UNIVERSITY OF INDONESIA

IMMOBILIZATION OF LIPASE IN MEMBRANE MICROREACTOR FOR TRANSESTERIFICATION OF TRIOLEIN TO METHYL OLEATE

DISSERTATION

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FACULTY OF ENGINEERING
CHEMICAL ENGINEERING DEPARTMENT
DEPOK
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Submitted to the Chemical Engineering Department,
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DOCTOR

ACHMADIN LUTHFI MACHSUN
8405002013

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STATEMENT OF ORIGINALITY

I declare that the dissertation with the title of:

IMMOBILIZATION OF LIPASE IN MEMBRANE MICROREACTOR FOR TRANSESTERIFICATION OF TRIOLEIN TO METHYL OLEATE

is not a duplication of another dissertation that was used to obtain a doctorate in educational institutions in the world, except the passages quoted or referred to which I have stated correctly

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PREFACE

In the name of Allah, the Beneficent, the Merciful.

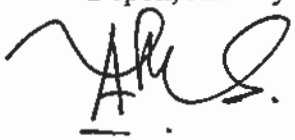
Five years ago, in January 2006, I went to University of Indonesia in Depok, in order to speak with Dr.-Ing. Misri Gozan and Prof. Mohammad Nasikin about the possibility to do my PhD studies at the Department of Chemical Engineering. I was Lucky; just around that time some funding had become available for a project on biodiesel and bioethanol. Since then I have spent five years in Department of Chemical Engineering.

First and foremost and I would like to acknowledge and thank my advisor Prof. M. Nasikin for all of his guidance and direction, and my co-promoters Dr.-Ing. Misri Gozan and Dr. Siswa Setyahadi for their assistance. I am especially grateful to Prof. Bambang Prasetya, Prof. Setijo Bismo, Sutrasno Kartohardjono, PhD and Ariyanti Oetari, PhD for their input and review various parts of my dissertation for me. During my study in Chemical Engineering Department I had the opportunity to cooperate with various researcher. The first immobilization experiments were carried out together with Dr. Heri Hermansah and Praswati Wulan. I am grateful to both of them for their hospitality and support.

Next, I would like to thank Viola and Renny from the Bioseparation Laboratory - BPPT. They both have provided countless hours of support to my research and have helped me through many challenging problems. The analysis of triolein reported in this dissertation was performed in analytical laboratory and worked together with Arie Bachtiar and I especially want to thank him.

Finally, I would like to thank my family for their support and apologize for "not being there" all those night and holydays that I spent in the laboratory and on writing this dissertation.

Depok, January 2011



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Achmadin Luthfi Machsun

ABSTRACT

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Program study : Chemical Engineering, Engineering Faculty, University of Indonesia
Judul : Immobilization of lipase in membrane microreactor for Transesterification of triolein to methyl oleate

Microreactors have become a promising technology in the biotechnology and chemical engineering field. In this study a new concept of biocatalytic membrane microreactor was developed for continuous transesterification reaction by utilizing membrane pores as a kind of microreactor. The membrane pores were coated with lipase from *Pseudomonas sp* by simple adsorption and continues with pressure driven filtration. A lipase solution was allowed permeating through the membrane and lipase molecule adsorbed on the inner wall of the membrane pores. Membranes made of mixed cellulose ester (MCE) and polyethersulfone (PES) were used for lipase immobilization studies but only PES membranes were used as microreactor for transesterification studies.

The catalytic properties of biocatalytic membrane microreactor (BMM) have been studied in biodiesel synthesis through transesterification of triolein with methanol. Transesterification was carried out by passing solution of triolein and methanol through pores of the membrane. The maximum conversion of triolein with lipase-membrane microreactor was approximately 80% with reaction time 20-30 minutes. The biocatalytic membrane microreactor system with lipase as biocatalysts showed far superior activities compared to those of free lipase, i.e. 12-25 fold. No decrease in flux and activities were observed over a period of 12 days of continuous operation. These biocatalytic membrane microreactor is of great potential to be applied in the process of transesterification of triglycerides for commercial biodiesel production since it would reduce waste in large scale and has a much smaller reaction time.

Keywords: *Catalytic membrane, membrane microreactor, lipase, immobilization, transesterification, biodiesel*

ABSTRAK

Nama : Achmadin Luthfi Machsun
Program studi : Teknik Kimia, Fakultas Teknik, Universitas Indonesia
Judul : Immobilisasi lipase pada membran mikroreaktor untuk Transesterifikasi triolein menjadi metil oleat

Microreaktor telah menjadi teknologi yang menjanjikan dalam bidang bioteknologi dan teknik kimia. Dalam penelitian ini dikembangkan konsep baru biokatalis membran mikroreaktor (BMM) untuk reaksi transesterifikasi secara kontinyu dengan memanfaatkan pori-pori membran sebagai mikroreaktor. Pori-pori membran yang dilapisi dengan enzim lipase dari *Pseudomonas sp* dengan cara adsorpsi sederhana dan dilanjutkan dengan filtrasi bertekanan. Suatu larutan lipase dibiarkan mengalir pada membran dan merembes melalui pori-pori dan molekul lipase molekul teradsorpsi pada dinding pori-pori bagian dalam. Membran yang terbuat dari *mixed cellulose ester* (MCE) dan *polyetersulfone* (PES) digunakan untuk studi immobilisasi lipase tetapi hanya PES membran digunakan sebagai mikroreaktor untuk studi transesterifikasi.

Sifat katalitik biokatalis membran mikroreaktor (BMM) telah dipelajari dalam sintesis biodiesel melalui reaksi transesterifikasi triolein dengan metanol. Transesterifikasi dilakukan dengan melewatkan larutan triolein dan metanol melalui pori-pori membran yang telah dilapisi lipase. Konversi maksimum triolein dengan BMM sekitar 80% dengan waktu reaksi 20-30 menit. Sistem biokatalis membran mikroreaktor dengan lipase sebagai biokatalis menunjukkan aktivitas yang jauh lebih unggul dibandingkan dengan lipase bebas, yaitu 12-25 kali lipat. Tidak ada penurunan fluks dan aktivitas yang diamati selama 12 hari operasi terus-menerus. Biokatalis membran mikroreaktor memiliki potensi yang besar untuk diterapkan dalam proses transesterifikasi trigliserida pada produksi biodiesel komersial karena akan mengurangi limbah dalam skala besar dan memiliki waktu reaksi yang jauh lebih kecil.

Kata kunci: Membran katalis, membran mikroreaktor, lipase, amobilisasi, transesterifikasi, biodiesel

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SYMBOLS

p	:	Amount of Immobilized lipase (g/m^2)
p_m	:	Amount of Immobilized lipase (g)
C	:	Concentration of Lipase (g/mL)
V	:	Volume of Lipase (mL)
A	:	Membrane Area (m^2)
HRT	:	Hydraulic Residence Time (h)
V_m	:	Membrane volume (mL)
F	:	Flow rate of permeate (mL/h)
P_{cat}	:	Immobilized enzyme productivity ($mmol/h.mg_{lipase}$)
C_p	:	Concentration of Methyl Oleate (mmol/mL)
t	:	Time (s)
L	:	Channel width (m)
D	:	Diffusion coefficient (m^2/s)
X_c	:	Conversion degree (%)
C_i	:	Initial concentration of triolein (ppm)
C_s	:	Unconverted triolein (ppm)

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A. National Conference

1. Gozan, M., & Luthfi, A. (2007, April). *Sintesa biodiesel melalui jalur enzimatik.* Paper presented at Seminar Nasional Teknik Kimia, Teknologi Ramah Lingkungan berbasis SDA Indonesia, Bandung.
2. Mediariska, V., Machsun, A.L., Setyahadi, S., Gozan, M., & Nasikin, M. (2007, November). *Immobilisasi enzim lipase pada membran.* Paper presented at Seminar nasional Fundamental dan aplikasi teknik kimia Surabaya.
3. Hermansyah, H., Prabu, D., Rejoso, M.T., Wulan, P.P.D.K., Machsun, A.L., Wijanarko, A., Gozan, M., Arbianti, R., & Utami, T.S. (2007, August). *Model Kinetika sederhana untuk reaksi hidrolisis minyak zaitun menggunakan lipase.* Paper presented at Seminar Tjipto Utomo, Bandung.
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5. Luthfi, A., Setyahadi, S., Gozan, M., & Nasikin, M. (2007, August). *Immobilization of lipase in membrane and its application in synthesis of biodiesel.* Paper presented at The 4th Indonesian Biotechnology Conference" IPB International Convention Centre, Bogor.
6. Luthfi, A., Setyahadi, S., Gozan, M., & Nasikin, M. (2008, November) *Pengembangan reaktor membran berkatalis untuk sistesis biodiesel.* Paper presented at Seminar Nasional Fundamental dan Aplikasi Teknik Kimia, Surabaya.
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B. National Journal

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C. International Conference

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adsorption and cross-linking methods. Paper presented at 14th RSCE, Yogyakarta.

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D. International Journal

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CHAPTER 1
INTRODUCTION

1.1. Background of The Study

Biodiesel is defined as fatty acid methyl ester and represents a promising alternative fuel for use in compression-ignition (diesel) engines. Biodiesel is a renewable fuel that can be manufactured from vegetable oils, animal fats or even recycled greases from food industry (Kiss *et. al.,* 2006). Biodiesel today is produced by transesterification of the triglycerides with short chain alcohols in the presence of a suitable catalyst (Barnwal and Sharma, 1999; Pinto *et al.*, 2005). The production of biodiesel by transesterification employing acid or base catalyst has been industrially accepted for its high conversion and reaction rates (Bisen *et al.,* 2010).

Although alkali-catalyzed methanolysis of a triglyceride is one of the most powerful methods for the production of commercial biodiesel, the reaction has several drawbacks: it is energy intensive; the recovery of glycerol is difficult; the catalyst has to be removed from product; alkaline waste-water requires treatment and free fatty acids (FFA) and water interfere with the reaction (Fukuda *et al.,* 2001). Downstream processing costs and environmental problems associated with biodiesel production and byproducts recovery has led to the search for alternative production methods.

Recently, enzymes have been proposed to overcome the drawbacks facing the conventional chemically catalyzed biodiesel production methods. Lipases have been used for biodiesel production with promising results; all FFA contained in feedstock are converted to biodiesel (Fjerbaek *et al.,* 2008). In addition, by-product glycerol can be easily recovered, and the purification of biodiesel is simplified using biological catalyst (Kaieda *et al.*, 1999; Fukuda *et al.*, 2001; Meher *et al.*, 2006; Kumari *et al.*, 2007).

Although enzymatic approaches have become more and more attractive, they have not been appliedin industries mainly due to high price of lipase and inactivation of lipase by methanol and glycerol (Fukuda *et al.,* 2001; Haas *et al.,* 2006; Su and Wei, 2008; Bisen *et al.,* 2010). In order to solve the problem of

lipase inactivation, several researchers proposed the following three options, methanol stepwise addition (Shimada *et al.*, 2002; Chen *et al.*, 2006), acyl acceptors variations (Xu *et al.*, 2003; Wei *et al.*, 2004) and solvent engineering (Ha *et al.*, 2007; Royon *et al.*, 2007; Park *et al.*, 2008). Although these studies have been successfully applied in laboratory, however, there are some disadvantages. They include the low of reaction rate, the complexity of large-scale application and increment of production costs. In addition, high price of lipase remains a barrier for its industrial implementation (Tan *et al.*, 2010). Therefore several attempts should be made to develop an effective system in order to reduce the lipase cost significantly. Immobilizing enzyme on porous material can be useful in preventing inactivation of enzyme and improving operational stability of enzyme (Mateo *et al.*, 2007).

The existence of mesoporous materials has been a significant breakthrough in the field of porous materials and opened up unprecedented opportunities for immobilizing enzyme (Zhao *et al.*, 2006). One example of porous materials for immobilization of enzyme is a membrane. A membrane is a permeable or semi-permeable phase, often in the form of a thin film, and made from a variety of materials ranging from inorganic solids to different types of polymers (Mulder, 1991). According to the IUPAC classification, porous membranes with average pore diameters larger than 50 nm are classified as macroporous, and those with average pore diameters in the intermediate range between 2 and 50 nm as mesoporous; micro- porous membranes have average pore diameters which are smaller than 2 nm.

Membranes were initially defined as filtration media. However, the high ratio of available area to volume allows membrane as support for enzyme immobilization. Mesoporous materials provide restricted nanospaces for enzyme immobilization (Lee *et al.*, 2009). Nanospace environment provide the upper limits in balancing the key factors that determine the efficiency of biocatalysts, including surface area, mass transfer resistance, and effective enzyme loading (Wang, 2006). In terms of catalytic applications, nanospace structures offer an optimized material efficiency due to the extremely high surface-to-volume ratio (Kim *et al.*, 2008).

There have been many research published on enzyme immobilized in membrane reactors (Tanigaki *et al.,* 1993; Giorno *et al.,* 2001; Lozano *et al.,* 2003). The main advantages of these reactors are presented in a recent review i.e. continuous mode, reuse of catalyst, reduction in substrate/product inhibition, control of product properties by enzyme (specificity) and single-step reaction and separation. Rios *et al.,* (2004) proposed the idea of using membrane pore as microreactor. Beyond its simple use as reactor, a membrane can be considered as a specific macrosystem resulting from the assembly of many microsystems. Indeed each pore could be looked upon as a particular microreactor. One of the important issues in catalytic microreactors is the proper incorporation of the active catalyst on surface (Rios *et al.,* 2004). A microreactor exhibits excellent performance in liquid – liquid phase reaction for large specific area, extremely high mass transfer rate and short molecular diffusion distance (Ehrfeld, 2000; Wörz *et al.,* 2001).

1.2. Problem Statement

This research studied the utilization of porous membrane as matrix for immobilizing lipase. We expected the enzyme molecules to be fully dispersed in the porous structure of a membrane material. Thus, the efficiency of immobilized enzyme could be improved. Therefore, the challenge was to develop an effective system to immobilize enzyme and deposited correctly within the microchannel of the membrane. A simple technique has been developed for immobilizing lipase on asymmetric membrane.

To the author's knowledge, there is no existing report on the use of polymeric membrane that utilizes porous membrane as a kind of microreactor. In a permeable membrane a challenge was to deposit enzymes correctly at the pore mouth and within the microchannel. Based on the concept of microreactor, the systems should allow to get high reaction rates.

1.3. Objectives

The reactor concept discussed in this dissertation is called "flow-through biocatalytic membrane microreactor". As the biocatalyst is immobilized in the

membrane, it can be included in the category of membrane reactors. The porous structures of asymmetric membrane are in the scale range of micrometers and even below, thus it can be considered a microreactor. Finally the reactant flows convectively through uniform catalytic channels, which is the flow-through mode.

The specific objectives of the research are:

(1) To develop biocatalytic membrane microreactors for continuous methanolysis of triolein.

(2) To evaluate the performance of microreactor system in term of methanol tolerance and operational stability of immobilized lipase.

(3) To investigate the performance of microreactor during continuous lipase-mediated methanolysis of triolein in term of productivity. The productivity defined as rate of methyl oleate per mass of lipase [mmol/h.mg_{lipase}].

1.4. Scope of This Study

The scope of the present work was to prepare biocatalyst membrane microreactor for continuous transesterification of triolein to methyl oleate. In order to accomplish the above objectives, three (3) studies were carried out.

(1) Immobilization of lipase within the membrane matrix by two-step methods: adsorption - filtration.

(2) Characterization the immobilized lipase with general techniques such as Scanning Electron Microscopy (SEM) for morphological studies and evaluation of enzyme distribution, Spectrophotometer for protein measurement with Lowry method and calculation of amount of immobilized lipase.

(3) Evaluation of microreactor performance in term of stability and productivity. The stability of immobilized lipase in membrane microreactor was monitored the activity of immobilized lipase. The enzyme activity was expressed as triolein conversion. While the selectivity was not measured in this study.

1.5. Benefit for Biodiesel Industry

Immobilized lipase-catalyzed biodiesel production is a very interesting topic in recent years as it allows the use of mild reaction conditions that are consistent with a green technology. The final result of this research is to obtain a biodiesel production system that is efficient and plausible technically and economically for commercial scale application. Moreover, the results obtained will provide information on the novel technology for producing biodiesel and will allow establishment of new technology in biodiesel industry.

CHAPTER 2
LITERATURE REVIEW

2.1. General View About Biodiesel Production

Biodiesel is the name given to a renewable diesel fuel that is produced from fats and oils (Haas *et al.,* 2005). This is an alternative fuel for diesel engines that is receiving great attention worldwide. Biodiesel has several advantages over other energy sources, because it is a renewable energy source, it emits lower amount of toxic exhaust gases and carbon dioxide compared to petroleum diesel, and it is biodegradable (Park *et al.,* 2006). Moreover, no engine modification is required. So, blending of petroleum diesel with biodiesel has been a common practice.

2.1.1. Conventional Production of Biodiesel

Current practice of biodiesel production is through transesterification of the triglycerides with short chain alcohols in the presence of a suitable catalyst. Conventional transesterification refers to a catalyzed chemical reaction involving vegetable oil and an alcohol to yield biodiesel and glycerol (Kaieda *et al.,* 1999; Srivastava and Prasad, 2000; Zhang *et al.,* 2003; Meher *et al.,* 2006). The transesterification reaction is preferred to the direct esterification of fatty acid because triglycerides are more available than free fatty acids. Transesterification, also called alcoholysis, is the displacement of alcohol from an ester by another alcohol in a process similar to hydrolysis, except that an alcohol is used instead of water (Otera, 1993). This process has been widely used to reduce the viscosity of triglycerides, thereby enhancing the physical properties of renewable fuel. A catalyst is used to improve the reaction rate and yield.

The transesterification reaction is represented by the general equation shown in Figure 2.1. The overall process is normally a sequence of three consecutive steps, which are reversible reactions. The first step is the conversion of triglycerides to diglyceride, followed by the conversion of diglyceride to monoglycerides, and of monoglycerides to glycerol, yielding one methyl ester molecule per mole of triglyceride at each step (Noureddini and Zhu, 1997). In all

these reactions methyl ester (biodiesel) are produced. The stoichiometric relation between alcohol and the oil is 3:1. However, an excess of alcohol is usually more appropriate to improve the reaction towards the desired. As seen below, the transesterification is an equilibrium reaction in which excess alcohol is required to drive the reaction close to completion. The transesterification of triglycerides can be performed using alkaline, acidic, and enzymatic catalysts. Depending on the undesirable compounds (especially free fatty acid and water), each catalyst has its own advantages and disadvantages.

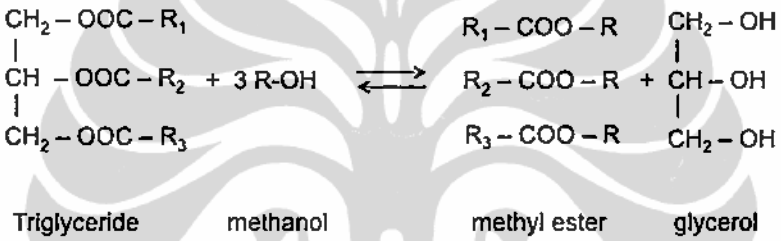

Figure 2.1. Transesterification Reaction

(Source: Ma and Hanna, 1999)

Converting triglyceride oils to methyl or ethyl esters through a transesterification process reduces the molecular weight to one-third that of the oil, reduces the viscosity by a factor of eight, and increases the volatility. This method has a long history of development and the biodiesel produced by this method is now available in North America, Japan and some western European countries (Barnwal and Sharma, 2005).

Several aspects, including the type of catalyst, alcohol/vegetable oil molar ratio, temperature, water content and free fatty acid content influence the course of transesterification. Transesterification reactions are conventionally alkali-catalyzed or acid-catalyzed. Comparison between chemically catalyzed processes and a non-catalytic supercritical methanol method, for biodiesel production from vegetable oils, is shown in Table 2.1.

Table 2.1. Comparison Between Chemically Catalyzed Processes and A Non-Catalytic Supercritical Methanol Method, for Biodiesel Production from Vegetable Oils

	Method		
	Alkali catalytic	**Acid catalytic**	**Supercritical**
Temperature (° C)	30 – 65	65	250 - 300
Pressure (Bar)	1	1	100 - 250
Reaction Time (min)	60 - 360	4140	7 - 15
Methyl Ester yield (%)	96	90	98
Purification	Glycerol, soaps	Glycerol	
FFA	Saponified product	Methyl Ester, Water	Methyl Ester, Water

(Source: Al-Zuhair, 2007)

Currently, almost all biodiesel is produced using alkali-catalyzed transesterification of the oil (Barnwal and Sharma, 2005). The main processing steps are as follows:

1. Reaction:
   a. Mixing of alcohol (methanol) and catalyst (NaOH).
   b. Adding oil and heating the mix.
2. Separation of biodiesel and glycerin phases
3. Alcohol removal from each phase
4. Glycerin neutralization by using an acid to neutralize unused catalyst and soaps.
5. Biodiesel purification by washing, with warm water, to remove residual catalyst or soaps. Sending the biodiesel to storage after drying.

The general equipment shown in Figure 2.2 represents the flow diagram of transesterification reaction. This process has been widely used to reduce the viscosity of triglycerides, thereby enhancing the physical properties of renewable fuel. A catalyst is usually used to improve the reaction rate and yield. In transesterification, when raw material have a high water or free fatty acid (FFA) content needs pretreatment with an acidic catalyst in order to esterify FFA

(Freedman *et al.*, 1984; Kaieda *et al.*, 1999; Zhang *et al.*, 2003). Pretreatment is necessary to reduce soap formation during the reaction and ease the extensive handling for separation of biodiesel and glycerol together with removal of catalyst and alkaline wastewater (Mittelbach, 1990; Meher *et al.*, 2006).

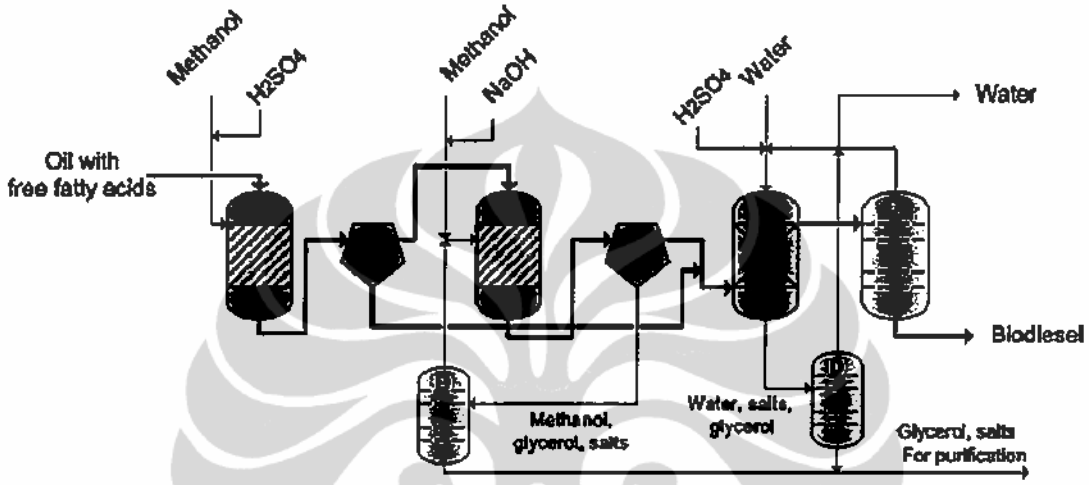

Figure 2.2. Conventional Biodiesel Process with An Acidic Pretreatment Step Followed by Alkaline Catalysis

(Adapted from: Fjerbaek *et al.*, 2008)

2.1.2. Enzymatic Production of Biodiesel

Although transesterification using a conventional alkali catalyzed process gives high conversion levels of triglycerides to their corresponding methyl esters in short times, the reaction has several drawbacks: it is energy intensive; recovery of glycerol is difficult; the catalyst has to be removed from the product; alkaline waste-water requires treatment and free fatty acids and water interfere with the reaction (Fukuda *et al.*, 2001). In order to minimize problems in a conventional process, attempts to use new catalyst in alcoholysis of triglycerides have been made. Enzymes have been proposed to overcome the drawbacks facing the conventional chemically catalyzed biodiesel production methods, and have shown promising results (Fjerbaek, et al., 2008).

Contrary to alkaline catalysts, enzymes do not form soaps and can esterify both FFA and triacylglycerol (TAG) in one step without the need of a subsequent washing step. Thus enzymes are an interesting prospect for industrial-scale production for reduction of production costs. This is especially the case when using feeds high in FFA such as rice bran oil (Lai *et al.*, 2005), non edible *Madhuca indica* oil or second-generation raw materials like spent oils, animal fat and similar waste fractions, with high FFA and water content and large variation in raw material quality (Kumari *et al.*, 2007).

Most importantly, glycerol can be easily recovered without any complex process, free fatty acids contained in the oils can be completely converted to methyl esters and subsequent wastewater treatment is not required (Janssen, *et al.*, 1996). Table 2.2 gives an overview of the most promising results for enzymatic biodiesel production to date (Al-Zuhair, 2007). There are many reports on biodiesel production using enzyme catalysis by free and immobilized lipase (Nielsen *et al.*, 2008). There are two major limitations of lipase-catalyzed biodiesel synthesis. One is higher cost of lipase and another is inactivation of lipase by methanol and glycerol (Sonare and Rathod, 2010).

2.2. Enzyme

Enzymes are specialized proteins, which are able to catalyze reactions with a very high substrate and product specificity. These proteins composed by a number of amino acid residues that range from a hundred to several hundreds (Illanes, 2008). They occur widely in animals, plants, yeast, bacteria, and fungi. Enzymes have been naturally tailored to perform under physiological conditions. Biocatalysts are *strictu sensu* the catalysts of cell metabolism, i.e. the enzymes. However, this concept has expanded beyond its physiological meaning, a biocatalyst being, in broader terms, any biological entity capable of catalyzing the conversion of a substrate into a product (Illanes, 1999). Biocatalysis refers to the use of enzymes as process catalysts under artificial conditions (*in vitro*), so that a major challenge in biocatalysis is to transform these physiological catalysts into process catalysts able to perform under the usually tough reaction conditions of an industrial process (Illanes, 2008).

Table 2.2. Biodiesel Production with Various Lipases

Lipase	Feedstock	Alcohol	References
Novozym 435	Soybean oil	Methanol	(Watanabe *et al.*, 2001) (Samukawa *et al.*, 2000) (Kaieda *et al.*, 2001)
	Soybean oil	Methyl acetate	(Wei *et al.*, 2004)
	Canola oil	Methanol	(Chang *et al.*, 2005)
	Rice bran oil	Methanol	(Lai *et al.*, 2005)
	Olive oil	Methanol	(Sanchez *et al.*, 2006)
	Vegetable oil	Methanol	(Shimada *et al.*, 2002)
	Waste ABE *)	Methanol 1-propanol, 1-butanol, iso-butanol, n-octanol, iso-amylalcohol	(Noureddini *et al.*, 2005)
Rhizopus delemar	Vegetable oil	Methanol	(Shimada *et al.*, 2002)
Rhizopus miehei	Vegetable oil	Methanol	(Shimada *et al.*, 2002)
	Palm oil	Methanol	(Zuhair *et al.*, 2006)
Candida rugosa	Waste ABE *)	Methanol 1-propanol, 1-butanol, iso-butanol, n-octanol, so-amylalcohol	(Noureddini *et al.*, 2005)
	Jatropha oil	Ethanol	(Shah and Gupta, 2006)
Candida lipolytica	Soybean oil	Methanol	(Kaieda *et al.*, 2001)
Klebsiella oxytoca	Soybean oil	Methanol	(Kaieda *et al.*, 2001)
Pseudomonas camembertii	Soybean oil	Methanol	(Kaieda *et al.*, 2001)
Pseudomonas fluorescens	Soybean oil	Methanol	(Kaieda *et al.*, 2001)
	Triolein	1-propanol	(Iso *et al.*, 2001)
	Jatropha oil	Ethanol	(Shah and Gupta, 2006)
Pseudomonas cepacia	Soybean oil	Methanol Ethanol	(Kaieda *et al.*, 2001)
	Jatropha oil		(Noureddini *et al.*, 2005)

Note: ABE = activated bleaching earth

(Modified from: Zuhair, 2007)

The application of enzymes in biocatalysis plays an increasing role in industrial processes, such as energy and fuels, fine chemicals, pharmaceuticals, and other commodity chemicals. Moreover, currently enzymes are becoming increasingly important in sustainable technology and green chemistry (Zhao, 2006). The major advantages of using enzymes in biocatalytic transformations are their chemo-, regio -, and stereo-specificity as well as the mild reaction conditions that can be used. Enzyme catalysts (biocatalysts), as any catalyst, act by reducing the energy barrier of the biochemical reactions, without being altered as a consequence of the reaction they promote (Illanes, 2008).

Enzymes can be considered to be "green" catalysts in that their reactions are environmentally not hazardous and are thus favored over chemical catalysts whenever possible (Montgomery, 2004). Increasingly, industry has been turning to the use of enzymes; for example, the starch industry has replaced the hydrolysis of starch by mineral acid catalysts with specific amylases (Michels and Rosazza, 1999; Patel, 2000; Drauz and Waldmann, 2002). Enzymes can catalyze reactions in different states: as individual molecules in solution, in aggregates with other entities, and as attached to surfaces. The attached—or "immobilized"—state has been of particular interest to those wishing to exploit enzymes for technical purposes. The term "immobilized enzymes" refers to "enzymes physically confined or localized in a certain defined region of space with retention of their catalytic activities, and which can be used repeatedly and continuously" (Katchalski-Katzir, 1993).

2.2.1. Lipase

Lipases, as a class of enzymes, are stable and extremely valuable catalysts for many practical / industrial applications (Bjorkling *et al.* 1991). They have been used to generate chiral entities from alcohols, carboxylic acid esters, cyanohydrins, chlorohydrins, diols, amines, diamines and amino alcohols, which are used as building blocks for a variety of pharmaceuticals and other fine chemicals (Jaeger *et al.* 1999). Many lipases are only moderately stable at high temperature and pH that can influence their usefulness in some interesting reactions. Using lipases from thermophilic microorganisms, whose resistance to

drastic conditions has been developed by nature, can solve this problem. At present, the majority of the thermophilic lipases that have been purified and characterized are obtained from *Bacillus* sp. (Nawani, *et al.* 2006).

Microbial lipases are widely studied for a variety of applications (Table 2.3), such as transesterification, ester synthesis, biosurfactants production and resolution of racemic mixtures to produce optically active compounds (Mustantra, *et al.*, 1992). Although lipases for lipid hydrolysis have been known for decades before the discovery of possible uses of enzymes in micro-aqueous systems, none of the industrial applications had really been used in practice (Xu, 2003). In 1984, *Zaks and Klibanov* published a paper in Science, indicating that enzymes could work in a solvent microaqueous medium at 100°C (Zaks and Klibanov, 1985). This is an important milestone in enzyme history. It means that enzymes can also be used for the catalysis of reverse reaction, i.e. esterification. This opens up a variety of potential applications. In the lipid area, a reaction such as interesterification (acidolysis, alcoholysis, and ester-ester exchange) is also possible with the catalysis of lipases in a probable hydrolysis-esterification two-step mechanism (Xu, 2003).

2.2.2. Stability of Biocatalyst

The efficient application of biocatalysts requires the availability of suitable enzymes with high activity and stability under process conditions (Bornscheuer and Pohl, 2001). Enzymes are biocatalysts, which are extremely important because they are highly specific and active under ambient conditions and are biodegradable. Since enzymes are labile molecules, their stabilization is central issue of biotechnology today. In most cases, enzymes have to be greatly improved before their use in industrial processes (Sharma *et al.*, 2007).

Table 2.3. Some Important Application of Lipase

Product	Application	Reaction and Operating condition	Reference
1,2-Propanediol mono-laurate	Emulsifiers	Esterification, 65°C, no solvent, Novozyme	Shaw, *et al.*, 2003
1,3-Propanediol-oleic acid	Surfactant	Esterification, Lipozyme	Castillo, *et al.*, 1997
Trimethylolpropane-oleic acid	Lubricant	Alcoholysis, no solvent, immobilized lipase	Linko, *et al.*, 1997
Omega-3 Poly Unsaturated Fatty Acid (PUFA)	Food Industry	Alcoholysis	Zuyi and Ward, 1993
Tetraconazole	Pesticide		Bianchi, *et al.*, 1990
Methyl Oleate	Biodiesel	Transesterification, 50°C no solvent, immobilized lipase	Iso, *et al.*, 2001
Methyl Ester	Biodiesel	Transesterification Fixed bed reactor	Watanabe, *et al.*, 2001
Precursor of vitamin D	Biomedical		Fernandez *et al.*, 1995

The engineering of enzymes, from biological to chemical industries, is one of the most exciting, complex, and interdisciplinary goals of biotechnology. The large-scale implementation of enzymes as industrial catalysts requires a multi-disciplinary utilization of very different techniques (Guisan, 2006):

- Screening of enzymes with suitable properties
- Improvement of enzyme properties via techniques of molecular biology
- Improvement of enzyme properties via immobilization
- Improvement of enzyme properties via reaction and reactor engineering

Biocatalyst stability is a key factor for successful industrial bioprocesses (Polizzi, *et al.* 2007). Concerns with enzyme stability in the presence of organic solvents, surfactants, extreme pH values, or high temperatures have stimulated much research from both academia and industrial R&D scientists to provide substantial improvements for many biocatalysts (Costa, *et al.* 2009). There have been many approaches to improve the enzyme stability (Kim, *et al.* 2006):

enzyme immobilization, enzyme modification, protein engineering and medium engineering. Out of these approaches, immobilization is perhaps the most used strategy to improve the stability of enzymes (Illanes, 1999).

The ability to make high cost enzymes reusable and stable has meant that immobilized enzymes have attracted a greater deal of attention (Blandino, *et al.*, 1999). Various techniques have been developed for immobilization like adsorption, encapsulation and covalent linkage to an insoluble support. Some studies reported recent developments in the field of nanotechnology for enzyme stabilization (Kim *et al.* 2006, Lee *et al.* 2009). Various nanomaterials, such as nanoparticles, nanofibers, nanotubes and nanoporous matrices, have shown potential for revolutionizing the preparation and use of biocatalysts (Wang, 2006). Beyond their high surface area: volume ratios, nanoscale biocatalyst systems exhibit unique behaviors that distinguish them from traditional immobilized systems. The Brownian motion of nanoparticles, confining effect of nanopores and self-assembling behaviors of discrete nanostructures are providing exciting opportunities in this field (Kim *et al.*, 2008).

2.2.3. Immobilization of Enzyme

Immobilization of enzyme has been prepared with various methods. The main goal of enzyme immobilization is the industrial re-use of enzymes for many reaction cycles. In this way, simplicity and improvement of enzyme properties have to be strongly associated with the design of protocols for enzyme immobilization. There are many protocols for immobilization of enzymes but very few are also very simple and very capable of improving enzyme properties (Guisan, 2006).

The most frequently used immobilization techniques fall into five categories (Figure 2.3):

a. Noncovalent adsorption or deposition
b. Covalent attachment
c. Entrapment in a polymeric gel
d. Cross-linking of an enzyme
e. Encapsulation in a membrane, or capsule

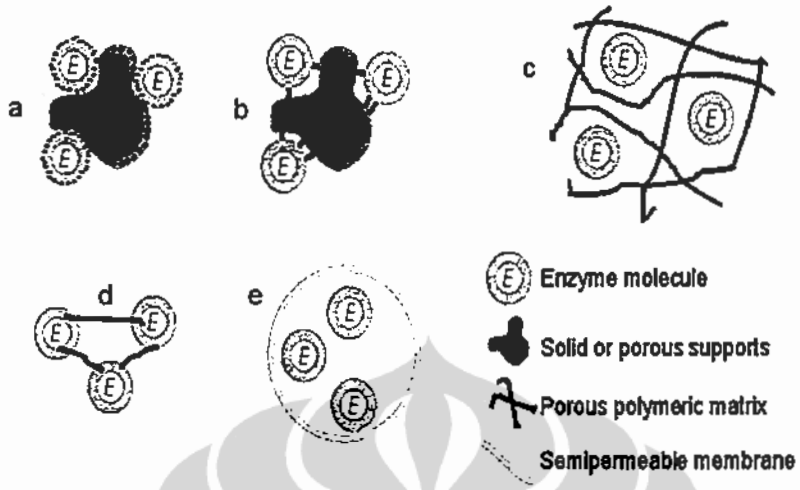

Figure. 2.3. Methods of Enzyme Immobilization

(Modified from: Chaplin and Bucke, 1990)

Adsorption; This is perhaps the simplest of all the techniques and one that does not grossly alter the activity of the bound enzyme. Catalysts immobilized by adsorption mainly rely on weak van der Waals interactions. Thus, the catalyst will readily leach into the reaction medium during use. The stability of the immobilized catalyst can be improved by modifying the catalyst and support to enable hydrogen bonding to occur. Adsorption is by far the most widely used method for enzyme immobilization onto matrix material because it is simple and no further treatment of the support is needed. Thus denaturation of the enzyme is avoided. The most prominent advantages of adsorption include: little or no conformational change of the enzyme or destruction of its active center, usually no reagents and only a minimum of activation steps are required, simple and cheap (D'Souza, 1989).

Covalent; Covalently binding enzymes to a suitable carrier is the second method developed for enzyme immobilization. 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. Since the 1950s covalent immobilization of enzyme has flourished and is

now an important method of enzyme immobilization, because covalent bonds usually provide the strongest linkages between enzyme and carrier, compared with other types of enzyme immobilization method such as noncovalent adsorption-based enzyme immobilization (Cao, 2005). Thus, leakage of enzyme from the matrix used is often minimized with covalently bound immobilized enzymes. Enzymes like glucose oxidase, peroxidase, invertase, etc. have been immobilized using this technique (D'Souza, 1989).

Entrapment; Entrapment technique is one of the simplest methods for immobilization of enzymes and whole cell-based immobilized enzymes. Historically, entrapment of enzyme was probably the third developed enzyme immobilization method after covalent enzyme immobilization and adsorption. This method is also characterized in that more than one enzyme can be immobilized simultaneously (Wei, *et al.,* 2002). By definition, entrapment of enzymes refers to the processes by which the enzymes are embedded in a matrix formed by chemical or physical means such as cross-linking or gelation. In general, the entrapment matrix is generally formed during the immobilization process. Thus, it is not surprising that the precursors of the gel matrix and the conditions used for the formation should be compatible with the enzyme molecules. The enzyme molecules can be physically embedded or covalently linked to the matrix. Thus, entrapment can be also classified as covalent entrapment and chemical entrapment (Cao, 2005).

Encapsulation; Encapsulation of an enzyme is the formation of a membrane-like physical barrier around an enzyme preparation. Chang pioneered use of this concept for enzyme immobilization in 1964 (Chang, *et al.,* 1996). Thus, other encapsulation techniques such as the sol–gel process are put into the category entrapment, because here the enzyme is enclosed in a matrix not in the membrane, although the microscopic structure of the enzyme in the sol–gel matrix may resemble that of the encapsulated enzyme (Cao, 2005).

Cross-linking; The fifth type of immobilization, via cross-linking of enzyme molecules with a bifunctional cross-linking agent, most commonly glutaraldehyde, does not suffer from this disadvantage. Biocatalysts can also be immobilized through chemical cross-linking using homo- as well as hetero

bifunctional cross-linking agents (Hartmier, 1988). Among these, glutaraldehyde that interacts with the amino groups through a base reaction has been extensively used in view of its GRAS (Generally Recognizes As Safe) status, low cost, high efficiency, and stability (Nakajima, *et al.,* 1993). The enzymes or the cells have been normally cross-linked in the presence of an inert protein like gelatine, albumin, and collagen (D'Souza, 1989; Kolot, 1981). Because the molecular weight (MW) of the cross-linking agent is negligible compared with that of the enzyme the resulting biocatalyst essentially comprises 100% active enzyme (D'Souza, 1989).

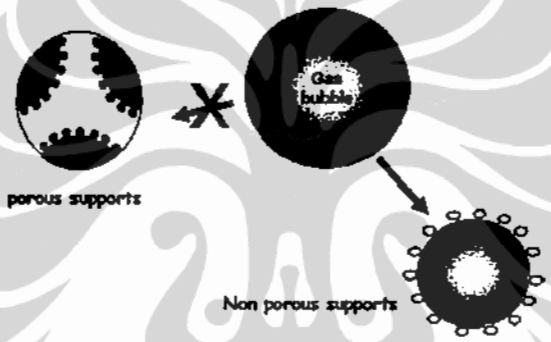

Figure 2.4. Effect of The Type Of Support on The Immobilized Enzyme Stability Against Inactivation by Gas Bubbles

(Source: Mateo *et al.*, 2007)

Although the basic methods of enzyme immobilization can be categorized into a few different methods only, hundreds of variations, based on combinations of these original methods, have been developed (Cao, 2005). There is still no rule of thumb for selecting the best type of immobilization technique that guarantees the highest activity, being still necessary an approach of trial and error to determine the best option. Correspondingly, many carriers of different physical and chemical nature or different occurrence have been designed for a variety of bio-immobilizations and bio-separations (White and Kennedy, 1980; Gemeiner, 1992). 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 (Akgol *et al.,* 2001).

An approach to overcome these problems is the integration of different techniques. Immobilization of enzymes inside the porous structure of a solid may permit to have the enzyme molecules fully dispersed and without the possibility of interacting with any external interface. Thus, this immobilization will stabilize the enzyme against interaction with molecules from the enzymatic extract, preventing aggregation, autolysis or proteolysis by proteases from the extract. Moreover, the immobilized enzyme molecules will not be in contact with any external hydrophobic interface, such as air bubbles originated by supplying some required gases or promoted by strong stirring, necessary to control pH (Mateo *et al.*, 2007). These gas bubbles may produce enzyme inactivation of soluble proteins (Bommarius and Karau, 2005), but cannot inactivate the enzymes immobilized on a porous solid (Bolivar, *et al.* 2006) (Fig. 2.4).

2.2.4. Protein Fouling Phenomenon

Membrane technologies have been successfully applied in the biotechnological and chemical industries. However, there are still limited major problem concerning flux decline with time; this is due to particle deposition and protein fouling (Song and Elimelech, 1995).

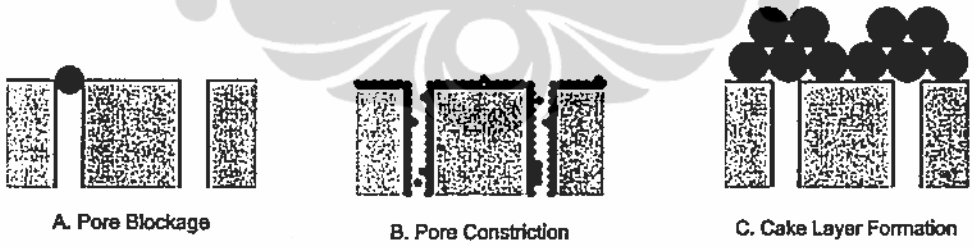

Figure 2.5. Schematic Drawing of The Fouling Mechanism (a) Pore Blockage, (b) Pore Constriction, and (c) Cake Layer Formation

(Modified from: Belfort *et al.*, 1994)

The membrane fouling mechanisms by small molecular substances, macromolecules and colloids are all different. The small molecular substances can pass through the membrane and become absorbed inside the membrane pores. Macromolecules and colloids are rejected on the membrane surface and tend to

form a cake layer. The models for the membrane fouling that occur during protein microfiltration have generally been based of the following phenomena (Belfort, *et al.,* 1994): (a) pore blockage, (b) pore constriction, and (c) cake formation. These three cases are shown schematically in Figure 2.5.

From the immobilization point of view, the membrane fouling is attractive because of the possibility to implement this phenomenon as a new immobilization method. Since the membrane is porous material the immobilized enzyme molecules will not be in contact with any harsh environment. The advantages of immobilizing enzymes on the porous support have been discussed in section 2.2.3 and this section focused on the membrane. Assuming membrane pore is *dp* and enzyme molecules is d, so in order to get very efficient contacting conditions, the membrane pores should bigger than enzyme molecules ($dp >> d$) and thus deposited correctly within the microchannels.

2.3. Membrane

Membrane science and technology is interdisciplinary, involving polymer chemists to develop new membrane structures; physical chemists and mathematicians to describe the transport properties of different membranes using mathematical models to predict their separation characteristics; and chemical engineers to design separation processes for large scale industrial utilization. The most important element in a membrane process, however, is the membrane itself (Mulder, 1991).

Membrane is a selective barrier between two phases (Mulder, 1991). Membranes can be classified according to different viewpoint. The first is by nature, i.e. biological membrane and synthetic membrane. The two types of these membranes differ completely in structure and functionally. This thesis deals only with synthetic membranes or synthetic selective barrier. A synthetic membrane is a barrier, which separates two phases and restricts the transport of various chemical species in a rather specific manner (Porter, 1990). Another means of classifying membrane is by morphology or structure, two types of membranes may be distinguished, i.e. symmetric and asymmetric membrane. The two classes can subdivide further as shown schematically in Figure 2.6.

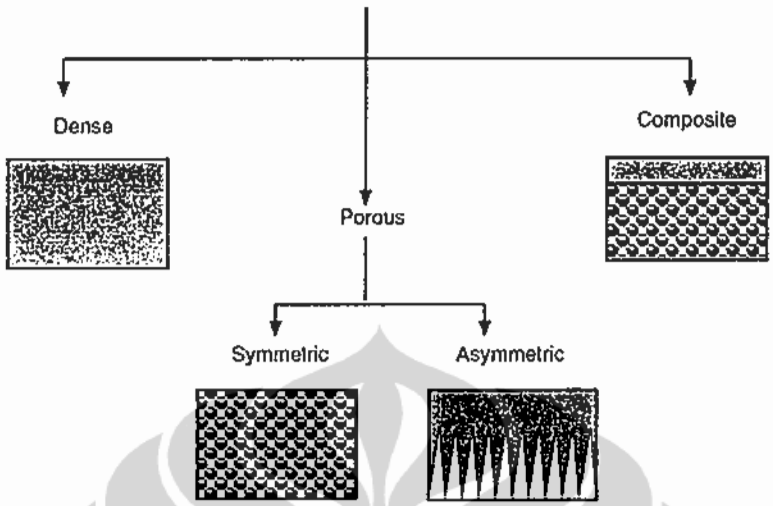

Figure 2.6. Membrane Classification According to Morphology

(Source: Nunes and Peinemann, 2006)

The preparation of asymmetric cellulose acetate membranes in the early 1960s by Loeb and Sourirajan is generally recognized as a pivotal moment for membrane technology (Drioli, 2001). They discovered an effective method for significantly increasing the permeation flux of polymeric membranes without significant changes in selectivity, which made possible the use of membranes in large scale operations for desalting brackish water and seawater by reverse osmosis and for various other molecular separations in different industrial areas.

2.3.1. Structures of Synthetic Membrane

Most of the presently available membranes are porous or consist of a dense top layer on a porous structure. The preparation of membrane structures with controlled pore size involves several techniques with relatively simple principles, but which are quite tricky (Nunes and Peinemann, 2006). The breakthrough of the membrane technology came first in the 1960s with the development of the asymmetric porous membranes by Loeb and Sourirajan (Loeb and Sourirajan, 1962). The asymmetric membranes combine high permeant flow, provided by a very thin selective top layer and a reasonable mechanical stability, resulting from the underlying porous structure. An asymmetric structure

characterizes most of the presently commercially available membranes, which are now produced from a wide variety of polymers.

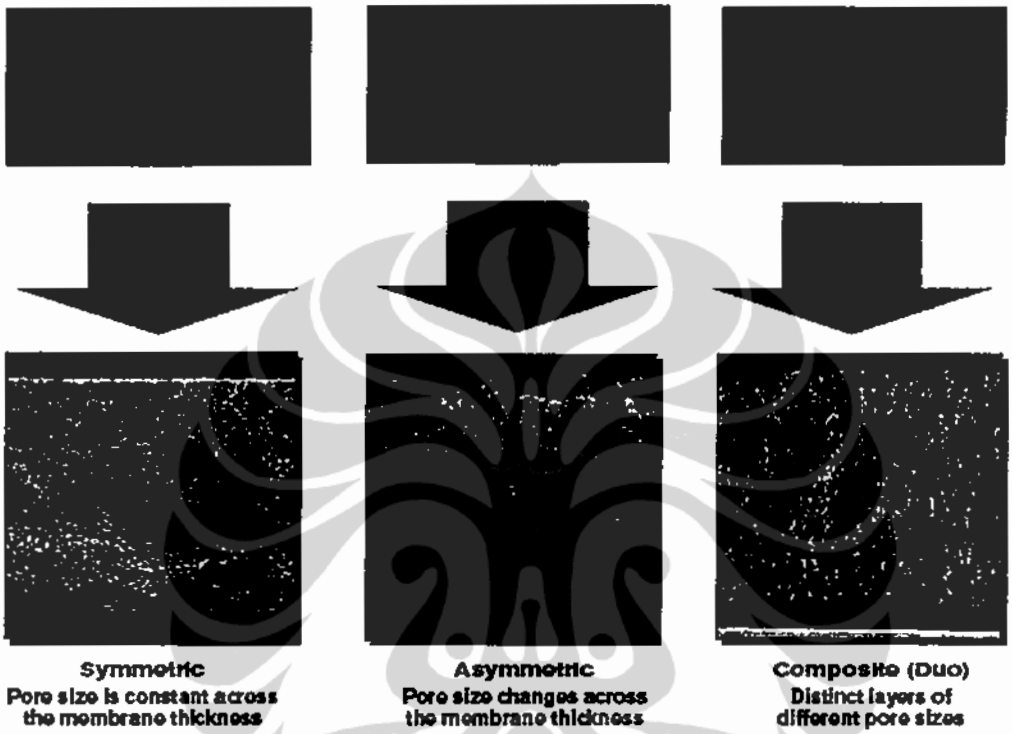

Figure 2.7. Schematic Representation and SEM Cross-Section of Symmetric, Asymmetric and Composite Membrane Structure

(Source: Vitorino *et al.*, 2009)

Symmetric membrane; The most important commercially available, symmetric, microporous membranes are prepared by the so-called phase inversion process (Kesting, 1971). In this process, a polymer is dissolved in an appropriate solvent and spread as a 20 to 200 μm thick film. A precipitant such as water is added to this liquid film from the vapor phase, causing separation of the homogeneous polymer solution into a solid polymer and a liquid solvent phase. The precipitated polymer forms a porous structure containing a network of more or less uniform pores. This type of membrane can be made from almost any polymer that is soluble in an appropriate solvent and can be precipitated in a non-solvent (Strathmann, 1985). By varying the polymer, the polymer concentration, the precipitation medium, and the precipitation temperature, microporous phase

inversion membranes can be made with a very large variety of pore sizes (from less than 0.1 to more than 20 μm) with varying chemical, thermal, and mechanical properties. These membranes were originally prepared from cellulosic polymers by precipitation at room temperature in an atmosphere of approximately 100% relative humidity (Zsigmondy, 1922).

Asymmetric Membranes; The most important membrane used today in separation processes is composed of a rather sophisticated asymmetric structure. In this membrane, the two basic properties required of any membrane, i.e., high mass transport rates for certain components and good mechanical strength, are physically separated. An asymmetric membrane consists of a very thin (0.1 to 1 μm) selective skin layer on a highly porous (100 to 200 μm) thick substructure, as indicated in the schematic drawing of Figure 2.7, which shows the cross section of symmetric, asymmetric and composite membrane. The very thin skin represents the actual membrane. Its separation characteristics are determined by the nature of the polymer and the pore size while the mass transport rate is determined by the membrane thickness, since the mass transport rate is inversely proportional to the thickness of the actual barrier layer.

2.3.2. Catalytic Membrane and Membrane Reactor

Membrane reactors combine selective mass transport with chemical reactions, and the selective removal of products from the reaction site increases the conversion of product-inhibited or thermodynamically unfavorable reactions (Giorno and Drioli, 2000). Membrane reactors using biological catalysts can be used in production, processing and treatment operations. In some cases where the membrane acts as a support for a catalyst or as the catalyst itself, the feed stream is permeated through the membrane in order to promote the desired reaction and there is no retentate stream. A schematic representation of such a process is presented in Figure 2.8.

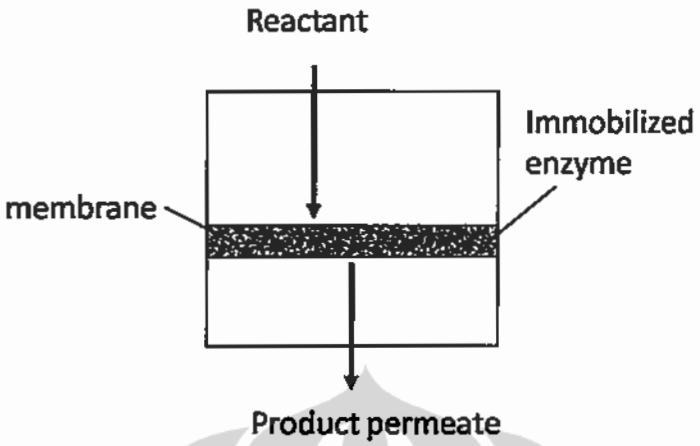

Figure 2.8. Configuration of a Reactor with The Membrane Active as a Catalytic and Separation Unit

(Adapted from: Giorno and Drioli, 2000)

The most common application of catalytic membrane found in literatures is in the field of enzymatic-catalyzed reactions. In this case, enzymes are initially immobilized in a membrane and then used as the catalytic medium to perform biochemical conversions (Butterfield *et al.*, 2001). Beyond its simple use as reactor, a membrane can be considered as a specific macrosystem resulting from the assembly of many microsystems. Indeed each pore could be looked upon as a particular microreactor. In a permeable membrane a challenge could consist in depositing enzymes correctly at pore mouth and within the microchannels, in order to get very efficient contacting conditions, i.e. a very high probability of capture of substrate molecules by catalyst as well as an improved contact time (Rios *et al.*, 2004).

2.3.3. Microreactor

A microreactor is generally defined as a miniaturized reactor with characteristic dimensions in micrometers and reaction volumes in the nanoliter to microliter range (Zhang *et al.*, 2004). Today microreactors raise new opportunities for applications of the reaction engineering toolbox, and microreaction technology is expected to have a number of advantages for chemical production.

Microreactors represent a new efficient tool for optimum reactor design (Ehrfeld, 2000; Jensen, 2001). Microfabrication techniques and scale-up by replication have already fuelled spectacular advances in the electronics industry. The outstanding property of a microreactor is its extremely large surface-to-volume ratio. The difference with a classical reactor may be as high as 10^5 (Wörz *et al.*, 2001).

The idea of using a microfluidic system to perform chemical synthesis was first introduced at a workshop in Mainz in 1995 (Ehrfeld, 2000). Since then, microfluidic devices have been widely used in synthetic chemistry. Before the appearance of the microreactor, conventional vessel reactors with volumes ranging from a few milliliters to a thousand liters were widely used in a variety of fields. Productivity is the most significant aspect to be considered in the synthesis of substances (Liu, 2008).

Microfabrication techniques and scale-up by replication have fueled spectacular advances in the electronic industry and more recently in microanalysis chips for chemical and biological applications. For a reaction engineer, the term of microreactor traditionally has meant a small tubular reactor for testing catalyst performance, but with the widening use of microreactor technologies, the "micro" prefix generally designates chemical system fabricated with technology originally developed for electronic circuits. The reduction in size and integration of multiple functions has the potential to produce structures with capabilities that exceed those of the conventional macroscopic systems and to add new functionally while potentially making low cost, mass production possible (Jensen, 2001).

Attractive advantage of implementing a microreactor is given by the reduced total size of the system. Resulting characteristics of microreactor are (Westermann, 2009):

- Virtually always laminer flow pattern
- High heat transfer rate, allowing for ishothermal or periodic operation
- Immproved mass transport due to fast diffusive mixing
- Inherently safe operation of potentially dangerous reactions
- Exact reaction control due to narrow residence time distribution
- High pressure stability
- Reduced time-to-market by numbering up instead of scale up

These advantages can be attributed to the dramatic reduction in scale leading to unique operating conditions such as the spatial and temporal reagent control obtained under a nonturbulent, diffusive mixing regime and a high surface-to-volume ratio. There is no doubt that micro-fluidic and Lab-on-a-Chip technology can be used as a platform for a wide range of applications such as chemical and biological analysis, chemical synthesis, materials chemistry and biotechnology (Zhang and Haswell, 2007).

CHAPTER 3
EXPERIMENTAL METHODS

3.1.Materials

The immobilization matrix membrane used in this study was asymmetric membrane having NMWL of 300 kDa, diameter of 63.5 mm and thickness of 280 μm, are PES 300 and MCE 300 (Millipore Inc., USA). According to the manufacture's specification, the sponge layer is porous non-woven polymer material, in which pores are significantly larger than those in the thin layer. Lipase AY from *Candida rugosa* was purchased from Sigma-Aldrich (Japan). Lipases PS from *Pseudomonas cepacia* and Lipase AK from *Pseudomonas fluorescens* were purchased from Amano Enzyme (Nagoya, Japan). Triolein from Sigma-Aldrich (Belgium) was used as substrate triglyceride. All the chemicals were analytical grade and used without further purification.

3.2.Preparation of Immobilized Lipase

Enzyme immobilization withinthe membranes poreswas carried out following the two steps: could achieved by adsorption of the enzyme on the support layer and its filtration through membrane pores (Fig. 3.1). Two steps were carried out, firstly, membrane was fixed in a filtration cell and cleaned with pure water of reverse osmosis then stored overnight for drying. In order to deposit the lipase in the sponge layer, the membrane was placed upside down at the bottom of vessel with the sponge layer facing upward. The vessel was charged first with 10 ml lipase solution containing 0.5–0.75 g of crude lipase in 10 ml of 50mM phosphate buffer (pH 7.0). Membrane samples were left to stand with lipase solution in a incubation time at 4°C for deposition and adsorption. Then the solution was filtered from the sponge layer through thin layer of the membranes by pressure driven of nitrogen gas at a pressure of 3 kPa. After pressure driven immobilization, membranes were stored overnight for drying, then rinsed twice with 10 ml of 50mM phosphate buffer (pH 7). The membrane loaded with lipase was taken out from the vessel and used as a biocatalytic membrane microreactor.

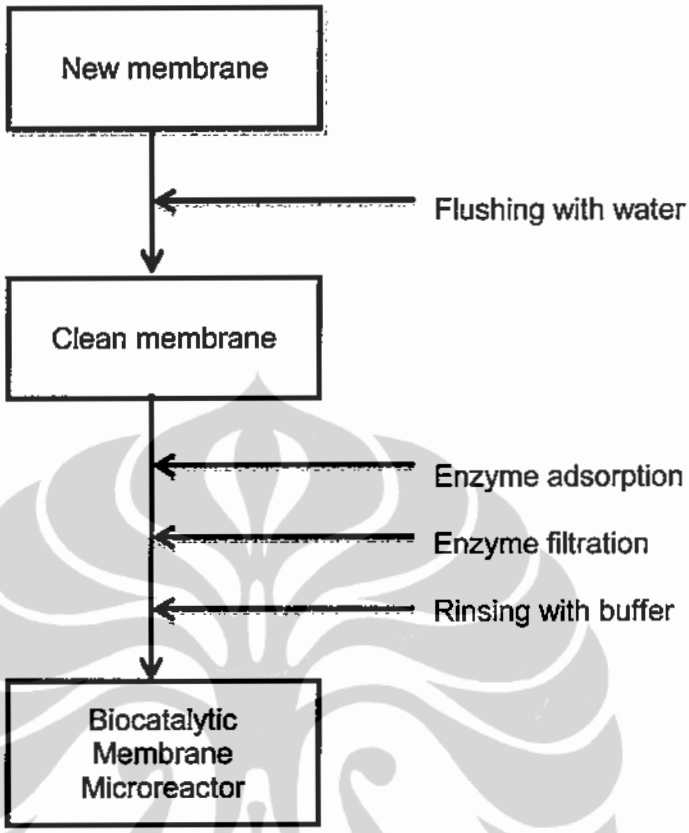

Figure 3.1. General Procedure of Preparation of Immobilized Lipase in The Membrane Pores

In order to calculate the amount of immobilized lipase during immobilization, the filtrate was collected and was measured. The amount of lipaseattached into the microstructure membrane (p) was determined from the difference in the protein concentration, before and after immobilization as well as total volume of the enzyme solution.

$$p \left(g/m^2\right) = \frac{(C_0 - C).V}{A} \tag{3.1}$$

where C_0 and C are the concentrations of lipase solution before and after immobilization (g/mL), respectively. V is the initial volume of lipase solution (mL) and A is the membrane area (m^2).

In an attempt to increase amount of immobilized lipase embedded into membrane pores, the following studies were carried out:

1. Effect of adsorption time (6, 12, 18 and 24 hours) at 4°C, using an initial enzyme concentration of 25.0 mg/mL. In this study Mixed cellulose ester (MCE) and Polyethersulfone (PES) membranes have been explored.
2. Effect of the initial enzyme concentration between 25 and 75 mg/mL and with incubation time of 18 hours at 4°C. In this study PES membrane has been explored.
3. In order to investigate the membrane structure, SEM characterization of the untreated and prepared biocatalytic membranes has been carried out by JSM-6301F scanning electron microscope. For this purpose, the membrane samples were fractured in liquid nitrogen and then coated with Au–Pd under vacuum conditions.

3.3.Experimental Setup for Transesterification System

Figure 3.2 is a diagram of experimental setup, which consisted of a filtration cell, a hot plate magnetic stirrer and peristaltic pump to provide driving force (vacuum) for permeation. The membrane microreactor was designed on an asymmetric membrane as enzyme-carrier material. The membrane microreactor consists of lipase in the membrane with an effective area of 28.7 cm^2 and was operated by settled at the bottom of filtration cell with thin layer facing upward. The transesterification reaction was monitored by quantifying the triolein substrate with HPLC. While, the methyl oleate reaction products were identified by GC/MS.

The membrane microreactor was operated in flow-through mode. Transesterification was started by forcing the solution passes through the membrane microreactor and the product was sucked from the vessel outlet. The reaction is carried out in situ within the membrane pores, so that its pores act as a kind of microreactor during formation of the product material. Triolein solution in methanol was diffused through the biocatalytic membrane microreactor then converted to methyl oleate and glycerol. Continuous agitation was provided just above the membrane microreactor surface by a magnetic spin bar suspended from

the cell top and driven by an external magnetic stirrer. The peristaltic pump was used for driving forces (vacuum) of permeate streams at a certain flow rate. Forty (40) milliliters of triolein and methanol in a selected molar ratio were used as a feed stream. The feed reservoir were kept at a constant temperature.

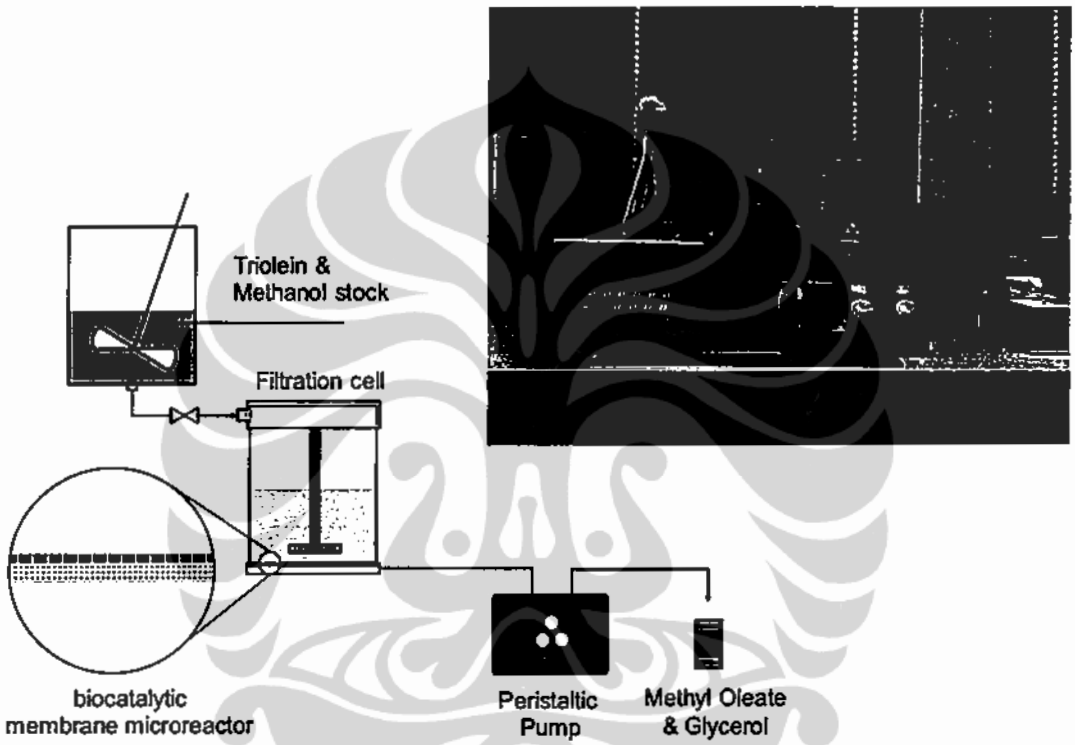

Figure. 3.2. Experimental Apparatus for Continuous Process

Using this system the following studies were carried out:

1. Effect of methanol molar ratio at 35±1 °C and constant flow rate of 2 ml/h. The triolein to methanol molar ratio was changed from 1:3 to 1:6.
2. Effect of temperature between 35 and 50 °C, taking 3 mol methanol and flow rate of 2 mL/h.
3. Effect of residence time (HRT) using 3 mol methanol and constant temperature 35±1 °C. Flow rate of permeate streams was changed between 2 and 7 ml/h.

4. The operational stability of the catalyst in the continuous process was tested at triolein to methanol molar ratio 1:3 at constant temperature 35 ± 1 °C and a flow rate of 2 ml/h. The system was operated over one week.

All of the reactions were performed in triplicate, and the results were reported as mean values. The permeate rate was measured as a function of time.

Transesterification reactions occurred as a result of catalytic action of immobilized lipase inside the membrane. Based on membrane volume, the residence time (*HRT*) of the substrate solution was defined as follow:

$$HRT = V_m / F \tag{3.2}$$

where V_m is the volume of membrane (ml) and F is flow rate of permeate (ml/h).

3.4. Analytical Methods

3.4.1. High Pressure Liquid Chromatography (HPLC)

The concentrations of residual triolein in permeate product were determined using HPLC with a UV detector at 205 nm. The analytical HPLC system consisted of two LC-9A pumps (Shimadzu) and a SPD - 10A variable-wavelength UV detector (Shimadzu, Kyoto). A LiChroCART RP-C18 analytical column of 2504 mm (Merck, Darmstadt, Germany) was employed. The mobile phase consisted of three different components: hexane, isopropanol and methanol. Reservoir A contained methanol and reservoir B contained a mixture of isopropanol and hexane (5:4, v/v). The gradient went from 100% A to 50% A - 50% B linearly over 30 min. The flow rate of the mobile phase was 1 ml/min and the sample injection volume was 10 µl. This nonaqueous RP-HPLC method was modified from the method reported by Holcapek et al. (Holcapek, 2002).

Triolein degree of conversion (X_c) was determined using the following equation:

$$X_c = \left(1 - C_s / C_i\right) 100\% \tag{3.3}$$

This equation is based on asummption that no triolein was adsorbed onto the membrane surface, so C_i is the amount initial triolein in the feed, and C_s is the triolein concentration that did not convert to the product. Membrane microreactor product permeability was determined from the flow rate per unit area of membrane and expressed as a flux (J_s) in L/m^2.h

3.4.2. Gas Chromatography / Mass Spectrometry (GC/MS)

The concentrations of methyl oleate in permeate product were determined using gas chromatography/mass spectrometry (GC/MS) techniques (Hewlett-Packard 5890) equipped with an MS and a 15-m capillary column (DB-1; Agilent Technologies Inc., Palo Alto, CA). The flow rate of He (carrier gas), air, and H_2 were 16, 360 and 30 mL/min, respectively. Temperatures of the injector and MS were 70 and 350 °C, respectively. The column temperature was increased from 70 to 200 °C at 20 °C/min. The use of mass spectrometric detectors would eliminate any ambiguities about nature of the eluting materials since mass spectra unique to individual compounds would be obtained (Knothe, 2001). In this case, sample derivatization was not necessary. The immobilized enzyme productivity (P_{cat}) were defined as follows:

$$P_{cat}\left(\frac{mmol}{h.mg_{lipase}}\right) = \frac{C_p \times F}{p_m} \qquad (3.4)$$

Where C_p was the concentration of the methyl oleate (mmol/mL), and F was the flow rate of the substrate solution (mL/h).p_m was amount of lipase immobilized on the membrane (mg). In the batch reaction, lipase productivity was defined as the initial formation rate of methyl oleate per milligram of lipase [mmol/(h.mg$_{lipase}$)]. The batch reactions were carried out using native lipase. Reactions were carried out in a 50-mL Erlenmeyer with an agitation speed of 200 rpm and temperature 35±1°C.

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Properties of Immobilized Lipase

In this research, immobilization of lipase on symmetric and asymmetric membranes was explored. Many studies utilized the membrane as a matrix for the enzymes immobilization. Enzymes were absorbed not only on the outer surface but also within the pores. However, the mechanism of how enzymes can deposit themselves right in the pores has not been clarified. The "pore constriction model" can explain this phenomenon, which was termed by Belfort *et al.* (1994). The model comes from the problems that occurred in the application of membrane in protein purification called fouling. When enzyme solution was passed through a membrane, the enzyme becomes adsorbed onto the sponge layer and the membrane pores (Belfort *et al.*, 1994). Inthis study, it is noteworthy that we took advantage of the protein fouling as immobilization method.

According to pore constriction model the enzyme could be deposited correctly withinthe microchannel and was adapted for enzyme immobilization, therefore denaturation of the enzyme was avoidedThe diameter of an enzyme is expressed as d, and the diameter of the membrane pores is d_p. When $d<<d_p$ the enzyme can enter most membrane pores, then deposited itself on the pore walls, and thus the enzyme will be confined within the pores. In order to test the performance of immobilized lipase in the membrane, it will be discussed in the next section.

4.1.1. Effect of Adsorption Time on The Amount of Immobilized Lipase

Figure 4.1 shows the effect of adsorption time on the amount of immobilized lipase in g/m^2. Experiments on adsorption time were done by adding a concentration of 25 lipase mg/mL in the MCE and PES membranes. Variation of adsorption time taken was 6, 12, 18 and 24 hours. The amount of lipase immobilized into the microstructure membrane was defined as protein quantity and calculated from the protein mass balance of the solutions (Eq. 3.1). In general, the amount of immobilized lipase in the membrane increased with the adsorption

time and after 18 hours the adsorption process should be completed. Figure 4.1 also shows that the type of membrane affected the amount of immobilized lipase, whereby the amount of immobilized lipase on PES membrane was higher than the amount of immobilized lipase on the MCE membrane. Amount of immobilized lipase approached 1.4 g/m^2 in MCE membrane and 3.0 g/m^2 in PES membrane. This can beeasily understood because the PES membrane is thicker than MCE membrane.

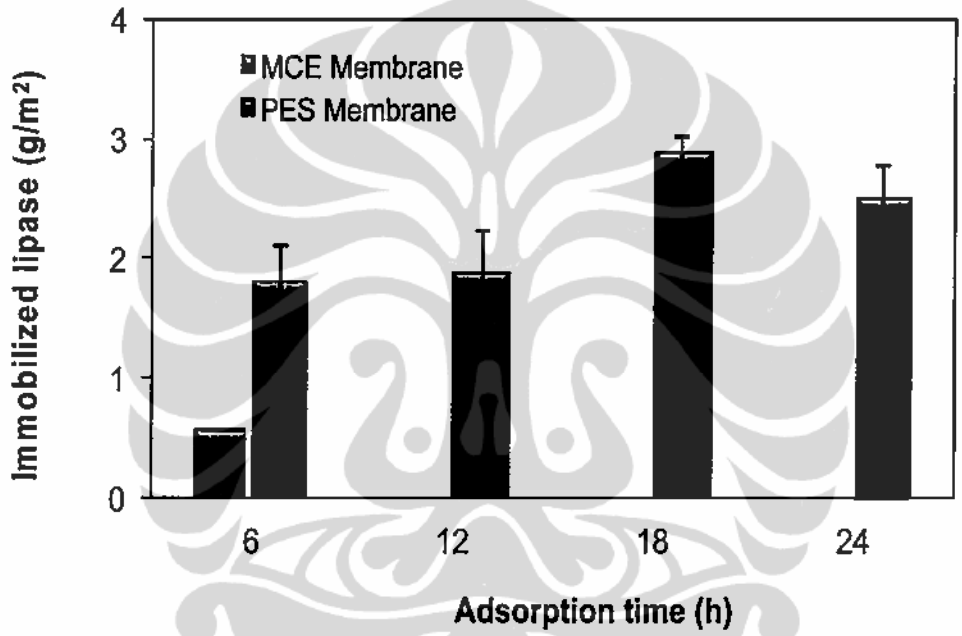

Figure 4.1. Effect of Adsorption Time on The Amount of Immobilized Lipase with 25 g/L of Initial Lipase Concentration

The increment of immobilized lipase on the membrane was caused by protein adsorption, which involves a specific interaction between the protein (lipase) and the membrane polymer that occurs in the absence of any convective flow through the membrane. When all the sorption sites were occupied, a pseudo-steady state was reached. In these experiments, it was found that 18 h was required to reach the adsorption equilibrium in lipase–membrane system over the studied range of lipase concentration. It took longer time to reach the balance than was reported by Hilal *et al.,* (2006), which took 8 h. In this study, the amount of immobilized lipase was greater than those reported by Hilal *et al.,* (2006), i.e.

0.034 - 0.68 g/m^2. They used similar type of membrane but different type of lipase, which was produced from *Candida rugosa*.

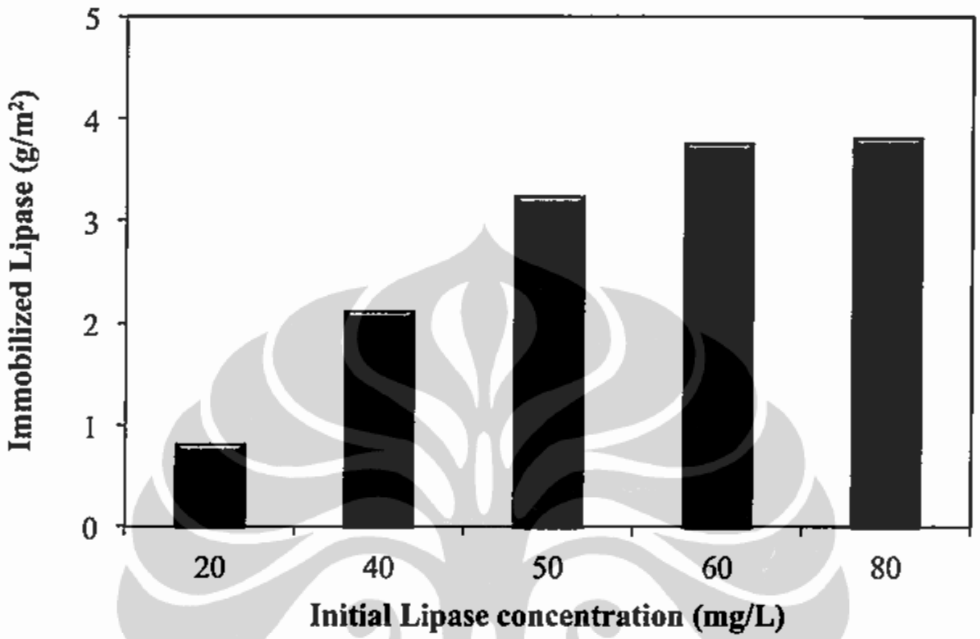

Figure 4.2. Effect of Initial Enzyme Concentration on The Amount of Immobilized Lipase, with 18 h of Adsorption Time

4.1.2. Effect of Initial Concentration on Immobilized Lipase

Figure 4.2 shows the effects of initial enzyme concentration on the amount of immobilized lipase (g/m^2) in PES membranes. The amount of immobilized lipase in the membranes increased with the enzyme concentration. High amount of immobilized lipase was obtained on PES membrane with an initial concentration of 60 - 80 g/L, which was equal to 3.75 g/m^2. According to Parfitt and Rochester (1983) it is well known that the time needed to reach the adsorption equilibrium depends on the initial adsorbate concentration in adsorbate–adsorbent system.

4.1.3. Enzyme Distribution

The SEM images were carried out to examine the enzyme distribution within the support material. Immobilized lipase on the MCE and PES membranes

are presented in Figure 4.3 and 4.4, respectively. Figure 4.3 shows the difference of new membrane and the membrane containing the lipase (A-D). The images show that most of the enzyme molecules were able to distribute into the membrane channel uniformly after incubation and pressure driven filtration. The uniform distribution of enzyme in this study may be due to the pressure driven which was applied during immobilization process. Belfort et al. (1994) noted that in pressure driven filtration the interaction between membrane and enzyme would follow the pore constriction model.

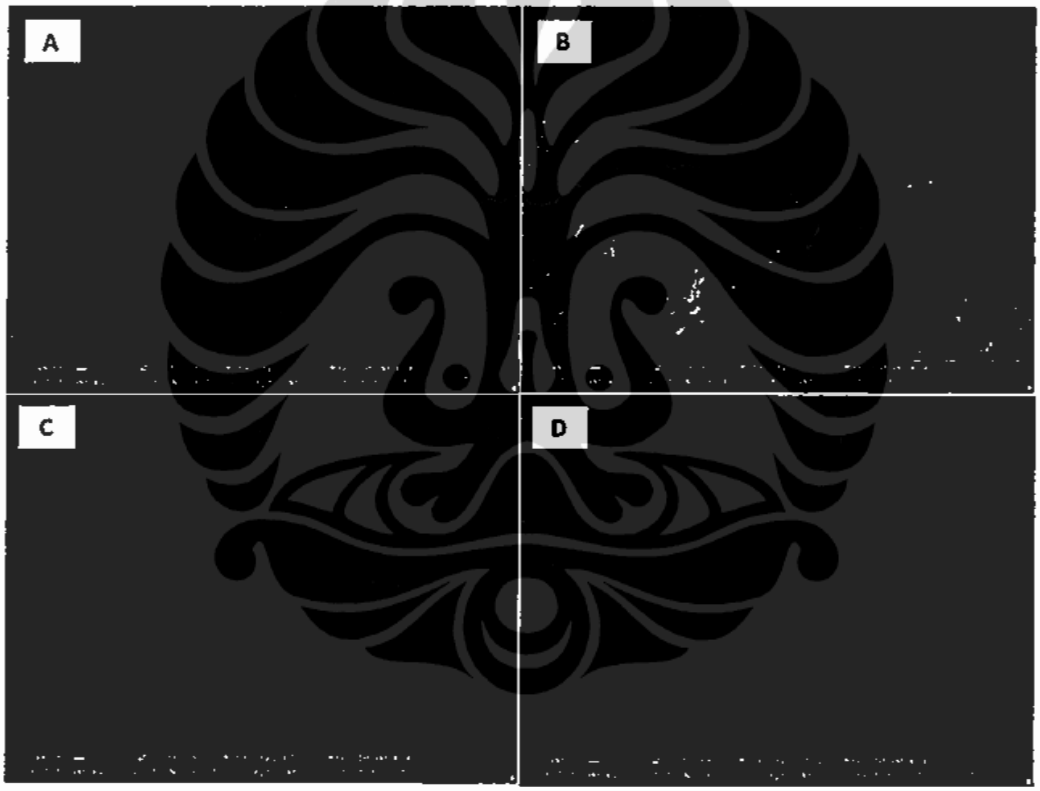

Figure 4.3. SEM Image Matrix MCE Membrane with Pore Size 0.22 μm and Immobilized Lipase by Incubation of Different Treatments: (**A**) New Membrane, (**B**) 2-Hours of Adsorption Time, (**C**) 12 Hours of Adsorption Time and (**D**) 24 Hours of Adsorption Time

According to the pore constriction model by Belfort *et al.* (1994), if protein diameter is much smaller than the membrane pore diameter, the protein could enter every pore and will be deposited on the pore walls, thus resulted in the enzyme molecules distributed uniformly within the membrane pores. In this study

we used MCE membrane, with pore size of 0.22 μm. Since the molecular weight of Lipase AK equals to 15 kDa (the particle diameter approximately ≈ 3 nm), the protein were deposited correctly and distributed uniformly within the microchannel.

However, without pressure driven filtration the enzymes were unable to move into the membrane pores. Pederson *et al.* (1985) studied the enzymes which were adsorbed in carriers by physical adsorption, as exemplified by immobilization of β-galactosidase on porous supports. It was found that most enzyme molecules were unable to move into half of the beads after incubation for 10 h. Based on this report, Cao (2005) noted that immobilization of an enzyme in a porous carrier usually follows the shrinking core theory – the enzyme molecules first occupy the outer shell of the carrier and move slowly to the interior of the carrier when the nearest part of the carrier is occupied.

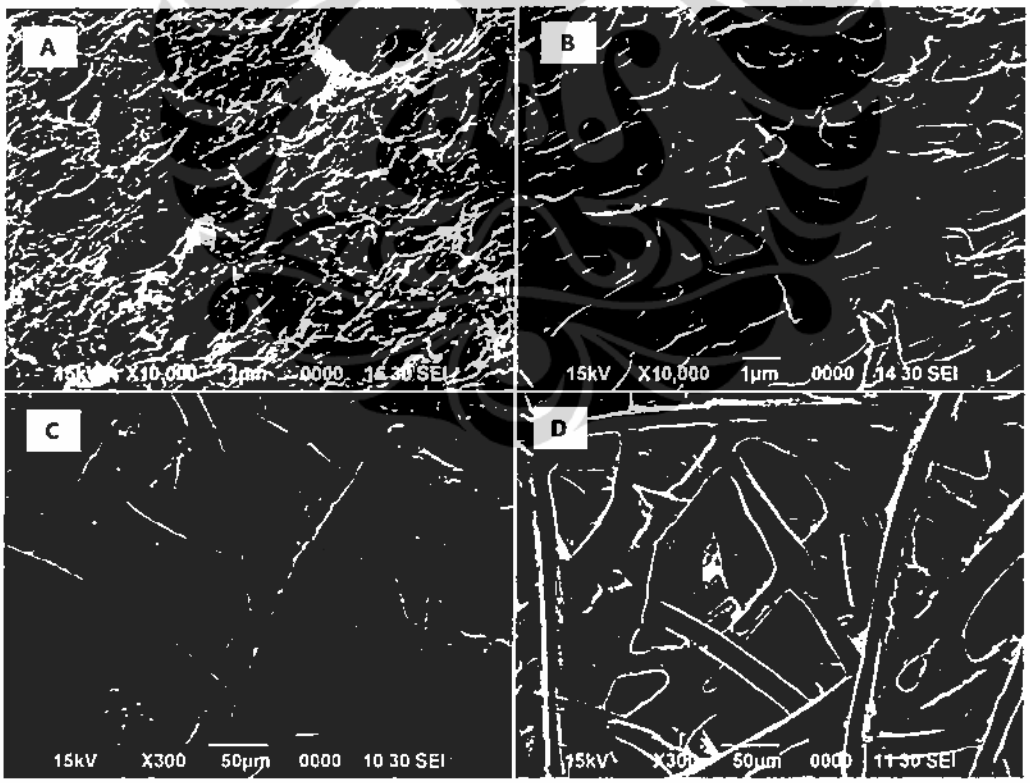

Figure 4.4. SEM Images of Cross Section Asymmetric PES Membrane NMWL 300 kDa: (A) Thin-Layer of Membrane Without Treatment, (B) Thin-Layer Immobilized Lipase, (C) Sponge Layer of Membrane Without Treatment and (D) Sponge Layer of Immobilized Lipase

Figure 4.4 shows the SEM images of morphological structure of the PES membrane with and without lipase. The images show that the enzyme molecules were able to distribute into the membrane channel uniformly within thin layer and sponge layer after incubation and pressure driven filtration. Changes of morphological structure of the PES membrane can be clearly seen by the presence of a good adsorption of lipase within the thin layer and sponge layer. Mateo *et al.* (2007) stated that Immobilization of enzymes inside the porous structure allows the enzyme molecules to be fully dispersed and without the possibility of interacting with any external interface. Therefore, immobilized lipase in this study is expected to increase methanol tolerance and operational stability due to the immobilized lipase molecules not in contact with any external hydrophobic interface. This will be discussed in more detail in section 4.2.

4.2. Principle of Biocatalytic Membrane Microreactor Operation

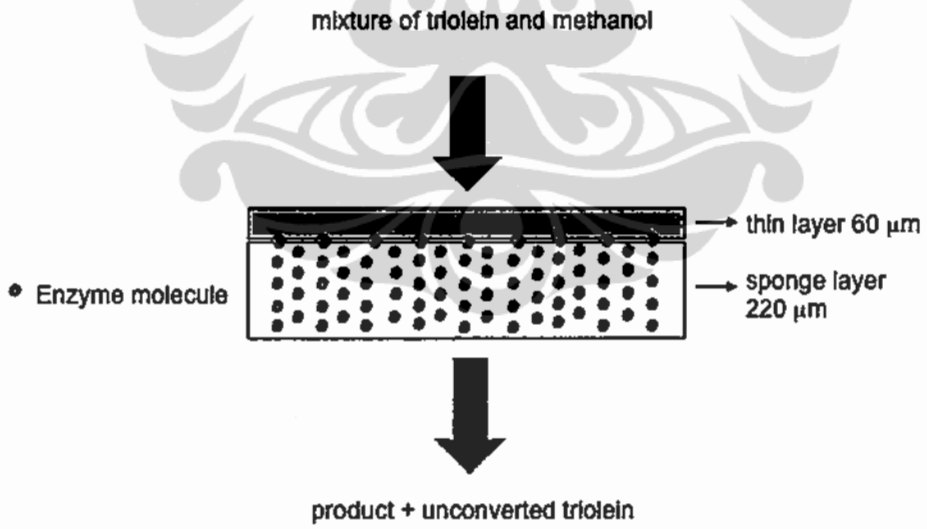

Figure 4.5. Transport Processes During Continuous Transesterification of Triolein with Immobilized Lipase in Membrane Microreactor

The principle of membrane microreactor operation is depicted in Figure 4.5.The reactants are forced to flow through the membrane. The main components of the catalytic membranes are the microstructure of membrane porous made from

PES activated with immobilized lipase. PES was chosen because of its asymmetric structure and excellent compatibility with enzyme. The reaction is carried out *in situ* within the pores of the membrane material, so that its pores act as a kind of microreactor during formation of the product material.

4.2.1. Catalytic Behavior of Membrane Microreactor

The catalytic behavior of triolein conversion degree in membrane microreactor was shown in Figure 4.6. Flux value was relatively constant of 0.5 L/m^2.h indicating almost no adsorption of glycerol on the surface of the immobilized lipase during continuous operation for 12 hours.

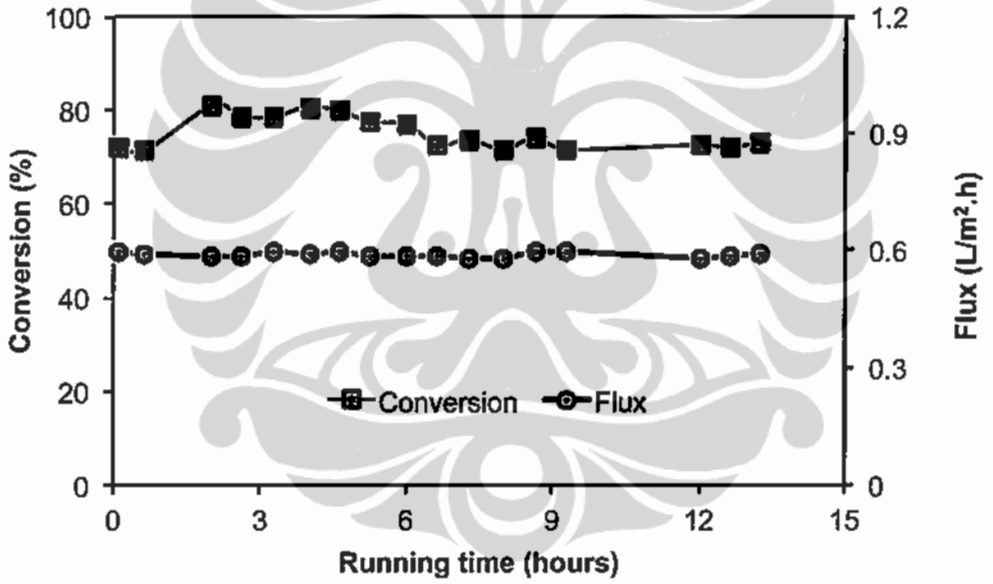

Figure 4.6. Time Course of Continuous Transesterification Reaction in a Biocatalytic Membrane Microreactor with 2.129 g/m^2 of Immobilized Lipase

In this study constant conversion value about 80% triolein into methyl oleate was observed using membrane microreactor with the amount of 2.129 g/m^2 of immobilized lipase. The transport process of reactants across microreactor was in flow-through mode with constant residence time at 30 minutes as defined in equation (3.2). The continuous process in microreactor was performed at constant residence andmaintained for 12 hours. During the experiment there was no

decrease in lipase activity, which was indicated by stable triolein conversion value. Temperature was controlled at 35 ± 1°C, with molar ratio of triolein to methanol as 1:3.

4.2.2. Effect of Triolein to Methanol Ratio

The effect of triolein to methanol molar ratio on conversion of triolein was studied, since it is an important parameter in the enzymatic transesterification reaction. Experiments were conducted with different molar ratio of triolein to methanol ranging from 1:3 to 1:6. In the transesterification reaction as per stoichiometry, three moles of methanol react with one mole of triolein to yield three moles of methyl oleate and one mole of glycerol. Results obtained are shown in Figure 4.7. The results show that at any triolein to methanol ratio, there is no decrease in conversion of triolein. The increasing ratio of methanol did not cause activity loss of enzyme. This might be due to protection of enzyme, by membrane pores in support layer. It is noteworthy that the use of biocatalytic membrane microreactor has been providing solutions to the problems of deactivation of lipaseby insoluble methanol.

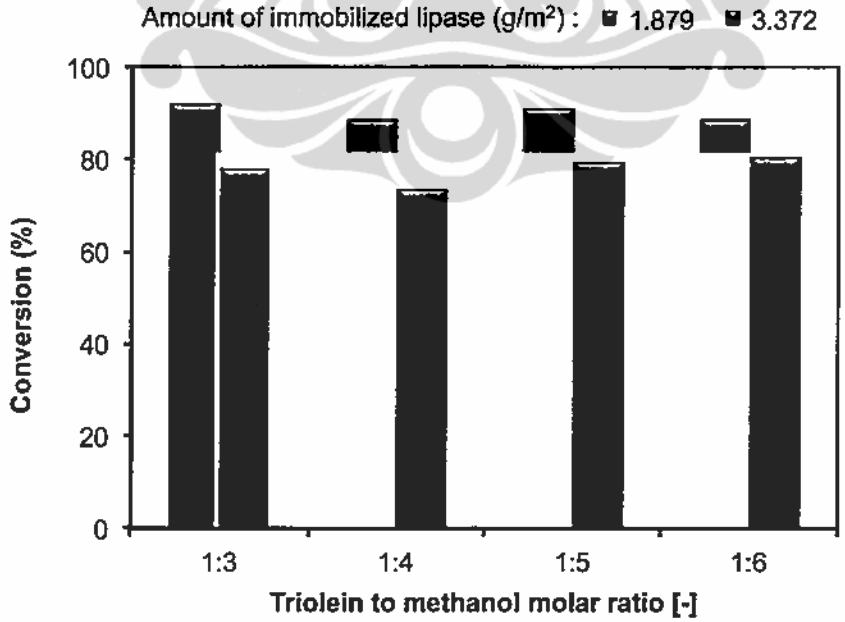

Figure 4.7. Effect of Molar Ratio of Methanol to Triolein by Biocatalytic Membrane Microreactor with Different Amount of Immobilized Lipase

It is well known that lipase inactivation caused by methanol was the major obstacle for the enzymatic biodiesel production (Fukuda *et al.*, 2001; Haas *et al.*, 2006; Su and Wei, 2008; Bisen *et al.*, 2010). To solve this problem, other researchers have selected the following three options, i.e. methanol stepwise addition, acyl accept or alterations and solvent engineering (Tables 4.1).

Although these studies have been successfully applied in laboratory, however the complexity of large-scale application and increment of production costs are still the barrier for its industrial implementation (Tan *et al.*, 2010). In this study, that problem has been successfully overcome by methanol inactivation with a simple membrane microreactor.

Table 4.1. Summary of The Options to Avoid Lipase Inactivation Caused by Methanol

Options	Conditions	Advantages and Disadvantage	References
Methanol stepwise addition	Three-step or two-step methanol addition	(+) Higher yield is obtained without inactivation to the lipase (-) The operation is relative complicated in large-scale production.	Chen *et al.*, 2006; Lu *et al.*, 2007; Shimada *et al.*, 1999; Soumanou and Bornscheuer, 2003
Acyl acceptor alterations	Methyl acetate, ethyl acetate	(+) No inactivation effect occurs and no glycerol is produced. (-) The reaction rate is low and the acyl acceptor cost is high.	Xu *et al.*, 2003
Solvent engineering	With t-butanol, 1,4-dioxane, ionic liquid as solvents	(+) Good solvents of methanol and glycerol, so methanol inactivation and glycerol deposit are avoided. (-) Increment of the solvent recovery cost.	Ha *et al.*, 2007; Iso *et al.*, 2001; Royon *et al.*, 2007

(Source: Tan *et al.* 2010)

4.2.3. Effect of Residence Time (HRT)

Figure 4.8 shows the effect of residence time on the triolein conversion with different amount of immobilized lipase. It could be seen that, with the increase of residence time from 5 – 15 minutes the conversion of triolein also increased. However, prolonging the residence time over 30 minutes did not have much effect on triolein conversion.

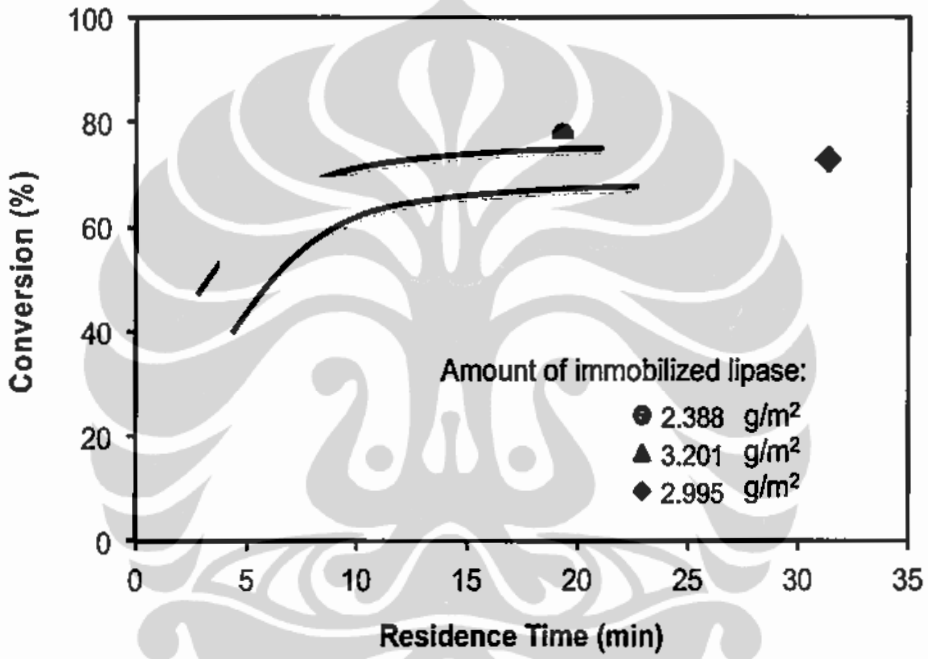

Figure 4.8. Effect of Residence Time on Triolein Conversion During Continuous Transesterification with Different Amount of Immobilized Lipase

Figure 4.8 also shows that the effect of residence time was apparent on the immobilized enzyme with catalyst amount of 2.388, 3.201 and 2.995 g/m^2. Regardless of the adsorption values, the maximum conversion of triolein ranged at 80% with 20-30 minutes of reaction time. These results clearly indicate that the biocatalytic transesterification reaction time on the membrane microreactor system is faster than the conventional system. From an economical point of view, the best process should allow to obtain the maximum conversion value in the shortest time.

It is well known (Zhang and Haswell, 2007) that the flow within microchannel lies in the laminar flow regime. Under laminar flow conditions, the mass transfer will be dominated by diffusion. Based on Fick's law (Fick, 1855), the relationship between the channel width *(L)* of a molecule by diffusion and time *(t)* can be simplified as:

$$t = L^2/2D \tag{4.1}$$

where D is the diffusion coefficient.

Equation (4.1) shows that by scaling down the dimension in which diffusive mixing occurs, the time it needs to achieve complete mixing is reduced significantly. Reduction by a factor of two in the channel width will result in reduction by a factor of four in time, and thus, one could design the reaction channel width as narrow as possible.

Table 4.2. Biodiesel Production with Various Lipases

Lipase	Reactants	HRT	References
Pseudomonas fluorescens	Sunflower oil Methanol	24 h	Soumanou and Bornscheuer, 2003
Burkholderias cepacia	Grease Ethanol	48 h	Hsu *et al.*, 2004
Pseudomonas cepacia	Mahua oil Ethanol	2.5 - 6 h	Kumari *et al.*, 2007
Pseudomonas cepacia	Jatropha oil Ethanol	8 h	Shah and Gupta, 2007
Candida antarctica	Tallow Methanol	72 h	Lee *et al.*, 2002
Rhizopus oryzae mixed with *Candida rugosa*	Soybean oil Methanol	21 h	Lee *et al.*, 2006
Thermomyces lanuginose mixed with *Candida antarctica*	Rapeseed Waste oil Methanol	12 h	Li *et al.*, 2006
Pseudomonas fluorescens	Triolein Methanol	20-30 min	This study

The results of this study have shown that the biocatalytic membrane microreactor system could finish the reaction time not more than an hour. Table 4.2 shows the progress of research conducted by other researchers in the field of biodiesel production using lipase as catalyst.

4.2.4. Effect of Amount of Immobilized Lipase

The amount of enzyme used is a crucial factor for successful industrial application. Therefore, the effect of immobilized enzyme amount was examined. To study this effect, membranes with various immobilized lipase were prepared. Reaction temperature was controlled at 35 ± 1°C, with molar ratio of oil to methanol as 1:3. Figure 4.9 demonstrates that the amount of immobilized lipase was in the range of 0.814 – 5.576 g/m^2.

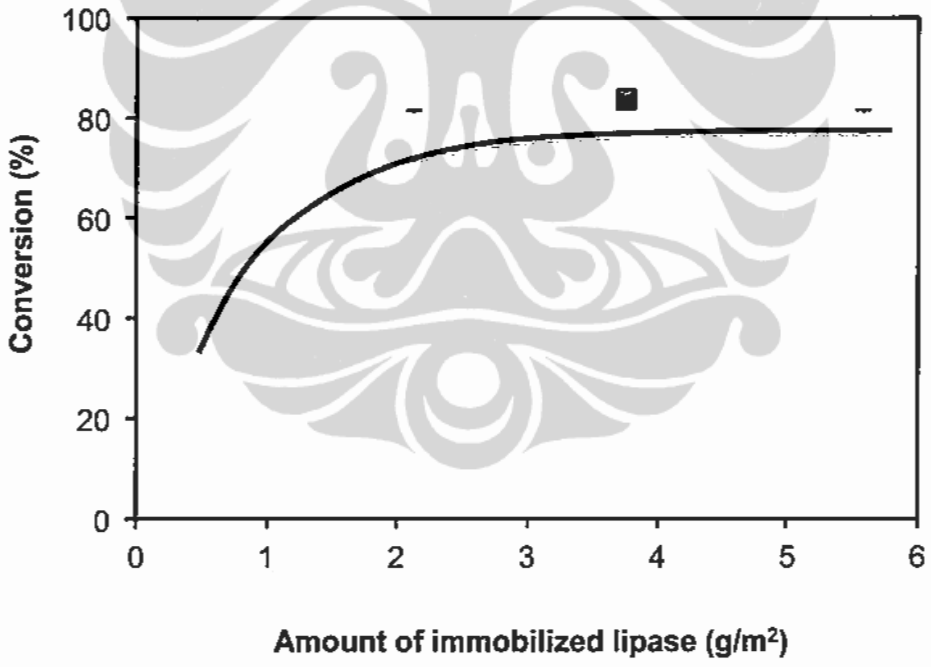

Figure 4.9. Effect of Amount of Immobilized Lipase on The Conversion of Triolein During Continuous Transesterification by Biocatalytic Membrane Microreactor

From Figure 4.9, it is seen that there was an increase in the triolein conversion from 55% to 77% by increasing the amount of immobilized lipase from 0.814 g/m^{2}to 2.129 g/m^2. However, further increase in the amount of

immobilized lipase did not have much effect on triolein conversion. This means that only a small amount of the immobilized enzyme within the membrane contributes to the reaction. These results suggested that the amount of enzyme added was much greater than required. This behavior of the immobilized lipase was consistent with those reported by other researchers (Noureddini *et al.*, 2005; Hilal *et al.*, 2006). Therefore, excessive used of enzymes when using the membrane microreactor should be avoided.

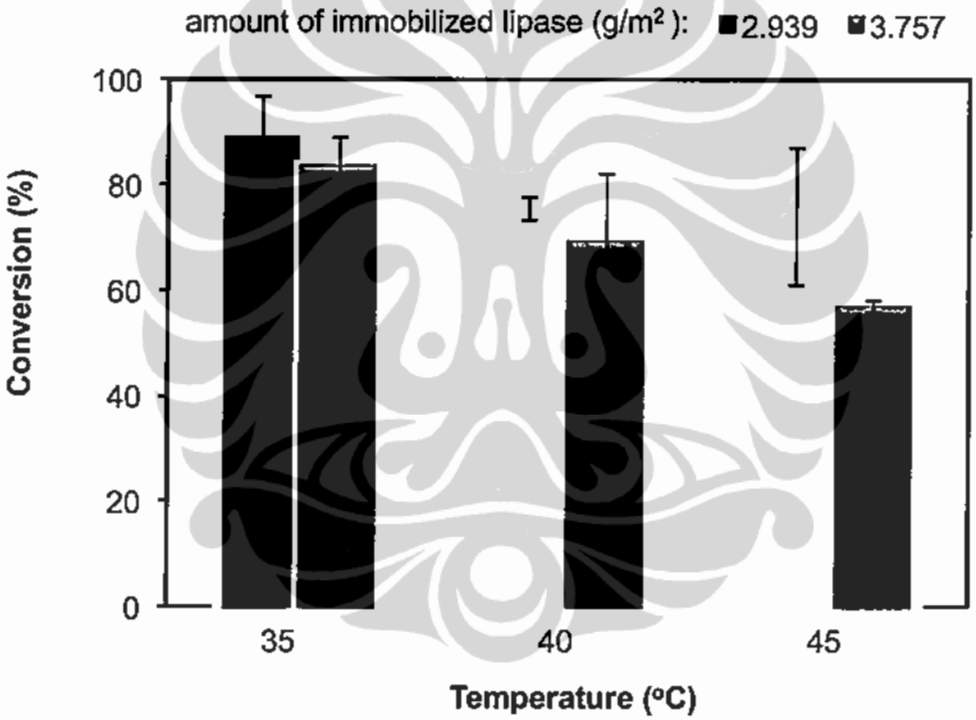

Figure 4.10. Effect of Temperature on Continuous Transesterification in Catalytic Membrane Reactor with Different Amount of Immobilized Lipase

4.2.5. Effect of Temperature

The effect of temperature on the catalytic activity in the membrane microreactor was investigated using two different amounts of immobilized lipases, e.g. 2.939 g/m^2 and 3.757 g/m^2. Temperatures were varied in the range from 35°C to 45°C with 5°C increments at triolein to methanol with a ratio of 1:3. The highest conversion yields were obtained in 35°C (Figure 4.10). Catalytic

activity decreased at temperature above 40 °C, indicating a possible thermal deactivation of the biocatalyst. Because the inactivation of the lipase was significantly greater at higher temperatures, we selected 35°C for subsequent experiments with all the studied lipase.

4.2.6. Stability of Biocatalytic Membrane Microreactor

One of the main purposes that researchers use enzyme immobilization is to achieve enzyme stabilization. To verify the stability of enzyme in membrane microreactor, we performed long-term enzymatic transesterification reaction using 0.814 g/m^2 of immobilized lipase. The stability of enzyme was monitored for 12 days and the results are presented in Figure 4.11. The conversion of triolein was stable at about 55% and this value run for 12 days continuously. These results indicated that there was no loss of enzyme activity during the time span of operation.

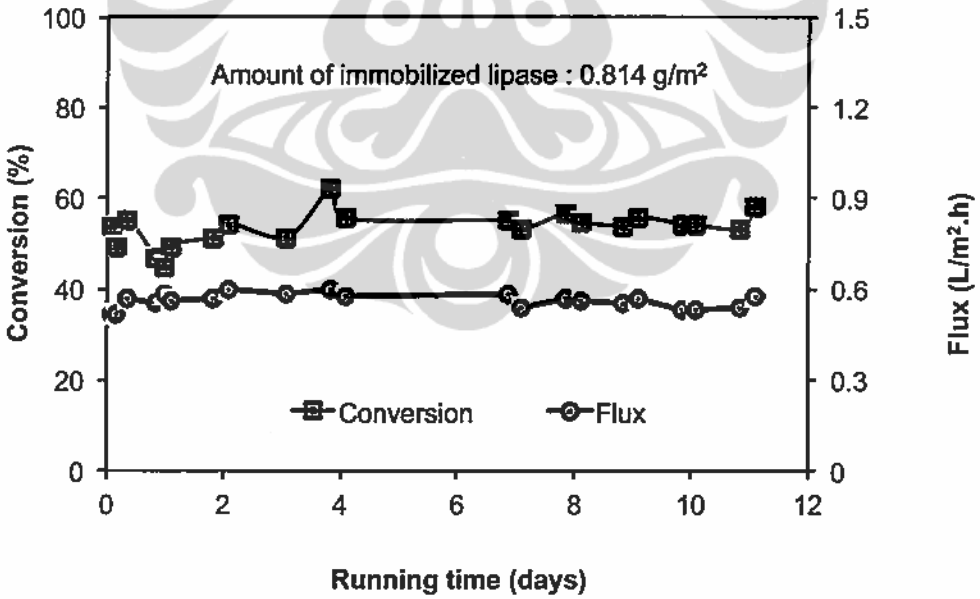

Figure 4.11. Stability of Immobilized Lipase in Membrane Microreactor During Continuous Transesterification of Triolein

To study the performance of the membrane microreactor for transesterification of olive oil we carried out an investigation using 3.447 g/m^2 of immobilized lipase. Reaction temperature was controlled at 35 ± 1°C, with molar

ratio of oil to methanol at 1:3 and 30 minute of residence time. The results are presented in Figure 4.12. It can be seen that there is no decrease in permeate flux and olive oil conversion. These results indicated that there was no loss of enzyme activity and no adsorption of glycerol.

Product permeability of the membrane microreactor was determined from flow rate per membrane area and expressed as flux, J_s in (L/m^2.h). The flux of product permeate in membrane microreactor was monitored and it was constant for 12 days. These results indicated that there was no glycerol adsorption during the time span of operation. In order to calculate the half-life of the stabilized enzyme, more experiments will be needed with longer time of stability test; nevertheless the results show that microreactor maintained good stability for at least 12 days.

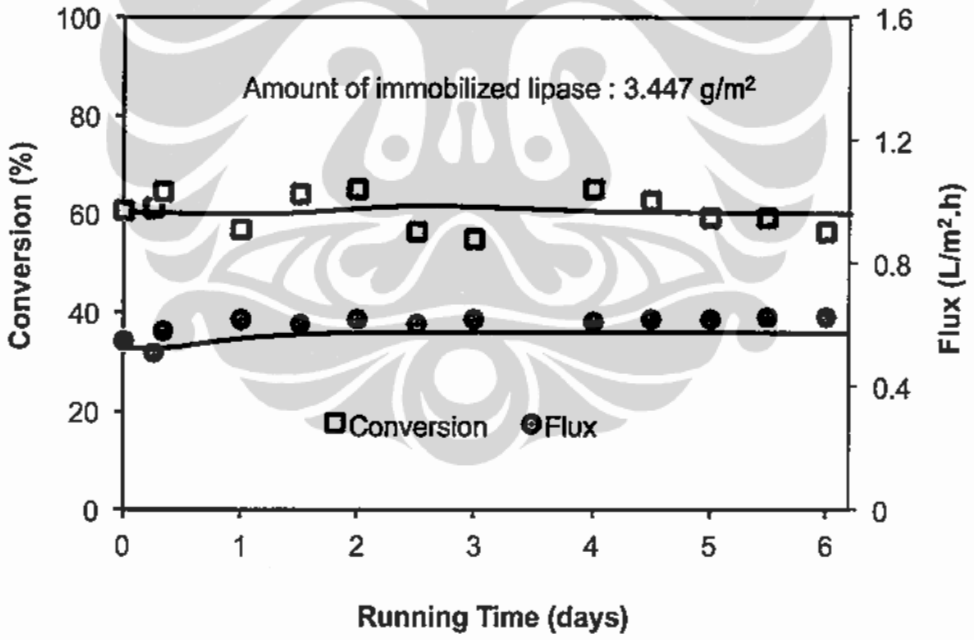

Figure 4.12. Stability of Immobilized Lipase in Membrane Microreactor During Continuous Transesterification of Olive Oil

4.2.7. Productivity of Biocatalytic Membrane Microreactor

To determine productivity of the biocatalyst in this continuous membrane microreactor system were used equation (3.3). In control experiments, batch

reaction were carried out using native free lipase. Figure 4.13. shows the productivity of enzyme in control (batch without membrane) and lipase loaded membrane with triolein and olive oil substrate. The productivityof membrane microreactor in this study was 12-25 times higher than the original free lipase. These results show that the use of membranes as biocatalysts system can increase the productivity and usefull for transesterification. These results confirmed the results of Goto *et al.* (2006) in term of productivity. However, there are different condition of experiments, i.e. for esterification reaction.

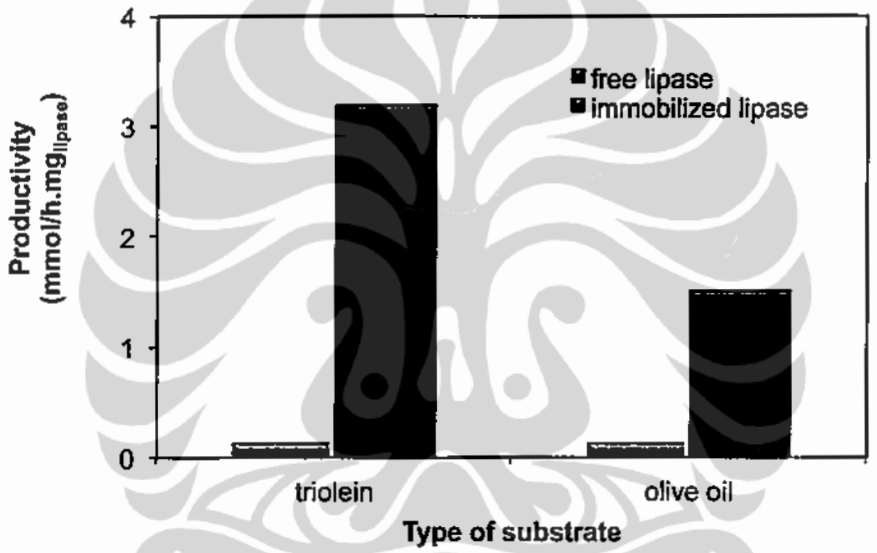

Figure 4.13. Productivity of Biocatalytic Membrane Microreactor vs Batch Reactor for Continues Transesterification with Diffrent Substrate

CHAPTER 5
CONCLUSION

5.1. Conclusion

In this study, a new concept of biocatalytic membrane microreactor was developed for transesterification reaction. In such concept a membrane pore could be considered as a microreactor and the membrane does not act as selective barrier but rather as microreactor with lipase inside. The conversion of triolein to methyl oleate occurred during the permeation of the substrate solution through the biocatalytic membrane. The maximum conversion of triolein with lipase-membrane microreactor was approximately 80% with reaction time 20 – 30 minutes. The biocatalytic membrane microreactor system with lipase as biocatalyst showed far superior activities compared to those of free lipase, i.e. 12-25 fold. No decrease in flux and activities were observed over a period of 12 days of continuous operation. This biocatalytic membrane microreactor system is of great potential to be applied in the process of transesterification of triglycerides for commercial biodiesel production since it would produce biodiesel in large scale and has a much smaller reaction time.

5.2. Outlook

The study on the immobilization of lipase in the membrane microreactor, as presented in this dissertation, is needs further study. In order to provide fully functional technical solution for industrial application more investigation should be carried out in different configuration of membrane module, i.e. hollow fiber membrane. Recently, some studies some published on the utilization of hollow fiber membrane as carrier for lipase immobilization (Balcao and Macata, 1998; Calabro *et al.*, 2002 and Goto *et al.*, 2006). However, no verification is made for utilization of pores as microreactors. In our case, the membrane pores were considered as microreactors and gave satisfactory results.

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APPENDICES

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RESEARCH PAPER

Membrane Microreactor in Biocatalytic Transesterification of Triolein for Biodiesel Production

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Abstract Transesterification is a principal chemical reaction that occurs in biodiesel production. We developed a novel biocatalytic membrane microreactor (BMM) for continuous transesterification by utilizing an asymmetric membrane as an enzyme-carrier for immobilization. The BMM was developed by pressure driven filtration of lipase from *Pseudomonas fluorescens*, which is suitable for highly efficient biocatalytic transesterification. Lipase solution was allowed to permeate through an asymmetric membrane with NMWL 300 kDa composed of polyethersulfone. The performances of BMM were studied in biodiesel synthesis *via* transesterification of triolein with methanol. Transesterification was carried out by passing a solution of triolein and methanol through the asymmetric membrane. The degree of triolein conversion using this microreactor was ca. 80% with a reaction time of 19 min. The BMM system displayed good stability, with no activity decay over a period of 12 day with continuous operation. Results from triolein transesterification clearly demonstrate the potential of an asymmetric membrane as an enzyme carrier material. Enzyme activity (mmol/h·g_{lipase}) was approximately 3 fold higher than that of native free lipase.

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1. Introduction

The utilization of enzymes as biocatalysts has become increasingly important in sustainable technology and green chemistry [1]. Lipases are versatile group of enzymes, which have the ability to catalyze many different, industrially important reactions such as hydrolysis, esterification and transesterification [2]. Lipases are too costly for single use, so for industrial applications they are preferred in the immobilized state, allowing easy separation of the catalyst and product [3]. Immobilized lipases have a number of advantages over the use of soluble catalysts, such as the possibility of recovery and re-use, operational simplicity, improved stability and enhanced activity [4].

Several different methods of immobilization are available and each of them associated with varying degrees of complexity and efficiency [5-7]. Although there are hundreds of immobilization protocols, designing new protocols that may improve enzyme properties is still required [6]. Recently, we developed a simple method of immobilizing enzymes on to asymmetric membranes using an adsorption technique in the sponge layer with pressure-driven filtration through the thin layer [7]. The membranes had an asymmetric structure with a thin layer at the top supported by a sponge layer at the bottom. A membrane can be considered as a specific macrosystem resulting from the assembly of many microsystems [8]. Although membranes were initially defined as filtration media, the high ratios of available area to volume have permitted their use as supports for enzyme immobilization [9]. In such systems, the membrane does not act as a selective barrier but rather

as a microreactor containing lipases.

To the best of our knowledge, the application of lipase-containing membranes as microreactors in biocatalytic transesterification for biodiesel production has not been reported. The use of lipases as catalysts in transesterification for biodiesel production has been widely studied over the last decade, due to the waste-free process [10]. In these studies, the main obstacle was enzyme inactivation due to immiscibility of methanol with triglycerides [11]. To address this, three-step flow transesterification in a fixed-bed bioreactor packed with lipase was proposed. However, a very slow reaction was observed, taking nearly 36 h to complete the reaction for biodiesel production [12]. Therefore it is important to develop an effective system to successfully commercialize enzyme applications in the biodiesel production industry.

We present a novel biocatalytic membrane microreactor developed for the transesterification process. We demonstrate that the method can overcome the problem of miscibility of methanol in oil and that insoluble methanol can cause deactivation of lipase in biocatalytic transesterification by immobilizing the lipase on to the asymmetric membrane. In addition, by using the membrane as an enzyme-carrier, the efficiency of the immobilized enzyme can be improved [13], since reduction in the size of the membrane matrix results in a short diffusion length. In this study, we used a filtration cell as a vessel with lipase-containing membranes as a microreactor.

2. Materials and Method

2.1. Materials

The immobilization matrix membrane used in this study was an asymmetric membrane with an NMWL of 300 kDa, diameter of 63.5 mm and thickness of 280 μm, consequently termed PES 300 (Millipore Inc., USA) for the rest of this article. The sponge layer is a porous non-woven polymer in which pores are significantly larger than those in the thin layer. Lipase AY from *Candida rugosa* was purchased from Sigma-Aldrich (Japan). Lipase PS from *Pseudomonas cepacia* and Lipase AK from *Pseudomonas fluorescens* were purchased from Amano Enzyme (Nagoya, Japan). Triolein from Sigma-Aldrich (Belgium) was used as the triglyceride substrate. All the chemicals were of analytical grade and used without further purification.

2.2. Preparation of microreactor

The PES 300 asymmetric membrane was used as support. Firstly, the membrane was fixed in a filtration cell and cleaned with pure water for reverse osmosis and stored overnight for drying. Since the lipase was aimed for loading from the sponge layer, the membrane settled upside down at the bottom of the vessel with the sponge layer facing upwards. The vessel was primed with 50 mL lipase solution containing 0.5 ~ 0.75 g crude lipase in 50 mL 50 mM phosphate buffer (pH 7.0). Membrane samples were left to stand in the lipase solution for 18 h at 20°C for deposition and adsorption. After standing for 18 h, the solution was filtered from the sponge layer through the thin layer of the membrane by pressure-driven nitrogen gas at 2 kPa. After pressure-driven immobilization, membranes were stored overnight for drying and then rinsed with 50 mM phosphate buffer (pH 7). The membrane, loaded with lipase, was then removed from the vessel and used as an enzyme microreactor.

In order to calculate enzyme loading during microreactor preparation, the filtrate was collected and measured. The amount of protein attached to the microstructure membrane (L_i) was determined by calculating the difference in protein concentration, before and after immobilization, as well as the total volume of the enzyme solution, as represented by the equation below:

$$L_i(kg/m^2) = \frac{(C_0 - C)V}{A} \quad (1)$$

C_0 and C are the concentrations of lipase solution before and after immobilization (kg/m^3), respectively. V is the initial volume of lipase solution (m^3) and A is the membrane area (m^2).

2.3. Experimental setup for continuous transesterification

Fig. 1 shows a diagrammatic representation of the experimental design, which consisted of a filtration cell, a hot plate magnetic stirrer and peristaltic pump to provide the vacuum for permeation. The membrane microreactor was designed on an asymmetric membrane as the enzyme-

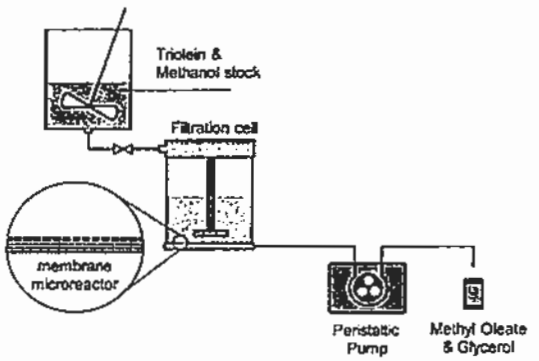

Fig. 1. Experimental apparatus for the continuous transesterification process.

carrier. The membrane microreactor consisted of lipase in the membrane with an effective area of 28.7 cm^2 and was operated by locating at the bottom of filtration cell with thin layer facing upward. The temperature of the system was maintained at 35 1°C. The transesterification reaction was monitored by quantifying the triolein substrate with HPLC.

The membrane microreactor was operated in flow-through mode. Transesterification was initiated by forcing the solution through the membrane microreactor and extracting the product from the vessel outlet. Each pore was considered as a particular microsystem. The reaction was carried out *in situ* within the membrane pores, so that the pores could act as microreactors during the formation of the material. Triolein solution in methanol was diffused through the BMM and converted to methyl oleate and glycerol. Continuous agitation was provided just above the membrane microreactor surface by a magnetic spin bar suspended from the cell top and driven by an external magnetic stirrer. The peristaltic pump was used for permeating streams at a flow rate of 5 mL/min. Fifty milliliters of solution containing triolein in methanol was used as the feed stream. The feed and receiving reservoirs were kept at a constant temperature of 35 ± 1°C and experiments were carried out continuously for 12 days.

Transesterification reactions occurred as a result of the catalytic action of immobilized lipase inside the membrane. The product was received in a reservoir for measurements of flow rate and triolein concentration. Based on membrane volume, the residence time (τ) of the substrate solution was defined as follows:

$$\tau(h) = V_m/v \quad (2)$$

V_m is the volume of membrane (L) and v is flow rate of permeate (L/h).

2.4. Analysis methods

The concentrations of methyl oleate and residual triolein in the permeate product were determined by using HPLC with a UV detector set at 205 nm. The analytical HPLC system consisted of two LC-9A pumps (Shimadzu, Japan) and a SPD-10A variable-wavelength UV detector (Shimadzu, Japan). A LiChroCART RP-C18 analytical column of 2,504 mm (Merck, Germany) was employed. The mobile phase consisted of three different components, which were hexane, isopropanol and methanol. Reservoir A contained methanol and reservoir B contained a mixture of isopropanol and hexane (5:4, v/v). The gradient went from 100% A to 50% A to 50% B linearly over 30 min. The flow rate of the mobile phase was 1 mL/min and the sample injection volume was 10 μL. This nonaqueous RP-HPLC method was modified from the previously reported method [14].

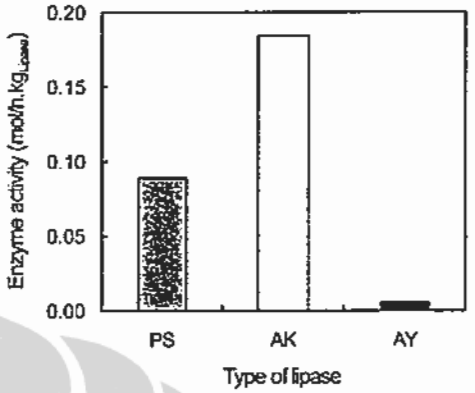

Fig. 2. Lipase screening on transesterification reaction. PS: lipase from *Pseudomonas cepacia*, AK: lipase from *Pseudomonas fluorescens*, AY: lipase from *Candida rugosa*.

3. Results and Discussion

3.1. Screening of the enzyme

Reactions were carried out in Erlenmeyer flasks (100 mL) containing a mixture of 10 g triolein, 3 × 0.37 g methanol (methanol to oil molar ratio of 3), and 0.25 g dried lipase. The reaction mixture was incubated at 35°C for 24 h at 220 rpm in a shaker incubator. Screening results are presented in Fig. 2. Lipase activity was presented as the rate of methyl oleate per kilogram of lipase (mol/h·kg$_{lipase}$).

Lipases were screened for their transesterification ability of triolein with short-chain alcohols to alkyl esters. Lipase AK was most efficient for converting triolein to methyl oleate with primary alcohols, whereas the Lipase AY was not efficient for transesterification of triolein with primary alcohols to give methyl oleate.

3.2. Characterization of microreactor

The main component of the microreactor was the microstructure of membrane pores made from polyethersulfone (PES). PES was chosen because of its asymmetric structure and excellent compatibility with enzymes. When enzyme solution was passed through a membrane, the enzyme adsorbed on to the sponge layer and the membrane pores. Adsorption is the most widely used method for enzyme immobilization because enzyme denaturation is avoided. Size matching between membrane pore size and molecular diameter of enzymes plays a key role in achieving high enzymatic stability [15]. A membrane with NMWL of 300 kDa was characterized by a pore size of 20 nm. Lipases with diameter d ≈ 3 nm (equal to 15 kDa) and a membrane

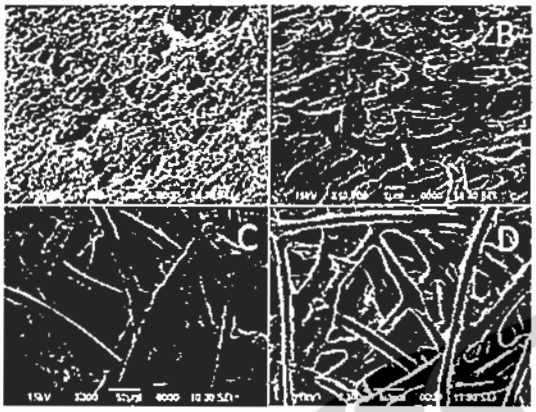

Fig. 3. SEM photographs of cross section of asymmetric membrane made of polyethersulphone with NMWL 300 kDa: (A) thin layer of initial membrane, (B) thin layer with immobilized lipase, (C) sponge layer of initial membrane, and (D) sponge layer with immobilized lipase.

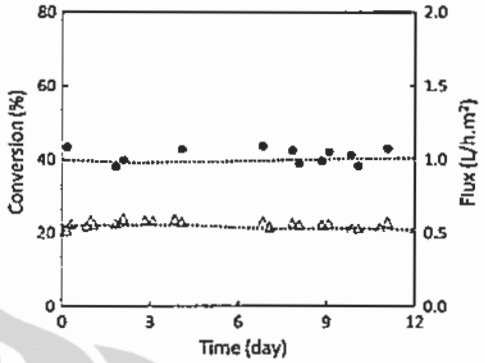

Fig. 4. Operational stability of the reactor during a 12 day transesterification reaction with enzyme loading of 0.81×10^{-3} kg/m^2 and residence time of 0.45 h. Conversion (●), permeate flux (△).

pore of diameter $d_p \approx 20$ nm (equal to 300 kDa) allow protein entry when $d < d_p$ and therefore deposit the enzyme on the pore walls, and thus increase enzyme loading [16]. The amount of protein attached in the microstructure membrane (enzyme loading) was determined by using equation (1) and a large amount of enzyme was bound to the microstructure of the membrane ($2.0 \sim 3.2\ 10^{-3}$ kg/m^2).

Fig. 3 shows SEM images of the new membrane and lipase-immobilized membrane exposed as a cross section. These images showed the morphological structure of the membrane with and without immobilized lipase. The change in morphological structure clearly showed that lipase was adsorbed on both the thin and sponge layer of the membrane. Thus, immobilization of lipase inside the membrane may permit stability of the enzyme in an operational application. However, this stabilization is not associated to the intrinsic stability of the enzyme.

3.3. Continuous transesterification and operational stability

A filtration cell was used as a flow-through reactor equipped with lipase-immobilized membrane as the microreactor. To study continuous conversion, experiments were carried out continuously over a 12 day period. The degree of triolein conversion (X_c) was determined using the following equation:

$$X_c(\%) = \left(1 - \frac{C_s}{C_i}\right) \times 100 \quad (3)$$

C_i is the initial quantity of triolein in the feed stream and C_s is the concentration of unconverted triolein in the product stream. Product permeability of the membrane microreactor was determined from flow rate per membrane area and expressed as flux, J_s in (L/m^2·h). The degree of triolein conversion and product permeate flux are shown in Fig. 4. The flux was constant at about 0.5 L/m^2·h, indicating that almost no glycerol was adsorbed onto the lipase during continuous operation for 12 days.

The yield of conversion was constant at about 40% and the transesterification of triolein into methyl oleate was observed with enzyme loading of 0.81×10^{-3} kg/m^2 in the flow-through microreactor in 27 min of residence time as defined by the equation (2). The steady state of the reactor at constant τ could be maintained continuously for 12 days. During the course of the experiments there was no loss of enzyme activity. In order to calculate half-life, experiments need to be carried out for a longer time. Nevertheless, the results show that the microreactor maintained good stability for at least 12 days.

In order to determine conversion speed, we calculated immobilized enzyme activity (P_c) as follows:

$$P_c\left(\frac{\text{mol}}{\text{h}\cdot\text{kg}_{\text{lipase}}}\right) = \frac{C_p \times J_s}{L_i} \quad (4)$$

C_p was the concentration of methyl oleate (mol/L), J_s was flux of the permeate solution (L/m^2·h) and L_i was lipase immobilized in the membrane (kg/m^2). In the batch reaction, lipase activity was defined as the initial formation rate of methyl oleate per kilogram of lipase (mol/h·kg$_{lipase}$) as shown in Fig. 2. The activity of immobilized lipase in the microreactor was based on unit mass of lipase (mol/h·kg$_{lipase}$) defined by equation (4), and was nearly 3 fold higher than that of native lipase as shown in Fig. 5. These results clearly show that confining lipase inside the membrane make the transesterification reaction efficient.

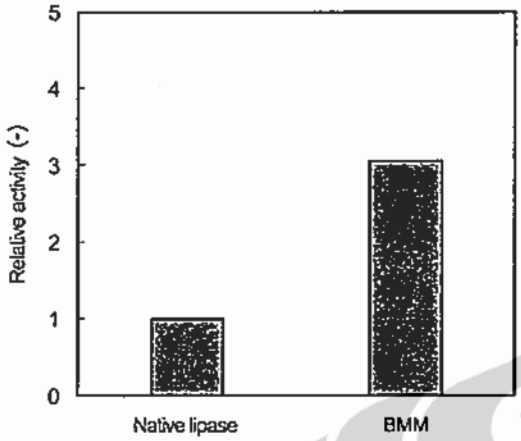

Fig. 5. The activity of immobilized lipase in microreactor (BMM) based on unit mass of lipase (mol/h·kg$_{lipase}$). The values were normalized by the corresponding value for native free lipase in a batch reaction.

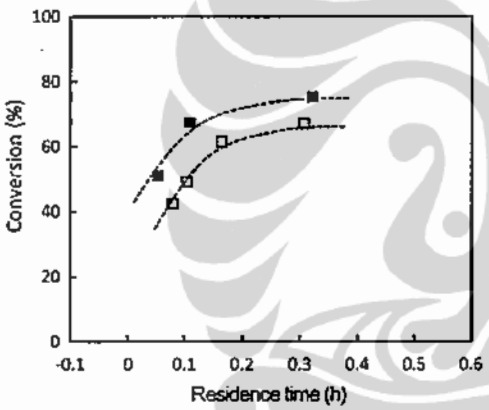

Fig. 6. Influence of residence time on the conversion of triolein. Enzyme loading of 2.4 × 10^{-1} kg/m^2 (■), 3.2 × 10^{-3} kg/m^2 (□).

3.4. Influence of residence time

Since transesterification was initiated by forcing the solution phases through the BMM, the conversion of triolein to methyl oleate occurred within the sponge and thin layer, during the permeation of the substrate solution through the membrane microreactor. The membrane microreactor was operated in flow-through mode in which residence time is an important parameter [17]. The reaction was carried out using 10 g triolein in 3 g methanol and the vacuum pump scale was between 1 and 3, corresponding to a residence time of 5 ~ 30 min. Residence times were plotted versus conversion as illustrated in Fig. 6. Also, the performance of the transesterification reaction with enzyme loading of 2.3 × 10^{-3} kg/m^2 was better than 3.2 × 10^{-3} kg/m^2.

Fig. 6 also demonstrates that at a higher residence time the conversion increased, and this effect was clear for 2.3 × 10^{-3} kg/m^2 and 3.2 × 10^{-3} kg/m^2 of immobilized enzyme. From these values, the corresponding residence time was calculated and maximum triolein conversion using these microreactors was approximately 80% with a reaction time of 19 min. The results clearly show that the reaction time of biocatalytic transesterification in the membrane microreactor system was faster than in a conventional system. In order to achieve product yield of ≥ 90% it was necessary to utilize a reaction time of greater than 40 h [18].

4. Conclusion

In this study, a novel biocatalytic membrane microreactor (BMM) was developed for the transesterification reaction. The membrane pores could be considered as microreactors, and the membrane acted as a microreactor with lipase inside rather than as a selective barrier. The conversion of triolein to methyl oleate occurred during permeation of the substrate solution through the biocatalytic membrane. Maximum triolein conversion was approximately 80% with a reaction time of 19 min. Most significantly, the BMM exhibited far superior activity than the native lipase. The activity of immobilized lipase in the BMM was based on unit mass of lipase (mol/h·kg$_{lipase}$) defined by equation (4), which was nearly 3 fold higher than that of native lipase. In a packed-bed reactor enzyme activity decreased since the glycerol remained adsorbed on the enzymatic support [19]. By using the BMM, the good stability was observed with no activity decay during a 12 day period of continuous operation. This microreactor system is suitable for triglyceride transesterification (commercial production of biodiesel), producing no waste stream.

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