Transesterification of waste vegetable oil by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5

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Transesterification of waste vegetable oil by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5

Abstract
In this thesis, the effect of organic solvent-cosolvent system, temperature, methanol concentration, water content and used enzyme washing method on transesterification of waste vegetable oil catalyzed by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5 was investigated. Four organic solvents, one co-solvent, three reaction temperatures, three methanol addition methods, four different water content and two used-enzyme washing methods were assessed. Optimal conditions were obtained with n-hexane as solvent, 5 vol.% tert-butanol as co-solvent, 25°C reaction temperature, batch addition of 3 equivalent molar methanol, water content equivalent to 7.5% by weight, and used-enzyme washing method B. It was also confirmed that the transesterification reaction catalyzed by used Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5 was limited by the concentration of acyl acceptor, and that active water loss was related to enzyme activity lost after every reaction cycle.

Keywords
Alternative Energy, Energy, Engineering, Chemical, Chemical Engineering
TRANSESTERIFICATION OF WASTE VEGETABLE OIL BY THERMOMYCES LANUGINOSUS LIPASE IMMOBILIZED ON ZEOLITE ZSM-5

BY

YE DENG

B.S. in Biochemical Engineering, Tianjin University, 2010

THESIS

Submitted to the University of New Hampshire

In Partial Fulfillment of

The Requirements for the Degree of

Master of Science

In

Chemical Engineering

September, 2012
This thesis has been examined and approved.

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Date
DEDICATION

I would like to dedicate this work to my parents.
ACKNOWLEDGEMENTS

First, I want to thank my advisor Prof. Vasudevan. I could not have completed this thesis without his great mentoring and support. He has been the source of knowledge throughout my Master's degree study. Thank you very much, Prof. Vasudevan.

Also I would like to thank Prof. Gupta and Prof. Li for serving on my thesis committee. I very appreciate their guidance and help in my academic program.

Eventually I want to thank my parents and my girlfriend. Their encouragements support me throughout my life. Also, Michael, Wenxin, Jingwen, Liang and Prof. Teng, I want to express my sincere thanks for their support these years.
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ABSTRACT

TRANSESTERIFICATION OF WASTE VEGETABLE OIL BY THERMOMYCES LANUGINOSUS LIPASE IMMOLIZED ON ZEOLITE ZSM-5

by

Ye Deng

University of New Hampshire, September 2012

In this thesis, the effect of organic solvent-cosolvent system, temperature, methanol concentration, water content and used enzyme washing method on transesterification of waste vegetable oil catalyzed by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5 was investigated. Four organic solvents, one co-solvent, three reaction temperatures, three methanol addition methods, four different water content and two used-enzyme washing methods were assessed. Optimal conditions were obtained with n-hexane as solvent, 5 vol.% tert-butanol as co-solvent, 25°C reaction temperature, batch addition of 3 equivalent molar methanol, water content equivalent to 7.5% by weight, and used-enzyme washing method B. It was also confirmed that the transesterification reaction catalyzed by used Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5 was limited by the concentration of acyl acceptor, and that active water loss was related to enzyme activity lost after every reaction cycle.
CHAPTER 1

INTRODUCTION

Energy is always an important issue in the world. For hundreds of years, fossil fuel has been the main source of energy in transportation. However, according to the Energy Information Administration (EIA), the consumption of petroleum products in 2011 was 83,760,391 barrels per day while the total amount of fossil oil reserves worldwide is 1,119 billion barrels to 1,317 billion barrels, and at this level, the fossil oil will be exhausted in 50 years. As a result, looking for alternative sources of energy has attracted more and more attention, and of these potential energy sources, biodiesel is one of the most promising one.

Biodiesel, defined by National Biodiesel Board (USA), is a vegetable oil- or animal fat-based diesel fuel, which consists of mono long-chain alkyl (methyl, propyl or ethyl) esters. Biodiesel can be applied in transportation as pure form (B100) or be blended with fossil diesel in the volume ratio of 5% (B5), 10% (B10) and 20% (B20), of which B20 is the current commercial biodiesel employed in the U.S. The technical standard of biodiesel is ASTM International D6751, which determines the main physical and chemical characteristics of biodiesel. All commercial biodiesel employed in the U.S. should meet ASTM International D6751.

Application of biodiesel has lots of advantages: i) biodiesel is more environmentally
friendly than fossil oil. Biodiesel has a large oxygen content of 12 wt%, which makes it easier to combust completely to produce less CO. And without sulfur and nitrogen molecule in biodiesel, there is no SO₂ or NOₓ produced in biodiesel combustion; ii) biodiesel has good energy efficiency. The calorific value of biodiesel is 37.27 MJ/kg, which is 9% lower than the common petroleum oil. However, the better lubricity and more complete combustion of biodiesel can enhance the engine output energy and make the actual energy density of biodiesel almost the same as petroleum oil; iii) biodiesel is easily produced and applied. Only one or two reactions are needed to produce biodiesel, which makes the synthesis process of biodiesel easy to design and modify. Besides, no extra modification to current vehicle engine is necessary in applying biodiesel since the characteristics of commercial biodiesel (B20) is very similar to No. 2 petroleum diesel; iv) biodiesel is a safe fuel. Biodiesel's higher boiling point (238°C in atmospheric pressure), higher flash point (130°C), non-toxicity and smaller density make biodiesel easy to store and transport compared to petroleum oil.

The production of biodiesel is mainly based on transesterification reaction, which is shown in Figure 1.1.

Figure 1.1 Transesterification of triglyceride with alcohol
Triglyceride is the main source of acyl-provider in the transesterification reaction, which can be derived from vegetable oil\(^4\) and animal fat\(^5\). In addition, free fatty acid (FFA), which is the hyrolysis product of triglyceride derived from waste cooking oil\(^6\), can also work as acyl-provider to produce biodiesel by esterification reactions as shown in **Figure 1.2**.

\[
\begin{align*}
R_1\text{COOH} & \quad \text{Catalyst} \quad R_2\text{OH} & \quad \text{EE} \quad R_1\text{-OOC-R}_2 & \quad H_2O \\
\text{FFA} & \quad \text{Alcohol} & \quad \text{Alkyl Ester} & \quad \text{Water}
\end{align*}
\]

**Figure 1.2** Esterification of FFA with alcohol

Methanol is currently the common acyl-acceptor used in industry because of the low cost. Transesterification reaction based on methanol is also called as methanolysis reaction, and biodiesel produced by methanolysis reaction is fatty acid methyl ester (FAME). Other alcohols and ketones such as ethanol, propanol, butanol and actone are also employed in academic research as potential acyl-acceptors because of their low toxicity and low polarity\(^4\).

The catalyst is the most important part in the production of biodiesel. Common catalysts applied in the industry are acid / base catalysts, such as H\(_2\)SO\(_4\)\(^9,10\), NaOH\(^11\) and KOH\(^12\). These catalysts are highly efficient and cheap\(^13\). However, there are many shortcomings in acid / base catalysis. For example, in homogeneous base catalyzed transesterification reactions, there is a significant saponification phenomenon, which reduces the yield of biodiesel and increases difficulty of the product separation process\(^14\). Besides, FFA and water produced by hydrolysis of
vegetable oil or animal fat can increase the saponification reaction rate to make things worse. Thus, in homogeneous base catalyzed reactions, the triglyceride substrate is extremely limited because there is large content of FFA in used cooking oil. Homogeneous acids can only catalyze the esterification reaction\(^\text{15}\). Thus, a substrate pre-treatment process to transfer triglyceride to FFA is necessary in homogeneous acid catalyzed reactions, which will increase the cost of biodiesel production. Heterogeneous catalysts suffer from a number of drawbacks as well. For example, the efficiency of heterogeneous base / acid catalysts is much lower than homogeneous catalysts\(^\text{15}\). Thus, higher temperatures (>150°C) are needed in heterogeneous acid / base catalyzed reactions to meet the industry requirement, which increases the total cost and the safety risk. Furthermore, heterogeneous catalysts have a low tolerance to water\(^\text{16}\), which means a dehydration process is also needed in heterogeneous acid / base catalyzed transesterification reactions.

One promising catalyst to produce biodiesel is the enzyme lipase. Lipase has high catalyst efficiency in both transesterification and esterification reactions, and unlike acid / base catalysts, there is no soap formed in lipase catalyzed reactions.\(^\text{17,19}\) The temperature required in lipase catalyzed reactions is low (20°C~60°C), and the amount of alcohol needed in lipase catalyzed transesterification reactions is much less\(^\text{17,18}\). Lipase is also re-usable, which means it can be collected and used after each reaction cycle despite some loss in activity. However, there are still two obstacles in lipase application in the industry. First, lipase is expensive. Lipase is mainly produced from microbial fermentation, which makes its price much higher than acid
or base catalysts. Second, storage and transportation of large amounts of lipase is still difficult. Lipase is a protein derived from bacteria or mold, and is biologically unstable. It loses activity at room temperature due to protein denaturation and/or contamination. Thus, low temperature (< 0°C) is required in the storage and transportation process, which also increases the cost of biodiesel and limits the application of lipase in the industry.

Lipase immobilization is one good method to overcome these obstacles. By enzyme immobilization technique, lipase can be linked to an insoluble support, which makes immobilized lipase easily to collect and reuse. Furthermore, Macario et al. have demonstrated that the stability and productivity of lipase is enhanced by immobilization. Immobilization techniques can be divided into physical methods and chemical methods. Physical immobilization methods include adsorption, entrapment and encapsulation, while chemical immobilization method is linking the enzyme to an inert support by covalent bonding. Both immobilization methods require supports. A good support can also provide a heterogeneous micro-environment to benefit reactions. In addition, a support with nanostructure has an extremely large surface area to achieve good enzyme-support attachment and can be well dispersed in the reaction system to make a good catalytic interface.

Zeolite ZSM-5 is a novel pentasil-family alumino-silicate zeolite with unique channel shape. The chemical form of ZSM-5 is NaₙAlₙSi₉₆₋ₙO₁₉₂·16H₂O in which 'n' varies from 0 to 27, and these molecules form several pentasil chains by oxygen
bridges. The pentasil chains are also interconnected by oxygen bridges to form 10-ring channels. In these rings, Al or Si molecule form the vertices, which are bonded by oxygen molecule (See Figure 1.3). The pore size of ZSM-5 is estimated to be 5.4–5.6 Å.

[Image]

Figure 1.3 The pentasil-ring structure of ZSM-5

Zeolite ZSM-5 is a good immobilization support for catalysts due to its nano-scaled channel and its high silicon to aluminum ratio. Furthermore, control experiments with waste vegetable oil in our laboratory have conclusively shown that zeolite ZSM-5 itself has no catalyst activity in the transesterification reaction. Aldehyde functional group is the common group used in lipase immobilization and it can be attached to the zeolite ZSM-5 by a Si-NH covalent bonding. When modified with aldehyde functional group, zeolite ZSM-5 can be easily linked to lipase to form a stable zeolite-lipase immobilized enzyme. There is very limited work on enzymatically catalyzed transesterification reactions using ZSM-5 as support.

This thesis systematically investigates the characteristics of Thermomyces lanuginosus lipase, a common lipase used in transesterification reactions,
immobilized on zeolite ZSM-5. In this study, four organic solvents (n-pentane, n-hexane, n-heptane and iso-octane), with and without co-solvent (5 vol.% tert-butanol), three system temperatures (25°C, 30°C, and 40°C), three methanol addition methods (stepwise, 3:1 batch and 6:1 batch), two used enzyme washing methods (A and B - which are defined later in the thesis) and four different water content (2.5%, 5%, 7.5% and 10% by weight of enzyme) were investigated in the synthesis of biodiesel. The re-use efficiency of Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5 in the methanolysis of waste vegetable oil was investigated to gain a comprehensive understanding of the reaction and to ascertain the optimal conditions that achieve the best lipase performance.

Based on these results, the best reaction condition with specific solvent-cosolvent system, reaction temperature and methanol addition method is recommended in order to obtain the highest biodiesel production and enzyme re-use efficiency. The limiting factors related to the performance of Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5 in the transesterification reaction are also examined.

The organization of this thesis is as follows: Chapter 2 provides some Background on Transesterification Reactions; Chapter 3 deals with the Experimental Method; Chapter 4 discusses the Experimental Results; and Chapter 5 contains Conclusions and Recommendations for future work.
CHAPTER 2

BACKGROUND

2.1 Introduction

The background information in this chapter can be divided into five parts:

i) characteristics of the substrates; ii) kinetics of biodiesel synthesized transesterification reaction; iii) solvent-cosolvent system effect on the transesterification reaction; iv) temperature and mixing effect on the biodiesel synthesized transesterification reaction; v) effect of active water content on biodiesel synthesized reaction.

2.2 Characteristics of Substrates

2.2.1 Triglyceride

The most common triglyceride used in biodiesel industry is plant oil or vegetable oil, such as soybean oil, canola oil, sunflower oil, palm oil, olive oil, cottonseed oil and jatropha oil\(^{27-30}\). FAME yields produced from these edible or inedible oils can be higher than 0.90 g/g oil. Animal fats, such as animal tallow, grease, fish oil and lard,\(^ {31-33}\) are also a good source of triglyceride. FAME yield produced from animal fat can also be as high as 0.8 g/g oil.
However, the high cost of these edible or inedible oils severely limits their application in biodiesel production. In addition, there is a lot of pressure on the agriculture industry, especially in third world countries, not to produce edible oil for biodiesel synthesis. Both these factors serve as an incentive to find alternative cheap sources of triglyceride. Waste vegetable oil or used oil is a good source for a number of reasons. There is a large amount of waste oil produced everyday from factories and restaurants, and the use of waste oil in the development of catalysts provides a good indication of their robustness. The triglyceride and FFA content in waste oils is high, and according to the research of Lara and Park, the yield of methyl ester produced from waste oil can be as high as 0.96 g/g oil. In the U.S., more than 11 billion liters of waste oil is produced per year, and 1% of petroleum oil consumption would be offset if all waste oil could be converted to biodiesel. This number, though small represents significant progress in our efforts to wean ourselves from dependence on fossil fuels. This number is still increasing with the development of more fast food restaurants and the increase in petroleum price. The only problem is the high content of water and FFA in waste oils. The production of soap when a base catalyst is used can be solved by the application of lipase or through a pretreatment process.

2.2.2 Acyl-acceptor

The source of acyl-acceptor is also an important issue in biodiesel synthesis even though methanol is widely used in industry. The acyl-acceptor can be an alcohol or
an acetate. Some examples are methanol, ethanol, propanol, butanol and methyl acetate\textsuperscript{13,34}. The most common acyl-acceptor is methanol because of its low cost. However, there are many shortcomings in the use of methanol in biodiesel synthesis: i) there is the mass transfer issue between the hydrophilic methanol and the hydrophobic triglyceride; ii) the high polarity of methanol can strip water from enzyme and deactivate the enzyme; iii) high toxicity and high volatility of methanol poses a risk on the health of workers and environment; iv) the main source of methanol is fossil oil, which can be a big problem if the goal is to reduce the consumption of fossil oil.

2.3 Kinetics of Biodiesel Synthesis

From the transesterification reaction (shown in Figure 1.1), one mole of triglyceride reacts with three moles of alcohol to produce three moles of alkyl ester (biodiesel) and one mole of byproduct glycerol. And if the alcohol is methanol as is common, the product will be methyl ester.

Tan, Nie and Wang have shown that the mechanism of the transesterification reaction catalyzed by enzyme has three reversible steps\textsuperscript{13,35}: i) one mole of triglyceride reacts with one mole of methanol to produce one mole of methyl ester and one mole of diglyceride; ii) one mole of diglyceride reacts with one mole of methanol to produce one mole of methyl ester and one mole of monoglyceride; iii) one mole of
monoglyceride reacts with one mole of methanol to produce one mole of methyl ester and one mole of glycerol, as shown in Figure 2.1.

\[
\begin{align*}
\text{Triglyceride} & \quad + \quad \text{Methanol} & \quad \xleftrightarrow{\text{Catalyst}} & \quad \text{Methyl Ester} \\
\text{Diglyceride} & \quad + \quad \text{Methanol} & \quad \xleftrightarrow{\text{Catalyst}} & \quad \text{Methyl Ester} \\
\text{Monoglyceride} & \quad + \quad \text{Methanol} & \quad \xleftrightarrow{\text{Catalyst}} & \quad \text{Glycerol}
\end{align*}
\]

\text{Figure 2.1 Three reversible step mechanism in transesterification reaction}

For these reversible reactions, a high concentration of methanol can result in high conversion of triglyceride to give a high yield of biodiesel. However, due to its high polarity, high concentration of methanol is toxic to the enzyme and will inhibit the enzyme's re-use efficiency. One good solution is the stepwise methanol addition method of Nie et al.\textsuperscript{17}, which means adding methanol stepwise. Their results have shown that 3-times stepwise addition is the most optimal, and that more than 1.5 molar equivalent methanol added every time will deactivate the enzyme. Another solution is the application of other acyl-acceptors with lower polarity than methanol,
such as ethanol, tert-butanol and methyl acetate. Of these acyl-acceptors, ethanol is the most promising one because it can be produced from biomass via fermentation. Besides solvent source, solvents with a higher hydrophobicity and energy content than methanol also are beneficial and the application of ethanol compared to methanol falls in this category\textsuperscript{27,36}. However, the shortcoming of ethanol is also evident, which is the higher cost and higher viscosity.

Actually, application of organic solvent-cosolvent system is also a good solution to the methanol's high polarity problem, which will be illustrated in the next section.

2.4 Effect of Solvent-Cosolvent System

From what is illustrated in sections 2.2 and 2.3, there are two main problems in the transesterification reaction: i) the mass-transfer issue between hydrophobic triglyceride and hydrophilic alcohol; ii) the deactivation of lipase by alcohol with high polarity, such as methanol. A selected organic solvent-cosolvent system added to the reaction system can solve both the problems in the following way, i) a selected organic hydrophobic solvent and hydrophilic co-solvent system can increase the solubility of both the triglyceride and the methanol and overcome the mass-transfer problem raised by the highly viscous triglyceride; ii) the high solubility of methanol in the solvent-cosolvent system as well as the optimal polarity of co-solvent can reduce the concentration of methanol surrounding the lipase. Furthermore, the
addition of a hydrophobic solvent can create a hydrophobic microenvironment around the lipase, which can protect the active site of lipase and increase the concentration of triglyceride surrounding the lipase. Thus, a properly selected solvent-cosolvent system is a good method to improve the final biodiesel yield and enzyme re-use performance.

The selection of solvent is based on the solvent's density, viscosity and polarity. In general, a solvent with low viscosity, low polarity and high density is good for the transesterification reaction. However, according to the study of Soumanou and Bornscheuer, different solvents have different effects on lipases from different sources, which means the optimal solvent for a particular lipase is not constant. Tan et al. tested the activity of Candida sp. 99-125 lipase in different hydrophobic and hydrophilic solvents, and they reported that the lipase presented as a suspension in a hydrophobic solvent showed higher activity. Royon et al. reported that the use of hydrophilic solvents such as tert-butanol and tert-pentanol could result in high biodiesel yields due to their miscibility with methanol by reducing the concentration of methanol surrounding the enzyme.

To fully use the hydrophobic solvent’s “concentrating triglyceride” capability, and the hydrophilic solvent's “diluting methanol” capability, Su and Wei proposed a hydrophobic solvent and hydrophilic co-solvent mixture system method. In their study, they designed a solvent and co-solvent mixture system, which contained 75
vol.% iso-octane and 25 vol.% tert-butanol and the final biodiesel yield reached a maximum of 0.6 g/g oil.

2.5 Effect of Temperature and Mixing

In the transesterification reaction catalyzed by lipase, the reaction temperature usually varies from 20°C to 65°C. Other than the effect on the activity of lipase, temperature also has an effect on solvent viscosity and volatility. With the increase of reaction temperature, the viscosity of solvent will decrease and the mass transfer coefficient between triglyceride and methanol will be enhanced. However, the increase of temperature will also increase the cost of production of biodiesel. Furthermore, previous studies proved that due to the low heat of reaction (-18.5 kJ/mol FAME at 25°C), the conversion of biodiesel in transesterification reaction wouldn’t change much with change in temperature.

Mixing rate is also a factor that affects the biodiesel synthesis. In batch reaction, the parameter related to mixing rate is stirring speed. Increase of stirring speed will effectively increase the mass transfer coefficient between triglyceride and methanol to benefit the reaction. However, lipase can be deactivated by the shear stress caused by high stirring speed. The common stirring speed employed in research is in the range of 50-250 rpm. However, Shen and Vasudevan reported that there was no significant difference in biodiesel yield with stirring speed in the range 150 rpm to
400 rpm\textsuperscript{18}, and 150 rpm was recommended to diminish possible lipase deactivation.

2.6 Effect of Active Water Content

In addition to solvent-cosolvent systems, source of substrate, temperature and mixing rate, active water content of lipase is also an important factor related to biodiesel production and enzyme re-use efficiency.

Lipase is usually a hydrophilic protein and there is a necessary amount of water surrounding the active site of lipase, namely active water, to protect the activity of lipase and provide necessary microenvironment for hydrogen bonding inside the protein. In transesterification reactions catalyzed by enzyme, addition of water can also form a water-oil interface to enhance the activity of enzyme and protect the enzyme from deactivation by methanol\textsuperscript{17}. However, large amount of added water can also increase the saponification reaction rate to reduce biodiesel conversion. Thus, there is an optimal water addition amount in transesterification reactions to maximize the biodiesel yield.

Water content is the common parameter to measure the amount of added water, which can be based on solvent, substrate and amount of enzyme by weight or volume\textsuperscript{3,39}. For the transesterification reaction catalyzed by lipase, the optimal water content based on lipase from different sources and different solvents is different\textsuperscript{39}. 
Al-Zuhair et al.\textsuperscript{41} reported that for lipase from \textit{Mucor miehei}, maximum biodiesel yield emerged at water content less than 10\% (based on volume of solvent). Chen et al.\textsuperscript{42} studied the methanolysis of waste cooking oil catalyzed by \textit{Rhizopus oryzae} lipase and reported that the optimal water content is 50\% based on weight of substrate.
CHAPTER 3

EXPERIMENTAL METHODS

3.1 Introduction

In this thesis, the effects of four parameters (solvent-cosolvent system, temperature, methanol molar ratio and active water content) and two used enzyme washing methods on the performance of *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5 in the transesterification of waste vegetable oil were investigated. Specially, the experiments steps consisted of the following:

a) Immobilization of *Thermomyces lanuginosus* lipase on zeolite ZSM-5 by different techniques;

b) Effect of organic solvent-cosolvent system on the transesterification reaction of waste vegetable oil catalyzed by *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5. The four organic solvents were *n*-pentane, *n*-hexane, *n*-heptane and *iso*-octane and the co-solvent was 5 vol.% *tert*-butanol;

c) Effect of operation temperature (25°C, 30°C, and 40°C) on the transesterification reaction of waste vegetable oil catalyzed by *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5;

d) Effect of methanol molar ratio and addition method (stepwise, 3:1 batch and 6:1 batch) on the transesterification reaction of waste vegetable oil catalyzed by *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5;
e) Effect of active water content (2.5%, 5%, 7.5% and 10% by weight of enzyme) of enzyme on the transesterification reaction of waste vegetable oil catalyzed by *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5;

f) Comparison of two washing methods (A and B – which are defined later) of used *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5.

3.2 Materials

Zeolite ZSM-5 was purchased from Zeolyst International, Inc., Conshohocken, PA, USA. 4-Trimethylsiloxybenzaldehyde was purchased from Gelest, Morrisville, PA, USA. Used canola oil was purchased from ConAgra Food, Inc., Omaha, NE, USA. Lipase from *Thermomyces lanuginosus* (solution) with approximate activity of 100,000 U/ml (1 U is defined as the amount of lipase that catalyzes the conversion of 1 micro mole of triglyceride per minute) and glyceryl trioleate (practical grade, approximate 65%) were all purchased from Sigma-Aldrich, St. Louis, MO, USA. Pure tert-butanol and methyl acetate were purchased from Acros Organics, Geel, Belgium. HPLC grade n-hexane, n-heptane, n-pentane and iso-octane were purchased from the Fisher Scientific, Pittsburgh, PA.

3.3 Immobilization of *Thermomyces Lanuginosus* Lipase on Zeolite ZSM-5

Zeolite ZSM-5 modified with aldehyde functional group was obtained from Dr. Gonghu Li’s lab. 40 ml glass vials with PTFE/silicone septa from Kimble, Vinland,
NJ, USA were used as reactors to immobilize *Thermomyces lanuginosus* lipase on modified zeolite ZSM-5. The reaction system contained 5 ml lipase solution (about 500,000 U lipase) and 3 g zeolite modified with aldehyde functional group. The vial was put on the refrigerator at −20°C. After 24 hours, the product was filtered on a filter paper (90 mm diameter) from Whatman Inc., Kent, UK for 30 minutes and then dried in the vacuum desiccator for 48 hours.

3.4 Effect of Various Parameters on *Thermomyces Lanuginosus* Lipase Immobilized on Zeolite ZSM-5 in Catalyzing Transesterification Reactions

Even though the experimental methods and results are presented in the following order, please note that the experiments were not performed in the same chronological order.

3.4.1 Study of Solvent Effect on Performance of *Thermomyces Lanuginosus* Lipase Immobilized on Zeolite ZSM-5

40 ml glass vials with PTFE/silicone septa from Kimble, Vinland, NJ, USA were used as reactors to produce biodiesel. The reaction system contained 0.2 g immobilized *Thermomyces lanuginosus* lipase, 4 ml solvent (n-pentane, n-hexane, n-heptane and iso-octane), 200 μl tert-butanol, and 1 ml triglyceride (65 vol.%). To minimize the denaturation of lipase caused by methanol, exactly 27 μl methanol (molar ratio of methanol to triglyceride = 1:1) was added stepwise to the reactor at 0 hour, 4 hour and 8 hour, respectively. Hence the total molar ratio of methanol to
triolein was kept at 3:1 (81 μl methanol). The reaction was carried out at a constant temperature water bath at 30°C and a stirring speed of 250 rpm. The reaction was stopped after 12 hours. After the completion of each cycle, the enzyme was washed by washing method B (see chapter 3.5) and re-used. Two cycles were employed with each solvent.

3.4.2 Study of Co-Solvent Effect on Performance of Thermomyces Lanuginosus Lipase Immobilized on Zeolite ZSM-5

40 ml glass vials with PTFE/silicone septa from Kimble, Vinland, NJ, USA were used as reactors to produce biodiesel. The reaction system contained 0.2 g immobilized Thermomyces lanuginosus lipase, 4 ml iso-octane as solvent, 1 ml triglyceride (65 vol.%) and 81 μl methanol. To test the effect of co-solvent, one set contained 200 μl of tert-butanol as co-solvent, while the comparison set contained 200 μl iso-octane. The reaction was carried out in a constant temperature water bath at 30°C and a stirring speed of 250 rpm. The reaction was stopped after 12 hours. After each cycle, the enzyme was washed by washing method B (see chapter 3.5) and re-used. Four cycles were employed with each set.

3.4.3 Study of Temperature Effect on Performance of Enzyme Immobilized on Zeolite ZSM-5

40 ml glass vials with PTFE/silicone septa from Kimble, Vinland, NJ, USA were
used as reactors to produce biodiesel. The reaction system contained 0.2 g immobilized *Thermomyces lanuginosus* lipase, 4 ml *iso*-octane as solvent, 200 μl *tert*-butanol as co-solvent, and 1 ml triglyceride (65 vol.%). Twenty seven μl methanol (molar ratio of methanol to triglyceride = 1:1) were added stepwise to the reaction system at 0, 4 and 8 h, and the total amount was 81 μl. The temperature of reaction was set at 25°C, 30°C and 40°C. The reaction was carried out at a constant stirring speed of 250 rpm. The reaction was stopped after 12 hours. After each cycle, the enzyme was washed by washing method B (see chapter 3.5) and re-used. Two cycles were employed at each temperature.

3.4.4 Effect of Methanol Molar Ratio on Performance of Immobilized Enzyme

40 ml glass vials with PTFE/silicone septa from Kimble, Vinland, NJ, USA were used as reactors to produce biodiesel. The reaction system contained 0.2 g immobilized *Thermomyces lanuginosus* lipase, 4 ml *iso*-octane as solvent, 200 μl *tert*-butanol as co-solvent, and 1 ml triglyceride (65 vol.%). Methanol was added in the following three ways: a) 27 μl methanol (molar ratio of methanol to triolein = 1:1) was added stepwise to the reactor at 0, 4 and 8 h, respectively, and the total molar ratio of methanol to triolein was kept at 3:1; b) 81 μl methanol (molar ratio of methanol to triolein = 3:1) was added at 0 h; c) 162 μl methanol (molar ratio of methanol to triolein = 6:1) was added at 0 h. The reaction was carried out at a constant temperature water bath at 40°C and a stirring speed of 250 rpm. The reaction was stopped after 12 h. After each cycle, the enzyme was washed by
washing method B (see chapter 3.5) and re-used. Two cycles were employed with each set.

3.4.5 Effect of Active Water Content on Immobilized Enzyme Performance

Prior to the experiment, immobilized Thermomyces lanuginosus lipase was divided into five vials, each with 0.2 g. Every vial was dried in a vacuum desiccator for 24 h, and then 0, 5, 10, 15 or 20 μl deionized water (water content 0, 2.5%, 5%, 7.5% and 10% by weight of enzyme) was added. 40 ml glass vials with PTFE/silicone septa from Kimble, Vinland, NJ, USA were used as reactors to produce biodiesel. Every vial contained 0.2 g enzyme, 4 ml iso-octane as solvent, 200 μl tert-butanol as co-solvent, 81 μl methanol and 1 ml triglyceride (65 vol.%). The reaction was carried out in a constant temperature water bath at 30°C and a stirring speed of 250 rpm. The reaction was stopped after 12 h.

3.4.6 Study of Active Water Effect on Performance of Enzyme Immobilized on Zeolite ZSM-5

Prior to the experiment, 0.2 g immobilized Thermomyces lanuginosus lipase was dried in vacuum desiccator for 48 h, and then 15 μl deionized water (7.5% by weight of enzyme) was added. 40 ml glass vials with PTFE/silicone septa from Kimble, Vinland, NJ, USA were used as reactors to produce biodiesel. The reaction system contained 0.2 g enzyme, 4 ml iso-octane as solvent, 200 μl tert-butanol as co-solvent, 81 μl methanol and 1 ml glyceryl trioleate. The reaction was carried out in a constant
temperature water bath at 30°C and a stirring speed of 250 rpm. The reaction was stopped after 12 h. After each cycle, the enzyme was washed by washing method B (see chapter 3.5) and re-used. Four cycles were employed with each set.

3.5 Comparison of Two Used Enzyme Washing Methods

After the transesterification reaction, two methods to wash the used immobilized *Thermomyces lanuginosus* lipase were employed: in the first method A) all liquid was decanted from the reaction vial by pipette and 4 ml iso-octane was added to the vial to immerse the used enzyme for 15 minutes. This procedure was repeated four times. Finally, all liquid was removed by pipette and the washed used enzyme was air dried for 10 h; in the second method B), the liquid was decanted from the reaction vial by pipette and 4 ml iso-octane was added to the vial to immerse the used immobilized enzyme. After 15 minutes, all liquid was removed by pipette and 4 ml tert-butanol was added to the vial. The vial was kept at 30°C and stirred at 250 rpm for 15 minutes. Finally, all liquid was removed by pipette and 1 ml iso-octane was added to the vial. Then the vial was kept at 30°C, 250 rpm for 45 minutes. After 45 minutes, all liquid was removed by pipette and the washed used enzyme was air dried for 10 h.

3.6 Analysis Method

The concentration of methyl ester was measured by a HP 5890 gas chromatography
with a Restek RTX-1 column (15 m × 0.32 mm × 3 μm). Helium with a purity of 99.99% was chosen as the carrier gas. The column was initially set at a temperature of 185°C and ramped up to 200°C in 1.5 minutes and then maintained at 200°C. The temperature of injector and flame ionization detector were set as 275°C. Methyl oleate was employed as biodiesel standard in GC analysis.

The GC analysis indicates that the peak of methyl ester appears around 18 minutes (see Figure 3.1). The calculation of methyl ester amount is based on the peak area of methyl ester and the corresponding standard curve. The yield of methyl ester was determined as the mass of methyl ester produced per initial mass of oil (g methyl ester/ g oil).

![Figure 3.1 peak of methyl ester tested by GC machine](image)

In this study, methanol is the acyl-acceptor, which means the biodiesel essentially consists of mono fatty acid methyl ester (MFAE) (shown in Figure 1.1). Thus, the peaks of biodiesel tested by GC are similar to Figure 3.1 which was calculated by the calibration method outlined in Appendix A.
To determine the appropriate time of sampling, 30μl samples were collected at 0, 3, 6, 12 and 24 h, and then diluted with 1 ml iso-octane for GC analysis. A plot of the yield (calculated from peak area) versus time as shown in Figure 3.2 revealed that the optimum time for sample collection is 12 h.

![Figure 3.2 Determination of optimum sampling time](image.png)

In this thesis, samples were collected at 12 h, and then diluted with 1 ml iso-octane prior to GC analysis. Every experiment was repeated twice. Thereby, each data point shown in tables in the results section is the mean value.
CHAPTER 4

EXPERIMENT RESULTS

4.1 Introduction

In this chapter, results are discussed in five parts:

i) Effect of organic solvents (n-pentane, n-hexane, n-heptane and iso-octane) and co-solvent (tert-butanol) was evaluated as possible media for transesterification of used cooking oil catalyzed by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5.

ii) Effect of temperature (25°C, 30°C, and 40°C) was evaluated to determine optimum system temperature for transesterification of used cooking oil catalyzed by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5.

iii) Effect of methanol addition method (stepwise, 3:1 batch and 6:1 batch) was evaluated for transesterification of used cooking oil catalyzed by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5.

iv) Effects of active water content of enzyme on the transesterification of used cooking oil catalyzed by Thermomyces lanuginosus lipase immobilized on
zeolite ZSM-5 was investigated.

v) Effect of used enzyme washing method on the transesterification of used cooking oil catalyzed by Thermomyces lanuginosus lipase immobilized on zeolite ZSM-5 was investigated.

4.2 Effect of Organic Solvent-Cosolvent system

Four organic solvents were employed in this study (n-pentane, n-hexane, n-heptane and iso-octane), and the density, viscosity, biodiesel yield after the first and second runs as well as the enzyme re-use efficiency are shown in Table 4.1. The enzyme re-use efficiency for different solvents are also shown in Figure 4.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Density$^a$</th>
<th>Viscosity$^b$</th>
<th>1$^{st}$ Yield$^c$</th>
<th>2$^{nd}$ Yield</th>
<th>Re-use Efficiency$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n-pentane</td>
<td>0.626</td>
<td>240</td>
<td>0.47±0.02</td>
<td>0.40±0.02</td>
<td>0.84±0.02</td>
</tr>
<tr>
<td>n-hexane</td>
<td>0.655</td>
<td>294</td>
<td>0.47±0.02</td>
<td>0.41±0.02</td>
<td>0.86±0.02</td>
</tr>
<tr>
<td>n-heptane</td>
<td>0.680</td>
<td>386</td>
<td>0.51±0.02</td>
<td>0.32±0.02</td>
<td>0.64±0.02</td>
</tr>
<tr>
<td>iso-octane</td>
<td>0.692</td>
<td>469</td>
<td>0.49±0.02</td>
<td>0.31±0.02</td>
<td>0.65±0.02</td>
</tr>
</tbody>
</table>

$^a$Density is tested at 25°C, and the unit is g/ml.
$^b$Viscosity is tested at 20°C, and the unit is μPa*s.
$^c$The 1$^{st}$ and 2$^{nd}$ yield is tested at 12 h and the unit is g FAME/g triglyceride.
$^d$The re-use efficiency is calculated as the ratio of 2$^{nd}$ yield/ 1$^{st}$ yield.
From Table 4.1, it can be observed that there was no significant difference in first run yield among the different solvents, while the re-use efficiency changed significantly between n-pentane/n-hexane group and n-heptane/iso-octane group. And Figure 4.1 clearly shows that the highest re-use efficiency was obtained with n-hexane as the solvent. Thus, n-hexane is recommended as the best organic solvent for transesterification reaction catalyzed by *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5.

The probable reason is that n-hexane provides the best suspension conditions for *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5. From Table 4.1, it can be observed that the density and viscosity of these alkane solvents increase with increase in carbon number. n-Hexane has a density similar to the immobilized enzyme and a relatively low viscosity provides the best suspension conditions for *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5. Please note that the experiments were not performed in the same chronological order as reported in
the results section.

The effect of hydrophilic co-solvent was also tested in this study. The result of final yield in consecutive batch reactions with re-used enzyme with or without co-solvent adding is shown in Table 4.2 and Figure 4.2.

Table 4.2 Consecutive batch reactions with re-used enzyme with and without addition of co-solvent

<table>
<thead>
<tr>
<th>Name</th>
<th>1st Yield</th>
<th>2nd Yield</th>
<th>3rd Yield</th>
<th>4th Yield</th>
<th>Average Re-use Efficiency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>tert-butanol</td>
<td>0.52±0.02</td>
<td>0.44±0.02</td>
<td>0.36±0.02</td>
<td>0.28±0.02</td>
<td>0.78±0.01</td>
</tr>
<tr>
<td>No Co-Solvent</td>
<td>0.54±0.02</td>
<td>0.29±0.02</td>
<td>0.12±0.02</td>
<td>0.06±0.02</td>
<td>0.49±0.01</td>
</tr>
</tbody>
</table>

*The average re-use efficiency is calculated by \( \frac{2\text{nd yield}}{1\text{st yield}} + \frac{3\text{rd yield}}{2\text{nd yield}} + \frac{4\text{th yield}}{3\text{rd yield}} \)/3.

From Table 4.2 and Figure 4.2, it is observed that addition of tert-butanol as co-solvent did enhance the performance of Thermomyces lanuginosus lipase.
immobilized on zeolite ZSM-5 in recycle use. The plausible reason is that methanol is miscible with the hydrophilic tert-butanol, and addition of a small amount of tert-butanol can dilute the methanol and control the diffusion rate of methanol to the active site of enzyme.

4.3 Effect of Temperature

As discussed in Chapter 2, temperature has a significant effect on enzyme activity and reaction system mass transfer. In this study, three temperatures were evaluated (25°C, 30°C, and 40°C) and the results are shown in Table 4.3 and Figure 4.3.

<table>
<thead>
<tr>
<th>Name</th>
<th>1st Yield</th>
<th>2nd Yield</th>
<th>Re-use Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>25°C (Room Temperature)</td>
<td>0.57±0.02</td>
<td>0.29±0.02</td>
<td>0.51±0.02</td>
</tr>
<tr>
<td>30°C</td>
<td>0.49±0.02</td>
<td>0.26±0.02</td>
<td>0.53±0.02</td>
</tr>
<tr>
<td>40°C</td>
<td>0.44±0.02</td>
<td>0.09±0.02</td>
<td>0.21±0.02</td>
</tr>
</tbody>
</table>

*The reaction conditions are: 0.2 g immobilized enzyme, 400 ml iso-octane, 200 μl tert-butanol, 1 ml triglyceride (65 vol.% pure), stepwise addition of 3 equivalent mole methanol (81 μl total), 250 rpm. Reaction time is 12 h.*
The results clearly showed that reaction at 25°C and 30°C resulted in good re-use efficiency. Since the difference in yields is quite small, a temperature of 25°C is recommended, and this is likely to result in energy savings as well.

### 4.4 Effect of Methanol Addition Method

The concentration of methanol in the reaction medium has a significant effect on biodiesel synthesis, because it is an important substrate in the transesterification reaction and its high polarity has a deleterious effect on lipase activity. In this study, three methanol addition methods were employed: i) 1 equivalent mole methanol was added stepwise at 0, 4 and 8 h, and the total content was 3 equivalent molar; ii) 3 equivalent moles of methanol were added at 0 h; iii) 6 equivalent moles of methanol were added at 0 h. The results are shown in Table 4.4 and Figure 4.4.
Table 4.4 Results of different methanol addition methods

<table>
<thead>
<tr>
<th>Name</th>
<th>1st Yield</th>
<th>2nd Yield</th>
<th>Re-use Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stepwise 3 equivalent mole</td>
<td>0.48±0.02</td>
<td>0.31±0.02</td>
<td>0.64±0.02</td>
</tr>
<tr>
<td>Batch 3 equivalent mole</td>
<td>0.52±0.02</td>
<td>0.44±0.02</td>
<td>0.85±0.02</td>
</tr>
<tr>
<td>Batch 6 equivalent mole</td>
<td>0.12±0.02</td>
<td>0.03±0.02</td>
<td>0.27±0.02</td>
</tr>
</tbody>
</table>

*In stepwise addition method, we assumed the methanol is consumed up in 4 h, thus the molar ratio of methanol is considered to be 1. Reaction conditions: 0.2 g immobilized enzyme, 400 ml iso-octane, 200 μl tert-butanol, 1 ml triglyceride (65 vol.% pure), 25°C and 250 rpm. The reaction time is 12 h.

Figure 4.4 Results of different methanol addition methods

The results showed that batch addition of 3 equivalent moles of methanol resulted in the best results. The difference between this result and what is reported in the literature is probably due to the addition of a co-solvent and the re-establishment of an active water layer. Addition of hydrophilic co-solvent can control the diffusion rate of methanol to the active site by diluting the methanol, and re-establishment of the active water layer near the enzyme's active site before each cycle can offset the
damage of high concentration of methanol. However, if the concentration of methanol is too high, not only the active water but also the enzyme itself will be affected, which will result in a low biodiesel yield as well as low re-use efficiency. Thus, the maintenance of an active water layer certainly enhances the performance of the catalyst and allows use of a higher concentration of methanol, which in turn results in a higher biodiesel yield. In our case, the 3:1 batch addition resulted in higher yields compared to stepwise addition. The addition of tert-butanol also to some extent prevents the active water layer from being stripped.

4.5 Effect of Active Water Content

Active water content is an important factor in enzyme activity. From Chapter 3, we know that one step in enzyme immobilization is drying the enzyme in a vacuum desiccator, which may strip some active water from the enzyme. Furthermore, the acyl-acceptor methanol, and washing step in enzyme re-use process will also strip the active water. Thus, addition of optimal amount of deionized water is a good method to enhance enzyme’s activity.

4.5.1 Optimal Content of Deionized Water Addition

After testing, it was ascertained that there was almost no activity of enzyme after 24 hours treatment in a vacuum desiccator, and the enzyme was assumed to be totally dry at this stage. In this study, different amounts of deionized water (0 µl, 5 µl, 10 µl, 15 µl and 20 µl) were added to totally dried enzyme. The biodiesel yield for each
case is shown in Figure 4.5.

![Figure 4.5](image)

**Figure 4.5** Biodiesel yield catalyzed by enzyme with different amount of active water. Reaction condition: 0.2 g immobilized enzyme, 400 ml iso-octane, 200 μl tert-butanol, 1 ml triglyceride (65 vol.% pure), 3 equivalent mole methanol, 40°C and 250 rpm. Reaction time is 12 h.

From the results, we observe that with 0.2 g enzyme, 15 μl deionized water addition (7.5% by weight of enzyme) result in the highest biodiesel yield and the yield decrease with additional water addition.

4.5.2 Effect of Deionized Water Addition Method on Enzyme Re-use

In this thesis, the effect of deionized water addition method on performance of re-used enzyme was investigated. The results are shown in Table 4.5 and Figure 4.6. It is evident that addition of optimum amount of deionized water certainly promote enzyme re-use performance.
Table 4.5 Effect of deionized water addition methods on performance of re-used enzyme

<table>
<thead>
<tr>
<th>Name</th>
<th>1st Yield</th>
<th>2nd Yield</th>
<th>3rd Yield</th>
<th>4th Yield</th>
<th>( \text{Average Re-use Efficiency} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>With deionized water addition</td>
<td>0.52±0.02</td>
<td>0.44±0.02</td>
<td>0.37±0.02</td>
<td>0.28±0.02</td>
<td>0.82±0.01</td>
</tr>
<tr>
<td>Without deionized water addition</td>
<td>0.54±0.02</td>
<td>0.29±0.02</td>
<td>0.12±0.02</td>
<td>0.07±0.02</td>
<td>0.49±0.01</td>
</tr>
</tbody>
</table>

Figure 4.6 Effect of deionized water addition methods on performance of re-used enzyme

4.6 Comparison of Used Enzyme Washing Methods

In this study, two used enzyme washing methods were tested. The enzyme lost and re-use efficiency is shown in Table 4.6.

Table 4.6 Effect of different used enzyme washing methods

<table>
<thead>
<tr>
<th>Name</th>
<th>1st Yield</th>
<th>2nd Yield</th>
<th>Weight Loss % of Enzyme</th>
<th>Re-use Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

35
<table>
<thead>
<tr>
<th></th>
<th>Method A&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yield</td>
<td>0.49±0.02</td>
<td>0.49±0.02</td>
</tr>
<tr>
<td>Weight</td>
<td>0.26±0.02</td>
<td>0.31±0.02</td>
</tr>
<tr>
<td>Loss %</td>
<td>14.8±0.1%</td>
<td>7.6±0.1%</td>
</tr>
<tr>
<td>Efficiency</td>
<td>0.53±0.02</td>
<td>0.64±0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup>Both 2<sup>nd</sup> yields were calculated after adjusting for weight loss. For example, in the 2<sup>nd</sup> run of Method A, the yield from GC analysis was 0.22614 g/g, the weight loss% of immobilized enzyme was 14.8%. Thus, the final yield is 0.22614/(1-14.8%) = 0.26542 g/g. This calculation method was employed in all data of this thesis.

<sup>b</sup>In both runs, the initial immobilized enzyme’s weight was 0.2 g. To maintain the same conditions, weight loss was determined after drying and addition of water at the end of the first run.

<sup>c</sup>The explanation of washing method A & B and the reaction conditions are illustrated in Section 3.5.

The results clearly demonstrate that washing method B is better on two counts: lower enzyme loss and higher enzyme re-use efficiency.
CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Conclusions

In this thesis, the effects of four organic solvents (n-pentane, n-hexane, n-heptane and iso-octane), co-solvent (tert-butanol), three temperatures (25, 30 and 40°C), three methanol addition methods (3:1 stepwise, 3:1 batch, 6:1 batch), four water content (2.5%, 5%, 7.5% and 10% by weight of enzyme) and two used enzyme washing methods (A and B) on the transesterification reaction catalyzed by *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5 were investigated. The optimum conditions for transesterification of waste vegetable oil by *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5 were as follows: n-hexane as solvent, 5 vol.% tert-butanol as co-solvent, room temperature of 25°C, batch wise addition 3 equivalent mole methanol addition, washing method B and 7.5% by weight of enzyme deionized water added to totally dried enzyme.

The concentrations of the acyl-acceptor and solvent play a critical role in the performance of the enzyme catalyst. The highest enzyme re-use efficiency was obtained with n-hexane, which resulted in the best enzyme suspension. A 3:1 methanol molar ratio with all the methanol added at the beginning of the reaction was better than stepwise addition method. This is contrary to what has been reported
in the literature and is perhaps due to the presence of the co-solvent, tert-butanol. Thus, the combination of a good solvent-cosolvent system and optimal substrate concentration are very important to enhance the performance of *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5.

The results of experiments to determine active water content showed that active water loss was a reason for lower activity of *Thermomyces lanuginosus* lipase immobilized on zeolite ZSM-5 after each cycle. The maintenance of an active water layer certainly enhances the performance of the catalyst and allows use of a higher concentration of methanol, which in turn results in a higher biodiesel yield. In our case, the 3:1 batch addition resulted in higher yields compared to stepwise addition.

### 5.2 Recommendations for Future Work

Here are four aspects of future work recommended:

i) Additional solvents should be investigated. Other than organic solvents, ionic solvents are also excellent. From the study of Ha et al.\(^4^3\) and Gamba et al.\(^4^4\), many ionic such as [EMIM].[TfO], [BMIM].[THF\(_2\)], [BMIM].[PF\(_6\)] have shown good performance in transesterification reactions.

ii) Effect of different co-solvents need to be evaluated. In addition to 5 vol.% tert-butanol, other hydrophilic organic solvents and tert-butanol of other
concentrations should be investigated. For example, methyl isopropyl ketone may be a good co-solvent.

iii) Other source of acyl-acceptors should be investigated. Ethanol, methyl acetate and tert-butanol can also be good source of acyl-acceptor. In addition to their lower toxicity to enzymes, the benefit of these low hydrophilic acyl-acceptors is that the concentration of these acyl-acceptors can be high, and some of these acyl-acceptors can also work as the co-solvent to promote the rate of triglyceride conversion.

iv) Reaction run in PFR should be investigated. The use of a continuous process could result in higher yields and increased productivity.
REFERENCES


37. Soumanou, M. M.; Bornscheuer, U. T.: Improvement in lipase-catalyzed synthesis of fatty acid methyl esters from sunflower oil. *Enzyme and Microbial


APPENDIX A

PERPARATION OF CALIBRATION CURVES

As illustrated in Chapter 3, in this study methyl oleate was selected as the biodiesel standard for GC analysis. *iso*-Octane was selected as dilution solvent to get different concentrations of methyl oleate and *iso*-octane solution.

Calibration curves were prepared as follows:

i) 20 µl methyl oleate was added to 2 ml *iso*-octane to get the "specimen 0"; ii) After adequate shaking of specimen 0, 0.8 ml mixture was extracted and mixed with 1 ml *iso*-octane to get the "specimen 1"; iii) 1 ml of mixture specimen 1 was extracted and mixed with 1 ml *iso*-octane to get "specimen 2"; iv) 1 ml of mixture was extracted from specimen 2 and mixed with 1 ml *iso*-octane to get "specimen 3"; v) 1 ml of mixture was extracted from specimen 3 and mixed with 1 ml *iso*-octane to get "specimen 4".

Except specimen 0, the mass concentration of specimen 1-4 was calculated by Equation A.1:

\[ W_i = \frac{W_{i-1} \cdot m_i}{m_i \cdot (1 - W_{i-1}) + I_i} \]

(Equation A.2)
$W_i \text{ (g/g)}$ and $W_{i-1} \text{ (g/g)}$ is the mass concentration of specimen $i$ and specimen $i-1$, respectively; $m_i \text{ (g)}$ is the weight of mixture extracted from specimen $i-1$; $l_i \text{ (g)}$ is the weight of iso-octane added to the mixture. Concentration of specimen 0 was calculated by weight of methyl oleate divided by added iso-octane.

30 μl samples of specimen 1-4 were extracted and test by GC analysis. Software *Origin* was used for calculating the area of peaks. Finally, these area values were correlated with the corresponding mass concentrations in the calibration curve as shown in Figure A.1. In this study, calibration curve was tested every week with $R^2 > 0.95$.

![Figure A.1 Calibration curve for methyl oleate](image-url)