.

3.2 Research Procedures

3.2.1 Immobilization of lipase

Crude lipase from *C. rugosa* (1.50 g) was dispersed into distilled water (30 ml). The mixture was stirred for an hour then the immobilization of lipase was carried out by continuous shaking at 100 rpm of kaolin (4.00 g) with partially purified lipase solution (15 ml) for 1 h at room temperature. The immobilized lipase was then separated by filtration and washed with distilled water to remove the unabsorbed soluble enzyme. The immobilized lipase was then lyophilized in freeze drier.

3.3 Procedures Analysis

3.3.1 Lipase Assay

The lipase activity was determined by soap copper colorimetry (Kwon and Rhee, 1986). Lipase (1ml) was shaken with 5 ml of olive oil and 20 μL of 0.02 Molar $CaCl_2$ in a water bath shaker at an agitation rate of 150 rpm.

3.4 Characterization of the immobilized lipase on the esterification activity

3.4.1 Effect of Temperature

The mixtures were reacted at different temperatures (30°C, 40°C, 50°C and 60°C) for 30 minutes at 150rpm in a waterbath shaker. The relative activities are determined as percentage yield of activities at different temperature compared to the activity of reaction at 40°C

$$\text{Relative activity (\%)} = \frac{\text{Activity at different temperature}}{\text{Maximum \% activity (40 }^{\circ}\text{C)}} \times 100$$

3.4.2 Effect of pH

The mixtures were reacted at different pH (6, 7, 8, and 9) for 30 minutes at 150rpm in a waterbath shaker. The relative activities are determined as percentage yield of activities at different temperature compared to the activity of reaction pH 8.

$$\text{Relative activity (\%)} = \frac{\text{Yield at different pH}}{\text{Maximum \% activity (pH 8)}} \times 100$$

3.5 Analytical Procedures

3.5.1 Protein assay (Bradford method)

The amount of protein content before and after immobilization was determined by using the method of Bradford Coomassie brilliant blue assay procedure using bovine serum albumin as standard (Bradford, 1976).

3..5.2 Bradford Method procedure

Bradford Reagent was prepared by dilution of Coomassie protein assay reagent to distilled water with ratio 1:4. Standard curve then was prepared by preparing a series of dilution BSA stock solution at concentration of 200, 500, 1500 and 2000 µg/ml. 1 ml of Bradford reagent was added into 0.1 ml of each concentration of BSA. The mixture was mixed well and leave at room temperature. After 5 minutes, optical density (OD) of mixture was measured at 595 nm against blank. Calibration curve of OD versus concentration for each BSA concentration then was plotted. The same procedure was repeated to determine protein content in sample by replacing the 0.1 ml of BSA solution with 0.1 ml of sample.

3.5.3 Esterification Assay for Enzyme Activity

3.5.3.1 Standard Curves of Free Fatty Acids.

A sample containing 2.0-1000.0 µmole free fatty acids, oleic acids was prepared by dissolving them in test tubes with 5 ml of hexane. Then 1.0 ml of cupric-pyridine reagent was added and the two phases thus formed were mixed vigorously for 90 seconds using a vortex mixer. The mixture was allowed to stand still for about 10-20 seconds until the aqueous phase was formed clearly from the solution of hexane and fatty acid. The standard curves of free fatty acids vs. absorbency were determined by measuring the absorbance of hexane solution at 715 nm against the control which contains no free fatty acids. One unit of lipase activity was defined as the amount of enzyme that liberated 1 µmol of free fatty acid per min

3.5.3.2 Determination of Lipase Activity

The reaction was shaken for 30 min at determined temperature (30°C, 40°C, 50°C and 60°C). The enzyme reaction in the emulsion system was stopped by adding 6 M HCl (1 ml) and hexane (4 ml), followed by mixing using a vortex mixer for 30 s. The upper hexane layer containing the fatty acid was transferred to a test tube for analysis. Copper reagent (1 ml) was added and again mixed with a vortex mixer for 30 s. The reagent was prepared by adjusting the solution of 5 % (w/v) copper reagent to pH 6.1 with pyridine.

CHAPTER 4

RESULT AND DISCUSSION

4.1 Introduction

To immobilize *Candida rugosa* lipase (CRL) there are proven patents and study about methods of immobilization natural clay. However the main concern will be chemical compositions that exist in the particular natural substances. As in natural clays, water molecules were replaced with lipase molecules; calcined- kaolin has no water molecules for lipase to replace with. (A.Rahman *et al*.,2005). But with shrinking effect of calcined kaolin, perhaps pores will emerged for lipase molecules to stick into them.

4.2 Immobilization of lipase

Using the Bradford method, the protein content in supernatant of crude lipase before immobilization was determined.

% of immobilization

$$= \frac{(\text{Total amount of protein in supernatant before immobilization} - \text{total amount of protein in supernatant after immobilization})}{\text{Total amount of protein in supernatant before immobilization}} \times 100$$

Below is the table of data from spectrophotometer and use for construction of standard curve of Bradford method

Table 4.1: Concentration of BSA versus Optical Density

Concentration BSA(µg/mL)	Reading			
	1st	2nd	3rd	Average
Blank	0.00	0.00	0.00	0.00
200	0.421	0.418	0.414	0.417
500	0.632	0.628	0.622	0.627
1000	1.272	1.253	1.247	1.257
1500	1.658	1.662	1.662	1.660
2000	2.012	1.982	1.971	1.988

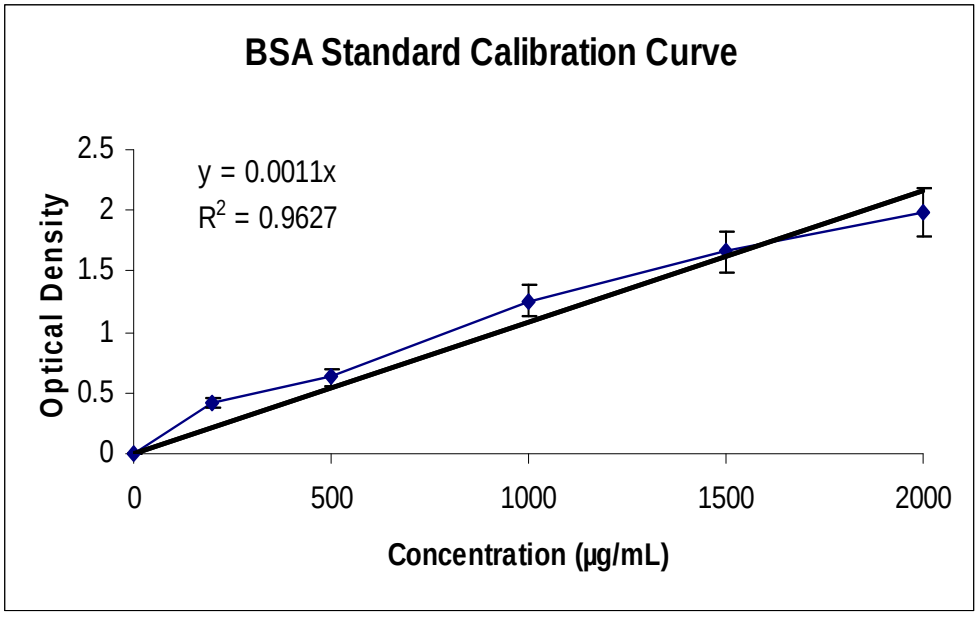

Figure 4.1: Concentration of BSA versus Optical Density

After immobilization, the protein content in supernatant was found. By applying the formula (refer at section **4.1**), it was estimated that 68% of protein in supernatant from *C. rugosa* have been immobilized onto the support. The lipase molecules may generally be immobilized on the surfaces and within the support as calcined-kaolin possess pores due to the shrinking effects as it was heated at high temperatures. (Sabir *et al*.,2001). Compared to usage of natural kaolin, the percent of immobilized lipase is higher (77%). It is believed this is because of the protein molecules of lipase replacing the molecules of water. (A.Rahman *et al*.,2005)

Kaolin provides good distribution of lipase on mass transfer and preventing lipase particles from aggregation thus helps dispersion of lipase in the reaction media. In comparison, native lipase was not easily dispersed as they tend to aggregate, thus causing a decrease in their activity. In spite of that feasibility of support for immobilized lipase cannot be determine solely on function of protein loading. The resistance of immobilized lipase towards extreme condition such as temperatures and pH need to consider as well as protein loading capabilities.

4.3 Immobilized lipase activity

Immobilized lipase and free lipase were reacted at temperatures varying from 30 ^{o}C to 60 ^{o}C with 10 ^{o}C interval. Lipase activity was determined by measuring the amount of free fatty acids from the standard curves of free fatty acids. Unit activity (U/g) of lipase is defined as the amount of enzyme capable of producing 1 μmol of fatty acid per 1 min. The standard curves of free fatty acids vs. absorbency were determined by measuring the absorbance of hexane solution at 715 nm against the control which contains no free fatty acids.

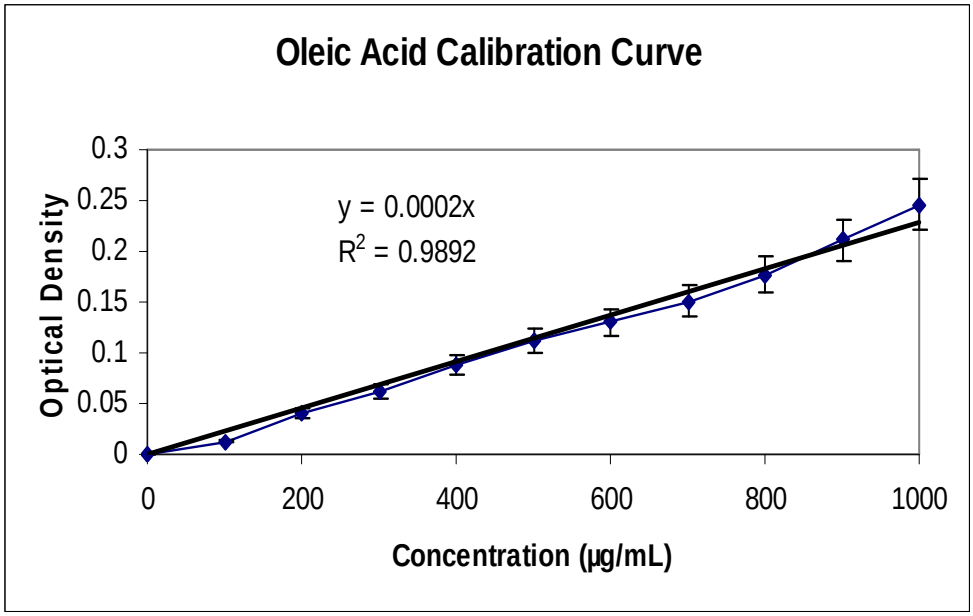

Figure 4.2: Concentration of Oleic acid versus Optical Density

4.4 Characterization of the immobilized lipase

4.4.1 Effect of temperature on activity of lipase

The mixtures were reacted at different temperatures (30 °C, 40 °C, 50 °C, and 60 °C) for 30 minutes at 150 rpm in a water bath shaker.

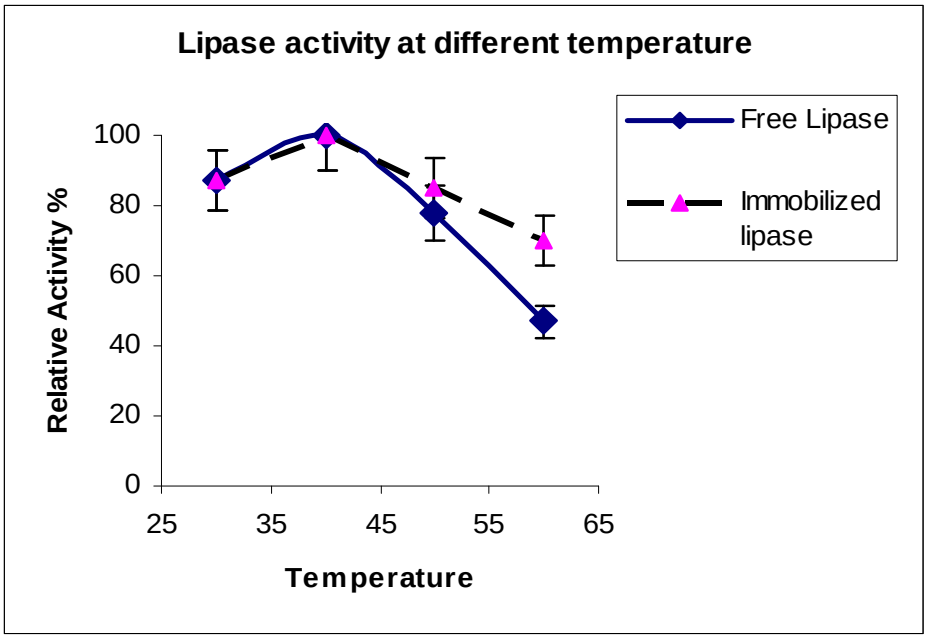

Figure 4.3: Temperature lipase assay versus relative activity

It is expected lipase to be active at optimum temperature of 37°C and eventually becoming inactive due to denaturation towards 60 °C (A.Rahman *et al*.,2005). The heat energy from the reaction temperature may affect enzymatic rate and functional group of substrate involved in the reaction. The excess heat energy provided to the enzymatic system has inhibited the conversion of product because lipase may become inactivated by denaturation.

Usage of calcined-kaolin is proved to be suitable as kaolin possess ceramic properties which display excellent medium for heat resistance. Excess heat energy was transferred and dispersed evenly along the support hence will not denatured the lipase. As calcined-kaolin also known as inert substance, exposure to heat will not chemically change the structure and properties of calcined-kaolin. This is important as if solid support material reacted to heat effect, it will eventually affect the lipase as a whole. This is because when a lipase molecule adsorp to calcined-kaolin, it will act as a complex known as immobilized lipase where it known and acts as a single entity.

4.4.2 Effect of pH on activity of lipase

The mixtures were reacted at different pH (6, 7, 8 and 9) for 30 minutes at 40°C and at 150rpm in a waterbath shaker. The relative activities are determined as percentage yield of activities at different temperature compared to the activity of reaction pH 8.

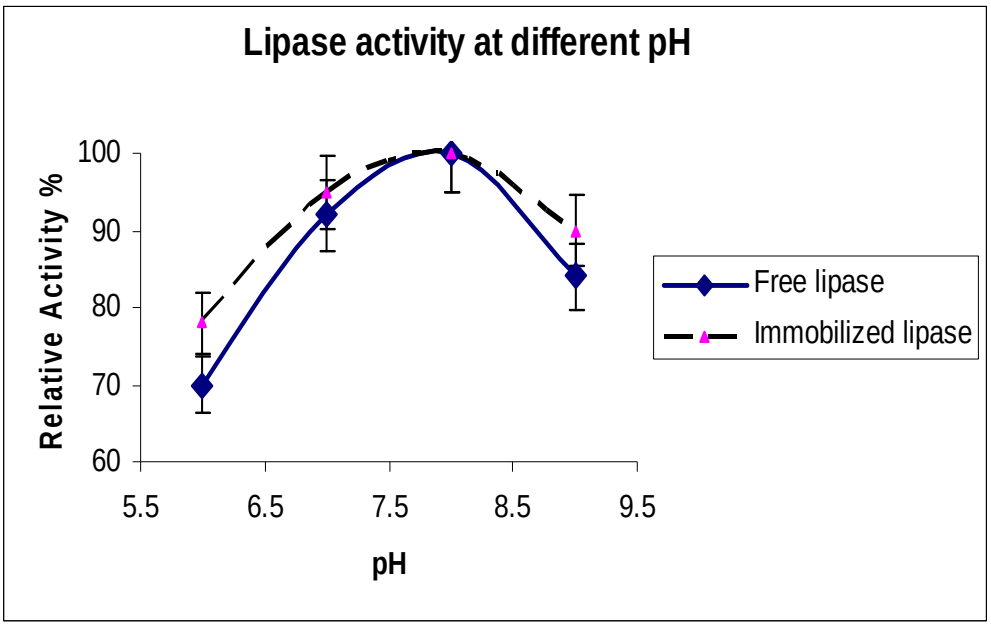

Figure 4.4: pH of lipase assay versus relative activity

It is expected lipase to be active at optimum pH of 8 and eventually becoming inactive due to denaturation. (Hong *et al*.,2005). It was observed that the higher pH, the higher activity was obtained. It was known that the instantaneous neutralization of fatty acid by OH− at reaction site accelerated the extraction of hydrolytic products by aqueous phase. The increase of OH− concentration would enhance the driving force for the diffusion of OH− from bulk to reaction front, the transformation of fatty acid RCOOH to RCOO− at reaction site and the diffusion of RCOO− from reaction front to aqueous phase bulk. All of these contributed to the high catalytic efficiency at high pH.

Despite of that, extreme pH will affect the configuration of lipase. As 3 dimension globular form of enzyme is determined by interaction of charges of its constituents, extreme pH will absolutely change the structure. Immobilized lipase complex will avoid the denaturation by mechanism of calcined kaolin engulfing the lipase and avoid direct contact of extreme pH. Though the mechanism will somehow lower lipase activity, it will preserve immobilized lipase at harsh condition. It is because as long as activity concern, the main part of lipase structure that matter is active site that made from interaction of lipase constituent. The base line is calcined-kaolin proven to decreased denaturation of lipase due to extreme pH.

CHAPTER 5

CONCLUSION AND RECOMMENDATION

5.1 Conclusions

Immobilized lipase is done successfully using *Candida rugosa* lipase (CRL) as lipase source and calcined-kaolin as solid support material. 68% of free lipase was immobilized onto calcined-kaolin as solid support material. Calcined kaolin also displayed good properties as support to the effects of temperature and pH. Profile of free lipase and immobilized lipase to effect of temperatures and pH were determined and objectives of this research were met. With the cost of lipase rather high; the only ways to cut the cost are solid support material which is used and method of immobilization. Although a physical adsorption method works well to immobilize the enzyme, combination of methods and pre-treatment or post-treatment will enhanced the immobilization process.

Future of immobilization of lipase is bright because it is feasible to immobilize enzyme rather modification of enzyme. With abundance natural resources in Malaysia particularly, (clay, straw, paddy husk) and ever growing interest toward cheaper solid support material, it is believe immobilized lipase using natural solid support material have huge potential. In addition, calcined-kaolin display potential in bigger scale processes as it possesses characteristic that able to withstand higher pressure as the nature of ceramic. This permitted calcined-kaolin to be applied in reactor where pressure is high; rather than usage of other conventional solid support material; like gel-types.

5.2 Recommendation

This experiment was merely to prove whether calcined- kaolin can be considered to be used as commercial solid support material. Further extensive research need to be done to clarify the feasibility of calcined-kaolin. As for future, it is advisable to prepare calcined-kaolin for natural clay as strong acid treatment and heat treatment can be monitored and studied. Optimization of lipase need to be done by manipulating enzyme concentration, optimum temperature and pH before feasibility of calcined-kaolin can be proven.

As for lipase activity determination, it is recommended to use analytical equipments such as Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC) to replace conventional chemical-method determination procedures. Application of GC and HPLC in protein determination had being used widely and quite numbers of journals stating the standard procedures to for operating condition of them. The main hurdle might be the access to GC and HPLC as both of them are rather expensive to buy.

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APPENDIX

% of immobilization

$$= \frac{\text{(Total amount of protein in supernatant before immobilization - total amount of protein in supernatant after immobilization)}}{\text{Total amount of protein in supernatant before immobilization}} \times 100$$

$$= \frac{(2625.5\ \text{mg} - 840.19\text{mg})}{2625.5\text{mg}} \times 100$$

= **68%**

Relative activity

Effect of temperature

OD reading at $40^{o}C$= 0.188
Base on BSA standard curve, concentration = 940 μg/ml

So, enzyme activity = $\frac{940\ \mu g/ml}{30(min)}$

= **31 μg/ml/min**

Free lipase

Temperature	**OD(nm)**	**Concentration (μg/ml)**	**enzyme activity (μg/ml/min)**	**Relative Activity (%) with base condition ($40^{o}C$)**
30	0.162	810	27	87
40	0.188	940	31	100
50	0.145	725	24.2	78
60	0.088	440	14.6	47

Immobilized lipase

Temperature	OD(nm)	Concentration (µg/ml)	enzyme activity (µg/ml/min)	Relative Activity (%) with base condition (40^oC)
30	0.162	810	27	87
40	0.188	940	31	100
50	0.158	790	26.3	85
60	0.130	650	21.7	70

Relative activity

Effect pH

OD reading at pH 8 = 0.175
Base on BSA standard curve, concentration = 875 µg/ml

So, enzyme activity = $\frac{875\ \mu g/ml}{30(min)}$

= **29 µg/ml/min**

Free lipase

pH	OD(nm)	Concentration (µg/ml)	enzyme activity (µg/ml/min)	Relative Activity (%) with base condition (pH 8)
6	0.122	610	20.3	70
7	0.160	800	26.7	92
8	0.175	875	29	100
9	0.146	730	24.3	84

Immobilized lipase

pH	OD(nm)	Concentration (µg/ml)	enzyme activity (µg/ml/min)	Relative Activity (%) with base condition (pH 8)
6	0.136	680	22.6	78
7	0.165	825	27.5	95
8	0.175	875	29	100
9	0.156	780	26	90