IMMOBILIZATION OF LIPASE FROM *Candida rugosa* ON CHITOSAN BEADS FOR TRANSESTERIFICATION REACTION

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I declare that this thesis entitled "*Immobilization of Lipase from Candida rugosa on Chitosan Beads for Transesterification Reaction*," 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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Signature	: ……
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Date	: 2nd May 2008

DEDICATION

Special Dedication to This Grateful Feeling to My..

***Beloved Parents**:*
Mr. Said B. Musa
Mrs. Ramlah Bt Abd Talib

Loving Brothers and Sister:
Halimie B. Said
Norliza Bt. Said
Hasnizam B. Said
Mohd Akmal B. Said

My fellow colleague:
Pioneer BKB's Students
(2004 – 2008)

And to my special one…

For Their Love & Care, Supports and Encouragements

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ABSTRACT

The objective of this study was to evaluate the immobilization of lipase on a chitosan support by physical adsorption, aiming its application in transesterification reactions. Immobilization of lipase give several advantages such as improved stability, reuse, continuous operation and the possibility of better control of reactions. Chitosan offers several advantages as an enzyme immobilization carrier such as versatility of available physical forms (flakes, porous beads, gel, fiber and membrane), low biodegradability and easy handling. In this study, bead porous of chitosan was used for immobilizing lipase from a microbial source of *Candida rugosa.* Lipase was immobilized by physical adsorption on chitosan following a previously developed methodology by Carneiro da Chunca *et al*. (1999). The ability of immobilized lipase on chitosan beads to catalyze transesterification of cooking oil and methanol was investigated. The important parameters like reaction time and oil to methanol molar ratios were studied to indentify the comparison between free lipase and immobilized lipase on transesterification reaction. From the study it was found that maximum conversion of ester using immobilized lipase and free lipase were 72.25% and 76.5% obtained at the optimum conditions of 1:4 molar ratios and reaction time of 48 hour. Therefore, the conversion of ester for free lipase was higher than immobilized lipase. As a conclusion, the chitosan beads were appear as a suitable support for immobilized lipase on trasnesterification reaction even though the ester conversion was lower than free lipase. On the other hand, immobilized lipase was provided an important advantage such as easy separation from the product and has a high potential to reuse.

ABSTRAK

Objektif di dalam kajian ini adalah untuk menilai pengimobilan lipase terhadap bahan penyokong chitosan melalui penjerapan fizikal, mengaplikasikannya untuk tindak balas transesterifikasi. Pengimobilan lipase mempunyai beberapa kelebihan, antaranya adalah meningkatkan kestabilan, boleh digunakan semula, dan berkemungkinan boleh mengawal tindak balas dengan baik. Chitosan menawarkan beberapa kelebihan sebagai bahan penyokong pengimobilan enzim seperti kepelbagaian bentuk fizikal (kepingan, manik poros, gel, jaringan dan penapis), penguraian biologi yang rendah dan mudah untuk dikawal. Dalam kajian ini, manik poros chitosan telah digunakan untuk pengimobilan lipase yang diperolehi daripada *Candida rugosa*. Pengimobilan Lipase telah dilakukan dengan menggunakan teknik penjerapan fizikal mengikut kaedah yang pernah digunakan oleh Carneiro da Chunca *et al.* (1999). Kebolehan lipase terimobil di atas manik poros chitosan diuji sebagai katalis untuk tindak balas transesterifikasi minyak masak dan metanol. Parameter yang penting seperti tempoh tindak balas dan nisbah molar minyak terhadap metanol turut diuji untuk menentukan perbezaan prestasi diantara lipase terimobil dan lipase bebas. Daripada eksperimen ini, penukaran dari minyak masak ke ester yang maksimum diperolehi pada keadaan nisbah molar 1:4 dengan 48 jam masa tindak balas dengan menggunakan lipase terimobil dan lipase bebas adalah 72.25% dan 76.5%. Oleh itu, penukaran produk ester bagi lipase bebas adalah lebih tinggi jika dibandingakan dengan lipase terimobil. Kesimpulanya, manik poros chitosan merupakan salah satu penyokong yang sesuai untuk pengimobilan lipase bagi diaplikasikan sebagai katalis dalam proses transesterifikasi minyak masak walaupun peratus penukaran ester lebih rendah jika dibandingkan dengan lipase bebas. Walaubagaimanapun, lipase terimobil mempunyai kelebihan yang lain seperti mudah dalam proses pemisahan antara produk dan juga mempunyai potensi yang baik untuk digunakan semula.

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LIST OF SYMBOLS

BSA	-	Bovine Serum Albumin
g	-	gram
E	-	Enzyme
S	-	Substrate
P	-	Product
U/g	-	Activity per gram
e.g.	-	Example
%	-	Percentage
mg	-	milligram
PCB	-	Porous chitosan beads
PPL	-	Porcine Pancrease lipase
µmol	-	micro mol
g/mmol	-	gram per millimol
h	-	hour
M	-	Molarities
V	-	Volume
mm	-	millimeters
˚C	-	Degree Celsius
v / v	-	Volume per volume
w / w	-	Weight per volume
rpm	-	Rotation per minute
ml	-	milliliter
TLC	-	Thin layer chromatography
µg/ml	-	microgram per milliliter
VII	-	Six
NaOH	-	Sodium hydroxide
ρ	-	Density

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Lipases enzyme are widely used in industrial applications due to the wealth of reactions they catalyze. Lipase has unique characteristics such as can catalyze reactions involving insoluble organic and aqueous phases and are able to preserve their catalytic activity in organic solvents, biphasic system and in micellar solutions (Hung *et al*., 2003). Due to their capability, many researches have been done to see potential usage of lipase in the future. One of them is the capability of lipase as biocatalyst in transesterification process to produce biodiesel. In order to use the enzyme economically and efficiently in aqueous as well as in non-aqueous solvents, their activity, selectivity, and operational stability can be modified by immobilization.

Various methods for enzyme immobilization can be divided into two general classes; chemical methods, where covalent bonds are formed with the enzyme, and physical methods, where weak interactions between support and enzyme exist (Chiou and Wu, 2004). Type of support as well as the method of immobilization influences the activity and operational stability of immobilized lipases (Hung *et al*., 2003). One of the advantages using immobilized lipase is high possibility of regeneration and reusability the immobilized enzyme. Beside, separation of product will be easier using the immobilized lipase. The important thing is to find the best and suitable support for lipase immobilization.

In this study, chitosan has been chosen to be a support for lipase. Chitosan, which is a poly-N-acetylglucosamine, is a transformed oligosaccharide obtained by deacetylation of chitin, and is the second after natural cellulosic carbohydrate polymer abundance (Chiou and Wu, 2004). Chitosan has many significant biological and chemical properties such as biodegradable, biocompatible, bioactive and polycatonic properties (Tang *et al.,* 2007). Therefore chitosan is a suitable support to used in immobilized enzyme due to its widely used in industrial application such us for water treatment and also in biomedical. Since chitosan is derived from natural resource it is an economical support compare to the polymer support.

1.2 Problem Statement

Pandey *et al.* (1999) has stated that lipase enzyme can catalyze hydrolysis, intersterification, esterification, alcoholysis, acidsolysis, and aminolysis reaction. Those are the capability of lipase as a catalyst in the particular reaction. For many applications lipase enzymes are preferably used in an immobilized state in order to easily separate the catalyst from the product stream (Pereira *et al.,* 2003). With immobilized lipases, improved stability, reuse, continuous operation, the possibility of better control of reactions and hence more favorable economical factors can be expected (Frence *et al.,* 1996; Tischer and Wedekind, 1999).

Many different methods of lipase immobilization are available, each involving a different degree of complexity and efficiency (Malcata *et al.,* 1990). In addition, attachment method of lipase to the support is an important criterion in order to sustain the stability of immobilized lipase itself. Beside, suitable support for immobilized enzyme is also important to enhance the reaction and achieve high performance during the reaction occurs.

However, research on application of immobilized lipase is still lacking in transesterification process. So, there is a need to have an initial study on producing immobilized lipase by using support material like chitosan beads and to study transesterification process while using immobilized lipase as a catalyst.

1.3 Objectives of the Research

Objective of this research are as follows

- To produce immobilized lipase from *Candida rugosa* on chitosan beads.
- To study the comparison between free lipase and immobilized lipase on the effect of reaction time and oil to methanol molar ratio on transesterification reaction.

1.4 Scope of Research Work

Scopes of this research work are as follows

- Preparation of chitosan beads as a support for immobilized lipase.
- Immobilization of lipase on chitosan beads by physical adsorption method.
- Observe the transesterification reaction catalyzed by free lipase and immobilized lipase to determine the enzyme assay and enzyme activity assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Enzyme

2.1.1 Introduction

A catalyst is a substance that lowers the activation energy of a reaction, thereby increasing the rate of reaction (Shuler and Kargi, 2002). Catalysts facilitate reactions but are themselves not consumed or transformed by these reactions. It is important to note that catalyst do not affect the energetic or the equilibrium of a reaction; catalyst affect only the speed at which reaction proceed. Most reactions in living organisms would not occur at appreciable rates without catalyst. The catalysts of biological reactions are proteins called enzymes. Enzymes are highly specific in the reactions that they catalyze. Each enzyme catalyzes only single type of chemical reaction, or in the case of certain enzymes, a class of closely related reactions. This specificity is related to the precise three-dimensional structure of the enzyme molecule. In an enzyme-catalyzed reaction, the enzyme temporarily combines with the reactant, which is termed a substrate (S) of the enzyme, forming an enzyme-substrate complex. Then, as the reaction proceeds, the product (P) is released and the enzyme (E) is returned to its original state (Mckee and James, 2003):

$$E + P \longleftrightarrow E - S \longleftrightarrow E + P \tag{2.1}$$

The catalytic power of enzymes is impressive. Enzymes typically increase the rate of chemical reactions from 10^8 to 10^{20} times the rate that would occur

spontaneously. To catalyze a specific reaction, an enzyme must do two things: first is bind the correct substrate, and secondly is position the substrate relative to the catalytically active groups at the enzyme's active site. Binding of substrate to enzyme produces the enzyme-substrate complex. This serves to align reactive groups and places strain on specific bonds in the substrate (S). The result of enzyme-substrate complex formation is a reduction in the activation energy required to make the reaction proceed with the conversion of substrate (S) to product (P) (Mckee and James, 2003).

Enzymes can also catalyze energy-requiring reactions, converting energy-poor substrates to energy-rich products. In this case, not only must an activation energy barrier be overcome, but sufficient free energy must also be put into the system to raise the energy level of the substrates to that of the products. Although theoretically all enzymes are reversible in their action, in practice, enzymes catalyzing highly exergonic or highly endergonic reactions are essentially unidirectional. If a particular exergonic reaction needs to be reversed during cellular metabolism, a distinctly different enzyme is frequently involved in the reaction (Mckee and James, 2003).

2.1.2 Structure of enzyme

Many enzymes contain small non-protein molecules that participate in the catalytic function but are not considered substrates in the usual sense. These small enzyme associated molecules are divided into two categories on the basis of the nature of their association with the enzyme: prosthetic groups and coenzymes. Prosthetic groups are bound very tightly to their enzymes, usually permanently. Coenzymes are bound rather loosely to enzymes, and a single coenzyme molecule may associate with a number of different enzymes at different times during growth. Most enzymes are derivatives of vitamins (Shuler and Kargi, 2002).

Enzymes are named either for the substrate they bind or for the chemical reaction they catalyze, by addition of the combining form–*ase*. Thus, cellulose is

and enzyme that attacks cellulose, glucose oxidase is an enzyme that catalyzes the oxidation of glucose, and ribonuclease is an enzyme that decomposes ribonucleic acid. A more formal nomenclature system employing a specific numbering system is used to classify enzymes more precisely (Shuler and Kargi, 2002).

2.1.3 Lipase enzyme

Lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) are enzymes widely distributed among animals, plants, and microorganisms that catalyze the reversible hydrolysis of glycerol ester bond and also the synthesis of glycerol esters. In nature, lipase is used only for hydrolysis. Under certain circumstances, lipases also catalyze a number of transesterification reactions. Lipases can be used in low-water environment as excellent tool for the transformation of commercial triglycerides, and or their derivate, to synthesize a growing range of products of potential industrial interest (Pirozzi, 2003). Lipases have been successfully used as catalysts for the synthesis of esters, both in small-scale work and on an industrial scale. The mild reaction conditions in the enzymatic reactions make it possible to obtain products of very high purity. Another advantage of enzymes is their selectivity. For example, some lipases have selectivity towards the length of the fatty acids or the number and location of fatty acids

The industrial applications of lipases have grown rapidly in recent years and are likely to markedly expand further in the coming years. Lipase may be used to produce fatty acids (Linder *et al*., 2002), biosurfactants (Edmundo *et al.,* 1998), aroma and flavor compounds (Athawale *et al.,* 2003), lubricant and solvent esters (Hills, 2003), polyesters (Kumar and Gross. 2000) and amides, thiol esters (Gandhi, 1997).

There are at least 35 lipases available commercially, but only a few can be obtained in industrial quantities. Some of the most promising commercially available microbial lipases are *Candida antarctica*, *Candida rugosa* (ex. *Candida*

cylindracea), *Rhizomucor miehei*, *Pseudomonas fluorescens*, *Aspergillus niger,* and *Chromobacterium viscosum* (Wu *et al.,* 1996).

Lafuente *et al.* (1998) reported that lipases have triglycerides as natural substrates. Because of triglyceride is a key role in the metabolism of fats, lipases are widely spread in nature. Interestingly, in addition to triglycerides, lipases are also able to catalyze the hydrolysis or synthesis of wide range of soluble or insoluble carboxylic acid esters and amides. In this way, lipase-catalyzed reactions can be applied for a great variety of purposes in industry. Iso *et al.* (2001) has demonstrated that lipase can be used as biocatalyst in transesterification reaction. Besides, when an enzyme is immobilized, its active sites become more effective, as each and every enzyme is dispersed on the surface of the carrier particle. Therefore, the activity of immobilized lipase is highly increased in comparison with free lipase.

2.1.4 *Candida rugosa* lipase

Candida rugosa is a well-known lipase producing yeast. Its extracellular lipase has been reported to be non-specific with respect to the glycerol position (Fadiloglu and Erkmen, 2002). Moreover, lipase from *Candida rugosa* has been found to be a highly stereospecific catalyst suitable for the preparative resolution of racemic acids and alcohols (Brockerhoft and Jensen, 1974). Below is the summary about *Candida rugosa* lipase and Table 2.1 is the specification of lipase sources from *Candida rugosa*.

Type	: Monotopic/peripheral (4 classes)
Class	: Alpha/Beta monotopic/peripheral (18 superfamilies)
Family	: Fungal lipase (12 proteins)
Species	: *Candida rugosa* (3 proteins)
Localization	: Secreted (260 proteins)

Table 2.1: Specification of lipase sourced from *Candida rugosa* (Fadiloglu and Earkmen, 2002)

Criteria	Specification
Lipase activity (U/g)	115,000 (+/- 10%)
Total viable count (cfu/g)	<50,000
Total coliforms (cfu/g)	<30
Salmonella (in 25 g)	Negative by test
Escherichia Coli (in 25 g)	Negative by test
Antibiotic activity	Negative by test
Heavy metals as Pb (mg/kg)	<30
Lead (mg/kg)	<5
Arsenic (mg/kg)	<3

2.2 Enzyme Immobilization

2.2.1 Introduction

Immobilized enzymes have been defined as enzymes that are physically confined or localized, with retention of their catalytic activity, and which can be used repeatedly and continuously (Zaborsky, 1973). There are varieties of methods by which enzymes can be localized, ranging from covalent chemical bonding to physical entrapment (Messing, 1975; Trevan, 1980) however they can be broadly classified as follows (Carr and Bowers, 1980):

- Covalent bonding of the enzyme to a derivative, water-insoluble matrix.
- Intermolecular cross-linking of enzyme molecules using multi-functional reagents.
- Adsorption of the enzyme onto a water-insoluble matrix.
- Entrapment of the enzyme inside a water-insoluble polymer lattice or semi-permeable membrane.

The attractions of immobilized enzymes from an analytical standpoint are primarily their reusability, and hence cost saving, and the greater efficiency and control of their catalytic activity (e.g., potentially longer half-lives, predictable decay rates and more efficient multi-step reactions). The immobilized form of an enzyme can be presented for use in three distinct forms (Worsfold, 1995):

- Solid-phase immobilized enzyme reactors (packed bed and open tubular) for use in continuous flow techniques such as flow injection analysis and post-column derivatization in liquid chromatography.
- Immobilized enzyme membranes incorporated into sensors such as potentiometric enzyme electrodes and optical sensors.
- Solid-phase immobilized enzyme films for use in disposable, dry reagent kits with photometric detection.

There are a number of advantages to attach enzymes to a solid support and several major reasons such as multiple or repetitive use of a single batch of enzymes. Enzymes that attach to the support can be used for many times. Nie *et al.* (2006) has demonstrated that the operational stability of the immobilized enzyme is more than 20 days. Beside, enzyme immobilization has ability to stop the reaction rapidly by removing the enzyme from the reaction solution (or vice versa). The immobilized enzyme is in small particle, so that it easily to take out or remove from the mixture of solution for the reaction.

In addition, enzymes are usually stabilized by binding. The stability of enzyme can be improved by attachment the enzyme to the suitable support. Moreover, product is not contaminated with the enzyme, especially useful in the food and pharmaceutical industries. Normally at the end of reaction, there is a layer that distinguishes the product and the immobilized enzyme. Therefore, immobilized enzyme will not contaminate the product. It is easily separation of enzyme from the product compare to the soluble enzyme (Nie *et al.,* 2006).

2.2.2 Immobilized enzyme characteristics

Worsfold (1995) has reported that enzyme activities for immobilized enzymes are defined in the same way as for free enzymes. This is the most important information for comparing immobilization methods and is often not provided. The percentage of enzyme immobilized is usually calculated by measuring the amount of enzyme remaining in the supernatant after immobilization and subtracting this from the amount originally present. The absolute enzyme activity remaining on the support after immobilization is more difficult to determine and an apparent activity is usually measured which takes into account mass transfer and diffusional restrictions in the experimental procedure.

Beside, Worsfold (1995) has also emphasized the other critical performance indicator is the stability of the immobilized enzyme with respect to time, temperature and other storage conditions and experimental variables. Clearly this can be expressed in a number of ways but the recommended procedure is to store the enzyme under normal operating conditions (e.g. ambient temperature (20 ^{0}C) in an appropriate buffer and monitor its activity after fixed periods of time using the same procedure as that used for determining the activity remaining after immobilization. For analytical purposes the effect of the controlled introduction of synthetic standards, reference materials and samples at predefined intervals and frequencies must be determined in order to specific the minimum number of analysis possible and the lifetime of the immobilized enzyme. The effect of storage conditions, e.g. pH, temperature and ionic strength and of impurities incorporated during the immobilization step must also be considered.

2.2.3 Recently study on immobilization of lipase

Selection of the suitable support should greatly improve the performance of the enzymes allowing for a continuous use or re-use. In addition, lipases, like most enzymes, are not perfect chemical catalysts. Lipases may be unstable and they may not have the optimal activity nor the optimal enantio or regioselectivities. In this