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ENGINEERING LIPASES AND SOLVENTS FOR TRANS/-ESTERIFICATION OF USED VEGETABLE OILS

BY

MICHAEL DORE GAGNON

B.S. in Chemical Engineering, University of New Hampshire, 2009

DISSERTATION

Submitted to the University of New Hampshire

in Partial Fulfillment of

the Requirements for the Degree of

Doctor of Philosophy

in

Chemical Engineering

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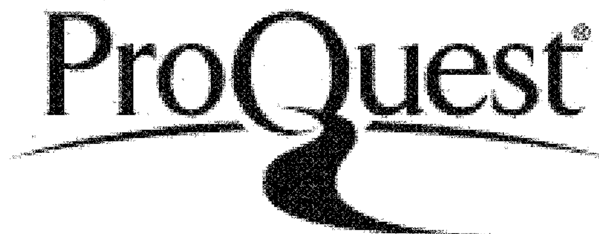

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DEDICATION

I lovingly dedicate this dissertation to my wife and parents.

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ABSTRACT

ENGINEERING LIPASES AND SOLVENTS FOR TRANS/-ESTERIFICATION OF USED VEGETABLE OILS

by

Michael Dore Gagnon

University of New Hampshire, May 2013

Diminishing petroleum reserves and increasing environmental awareness has led to an urgent need to develop alternative fuels, such as biodiesel. However, the conventional method to produce biodiesel uses environmentally harmful chemical catalysts. A relatively new development in the production of biodiesel is through enzymatic trans/-esterification with a lipase catalyst. Despite several advantages, there are a few technical and economical obstacles that limit this process: (1) immiscibility of the hydrophilic methanol and hydrophobic triglyceride which results in the formation of an interface leading to mass transfer resistance, (2) insufficient availability of large quantities of inexpensive lipase suitable for catalysis, and (3) stripping of essential water from the active site by the strong polarity of methanol causing a reduction in enzyme activity after multiple reuses. Each obstacle is addressed by:

- Examining the effect of 15 organic solvents on activity of lipase from three sources, namely *Candida antarctica*, *Pseudomonas cepacia*, and *Thermomyces lanuginosus*, in the trans/-esterification of used vegetable oil with the goal of

recommending the best solvent through solvent engineering.

- Cloning and over-expressing recombinant lipase from *T. lanuginosus* in tobacco for the enzymatic production of biodiesel in order to develop an abundant inexpensive biocatalyst.
- Investigating the effects of reaction parameters on the trans/-esterification of used vegetable oil and their effects on enzymatic activity over consecutive reactions with a view to lowering costs.

The major findings are: (i) there appears to be a correlation between the solvent's hydrophobicity (log P) and biodiesel yield, (ii) the choice of lipase can have a considerable effect on the reaction kinetics and biodiesel yield, (iii) a thermophilic fungus lipase gene can be constitutively expressed in tobacco without adversely affecting plant growth or development, (iv) plants systems offer a promising platform for producing recombinant enzymes for biodiesel production, (v) the type of methanol addition does not appear to greatly affect the biodiesel yield when fresh enzyme is used, but does significantly affect the enzyme's activity during subsequent reuse, and (vi) optimization of reaction parameters such as methanol addition, reaction temperature, and solvent can minimize enzymatic deactivation and increase enzyme reusability without significantly affecting biodiesel yield.

CHAPTER 1

INTRODUCTION

1.1 Energy and the Environment

According to the Energy Information Administration (EIA), current estimates of worldwide recoverable reserves of petroleum and natural gas are estimated to be 1.34 trillion barrels and 6,289 trillion cubic feet, respectively.[1] The world consumes a total of 87.3 million barrels per day of oil and 112.6 billion cubic feet per day of natural gas.[1] The U.S. consumes 21.6 % of the world's petroleum, 21.1% of the world's natural gas, and 42% of the world's gasoline. At current consumption levels, the worldwide reserves of oil and natural gas will be exhausted in about 40 and 60 years, respectively.

While petroleum supplies dwindle, the worldwide demand has increased over the past decade. As a result, the price of petroleum has increased dramatically in the past decade to the point where it reached a record high at $147.27 per barrel of crude oil in July 2008. This is an increase of 1190 % from a decade earlier when the price of crude oil was $12.38 per barrel in July 1998.[1] The rapid rise in the price of oil has resulted in gasoline increasing from $1.08 to $4.09 per gallon during the same time period, representing an increase of 379%.

In addition, carbon dioxide (CO_2) is gaining a great deal of attention from scientists studying climate change. CO_2 is a heat-trapping greenhouse gas that is primarily produced by the combustion of fossil fuels, such as oil, coal, and natural gas. Human activities have changed the atmospheric concentrations of CO_2 that far exceed its natural

fluctuation (180 – 300 ppm) over the past 800,000 years.[2] In 2010, the total CO_2 gas emissions in the United States were 6,821.8 million metric tons, an increase of 10.5 % from 1990.[3] As a result, the increase in greenhouse gases, along with deforestation, has increased the Earth's surface temperature throughout the 20th century. The second warmest temperature rise on record was just recorded in 2010.[2] The issue of climate change is one of the key challenges facing us and it is imperative that steps are taken to reduce greenhouse gas emission. The combination of diminishing petroleum and the deleterious environmental consequences of greenhouse gases has led to an urgent and critical need to develop alternative, renewable and environmentally friendly fuels, such as biodiesel.

1.2 Biodiesel

1.2.1 Diesel engines & fuel

The first account for biodiesel production was in 1937 by Professor G. Chavanne of the University of Brussels, who applied for a patent (Belgian Patent 422,877) for the 'Procedure for the transformation of vegetable oils for their uses as fuels'.[4] Biodiesel as a fuel is clean burning, non-toxic, and biodegradable with a chemical structure of a fatty acid alkyl ester (FAAE). The combustion of biodiesel greatly reduces visible smoke, hazardous particulate matter, total hydrocarbons, sulfur dioxide (SO_2), carbon monoxide (CO), and CO_2, which greatly reduces health risks when compared to petroleum diesel.[5]

In 1893, the first diesel engine was invented by the German mechanical engineer, Rudolph Diesel.[6] The diesel engine is an internal combustion engine that uses the heat of compression to ignite the fuel, instead of a separate ignition system used in gasoline engines.[6] A higher compression ratio is required for a diesel engine to cause auto-

ignition, which is more efficient and uses less fuel than a similar gasoline engine. Additionally, diesel fuel (typically $C_{14}H_{30}$) has a higher energy density than gasoline (typically C_9H_{20}), containing approximately 155 MJ (147,000 BTU) compared to 132 MJ (125,000 BTU), respectively.[7] Improved engine efficiency and higher fuel energy density explains why diesel vehicles get up to 40 % more miles per gallon (mpg) than their equivalent gasoline counterparts. European markets consist of over 40% of diesel vehicles due to a large influx of highly efficient diesel engines used in small cars. The United States is just beginning to see the next generation of diesel cars arrive with the Audi (A3 & Q7), BMW (335D & X5 xDrive35d), Mercedes-Benz (350 series), and Volkswagen (TDI series).

1.2.2 Alternative diesel fuel

Biodiesel is defined by the National Biodiesel Board (NBB) as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats, designated B100, and meeting the requirements of American Society for Testing Materials (ASTM) D6751.[8] The ASTM standard specifications set the required physical and chemical properties of biodiesel determined by chemical analysis.[9] This specification applies to pure biodiesel (B100) that can be used as a blending component with petroleum diesel at ratios of 2 % (B2), 5 % (B5), or 20 % (B20). This ensures that biodiesel meets a specific minimum quality standard for commercial use with existing diesel engines that are $\leq$ 15 years old, without any engine modifications. Older engine systems may require replacement of fuel lines and other rubber components in order to operate with biodiesel. The interchangeable nature of biodiesel with petroleum diesel

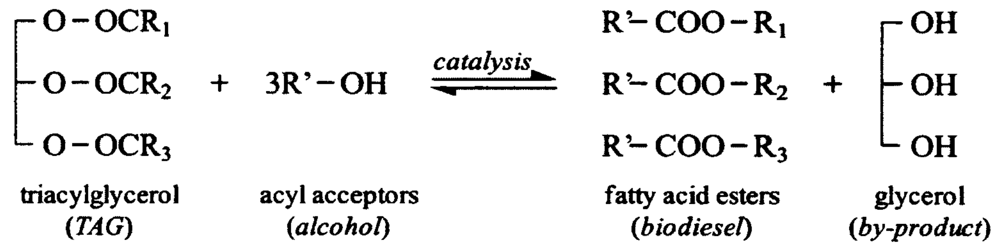
allows for the use of the current diesel infrastructure and allows for easier widespread implementation.

In 2006, the Environmental Protection Agency (EPA) limited sulfur emission in diesel fuels to 15 ppm. Additionally, new trucks and buses with diesel engines, from model year 2007, are now required to use only ultra low sulfur diesel (ULSD) with new emissions control equipment.[10] However, the processing required to reduce sulfur from 500 ppm (previous limit) to 15 ppm also removes naturally occurring lubricating agents in the diesel fuel. Biodiesel is naturally oxygenated and therefore a better lubricant and has similar material compatibility to ULSD. Many countries are utilizing biodiesel's lubrication properties to blend with ULSD to replace expensive lubricating additives.[11]

1.2.3 Chemistry of biodiesel synthesis

The production of biodiesel is based on the transesterification reaction of triglycerides (TAGs) or by the esterification of free or unesterified fatty acids (FFAs). TAGs are the main component of grease, vegetable oil, and animal fat. The transesterification of the TAGs with an alcohol and a catalyst will result in FAAE (biodiesel) and glycerol as a by-product, as shown in Figure 1a.[12] FFAs are naturally present in virgin vegetable oils in small quantities. Used cooking oils and fats, such as restaurant grease, spent frialator oil, and trap grease, have higher levels of FFAs due to the hydrolysis and decomposition of TAG molecules. The esterification reaction of these FFAs with an alcohol and catalyst will also result in a FAAE and have water as a by-product, as shown in Figure 1b.[13]

a) transesterification



b) esterification

Figure 1. Production of biodiesel by (a) transesterification and (b) esterification.

The alcohol works as an acyl-acceptor for biodiesel synthesis and is typically a short chained alcohol, such as methanol, ethanol, propanol, or butanol.[14] Methanol is the most common acyl-acceptor used in research and industry because of its low cost. The use of methanol in the transesterification reaction is called methanolysis and produces a fatty acid methyl ester (FAME) as biodiesel. All of the biodiesel available in the United States is currently based on FAME. Ethanol is the second most commonly used acyl-acceptor because it can be produced from biomass via fermentation, whereas methanol is mainly from fossil fuels. As a result, biodiesel produced from ethanol is more of a renewable fuel and has a higher energy content than methanol. However, the cost of pure ethanol (200 proof) is about twice as much as anhydrous methanol (for the same quantity). Other acyl-acceptors that have been used to produce biodiesel include propanols, butanols, methyl acetate, and other short chain acetates.[15] Acetates have become attractive because they prevent the formation of glycerol which can have some detrimental effects, but again cost becomes the main factor in finding a replacement to methanol.

1.3 Feedstocks

1.3.1 Increasing demand

Biodiesel synthesis primarily involves two substrates, alcohol and TAG/FFA. The latter is produced from renewable sources, such as oils and fats, and are often used interchangeably referring to the feedstock for biodiesel production. Recent technological advancements convert a wide variety of biomass into biodiesel feedstocks. This has resulted in a significant increase in the production of biodiesel within the United States[16] and European[17] countries over the past decade, as shown in Figure 2. Even with such an increase, large-scale production of biodiesel as an alternative fuel faces several challenges that need to be addressed. One of the main challenges with any renewable fuel is the acquisition of large quantities of low cost raw materials. Biodiesel's profitability relies heavily on feedstock prices, which can account up to 75 % of the final cost per gallon of biodiesel.[18]

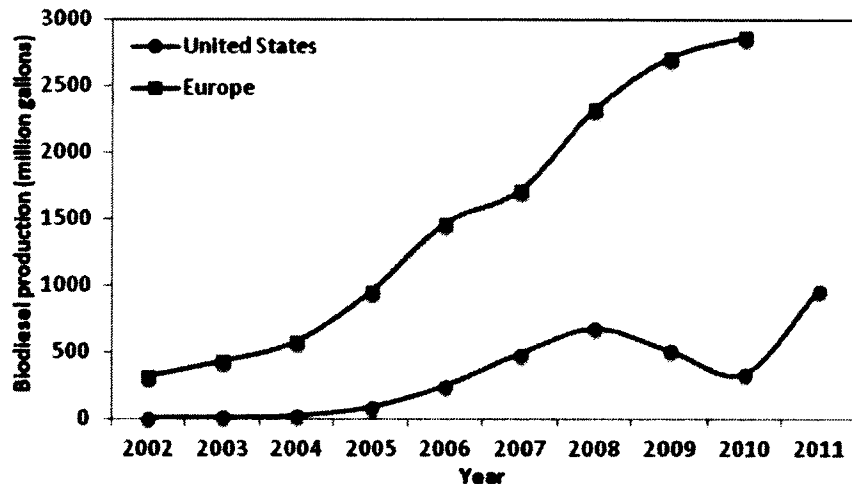


Figure 2. Annual production of biodiesel in the United States and Europe, adapted from the U.S. Energy Information Administration and European Biodiesel Board.[16-17]

1.3.2 Virgin oils & edible sources

There are many feedstocks available for biodiesel production, such as virgin vegetable oils, biomass, algae, and waste oils. A considerable amount of the research to produce biodiesel has been done using edible sources of virgin vegetable oils, like soybean,[19] rapeseed,[20] sunflower seed,[21] and canola oils.[22] Most of these feedstocks vary with location, climate, and growing conditions. Soybean oil is currently the dominant feedstock for the production of biodiesel in the United States. This has resulted in crops being sold as fuel crops instead of for food related products. Therefore, reducing the food supply has increased the price of soybeans from 16.46 cents/lb back in 2001 to 54.5 cents/lb in 2011.[23] A recent United Nations report states that the global rush to switch from oil to energy derived from plants will drive deforestation, push small farmers off their land, increase poverty, and lead to serious food shortages unless it is carefully managed.[24] This has resulted in the so-called "food versus fuel" controversy, which has been greatly exaggerated in many cases. Nevertheless, the focus of biodiesel production needs to be on more sustainable feedstocks, such as waste oils and greases, animal fats, and non-edible vegetable oil sources.

1.3.3 Algae oils

There is a growing interest in using algae oils as a feedstock for biodiesel production within the United States. Algae have become an appealing feedstock due to their ease of handling, rapid growth, and high cell densities in an aquatic environment. As a result, their photosynthetic efficiency is significantly higher than the average land based plants.[15] However, the algae oil yields reported are quite low in comparison to the potential yield based on the quantum limits of photosynthetic efficiency, as well as

compared to other means for harnessing solar energy.[15] Interestingly, algae that produce higher oil stores reproduce at a much slower rate than the conventional algae. The Department of Energy's (DOE) Aquatic Species Program also found that the overall oil yield decreased as the algae's oil storage increased.[25] The current research for algae growth for fuel production is being done using photobioreactors. Unfortunately, the reactor designs require a high capital cost, which makes large-scale production uneconomical until a low cost design or new method of production is discovered.[15, 26]

1.3.4 Non-edible oil sources

There are more than 300 oil-bearing plant species, and of them 63, belong to 30 plant families, hold promise for bio-diesel production. From these, there are a few that are non-edible and high oil producing that have captured the attention of the biodiesel community, such as Jatropha (*Jatropha curcas*), Karanj (*Pongamia pinnata*), Castor (*Ricinus communis*), and Rubber (*Hevea barasiliensis*) seeds.[27] For example, Jatropha produces a significant amount of oil from its seeds (30 – 50 % by weight) and has the greatest potential due to its characteristics and growth requirements.[28] It grows in arid, semi-arid, and in tropical regions around the world and can survive in poor soil conditions, such as stony, gravelly, sandy or saline soils. Jatropha is also pest resistant, drought resistant (requires as little as 25 cm of rain per year), and can live over 50 years. Most importantly, it is a rapidly growing tree that is easily propagated and can bloom and produce fruit throughout the year with high seed yields. Some varieties of Jatropha can produce as much as 1600 gallons of biodiesel fuel per acre-year and can capture 4 tons of CO_2 per acre with negligible greenhouse gases emissions.[29] However, the government of

India is having difficulty with developing large scale ventures into Jatropha plantations, with a confusing array of mixed reports from the field.

1.3.5 Waste fats and oils

Waste cooking oils and animal fats are available in huge quantities throughout the world, especially in developed countries. Disposal of these waste oils and fats pose a significant challenge because of possible contamination of water and land resources. Large quantities are discharged into the environment, by being dumped into rivers and landfills.[30] The use of waste cooking oil as a feedstock offers significant advantages over virgin vegetable and algae oils because it enhances the economic viability of biodiesel production. Used vegetable oils and greases (like frialator oils) are typically collected from restaurants and fast-food establishments. In the United States, the EPA estimates that about 3 billion gallons of waste cooking oil are produced per year.[31]

Food processing and service facilities also produce greases, fats, and oils in large quantities, some of which are currently sold as inexpensive animal feed. These recycled greases are generally placed into one of three categories: animal fats, yellow grease, and brown grease. Animal fats are mainly a byproduct of meat processing facilities that include tallow, lard, choice white grease, and poultry fats.[32] The collection and distribution of these animal fats is well established and is a promising feedstock for biodiesel. Yellow grease is spent cooking oils and fats produced from industrial cooking manufacturers.[32] Yellow grease is a low value byproduct that often sells for $0.09 – 0.20 per pound which makes it a prime candidate for biodiesel.[31] Brown grease, also known as trap grease, is collected from grease traps that are used to separate grease and oil from waste water in commercial, industrial, and municipal sewage facilities.[32] Trap grease is

not currently collected; however, it would make a very low cost feedstock and could make a strong candidate for biodiesel production. All of these waste oils, fats, and greases provide an economic opportunity to provide a sustainable, low cost, waste product and turn it into a viable feedstock for use in a growing biodiesel market.

1.4 Comparison of Technology/Production

1.4.1 Background

Rudolf Diesel wanted to provide farmers the opportunity to produce their own fuel and firmly believed vegetable oil to be the real future fuel for his engine. He demonstrated this at the World Exhibition in Paris in 1900 by running his engine on pure peanut oil. [6] However, the characteristics of vegetable oil, such as high viscosity, low volatility, and high cloud and pour points, make direct use in a diesel engine problematic.[33] In order to avoid these problems, the vegetable oil is chemically modified to FAAEs which have properties more similar to conventional petroleum diesel. There are three main methods in which FAAEs are produced from vegetable oil; pyrolysis, microemulsification, and transesterification.[33] The pyrolysis method uses the application of extreme heat to chemically reduce TAG molecules to FAAEs. Microemulsification method uses solvents to physically reduce the viscosity of the vegetable oil. Transesterification has been demonstrated as the simplest, most efficient, and ecofriendly route to produce FAAEs in large quantities and has become the most popular biodiesel production method.

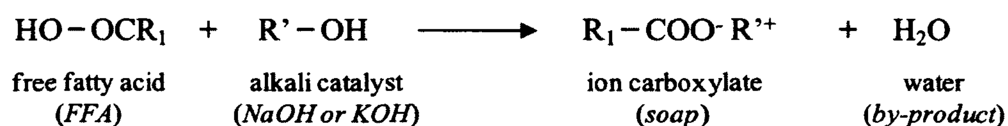
1.4.2 Base catalysis

Most of the biodiesel commercially produced today is by alkali-catalyzed transesterification of vegetable oils, using a homogenous base catalyst, such as sodium

hydroxide (NaOH) or potassium hydroxide (KOH). Due to solubility considerations, NaOH is used as the catalyst when methanol is the acyl acceptor, and KOH is used with ethanol.[26] This method has proved to be the most economical because of the high conversion rate (upward of > 98 % ester yield) within a short reaction time (< 1 h) under a low temperature (66 °C) and pressure environment(1.4 atm).[34]

The main drawback of this process is the sensitivity of alkaline catalysts with respect to feedstock purity. The vegetable oil and alcohol must be substantially anhydrous, and have low FFA content because water and/or FFA promote soap formation. The formation of soap lowers the yield of esters and renders the downstream separation of the products difficult, as shown in Figure 3.[35] A pretreatment step becomes necessary before the transesterification process to reduce the acid and water concentrations below an optimum threshold limit; FFAs < 1.0 wt% and water < 0.5 wt%.[36] FFA pretreatment can be accomplished by acid esterification, ion exchange resins, or by alcohol extraction.

a) saponification



b) hydrolysis

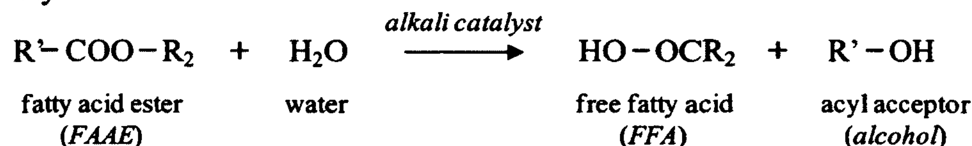


Figure 3. Undesired reactions with an alkaline catalyst (KOH); (a) saponification of a FFA, (b) hydrolysis of a fatty acid ester.

Besides pretreatment, alkaline transesterification requires multi-step purification of the end products where the glycerol byproduct contains some of the excess methanol, most of the catalyst, and soap.[37] The resulting low quality glycerol has very little value and

requires to be treated as a hazardous waste due to the methanol content. In addition, approximately 0.2 ton of waste water is also produced from this process per ton biodiesel produced and must be treated.[33] The need for extensive treatments and processing makes alkaline transesterification expensive and not environmentally friendly.

1.4.3 Acid catalysis

The second most commonly used commercial process to produce biodiesel is the acid-catalyzed transesterification. The most commonly employed acid catalysts are sulfuric acid (H_2SO_4) and hydrochloric acid (HCl). Acid catalysis is considered a viable alternative to alkaline catalysis because it is not susceptible to soap formation due to strong catalytic activity for esterification. This results in easier product separation and produces a relatively high quality glycerol byproduct. However, the reaction is much slower than alkali catalysis because the acids have a low catalytic activity for transesterifications and can result in a lower yield, ranging from 56.8 – 96.4 % depending on the feedstock.[26, 37] In addition, the typical reaction conditions require a higher temperature (100 °C) and pressure (5 atm) as well as larger amount of alcohol than alkali-catalyzed transesterification.[38] The higher temperatures also increase the formation of unwanted secondary products, such as dialkylethers or glycerol ethers, which requires additional downstream processing. Due to the high temperature requirements and slow reaction rates for transesterification, acid catalysis is typically used for esterification reactions only. The use of acid catalysis has been found to be useful for pretreating high FFA feedstocks prior to the alkaline catalyzed transesterification reaction. Both the alkali and acid catalysis processes are energy intensive and require extensive downstream processing.

1.4.4 Enzyme catalysis

A relatively new and attractive method for biodiesel production is by enzymatic transesterification and esterification. Enzymes offer several advantages over chemical catalyst, such as high substrate selectivity and operate with mild temperature (20 – 60 °C), pressure (1 atm), and pH conditions.[39] Enzymatic catalysis is an attractive method for biodiesel production because it eliminates many of the problems associated with conventional chemical catalysts as well as other environmental benefits. Unlike homogeneous chemical catalysts, enzymes are often immobilized, which simplifies the separation of products, produces a high quality glycerol, and allows for the reuse of the catalyst.[33] Waste oils, greases, lard, and other low cost feedstocks that are higher in FFAs can be used for biodiesel production without extensive processing.[39] Moreover, enzymes require much less alcohol to perform the reaction when compared to chemical catalysts, usually close to the stoichiometric ratio. Excessive amounts of alcohol can interfere with glycerol separation because of the increased solubility with alcohol.[40]

Lipases are enzymes that naturally catalyze the hydrolysis of lipids and are a versatile biocatalyst that can often express various other activities like phospholipase, cholesterol esterase, and amidase.[41] The versatility of lipases are used extensively within industry for the production of detergents, baking, organic synthesis, hard surface cleaning, leather, and paper.[42] The lipases discussed in this thesis have triacylglycerol activity (EC 3.1.1.3 group) and are capable of catalyzing both transesterification and esterification reactions (trans/-esterification).[41] Several microbial strains of lipases have been found to have high trans/-esterification activity; *Candida antarctica*,[43] *Pseudomonas cepacia*,[44] and *Thermomyces lanuginosus*[45] are a few that have been reported.

Despite numerous advantages, enzymatic catalysis does have several drawbacks and challenges to overcome. The enzymatic process requires a longer reaction time when compared to chemical catalysis, some taking several days to complete. Higher catalyst concentrations are required to complete the reaction than that of chemical catalysts and enzyme inhibition can result in lower ester yields.[33] A challenge in enzymatic process is the immiscibility of the two substrates, alcohol and oil. This forms an interface between the hydrophilic methanol and hydrophobic triglycerides which results in a resistance to mass transfer. In addition, strong polarity of methanol tends to strip the essential water from the active site of the enzyme, resulting in deactivation of the enzyme. The addition of an organic solvent to the reaction medium can overcome these two limitations by enhancing the solubility of methanol and oil while simultaneously limiting the methanol concentration surrounding the enzyme's active site.[46]

A second challenge is the diversity and availability of enzymes in sufficient quantities for testing and scale-up. Currently, there are a limited number of lipases that have been screened for trans/-esterification activity and even fewer that are acceptable for biodiesel catalysis. Recent advances in biotechnology and genomics have allowed researchers to identify novel genes, clone them, alter their sequences, and over-express them in a variety of host organisms, such as bacteria, yeast, and plants.[47] Therefore, the genes encoding novel or known lipases from a diverse group of plant and microbial organisms requires screening for trans/-esterification activity that is suitable for biodiesel production. In addition, a variety of host expression systems needs to be investigated to determine the most cost effective method to produce sufficient quantities of these biocatalysts.

The third challenge is increasing the number of times the immobilized enzymes can be reused before it loses a significant amount of its activity. Immobilized lipases currently cost around $1000 per kg, whereas NaOH is only $0.62 per kg.[33] The sustained reuse of immobilized enzymes can significantly reduce production costs. However, there are several factors that can reduce enzyme activity over multiple reuses, such as glycerol inhibition, methanol inhibition, and thermal degradation. Optimizing reaction conditions and enzyme treatments may be able to reduce these effects and increase the overall effective lifetime of the catalysts.

1.5 Overview

The production of biodiesel by enzymatic trans/-esterification has been extensively studied, however, this technology has not received much commercial attention due to costs. The production of lipases in sufficient quantities from microorganisms is very expensive and requires purification and immobilization. The key to economical feasibility of commercializing this technology is to lower the cost associated with using enzymes. In order to make enzymatic trans/-esterification competitive on an industrial scale, there are several issues that have to be addressed: solvent engineering, low-cost lipase production, and extended enzyme reuse. This dissertation will try to offer solutions to the current problems and look to new perspectives in the enzymatic trans/-esterification of used vegetable oil. The organization of this dissertation is as follows:

Chapter 2 reports the results of solvent engineering studies on the effect of choice of organic solvent on activity of lipase from three sources, namely *Candida antarctica*, *Pseudomonas cepacia*, and *Thermomyces lanuginosus*. In particular, fifteen hydrophobic and hydrophilic solvents from four organic groups were evaluated as possible media in

the methanolysis of used vegetable oil. In this study, the solvent hydrophobicity (log P) was correlated with the corresponding biodiesel yields, with the goal of investigating their potential relationship to enzyme activity.

Chapter 3 reports the results of genetically engineering plants to constitutively over-express lipases for biodiesel production from spent oils. The gene of a lipase with known trans/-esterification activity was cloned from a thermophylic fungus, *T. lanuginosus*. The gene was inserted into a cloning vector and sequenced to confirm its identity and then inserted into a plant destination vector. The transformed plasmids were inserted into *Agrobacterium tumefaciens*, which was used to insert the into *Nicotiana tabacum* (tobacco) by *Agrobacterium*-mediated transformation and into *Arabidopsis thaliana* by the floral dip method. The recombinant enzyme was collected from the transgenic plants, purified, and tested for its activity. This approach should provide a relatively cheap and environmentally safe source of lipase for use as a biocatalyst.

Chapter 4 reports the effects of various conditions on the reuse of immobilized *T. lanuginosus* lipase in the trans/-esterification of used vegetable oil in a solvent system. In particular, the type of methanol addition, reaction temperature, and post-reaction solvent washes were examined. The partition coefficient (log P) was evaluated to correlate hydrophobicity of the solvents used to wash the enzymes with enzyme activity over five consecutive runs. Optimization of these parameters can minimize enzymatic deactivation and increase enzyme reusability without significantly affecting FAME yield.

Chapter 5 reports the Summary, including an overview, conclusions, and recommendations for future work.

CHAPTER 2

EFFECTS OF SOLVENT AND ENZYME SOURCE ON TRANS/-ESTERIFICATION ACTIVITY

2.1 Introduction

2.1.1 Lipase as biocatalysts

Lipases are natural protein catalysts and are considered hydrolases, which hydrolyze triacylglyceride (TAG), that are instrumental in the deposition and mobilization of fats used for energy storage. Over the past decades, many practical applications of lipase have been developed for industrial manufacturing, such as food products, cosmetics, and detergents. The synthesis of isopropyl myristate and myristyl myristate are produced on a large-scale using *Candida antarctica* lipase B and widely used in cosmetic formulations.[48] Lipases are also very powerful enzymes for organic synthesis, as they operate at an oil-water interphase and can react with non-lipid substrates. Researchers have found a number of unusual substrates by employing the enantio- and stereo-specificity of lipases for synthesis; including asymmetric synthesis of pyrrolizidine alkaloids,[49] and tertiary carbinols,[50] kinetic resolutions of racemic alcohols,[51] cyclophanes,[52] and thioesters.[50] The versatility of lipases is due to the enzyme's ability to catalyze alternative reactions that differ from their natural physiological reaction.

Lipases (EC 3.1.1.3) constitute an immense and diverse family of enzymes which are produced by bacteria, yeasts, fungi, plants and mammals. Some of the lipases isolated from these sources have been commercially exploited in a wide range of industrial

applications, as shown in Table 1. The future potential of lipases as biocatalysts to replace the conventional chemical catalysts is just being recognized. Several lipases produced from bacterial sources have been suggested for use in biodiesel production; such as *Achromobacter lipolyticum*, *Alcaligenes denitrificans*, *Chromobacterium viscosum*, *Pseudomonas cepacia*, *P. fluorescens*, and *P. putida*.[33, 53]

Table 1. Commercially available lipases and their industrial applications.

Source*	**Trade Name / Supplier**	**Application**
Pseudomonas mendocin^B	Lumafast/ Genencor International	Industrial detergent
Pseudomonas alcaligenes^B	Lipomax/ Genencor International	Industrial detergent
Pseudomonas fluorescens^B	Lipase AK/ Amano	Chiral compound resolution
Pseudomonas cepacia^B	Lipase PS/ Amano	Chiral compound resolution
Candida antarctica^Y	Novozyme 735/ Novozymes	Removal of size lubricants
Candida antarctica^Y	Novozyme 435/ Novozymes	Isopropyl myristate production
Candida rugosa^Y	Resinase/ Novozymes	Control of pitch
Candida cylindracea^Y	Lipomod 34PL034P/ Biocatalysts	Delipidation of egg white
Penicillium roquefortii^F	Lipomod 338PL338P/ Biocatalysts	Enzyme modified cheese
Thermomyces lanuginosa^F	Lipozyme TL IM/ Novozymes	Vegetable oil interesterification
Rhizopus oryzae^F	Lipomod 627PL627P/ Biocatalysts	Improvement of dough texture
Rhizomucor miehei^F	Lipozyme RM IM/ Novozymes	Vegetable oil interesterification
Wheat germ^P	L-3001/ SigmaAldrich	Analytical enzymes
Papaya^P	P-4880/ SigmaAldrich	Analytical enzymes
Porcine pancreas^M	L-0382/ SigmaAldrich	Chiral compound resolution
Human pancreas^M	L-9780/ SigmaAldrich	Analytical enzymes
Horse pancreas^M	PNLIP/ Cusabio	Analytical enzymes

* Type of organism responsible for the lipase is annotated with a superscript; (B) bacterial, (Y) yeast, (F) fungi other than yeast, and (M) mammalian.

Yeasts are eukaryotic microorganisms that are classified as fungi and constitute approximately 1 % of the total fungi kingdom.[54] For the purpose of this paper, yeasts will be named separately from the other forms of fungi due to some of their unique properties and uses. Lipases produced by various yeast strains have been suggested as

effective biocatalysts for trans/-esterification; such as *Candida antarctica* lipase A, *Candida antarctica* lipase B, *Candida cylindracea*, *Candida rugosa*, *Geotrichium candidum*, *Pseudozyma antarctica*, and *Yarrowia lipolytica*.[55] In addition to yeasts, other fungal sources of lipase have also been well studied by researchers, exploited by industry, and show promise for biodiesel production; such as *Aspergillus niger*, *Penicillium roquefortii*, *Rhizomucor miehei*, *Rhizopus chinensis*, *Rhizopus oryzae*, and *Thermomyces lanuginose*.[33]

In plants, lipases are primarily found in the early stages of seed germination in oleaginous seeds (oilseeds) and other cereal seeds.[47] During germination the lipolytic activity is very high as oil reserves are hydrolyzed to produce energy for embryonic growth.[56] Researchers have isolated some plant lipases which can be used as industrially important enzymes; such as *Caesalpinia bonducella* seeds, *Carissa carandas* fruit, *Castor bean*, *Hibiscus cannabinus*, *Moringa olifera*, and *Triticum aestivum*.[57] The wheat germ lipase is one of the few that is available commercially, but this lipase is limited in use due to its selectivity towards very short chained TAGs; such as triacetin and tributyrin.[56] Plant seed lipases are usually present at very low concentrations in their native source and require a germination time which is a major drawback for large-scale applications for biodiesel production.

Mammalian lipases are found in at least three tissue-specific organs: pancreatic, hepatic, and gastric/lingual.[58] The most thoroughly studied are lipases that are secreted from the pancreas and are the primary lipases used in the digestive system to break down (hydrolyze) dietary fat molecules.[59] Porcine pancreatic lipase is one of the earliest recognized mammalian lipase and is still the best understood. Important sources of

animal lipase are from the pancreas of cattle, sheep, and pigs.[55] In humans, gastric lipase and pancreatic lipase are predominantly the sources of isolated lipases. At present, lipases are widely used in digestive medicine, pancreatic disease treatments, and diagnostic reagents for assay of neutral lipids and cholesterol esters in blood. These are not currently being looked at for biofuel production.

2.1.2 Lipase structure

In 1981, the first amino acid (aa) sequence of a lipase (E.C. 3.1.1.3) was determined by De Caro *et al.*[60] Since then, a great number of lipases have been sequenced from various organisms, showing their diversity in aa sequences. It was not until 1990 that the first high resolution crystallographic study of a lipase clearly showed the three-dimensional structure.[60] In contrast to their diversity, there appears to be much greater conservation in the secondary and tertiary structures of lipases. Currently, more than 36 organisms with X-ray structures of lipases are available in the protein database (PDB), of which 15 are bacterial, 13 are fungal, and 7 are mammalian.[61]

Lipases are typically globular proteins that range in size between 20 – 60 kDa[62] with an overall structure consisting of a central β-sheet surrounded by several α-helices. Furthermore, the catalytic mechanism of a lipase is composed of a nucleophilic serine (Ser) activated by a hydrogen bond in relay with a histidine (His) and an aspartate (Asp) or glutamate (Glu), forming a catalytic triad.[63] Within the active site are two functional groups that have been identified as being particularly important to the catalytic process; a hydroxyl group that acts as a nucleophile and a nitrogen atom of an amine group that acts as a proton acceptor and donor.[64] Protecting this active site from external environmental conditions is a helical surface loop or "lid" that is found on the majority of lipase.[65] In an

aqueous solution, the lid is in a closed conformation and has little to no activity. In the presence of fats and oils, the hydrophilic and hydrophobic domain of the lid undergoes a conformational change which moves the lid into an open position. The open conformation of the lid allows the substrate access to the active site while exposing a large hydrophobic region and simultaneously burying a hydrophilic domain with the protein.[63] This process is often referred to as interfacial activation and can also be affected by the ionic strength or dielectric constant of the environment, as shown in Figure 4.[41, 65] However, interfacial activation does not include all lipases; *Candida antarctica* lipase B and *Candida rugosa* lipase do not have a "lid" and therefore lacks interfacial activation.[66] These enzymes constitute a link between lipases and esterases.[67]

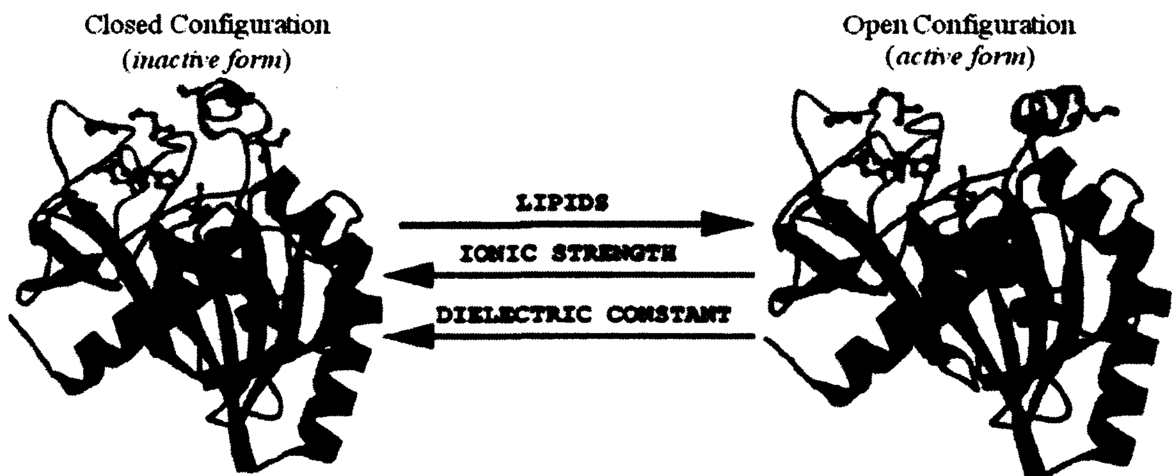


Figure 4. Interfacial activation of lipase from *Thermomyces lanuginosus* caused by environmental factors.[41, 65]

2.1.3 Catalytic specificity of lipases

Enzymes can exhibit high selectivity towards specific location on a particular substrate, which can be an important catalytic feature. The specificity of lipases used for enzymatic trans/-esterification are classified by their positional catalysis, which refers to their selectivity for the acyl position on the glycerol backbone, also known as

regioselectivity. Since lipase have a preference for TAG, the regioselectivity of the enzyme refers to the position on glycerol backbone it hydrolyzes the ester bond. The TAG molecule is labeled by position using stereospecific numbering (*sn*) from 1 to 3 due to the lack of rotational symmetry,[47] as shown in Figure 5. There are three types of regioselectivity found in lipases; *sn*-1,3-specific, *sn*-2-specific, and non-specific (*sn*-1,2,3-specific).[42] Lipases are generally highly specific and can differ by the type of organism from which they originated.

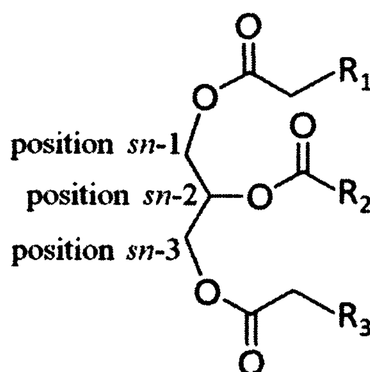


Figure 5. General structure of a triacylglycerol (TAG) with stereospecific numbering, where R represents a saturated, unsaturated, or poly-unsaturated fatty acid.[47]

In general, lipases from bacteria, such as *Bacillus amyloliquefaciens*, *B. subtilis*, and *C. viscosum* are non-specific.[68] Other lipases from bacteria, such as *A. denitrificans*, *P. cepacia*, and *P. fluorescens* are also non-specific, but prefer hydrolyzing ester bonds at the *sn*-1,3-position more than that at the 2-position.[68] Yeast, such as *C. antarctica* (lipase A & B), *C. cylindracea*, *C. rugosa*, and *Trichosporon fermentans* also produce non-specific lipase.[55, 69] A minor component of *G. candidum* lipase and *C. antarctica* lipase A are reported to preferentially recognize ester bond at the 2-position of TAG.[68] Although yeasts are within the fungi kingdom, interestingly, other fungi such as *A. niger*, *R. miehei*, *R. oryzae*, and *T. lanuginosus* are highly *sn*-1,3-position specific.[55] Lipases

from plant seeds, such as *Linum usitatissimum* (linseed),[70] *Cocos nucifera* (coconut),[71] and *Ricinus communis* (castor bean)[72] have been found to be *sn*-1,3-position specific. The lipase from oat seeds, however, exhibit almost no positional specificity but does preferentially recognize the 2-position similarly to *G. candidum* lipase.[73] The regioselectivity of porcine pancreatic lipase and human gastric lipase were found to be *sn*-1,3-specific,[74] whereas bile-salt stimulated lipase (BSSL) and pancreatic lipase-related protein 2 (PLRP2) from rodents were found to be non- specific.[75] To date, no lipase has been found to be strictly *sn*-2-possition specific in any organism.

A lipase with *sn*-1,3-regiospecificity can only catalyze those fatty acids in the sn-1 and sn-3 positions on a TAG leaving a monoacylglyceride (MAG) with a FA at the *sn*-2. Accumulation of MAG within the reaction medium would result in a theoretical yield of 66.7 %.[76] However, this is not the case. A number of *sn*-1,3-specific lipase have been reported to obtain ester yields higher than the theoretical yield; such as 92 % with *R. orzyae*, 90 % with *T. lanuginosus*, 92.2 % with *R. miehei*, and 99.2 % with *M. miehei* have been reported and summarized in several review articles.[14, 42, 77] The reason for the higher yields are due to a phenomenon called acyl migration, which is not related to the enzyme. The process of acyl migration involves the spontaneous exchange of the acyl group in the *sn*-2 position with a hydroxyl group of the *sn*-1 or 3 positions, which then can be catalyzed by a *sn*-1,3-specific lipase. There are several factors which can have an effect on acyl migration; such as enzyme support material, solvent polarity, water content, reaction temperature, and pH.[78]

2.1.4 Enzyme kinetics and mechanism

One of the main advantages of lipase is that it can catalyze reactions with both TAG and FFA to produce biodiesel, as previously shown in Figure 1. The main difference between the esterification of FFAs and transesterification of TAGs is in the mechanism of catalysis. In esterification, the O-H bonds are broken and water is produced as a byproduct.[64] Whereas in transesterification, there are three consecutive reversible reactions in which each of the ester bonds are cleaved, as shown in Figure 6.[14] Each reaction produces a glycerol moiety of diacylglycerol (DAG), monoacylglycerol (MAG), and finally glycerol which accumulates as the reaction progresses. The regioselectivity and position of the TAG molecule relative to the enzyme's active site dictates the positional order in which the FA chains are cleaved.

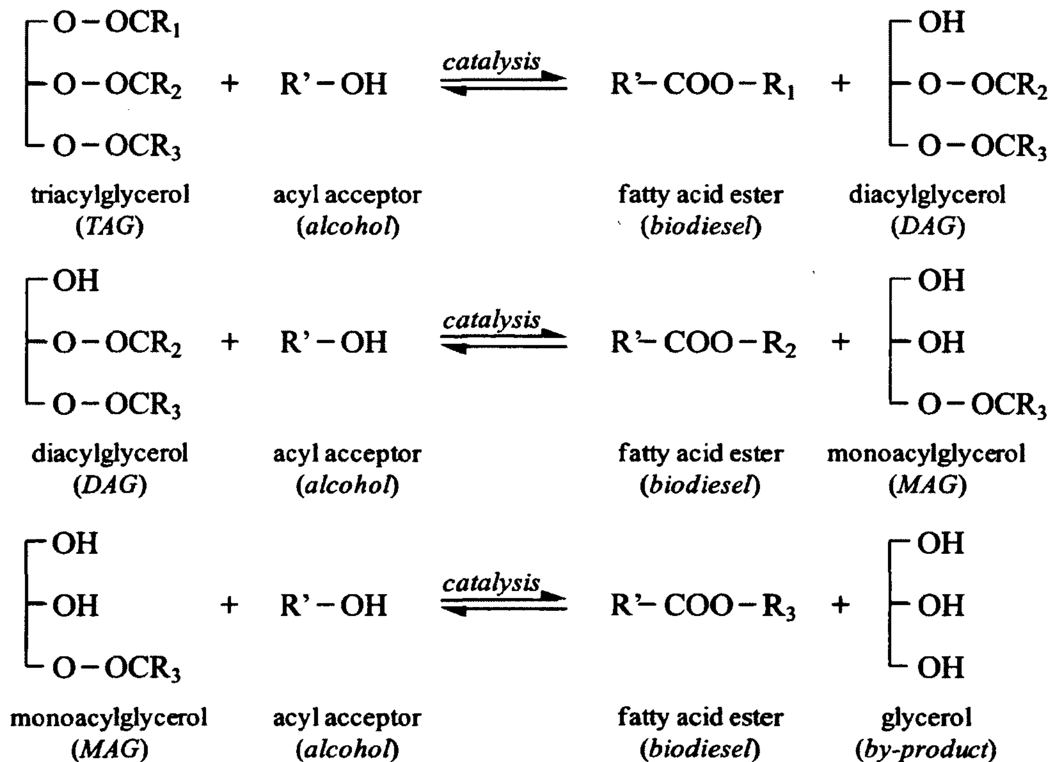


Figure 6. The series of three consecutive reversible reactions involved in the transesterification reaction.[14]

The rate behaviors of enzymes are typically modeled after classical Michaelis-Menten kinetics, where increasing substrate concentration exhibits a hyperbolic character. However, this mechanism cannot be applied to lipase-catalyzed trans/-esterification due to the complex nature of the bi-substrate reaction.[79] Additionally, Michaelis-Menten approach assumes that the enzymatic reaction takes place in an isotropic medium, where lipases act at the water-lipid phase interface.[80] The best possible kinetic mechanism that describes the catalytic action of lipases found by researchers is the Ping Pong Bi Bi model. Earlier kinetic studies assumed that FAAE were the first product to be released from the enzymatic reaction, followed by the by-product (either water or glycerol moiety).[81] More recent studies have determined that the by-product is actually produced first, followed by FAAE, as shown in Table 2.

Table 2. Ping Pong Bi Bi mechanismic steps of trans/-esterification of vegetable oils.

Esterification of FFA[79]		**Transesterification of TAG**[64]	
$E + S_{FFA} \leftrightarrow E{\bullet}S_{FFA}$	(E1)	$E + S_{oil} \leftrightarrow E{\bullet}S_{FFA}$	(T1)
$E{\bullet}\ S_{FFA} \leftrightarrow E{\bullet}FW$	(E2)	$E{\bullet}\ S_{oil} \leftrightarrow E{\bullet}FG$	(T2)
$E{\bullet}FW \leftrightarrow E{\bullet}F + W$	(E3)	$E{\bullet}FG \leftrightarrow E{\bullet}F + G^*$	(T3)
$E{\bullet}F + A \leftrightarrow E{\bullet}FA$	(E4)	$E{\bullet}F + A \leftrightarrow E{\bullet}FA$	(T4)
$E{\bullet}FA \leftrightarrow E{\bullet}B_D$	(E5)	$E{\bullet}FA \leftrightarrow E{\bullet}B_D$	(T5)
$E{\bullet}B_D \leftrightarrow E + B_D$	(E6)	$E{\bullet}B_D \leftrightarrow E + B_D$	(T6)

E is the enzyme, S_{FFA} is FFA, W is water, A is alcohol, S_{oil} is an oil substrate (TAG, DAG, or MAG), G^* is a glycerol moiety, and B_D is a FAAE (biodiesel). $E{\bullet}S_{FFA}$ & $E{\bullet}S_{FFA}$ are enzyme-substrate complexes, E•FW, E•FG, and $E{\bullet}B_D$ are enzyme intermediates after a proton exchange from the conjugate acid of the amine, E•F is an acylated enzyme complex, and E•FA is an acelated enzyme alcohol complex.

The mechanistic inference agrees with most of the kinetic studies on lipase-catalysed esterifications and transesterification of FFA and TAG, respectively.[64, 79-80, 82] However, there are conflicting views on the exact mechanism due to the complex nature of lipase and their wide array of substrates available for catalysis. The main difference between

various rate expressions and kinetic models that use the aforementioned mechanism is the diversity of physical parameters studied: examples include lipase source, support material, immobilization method, temperature, and pH. Additionally, these models incorporate different chemical parameters and their significance on reaction rate, such as water content, substrate inhibition, and solvent effects.

Trace amounts of water can enhance enzyme activity and stability in general due to a water-oil interface required for catalytic action.[83] However, water has also been found to inhibit trans/-esterification reactions by facilitating the hydrolysis of esters, which will reduce FAAE yield and has been treated as a competitive inhibitor in some kinetic models.[82, 84] It is generally recognized that low molecular weight alcohols (such as methanol and ethanol) can competitively inhibit the enzyme directly to produce a dead-end enzyme–alcohol complex and is usually accounted for in kinetic models.[80, 83, 85] Inhibition by methanol in organic media[79, 86] and solvent-free media[85] has been reported at alcohol to oil ratios above 3:1 and 1.5:1, respectively. The strongly polar and hydrophilic nature of these alcohols tends to disrupt the three dimensional architecture of the lipase arising from their capacity to strip away essential water molecules within the active site.[14, 33, 42, 46, 64] The addition of organic solvents have been found to affect the intrinsic kinetic parameters of enzymes.[87] Explanations of solvent dependence on lipase activity have been reported in terms of predictable effects on substrate salvation to model the esterification in toluene, hexane, trichloroethane, and diisopropyl ether.[88]

Refinement of the kinetic model continues to be ongoing due to the complexity of additional parameters involved in the enzymatic trans/-esterification. Simplifications are commonly employed to avoid the over-parameterization associated with the generic Ping

Pong Bi Bi mechanism. In regards to biodiesel production, the main interest is the transesterification of TAG due to the predominance found in fats and oils. However, the esterification of FFA should not be ignored due to the abundance in waste oils and the production of water as a by-product. To date, there are a very limited number of kinetic models that incorporate both reactions mechanisms.[89]

2.1.5 Organic solvent addition

Lipase are developing into the most widely used class of enzymes in biotechnology and synthetic organic chemistry because of their ability to catalyze a diverse range of alternative reactions in aqueous and non-aqueous media.[90] The use of organic solvents as reaction media can greatly expand the repertoire of enzyme catalyzed transformations and offers an important advantage for many industrial applications. In biodiesel production, the addition of organic solvents as the reaction medium for the enzymatic trans/-esterification may simultaneously overcome three limitations: (i) immiscibility of the hydrophobic oil (TAG & FFA) and biodiesel (FAME) and the hydrophilic alcohol (methanol) and glycerol, (ii) enzyme deactivation caused by the stripping away of essential water from the active site by highly polar alcohols, and (iii) the relatively high viscosity of oil that can lead to mass transfer limitations and flow difficulties.

The solvent effects on the micro-environment around the enzyme during trans/-esterification is shown in Figure 7.[46] In a solvent-free system, the insoluble alcohol forms an emulsion where high concentrations can encircle the enzyme molecules and strip tightly bound water that is essential for catalytic activity.[15, 42, 46, 91] In addition, the difference in polarity between TAG and methanol forms an interface around the enzyme which restricts mass transfer.[46, 91] The addition of an organic solvent alters this micro-

environment by eliminating methanol and oil emulsion while simultaneously diluting the methanol concentration within the media. This will protect enzyme activity by limiting water stripping by methanol as well as improve mass transfer by reducing the viscosity during the reaction. The addition of a hydrophobic solvent into the non-polar micro-environment can attract the hydrophobic TAG and FFA and thus increase concentration surrounding the enzyme, which is beneficial to trans/-esterification.[46, 92]

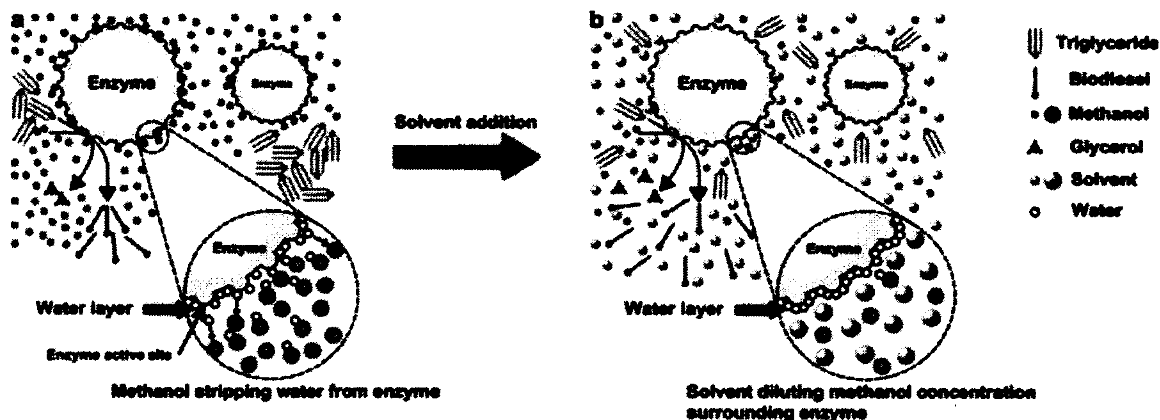


Figure 7. The effects of the micro-environment surrounding the enzymes during trans/-esterification in (a) solvent-free and (b) solvent systems.[46]

The relevance of hydrophobicity to the organic solvent effect in enzymology has been employed in the lipase-catalyzed synthesis of biodiesel. The properties and characteristics of a solvent's hydrophobicity have been determined by various parameters; such as Hildebrand solubility parameter, dielectric constant, and logarithm of the partition coefficient. The Hildebrand solubility parameter (δ) provides a good indication of a solvent's solubility and cohesion energy density. The δ equation for liquid solvents is estimated by

$$\delta = \sqrt{\frac{\Delta H_{vap} - RT}{V_m}} \quad \text{Equation 1}$$

where ΔH_{vap} is the enthalpy of vaporization, R is the gas constant, T is the reaction temperature, and V_m is the molar volume.[46, 93] Solvents with similar δ values are most likely to be miscible with each other.[46]

The dielectric constant (ε) is a measure of the solvent's ability to separate ions and is primarily used to assess the polarity of a solvent; $\epsilon > 50$ is polar, $20 \leq \epsilon \leq 50$ is considered semi-polar, and $\epsilon < 20$ is non-polar.[46] There are several methods to calculate the dielectric constant by means of computational simulations, the most widely used is

$$\frac{4\pi\langle M^2\rangle}{9kVT} = \left(\frac{\varepsilon-1}{\varepsilon}\right)\frac{2\varepsilon+1}{2\varepsilon+\varepsilon} \qquad \text{Equation 2}$$

where V is the volume of the system, k is the Boltzmann constant, and $\langle M^2 \rangle$ is the average square of the total dipole moment of the system.[93] There is a correlation between the dielectric constant of alcohols, esters, and ketones with their molecular weights (MW), where ε decreases as their MW increases.[93] The logarithm of the partition coefficient (log P) is the concentration ratio of a compound in an *n*-octanol/water two phase system and is calculated by

$$\log P = \log\left(\frac{[C]_{octanol}}{[C]_{water}}\right) \qquad \text{Equation 3}$$

where $[C]_{octanol}$ and $[C]_{water}$ refers to the concentration of un-ionized compound in the *n*-octanol and water phase, respectively.[93-94] Another method for determining log P values was developed at Molinspiration (miLog P 2.2 - November 2005) and was based on group contributions. This prediction model has been obtained by fitting calculated log P with experimental log P for more than 12,000 molecules, as well as 35 small basic fragments and 185 larger fragments.[95] An organic solvents log P value corresponds to its relative hydrophobicity; $\log P < 2$ is hydrophilic, $2 \leq \log P \leq 4$ has moderate hydrophobicity, and $\log P > 4$ is hydrophobic.[94]

Brink and Tramper[96] were the first to investigate the influence of organic solvent properties on the fundamental aspects of biocatalytic conversions. The study used the Hildebrand solubility parameter to relate the solvent polarity of propene and 1-butene on the retention of immobilized-cell activity. They concluded that higher activity was favored by low polarity ($\delta < 8$) organic solvents with a high molecular weight (MW > 150 g/mol). A groundbreaking paper by Laane *et al.*[97] investigated 107 solvents to correlate enzyme function with physicochemical properties of organic solvents, such as δ, ε, and log P. This study measured the rates of lipase-catalyzed transesterification of tributyrin with heptanol using pancreatic, yeast, and mold lipase in an organic solvent. They found no correlation between δ and ε, but did find a positive correlation between the activity of lipase and increased log P values of the solvent. This result was in agreement with Lu *et al.*[98], who investigated 12 different organic solvents on the methanolysis of glycerol trioleate with lipase *Candida* sp. 99-125 immobilized on textile fibers. They found more hydrophobic solvents (log P > 3) resulted in a higher ester yield and were more suitable solvents for enzymatic transesterification.

Soumanoua and Bornscheuer[86] investigated the enzymatic alcoholysis of sunflower oil with methanol by crude and immobilized lipases from different microorganisms in organic solvent and in a solvent-free system. The crude lipase resulted in almost no activity, with the highest conversion of 48 % after 24 h was found with *P. fluorescens* lipase. Immobilized lipase from *C. antarctica* (A & B), *P. cepacia*, *P. fluorescens*, *R. miehei*, and *T. lanuginosus* were studied their initial alcoholysis rate and conversion. They found that *R. miehei* gave the highest conversion of 96.3 % despite a low initial rate of 1.6 μmol/mg/min. The highest initial rates were found with *T. lanuginosa* and *P.*

fluorescens at 6.2 and 3.3 μmol/mg/min, respectively. In general, the immobilized lipase they tested exhibited a high conversion in non-polar solvents and significantly lower conversion with polar solvents.

A comment on the paper by Soumanoua and Bornscheuer,[86] the five commercial lipases and two in-house lipases evaluated utilized different support carriers and immobilization techniques. The significance of this lies in their testing methodology, where they use 10 % (w/w sunflower oil) to conduct their experiments. The measurement of the weight of an enzyme differs considerably when compared to the activity of an enzyme. Weight depends on size, weight, and surface area of the support carrier as well as number of lipase immobilized to it. Enzyme activities are determined by measuring the amount of product that is formed when an enzyme acts upon a specific substrate and are a much better comparison than weight. For this reason, a unit of enzyme activity was defined in 1964 by the International Union of Biochemistry; 1 unit (U) is the amount of enzyme activity which will catalyze the transformation of 1 μmole of the substrate per minute under standard conditions of temperature, optimal pH, and optimal substrate concentration.[99] The exact definition may differ by method, procedure, substrates, reaction conditions, and manufacturer. Therefore, it is imperative to ensure a uniform definition for enzyme activity during evaluations. Assay methods have been developed for determining enzyme activity by testing the free fatty acid formed under specific conditions. In general, the tributyrin titration assay is the most commonly used method for estimating lipase activity. Other lipase assay procedures are spectrophotometric, radiolabeling, fluorescence, HPLC, and surface tension assays.[100]

In fairness, Soumanoua and Bornscheuer[86] are not the only researchers that study enzyme activity by weight comparisons. Tamalampudi et al.[101] reported that whole cells of lipase producing *Rhizopus oryzae* can catalyze the alcoholysis of Jatropha oil more effectively than Novozym 435. This study immobilized *R. oryzae* lipase onto biomass support particles (BSPs) made of reticulated polyurethane foam and compared 0.2 g to the same weight of Novozyme 435, which was the basis of their claim. A more recent study, Mendes et al.[102] evaluated five microbial lipases on poly-hydroxybutyrate beads to catalyze biodiesel synthesis. The study did measured the hydrolytic activity (U/g) of the immobilized enzymes, however, they disregarded this activity when evaluating the catalytic activity for trans/-esterification and used 10 wt. % in relation to the total weight of reactants involved in the reaction media. These are just a few examples of many that incorrectly compare the effects of support carriers and enzymes for the synthesis of biodiesel.

In a previous study, Fu and Vasudevan[46] investigated the effect of organic solvents on the enzymatic transesterification of fresh canola oil. The study evaluated 28 hydrophilic and hydrophobic solvents from 7 organic groups as possible media in the transesterification of canola oil by *Candida antarctica* lipase B immobilized on macroporous resin (Novozyme 435). Their results coincided with the other studies that found a positive correlation with enzymatic activity and overall yield with a solvents log P value, but no correlation with δ and ε. The ideal solvent in this study appeared to be isooctane due to its hydrophobicity (log P = 3.668) and its unique three methyl side-chain molecular structure. Relatively high product yields were also obtained with several

hydrophilic solvents possessing high miscibility with methanol, such as dimethoxyethane, methyl iso-propyl ketone (MIPK), and acetone.

In this chapter, we examined the effect of 15 organic solvents on the enzymatic trans/-esterification of used soybean vegetable oil. Solvents were selected from four distinct functional groups: alkanes, ketones, ethers, and cyclic hydrocarbons. Evaluation of the solvent's log P was used to correlate hydrophobicity of the solvents with corresponding biodiesel yields. In addition, the effect of solvent on trans/-esterification activity using three different lipases from *Candida antarctica* (lipase B), *Pseudomonas cepacia*, and *Thermomyces lanuginosus* were also investigated.

2.2 Experimental Section

2.2.1 Materials

Used soybean vegetable frying oil was obtained from local restaurants and contained 2.3 wt.% FFA as determined by titration and confirmed by NMR analysis.[103] *Pseudomonas cepacia* lipase immobilized on Immobead 150 with an activity of 900 U/g; *Thermomyces lanuginosus* lipase immobilized on Immobead 150 with an activity of 3000 U/g, and *Candida antarctica* lipase B immobilized on Immobead 150 with an activity of 5500 U/g, (in each case, 1 U corresponds to the amount of enzyme that liberates 1 μmol butyric acid per minute at pH 7.5 and 40 °C with tributyrin, Fluka No. 91010, as substrate),[104] methyl oleate (> 98 %), ACS grade toluene (99.5 +%), 1,2-dimethoxyethane (≥ 99 %), 4-heptanol (98 %), dodecane (99 +%), anhydrous dibutyl ether (99.3 %), decane (≥ 99 %), HPLC grade 2,2,4-trimethylpentane (isooctane), and 2-butanone were all purchased from Sigma-Aldrich Co. Reagent grade cyclohexane, ACS grade mixed-xylenes, HPLC grade methanol, and methyl-tert butyl ether (MTBE) were

purchased from Fisher Scientific. Methyl acetate (98 %), extra dry n-hexane (96 %), 3-methyl-2-butanone (MIPK), anhydrous diisopropyl ether (99.0 %), and 1,4-dioxane (99.0 %) were purchased from Acros Organics. Acetone was purchased from EMD Chemicals Inc.

2.2.2 Biodiesel synthesis

The used vegetable oil was heated to 100 °C and gravity filtered through an 11.0 cm Whatman 40 filter paper to remove particulates. Davison molecular sieves with a 4 Å effective pore size (grade 512) were added to the oil to maintain anhydrous conditions. The addition of water can enhance enzyme activity and stability in general. However, in this case, the presence of water can also facilitate the hydrolysis of esters, which will reduce the product yield. The main reason for the use of the molecular sieves was to remove any excess water present in the waste oil. Reactions took place in 40 mL glass vials with PTFE/silicone septa from Kimble, Vineland, NJ, USA. The reaction system contained 500 U enzyme particles, 4 mL organic solvent, 1 mL used vegetable oil, and a molar ratio of methanol to oil of 3.0 (125 μL methanol). The reaction was maintained at a constant temperature of 40 °C and a stirring speed of 250 rpm. The reaction was stopped after 24 h.

2.2.3 Analysis method

Samples of 30 μl were collected at 4 h, 8 h, and 24 h, respectively, and then diluted with 1 ml isooctane for GC analysis.[46] The concentration of fatty acid methyl esters (FAME) was measured by a HP 5890 gas chromatograph 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 this value. The temperatures of injector and flame ionization detector were maintained constant at 275 °C. Methanol was selected as the acyl-acceptor in the trans/-esterification reaction due to its cost, purity, and industrial use. Therefore, the corresponding biodiesel formed were fatty acid methyl esters (FAME). Oleic acid methyl ester (methyl oleate) was a specific FAME used as the biodiesel standard for GC analysis to generate a calibration curve, as shown in Appendix A. GC analysis indicated the peak of methyl esters appeared around 17 – 19 min, as shown in Figure 8. The concentration of FAME in the sample was calculated based on the peak area of methyl ester and the corresponding calibration curve. The yield of FAME was determined as the mass of FAME produced per initial mass of used vegetable oil added (g of FAME/g of oil). Nonlinear regression was used to fit the data by minimizing the sum of squares of error between the data and the model's predictions, similar to the Michaelis–Menten model, to produce a hyperbolic curve using Polymath 6.10 software. Sample calculations of GC analysis and FAME yield are shown in Appendix B.

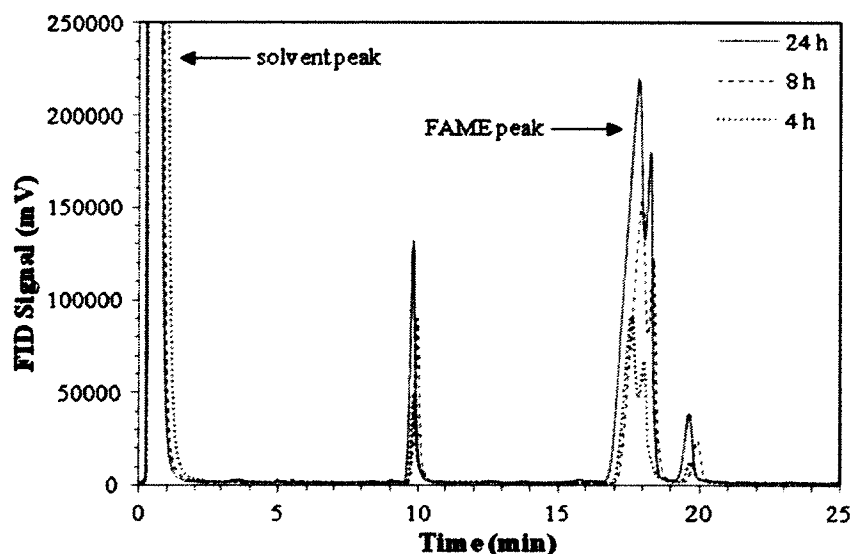


Figure 8. GC analysis of FAME peaks produced in the enzymatic trans/-esterification at 4 h, 8 h, and 24 h, respectively.

2.2.4 Enzyme selection

The following microbial strains of lipases have been found to have transesterification activity: *Candida antarctica*[105] (CA), *Pseudomonas cepacia*[106] (PC), and *Thermomyces lanuginosus*[45] (TL). The following criteria were used to ensure uniformity in comparison: (i) all three enzymes were purchased from Sigma-Aldrich ensuring similar immobilization procedures and techniques, (ii) immobilization was on the same support carrier, Immobead 150, which is a macroporous acrylic polymer bead that was covalently bound to the enzyme and having a particle size 150 – 300 μm,[107] and (iii) a standard enzymatic activity was maintained constant at 500 U for each enzyme for each experimental runs.

2.2.5 Solvent selection

The selection criteria for the organic solvents were based on the following characteristics: (i) functional group: several solvents were selected from alkanes, ketones, ethers, and cyclic hydrocarbons; (ii) boiling point: typical reaction temperatures for enzymatic processes is 20 – 60 °C,[42] and consequently, the solvents selected had a boiling point above this range, (iii) viscosity: typically, biodiesel has a dynamic viscosity of 4 – 6.0 cP at 20 to 40 °C,[46] and soybean oil has a viscosity of 31.8 cP at 37.8 °C;[108] therefore, the desired viscosity for a solvent should be 4 cP or lower, and (iv) partition coefficient (log P) or solvent hydrophobicity, due to the correlation between log P value of a solvent and biodiesel yield.[42, 46, 97] Organic solvents were chosen with log P values between 0 and 7, as shown in Table 3. Solvents with a negative log P value are too hydrophilic and aggressively strip away essential water from the enzyme and were not considered for evaluation.

Table 3. Log P values of organic solvents.[95]

ALKANES		KETONES	
Solvent	**Log P**	**Solvent**	**Log P**
n-Hexane	3.657	Acetone	0.234
Isooctane	3.668	Butanone	0.736
Decane	5.678	MIPK	0.980
Dodecane	6.689	4-Heptanone	2.358
ETHERS		**CYCLIC HYDROCARBONS**	
Solvent	**Log P**	**Solvent**	**Log P**
Dimethoxyethane	0.093	Toluene	2.386
MTBE	1.482	Xylenes	2.821
Diisopropyl ether	1.775	Cyclohexane	3.205
Diisobutyl ether	3.173		

2.3 Results and Discussion

2.3.1 Solvent effect of alkanes

Alkane solvents are saturated compounds that are strongly hydrophobic (high log P), have excellent solubility for organic compounds, and are chemically stable.[93] The effects of four alkanes were investigated, namely *n*-hexane, isooctane, decane, and dodecane on each of the three lipases, as shown in Figure 9. Lipase CA, PC, and TL obtained the highest FAME yields of 0.804 g/g oil, 0.850 g/g oil, and 0.866 g/g oil, respectively, with *n*-hexane as solvent. However, it is evident from Figure 9 that the fastest reaction rates were obtained with isooctane as solvent. In the case of lipase PC, the addition of isooctane accelerated the reaction rate so that the maximum FAME yield was obtained in about 4 h where the other solvents achieved this in about 8 h.

The addition of an organic solvent into the reaction media can significantly increase the reaction rate for enzymatic catalysis over solvent free systems. Previous studies of immobilized lipase from CA,[85] PC,[109] and TL[110] in solvent free systems have required 48 h, 24, and 48 h, respectively, to reach their maximum potential yields. Even though lipase PC with isooctane indicated the fasted reaction rate, it is believed that lipase TL

has the best performance of the three enzymes with isooctane and *n*-hexane. This was due to relative reaction rate, overall yield of FAME, and enzyme activity, where lipase TL has 3000 U/g and lipase PC has 900 U/g. Lipase CA showed much lower reaction rates with alkane solvents and required a total reaction time of 24 h to obtain yields equivalent to that of lipase PC and TL. The lag observed with dodecane may be attributed to reduction in mass transfer due to its relatively higher viscosity.

2.3.2 Solvent effect of ketones

Solvents with the ketone functional group are widely used in industry as solvents and as biotransformation reaction media.[111] The effects of four ketone solvents, namely acetone, butanone, MIPK, and 4-heptanone, on biodiesel yield were investigated (Figure 10). The highest FAME yields of 0.660 g/g oil and 0.569 g/g oil were achieved with 4-heptanone on lipase PC and TL, respectively. Lipase CA obtained similar high yields with 4-heptanone and MIPK of 0.634 g/g oil and 0.645 g/g oil, respectively. The higher yields with 4-heptanone are most likely due its higher log P value in comparison to the other ketone solvents, as shown in Table 3. The higher log P value minimizes the solvent's ability to strip the enzymes of essential water. Whereas acetone and butanone were more hydrophilic with the lowest log P values and had a strong tendency to strip the essential water from the enzyme's active sites, resulting in a lower yield. This was especially prevalent for lipase TL, which showed almost no enzyme activity for trans/-esterification in these two solvents.

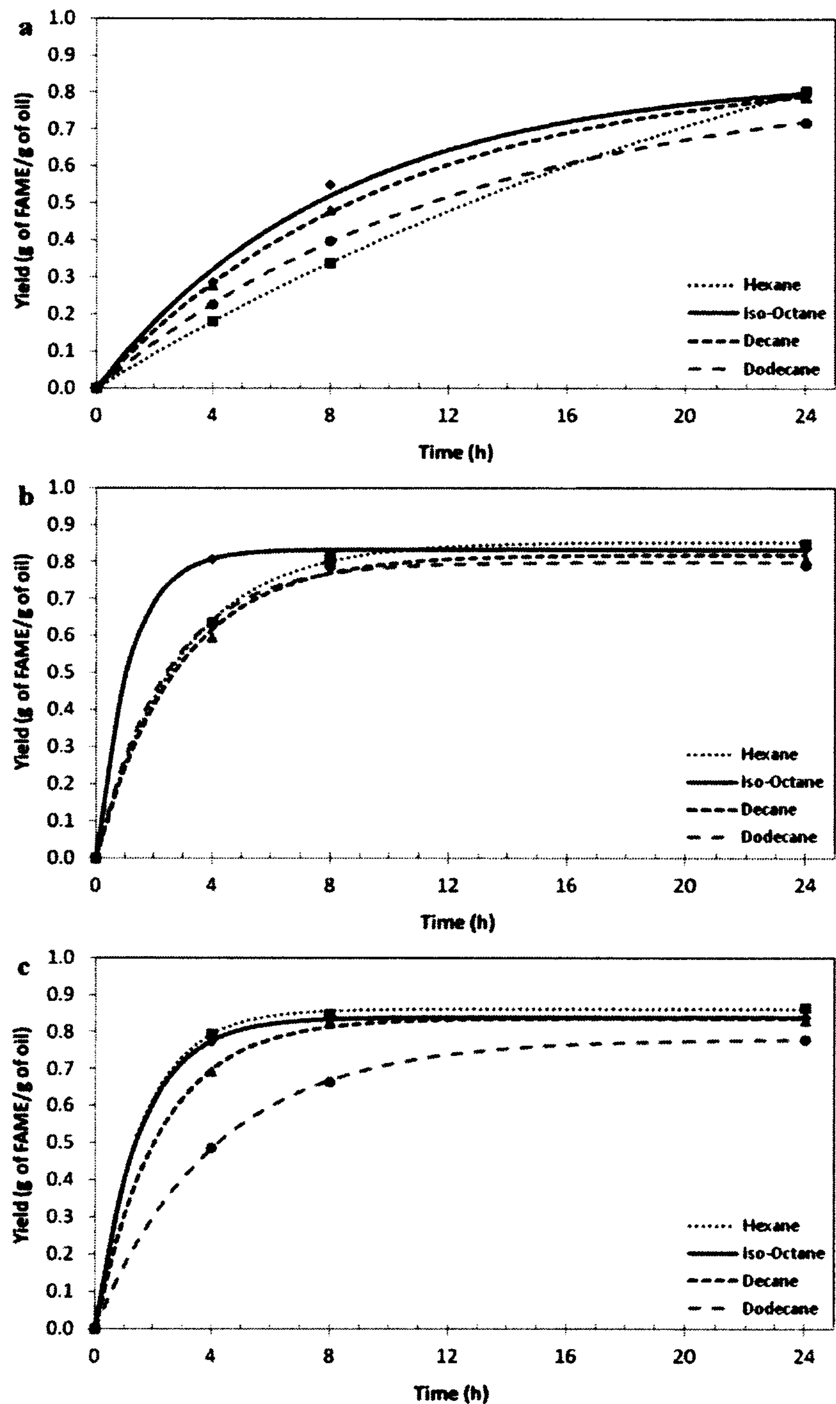


Figure 9. Effect of alkane solvents on the enzymatic transesterification of used vegetable oil. Reaction conditions: 1 mL of used vegetable oil, 4 mL of solvent, 500 U of lipase, 40 °C, stir speed of 250 rpm, and 3.0 mol equivalent of methanol (125 μL). (a) *Candida antarctica* lipase, (b) *Pseudomonas cepacia* lipase, (c) *Thermomyces lanuginosus* lipase.

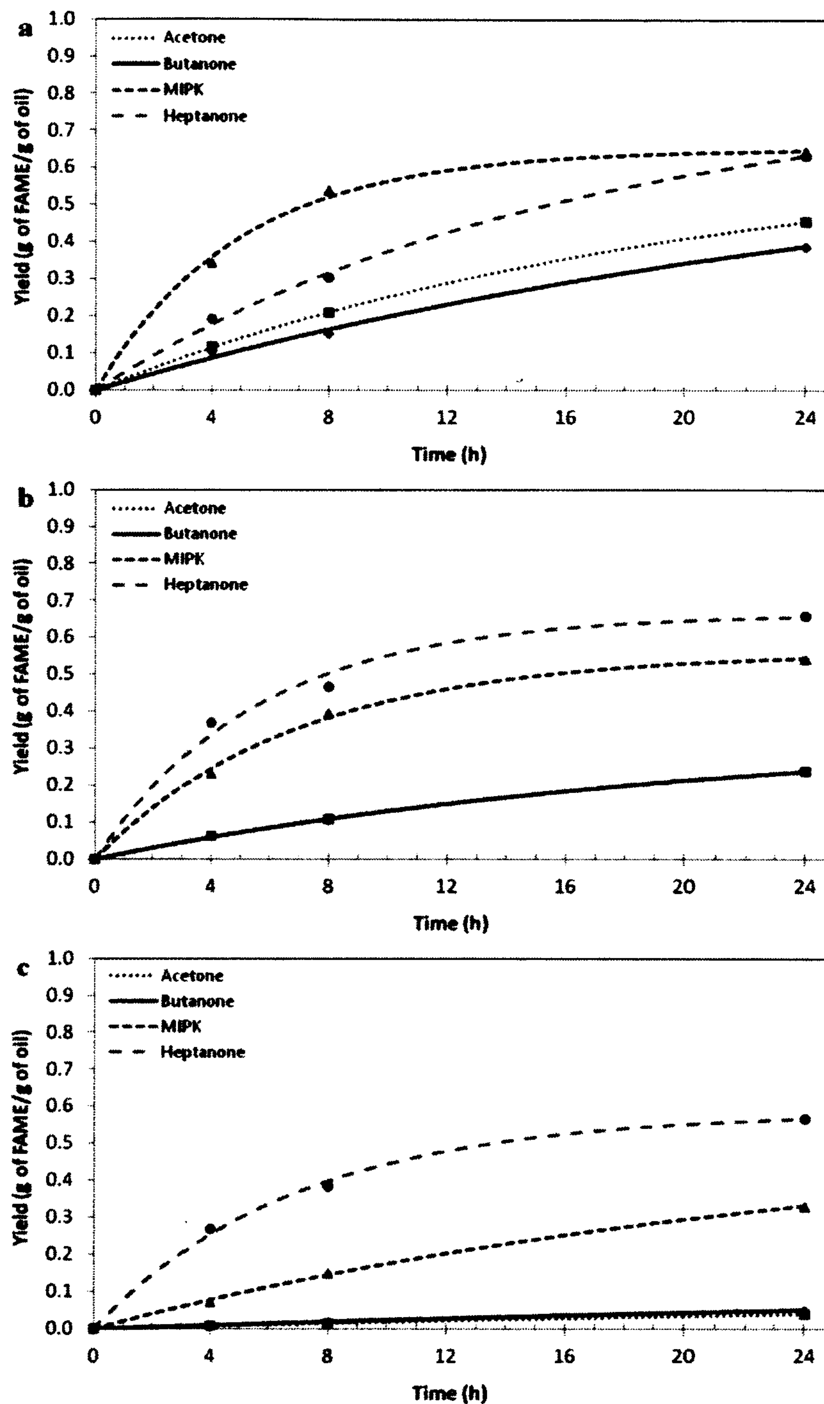


Figure 10. Effect of ketone solvents on the enzymatic transesterification of used vegetable oil. Reaction conditions: 1 mL of used vegetable oil, 4 mL of solvent, 500 U of lipase, 40 °C, stir speed of 250 rpm, and 3.0 mol equivalent of methanol (125 μL). (a) *Candida antarctica* lipase, (b) *Pseudomonas cepacia* lipase, (c) *Thermomyces lanuginosus* lipase.

2.3.3 Solvent effect of ethers

Ether solvents are widely used for a variety of organic compounds and reactions due to their relatively non-reactive nature.[111] The effects of four ether solvents on biodiesel yield were investigated, namely dimethoxyethane, MTBE, diisopropyl ether, and diisobutyl ether, as shown in Figure 11. The ether solvents in this study covered the largest span of hydrophobicities (log P 0.093 – 3.173). The effect of hydrophobicity can be shown with lipase PC and to a greater extent with lipase TL. These lipases obtained a wide range of biodiesel yields between 0.180 – 0.751 g/g oil and 0.027 – 0.763 g/g oil, respectively. The highest yields of both of these lipases were with diisobutyl ether as the solvent, which had the highest log P value of the ketone solvents. Whereas, the FAME yield was in the range 0.604 – 0.744 g/g oil with lipase CA, which indicated that the hydrophobicity of the four ketone solvents does not appear to have much of an effect on enzyme activity.

It was interesting to note that dimethoxyethane gives a relatively high FAME yield with lipase CA and was similar to what has been reported by Fu and Vasudevan[46] with fresh canola oil. However, dimethoxyethane had resulted in very low yields using lipases PC and TL. This indicated that specific biocatalysts have distinct tolerances for the same organic solvent with respect to their particular active sites, but the exact reason for the difference is still unclear. The overall FAME yield and reaction rate with diisopropyl and diisobutyl ethers were fairly similar to each other for all three lipases, although lipase PC and TL had almost twice the reaction rate than lipase CA. Interestingly, diisobutyl ether had a log P value almost twice that of diisopropyl ether. Therefore, the structure of the ether molecule may play a more important role than the solvent's hydrophobicity.

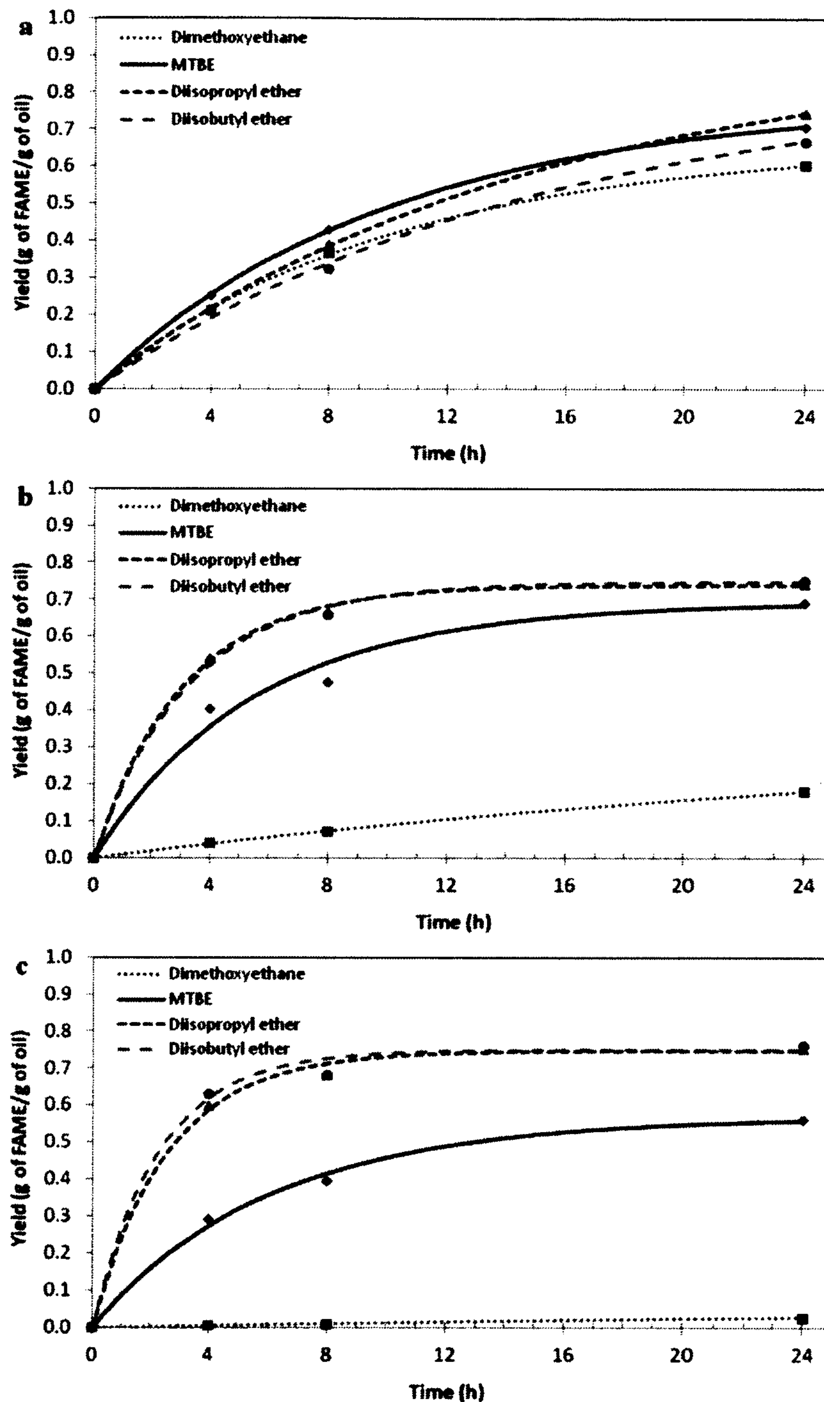


Figure 11. Effect of ether solvents on the enzymatic transesterification of used vegetable oil. Reaction conditions: 1 mL of used vegetable oil, 4 mL of solvent, 500 U of lipase, 40 °C, stir speed of 250 rpm, and 3.0 mol equivalent of methanol (125 μL). (a) *Candida antarctica* lipase, (b) *Pseudomonas cepacia* lipase, (c) *Thermomyces lanuginosus* lipase.

2.3.4 Solvent effect of cyclic hydrocarbons

A vast number of organic solvents are cyclic compounds with a six-carbon ring and are commonly used throughout industry.[93] The effects of three common cyclic solvents on biodiesel yield were investigated, two of which were aromatic hydrocarbons (toluene and mixed-xylenes) and the third a cycloalkane (cyclohexane), as shown in Figure 12. Cyclohexane resulted in the highest overall FAME yield of 0.559 g/g oil, 0.870 g/g oil, and 0.849 g/g oil for lipase CA, PC, and TL, respectively. In addition, the fastest reaction rates were also seen with cyclohexane with all three lipases and had a similar overall performance as the alkane solvents.

It was interesting to note that the aromatic solvents performed comparably to each other but differed greatly depending on the enzyme. Lipase TL showed an enzymatic activity that coincides with cyclohexane and non-cyclic alkanes. Whereas, lipase CA demonstrated a much lower reaction rate and FAME yield in comparison to cyclohexane. The benzene ring of the aromatic hydrocarbon may have interfered with the active site of lipase CA causing reduced enzyme activity. Lipase CA does not have a lid to protect its active site and may be the reason why this effect was not be seen on the other two lipases. Lipase PC seems to fall somewhere in between the performances of lipase CA and TL with moderate reaction rates and yield but noticeably lower than cyclohexane.

2.3.5 Solvent effects on biodiesel synthesis

Organic solvents can have a remarkable influence on the enzymatic trans/-esterification of used vegetable oil with methanol. The yields of FAME varied greatly depending on the choice of solvent that ranged from 0.125 – 0.804 g/g oil, 0.180 – 0.869 g/g oil, and 0.027 – 0.866 g/g oil from lipases CA, PC, and TL, respectively. The study

indicated that isooctane and *n*-hexane to be the best choice of organic solvent based on reaction rate and overall FAME yield for all three enzymes.

The relationship between the log P of a solvents and the corresponding FAME yield after 24 h was presented in Figure 13. There appears to be no clear correlation between log P and yield with lipase CA which indicated that the hydrophobicity of organic solvents does not have much of an effect it enzyme activity. However, despite their moderate log P values, it appears that aromatic hydrocarbons containing a benzene ring may interfere with enzyme activity probably caused by the lack of a protecting lid.

A positive correlation between log P of solvent and yield was observed with lipase PC and TL. Strongly hydrophilic solvents with a log $P < 1$ resulted in a low yield, as the solvents become more hydrophobic and as log P increased the FAME yield increased until it leveled off at a relatively constant value with a log $P > 3$. The lower yields obtained with hydrophilic solvents with a log $P < 2$ were probably due to essential water being stripped from the enzyme's active sites. In addition, interfacial activation of the lid may play a more critical role in the solvent effects, where hydrophilic solvent result in a more closed configuration. The hydrophobic solvents would maintain an open conformation and thus allowing full access to the enzymes active site. This could also explain why lipase CA does not appear to be affected by a solvents' hydrophobicity. Lipase TL exhibited significant adverse effects to solvents that were very hydrophilic with a log $P < 1$. The highest yields were with *n*-hexane and isooctane, which were more hydrophobic (log $P > 3$). There was a slight decrease in FAME yield with strongly hydrophobic solvents with a log $P > 5$. This may be due to immiscibility issues between the solvent and methanol, where small emulsions were formed.

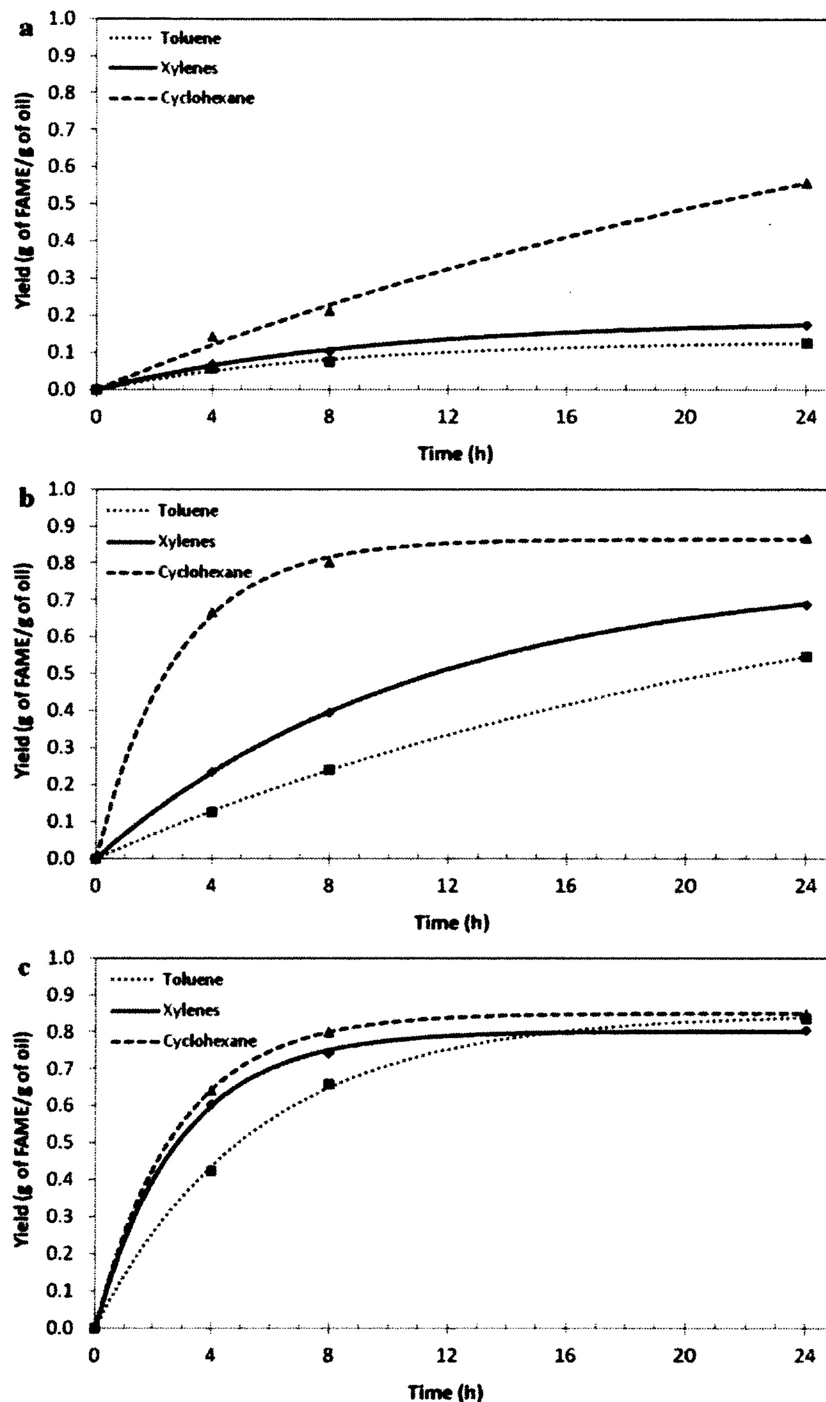


Figure 12. Effect of cyclic hydrocarbons solvents on the enzymatic transesterification of used vegetable oil. Reaction conditions: 1 mL of used vegetable oil, 4 mL of solvent, 500 U of lipase, 40 °C, stir speed of 250 rpm, and 3.0 mol equivalent of methanol (125 µL). (a) *Candida antarctica* lipase, (b) *Pseudomonas cepacia* lipase, (c) *Thermomyces lanuginosus* lipase.

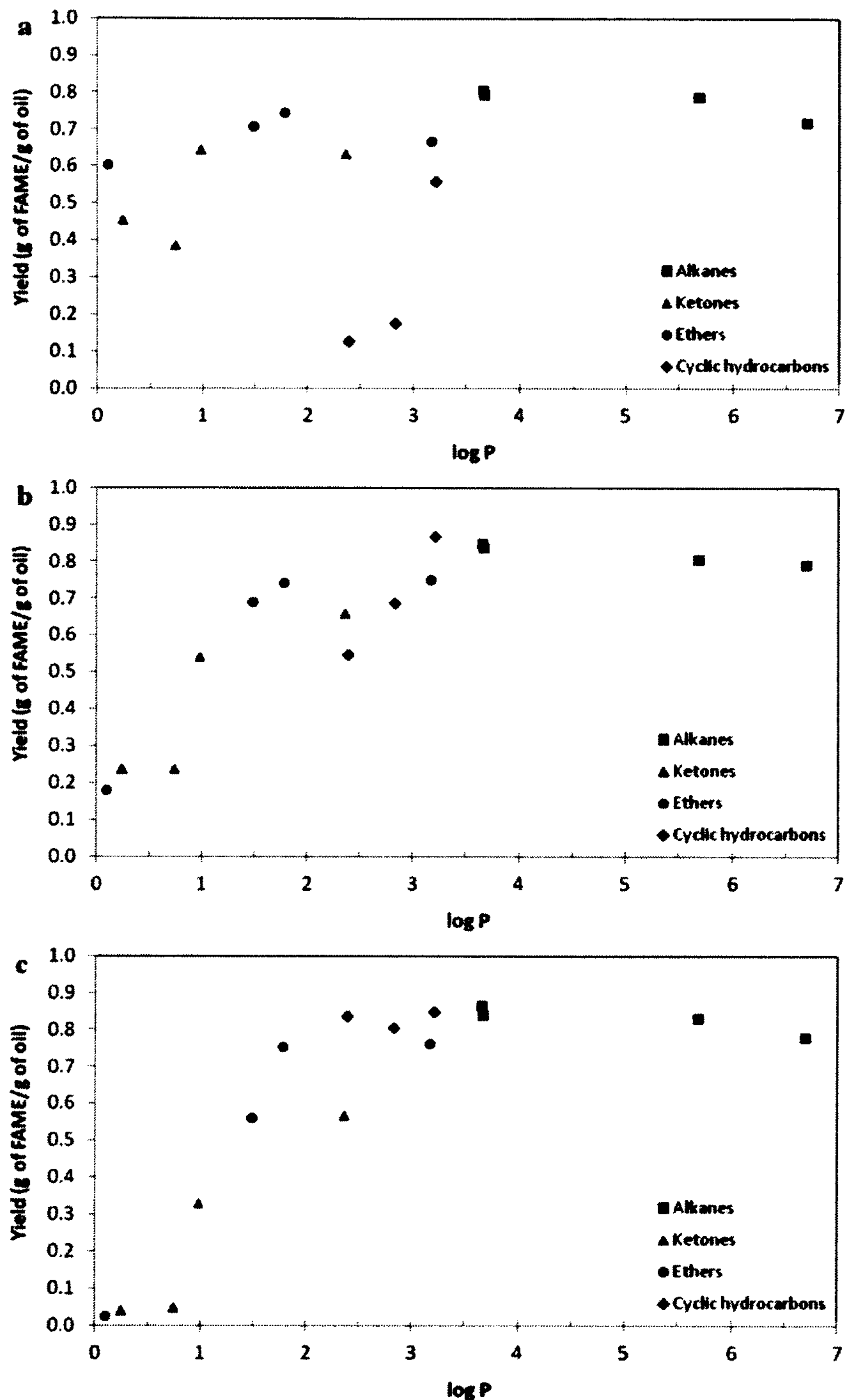


Figure 13. Scatter plot of log P's of solvents with the corresponding FAME yield from the transesterification of used vegetable oil after 24 h. Reaction conditions: 1 mL of used vegetable oil, 4 mL of solvent, 500 U of lipase, 40 °C, stir speed of 250 rpm, and 3.0 mol equivalent of methanol (125 μL). (a) *Candida antarctica* lipase, (b) *Pseudomonas cepacia* lipase, (c) *Thermomyces lanuginosus* lipase.

An optimum reaction system should include a solvent that will enhance the rate of trans/-esterification by improving the solubility of methanol, oil, and glycerol byproduct. All three enzymes showed an optimum log P between 3.2 – 3.6, which resulted in the highest biodiesel yield. In general, these hydrophobic organic solvents protect the enzyme from being denatured by methanol, maintain proper enzyme conformation, and allow the acyl acceptor to diffuse to the enzyme's active sites to react.

2.3.6 Enzyme effects on biodiesel synthesis

The choice of enzyme can significantly affect the reaction rate and FAME yield and can depend heavily on the selection of an organic solvent. While the actual mechanism of catalysis may be similar, there are structural differences in the enzymes' active sites. The sources of lipases evaluated have different regioselectivities for the TAG substrate; *Candida antarctica* lipase B[112] and *Pseudomonas cepacia* lipase[69] are non-position specific and *Thermomyces lanuginosus* lipase[113] is a 1,3-position specific.

However, it has been reported that lipase PC is much more 1,3-specific[114] than lipase CA.[115] This may be due to the presence of a protective lid in lipase PC whereas lipase CA does not. In addition, the substrate binding pocket of lipase CA is a steep elliptical funnel, in contrast to lipase PC that does not have a narrow hydrophobic binding site and has a more shallow alcohol binding site.[116] These features of lipase PC over lipase CA may allow quicker access for substrate binding that resulted in the faster reaction rates. It has been reported that lipase CA has a large stiff binding pocket for catalysis, while lipase TL has a smaller, more flexible, active site.[117] The flexibility of an enzyme's active site may allows for faster binding of the substrate, which is indicated by the faster reaction rates of lipase TL over lipase CA. The substantial reorientation and secondary

structural adjustments of the binding pocket required for catalysis means that lipase TL is more susceptible to the hydrophobicity of its environment. This could possibly explain why the more hydrophilic solvents (log $P < 1$) significantly reduced the yield of FAME.

More hydrophobic solvents like alkanes have resulted in the highest yields of FAME for all three enzymes. The results obtained with lipase PC and TL displayed many similarities between them, with the exception of aromatic hydrocarbon solvents and to some extent ketone solvents. The most striking difference with the enzymes studied was the reaction rate and the time required to achieve the maximum possible yield. This time for lipase PC and TL was typically between 4 – 8 h in an alkane solvent, whereas it took around 24 h for lipase PC. The reduction in reaction time is crucial for industrial implementation and economic feasibility.

2.4. Conclusions

In this chapter, the effects of organic solvents and enzyme source on the enzymatic trans/-esterification of used vegetable oil with methanol were investigated. Fifteen hydrophilic and hydrophobic solvents with four distinct functional groups were evaluated on immobilized lipase from *Candida antarctica*, *Pseudomonas cepacia*, and *Thermomyces lanuginosus*. The results indicated that the choice of solvent can have a tremendous effect on both the reaction rate and FAME yield. The yield varied greatly depending on the solvent, ranging from 0.125 – 0.804 g/g oil, 0.180 – 0.869 g/g oil, and 0.027 – 0.866 g/g oil from lipases CA, PC, and TL, respectively. The reaction rate also varied depending on the solvent selected, with the time to attain equilibrium ranged from 4 h to >24 h. Alkane solvents were able to obtain the highest FAME yields and fastest reaction rates for all three lipase, with isooctane and *n*-hexane being the best performers.

There appeared to be a correlation between the solvent's hydrophobicity (log P) and overall FAME yield with the lipase PC and TL. However, no correlation could be made with lipase CA. In general, yield of FAME increased with an increase in log P value, implying that the solvent's hydrophobicity plays a more important role in enzyme activity than its distinct functional group. However, with certain solvents, the structure of the molecule may play a significant role compared to the hydrophobicity of the solvent alone. In some instances, a specific biocatalyst may have a distinct tolerance for a certain solvent. The choice of lipase can have a considerable effect on the reaction kinetics and FAME yield. This can be due to mechanistic and structural difference between proteins from different organisms. In addition, the hydrophobicity of an enzyme's environment can cause conformational changes that alter enzymatic activity. Of all three enzymes evaluated, the best performance was exhibited by lipase TL with isooctane or n-hexane due to its relative reaction rate, overall yield of FAME, and enzyme activity.

The production of biodiesel by enzymatic trans/-esterification has many industrial and environmental advantages. The addition of a moderately hydrophobic organic solvent to the reaction media can improve substrate immiscibility, mass transfer, and enzymatic activity. It can also dramatically improve the reaction rate and obtain high FAME yields > 0.85 g/g oil with 4 – 8 h, in comparison to solvent-free systems that require > 24 h. This minimizes one of the major economic hurdles associated with the enzymatic process, reaction time. However, enzymes like *Thermomyces lanuginosus* are expensive to produce in sufficient quantities by traditional methods. Novel biotechnological approaches using genetic engineering could provide a means to develop alternative methods to produce inexpensive enzymes in large quantities.

CHAPTER 3

TRANSGENIC EXPRESSION OF LIPASE IN PLANTS FOR THE ENZYMATIC PRODUCTION OF BIODIESEL

3.1 Introduction

3.1.1 Plant biotechnology

Over the past few decades, biotechnology has made significant progress through the application of scientific and technical advances in molecular biology. Novel biotechnological approaches provide a variety of innovative tools to manipulate biomolecular structures and properties for advanced applications; such as genetic engineering, metabolic engineering, protein and enzyme engineering, and recombinant DNA technology. Biopharmaceuticals produce drugs and medicines through the application of biotechnology and are currently the fastest growing segment of the pharmaceutical industry, accounting for approximately 10% of the pharmaceutical market in 2007.[118] Demands for vaccines, antibodies, and other medicines have increased sharply over the past decade. A limited manufacturing capacity has resulted in the recent shortages of important medicines; such as the H1N1 vaccine during the "swine flu" pandemic in late 2009.[118] Most biopharmaceuticals are produced using bacterial cells, like *Escherichia coli* (*E. coli*), or mammalian cells, like Chinese hamster ovary (CHO) cells.[119] However, the ability to identify novel genes, clone them, and alter their sequences with ease allows for a more versatile expression systems with the capability for incredible large scale production, such as plants.

In 1990, Sijmons *et al.* were the first to produce a plant-derived pharmaceutical protein (human serum albumin) in transgenic tobacco and potato plants.[120] Recent advances in plant biotechnology and genomics have resulted in the alteration of plants in the areas of nutrition, herbicide resistance, insect tolerance, and abiotic stress tolerance. Biotechnology has also allowed the production of large amounts of selected proteins including animal, human and bacterial proteins of commercial value.[47] A critical technology required for these modifications is plant genetic transformation. Various methods for stable introduction of novel genes into the nuclear genomes have been developed. Plant transformation has been successful with hundreds of different plant species, including tobacco, *Arabidopsis thaliana*, tomato, maize, wheat, grape, and rice.[121] The techniques for producing transgenic plants are commonly divided into two groups: indirect and direct methods. Indirect methods refer to the use of bacterial systems, such as *Agrobacterium*-mediated transformation. Direct methods are physical in nature and based on gene insertion by penetrating the plant's cellular wall.

The indirect method uses specialized bacteria to introducing specific DNA fragments into plant genomes to create transgenic plants. One such technique that is commonly used today is *Agrobacterium*-mediated transformation, which is most widely used with dicotyledonous crops. The gram-negative soil bacteria, *Agrobacterium tumefaciens*, naturally developed the ability to transfer a particular DNA segment into plant genome.[47] This exploits the plant's own bio-machinery to produce specific nutrients (opines and agropines) that can only be metabolized by the *Agrobacterium*, causing crown gall tumors.[122] The bacterium contains a large tumor-inducing plasmid (Ti plasmid) that consists of two main sets of genes that are vital for tumorigenesis. One set involves the

transfer of a defined segment of DNA from the bacteria and integrated into the plant genome, called the transfer-DNA or T-DNA. The T-DNA region is located between left and right border sequences that consist of 24 bp imperfect repeats and contain the genes for directing opine biosynthesis and uncontrolled cell division.[47] The other set is the virulence (*vir*) genes which are responsible for the process for transferring T-DNA from the bacterium into the plant cell. The *vir* genes consist of at least nine genetically identified operons. Wounded plant cells release phenolic compounds (such as acetosyringone) and sugars (monosaccharides), which are sensed by the *vir*A protein, causing it to autophosphorylate. This subsequently phosphorylates and thereby activates the *vir*G protein which induces expression of all the other *vir* genes located on the Ti plasmid to facilitate T-DNA transfer.[47, 122]

Great progress has been made in understanding the mechanisms of gene transfer in *A. tumefaciens*. Scientists have been able to modify the T-DNA by removing most of the natural genetic information and replacing it with desired genes to encode proteins of interest in plants. Curtis *et al.*[123] and Nakagawa *et al.*[124] have developed a series of new vector systems for *Agrobacterium*-mediated transformation. These vectors have been developed to facilitate fast and reliable DNA cloning with a collection of functional parameters; such as different promoters, terminators, and antibiotic resistances, as well as allowing gene fusion of a variety of reporter/purification tags. Another alternative to *A. tumefaciens* is *Agrobacterium rhizogenes* which infects the root system of a plant with a phenomenon called hairy roots. Root-inducing (Ri) plasmids are found in *A. rhizogenes* and a variety of vector systems have been constructed by researchers to take advantage of its root producing properties. There are a very limited number of organisms like

Agrobacterium capable of inter-kingdom gene transfer. However, non-*Agrobacterium* gene transfer systems are being explored using other microorganisms. A recent patent (US 7,888,552 B2) uses *Rhizobia*-mediated transformation of various crops, such as soybean, canola, corn, and cotton with bacterial strains *Rhizobium* sp., *Sinorhizobium* sp., and *Mesorhizobium* sp.[125]

The second method developed to transform plants directly penetrates the cell of the plant with a large amount of DNA. Direct gene transfer methods originated in the 1980's and there are several different technologies available that are commonly used today. One of the most commonly used methods is biolistics, also known as particle bombardment or gene gun technique, and was initially developed in 1987 at Cornell University.[121] This technique utilizes the acceleration of high density carrier particles covered with exogenous DNA into the plant cell and can be employed for nuclear and chloroplast transformation. The initial delivery system consisted of firing a .22 caliber nail gun cartridge as a propellant. Today a more sophisticated system is used, consisting of a high and low pressure chamber separated by a rupture disc and compressed helium or N_2.[47] One of the first to use this technique was Klein *et al.* who successfully expressed the β-glucuronidase gene in a tobacco plant and incorporate a chloramphenicol acetyltransferase gene into maize using biolistic transformation.[126] Since then, many other transgenic plants were successfully created using biolistic transformation, such as wheat, poplar, rice, algae, and soybean.[121] Other direct methods used by researchers are electroporation, vacuum infiltration, ultrasound, silicon carbide fibers, microinjection, macroinjection, laser microbeams, and electroporation.[47, 121]

3.1.2 Genetically enhancing oil accumulation

Lipid biosynthesis involves the formation of TAG as an energy reserve in developing seeds, pollen grains, anthers, flower petals, and fruit mesocarp in a number of plant species.[127] Vegetable oils are commonly used for human and animal consumption, along with chemical industries for the production of soaps, cosmetics, paints, lubricants, etc.[128] As shown in Figure 14, the metabolic pathway involved in the biosynthesis of TAG are comprised of three major steps: (1) fatty acid (FA) synthesis in the plastids (2) FA acyl chain elongation and the development of a fatty acyl-CoA pool in the cytoplasm and (3) TAG formation in the endoplasmic reticulum (ER).[127-129]

The first step takes place *de novo* in the stroma of the plastid, where FAs are synthesized using acetyl coenzyme A (acetyl-CoA) as the initial precursor and is carboxylated to form malonyl-CoA by the enzyme acetyl-CoA carboxylase (ACC) plus ATP. The malonyl CoA is linked to acetyl-CoA by the enzyme malonyl-CoA:ACP transacylase and transferred to an acyl carrier protein (ACP) of the fatty acid synthase (FAS) multi-enzyme complex.[129] In the second step, acetyl CoA undergoes a condensation reaction with malonyl-ACP to form 4-carbon intermediates and FA elongation occurs through the sequential addition of 2-carbon sub-units, catalyzed by FAS.[127] The synthesis ends by hydrolysis of the acyl moiety by thioesterase or the formation of a double bond on the acyl moiety by acyl-ACP desaturase.[47, 127-128] The final FAs are generally 16 or 18 carbons long and form the core of the fatty actyl-CoA pool within the cytoplasm. In the third step, TAG is formed in the ER and involves the acylation of glycerol 3-phosphate (G3P) with an actyl-CoA to form lysophosphatidic acid (LPA) catalyzed by glycerol 3-phosphate acyltransferase (GPAT).[47, 128] LPA is then

further acylated to form phosphatidic acid (PA) by lysophosphatidic acid acyltransferase (LPAAT). PA is then dephosphorylated by phosphatidic acid phosphatase (PAP) to form diacylglycerol. The last FA is transfered from the actyl-CoA pool to form TAG by the enzyme diacylglycerol acyltransferase (DGAT).

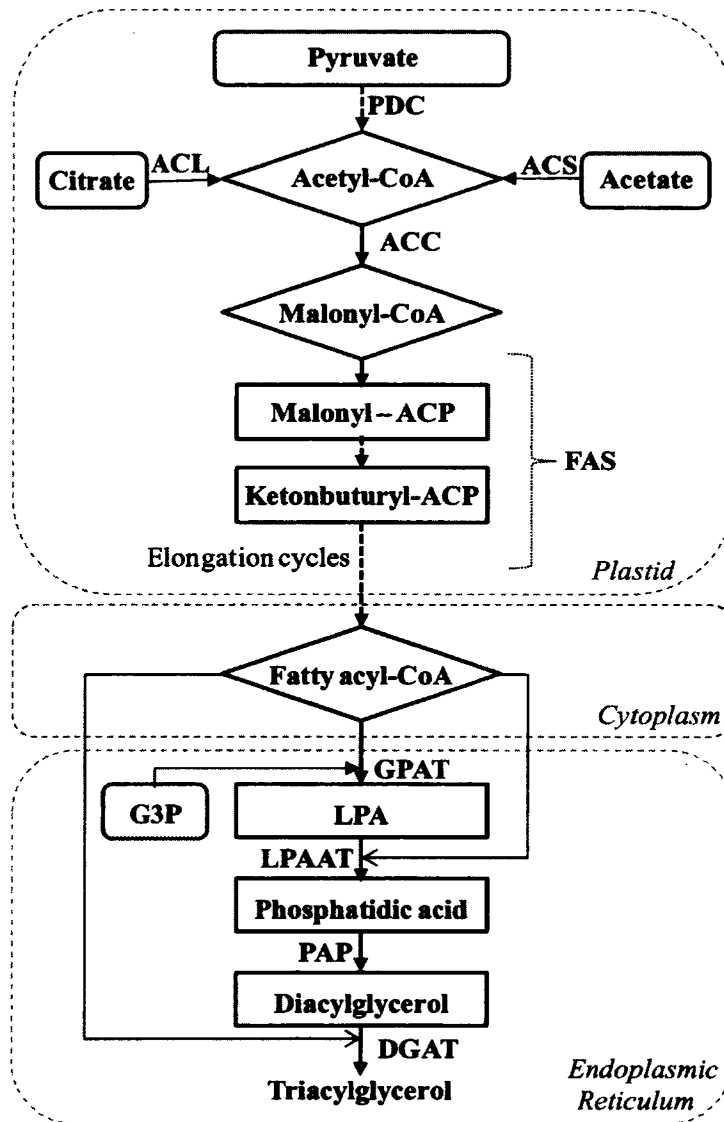


Figure 14. TAG biosynthetic pathway with the major intermediates and enzymes.[129]

Progress towards increasing seed oil content is economically desirable for edible foods, biodiesel, and other industrial uses. The wide range of seed oil percentages observed in nature suggests that metabolic engineering of key enzymes in the biosynthetic pathway of TAG can increase oil accumulation. A number of studies have been carried out on key enzymes in the metabolic pathway of TAG in order to increase the oil content. Rangasamy and Ratledge introduced a modified rat liver ACL gene into the genome of tobacco.[130] The over-expression of ACL resulted in a 4 times increase of ACL activity and a 16 % increase in FA composition. Page *et al.* demonstrated that ACC strongly controls FA synthesis in barley and maize using the herbicides fluazifop and sethoxydim as specific inhibitors.[131] A 6 % increase in FA content was reported by Roesler *et al.* after over-expressing an ACC gene from Arabidopsis in *Brassica napus* (rapeseed).[132] However, these studies have not resulted in an increase in TAG which suggests additional steps are limiting the conversion of FA to TAG.

The accumulation of TAG has been associated with increased DGAT activity in developing and germinating seeds of oilseed plants. Jako *et al.* were the first to report enhanced seed oil deposition and seed weight by over-expression of a DGAT in plants.[133] Over-expression of a seed-specific DGAT gene in wild-type Arabidopsis resulted in an increased activity of 10 – 70 % which was correlated to a 40 – 100 wt.% increase in oil content and seed weight. Bouvier-Navé *et al.* transformed yeast and tobacco plants with a cloned DGAT gene from Arabidopsis.[134] Analysis of the transgenic tobacco plants detected an increase of TAG up to 7 times the normal level with varying success with the transformed yeast strains. Zou *et al.* was the first to increase seed oil content by enhancing LPAAT expression in Arabidopsis and rapeseed.[135] The resulting transgenic

plants exhibited elevated LPAAT activity and showed an 8 – 48 % increase in seed oil content.

The metabolic pathway for TAG synthesis competes with other pathways for common substrates and precursors within the plant cell. Coleman and Lee discovered that the biosynthesis pathway for phospholipids competes with TAG biosynthesis for a common substrate, PA.[129] The production of TAG could be increased by down-regulating the enzyme phosphatidate cytidylyl transferase (CTP) which converts PA to CDP-diacylglycerol, a precursor of phospholipid synthesis.[128] Another competitive pathway is the production of oxaloacetate from phosphoenolpyruvate, a precursor to pyruvate. Chen *et al.* was able to down-regulate the competing enzyme, phosphoenolpyruvate carboxylase (PEPC), by expressing antisense PEPC in rapeseed, resulting in a 6 – 18 % increase in oil content.[136]

Numerous studies have allowed researchers to gain a better understanding of the biosynthesis of TAG. Different species of plants have been investigated with some reporting a substantial enhancement of TAG accumulation by over-expressing key enzymes while others have had only mild success. A multi-gene approach of over-expressing more than one key enzyme while down-regulating others may lead to substantial increases in oil content that would be useful for food and biodiesel production.

3.1.3 Transgenic lipase production

The majority of industrial enzymes on the market are hydrolytic enzymes, such as proteases, amylases, amidases, esterases and lipases. In 2011, the global industrial enzyme market was approximately $3.5 billion, up from $2.8 billion in 2009.[137] The capability of lipases to perform an assortment of biotransformations has made them

increasingly popular in the food, cosmetic, detergent, pharmaceutical, and biofuels industries. As a result, the abundance of commercially available lipases has rapidly increased since 1980's due to extensive advancements in biotechnology.[138]

The majority of potential producers of lipases come from microorganisms, such as bacteria, yeast, and fungi. Microbial lipases have gained special industrial attention due to their regio-, stereo-, and substrate selectivity. Despite a relatively high number of microbial lipase source available, only limited amounts are commercially available. The growth conditions and media requirements of some of these microbes in their native form are complex and occasionally unknown.[139] As a result, these lipases are unavailable in sufficient quantities due to high production costs, which limit their potential applications in industry. Biotechnology can be used to overcome these limitations and lower the associated costs for the large scale industrial production of lipases. Using recombinant DNA technology, the gene of a suitable lipase is selected and cloned into an appropriate expression system to be produced by another organism in large quantities. The genes of many lipases have been cloned, sequenced, and made publically available on several online databases, such as the National Center for Biotechnology Information (NCBI), Genozymes Project Public Genomes, Universal Protein Resource (UniProt), and GeneNetwork. Recombinant DNA technology can reduce the amount of raw materials, water, steam, and electricity resulting in a 40 % savings when compared with that for the production by the native species.[139]

In 1988, Lipolase by Novozymes was the first lipase to be commercially produced by recombinant DNA technology. The lipase originating from *Thermomyces lanuginosus* was cloned and over-expressed in *Aspergillus oryzae*.[140] Prathumpai *et al.* investigated

two recombinant strains of *A. niger* as an expression system and also produced lipase from *T. lanuginosus*.[140] They concluded that using *A. niger* with a TAKA amylase promoter from *A. oryzae* is very well suited to produce heterologous fungal enzymes. Zheng *et al.* was able to clone, express, and characterize *T. lanuginosus* in *Pichia pastoris* under the control of a strong inducible promoter (AOX1).[141] Analysis of the physicochemical and catalytic properties of the lipase displayed high thermostability and alkali tolerance while maintaining hydrolytic and ester synthesis activity.

Thermomyces lanuginosus (formally *Humicola lanuginose*) is a frequently isolated thermophilic fungus found in organic soils, composts, and animal excrement throughout the world. *T. lanuginosus* can be cultured in temperatures ranging from 30 – 50 °C and was first isolated from potato by Tsiklinskaya in 1899.[142] Several strains of *T. lanuginosus* have been found to be hyper-producers of extracellular lipase.[140-141, 143] The lipase is a globular protein with a size of 35 x 45 x 50 Å that consists of 269 amino acids (aa) and has a molecular weight of 31,700 g/mol.[143] Interfacial activation of the lid is required to expose the active site that includes the typical Ser-His-Asp catalytic triad.[117] The lipase has been found to be highly active and is quite thermostable, maintaining catalytic activity up to 60 °C at pH 9. Novozymes offers two commercially available preparations of *T. lanuginosus* lipase: Lipolase and an immobilized form called Lipozyme TL IM. Both the free and immobilized lipase have been used in a variety of applications, such as the modification and hydrolysis of oils and fats; the resolution of racemic mixtures; and for industrial cleaning.[143] More recently, immobilized *T. lanuginosus* lipase has been extensively investigated for the enzymatic production of biodiesel.[45, 110, 141, 144]

Industrial biocatalytic processes often require specific properties that are different from those found in nature, such as selectivity, thermostablility, and stability in organic media. An attractive approach to modify the properties of existing wild-type enzymes is by using protein engineering technologies. The first example of protein engineering on a lipase was reported in the mid-1980's with modifications of *Pseudomonas mendocina* lipase.[41] Rational protein design is a technique of protein engineering that develops molecular models of enzymes using the available knowledge of the enzymatic function, kinetic mechanisms, and three-dimensional structure from X-ray crystallography.[139] This molecular model is used to predict and plan changes to the amino acid sequence of the protein to determine how it affects activity, stability, or selectivity of the enzyme. Site-directed mutagenesis is then used to generate mutant genes, which are transformed and expressed in a host organism. The recombinant variants are collected, purified, and analyzed for the desired characteristics.

Lipolase Ultra and LipoPrime are two variants of Lipolase by Novozymes that were developed using rational protein design. Both lipase variants are more effective cleansers than the original and are also expressed in *A. oryzae*.[139] Interfacial activation of the lid has been of great interest of researchers. Deletions or substitutions in the lid have been attempted in many lipases, including *T. lanuginosus* lipase. However, removal of the lid in the *T. lanuginosus* lipase has resulted in an inactive lipase.[41] Holmquist *et al.* used site-directed mutagenesis at Glu87 and Trp89 in the lid of *T. lanuginosus* to determine the functional role in the transesterification in organic solvents.[145] The Glu87 → Ala and the Trp89 → Phe mutations decreased the apparent maximum rate of the reaction ($V_{max,app}$) for tributyrin by a factor of 1.5 and 2.7, respectively.

An alternative to rational protein design is directed evolution and is used when the function, mechanism, and structure are not known for a particular enzyme. This technology uses random mutagenesis of genes to produce molecular diversity.[139] Generated mutant genes are transformed and over-expressed in a host organism. The recombinant variants are collected and screened for enhanced characteristics. The process is repeated several times until the desired properties are obtained. Various methods are used for directed evolution to generate the mutated variants, such as site-specific saturation mutagenesis, error-prone PCR, cassette mutagenesis, DNA shuffling, heteroduplex recombination, and random-priming recombination.[139]

Lipex is a third variant of Lipolase that was developed using directed evolution technology by Novozymes.[146] Liebeton *et al.* selected lipase from *Pseudomonas aeruginosa* to catalyze the hydrolysis of chiral molecules 2-methyldecanoic acid and p-nitrophenyl ester using error-prone PCR.[147] The enantioselectivity of the lipase increased from 1.1 for the wild-type to 25.8 for the best variant. Zhang *et al.* successfully improved the tolerance of *Candida antarctica* lipase B towards irreversible thermal inactivation through directed evolution.[148] Two of the mutants resulted in over a 20-fold increase in half-life at 70 °C compared with the wild-type lipase.

Recombinant DNA technology and protein engineering can enhance lipase production and improve catalytic characteristics. These strategies have already successfully been applied by industry to produce several commercial lipases. In order to develop a competitive strategy for the enzymatic production of biodiesel, it is imperative to develop an appropriate and effective biocatalyst system using both genetic engineering and biochemical strategies.

3.1.4 Plant protein production

Before the 1980s, most of the enzymes used were derived from native sources, such as plants and animals, which resulted in high costs and limited availability.[149] Today, desired enzymes are produced in large quantities for a variety of applications, thanks to genetic and protein engineering technologies. Recombinant proteins can be expressed in a wide array of host cells, such as in bacterial, yeast, mammalian, and plant systems. Each of these has its inherent advantages and drawbacks, which must be understood and examined when choosing an appropriate expression system. Other important factors to consider are protein function, structure, quality, quantity, and costs. The enzymatic production of biodiesel in an industrial process would require large quantities of inexpensive lipases to become economically feasible.

Bacterial expression systems for transgenic protein expression represents about 30 % of the enzymes produced commercially.[149] The biological and genetic understanding of bacteria has been well documented mainly in part to the ability to be rapidly grown with relative ease in high biomass concentrations on inexpensive media. *E. coli* is the most predominantly used bacterium for recombinant protein expression. According to Russo, Boyer and Cohen developed DNA cloning in the mid 1970s and were the first to successfully transform *E. coli* with non-native and interspecies DNA.[150] In the early 1980s, human insulin was the first recombinant pharmaceutical to be commercially produced from *E. coli* for the treatment of diabetes.[151] Despite being an effective expression host for simple proteins, *E. coli* lacks the ability to perform post-translational modifications that are often required in more complex proteins, such as glycosylation, acylation, phosphorylation, and disulfide bond formation.[118] Some proteins can form

inclusion bodies that are often inactive, insoluble, and difficult to isolate without denaturing.[149] Secretion of recombinant proteins into the medium provide several advantages, one of which is to minimize the formation of inclusion bodies. However, this is difficult to accomplish because *E. coli* is a gram-negative bacterium and does not naturally secrete proteins in high amounts. Other bacterial systems that are commonly used belong to the genus *Bacillus*, such as *B. subtilis and B. licheniformis*, are gram positive bacteria with the machinery to strongly secrete proteins.[152]

Yeasts are single cell eukaryotic fungal organisms that are often used to produce recombinant proteins that cannot be expressed in *E. coli*. Yeast and molds have produced > 50 % of the commercial enzymes in biopharmaceutical and industrial uses for the past two decades.[149] Similar to bacteria, yeasts are simple to cultivate and can rapidly achieve high cell density levels in fairly inexpensive media. However, unlike bacteria, yeast has the machinery to perform post-translational modifications and is able to secrete the recombinant proteins from the cell into the fermentation broth.[152] The most common yeast strain for producing recombinant proteins is *Saccharomyces cerevisiae*; the same yeast that has been used throughout human history to make bread and alcoholic beverages.[153] Since 1986, a vaccine for hepatitis B was licensed and produced from cell cultures of recombinant strains of *S. cerevisiae*.[152-153] The process of glycosylation occurs in yeasts at the correct locations on recombinant proteins, however, they tend to over-glycosylate mammalian proteins. The carbohydrate chain of the glycosylation process often contains a number of mannose sugars which can cause a reduction in activity.[151] Methylotrophic yeasts, such as *Pichia pastoris*, have become very attractive alternative to S. *cerevisiae* because it creates much shorter chain lengths of mannose

during glycosylation.[149] Several other yeast expression systems are also used for the industrial production of recombinant proteins, such as *Arxula adeninivorans*, *Scizosacchromyces pombe*, and *Hansanuela polymorpha*.[149, 152-153]

Mammalian expression system for producing recombinant proteins provides only 8 % of industrially available enzymes.[149] Mammalian cells are particularly useful for more complex proteins, like biopharmaceuticals, which require mammalian post-translational modifications; such as antibodies, viruses, vaccines, and other gene-therapy proteins. The reason mammalian cells are sometimes preferred to yeast cells is that they properly fold and secrete glycosylated proteins in their native form without the addition of excessive sugar residues.[154] The most commonly used mammalian expression system for the past two decades to produce stable recombinant proteins are Chinese hamster ovary (CHO) cells. In 1987, tissue plasminogen activator was the first recombinant therapeutic protein produced by a mammalian system using CHO cells that was approved for clinical trials.[155] Despite the wide variety of cell lines available, there is currently only a small number of mammalian expression systems used commercially, such as baby hamster kidney (BHK) cells, human embryonic kidney (HEK) cells, and mouse L-cells.[152] Mammalian cells are well suited for the production of high valued products, such as pharmaceuticals and therapeutics. However, they are not well suited for producing large quantities of inexpensive enzymes due to several drawbacks: (i) extremely delicate cultivation process with complex media (ii) very expensive to operate and (iii) often produce low levels of proteins.[151] Therefore, mammalian cell cultures would not be well suited for producing biocatalyst for biodiesel production.

A plant expression system is a fairly new platform that offers the ability to produce an inexpensive recombinant protein safely and easily. Since the 1980s, the term "molecular pharming" has appeared in literature which initially referred to transgenically expressing high-valued pharmaceuticals in animals and has evolved to focus on transgenic plant protein production.[118] Transgenic plants are theorized to offer a number of advantages over microbial sources in terms of cost, protein complexity, storage, and distribution.[149] The amount of recombinant proteins can be easily scaled up by using current agricultural technologies: providing simple nutrients and solar energy for photosynthesis. Another advantage to plant cells is that they are able to fold proteins and perform post-translational modifications that are necessary for many enzymes to maintain function, activity, stability, and specificity.[118] Over 300 types of post-translational modifications have been identified and are highly regulated and specific to the requirements of the protein and organism.[156] Therefore, when introducing foreign genes into another genome, it is important to recognize and understand the protein's structural requirements and the capabilities of the host organism to meet these requirements. As recently as 2006, Newcastle disease vaccine (Concert) became the first vaccine made by transgenic plants which was approved by the USDA.[157] Dow AgroSciences manufactures Concert by transgenically expressing a poultry viral antigen in tobacco plants.

N-Glycosylation in plant cells is almost as efficient as mammalian cells, with the exception that they have no terminal sialic acid and contain xylose.[151] Where this slight modification does not usually affect activity, function, or stability, it is important factor in therapeutic applications where there are some immunological concerns. Castilho *et al.* has demonstrated sialylation in *Nicotiana benthamiana* by introducing an entire

mammalian biosynthetic pathway.[158] They successfully expressed a functional monoclonal antibody by integrating and over-expressing the six mammalian proteins of the sialic acid pathway. O-Glycosylation is also similar in plants as it is in mammals. However, in plant cells N-glycans possess two antenna structures while mammalian N-glycans can accommodate multiple antennary glycans with terminal branches.[156] This deficiency was just recently overcome by Yang *et al.*[159] They were able to demonstrate mammalian O-glycosylation in transgenic tobacco plants by expressing a *P. aeruginosa* Glc(NAc) C4-epimerase and a human polypeptide GalNAc-transferase to produce human O-glycoprotein substrates. Although further improvements are needed, they believe that transgenic plants are capable of producing mammalian O-glycoproteins for pharmaceuticals and therapeutics.

Plant cells have the ability to regenerate from a single cell into an entire plant, called totipotency, a characteristic that differentiates from mammalian cells. This ability makes plants and plant cells a versatile production system which allows for the targeting different organelles and subcellular compartments, such as the endoplasmic reticulum (ER), chloroplast, cytoplasm, or vacuole.[47] Ahmad *et al.* were able to transgenically express a maltose-binding protein with glutathione-S-transferase (GST) affinity tag in the chloroplasts of tobacco.[160] In this study, a chloroplast 16S rRNA promoter and 5' untranslated region of phage T7 gene were used for transformation, resulting in expression levels approximately 37 % of the total soluble protein. Magee *et al.* has expressed an antibody fragment in the plastids of tobacco using a T7 promoter.[161] The importance of the plastid is that it possesses small circular double stranded DNA that is similar to that found in bacteria and directs the synthesis of a part of RuBisCO, the most

abundant protein in the world. Protein expression in the plastid has been to interest of researchers because protein yields have been greater than that produced by nuclear transgenes.[161]

In addition to targeting organelles, recombinant protein production can be in various plant organs; such as the leaves, roots, seeds, and fruit. Tobacco has been grown in the United States for hundreds of years and can generate up to 170 tons/ha fresh weight of tissue. As smoking is on the decline, tobacco has emerged as an ideal candidate for leaf-based protein expression. Ma *et al.* produced transgenic tobacco plants that expressed humanized secretory murine IgA1 (Guy's 13 SIgA-G) in the leaves.[162] This resulted in an accumulation of up to 500 μg/g leaf tissue of Guy's 13 SIgA-G. Targeting expression in seeds is another popular organ for protein expression because they are designed to store lipids, proteins, sugars, and other nutrients required by the embryo during germination. Parmenter *et al.* successfully over-expressed hirudin, a blood anticoagulant, in the seeds of *Brassica napus* (rapeseed), which was later used for commercial production by SemBioSys.[118, 163] There has been considerable interest in developing recombinant protein expression in ripening fruit, for the purpose of edible vaccines. Transgenic tomato fruit was developed by Chen *et al.* to express antiviral agent, polyomavirus capsid protein (VP1) to prevent enterovirus 71 that causes seasonal epidemics of hand-foot-and-mouth disease.[164] They successfully demonstrated the feasibility of using transgenic tomato as an oral vaccine to generate protective immunity in mice against EV71. However, an edible vaccine would require a controlled and standardized dosage for human consumption which remains a major obstacle for researchers.

Plant expression systems can encompass a diversity of forms which the other systems cannot, such as in the whole-plant, suspension cell cultures, and root cultures. Although whole-plants offer unlimited scalability, the concern of transgenic lines of biopharmaceuticals crossing with food crops can be mitigated through cell suspensions or hairy root cultures. Production takes place in bioreactors with relative high cell density and performs similarly to that of bacterial and yeast cultures. Pires *et al.* successfully produced a recombinant human erythropoietin in three different plant suspension cell cultures.[165] The glycosylated human hormone Erythropoietin was over-expressed and secreted in transgenic lines of *Arabidopsis thaliana*, *Medicago truncatula*, and tobacco. Other plant species have also been transgenically modified to produce recombinant proteins include rice, soybean, alfalfa, and tomato.[118] Hairy root cultures are preferred over suspension cultures due to their genetic, biochemical, and relatively low-cost culture requirements (no sunlight required). Pham *et al.* were able to use *Agrobacterium rhizogenes* to transform tobacco hairy root cultures to express and secrete a recombinant protein, thaumatin.[166] They were able to increase extracellular concentrations of thaumatin from 0.21mg/L to 2.63 mg/L by simply adding sodium chloride into the medium. Biotransformations of compounds through different hairy root systems have included ginseng, rapeseed, radish, basil, and various flowering plants.[167]

Much of the focus of producing recombinant proteins in plants has pertained to pharmaceuticals, due to high production value. Plants may also offer reductions in production costs, however, the high cost of pharmaceuticals lies in research and the clinical phases of drug development. In addition, there are immunological concerns that need to be overcome and proven safe for human use. However, industrial proteins do not

have the same concerns and costs associated with pharmaceuticals. Within industry, inexpensive enzymes and proteins are required in large quantities and are much more dependent on production costs. Therefore, the use of transgenic plants is ideal due to their ease of transformation, expression versatility, and speed of scale-up.[168]

In 1997, ProdiGene and Sigma Chemical Co. produced the first commercial plant-based recombinant protein (avidin) in maize.[168] The chicken oviduct avidin gene was introduced in maize for expression in seeds using an ubiquitin promoter. This resulted in 230 mg/kg of extractable protein from the seeds, which could maintain activity for up to 3 months if stored in the cracked and flaked kernels.[169] ProdiGene also produced other recombinant proteins in corn, such as aprotinin, trypsin, and β-glucuronidase (GUS). Biopolymers are also being considered as an environmentally friendly option to petroleum-based polymers. There are some bioplastics already being produced using plant materials, like cellulose and starches. However, recombinant methods are being applied to plants in order to produce more exotic polymer precursors. Dalton *et al.* have recently reviewed the properties and recombinant expression of these biopolymers in woody plants.[170] Bioplastics created from polyhydroxybutyrate can be synthesized in transgenic poplar by expressing three genes through metabolic and genetic engineering. Spider silk protein is another important polymer that researchers are trying to produce in transgenic plants due to its extraordinary physical properties. Yang *et al.* has been able to synthesize an analogue of spider dragline silk protein in *Arabidopsis* with up to 18 % of total soluble protein.[171] Additional non-pharmaceutical proteins that have been produced in transgenic plants and marketed are shown in Table 4.[172]

Table 4. Industrial recombinant proteins produced by transgenic plants.[168-170, 172]

Protein	Gene Source	Plant Host	Application	Company
Avidin	Chicken	Maize	Diagnostics	ProdiGene
Aprotinin	Bovine	Maize	Diagnostics	ProdiGene
GUS	Bacteria	Maize	Research	ProdiGene
Trypsin	Bovine	Maize	Diagnostics	ProdiGene
Aprotinin	Bovine	Tobacco	Diagnostics	Large Scale Biology
Lactoferrin	Human	Rice	Research	Ventria Bioscience
Lysozyme	Human	Rice	Research	Ventria Bioscience
Thyrotropin receptor	Human	Melon	Diagnostics	Nexgen
Peroxidase	Fungus	Maize	Paper	Applied Biotechnology Institute
Laccase	Fungus	Maize	Paper	Applied Biotechnology Institute
Cellulase	Fungus	Maize	Ethanol	Applied Biotechnology Institute
α-amylase	Microbial	Maize	Ethanol	Syngenta
Cellulase	Fungus	Maize	Ethanol	Infinite Enzymes

In terms of biofuel production, transgenic plants have been widely thought as the key to a sustainable and successful future. Genetic modifications to various metabolic and biosynthesis pathways have enhanced plants to become more suitable for use as biofuel feedstocks. Strategies for engineering stress tolerances can allow feedstocks to thrive in a variety of environmental conditions, such as temperature, water availability, and salinity.[47] These modifications can permit biofuel crops to be grown in areas where they are subjected to external factors which are not suitable for food crops. Another genetic engineering approach has been to enhance desirable traits within the feedstocks, for

example the hemi/-cellulose content for ethanol production and oil storage in seeds for biodiesel production.[129, 173] Finally, organisms have been genetically engineered to produce enzymes that are used in the catalytic process to produce biofuels. The majority of these enzymes are produced in microorganisms, such as bacteria and yeasts. Plant expression systems allow us the versatility and almost unlimited scalability to produce recombinant enzymes that are required for cellulosic ethanol and enzymatic biodiesel. Currently, companies like Infinite Enzymes, LLC and Applied Biotechnology Institute are taking advantage of plant-based expression systems to produce cellulases for ethanol production.[118, 172] However, to the best of the author's knowledge, there is no research being done to produce lipases in plants for the enzymatic production of biodiesel.

In this chapter, we examine the results of genetically engineering plants to constitutively express lipase for biodiesel production from spent oils and non-edible plant oils. The gene of a lipase with known transesterification activity from *Thermomyces lanuginosus*, a thermophylic fungus, was cloned and inserted into tobacco and *Arabidopsis* plant using *Agrobacterium*. The recombinant enzyme was collected from the genetically engineered plants, purified, and tested for its activity.

3.2 Experimental Section

3.2.1 Strains, plasmids, and materials

Thermomyces lanuginosus (strain 200065) was purchased from the American Type Culture Collection (ATCC). Endura™ Chemically Competent *E. coli* were kindly supplied by Lucigen (Cat. No. 60240-0). The plasmid pCR8®/GW/TOPO used for gene cloning and sequencing was purchased from Invitrogen (Cat. No. K250020). *Agrobacterium tumefaciens* (strain GV3101) used plant expression plasmid vectors

pGWB408 and pMDC83 that were kindly supplied by Tsuyoshi Nakagawa (Shimane University) and Mark Curtis (University of Zürich), respectively. *E. coli* strain DB3.1 was used to amplify stock expression vectors.

Luria Broth (LB) medium (ultrapure), soluble starch (hydrolyzed potato), agar (ultrapure, bacterial grade A), and Tris (ultrapure, grade MB) were purchased from Affymetrix. Agar (type A, plant cell culture tested), Gamborg's vitamin solution 1000x, 6-benzylamino-purine (BAP), α-naphthalene-acetic acid (NAA), Murashige and Skoog basal salt mixture (MS), K_2HPO_4 (ACS grade), $CaCl_2$ (99.0%), glycerol ($\geq$ 99%), sodium phosphate monobasic monohydrate (ACS grade), imidazol hydrochloride, Triton X-100, Protease inhibitor cocktail for plant cell and tissue extracts in DMSO solution (Cat. No. P9599), protease inhibitor cocktail for use in purification of Histidine-tagged proteins (Cat. No. S8830), NaCl (DNase, RNase, and protease, none detected, $\geq$ 98%), and lipase from *Thermomyces lanuginosus* solution ($\geq$100,000 U/g) were purchased from Sigma-Aldrich.

Prestained SDS-PAGE broad range standards (Cat. No. 161-0318), β-mercaptoethanol (Cat. No. 161-0710), Laemmli sample buffer (Cat. No. 161.0737), 10x Tris/Glycine/SDS buffer (Cat. No. 161-0732), Bio-safe Coomassie G-250 stain (Cat. No. 161-0786), and mini-PROTEAN TGX Gels (Cat. No. 456-1034) were purchased from Bio-Rad. Sodium phosphate dibasic heptahydrate (ACS grade), sodium hydroxide solution N/10 (Certified 0.1000 ± 0.005 N), $MgSO_4 \cdot 7H_2O$ (Cat. No. M80-212), and Spectrum Spectra/Por 3 RC Dialysis Membrane Tubing 3500 Dalton MWCO (Cat. No. 08-670-5A) were purchased from Fisher Scientific. Tributyrin (98 %) and D(+) – sucrose (Cat. No. 177140050) were purchased from Acros Organics. Spectinomycin

dihydrochloride (Cat. No. S742) and carbenicillin (Cat. No. C346) were purchased from PhytoTechnology Laboratories. Other chemicals used are Seakem LE agarose (Lonza, Cat. No. 50000), S.O.C. medium (Invitrogen, Cat. No. 15544-034), yeast extract (Bacto, Cat. No. 212750), $MgSO_4 \cdot 7H_2O$ (Fisher Scientific, Cat. No. M80-212), kanamycin monosulfate (MP Biomedicals, Cat. No. 150029), hygromycin B (Plant Media, Cat. No. 40810000-1), TAE buffer 50x (Eppendorf, Cat. No. 95515533-5), ammonium sulfate (ICN Biomedicals, Inc., Enzyme grade), and acetone (Alfa Aesar, HPLC grade).

3.2.2 Cloning of *T. lanuginosus* lipase cDNA

T. lanuginosus used in this study was rehydrated with deionized (DI) water and grown on PDA inducing medium containing: 50.0 g/L yeast extract, 20.0 g/L soluble potato starch, 18.3 g/L olive oil, 5.0 g/L K_2HPO_4, 0.15 g/L $CaCl_2$, 1.0 g/L $MgSO_4 \cdot 7H_2O$, and 15.0 g/L bacterial agar (grade A) for 48 – 72 h at 50 °C. The fungal tissue was then frozen in liquid nitrogen (N_2) and used for total RNA extraction using the ZR Plant RNA MiniPrep Kit according to the manufacturer's protocol (Zymo Research, Cat. No. R2024), as described in Appendix C. Concentration and quality of mRNA was confirmed by characterization using ThermoScientific Nanospectrophotometer (NanoDrop) 2000c.[174] The total RNA was reversed-transcribed to synthesize complementary DNA (cDNA) immediately after isolation using qScript cDNA Supermix kit according to the manufacturer's protocol (Quanta Biosciences, Cat. No. 95048-100) and a PTC 100 Programmable Thermal Controller (MJ Research Inc.), as described in Appendix C. The RNA and resultant cDNA were stored at -80 °C and -20 °C, respectively.

3.2.3 DNA amplification by Polymerase Chain Reaction (PCR)

A PCR was performed to amplify the lipase genes using the PTC 100 Programmable Thermal Controller (thermocycler). A typical reaction was 50 μL total volume with a final concentration of x1 buffer (Mg^{2+}), 200 μM dNTP mix, 0.2 μM forward and reverse primers, 2 ng/μL of total plasmid DNA or 2–3 ng/μL of genomic DNA, and 1.25 units of Takara *Ex Taq* DNA polymerase (Takara Bio Inc., Cat. No. RR001A).

Typical PCR conditions for various lipase constructs are described in Table 5. Other *Taq* polymerases and thermocyclers were also used for screening purposes with similar reaction conditions.

Table 5. PCR conditions for the amplification of *T. lanuginosus* lipase cDNA.

Step	**TL1 – A1, A2, & A3**	**TL1 – A4, TL2 – B1, B2, B3, & B4**
1 – Initiation	94 °C for 1 min	94 °C for 1 min
2 – Denaturation	94 °C for 30 sec	94 °C for 30 sec
3 – Annealing	56 °C for 1 min	52 °C for 1 min
4 – Elongation	72 °C for 1 min	72 °C for 1 min
Repeat steps 2 - 4	35 cycles	30 cycles
5 – Final elongation	72 °C for 2 min	72 °C for 2 min
6 – Final hold	4 °C for 24 h	4 °C for 24 h

3.2.4 Agarose gel electrophoresis

Analyses of DNA fragment size(s) were performed using agarose gel electrophoresis. The gels consisted of 1% LE agarose dissolved in 1x TAE buffer (40 mM Tris-acetate, 1 mM EDTA). Samples were mixed with 6x loading dye and then loaded into the gel along with an appropriate DNA size standard (e.g. NEB 2-log DNA Ladder or NEB 1 kb DNA Ladder). The gel was electrophoresed at 90 – 100 V for 45 – 60 min and then stained in 0.5 μg mL^{-1} of ethidium bromide (EtBr) for 15 min followed by 15 min of

destaining in distilled water. Afterward, the gel was visualized and photographed using Fotodyne gel-documentation system. The DNA fragments were analyzed visually in comparison to bands of molecular weight standards from a DNA ladder.

3.2.5 Cloning vector and heat-shock transformation

After the PCR amplification and gel confirmation, the *T. lanuginosus* lipase gene product was cloned into the pCR8/GW/TOPO vector according to the manufacturer's protocol (Invitrogen, Cat. No. 45-0642), as described in Appendix C. The plasmid was inserted into bacterial cells using the heat-shock transformation protocol, where 150 ng of the plasmid DNA was added to 50 µL of chemically competent *E. coli* cells (stored at -80 °C and thawed on ice for 30 min), mixed gently, and placed on ice for 30 min. The cells were then placed in the Eppendorf Thermomixer at 42 °C for 30.0 sec (without shaking) and put on ice for 2 min. Then 250 µL of room temperature S.O.C. Medium was added to the cell culture tube and placed horizontally a New Brunswick Scientific Controlled Environment Incubator Shaker (series 25) at 37 °C and 200 rpm for 1 h. Afterward, 50 µL of cell culture was spread onto two prewarmed LB agar plates with the appropriate antibiotic and incubated overnight at 37 °C.

3.2.6 Bacterial culture and plasmid DNA isolation

Liquid cultures of *E. coli* and *Agrobacterium* were grown in LB medium containing appropriate antibiotics, depending on the selection gene (ampicillin 100 µg/mL, spectinomycin 100 µg/mL, kanamycin 50 µg/mL, or hygromycin 50 µg/mL). Sterile pipette tips or applicators were used to start a 3 mL liquid culture of *E. coli* or 5 mL liquid culture of *Agrobacterium* from glycerol stocks or plates. For solid cultures, 1.3% agar was added to the medium prior to autoclaving. Solid or liquid cultures of *E. coli* and

Agrobacterium were incubated at 37 °C for 16 – 18 h and 28 °C for 48 – 72 h, respectively. Liquid cultures were grown on a shaker at 200 rpm.

Individual cell colonies from a successful transformation were randomly selected and grown overnight in separate test tubes containing 3 – 5 mL liquid LB culture with the appropriate antibiotic and placed in the incubator shaker at 200 rpm and at their optimum temperature (37 or 28 °C). The plasmid DNA isolation from the transformed *E. coli* was performed using the Zyppy Plasmid Miniprep Kit, according to the manufacturer's protocol (Zymo Research, Cat. No. D4020), as described in Appendix C. The plasmid DNA was then quantified by the NanoDrop 2000c, and was stored at -20°C.

3.2.7 Restriction digest

Typical restriction digestion was performed using enzymes from New England Biolabs (NEB). A reaction mixture contained a final concentration of 1x of the appropriate buffer, 1x bovine serum albumin (if required), 2 units/µg DNA of the enzyme, 150-200 ng of template DNA, and all brought to the volume of 10 µL by sterile distilled water. The reaction was incubated at 37 °C for 3 h and used immediately for gel electrophoresis or stored at -20°C.

3.2.8 DNA sequencing and analysis

Two sequencing reactions were prepared containing 300 ng of plasmid DNA, 5 pmol of primer (forward primer in one tube and reverse primer in the other), and diluted to 6 µL with sterile water. The sequencing was done at UNH Hubbard Genome Centre using a ABI 3130 DNA Analyzer (Foster City, CA) and the results obtained from sequencing were aligned and analyzed with the target sequences using BioEdit Sequence Alignment Editor (Hall 1999).

3.2.9 Glycerol stocks

Liquid cultures containing 3 mL of LB medium with appropriate antibiotics from the desired colonies were grown overnight at 200 rpm at their optimum temperature (37 or 28 °C). Equal parts liquid culture and glycerol stock solution were thoroughly mixed in two cryo-vials and preserved at -80 °C. The glycerol stock solution contained a 3:2 ratio (v/v) of LB medium and glycerol that was then sterilized.

3.2.10 Construction of the final destination vectors

Gateway compatible destination vectors contained the bacterial ccdB gene, which encodes an anti-DNA gyrase protein (ccdB gene). This binds the DNA gyrase so that it was unable to reseal the DNA resulting in bacterial death. The *E. coli* strain DB3.1 contains a mutation in the DNA gyrase gene so that the ccdB protein was unable to bind and was used to amplify the stock vectors to provide sufficient quantities for transformation. The DB3.1 cells were transformed with the stock vector using the heat-shock method followed by plasmid isolation. The plasmid DNA was confirmed by restriction digestion and gel electrophoresis.

After the gene was inserted into the cloning vector (pCR8/GW/TOPO) and confirmed through restriction digestion, electrophoresis, and sequencing, it was then subsequently transferred into two Gateway compatible destination vectors (pGWB408 & pMDC83) using Gateway LR Clonase II enzyme mix according to the manufacturer's protocol (Invitrogen, Cat. No. 11791-020), as described in Appendix C. The plasmid was transformed into *E. coli* by heat-shock method and confirmed by plasmid isolation, restriction digest, and gel electrophoresis. Afterward, the plasmids were transformed into *Agrobacterium tumefaciens* by electroporation.

3.2.11 Electroporation

Electroporation of *Agrobacterium* was performed using an Eppendorf model 2510 electroporator. A 2 µL of the isolated plasmid from the final destination vector was added to 50 µL aliquot of *Agrobacterium* cells thawed on ice. The mixture was mixed gently and transferred to prechilled cuvettes (1 mm gap) and electroporated at 1800 V. This was quickly followed by addition of 250 µL S.O.C medium and incubation in the New Brunswick Scientific G24 Environmental Incubator Shaker at 28 °C for 3 – 4 h at 200 rpm. Afterward, 10 – 30 µL of the cell culture was spread on solid LB medium with appropriate antibiotic and incubated at 28 °C for 48 – 72 h. The resulting colonies were selected and grown in liquid LB medium with appropriate antibiotic at 28 °C for 24 – 48 h and 200 rpm.

Screening of the transformed *Agrobacterium* was performed by centrifuging 500 µL of culture at 13200 rpm for 30 sec, discarding the supernatant, and resuspending the pellet in 50 µL sterile water. The suspension was then heated to 99 °C in the thermomixer for 10 min, centrifuged at 132000 rpm for 5 minutes, and 5 µL of the supernatant was used to run a PCR with the appropriate primers. Gel electrophoresis confirmed a successful transformation.

3.2.12 Agrobacterium-mediated transformation

Small aliquots of transformed *Agrobacterium* were used to initiate 3 mL liquid LB medium with appropriate antibiotic overnight at 28 °C and 200 rpm. *Agrobacterium* cultures were subcultured to 30 mL fresh medium and grown 24 – 28 h at 28 °C and 200 rpm. Acetocyringone was added at a final concentration of 50 µM within 12 h prior to transformation. The entire culture was pelleted at 4 °C for 5 min at 10000 rpm. The

supernatant was discarded and the pellet was resuspended with 0.9 % (w/v) NaCl to adjust the OD_{600} to 0.65 ± 0.05 before transformation.

For transformation, non-transformed (NT) tobacco leaves (from plants grown in Magenta boxes; no more than one-month-old) were cut into 1 cm^2 pieces and submerged in the *Agrobacterium* suspension for 15 min after wounding the tissue several times with the tip of a scalpel. The leaf tissue was then blotted dry with sterile paper towels (twice) and carefully placed onto basal media plates (30 g/L sucrose, 4.3 g/L MS basal salts, 500x vitamin solution, 1.0 mg/L BAP, 0.1 mg/L NAA, 8 g/L plant agar, and pH 5.6 ± 0.1). The plates were place in the growth room for two days; then the leaves were washed in a 300 mg/L carbenicillin solution for 10 min, blotted dry, placed onto selection plates containing basal media and the appropriate plant hormones and antibiotics, and put back into the growth room. The leaf segments were subcultured on the same medium at 4 – 6 week intervals.

3.2.13 Floral dip transformation method

Arabidopsis thaliana (ecotype Columbia – 0) plants were transformed by *Agrobacterium* using modified floral dip method[175]. Seeds were planted into 4 pots per transformation six weeks before dipping. By week six, the plants were prepared by clipping the primary bolt to encourage synchrony in branching and flowering. A week later, 320 mL culture of *Agrobacterium* was started in liquid LB with the appropriate antibiotic and grown for 24 h at 28°C on a 200 rpm shaker. The entire culture was pelleted at 4 °C for 10 min at 5000 x g. The supernatant was discarded and the pellet was resuspended with 5 % (w/v) sucrose containing 0.05% final concentration of L-77 Silwet (Lehle Seeds, TX #VIS-02) to adjust the OD_{600} to 0.8 ± 0.2 before dipping.

The unopened flower buds along with flowers were dipped into the bacterial suspension for 8 – 10 sec, avoiding contact with the basal leaves and soil. The pots were placed on their sides in a flat, covered with a clear plastic to prevent desiccation, and left overnight. The next morning, the plants were rinsed thoroughly under cold water and moved to their normal growth conditions. The same plants were re-dipped in a similar fashion after 7 days. The seeds (T1) were harvested from each pot separately after the siliques matured. Seeds were desiccated at room temperature for 5 – 7 days followed by sterilization and storage at 4°C.

3.2.14 Screening for transgenic lines

For tobacco, only the transgenic cells would grow and multiply in the presence of the antibiotic. Shoots were randomly selected from individual events growing on the selection media (i.e. shoots not emanating from the same location) and divided up onto fresh basal shooting media (30 g/L sucrose, 4.3 g/L MS basal salts, 500x vitamin solution, 1.0 mg/L BAP, 8 g/L plant agar, and pH 5.6 ± 0.1). Each event was clearly labeled and placed in the growth room for 2 – 4 weeks.

An alkali treatment procedure was used to rapidly obtain DNA for PCR screening[176]. Tobacco tissue (5 mm long piece of young leaf piece) was collected into sterile microfuge tubes. To the tube was added 40 µL of 0.25 M NaOH and the plant tissue was punctured several times with the pipette tip until the solution turned slightly green. The sample was incubated at 99 °C for 30 sec in the thermomixer and subsequently neutralized by 40 µL of 0.25 M HCI and 20 µL of 0.5 M Tris-HCI, pH 8.0, 0.25% (v/v) Nonidet P-40 (Sigma), before boiling for an additional 2 min. The tissue sample was

centrifuged briefly and 5 μL was used immediately for PCR with the appropriate primers. Gel electrophoresis confirmed a successful transformation.

3.2.15 Recombinant enzyme extraction

Young leaf tissue was collected and immediately frozen in liquid N_2. The tissue was then transferred to a pre-chilled mortar and pestle and ground into a fine powder, using additional liquid N_2 to prevent thawing. The powdered tissue was quickly transferred into a sterile 50 mL conical centrifuge tube, weighing the tube before and after in order to determine the mass of tissue collected. Afterward, a 3:1 ratio (mL buffer/g tissue) was added to the tissue and vortexed vigorously and left at room temperature for 10 – 15 min. Protease inhibitor cocktail was added to selected buffers at a ratio of 0.5 mL/g tissue to the extraction buffer. Both the buffer and the protease inhibitor cocktail were cooled to 4 °C on ice prior to extraction. The tissue was pelleted at 12,000 x g for 25 min at 4 °C. The collected supernatant (crude extract) was used for protein precipitation and purification procedures.

3.2.16 Acetone precipitation

A 4:1 volume ratio of cold acetone (-20 °C) was slowly added to the crude extract. The mixture was inverted gently and left for 12 – 16 h at 4 °C to allow for proteins to precipitate. Afterwards, the proteins were pelleted at 13,000 x g for 10 min at 4 °C. The acetone was removed by decanting and the tube was left open to allow any residual acetone to evaporate. Before the pellet was completely dry, it was resuspended in $1/5^{th}$ the volume of the crude extract with the extraction buffer.

3.2.17 Ammonium sulfate precipitation and dialysis

The volume of crude extract was carefully measured and the amount of solid ammonium sulfate was calculated for 30 % saturation at 4 °C. The solid ammonium sulfate was ground into a fine powder with a mortar and pestle and weighed to the calculated amount. The crude extract was placed into a 100 mL glass beaker with stir bar within the cold room at 4 °C. At a moderate mixing rate, the ammonium sulfate was slowly added to the extract, a process that took 20 – 40 min. Afterwards, the solution was allowed to mix for 1 h to equilibrate. The solution was placed into a sterile centrifuge tube and the proteins that precipitated were pelleted at 13,000 x g for 10 min at 4 °C. The supernatant was decanted into another sterile tube and the volume determined. The pellet was resuspended in $1/10^{th}$ the volume of the crude extract. The amount of ammonium sulfate was recalculated for 60 % sat at 4 °C and the process was repeated on the supernatant, and then again for 90 % saturation.

Dialysis tubing (18 mm flat diameter, MWCO 3.5 kDa) was rehydrated in buffer solution 1 – 2 h prior to dialysis. After rehydration, the dialysis tubing was tied off on one end and the precipitation fraction was added into the tubing with a pipette. A dialysis tubing clamp was used to seal the open end, ensuring that the solution was tightly packed in the tubing with minimal air bubbles. String was tied to each dialysis tubing clamp, labeled with a tag, and placed into a large container of buffer (1 – 2 L) containing a stir bar. Using a low mixing speed, the strings attached to the dialysis tubes were taped to the outside of the container to ensure the stir bar did not contact the tubing. The tubes were left in the buffer solution for 12 – 16 h, with one buffer change within the first 4 h. The dialysis procedure was conducted entirely in the cold room at 4 °C. After dialysis, the

tubes were removed from the buffer and the outside of the tubing was patted dry. The clamp was removed and the solution was removed via pipette into a sterile tube and used for purification, SDS-PAGE, or tributyrin assay immediately afterwards.

3.2.18 His-tagged protein purification

The His-tagged lipase was purified using His-Spin Protein Miniprep kit by Zymo Research (Cat. No. P2002). 450 μL of the sample was added to an equal volume of binding buffer. Three aliquots of 300 μL were added to the spin column and binding matrix, in case the concentration of lipase was very dilute. The remainder of the procedure followed the manufacturer's protocol, as described in Appendix C. The purified protein was eluted with 150 μL of elution buffer.

3.2.19 SDS-PAGE

Samples were prepared by adding 25 μL of the sample with 25 μL sample buffer. Sample buffer was prepared by adding 950 μL Laemmli Sample Buffer to 50 μL β-mercaptoethanol. The prepared sample was heated to 95 °C for 5 minutes in the Thermomixer and quickly vortexed and centrifuged. The Mini-PROTEAN Tetra Cell (Cat. No. 165-8000) and Mini-PROTEAN TGX gel was set up in accordance with the manufacturer's instructions, as described in Appendix C. 6 μL of the broad protein standard and 10 – 30 μL of the prepared samples were loaded into the gel wells. The gel was electrophoresed at 200 V for 30 min and the gel was carefully removed from plastic container and washed three times in 200 mL of DI water for 5 min. 50 mL of Bio-Safe Coomassie G-250 stain was applied to the gel and placed on a rocker for 1 h. Afterwards, the stain was removed and 200 mL of DI water was added to destain the gel,

which was placed back on the rocker for 3 – 12 h. The destaining process was aided by the addition of a Kim Wipe.

3.2.20 Tributyrin assay

A solution of 2 mL tributyrin and 5 mL of 100 mM potassium phosphate buffer, pH 7.3, and 20 mL of DI water were added to a 100 mL plastic beaker (Mettler Toledo, Cat. No. 22006). The beaker was attached to the Mettler Toledo T50 pH-stat and a jacketed 250 mL reactor containing 200 mL of DI water was raised to encase the plastic beaker. The jacketed reactor was attached to a Fisher Scientific Isotemp Refrigerated Circulator (Model 9100), which maintained a constant reaction temperature of 40 °C. The pH electrode was rinsed and placed into the sample holder, along with the autoburette tip and rotational motor containing a plastic mixing impeller.

The program was activated and the pH-stat automatically adjusted the pH of the solution to 7.5 using 0.1 M NaOH certified solution. Once the pH 7.5 was maintained, the program indicated when to add the sample containing the lipase. The enzyme was added through a hole in the top of the pH-stat and the start button on the touch pad was immediately pressed, starting the program, as described in Appendix C. After the program was completed, the R2 value displayed was the mL of titrant per min used through testing (mL/min). This value and the mass of sample added were used to determine the enzyme activity in tributyrin units per gram (TBU/g) based on the calibration slope that was generated using enzymes with a known activity, as shown in Appendix D. 1 TBU unit = 1 μmol butyric acid released per minute / g enzyme.

3.3 Results and Discussion

3.3.1 Approach and methodology

A relatively high number of microbial lipase sources have been found to have high trans/-esterification activity that are suitable for biodiesel production. The genes for a number of these lipases have been identified, and their sequences made publicly available. Genetic and protein engineering has allowed a few of these lipases to be produced commercially in bacteria and yeasts. The recent advances in plant biotechnology and genomics have allowed us to transgenically express recombinant proteins and enzymes in plants as well. However, no one has taken advantage of plant expression systems to produce microbial lipases; leading to the motivation for this research. Our aim was to generate a proof-of-concept for the long-term goal of developing transgenic plants to express various lipases for testing and producing large quantities of lipases for the enzymatic biodiesel production.

The initial approach was to start with a known lipase from a microbial source that has high trans/-esterification activity, detailed genetic information, and has been successfully cloned, transformed, and expressed in a host organism. As a result, the lipase from *Thermomyces lanuginosus*, a thermophilic fungus, was chosen for this research. Although the transformation of plants was fairly straight forward, the transgenes had to be expressed in an appropriate fashion, since the originating host was a fungus. In some cases, the expression of transgenes could result in metabolic energy waste, negative pleiotropic effects, and potential gene escape.[47] Moreover, we did not know if the accumulation of lipase within the plant cell would interfere with the plant's metabolic and biosynthesis pathways; this could adversely affect growth and development.

Therefore, a considerable amount of planning and experimental design was needed in order to properly express a recombinant enzyme without detrimental effects on the plant.

We focused on over-expressing cloned lipase genes in plants to produce a biocatalyst for biodiesel production using genetic engineering and biochemical strategies. The specific goals and objectives of the research were: (1) to clone and characterize a lipase gene with known trans/-esterification activity; (2) to genetically engineer the cloned lipase gene into higher plants, like tobacco and *Arabidopsis thaliana*; and (3) to extract and purify the recombinant lipase from the transgenic plants followed by activity analyses. An outline of the steps in the experimental approach is shown in Figure 15.

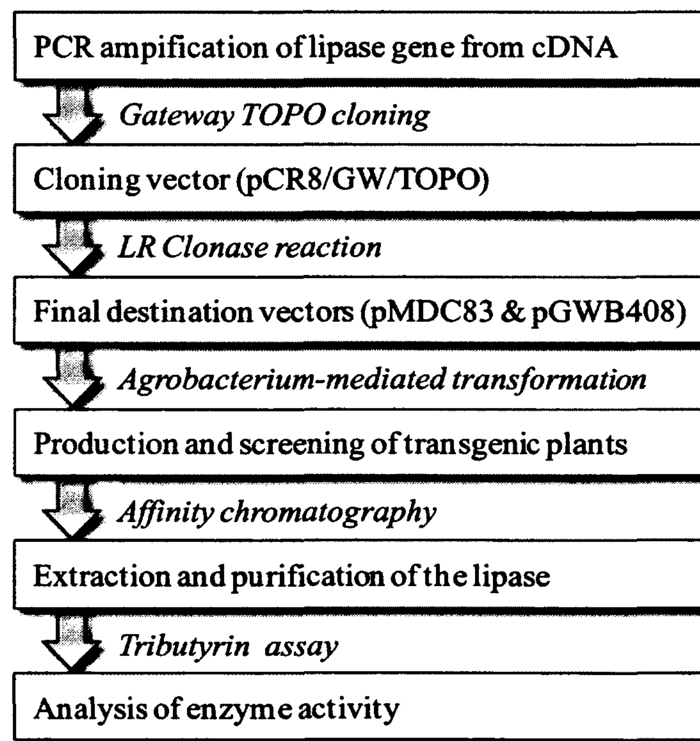


Figure 15. Flow chart for the strategy for the production of lipase in transgenic plants.

3.3.2 Cloning of *T. lanuginosus* lipase genes

There are currently 26 nucleotide listings on NCBI for lipase from *T. lanuginosus* and numerous other literature references. However, different strains of fungi can have slightly different genomic sequences and many of the strains referenced are locally acquired by research groups or provided by a company. Not having the correct fungal strain with the corresponding genetic information could cause difficulties in gene cloning. In April 2011, the entire genome for a commercially available strain of *T. lanuginosus* from ATCC (ATCC Strain 200065) was sequenced, annotated, and made accessible online by the Genozymes Project, Concordia University.[177] Analysis of their database identified two lipases that corresponded with other reported lipases listed on NCBI, matching both their coding sequence (CDS) and protein sequence, as reported in Table 6.

Table 6. *T. lanuginosus* (ATCC Strain 200065) lipase identified by NCBI BLAST.

Name	Protein ID[177]	Gene Length	Protein Length	NCBI access number with 100 % identity
TL1	Thela2p4_000465	1069	291	EU004196, EU370914
TL2	Thela2p4_000466	1061	291	CS793911, AF054513

The sample of *T. lanuginosus* we received from ATCC came freeze-dried in a glass vial and was rehydrated following protocol provided by ATCC. The rehydrated *T. lanuginosus* was cultured onto potato dextrose agar (PDA) medium containing olive oil to induce lipase expression and was incubated at 50°C for 72 h. The fungus was collected and frozen using liquid nitrogen in order to isolate the total RNA. The initial attempts to isolate the total RNA failed because the fungi grew into the agar media and was impossible to separate for isolation. This was easily corrected by subculturing the *T.*

lanuginosus onto a piece of sterile cellophane that was placed on top of the PDA medium, as recommended by Dr. Kirk D. Broders, Department of Biological Sciences.

Total RNA isolation was required because the genomic DNA (gDNA) indicated there were three introns in the genomic sequence, ranging between 58 – 74 bp each.[177] Although these introns were removed during the transcription process within the fungi, it was not guaranteed that they would be properly removed within plant cells. Therefore, cloning required isolating the total RNA and the reverse transcription of the mRNA into complementary DNA (cDNA) using reverse transcriptase. The mRNA of every protein being produced by the organism at the time of isolation had a corresponding double stranded cDNA segment with an identical sequence.

The lipase genes were amplified by PCR using specific primers. The primers were designed to construct three variations of the lipase gene, as shown in Table 7. The first constructs, A1 & B1, include the entire CDS with the predicted signal peptide that was 17 aa in length. The signal peptide at the N-terminus of the protein helped direct the protein to the proper post-translational pathway. The lipases of *T. lanuginosus* are extracellular and the signal peptide directed the protein to be secreted from the cell. However, it is unknown if the signal peptide or protein would function properly in a plant cell since the secretion mechanisms are different than that in yeasts. Therefore, constructs A2 & B2 were developed, in which the signal peptide was removed from the CDS. Since this removed the start codon (ATG), the primers were designed to add the start codon to beginning of the sequence. Recognition of initiator codons by eukaryotic ribosomes as the translational start site could also be affected by the removal of the signal peptide. Therefore, the third constructs, A3 & B3, were designed to replace the signal peptide

with the Kozak sequence: AGA ACC ATG GGC. This sequence was determined by Marilyn Kozak to improve translational recognition of the ribosome by introducing a modest amount of secondary structure to the beginning of the CDS.[178] Reverse primers removed the stop codon to allow for gene fusion with reporter/purification tag to the C-terminus.

Table 7. Primers used for cloning and sequencing of lipase from *T. lanuginosus*.

Construct / Lipase	Forward Primer (5' – 3')[a]	Reverse Primer (5' – 3')	T_M (°C)[b] Fwd/Rvs
A1 / TL1	ATGAGGAGCTCCCTTGTGCTGTT	ATCACACTCTGAAATGGGACCGAA	60.7 / 58.2
A2 / TL1	ATGCGGCCTGTTCGACGA	ATCACACTCTGAAATGGGACCGAA	59.9 / 58.2
A3 / TL1	AGAACCATGGGCCGGCCTGTT	ATCACACTCTGAAATGGGACCGAAATA	64.3 / 60.1
A4 / TL1	AGACGTAACGTGCTCGGAGAA	CATAGATGCGAAGGGATGTCT	58.1 / 54.2
B1 / TL2	ATGAGGAGCTCCCTTGTGCT	AAGACATGTCCCAATTAACCCGAA	58.5 / 56.6
B2 / TL2	ATGAGTCCTATTCGTCGAGAGGT	AAGACATGTCCCAATTAACCCGAA	56.9 / 56.6
B3 / TL2	AGAACCATGGGCAGTCCTATT	AAGACATGTCCCAATTAACCCGAA	56.0 / 56.6
B4 / TL2	AGTCCTATTCGTCGAGAGGTC	TACCATAGGTGCGCAGGGATA	56.6 / 57.7

[a] Underlined section is an addition to the CDS of the lipase.
[b] Melting temperature (T_M) determined by primer manufacturer; Integrated DNA Technologies (IDT).

The fourth pair of constructs, A4 & B4, were exploratory primer sets to amplified a conserved section of the CDS. These were designed in case any we were unable to amplify the lipase gene using the other primer sets. In addition, these primers could be used for screening purposes for all three of the previous constructs. After optimizing PCR conditions (with respect to annealing temperature), we were able to successfully clone all eight constructs. The optimum annealing temperature was found to be 56 °C for A1, A2, and A3, and 52 °C for the rest of the constructs (A4, B1, B2, B3, and B4). The results were determined by gel electrophoresis, as shown in Figure 16. In a collaborative effort, an undergraduate in biology, Jonathan D. Fogel, assisted in the project and focused

primarily on the TL1 constructs, while I focused on the TL2 constructs for the remainder of the project.

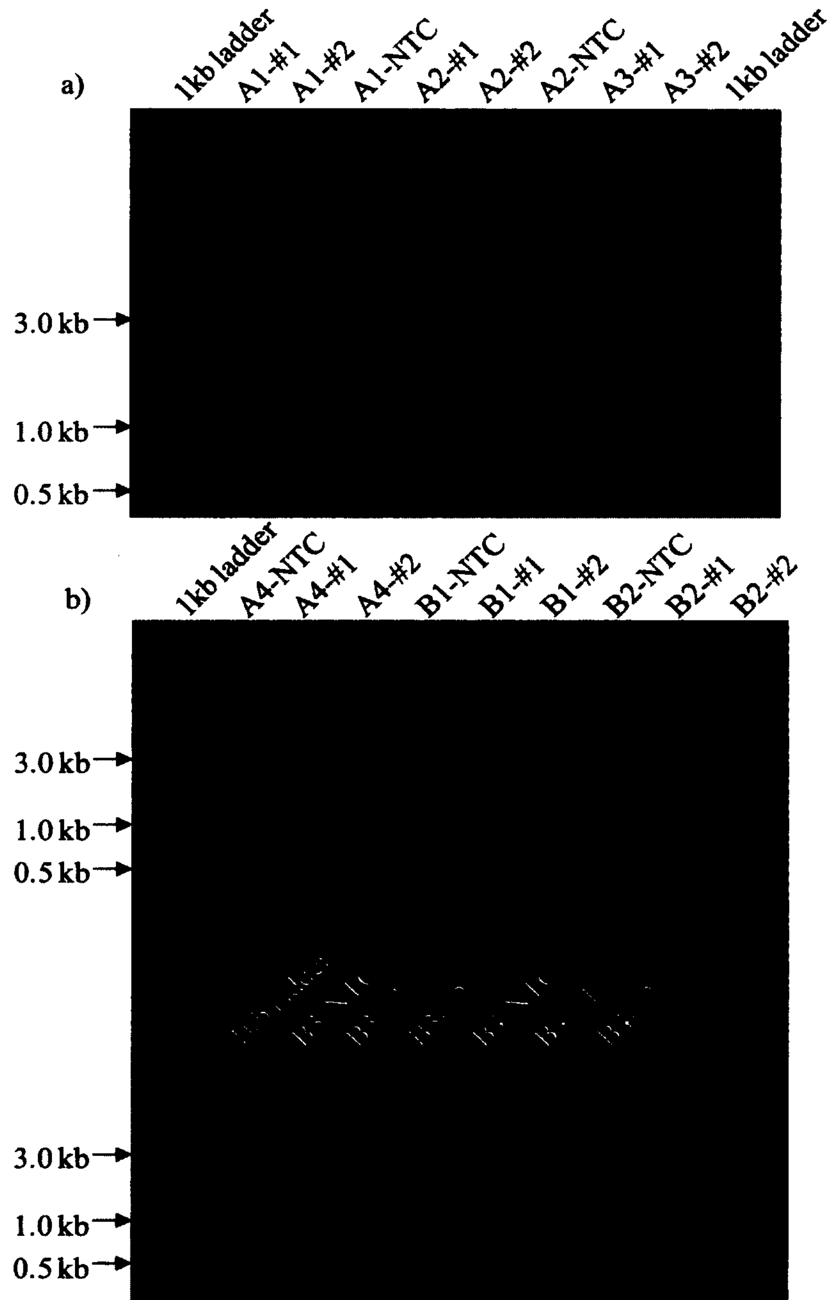


Figure 16. Gel analysis results of *T. lanuginosus* lipase amplification products using TAKARA *Taq* Polymerase. PCR conditions: i) initialization for 1 min at 94 °C, ii) denaturation for 30 sec at 94 °C, iii) annealing for 1 min at 56 °C for a) and 52 °C for b), iv) elongation for 1 min at 72 °C, repeat steps ii – iv for 35 cycles for a) and 30 cycles for b), v) final elongation for 2 min at 72 °C, and vi) hold at 4 °C for 24 h. Expected PCR product size: B1-873 bp; B2-825 bp; B3-834 bp.

3.3.3 Construction of the lipase vectors

The *taq* polymerase used for the PCR had a non-template dependent terminal transferase activity which added a single deoxyadenosine (A) to the 3′ ends of PCR products. This feature allowed us to simply insert our PCR products into a linearized Gateway recombination cloning vector platform, due to 3′-T overhangs for direct ligation. Insertion into a cloning entry vector (pCR8/GW/TOPO) offered *att* sites for recombination into a variety of Gateway compatible destination vectors without tedious sub-cloning. The pCR8/GW/TOPO – lipase constructs were transformed into chemically competent *E. coli* cells and grown overnight on LB media containing spectinomycin. The cloning vector contained the gene for spectinomycin resistance (SpnR), which is efficient for selection of transformed *E. coli*. However, directionality of the gene was important later for insertion into Gateway destination vectors and the simplicity of the pCR8/GW/TOPO allows the gene to be inserted backwards. Therefore, colonies growing on the selection medium need to be screened to ensure proper orientation.

Several individual colonies were chosen at random and grown overnight in liquid LB medium containing spectinomycin. These cultures were used to isolate and characterize the plasmids for purity and concentration. A restriction digest used restriction enzymes to cleave DNA molecules at specific sites. A plasmid map was generated for each construct using pDRAW32 and analyzed to select the most appropriate enzymes to determine (1) proper insert size and (2) correct orientation. The plasmid maps for pCR8/GW/TOPO - lipase constructs are shown in Figure 17 and the gel pictures from the corresponding plasmid restrictions are shown in Figure 18. Due to a series of defective products, Mr. Fogel was unable to successfully transform any of the TL1 constructs into

the cloning vector and obtain plasmids in the correct orientation before he graduated. Therefore, for the remainder of the dissertation the only lipase constructs discussed are those of TL2.

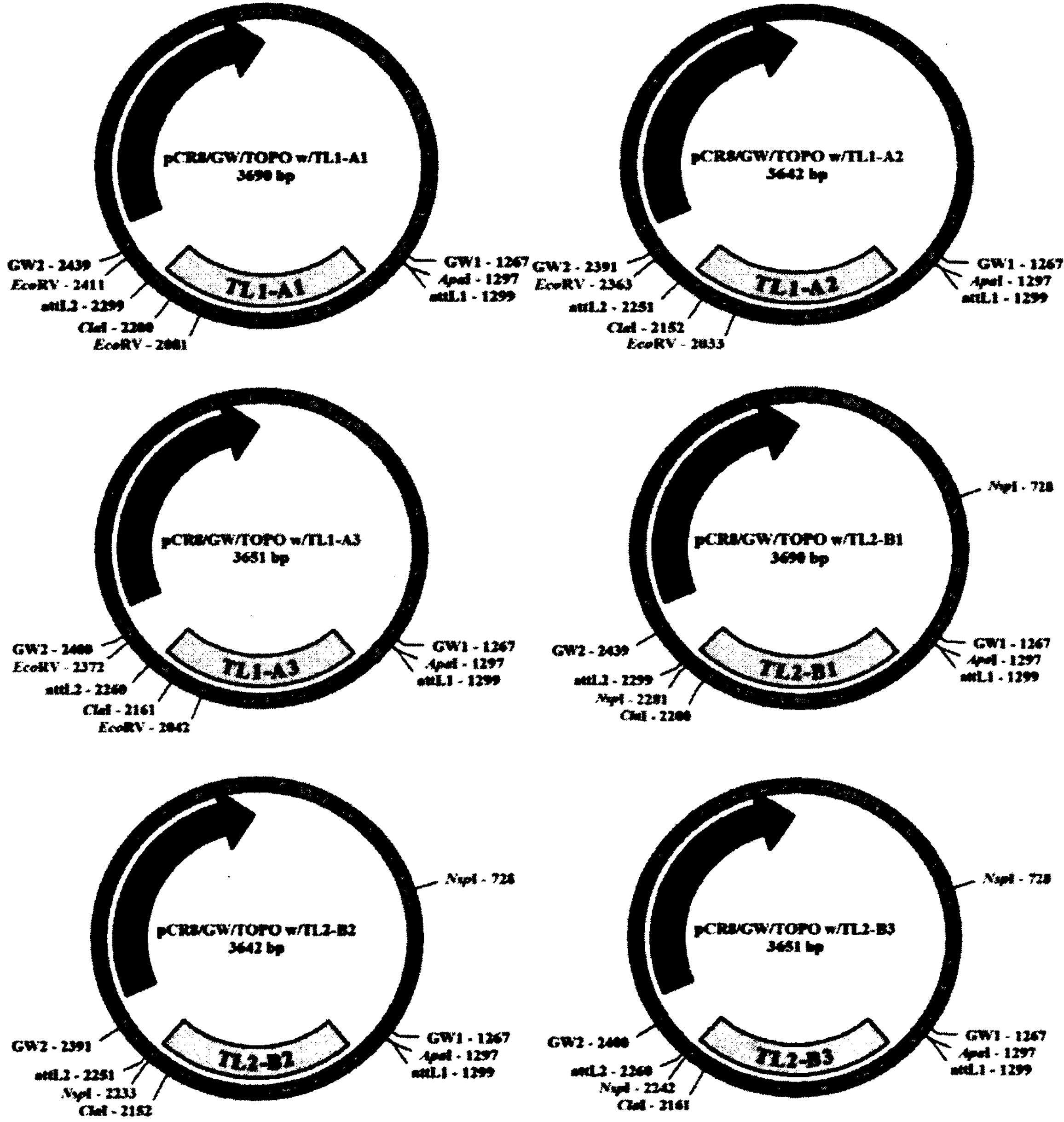


Figure 17. Plasmid maps of pCR8/GW/TOPO – lipase constructs with annotations of important features and enzyme restriction sites.

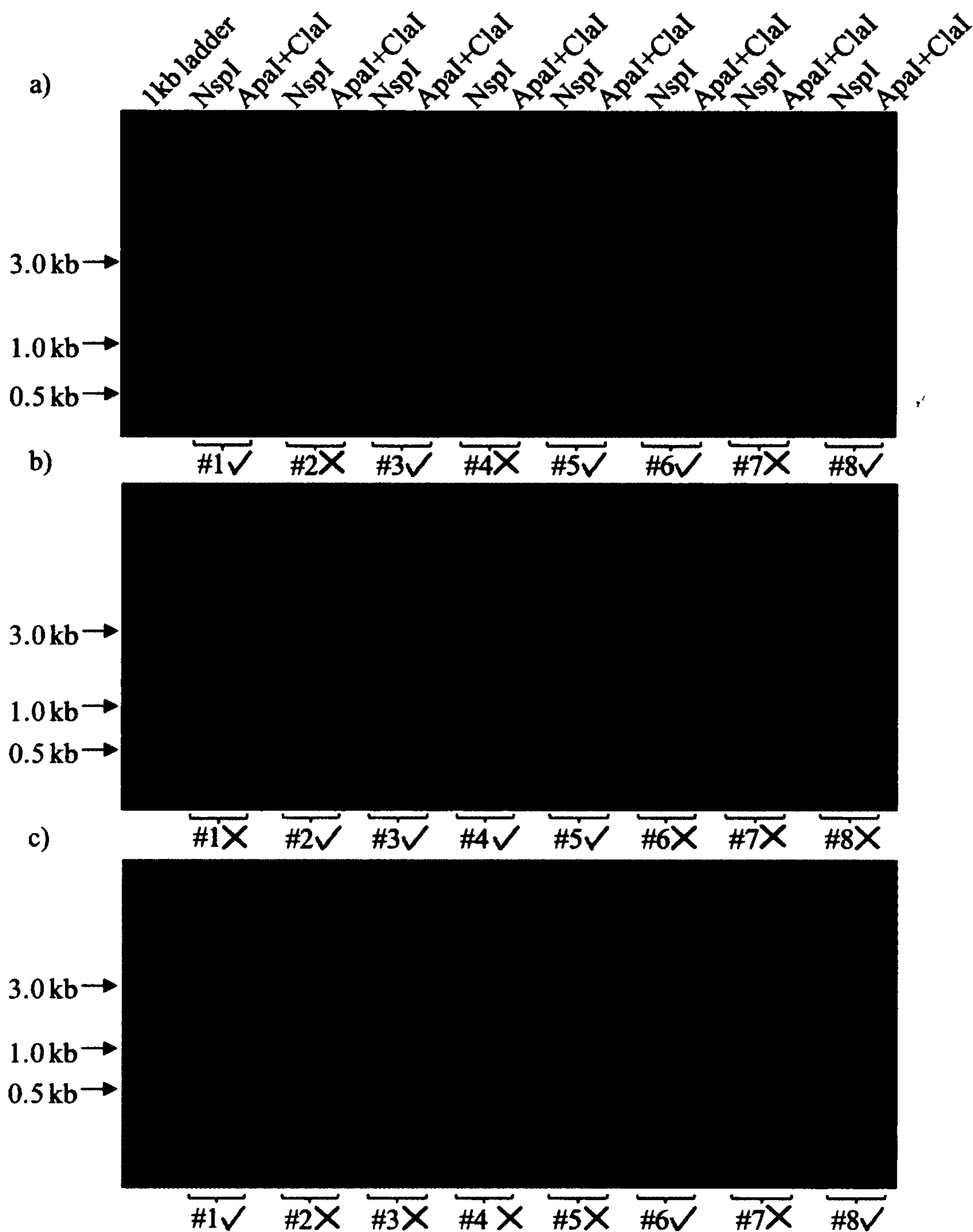


Figure 18. Gel analysis results from plasmid screening by restriction digest of pCR8/GW/TOPO – lipase constructs. Fragment sizes for correct orientation: Nspl: B1-2137, 1553 bp; B2-2137, 1505 bp; B3-2137, 1514 bp. ApaI + ClaI (mix): B1-2787, 903 bp; B2-2787, 855 bp; B3-2787, 864 bp. The green check mark indicates gene insertion in the correct orientation and red X represent plasmids with the lipase gene inserted in reverse, all plasmids indicated gene insertion.

The isolated plasmids that indicated the correct insert size and proper orientation by gel were sent for sequencing at the University of New Hampshire's Hubbard Center for Genome Studies (HCGS) DNA Sequencing Core Facility. While several of the sequences indicated some substitutions and deletions of the known sequence, there were a few colonies from constructs B1 and B2 that matched 100 % with the reported sequence from the Genozymes Project database, the results of which are shown in Appendix E. Construct B3 experienced a greater number of alterations its CDS. However, there was one vector analyzed that had minor alterations, which after analysis was determined not to affect the actual protein sequence and was used for further transformations. The other colonies sequenced indicated more substantial deviations from the known sequence, which affected the translated protein. After sequencing, glycerol stocks were made of each *E.coli* culture containing the cloning vector with the correct lipase gene (orientation and sequence).

Final destination vectors, in this case vectors for plant transformation, were used for *Agrobacterium*-mediated transformation of tobacco and *Arabidopsis*. These vectors were able to replicate in *Agrobacterium* and *E. coli*, contain selectable markers to identify successfully transformed bacteria and plants, and possess the left and right boarder sequences for *Agrobacterium*-mediated transformation to ensure proper integration of the genes of interest into the plant's genome.[47]

Producing transgenic plants is often hindered by the conventional cloning technology (i.e. restriction digestion and ligation), which is rather labor intensive. However, several research groups have developed several series of final destination vectors that are Gateway compatible and are freely available for academic purposes.[179] These vectors

were used for the expression of protein fusions to reporter/purification tags, such as GFP, β-glucuronidase (GUS), 6 x Histidines (His), and glutathione S-transferase (GST). In addition, these vectors could also be used to generate different promoter::reporter constructs to facilitate constitutive or inducible transgene expression.

Gateway Technology is a universal technology used to clone DNA sequences for functional analysis and expression in multiple systems using site-specific recombination properties of bacteriophage lambda.[180] This provides a rapid and highly efficient way to transfer DNA sequences by allowing recombinant sites to provide high specificity and activity. The use of the λ site-specific recombination system allows sites *att*L1 and *att*L2 (from the TOPO vector) to react only with the corresponding *att*R1 and *att*R2 (LR Clonase reaction), respectively, on the final destination vector by a mixture of lambda and *E. coli*-encoded recombination proteins.[181] Therefore, unlike the pCR8/GW/TOPO vector transformation, the orientation of the transferred gene was maintained. To ensure the desired vector was obtained, a negative selectable marker consisting of the bacterial *ccdB* gene was located between *att* sites, which encoded an anti-DNA glyrase protein. The DNA gyrase enzyme relieved the strain while double-stranded DNA is being unwound by helicase during transcription and replication.[179] Disruption of this enzyme results in bacterial death; however, *E. coli* strain DB3.1 contains a mutated DNA glyrase protein which was not susceptible to the *ccdB* protein and used to replicate the stock untransformed vector.

The two final destination vectors selected for this study were pGWB408 and pMDC83, which were developed by Nakagawa *et al.*[182] and Curtis and Grossniklaus[179], respectively. These two vectors both constitutively express the recombinant protein with

a fused 6xHis tag for rapid purification. The main difference between the two vectors is that the pMDC83 vector fuses a GFP reporter gene to the C-terminus before the 6xHis tag, while the pGWB408 vector just has a fused 6xHis tag on the C-terminus. Additional details on the two vectors are shown in Table 8. The use of two separate vectors and three gene constructs increased the chances for a successful transformation and expression. While the addition of GFP fused onto the lipase could interfere with its activity, it also allowed us to visually observe expression in transgenic plants. Furthermore, the GFP and 6xHis tag can be enzymatically cleaved and separated from lipase after purification. The pGWB408 vector had a higher probability of producing a lipase with a higher activity, since the 6xHis tag is a short segment and rarely interferes with enzyme activity.

Table 8. Final destination vectors selected for plant transformation.

Vector	Size[a] (bp)	Bacterial/Plant Resistances	Promoter	Reporter Gene	Purification Tag
pGWB408	11,703	Spectinomycin/Kanamycin	35S	N/A	6xHis
pMDC83	12,513	Kanamycin/Hygromycin	2x35S	GFP	6xHis

[a] Stock vector size without the gene of interest (does includes *ccdB* gene)

After confirmation by sequencing, the lipase gene was inserted into the final destination vectors (pGWB408 and pMDC83) by the LR Clonase reaction, transformed into E. coli by heat-shock method, and grown on LB plate containing the appropriate bacterial antibiotic overnight. Similar to the cloning vector, five individual colonies were selected and grown in liquid media overnight and the plasmids were isolated, characterized, and size confirmed by restriction digests. The plasmid maps for pGWB408/pMDC83 - lipase constructs are shown in Figure 19 and the gel pictures from the corresponding plasmid screening are shown in Figure 20. Analysis of constructs TL2-B1 and TL2-B2 in the pGWB408 vector showed the digest with restriction enzyme

NdeI resulted in the expected fragment sizes while the other restriction enzyme BsaI did not result with the expected sizes. A similar result was shown with the pMDC83

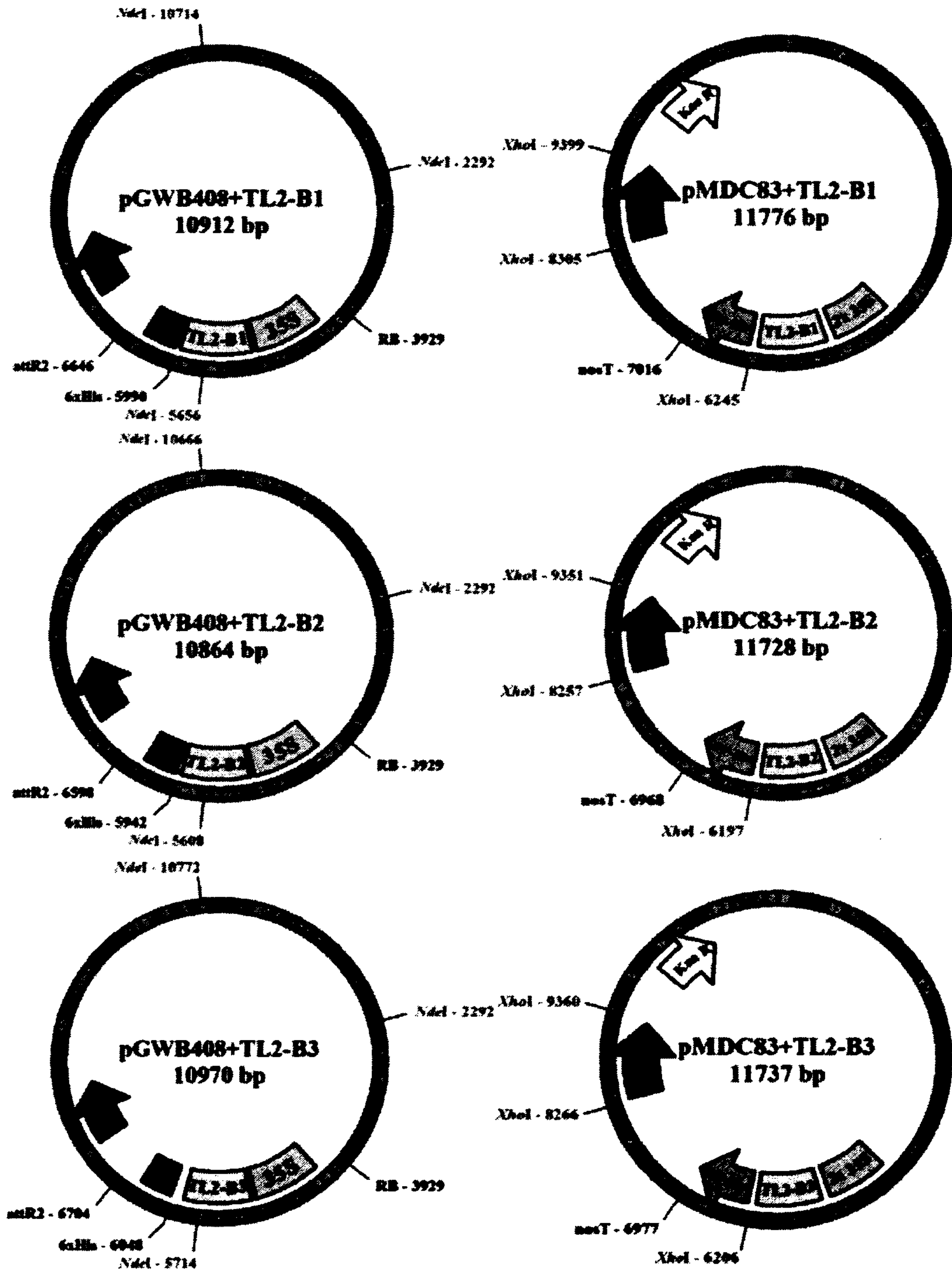


Figure 19. Plasmid maps of pGWB408 & pMDC83 – lipase constructs with annotations of important features and enzyme restriction sites.

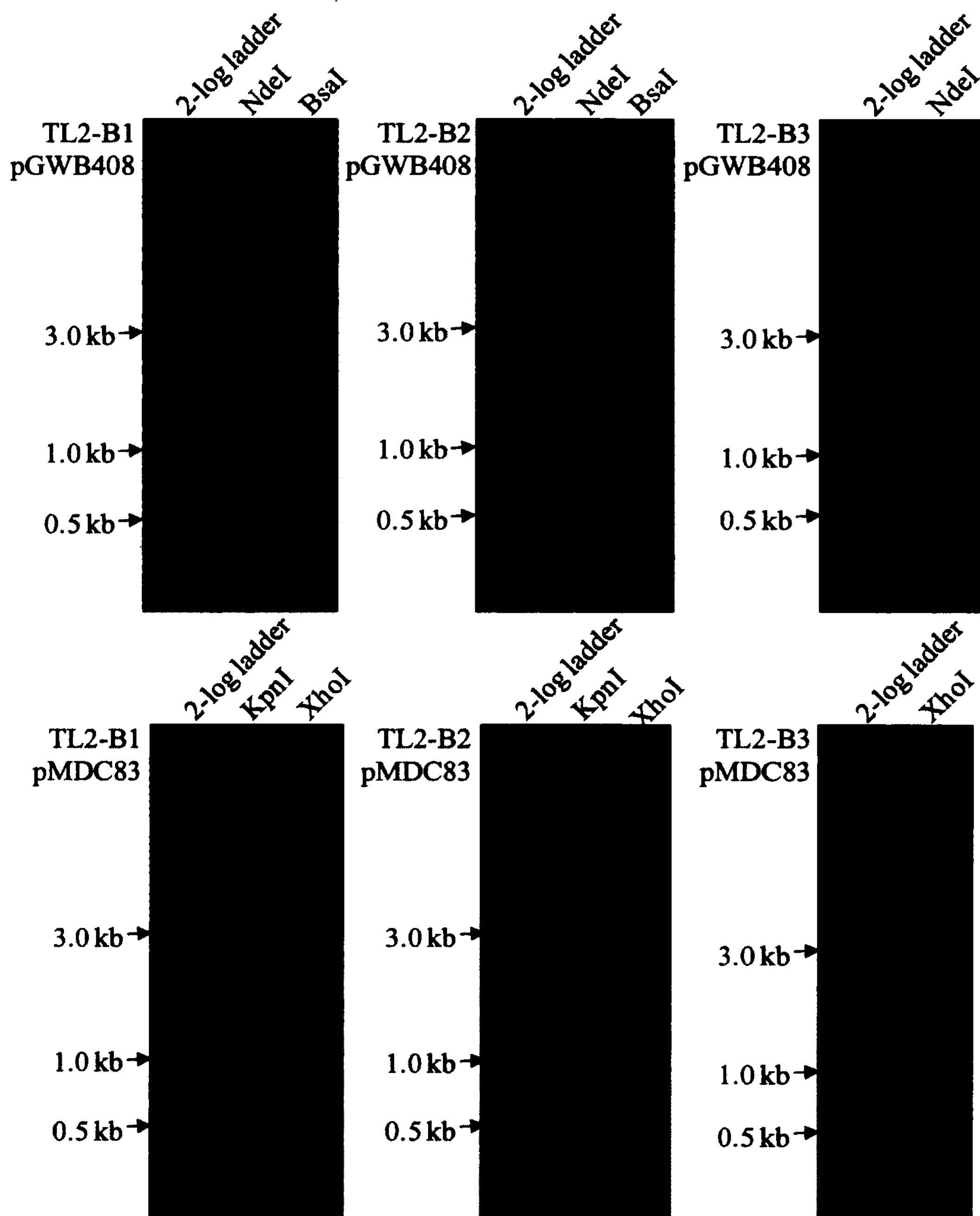


Figure 20. Gel analysis results from plasmid screening of final destination vectors, pGWB408 and pMDC83. Restriction expected sizes: NdeI: B1-5058, 3364, 2490 bp; B2-5058, 3316, 2490 bp; B3-5058, 3325, 2490 bp. BsaI: B1-6188, 3840, 884 bp; B2-6188, 3840, 836 bp. XhoI: B1-8622, 2060, 1094 bp; B2-8574, 2060, 1094 bp; B3-8581, 2060, 1094 bp. KpnI: B1-10758 and 1018 bp; B2-10758 and 970 bp.

vector with XhoI resulting in the expected sizes while KpnI did not. This may be due to problems with the restriction enzyme, buffers, preparation, or an incorrect plasmid map which was used to select the enzymes. Although the correct fragment sizes were shown with one enzyme, additional verification was needed. Therefore, instead of selecting another enzyme to test, which may run into similar problems, the presence of the lipase gene was confirmed using PCR. The results of the PCR using the constructs' corresponding primer set (Table 7) confirmed that the lipase gene was present in the final destination vectors, as shown in Figure 21. Until now, the destination vectors containing the TL2-B3 constructs were not analyzed. Therefore, after this point, they were analyzed using only restriction digest with NdeI and XhoI for vectors pGWB408 and pMDC83, respectively, to expedite the process. Glycerol stocks were made of each *E. coli* culture containing the destination vector with each lipase constructs.

Figure 21. Gel analysis results from PCR screening of pGWB408 and pMDC83 with TL2-B1 and TL2-B2 constructs. Expected PCR product size: B1-873 bp and B2-825 bp.

3.3.4 Transformation of plants

Each of the confirmed final destination vector constructs was transformed into *Agrobacterium tumefaciens* (strain GV3101) by electroporation and grown on LB plate media with the appropriate bacterial antibiotic for 48 – 72 h at 27 °C. Interestingly, the *Agrobacterium* with the pMDC83-lipase vector grew a sufficient number of colonies within 48 h. However, at that time only a few or no colonies with the pGWB408-lipase vectors grew and required an additional 24 h to obtain a sufficient number of colonies. Therefore, the choice in vector appeared to affect the selection and/or the replication rate of *Agrobacterium*, with the pMDC83 vectors resulting in efficient transformation or faster growth. A final verification was performed on selected *Agrobacterium* colonies to ensure the presence of the vector and the lipase gene. The individual colonies were grown for 48 h at 27 °C and screened by PCR after a crude DNA extraction using the corresponding lipase construct primer set (Table 7). The gel results indicated that the lipase gene was present in each colony tested, as shown in Figure 22. Glycerol stocks were made of each *Agrobacterium* culture containing the destination vector with each lipase constructs.

The *Agrobacterium* containing pGWB408 and pMDC83-lipase cultures were grown in liquid LB media with the appropriate antibiotic overnight. To assist in the transformation process, acetosyringone was added to the culture medium to a final concentration of 50 µM. Acetosyringone is a phenolic compound that is typically released by wounded plant cells and triggers the expression of the *vir* proteins in *Agrobacterium*. After incubation, the culture was diluted to OD_{600} ~ 0.7 with a 0.9 % (w/v) sodium chloride (NaCl) solution. Non-transformed tobacco (NT tobacco) leaves

were carefully cut into 1 cm^2 pieces and submerged into the diluted *Agrobacterium* solution. Each piece was wounded several times by puncturing the leave with the tip of a scalpel to allow access for the *Agrobacterium* to transform the plant cells. The tissue was left to soak in the solution for 10 – 15 min, padded dry with sterile paper towels, placed on a callusing basal medium plate without antibiotics, and left in the growth room for two days.

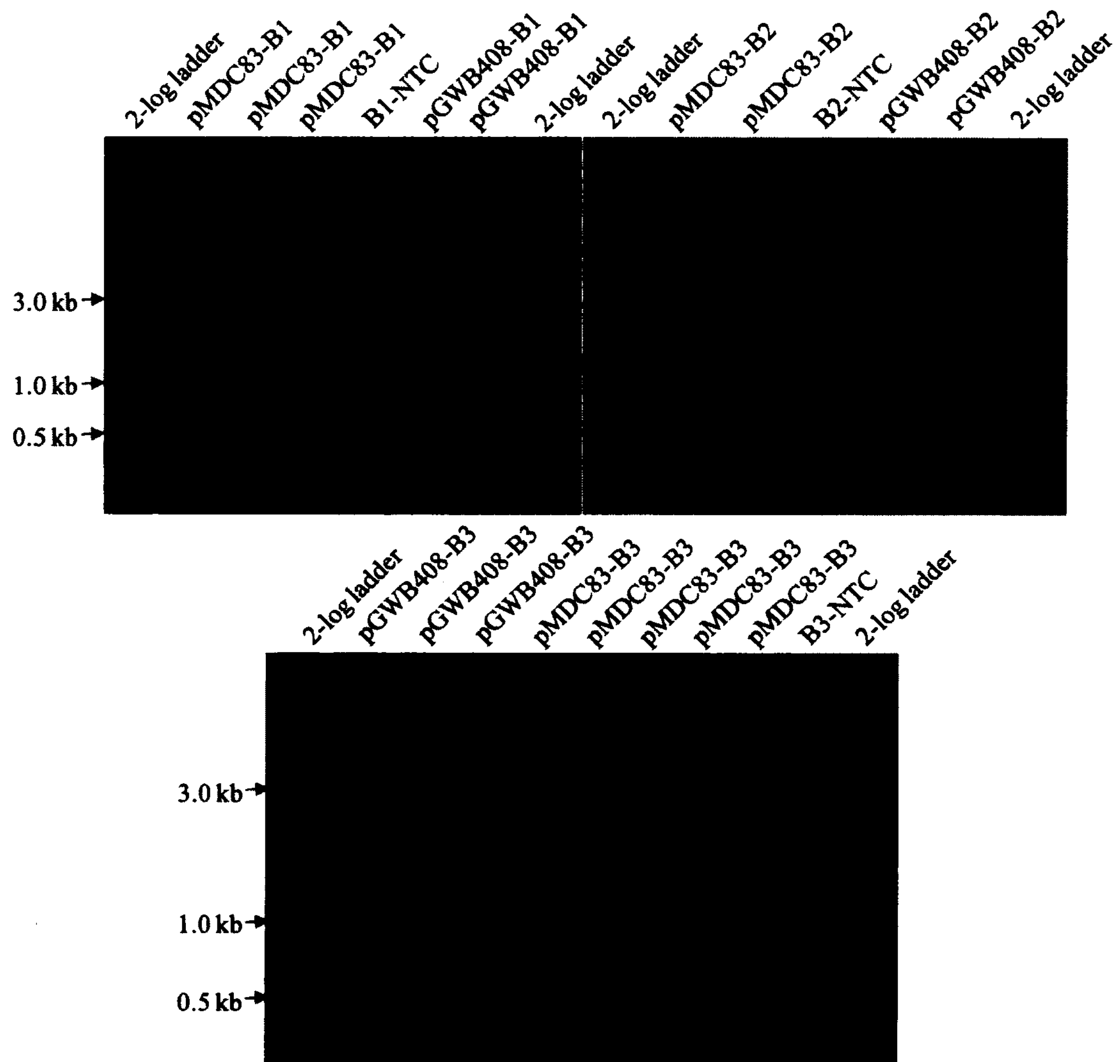


Figure 22. Gel analysis results of pGWB408/pMDC83-lipase constructs in *Agrobacterium*. PCR conditions: i) initialization for 1 min at 94 °C, ii) denaturation for 30 sec at 94 °C, iii) annealing for 1 min at 52 °C for b), iv) elongation for 1 min at 72 °C, repeat steps ii – iv for 35 cycles for a) and 30 cycles for b), v) final elongation for 2 min at 72 °C, and vi) hold at 4 °C for 24 h.

The two days on non-selection media allowed the *Agrobacterium* to transfer the TDNA and the plant leaf to begin callusing. This made the tissue more robust and firm, which was easier to handle in subsequent steps. After the two days, the tissue was removed and placed in a solution of 300 mg/L carbenicillin for 10 – 15 min to kill off *Agrobacterium*. The leaf pieces were padded dry with sterile paper towels, placed on a callusing basal media plate with the appropriate plant antibiotics, and placed in the growth room. A total of 36 leaf pieces were transformed per lipase construct and plated 6 per plate, totaling 216 leaf pieces transformed. To minimize chances of bacterial and fungal contamination, the interface between the bottom and top of the Petri dish were wrapped with microporous tape. The pieces were closely monitored for any signs of contamination and re-plated every 2 – 3 weeks onto new media. After 6 – 8 weeks, the leaf tissue began to callus and develop shoots, as shown in Figure 23.

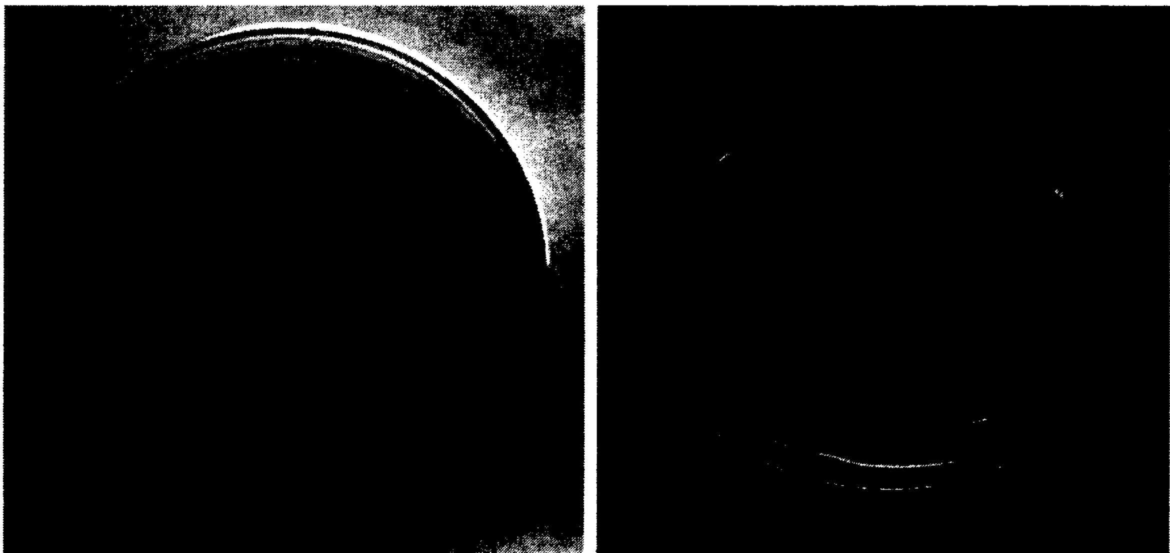


Figure 23. Early stages of producing transgenic tobacco by *Agrobacterium*-mediated transformation: a) leaf tissue growing on non-selection media during the transformation process; b) callus and shoot formation 8 weeks after transformation.

Arabidopsis thaliana was transformed using *Agrobacterium* containing the final destination vectors with the lipase constructs by the floral dip method, the same strains as used for tobacco. Non-transgenic seeds of Arabidopsis were sown in moist soil mix for 3 – 4 weeks. For each lipase construct, 3 pots were prepared one week prior to dipping by clipping the primary bolt to encourage synchrony in branching and flowering. The *Agrobacterium* preserved in glycerol stocks were used to grow 500 mL of culture in liquid LB medium with the appropriate antibiotic. These solutions were pelleted in a centrifuge and resuspended in a solution of 5 % (w/v) sucrose and 0.05 % (v/v) of L-77 Silwet to an OD_{600} of 0.8 ± 0.2 before dipping. The unopened flower buds along with flowers were dipped into the solution of *Agrobacterium* for 8 – 10 sec, avoiding contact with the basal leaves and soil. The pots were laid on their side overnight and covered with clear plastic wrap to prevent desiccation. The plants were then rinsed in cold water to remove any residual sugars and placed in a growth room. This dipping process was repeated 7 days later to ensure sufficient transformation. Afterward, the plants remained in the grow room for 4 – 6 weeks to allow for seed development. The seeds were collected and desiccated at room temperature for 2 – 3 days, followed by sterilization and storage at 4 °C. Pictures of various stages of the transformation process are shown in Figure 24.

The seeds were spread evenly on solid germination media containing the appropriate plant antibiotic. The plates were covered and placed in a dark room at 4 °C for 2 days. Afterward, they were removed from the cold storage and placed in the growth chamber. As expected, the vast majority of the seeds did not germinate or died after 1 week in the presence of the antibiotic. Of the three lipase constructs, only TL2-B1 (with both

vectors) showed growth for up to 20 days. However, these plants did not survive the transplant process from the germination medium to the moist soil.

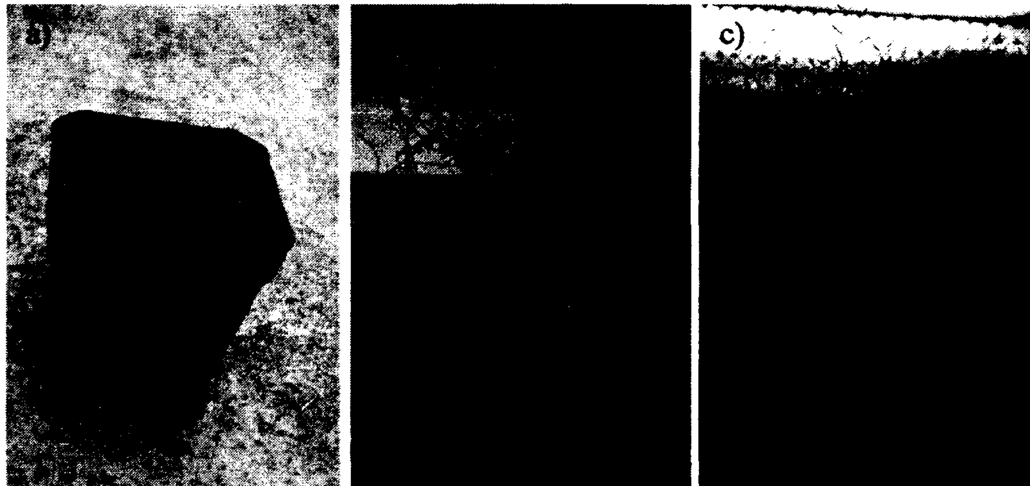


Figure 24. Stages of *Arabidopsis thaliana* floral dip transformation process: a) initial growth of Arabidopsis seeds, b) flowering stage in which the floral dip process is conducted, and c) development and maturity of transgenic seeds.

3.3.5 Screening transgenic plants

After the transgenic tobacco tissue began to calli, another 1 – 3 weeks was required in order to obtain well established shoots from the calluses. Individual events (*i.e.* a shoot with more than one leaf emanating from separate locations) were carefully removed and cultured onto rooting basal media. This allowed each event to establish a root system and develop into a full plant. At the time where the leaves were > 5 mm in size, one leaf was removed and screened for the presence of the lipase gene in its genome. A rapid DNA isolation was performed on the tissue followed immediately by a PCR with the corresponding lipase primers (Table 7). The results of the screening indicated that the majority of the plants did in fact have the lipase gene present. Examples of gel results from the PCR screening are shown in Figure 25.

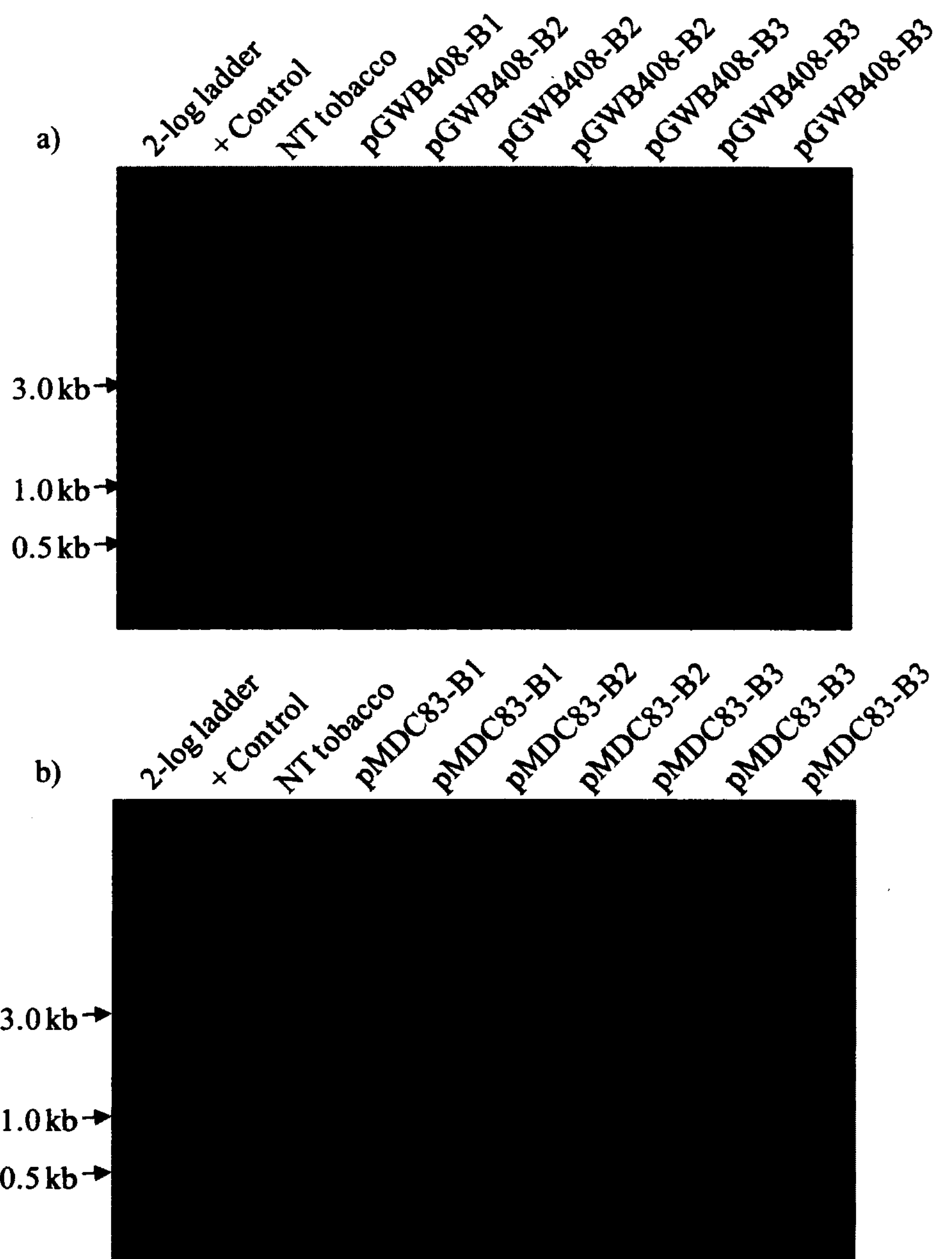


Figure 25. Gel analysis of PCR screening of transgenic tobacco for *T. lanuginosus* lipase gene: a) screening from pGWB408-lipase constructs and b) screening from pMDC83-lipase constructs. PCR product sizes: + Control (*Arabidopsis* spermidine synthase promoter + 5'UTR) – 696 bp, B1 – 873 bp, B2 – 825 bp, and B3 – 834 bp.

The plants positively identified in having the lipase gene were transplanted into a larger container (Magenta box) containing the solid basal plant media and the appropriate plant antibiotics. After another 4 – 6 weeks, nine of the plants were selected from each of the 6 constructs and transported to the University of New Hampshire's Macfarlane Greenhouse Facility. There the plants were transferred from the Magenta box and basal medium to 4" pots containing a moist soil mix. The plants were conditioned for 3 – 5 days in a humid environment and then transferred into the transgenic plant section of the greenhouse. After 4 – 6 weeks in the greenhouse, the plants were transferred into 6" round pots. It was interesting to note the speed in which the plants grew once in soil. In the Magenta boxes with basal media, the plants grew from 1 cm to 10 cm in 4 – 6 weeks. In that same time period, the same plants in soil will grow from 10 cm to 1 m high. This indicated that the plant's development was not affected by the constitutive expression of recombinant lipase.

3.3.6 Extraction, purification, and testing of the recombinant lipase

When extracting recombinant proteins from plant tissues, there are a wide variety of techniques, buffers, and protocols available to researchers. However, there are no real set of rules or guidelines in choosing one method over another and the procedure is usually tailored to the protein and expression system being used. Extraction methods for the recombinant expression of lipase in yeasts were fairly straightforward because the yeast normally secretes the lipase into the medium from which it can be easily filtered and purified.[140-141, 148] Lipases have also been extracted from the seeds of plants, where the early stages of germination produce the largest amount of lipase.[183] This procedure usually required steps to remove residual TAG molecules, since it is a major constituent

of the seed and a substrate for the lipases. However, the easiest and most desired method to extract recombinant proteins that showed constitutive over-expressed in transgenic tobacco is through the leaf tissue.

In general, the processing of recombinant proteins from plant expression systems consists of 4 steps: i) extraction, ii) solid-liquid separation, iii) pretreatment/conditioning, and iv) purification.[184] Extracts from green leaves contain large amounts of proteases, phenol oxidases, and thousands of other plant phenols and polyphenols, some of which can degrade or modify recombinant proteins.[185] Therefore, the process of extraction involves collecting the plant tissue and disrupting either fresh or deeply frozen tissue as quickly as possible by grinding, pressing, or homogenization of the leaf tissue and extracting the proteins in an aqueous environment. Improvements to the stability of the recombinant protein during and after extraction can be accomplished by carefully controlled extraction conditions and extraction buffers containing protease inhibitors, reducing agents, detergent, and antichelating agents.[184] The ionic strength and pH of the extraction buffer can also reduce protein and phenol interactions. However, many of the strategies commonly used to prevent recombinant protein degradation and increase product yield are also detrimental to the typical methods for purification, such as affinity chromatography. Therefore, great care was needed to develop an effective extraction and purification strategy.

Several common themes in protein extraction were observed in literature, a weak extraction buffer (phosphate or Tris) of pH 7 – 8 and a reducing agent, such as β-mercaptoethanol,[186] sodium bisulfate,[187] or dithiothreitol (DTT).[188] However, strong reducing agents, such as DTT, and metal-chelating agents, such as

ethylenediaminetetraacetic acid (EDTA), cannot be used in conjunction with the nickel (Ni) affinity columns used for His tag purification. Therefore, to increase our chances of successful purification, two different buffers were used to extract the recombinant lipase; (1) 25 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM β-mercaptoethanol, and plant protease inhibitor cocktail and (2) 10 mM sodium phosphate buffer pH 7.5, 1 % (w/v) sodium bisulfate, and plant protease inhibitor cocktail. Leaves from the greenhouse plants were frozen in liquid N_2 and ground with a mortar & pestle containing an extraction buffer with a ratio of 3:1 (mL buffer/ g tissue FW). Afterward, the extract was centrifuged and the supernatant collected. A portion of the extract was mixed with the binding buffer from the purification kit and then run through the spin columns in accordance with the manufacturers' instructions. To maximize purification, three column volumes of the sample were run through the column before elution. The crude extract and column elution sample were analyzed by SDS-PAGE. Unfortunately, no bands were observed on the purified sample of the SDS-PAGE gel for any of the samples analyzed, as shown in Figure 26. Several other plants were investigated from the greenhouse, with similar results.

After these results, we decided to backtrack and re-screen the plants in the greenhouse to determine if the lipase gene was being expressed. Additionally, the final destination vectors were sequenced to ensure the gene was correctly recombined and contains the start codon (ATG). The results from UNH HCGS DNA Sequencing Core Facility for both pGWB408 and pMDC83 with each lipase gene construct confirmed their proper insertion into the vectors. The greenhouse plants were re-screened for the presence of the lipase gene and surprisingly only 4 of the 9 plants indicated the presence of the lipase

gene, as shown in Figure 27. There was no clear reason to why some of the plants have lost the transgene. We can only speculate that there might have been contamination issues during the initial screening process or there was the possibility that there were some *Agrobacterium* cells remaining on the leaf tissue from the transformation process.

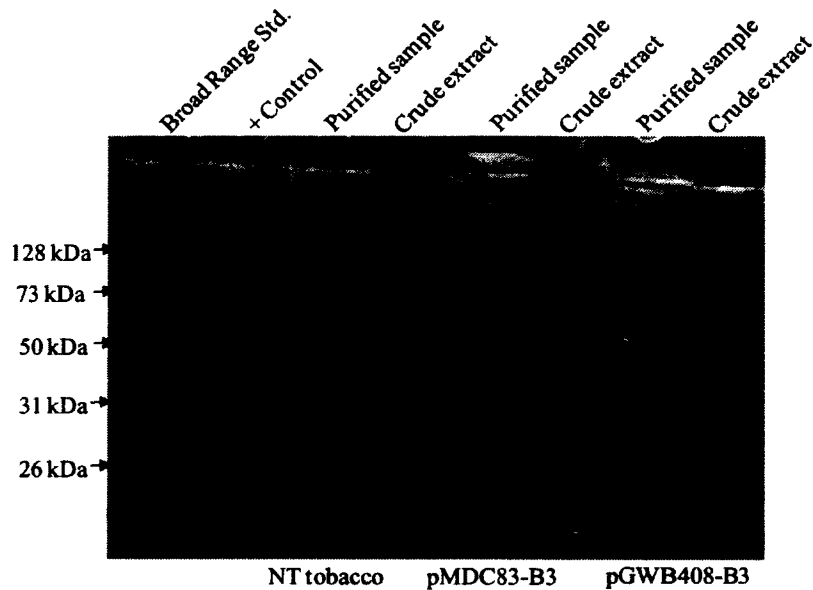


Figure 26. SDS-PAGE gel results from initial protein extraction and purification of the recombinant lipase. Positive control was a diluted solution of *T. lanuginosus* lipase that was commercially purchased. Expected fragment size for pGWB408-B3 is 32.3 kDa and for pMDC83-B3 is 61.4 kDa.

The 4 plants were analyzed to determine if they were expressing the lipase gene. In order to do this the total RNA was extracted from the plant tissue and deoxyribonuclease (DNase) was added to the column matrix and incubated at 37 °C for 30 min prior to column elution. This step removed any residual DNA so that only the RNA remained in the sample. Afterward, the total RNA was used to synthesize cDNA. Analysis of the following PCR with the corresponding primer set (Table 7) indicated that the lipase gene was being expressed in 3 of the 4 plants, as shown in Figure 28. Interestingly, only the pMDC83 constructs indicated gene expression and none by pGWB408 constructs.

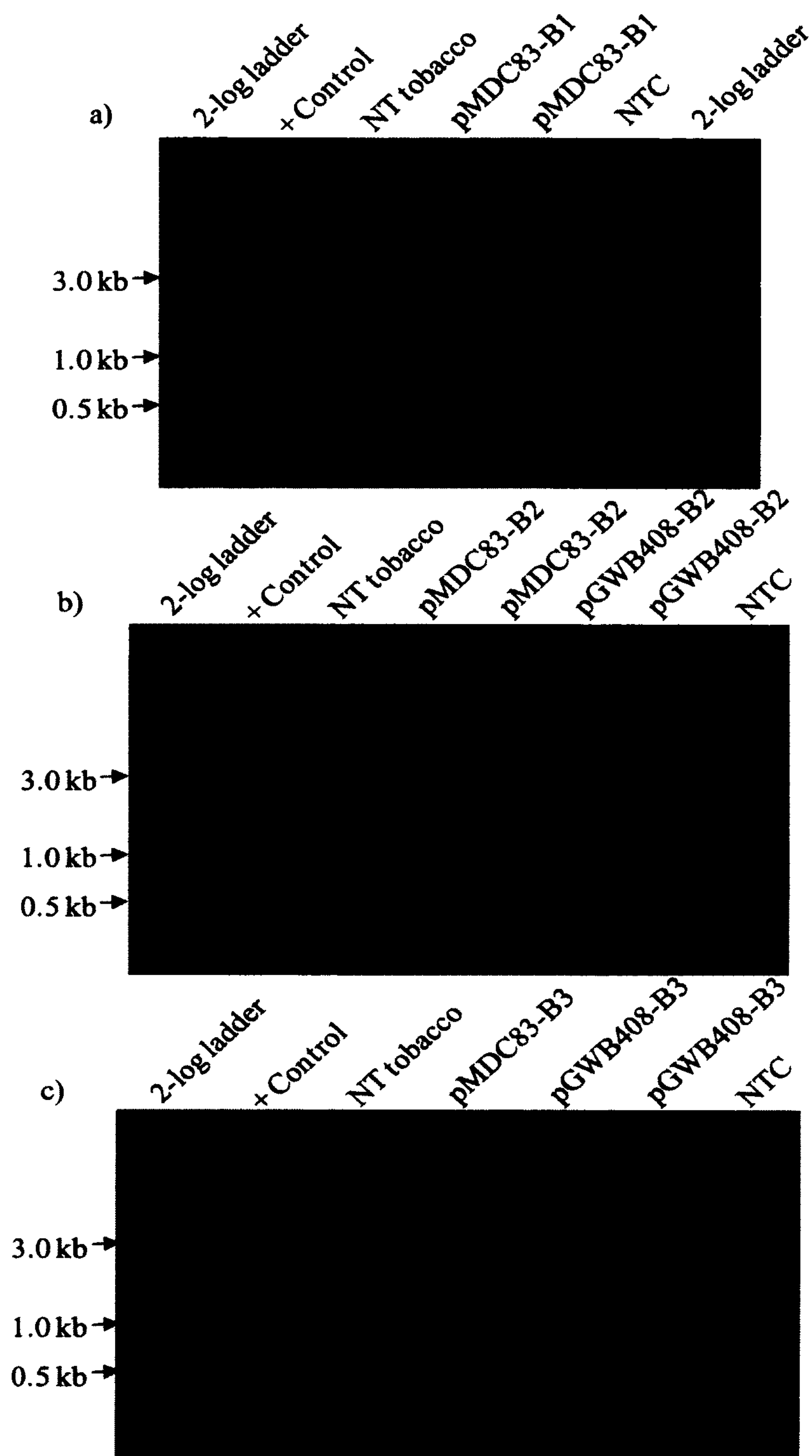


Figure 27. Gel analysis of PCR screening of transgenic greenhouse tobacco for recombinant lipase gene: a) TL2-B1 constructs – 873 bp, b) TL2-B2 constructs – 825 bp, and c) TL2-B3 constructs – 834 bp. Positive control is *Arabidopsis* spermidine synthase promoter + 5'UTR – 696 bp.

Figure 28. Gel analysis of expression screening of transgenic greenhouse tobacco for recombinant lipase gene using cDNA: Expected PCR product sizes: pMDC83-B1 constructs: 873 bp, pMDC83-B3 construct: 834 bp, and pGWB408-B2 construct: 825 bp.

Focus of recombinant protein extraction and purification was then placed on the 3 greenhouse-grown transgenic plants that were positive for lipase expression: pMDC83-B1-A2, pMDC83-B1-A7, and pMDC83-B3. To enhance the extraction process, the crude extracts were concentrated by protein precipitation. Although there were many options available to accomplish this, two of the methods most commonly seen in literature were used: ammonium sulfate and acetone precipitations.[183, 189] In addition, it was found that the plant protease inhibitor cocktail we were using for the first extraction contained EDTA, which was not compatible with the purification columns. Therefore, another protease inhibitor cocktail (PIC) was purchased that was specifically designed for His-tagged protein that contains no EDTA. Secondary metabolites can also accumulate in the vacuoles over time and can severely affect protein extraction and separation.[190]

Therefore, we used plant tissue only from younger/smaller leaves of the selected greenhouse plants.

The extraction process was re-designed to analyze two different buffer systems, with and without PIC, and concentrate the proteins by ammonium sulfate or acetone precipitation procedures (Figure 29). Ammonium sulfate precipitation acts upon the electrostatic forces of the water molecules surrounding a protein and accounts for about 60 % of the precipitation schemes conducted by researchers.[191] An excess amount of salt can cause a protein to precipitate by a phenomenon called *salting-out*. As the salt concentration increases, the ionic strength of the solution increases and the proteins will begin to precipitate out of solution depending on the properties of the specific protein. Often, different fractions with varying amounts of ammonium sulfate are collected sequentially in order to remove some of the unwanted proteins. Performing the procedure at lower temperatures (4 °C) increases the protein's stability and decreases the amount of ammonium sulfate required. Fractions of ammonium sulfate saturation levels were performed on the crude extract at 0 – 30 %, 30 – 60 %, and 60 – 90 % levels, as per Sagiroglu and Arabaci with lipase from sunflower seeds.[189] The precipitated proteins were pelleted by centrifugation and resuspended in buffer and the ammonium salts were removed by dialysis.

Precipitation by organic solvents, such as acetone, accounts for about 35 % of the precipitation schemes conducted by researchers.[191b] The general explanation is that the addition of a water-soluble organic solvent reduces the dielectric constant (ε) of the solution and increases the interaction between the proteins charged groups on the surface, thus leading to agglomeration and precipitation.[191a] However, van Oss discovered that

ethanol does not appreciatively decrease the dielectric constant of an aqueous solution.[192] Instead, he suggested that there was a stronger association between ethanol and water molecules than with protein molecules. Therefore, this dehydrated the protein at the surface which effectively de-shields charged groups and increased the protein-protein interactions. This allowed proteins to stick together more easily and eventually lead to precipitation. However the mechanism, the organic solvents were generally added to the solution cold, around -20 – 0 °C, in order to prevent inactivation and denaturing of the enzyme,[191a] as seen in Chapter 2. The precipitated proteins were pelletized by centrifugation and resuspended in buffer after the acetone was removed.

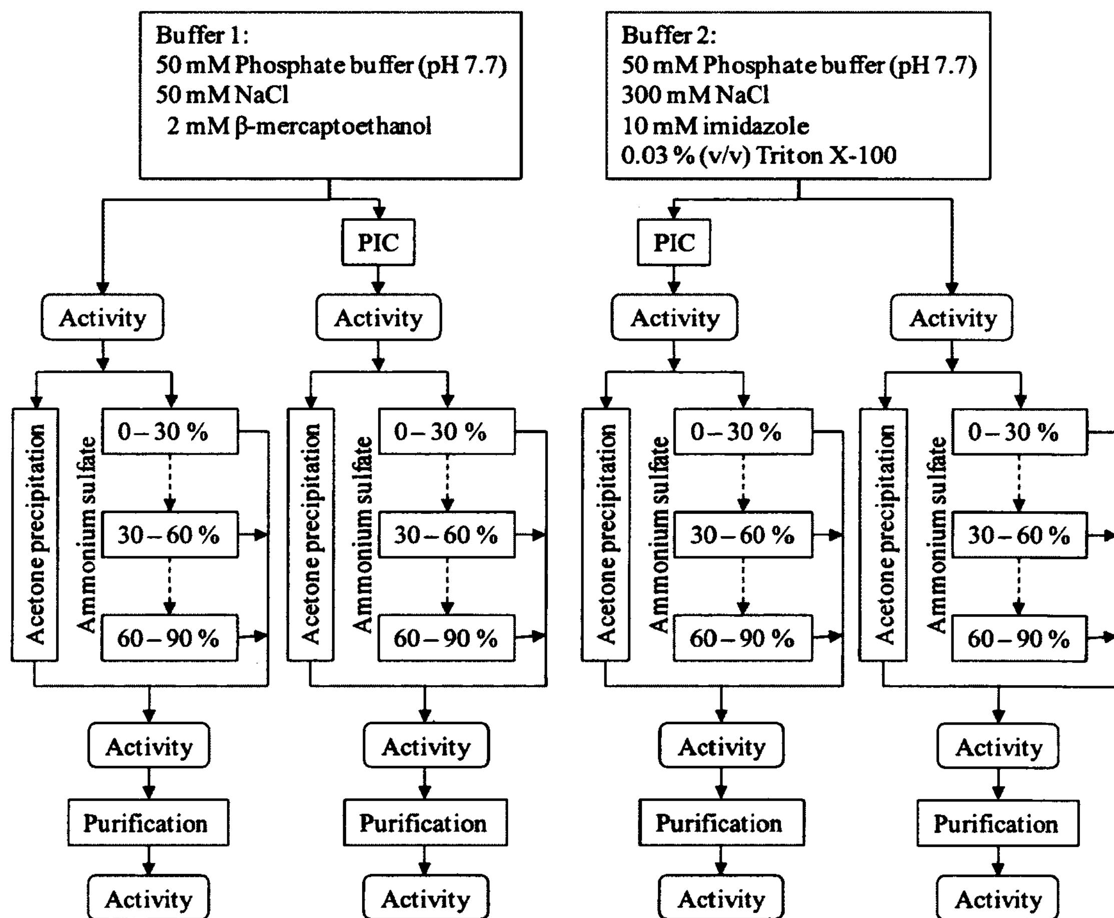


Figure 29. Extraction and purification schemes of recombinant lipase from transgenic tobacco plants.

The young tissue from the selected plants was frozen in liquid N_2 immediately after collection in order to prevent any adverse affects from proteases or any other detrimental compounds. Instead of adding the extraction buffer during the grinding process, the tissue was first ground into a fine powder in a mortar and pestle using liquid N_2 and then transferred to a conical tub. After weighing the tissue, the appropriate extraction buffer was added and vigorously vortexed and left at room temperature for 10 – 15 min. Afterward, the tube was centrifuged at 12,000 x g for 25 min and the supernatant carefully collected. From this point on, the precipitation and purification steps are conducted at 4 °C to maintain enzyme stability.

Ammonium sulfate was ground to a fine powder and slowly added to half of the crude extract, which was at a moderate mixing speed. After each fraction was completed, the solution was left to mix for 1 h and then centrifuged at 13,000 x g for 10 min. The supernatant was transferred to another container and the pellet was resuspended with buffer at $1/10^{th}$ the volume of crude extract. The procedure was repeated with the supernatant for the next two fractions. The resuspended pellet was placed inside dialysis tubing with a 3.5 – 5 kDa molecular weight cut off (MWCO). The tubing was closed tightly and placed in a large container (1 – 2 L) of the corresponding buffer for 12 – 16 h, with one buffer change. A portion of each fraction was run through the spin columns in accordance with the manufacturers' instructions.

The other half of the crude extract was used for acetone precipitation. The acetone was chilled to -20 °C and added slowly to the extract at a 4:1 volume ratio and left for 12 – 16 h at 4 °C to precipitate out. Afterward, the mixture was centrifuged at 13,000 x g for 10 min to pelletize the protein. Acetone was decanted out and pellet was left open

which allowed the residual acetone to mostly evaporate. The same volume of cold acetone was added and the solution was vortexed and left for 1 h. Then the protein was re-pelleted and the acetone was removed and evaporated. The pellet was then resuspended in 1/5th the volume of the crude extract. However, resuspension of the pellet proved difficult and the entire pellet was unable to remain suspended in the aqueous buffer. A portion of the resuspended pellet was also run through the spin columns in accordance with the manufacturers' instructions.

The samples collected were analyzed using the well-known pH-stat method, which was generally used to determine lipase activity.[193] Lipase assays are routinely performed by clinical and research laboratories and there are numerous other methods that can be used, such as spectrometric (photometric, fluorimetric, infra-red), radiometric, turbidometric, chromatographic, interfacial tensiometry, and conductimetric. [194] The titrimetric method using a pH-stat was chosen because we have developed the testing equipment and protocols in our lab and it was also the testing method for the commercial lipase used in Chapter 2 by Sigma-Aldrich. The pH-stat titrator consists of an autoburette, pH electrode, and propeller stirrer that connect to a computer to run the titration program, record results, and perform calculations of lipase activity. There were several commonly available TAG substrates used to determine lipase, such as triolein, tributyrin, and trioctanoin. Tributyrin was selected because it was the least expensive and the most frequently used substrate to determine lipase activity.

The pH-stat method was based on the rate at which the lipase hydrolyzes the tributyrin and forms butyric acid as a byproduct, as shown in Figure 30. The butyric acid was then titrated by the pH-stat with 0.1 N sodium hydroxide (NaOH) in order to

maintain the pH at 7.5 for 3 – 30 min. The consumption of NaOH was recorded as a function of time and was used to determine the tributyrin units per gram enzyme (TBU/g). A TBU was defined as the amount of lipase which releases 1 μmol titratable butyric acid per minute at pH 7.5 and 40 °C. The activity of a sample was calculated by

$$\boldsymbol{Enzyme\ activity}\ \left(\frac{TBU}{g}\right) = \frac{(R2)*(M)*1000}{W} \qquad \text{Equation 4}$$

where R2 is the average titrant rate (mL/ min), M is the molarity of the titrant (mol/L), and W is the weight of the sample (g).[195] A standard curve was generated with series of different dilutions of *T. lanuginosus* lipase in an aqueous solution with known activity of 100,000 TBU/g, as determined by Sigma-Aldrich, and plotted as TBU vs. R2, details of which are shown in Appendix D. The associated mean slope was used to determine the corresponding enzyme activity of the unknown samples.

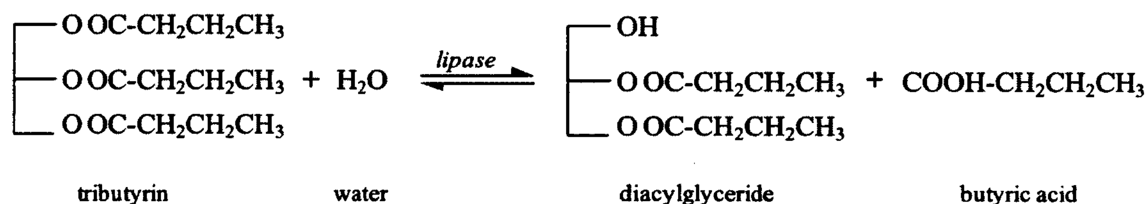


Figure 30. Enzyme hydrolysis of tributyrin.

The extraction and purification scheme (Figure 29) was performed on the greenhouse plant with pGWB408-B1#A7 and results from the tributyrin assay are shown in Table 9. The samples were analyzed soon after isolation in order to prevent or minimize any enzyme degradation or deactivation. The procedure was also carried out on a NT tobacco as a control and as expected, indicated no activity (data not shown). Although each sample was analyzed twice, the results were inconclusive. Only the crude extract using buffer #2 showed consecutive activity values of 229.9 and 183.0 TBU/g. The

corresponding samples to other activity readings indicated no activity. The problem may have been that the samples were too dilute for an accurate reading on the pH-stat. In addition, the samples that indicated lipase expression have a GFP molecule fused onto the C-terminus. This fusion may have interfered with the lipases' ability to catalyze the hydrolysis reaction and function properly, since the lipase and GFP molecules are approximately the same size at 31 kDa and 28 kDa, respectively.

Table 9. Lipase activity from the transgenic tobacco plant, pGWB408-B1#A2, by tributyrin assay.

Sample[a]	**Buffer #1 (TBU/g)**	**Buffer #1+PIC (TBU/g)**	**Buffer #2 (TBU/g)**	**Buffer #1+PIC (TBU/g)**
Crude - #1	0	0	229.9	0
Crude - #2	68.6	0	183.0	59.0
Acetone - #1	0	0	0	0
Acetone - #2	0	0	0	0
0 – 30 % - #1	0	0	0	0
0 – 30 % - #2	0	0	0	0
30 – 60 % - #1	0	156.5	0	0
30 – 60 % - #2	0	0	166.2	0
60 – 90 % - #1	0	0	0	0
60 – 90 % - #2	0	0	0	0

[a] Number values (#1 and #2) indicate replicate number of the sample analyzed.

The problems associated with detection and activity of the lipase could be due to several factors. Plant cells are very complex in comparison to bacteria or yeasts. Therefore, even though the transgene was being transcribed by the plant, it may not be properly translated by the ribosome. Alternatively, the plant cell may have proteases or other mechanisms to break down and destroy the foreign protein after translation. There could have been problems with the extraction from the green tissue. Proteases, phenolic

compounds, and secondary metabolites that interact, inactivate, or denature the recombinant protein. Lastly, Hey and Zhang also had problems in purifying proteins (antibodies) from green leafy tissue using Protein A affinity chromatography.[186] This was because green tissue contains large quantities of ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), which is involved in carbon fixation. RuBisCO has a molecular weight of approximately 560 kDa and consists of eight small and eight larger sub-units with molecular weights of 12.5 and 55 kDa, respectively. Both RuBisCO sub-units were present in all of their elution samples by Hey and Zhang, indicating some non-specific interactions were quite strong. The large and small RuBisCO sub-units could also be seen in our SDS-PAGE gels as well. In combination with the non-specific binding to affinity chromatography columns, the size of the large sub-unit was close to the expected size of the recombinant lipase, 61 kDa. Therefore, the resulting band in the control elution sample could mask any trace band from lipase from the transgenic plants.

3.4 Conclusions

In this chapter, we have genetically engineered plants to constitutively express a lipase for biodiesel production from spent oils and non-edible plant oils. The gene of a lipase with known trans/-esterification activity was cloned from *Thermomyces lanuginosus*, a thermophylic fungus. Cloning involved isolation of total RNA, reverse transcription of the mRNA into cDNA, and PCR amplification of the lipase gene using specific primers. Three lipase constructs were designed for gene fusion on the C-terminus: (1) entire CDS with signal peptide, (2) deletion of the signal peptide (B2), and (3) addition of the Kozak sequence after signal peptide removal. The gene was inserted into a cloning vector (pCR8/GW/TOPO) and sequenced to confirm its identity. Each of

the gene constructs were then inserted into *Agrobacterium*-compatible plant destination vectors via LR Clonase reaction: pGWB408 which fuses a 6xHis tag, and pMDC83 which fuses GFP and a 6xHis tag. *Agrobacterium tumefaciens* (strain GV3101) was used to transform *Nicotiana tabacum* (tobacco) by *Agrobacterium*-mediated transformation and into Arabidopsis thaliana by floral dip method. The transgenic plants were screened using selection media and PCR, nine of which were transferred to the Greenhouse.

Extraction and purification of the transgenic lipase proved to be more complex and difficult. Green plant tissue contains a myriad of phenolic compounds and secondary metabolites which can interact and deactivate recombinant proteins. Several extraction and purification methods were employed and analyzed by SDS-PAGE and tributyrin assay. The results showed no conclusive evidence for enzymatic activity or presence of lipase. However, there were positive indications that the transgene was being expressed within some of the plants transformed by the pMDC83 vectors.

Recent advances in biotechnology, genomics, and genetic engineering have allowed us to identify, clone, alter, and express gene sequences into plants to produce large amounts of recombinant proteins. The production of biodiesel by enzymatic trans/-esterification has many industrial and environmental advantages. One of the main drawbacks has been the cost and availability of the lipase catalyst. Transgenic expression of lipase within plants allowed us to produce large quantities at a relatively low cost, which made the enzymatic process more economically feasible. In this study, we were able to successfully transform and express lipase from *T. lanuginosus* in tobacco plants. However, we were not able to successfully extract and purify the lipase with any

significant activity. This may be due to problems in translation or protein storage within the plant cells, or with the extraction and purification processes.

Plant expression systems can play an important role in producing industrial grade enzymes in sufficient quantities for enzymatic processes, such as biodiesel. The inexpensive lipase produced by plants can be combined with solvents to allow for fast reaction rates with high product yields. However, further cost reductions are needed in order to compete with conventional biodiesel and petroleum diesel. Further optimization of reaction parameters may be able to significantly increase the useful lifetime of these lipases.

CHAPTER 4

EFFECT OF REACTION PARAMETERS ON ENZYME REUSE IN THE TRANS/-ESTERIFICATION OF WASTE OIL

4.1 Introduction

4.1.1 Reaction optimization

For the enzymatic process to achieve economic viability, the reaction parameters must be optimized for a given lipase and feedstock. As shown in Chapter 2, the choice of organic solvent into the reaction medium can significantly affect the kinetics and yield for biodiesel production. Moderately hydrophobic solvents, such as isooctane and hexane, resulted in the highest FAME yield with all three lipase studied. The lipase from *Thermomyces lanuginosus* was chosen as the optimum enzyme due to its kinetic performance, overall FAME yield, and enzyme activity. In addition to the choice of solvent and enzyme, the reaction parameters can also have a great affect on the yield of enzymatic trans/-esterification of biodiesel, such as temperature, mixing, acyl acceptor, and the molar ratio of alcohol to oil.

In general, increasing the temperature will increase the reaction rate of biodiesel synthesis. However, lipases are proteins and are bound by biological restraints concerning temperature. Daniel *et al.* does an excellent job in describing the effects of temperature on enzymatic structure and conformational stability.[196] They described enzymatic activity requires a sufficient amount of flexibility to perform substrate binding and catalytic functions. Increasing the temperature above an optimum temperature can lead to excessive flexibility, which is the first step towards enzyme denaturation and

inactivity.[197] Although an enzyme must have the flexibility needed for catalytic activity, it must also be sufficiently rigid to have a reserve of stability. Decreasing the temperature below optimum can cause the enzyme to become too stable and rigid, where it loses the flexibility required to perform catalytic functions. Therefore, some balance must be reached where the enzyme is flexible enough for catalysis but stabile enough for an effective lifespan.[197-198]

Reaction temperature enzymatic trans/-esterification can vary greatly depending on the lipase, ranging from 20 – 70 °C.[33] Kovács and Hancsók investigated the methanolysis of three commercially available immobilized lipases in a solvent free system of rapeseed oil.[199] They were able to obtain maximum FAME yields at 50 °C with Novozym 435 (*C. antartica*) and Lipozyme TL IM (*T. lanuginosus*) and at 40 °C with Lipozyme RM IM (*R. miehei*). However, optimum temperature can shift, depending on other system parameters. As an example, the optimum temperature of Novozym 435 has also been determine to be 60 °C by Sanchez and Vasudevan in a solvent free system with olive oil;[200] 35 °C was determined by Jeong and Park with rapeseed oil in a solvent system of *tert*-butanol.[33] Du *et al.* examined the effects of temperature from 30 – 50 °C on lipase from *T. lanuginosus* (Lipozyme TL IM) and found that the enzyme lost activity above 50 °C. Interestingly, they determined that the optimum temperature for a batch process was 40 – 50 °C, but observed a loss of activity at 40 °C during continuous operation and determined an optimum temperature of 30 °C.[42]

Mixing can be very important for the trans/-esterification reaction because of immiscibility issues between the hydrophobic TAG and FAME and the hydrophilic alcohol and glycerol. Ma *et al.* studied the effect of mixing on the transesterification of

beef tallow using the traditional NaOH catalyst.[40] Varying the stirring speeds from 110 – 330 rpm resulted in no significant effect on reaction yield and therefore concluded that the stir speeds investigated exceeded the mixing threshold. As previously described in Chapter 2, the addition of an organic solvent can significantly increase solubility and enhance mass transfer. The effects of mixing in a solvent system of n-hexane on enzymatic trans/-esterification of waste olive oil was investigated by Shen and Vasudevan.[201] In a similar fashion, there was no observable change in FAME yield when stir speeds ranged from 150 – 400 rpm.

Currently, the most common acyl-acceptor is methanol, due to its abundance and relative low cost. Ethanol is the second most common acyl-acceptor because it can be produced from the fermentation of biomass and is presently produced in large quantities for blending with gasoline. However, there is an extensive selection of other acyl-acceptors available for trans/-esterification, the choice of which can significantly influence enzyme catalysis. In addition, the FAAE associated with the corresponding alcohol can affect the cold flow properties, lubricity, cetane number, and the heat content of the fuel.[198, 202] Other than alcohols, methyl and ethyl acetate have been found to be an appropriate acyl acceptor in biodiesel synthesis by Xu and Wu.[33] The use of acetate allows for an ester-ester interchange in which triacetin is formed as a byproduct instead of glycerol. The formation of triacetin eliminates the risk of deactivation of enzyme by glycerol and can be used as a fuel additive to improve cold weather properties.[202] Fu and Vasudevan evaluated methyl and ethyl acetate, along with six other long chained and branched alcohols, on the trans/-esterification of canola oil in isooctane by Novozyme 435.[46] They found that the FAAE yield was much lower for methyl and ethyl acetate in

comparison to methanol and the other higher alcohols as the acyl-acceptor. In addition, the author has briefly investigated using acetates and observed little to no FAAE yield using all three lipase evaluated in Chapter 2. The effect of various other alcohols have also been investigated by Hsu *et al.* using immobilized lipase from *C. antarctica* (B), *P. cepacia*, and *T. lanuginosus* in a solvent free media with recycled restaurant grease, the results are shown in Figure 31.[203]

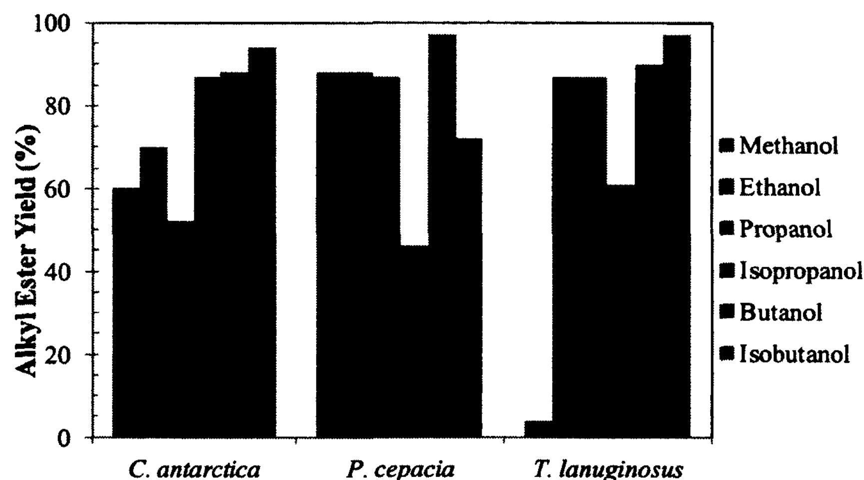


Figure 31. Screening of alcohols for the trans/-esterification of recycled restaurant grease by *C. antarctica (B), P. cepacia, and T. lanuginosus*, adapted from Nie *et al.*[203]

In conjunction with the type of acyl-acceptor, the concentration of the acyl-acceptor can significantly impact the reaction rate and overall yield. Generally, this concentration is referred to in terms of the molar ratio of acyl-acceptor to TAG, in which the stoichiometric ratio is 3:1. The molar excess can speed up the reaction rate and result in higher yields, but this could also lead towards enzyme inhibition, especially with methanol. The effects of the methanol to oil molar ratio on rapeseed oil with Novozyme 435 in *tert*-butanol were studied by Jeong and Park.[204] They determined that ratios between 2:1 and 5:1 resulted in a high conversion with no significant difference and that a ratio above 6:1 reduced conversion. Similar results were reported by Salis *et al.*, who

were able to reach almost 100 % conversions with ratios of 3:1 and 6:1 (butanol:triolein) with *P. cepacia*.[33] Du *et al.* determined the optimum molar ratio of methanol:soya bean oil in a solvent free system to be 4:1, which resulted in a 92 % FAME yield using lipase from *T. lanuginosus*.[205] Various other studies using different enzymes, alcohols, and oil sources have resulted in a general consensus that the optimum molar ratio of alcohol to oil to be around 3:1 and 4:1.[42, 112, 198, 202, 206] However, despite these relatively high yields, methanol and glycerol inhibitions are still a big problem.

4.1.2 Enzyme inhibition

In general, alcohols with less than three carbons are more likely to inhibit lipase due mainly by its polarity, which tends to strip away the essential water required for catalytic action.[144] Chen and Wu estimated that the degree of enzyme deactivation to be inversely proportional to the number of carbons of the alcohol.[33] Therefore, enzyme inhibition is the greatest with methanol. Shimada *et al.* determined the solubility of methanol and ethanol to be ½ and ⅓ of their respective stoichiometric amounts, respectively.[85] They also observed methanol inhibition with molar ratios greater than 1.5:1, supporting the theory of micro-droplets forming in a solvent free medium is the leading cause for deactivation. There are two solutions that have been suggested to overcome the effects of inhibition caused by methanol and ethanol: (1) addition of an organic solvent and (2) stepwise addition of the alcohol. Chapter 2 extensively described the effects and advantages of adding an organic solvent into the reaction media, mainly those that are moderately hydrophobic, like isooctane or hexane.

The most common method is the addition of two or three aliquots of alcohol into a solvent free system; this allows the stoichiometric amount of alcohol to be added without

inhibition. Shimada *et al.* added ⅓ molar equivalent of methanol at 0, 10, and 24 h into a solvent free reaction catalyzed by Novozyme 435.[85] The immobilized *C. antarctica* was found not to be inhibited by this method, whereas significant deactivation was observed using a single addition. They also had success in minimizing inhibition with a two-step methanol addition, with ½ molar equivalent added at 0 and 10 h. Nie *et al.* studied the effect of methanol addition to the reaction mixture on methanolysis by immobilized *Candida sp.* 99–125, where methanol was added from 1 to 10 times.[207] Their results showed that the FAME yield could not be further increased when the methanol was added stepwise more than three times. Several other studies have confirmed that the three additions of methanol are superior to batch addition, in terms of overall FAME yield.[42, 112, 202, 206] However, to the best of the author's knowledge, a stepwise addition into a solvent system or the continuous addition of methanol into the reaction media has not been investigated.

Other than the inhibition by the methanol substrate, the glycerol by-product is also hydrophilic, insoluble in oil, and can also inhibit enzyme activity. Dossat *et al.* recognized that glycerol molecules were adsorbing onto the surface of lipases that were immobilized on hydrophilic matrices.[42] The highly viscous glycerol adsorbing onto this surface was forming a hydrophilic coating over the enzyme, which inhibited its performance.[33] This coat disrupted the diffusion of the substrates to the enzyme's active site. In addition, methanol also begins to accumulate in the glycerol layer, because of its hydrophilic nature, which eventually results in higher concentrations and accelerates the enzyme's deactivation.[85] Therefore, the glycerol must be removed in a timely manner in order to maintain lipase activity and its operational stability. Typically, the glycerol

settles to the bottom and can be decanted easily because of its higher density. However, in continuous and repeated batch processes, reusing the same enzyme can amplify the effect of glycerol inhibition on the efficiency of trans/-esterification with immobilized enzymes.[42] There are two main solutions that have been suggested to overcome the effects of inhibition caused by glycerol: (1) mechanical removal and (2) chemical removal methods.

Shimada *et al.* developed a continuous flow reaction system to produce biodiesel by incorporating both the three-step methanol addition method with glycerol removal.[85] This was accomplished by setting up three packed bed reactors in series. A third molar equivalent of methanol was added prior to each reaction and the resulting mixture drained into a settling tank, where the glycerol was separated and removed. Bélafi-Bakó *et al.* experienced strong glycerol inhibition using Novozyme 435 and investigated *in situ* glycerol removal by dialysis.[208] Using a flat-sheet membrane module, they were able to achieve almost 100 % glycerol removal with an 85 mL/L flow rate at 50 °C. For larger production scales, they recommend using a membrane bioreactor to avoid glycerol inhibition. However, membrane reactors cost significantly more than packed bed and stir tank reactors.

Another approach uses the solubility of glycerol in hydrophilic solvents. Li *et al.* applied *tert*-butanol as an organic solvent for the trans/-esterification of rapeseed oil using Lipozyme TL IM and Novozyme 435.[42] As a fairly hydrophilic solvent (log P = 0.866), *tert*-butanol is a good solvent to prevent both glycerol and methanol inhibition. The adverse effects typically seen with the more hydrophilic solvents were not observed with *tert*-butanol, which may be due to its branched chemical structure. However, Fu and

Vasudevan also investigated *tert*-butanol and found that high concentrations, above 5 % (v/v), severely inhibited enzyme activity of Novozyme 435.[92] Du *et al.* studied the effect of hydrophilic organic solvents to remove glycerol from immobilized lipase.[205] They examined propanol, isopropanol, butanol, and *tert*-butanol and found isopropanol to be the most useful solvent, in terms of glycerol removal and enzyme activity. This result was supported by Xu *et al.*, who found rinsing the enzyme post-reaction with isopropanol maximized FAME yield and maintained activity after 15 repeated cycles.[206] An interesting study by Talukder *et al.* used a salt-based solution to control the concentration of methanol in the oil phase.[112] This is accomplished by dissolving methanol in $MgCl_2$ and LiCl salt solutions. In addition to preventing methanol inhibition, the glycerol also dissolved in the salt-solution phase which prevented glycerol inhibition. However, this process could generate large amounts of waste water and residue, which could significantly increase the cost of production.

4.1.3 Enzyme immobilization

An advantage to using enzymes as a biocatalyst is that they can be immobilized for ease of separation and reuse. By definition, immobilization of enzymes are confined or localized onto a solid support or on a carrier matrix, while retaining its catalytic properties.[202] Immobilization can often lead to some loss in enzyme activity. However, this is compensated by enhancing enzymatic properties; such as operational and thermal stability, activity in non-aqueous media. This allows for greater flexibility, which is why the immobilization method is commonly used industrially. In general, there are two basic categories for immobilizing enzymes: (1) chemical bonding and (2) physical retention, as

shown in Figure 32.[112] There are advantages and disadvantages to each method of immobilization; such as changes to enzymatic activity, stability, and reusability.

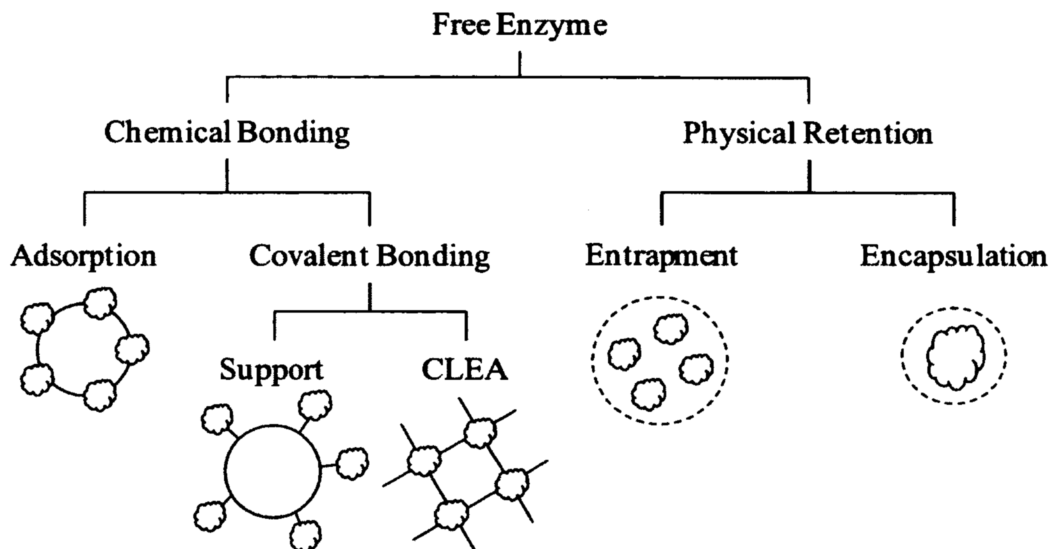


Figure 32. General methods of immobilizing enzymes.[112]

The most widely used method for immobilizing lipase is by adsorption, because it is easy to perform, conditions are mild, and are relatively inexpensive. The lipases are physically adsorbed to the support carrier without effecting catalytic activity by using weak linkages; mainly van der Waals, hydrogen bonds, and hydrophobic interactions.[209] A variety of support carriers have been used for immobilization; such as acrylic resin, alumina, celite, cellulose, ceramics, diatomaceous earth, hydrotalcite, polyethylene, sepadex, silica gel, textile membrane, and different type of zeolites.[112, 202, 209] Salis *et al.* compared the catalytic efficiencies of eight commercial lipases immobilised on macroporous polypropylene via physical adsorption. Namely lipases from *A. niger*, *C. rugosa*, *M. javanicus*, *P. cepacia*, *P. fluorescens*, *P. roqueforti*, and *R. oryzae* were studied.[210] Only those from *Pseudomonas* species were active; in particular *P. fluorescens* lipase reached the highest FAME yield of 96 mol% after a 49 h reaction with

soybean oil as the feedstock. Although adsorption has a high activity for biodiesel production using commercially available lipases and supports, the weak linkages can cause the enzyme to be stripped off and leak during the reaction by shear and other mechanical forces.[112] This can significantly reduce activity and stability of the immobilized lipase which limits reuse and may not be the best solution for enzymatically producing biodiesel.

Covalent bonding is becoming an attractive immobilization method because of its irreversible bonding of the lipase to the support matrix. This in turn increases enzyme stability and prevents leakage into the reaction mixture. There are numerous support carriers available for lipase immobilization by covalent bonding, such as polymer derivates of acrylic acid, porous alumina, cellulose, chitin, controlled pore glass, sepharose, and other synthetic polymers.[209] Immobilization by covalent bonding is much more complex than that of adsorption and usually consists of two stages: (1) activation or modification of the support because they do not naturally possess reactive groups and (2) attachment of the enzyme onto the activated support.[202] The covalent attachment of a lipase to a support carrier generally results in some loss of activity. However, a lipase's carbohydrate moiety, since it is a glycoprotein, can be covalently bound without effecting the enzyme's functionality and activity. Mendes *et al.* immobilized lipase from *T. lanuginosus* and *P. fluorescens* by multipoint covalent attachment on Toyopearl AF-amino-650M resin.[211] Results suggest that better catalytic properties where obtained when the support was activated with glycidol and epichlorohydrin for both lipase sources. Ultimately, they were able to obtain almost 30 times greater stability than the free lipase, with corresponding to trans/-esterification yields ranging from 86.6 to 94.9 %. Covalent

binding is used in many of the immobilized lipase available commercially, such as Novozym 435, Lipozyme RM IM, and Lipozyme TL IM, which are all immobilized onto acrylic resin. It should be noted that the lipase from *C. antarctica* (B), *P. cepacia*, and *T. lanuginosus* investigated in Chapter 2 were covalently immobilized on Immobead 150, which is a macroporous acrylic polymer.[144]

Cross-linking is a novel immobilization method based on intermolecular cross-linkages between enzyme molecules to form cross-linked enzyme aggregate (CLEA).[112] Immobilization by cross-linking allows for high activities in a carrier-free reusable enzyme. This method avoids the activity losses and costs associated with immobilizing onto support carriers.[212] The CLEA methodology essentially consists of a two step process: (1) precipitate the enzyme from an aqueous solution using chemicals like acetone, methanol, or polyethylene glycol to produce physical enzyme aggregates and (2) cross-link these aggregates covalently by means of cross-linking agents, such as glutaraldehyde, bisdiazobenzidine, and hexamethylene diisocyanate.[14] Sheldon compared enzyme activity and stability of CLEA from *C. antarctica* lipase B (CALB-CLEA) and Novozym 435.[213] Interestingly, the CALB-CLEA performed significantly better than Novozym 435 in water, however, the reverse was found for organic media. Other CLEA-lipases have been reported from *C. antarctica* (A), *C. rugosa*, *Mucor javanicus*, *P. cepacia*, and *Pseudomonas* sp.[14, 212] However, an intrinsic drawback for CLEA is the size of the aggregates, which are very small, generally < 10 μm.[112] This can cause separation problems and make recycling the CLEA catalyst difficult and expensive.

Immobilization by entrapment consists of entrapping an enzyme within an insoluble polymer matrix. The immobilized lipase by entrapment are more stable than that by

adsorption and much simpler than the covalent binding method.[214] A variety of matrices have been used for entrapment, such as calcium alignate, cellulose acetate, gelatin, polyacrylamide, and sol–gel powder.[215] These matrices are porous enough to allow the diffusion of the substrates and products while retaining the lipase. Nourenddini *et al.* entrapped lipase from *P. cepacia* within a sol–gel polymer matrix for the trans/-esterification of soybean oil with methanol and ethanol.[214] As a result, the methyl and ethyl ester yields obtained were 67 % and 65 %, respectively after 1 h. Although the reaction rate was relatively fast, the low conversion of esters was probably due to the poor diffusion through the matrix, a common result from using immobilized lipase by entrapment.[112, 202]

Similar to entrapment, the immobilization by encapsulation confines the enzyme within a porous membrane forming a bi-layer.[112] Encapsulation typically adsorbs the enzyme onto a support and entraps these particles within the polymer matrix, whereas encapsulation directly entraps the free lipase. Sawangpanya *et al.* compared the immobilization of *C. rugosa* lipase by adsorption onto $CaCO_3$ (CRLA), entrapment of $CaCO_3$-lipase in Ca-alginate matrix (CRLAE), and encapsulation in Ca-alginate matrix (CRLE) for the trans/-esterification of palm oil with ethanol.[216] After optimizing conditions, CRLA, CRLAE, and CRLE resulted in ethyl ester yields of 57.6 %, 42.7 %, and 74.2 %, respectively after 48 h. However, they were able to obtain a higher ester yield of 83.4 % within 24 h using just the free lipase. Encapsulation is plagued by the same challenges as entrapment, where there are diffusional limitations through the polymer matrix.[112] Another possibility is that the byproduct glycerol may be clogging the

pores of the matrix, thereby limiting access by the TAG molecules; however, this has not been investigated.

4.1.4 Enzyme reutilization

Enzymes are known to be more stable *in vivo* than in the harsh environments of reactor systems. High temperatures, inactivating impurities, sheer stresses, and other mechanical forces along with the detrimental effects of short chained alcohols and glycerol assist in enzyme deactivation, inhibition, and degradation.[33] As earlier discussed, immobilization can increase the stability of the enzyme and helps mitigate some of the detrimental effects imposed by its environment. However, maintaining enzyme stability and catalytic activity over multiple reuses has been a real challenge. A crucial factor in the enzymatic production of biodiesel is the effectiveness of enzyme reuse and recycling, as the high prices of lipases are one of the major constrains. In order to reduce production costs, enzymes must maintain high levels of activity over multiple reuses. Several methods have been suggested to improve lipase stability, including solvent engineering, molecular biology, protein and genetic engineering, chemical modification, reaction optimization, immobilization techniques, physical treatments, and reactor engineering.

Stepwise addition of short chained alcohols, as discussed earlier, has been shown to increase the longevity of immobilized lipases by reducing enzyme inhibition and deactivation. Shimada *et al.* conducted an extensive study of stepwise additions on enzyme reuse with Novozyme 435 on multiple substrates.[43, 85] Two and three step methanol additions initially indicated no significant difference in activity; however, the two step method experienced a rapid decrease in conversion after the 37th cycle while the

three step method maintained over 95 % conversion up to the 54th cycle. These tests were repeated with the methanolysis and ethanolysis of vegetable oil, waste edible oil, crude oil, and tuna oil using both batch and continuous reactors. As a result, the three step addition was found to be superior to the two step method in regards to enzyme reuse. It should be noted that the method of enzyme reuse in these papers were vaguely described as simply transferring the lipase into fresh media without any treatment. From the author's experience, the viscous nature of the oil and the glycerol byproduct renders this near impossible without solvent washes. In addition, Novozym 435 beads have a diameter of 0.3 – 0.9 mm,[217] making them very difficult to handle and transfer without some loss, which would be amplified with each sequential reuse.

Soumanou and Bornscheuer investigated the reuse of Lipozyme RM IM (*R. miehei*) and Lipozyme TL IM in a solvent free system of sunflower oil.[86] Applying a three step methanol addition method and found that Lipozyme RM IM could be used at least five times without any significant loss of activity, whereas Lipozyme TL IM lost almost 50 % of its activity the first time it was reused. After each batch reaction, they recovered the immobilized lipase by filtration, washed three times cold acetone, and allowed them to dry at room temperature prior to the next batch. Recall from Chapter 2, that lipase from *T. lanuginosus* is highly susceptible to strongly hydrophilic solvents, like acetone.[144] Washing the lipase with acetone may have cause irreversible conformational changes in the enzyme structure which resulted in such a drop in activity. Additionally, like with Novozym 435, Lipozyme TL IM is composed of small particles (0.25 – 1.0 mm) with an average size of 0.5 mm,[218] which makes them extremely difficult to handle without losses.

Du *et al.* also investigated Lipozyme TL IM and its reuse on the trans/-esterification of soya bean oil.[205] A dramatic drop in enzyme activity after the first couple of reuses, similar to Soumanou and Bornscheuer,[86] was also experienced and thought to be due to glycerol inhibition. After conducting studies on hydrophilic organic solvents, a new series was conducted where they rinsed the immobilized lipase with isopropanol after each cycle to remove glycerol. As a result, they were able to maintain the enzyme's relative activity over 10 consecutive batch reactions with only a 5 % drop in activity. They also investigated the effects of molar ratio of methanol on enzyme reuse, similar to Shimada *et al.*,[85] which confirmed that the three step process significantly improved enzyme activity over multiple reuses.

Although, the studies description of reuse was vague, it does signify that the significant loss in activity experience by Soumanou and Bornscheuer was most likely caused by the acetone effects on the lipase. Therefore, the methods and techniques used to regenerate the enzymes are just as important to reuse as the reaction conditions. Typically, in literature, enzyme reuse is the last events studied in a series of optimizations and are usually briefly described with little to no detail. However, to the author's knowledge, there are no studies that are explicitly focused on enzyme reuse. Understanding the reaction parameters and methods of reuse can result improved enzyme reuse, which will allow the enzymatic process of biodiesel production to be more economically feasible.

4.2 Experimental Section

4.2.1 Materials

Used soybean vegetable frying oil was obtained from local restaurants and contained 2.3 wt % FFA as determined by titration and confirmed by NMR analysis.[103] *Thermomyces lanuginosus* lipase immobilized on Immobead 150 with an activity of 3,000 U/g (1 U corresponds to the amount of enzyme that liberates 1 μmol butyric acid per minute at pH 7.5 and 40 °C with tributyrin, Fluka No. 91010, as substrate)[104], methyl oleate (>98%), anhydrous dibutyl ether (99.3%), decane (≥99%), and HPLC grade 2,2,4-trimethylpentane (isooctane) were all purchased from Sigma-Aldrich. Acetone was purchased from EMD Chemicals Inc. Methyl acetate (99%), isobutanol (99%), ethyl acetate (99.9%), and tert-butanol (99.5%) were purchased from Acros Organics. HPLC grade 2-propanol, HPLC grade methanol, and n-hexane (95%) were purchased from Fisher Scientific.

4.2.2 Synthesis of biodiesel

The used vegetable oil was heated to 100 °C and passed through an 11.0 cm Whatman 40 filter paper to remove particulates. Davison molecular sieves with a 4 Å effective pore size (grade 512) were added to the oil to maintain anhydrous conditions. The addition of water can enhance enzyme activity and stability in general. However, in this case, the presence of water can also facilitate the hydrolysis of esters, which will reduce the product yield. The main reason for the use of the molecular sieves was to remove any excess water present in the waste oil. Reactions took place in 40 mL glass vials with PTFE/silicone septa from Kimble, Vineland, NJ, USA. The reaction system contained 500 U immobilized *T. lanuginosus* lipase, 4 mL isooctane solvent, 1 mL used

vegetable oil and a molar ratio of methanol to oil of 3.0 (125 μL methanol). A Fisher Scientific syringe pump slowly added methanol to the reaction volume using a Hamilton Company Gastight 250 μL syringe (#1725) and needle (metal hub, 22 g, 6 ", PT2). The reaction was maintained at a constant temperature (40 °C, unless otherwise stated) and a stirring speed of 250 rpm. The reaction was stopped after 6 h.

4.2.3 Analysis method

Samples of 30 μl were collected and then diluted with 1 ml isooctane for GC analysis. The concentration of fatty acid methyl esters (FAME) was measured by a Perkin Elmer TotalChrom (v 6.3.2) and Clarus 480 gas chromatograph 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 this value. The injector and flame ionization detector temperature were maintained constant at 275 °C. Methyl oleate was employed as the biodiesel standard in the GC analysis, as shown in Appendix A. The yield of biodiesel was determined as the mass of FAME produced per mass of oil added (g of FAME /g of oil). Sample calculation of GC analysis and FAME yields are shown in Appendix B. Nonlinear regression was used to fit the data by minimizing the sum of squares of error between the data and the model's predictions, similar to the Michaelis–Menten model, to produce a hyperbolic curve using Polymath 6.10. The reuse ratio was determined by the ratio of the reaction yield over the reaction yield of the first run with fresh enzyme.

4.2.4 Reuse method

The reaction solution was decanted after each run, replaced with 4 mL of hydrophilic solvent (log P between 0 and 1) to remove glycerol, and stirred at 250 rpm at the same

reaction temperature as used in the run, for 15 minutes. Solvents with a negative log P value are too hydrophilic and strip away essential water from the enzyme and were eliminated from this study.[144] The solvent solution was then decanted and replaced with 4 mL of a hydrophobic solvent (log P $\geq$ 3) and stirred at 250 rpm at the reaction temperature for an additional 15 minutes. Afterwards, the solvent solution was decanted out and a slight flow of air was applied to the enzymes within the reaction vial overnight to evaporate any residual solvent. The reaction was restarted with the identical reaction protocol as the prior day. This reuse procedure was developed for a simple method of *in situ* washing the enzymes that would be easily scalable. Various reuse methods were explored but are not reported in this study.

4.3 Results and Discussion

4.3.1 Effect of methanol addition on enzyme reuse

Methanol (MeOH) is the most commonly used acyl-acceptor used in transesterification reactions to produce fatty acid methyl esters (FAME). Methanol is cheap, produced in large quantities, and is anhydrous. However, the addition of >1.5 molar ratio of MeOH:oil can have an inhibitory effect on lipases in solvent-free systems.[219] Step-wise addition of methanol has been commonly used by researchers to minimize inhibition.[42] However, the effect of continuous addition of methanol on enzyme reuse has not investigated in a solvent system. Organic solvents have been used to protect the enzyme from denaturation by high concentrations of alcohols, and also improve the miscibility of the two substrates, namely methanol and oil.[46]

In this study, we compared the various methods of methanol addition on the overall yield in a solvent system containing isooctane, as shown in Figure 33. The total

methanol added in each experiment was 130 μL (3:1 molar ratio of MeOH to oil), only the method of methanol addition into the reaction system varied. For single addition, the methanol was added all at once at the beginning of the reaction. For double and triple additions, the methanol was divided into equal portions and added at t = 0 h and t = 2 h for double addition and at t = 0 h, t = 1 h, and t = 3 h for triple addition. These additional aliquots of methanol were added once >95% of the methanol reacted in order to preserve enzyme activity. The continuous addition fed methanol at a steady rate of 32.5 μL/h for 4 h. It is interesting to note that the method of addition did not affect the overall FAME yield after 6 h. Since these results used new *T. lanuginosus* lipase for each addition method, the next step is to determine if the addition method has any effect of multiple uses of the enzyme.

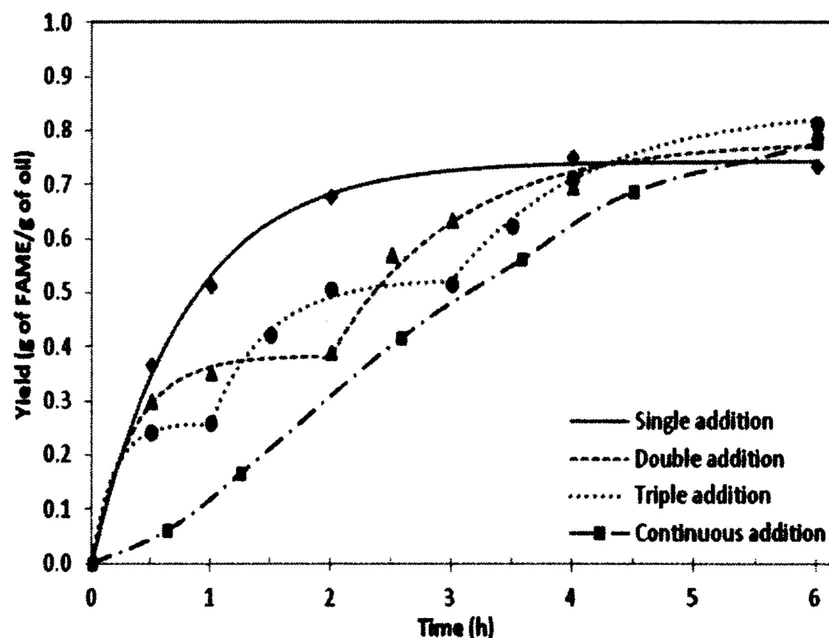


Figure 33. Effects of methanol addition on the transesterification of used vegetable oil by *T. lanuginosus* lipase. Reaction conditions: 1 mL used vegetable oil, 4 mL isooctane, 500 U lipase, 40 °C, stir speed 250 rpm, 3.0 mol equivalent total methanol. 130 μL MeOH added at t = 0 for single addition, 65 μL MeOH added at t = 0 and t = 2 h for double addition, 43.3 μL MeOH added at t = 0, t = 1 h, and t = 3 h for triple addition, and 130 μL MeOH added over 4 h for continuous addition at 32.5 μL/h.

Reusing enzymes is essential to make the enzymatic production of biodiesel economically feasible. Each methanol addition method was tested for a total of three runs with the same initial loading of 500 U of enzyme, the results of which are shown in Figure 34. It is interesting to note that the first two runs show very little change for all the addition methods. However, the third run shows a significant difference in yield based on the type of addition. As expected, the single addition of methanol has the greatest effect on enzyme activity, where the continuous addition has the least effect with over twice the yield on the third run. This could be due to the excess methanol from the batch addition deactivated the enzyme, whereas continuous addition is reaction limited and thus prevented any buildup. The results of the double and triple additions on the third run were about halfway between the single and continuous addition. However, there was very little difference between the double and triple additions. This may be due to the fact that both are at or under the 1.5 molar ratio threshold that has been reported.[219]

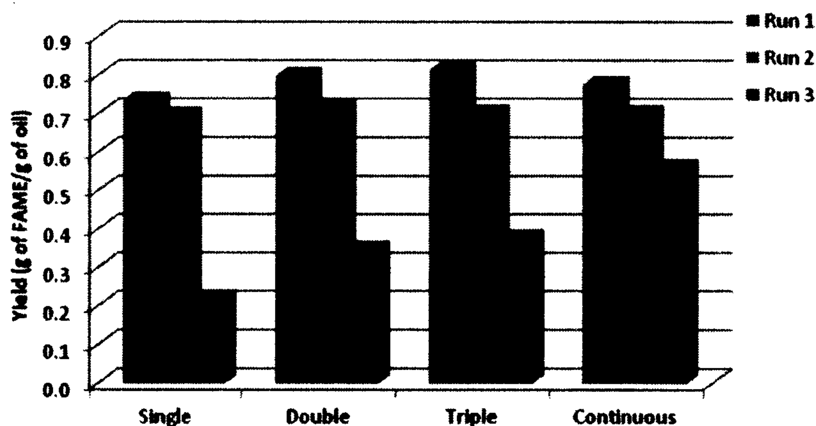


Figure 34. Effects of methanol addition on enzyme reuse from the transesterification of used vegetable oil by *T. lanuginosus* lipase. Reaction conditions: 1 mL used vegetable oil, 4 mL isooctane, 500 U lipase, 40 °C, stir speed 250 rpm, 3.0 mol equivalent total methanol. 130 μL MeOH added at t = 0 for single, 65 μL MeOH added at t = 0 and t = 2 h for double, 43.3 μL MeOH added at t = 0, t = 1 h, and t = 3 h for triple, and 130 μL MeOH added over 4 h for continuous at 32.5 μL/h.

The length of time of the methanol addition was varied in 1 h increments between 2 – 6 h. This corresponded to a change in methanol feed rate between 65.0 – 21.7 μL/h, respectively. The total amount of methanol added (130 μL) was the same in each case. The results of enzyme reuse ratio (defined as the ratio of the biodiesel yield at the end of each run to the yield after the first run) versus reuse number are shown in Figure 35. A reuse number of 0 corresponds to the first run with fresh enzyme. Lower feed rates resulted in an increase in enzyme reuse activity and can perhaps be attributed to less methanol being present near the active site that strip away the active water.

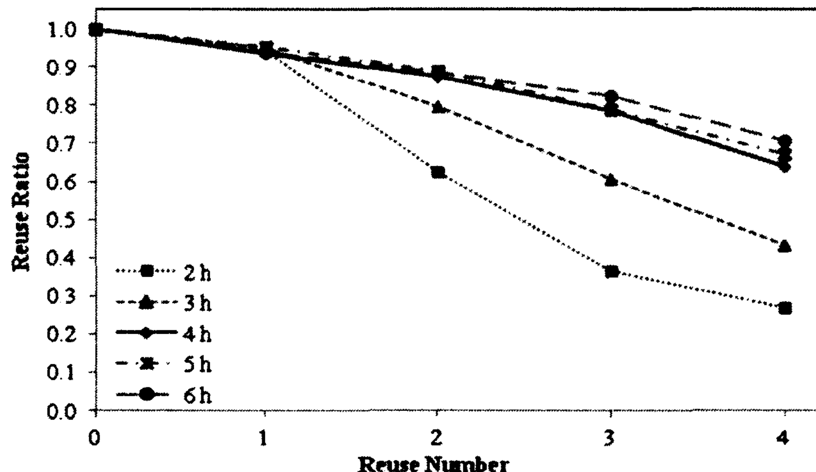


Figure 35. Effects of methanol feed rate on enzyme reuse from the transesterification of used vegetable oil by *T. lanuginosus* lipase. Reaction conditions: 1 mL used vegetable oil, 4 mL isooctane, 500 U lipase, 40 °C, stir speed 250 rpm, 3.0 mol equivalent total methanol (130 μL) fed continuously.

4.3.2 Effect of reaction temperature on enzyme reuse

Enzymes from thermophiles, like *T. lanuginosus*, are more heat stable than those from mesophiles when extracted and tested.[142] We found in recent work with this lipase that the optimum reaction temperature is 40 °C for a single run,[144] however, we wanted to determine if this was also the case with multiple reuses. Therefore, we investigated the

effect of reaction temperature from 25 – 45 °C on enzyme reuse with a 4 h continuous methanol feed, the results of which are shown in Figure 36.

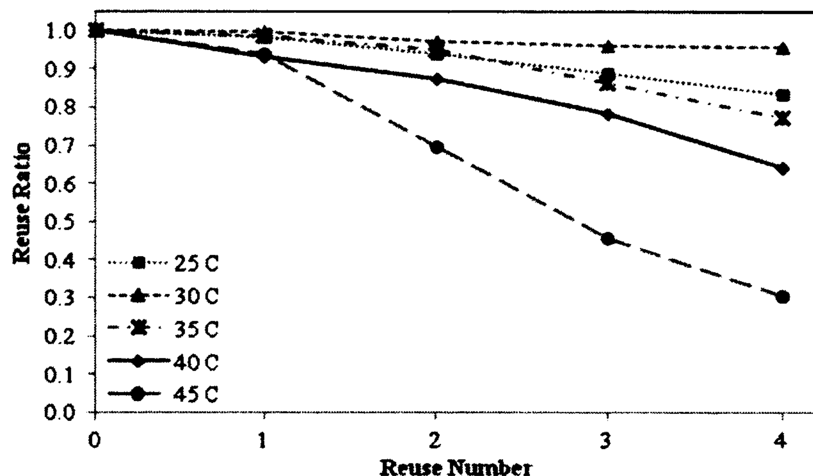


Figure 36. Effects of temperature on enzyme reuse from the transesterification of used vegetable oil by *T. lanuginosus* lipase. Reaction conditions: 1 mL used vegetable oil, 4 mL isooctane, 500 U lipase, 40 °C, stir speed 250 rpm, 130 μL MeOH added over 4 h for continuous at 32.5 μL/h.

We found that the enzyme reuse ratio increased as the temperature decreased, with an optimum reuse temperature around 30 °C. Reducing the temperature to 25 °C reduced the reuse ratio, which may be due to methanol accumulation near the enzyme active site caused by the lower reaction rate. Thermal denaturation was likely to play a major role in the loss of enzyme activity at higher temperatures, especially at 45 °C. The effect was not discernible during the first run, but was clearly evident over multiple reuses and can be explained by the conformational stability of the enzyme. The activity of the enzyme was dependent on its flexibility to alter its conformation for catalysis, but the stability of the enzyme increased with a more rigid structure.[196] The balance between the enzyme's flexibility and stability can be seen by looking at the two outermost temperatures in the study. The enzyme was too rigid at 25 °C and the limited flexibility made it an ineffective catalyst. At 45 °C, the enzyme was too flexible and loses its stability, which

resulted in a shorter lifetime. However, it was observed that enzymes are deactivated even at moderate temperatures over a period of time.

4.3.3 Effect of solvent wash on enzyme reuse

Solvents can enhance the rate of transesterification by improving the solubility of methanol, oil, and glycerol byproduct.[46, 144] After the bulk of the reaction media has been removed, some of these compounds remain and concentrate around the enzyme. This can lead to deactivation of the enzyme's active site and lower the product yield on subsequent runs and reduce the enzyme's overall life span. During the course of the transesterification reaction, the glycerol byproduct accumulated in the mixture and settled to the bottom of the reaction vessel with the immobilized enzymes. There, the glycerol formed a hydrophilic phase that inhibited the enzyme reaction, which was magnified over each reuse.[220] Glycerol's high viscosity and hydrophilic nature made it difficult to separate it from the enzymes. Therefore, a hydrophilic solvent wash must be used to remove the glycerol after each run.

The effect of four hydrophilic wash solvents, namely, *tert*-butanol, methyl acetate, ethyl acetate, and iso-propanol on enzyme reuse ratio were investigated (Figure 37). The highest reuse ratios after 5 runs were obtained by washing the enzymes with *tert*-butanol to remove residual glycerol; washes with ethyl acetate had similar results. These two solvents are less hydrophilic (higher log P) than either methyl acetate or iso-propanol, as shown in Table 10. The lower reuse ratios obtained with the more hydrophilic solvents (log $P < 0.5$) may be attributed to the essential water being stripped from the enzyme's active sites. Our previous research (Chapter 2) had shown *T. lanuginosus* lipase more

susceptible to stripping of the essential water from the active site by hydrophilic solvents.[144]

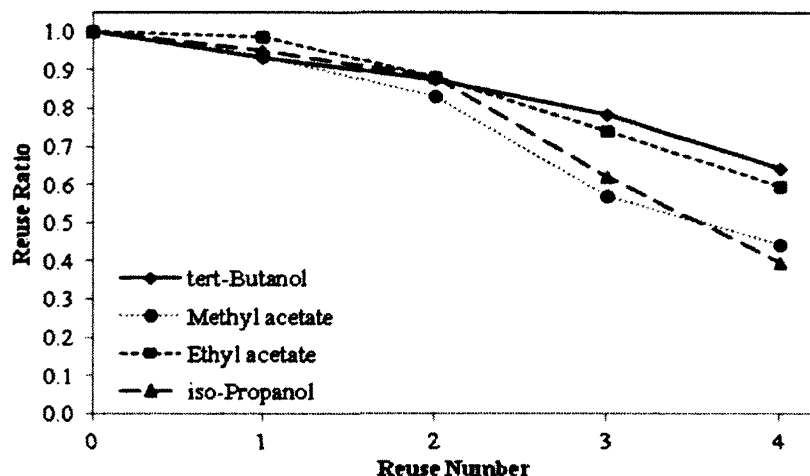


Figure 37. Effects of hydrophilic solvent washes on enzyme reuse from the transesterification of used vegetable oil by *T. lanuginosus* lipase. Reaction conditions: 1 mL used vegetable oil, 4 mL isooctane, 500 U lipase, 40 °C, stir speed 250 rpm, 130 μL MeOH added over 4 h for continuous at 32.5 μL/h.

The hydrophilic solvent wash of the enzymes removed the glycerol byproduct that can inhibit the enzyme's activity for subsequent runs. However, the hydrophilic change in the enzyme's environment may cause conformational changes that may negatively affect the enzymatic activity. Therefore, in a different set of experiments, we followed the hydrophilic solvent wash with a hydrophobic solvent (log P > 3) wash to remove any remaining oil residue and to restore enzyme conformation.

Table 10. Log P values of solvents for enzyme washes.

Hydrophilic solvents		**Hydrophobic solvents**	
Solvent	**Log P**[95]	**Solvent**	**Log P**[95]
Methyl acetate	0.384	Dibutyl ether	3.173
iso-Propanol	0.420	*n*-Hexane	3.657
Ethyl acetate	0.760	Isooctane	3.668
tert-Butanol	0.866	Decane	5.678

The effects of four hydrophobic wash solvents, namely, isooctane, *n*-hexane, dibutyl ether, and decane on enzyme reuse ratio were investigated (Figure 38). The highest reuse ratio after 5 runs was obtained by washing the enzymes with *n*-hexane after washing the enzyme with *tert*-butanol. The other three solvents had similar effects just below that of *n*-hexane. There does not appear to be any correlation between the log P value of the hydrophobic wash solvent (Table 10) and enzyme reusability, as observed with the hydrophilic solvents. In general, these hydrophobic solvents protect the enzyme from being denatured and increase reuse activity.

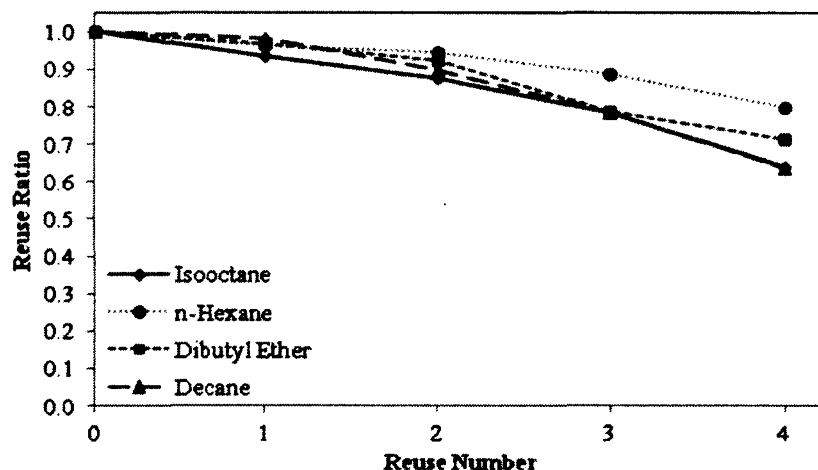


Figure 38. Effects of hydrophobic solvent washes on enzyme reuse from the transesterification of used vegetable oil by *T. lanuginosus* lipase. Reaction conditions: 1 mL used vegetable oil, 4 mL isooctane, 500 U lipase, 40 °C, stir speed 250 rpm, 130 µL MeOH added over 4 h for continuous at 32.5 µL/h.

4.4 Conclusions

In this chapter, we investigated the effects of methanol addition on the transesterification of used vegetable oil with isooctane by *T. lanuginosus* lipase. The method of methanol addition did not appear to affect the FAME yield when fresh enzyme is used, but did significantly affect the enzyme's activity during subsequent reuses. We determined the optimum method for methanol addition was to feed it continuously over

the course of 4 – 6 h into the reaction media. To further optimize our system, we investigated the effect of reaction temperature from 25 – 45 °C on enzyme reuse and determined that the enzyme reuse ratio increased as the temperature decreased, reaching an optimum at 30 °C. This temperature balanced the enzyme's flexibility, as demonstrated at higher temperatures, with the enzyme's stability, experienced at lower temperatures.

After the transesterification reaction, not all of the reaction media could be easily removed. The glycerol byproduct can adhere to the enzyme and accumulate over time, which results in inhibition. Therefore, we investigated various hydrophilic solvents to wash the enzymes to remove the glycerol after each reaction. There appeared to be a correlation between the solvent's hydrophobicity (log P) and the enzymatic activity over multiple reuses. We found that *tert*-butanol worked best to remove the glycerol without stripping away the essential water from the enzyme's active site. A similar correlation could not be found with hydrophobic solvents that were used to wash the enzyme after the hydrophilic solvent wash to remove the residual oil and restore enzyme conformation.

Optimization of reaction parameters such as methanol addition, reaction temperature, and solvent, can minimize enzymatic deactivation and increase enzyme reusability without significantly affecting FAME yield.

CHAPTER 5

SUMMARY

5.1 Overview

The worldwide consumption of transportation fuels has increased over the past few decades while the petroleum reserves have decreased. Furthermore, climate change and other environmental concerns have led to an urgent need to develop alternative and sustainable fuels, such as biodiesel. Biodiesel is a renewable, non-toxic, and biodegradable alternative fuel with several other desirable characteristics and advantages. Biodiesel is a fatty acid acyl ester produced from vegetable oil or animal fats and an acyl-acceptor by transesterification and/or esterification reactions. Conventional transesterification processes currently used to produce biodiesel requires environmentally harmful alkali or acid catalysts that can lead to soap formation.

Enzymatic production of biodiesel is a promising environmentally friendly alternative to the chemical processes. Enzyme reactions eliminate the possibility of soap formation, operate under milder conditions, and produce products that can easily be collected and separated with less waste. In addition, enzymes are typically immobilized and therefore can be reused multiple times, unlike alkali/acid catalyzed reactions. Several microbial strains of lipases have been found to have high transesterification and esterification activities and are ideally suited for producing biodiesel.

However, there are several technical and economical obstacles of enzymatic process which significantly affects its commercial viability, such as (i) immiscibility of the hydrophilic methanol and hydrophobic triglyceride which results in the formation of an

interface leading to mass transfer resistance, (ii) insufficient availability of large quantities of inexpensive lipase suitable for catalysis, and (iii) stripping of essential water from the active site by the strong polarity of methanol causing a reduction in enzyme activity after multiple reuses. This dissertation has addressed several of these challenges through solvent engineering, genetic engineering, and reaction optimization.

5.2 Conclusions

In Chapter 2, the challenge of immiscibility between the hydrophobic triacycleglycerides (TAG) and fatty acid methyl esters (FAME) and the hydrophilic methanol and glycerol was addressed through a series of solvent engineering studies. The effect of 15 organic solvents on reaction rate, overall yield, and activity from three sources of lipase, namely *Candida antarctica*, *Pseudomonas cepacia*, and *Thermomyces lanuginosus*, were studied in the trans/-esterification of used vegetable oil. What sets this study apart from others found in the literature was the uniformity and testing methodology. The variables were minimized by ensuring all three lipases were immobilized on the same support carrier and we compared them to each other using defined enzyme activity units, instead of by mass, which was most commonly cited and a grossly incorrect method of comparison.

As a result, we found that the choice in solvent can have dramatic effects on the reaction rate and FAME yield. Using moderately hydrophobic alkane solvents, such as isooctane and *n*-hexane, resulted in the fastest reaction rates and highest yields for all three lipases. Distinct functional groups were also analyzed and they appeared to follow the same trend based on their log P value. The most notable effect was seen with the aromatic hydrocarbons on *C. antarctica* and to a lesser extent *P. cepacia*. These two

enzymes have large, somewhat stiff, binding sites which may be interacted with the benzene ring which resulted in decreased activity. However, for the most part, the solvents' hydrophobicity played a more important role in enzyme activity than their distinct functional group.

It was also determined that enzyme structure and catalytic mechanism played a huge role in how much, if any, the solvent's hydrophobicity (log P) affected the enzyme. *C. antarctica* has a large stiff binding site with no protecting lid, therefore, it was less affected by the more hydrophilic solvents. However, *T. lanuginosus* required substantial conformational changes for TAG molecules to bind within the active site and was protected by an alpha-helical lid. The addition of hydrophilic solvents to its environments significantly deactivated the enzyme. Although it was significantly affected by hydrophilic solvents, the lipase from *T. lanuginosus* had the fastest reaction rate, highest yield and activity for all three lipases with the best solvents selected, isooctane and *n*-hexane.

In Chapter 3, the challenge of producing large quantities of inexpensive lipase that are suitable for biodiesel catalysis has been addressed through plant biotechnology and genetically engineering. A novel lipase production system in transgenic plants was developed for the enzymatic production of biodiesel. From Chapter 2, we know the lipase from *T. lanuginosus*, a thermophilic fungus, has high trans/-esterification activity and performs well in our reaction system. Therefore, we cloned the gene for this lipase by isolating the total RNA, using reverse transcription of the mRNA to synthesize cDNA, which was amplified by PCR using specific primers to develop three constructs. These

constructs alter the N-terminus end of the sequence to allow for an increased probability of proper expression.

The lipase genes were first inserted into a cloning vector, confirmed by restriction digest and sequencing, and then inserted into two final destination vectors by the LR clonase reaction. The destination vectors allowed the gene to fuse with reporter and/or purification tags on the C-terminus. *Agrobacterium*-mediated transformation and floral dip methods were used to transform tobacco and *Arabidopsis* plant cells, respectively. The resulting tobacco plants were separated and screened for the presence of the transgene. Several of these plants were selected and transferred to the UNH Greenhouse facility.

Multiple attempts were made to extract and purify the recombinant enzyme from the plant tissue. However, no visual signs of the lipase were seen on the SDS-PAGE gels. The greenhouse plants were then rescreened and only 4 of the 9 plants indicated the presence of the lipase gene. These 4 plants were further screened to determine if the gene was being expressed. To accomplish this, the total RNA was isolated from each plant and the resulting mRNA was synthesized to cDNA. PCR amplification indicated that 3 of the 4 plants were expressing the lipase gene. Additional protocols were performed to extract, precipitate, concentrate, and purify the recombinant lipase. A sample from each stage was analyzed by tributyrin assay to detect lipase activity. Unfortunately, the results were inconclusive and not repeatable.

We showed a proof-of-concept that a lipase gene with high trans/-esterification can be cloned from microbial sources, genetically transformed into a plant host, and over-expressed. Although we were unable to successfully purify the enzyme, we did

demonstrate a method to develop an inexpensive biocatalyst for biofuel production that had not previously been attempted. The use of plant expression systems could grant us the versatility and almost unlimited scalability that cannot accomplished by other expression systems.

In Chapter 4, the challenge of reducing adverse effects on enzyme activity after multiple reuses caused by methanol and glycerol inhibition have been addressed through a series of reaction parameter optimization. The effects of methanol addition, temperature, and solvent washes on the trans/-esterification of used vegetable oil and their effects on enzymatic activity over consecutive reactions were investigated. From the results in Chapter 2, we began our investigation using immobilized lipase from *T. lanuginosus* in an organic solvent system of isooctane. In addition to the other cost reduction methods described in this dissertation, increasing the number of reuses immobilized enzymes can tolerate without significant loss in activity can significantly lower production costs. The largest reason for enzymes to decrease in activity and become inactivated is due to both methanol and glycerol inhibition.

Initially, the method of methanol addition did not appear to affect the FAME yield when fresh enzyme is used. However, a significant effect was observed over multiple reuses, where an optimum was found by continuously feeding methanol into the reaction medium over the course of 4 – 6 h. This observation corresponded to the hydrophilic effects described in Chapter 2 with *T. lanuginosus* lipase. In addition, continuous addition was a more realistic scenario for large scale production over the 2 or 3 aliquot additions as described in most of the literature. Although *T. lanuginosus* is a thermophilic fungus that grows at 50 °C, the optimum temperature of its lipase to

maintain activity over multiple reuses was found to be 30 °C. This temperature allows the enzyme to have an increased stability, typically seen at lower temperatures, while maintaining the flexibility to properly perform catalysis.

Glycerol, the byproduct of the transesterification reaction, can accumulate in the reactor and difficult to remove due to its viscous nature. This accumulation can adhere to the enzyme and inhibit activity by covering the active site. Therefore, the hydrophilic glycerol should be easily removed using a hydrophilic solvent. However, as described in Chapter 2, hydrophilic solvents can have a detrimental effect on enzyme activity, especially with *T. lanuginosus*. Therefore, to maintain proper enzyme conformation, a following wash step was performed using a hydrophobic solvent. Various hydrophilic and hydrophobic solvents washes of the enzyme were investigated to maintain activity over multiple reuses. We found that *tert*-butanol worked best to remove the glycerol while maintaining enzyme activity. This may be due to the mild hydrophilic nature of *tert*-butanol along with its large branched structure helps minimize any adverse effects.

The enzymatic trans/-esterification of non-edible and waste oils is an effective and environmentally friendly process to produce biodiesel. Although this process has been well studied and proven technologically feasible, the real challenge lies with making it economically feasible and commercially accepted. Several challenging tasks in this process were investigated to improve immiscibility, increase availability, and extend the effective lifetime of the enzymes. Through solvent engineering, plant genetic engineering, and optimization, we were able to accomplish these tasks. The combination of all of these processes may significantly reduce production costs which could make the enzymatic production of biodiesel economically feasible in the near future.

5.3 Recommendations

Five recommendations are proposed:

1. Mechanical stirring can cause shear stresses and physical damage to the immobilized enzymes, which may result in the leaching of enzymes bound to the support carrier. This effect would be magnified over multiple reuses, indicating an apparent loss in activity. Switching from a batch reactor setup, it may be beneficial to investigate a packed bed reactor system. This not only prevents the need for mechanical stirring, but is a more practical industrial method to enzymatically produce biodiesel.

2. Several strains of lipases have been found to have high trans/-esterification activity from various organisms, as described in Section 2.1.1. Although the lipase from *T. lanuginosus* was determined to be the optimum biocatalyst in Chapter 2, it was also found to be the most susceptible to deactivation due to environmental changes. A more robust and stable enzyme, like *C. antarctica* lipase B, may be a better lipase to express, extract, and purify from transgenic plants. In addition, *C. antarctica* is a yeast and lacks introns in its genetic coding sequence, making the cloning process easier and more straightforward.

3. Plant cell cultures are another expression system to produce recombinant proteins, as stated in Section 3.1.4. In a research environment, it may be more beneficial to use these suspended cell cultures instead of producing entire lines of transgenic plants. The ease of transformation and most importantly the speed of growth can allow quick and easy screening of various lipase genes. In addition, the proteins can be tailored to

secrete into the medium, which would make extraction and purification much easier and simpler without the adverse compounds found in leafy tissue.

4. The immobilize d enzymes tested in Chapter 2 and 3 were immobilized on Immobead 150, where the enzymes are attached covalently to a macroporous acrylic polymer support. As stated in Section 4.1.3, there are other support carriers and methods for enzyme attachment. It may be interesting to investigate the effects of these various methods on enzyme reuse, using the same enzyme. It is important to note, like in Chapter 2, that the enzyme loading be compared by activity units and not by weight.

5. As describe in section 3.3.6, enzyme activity is generally determined by the tributyrin assay, an industry standard. However, a system using triolein, a TAG molecule with longer fatty acid chains, would be better suited to test activity and selectivity for biodiesel synthesis than tributyrin, a short-chained TAG molecule.

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APPENDICES

APPENDIX A: GC CALIBRATION CURVES

APPENDIX B: CALCULATION OF BIODIESEL YIELDS

APPENDIX C: MANUFACTURER'S PROTOCOLS

APPENDIX D: PH-STAT CALIBRATION

APPENDIX E: SEQUENCING DATA

APPENDIX A

GC CALIBRATION CURVES

In this dissertation, methyl oleate was used as the FAME standard for GC analysis, as stated in Sections 2.2.3 and 4.2.3. The methyl oleate was diluted with the hydrophobic solvent isooctane in order to obtain a series of concentrations. The series of dilutions were prepared to generate a calibration curve. The primary procedure to generate each standard is as follows:

1. "Standard 0" was generated by adding 20 μL methyl oleate (~0.0175 g) to 2 mL of isooctane (~1.3840 g) and inverted multiple times to ensure the solution was properly mixed. The weight of both methyl oleate and isooctane were determined by an electronic scale. The weight percentage of methyl oleate, W_o (g/g), in Standard 0 was calculated by;

   $$W_o = \frac{MO_o}{IO_o} \qquad \text{Equation A.1}$$

   where MO_o and IO_o are the initial weights (g) of methyl oleate and isooctane added to Standard 0, respectively.
2. "Standard 1" was generated by adding 0.8 mL of Standard 0 to 1 mL of isooctane and inverted multiple times to ensure the solution was properly mixed. The weight of both Standard 0 and isooctane were determined by an electronic scale.

3. Standards 2 – 4 were generated by adding 1 mL of the previous standard to 1 mL of isooctane, then inverting multiple times to ensure proper mixing. The weights of the standard added and the isooctane were determined by an electronic scale.
4. The weight percentage of methyl oleate, W_i (g/g), in each standard, *i*, were calculated by;

$$W_i = \frac{(m_{i-1})(W_{i-1})}{IO_i + m_{i-1}} \quad \text{Equation A.2}$$

where *i-1* represents a value from the previous standard, IO_i is the mass of fresh isooctane added, and m_{i-1} refers to the mass of the previous standard added to generate the new standard.

30 µL samples of Standards 1 – 4 were extracted and analyzed by the GC. Examples of the methyl ester peaks obtained by the GC are shown in Figure A1. The areas of these peaks were integrated by the software of *OriginPro* and *TotalChrom* for Chapters 2 and 4, respectively. The area values were graphed with the corresponding methyl oleate weight percentages to generate a calibration curve. In both studies, the calibration curve was re-established on a bi-weekly basis, with the $R^2 > 0.995$ for each calibration curve. Initial calibration was performed in triplicate, as shown in Figure A2.

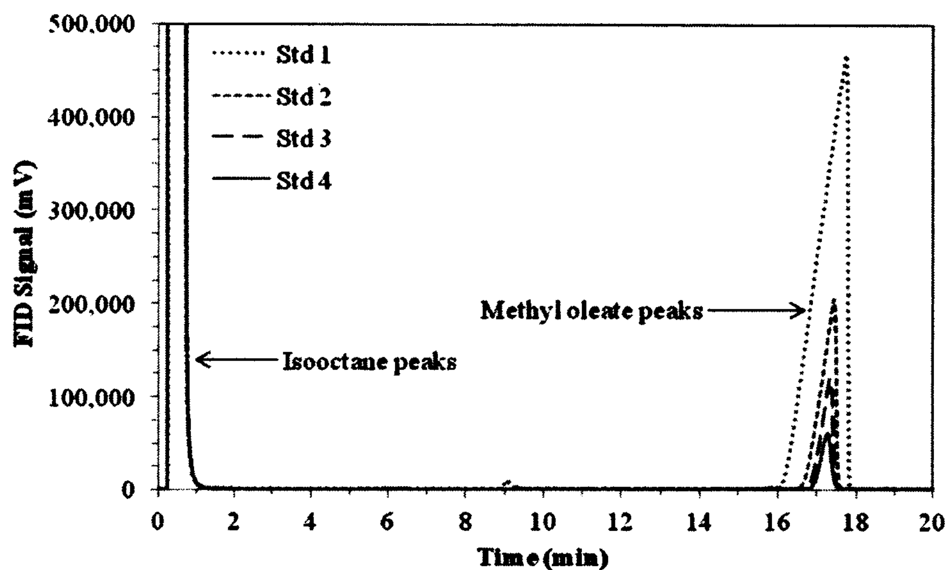


Figure A1. Methyl oleate GC calibration peaks for Standards 1 – 4.

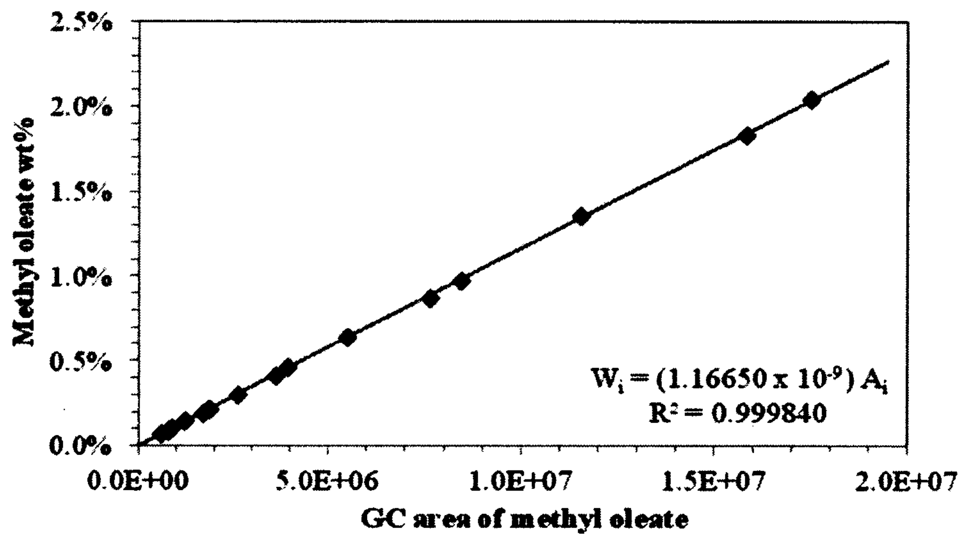


Figure A2. GC calibration curve.

APPENDIX B

CALCULATION OF BIODIESEL YIELD

The stoichiometric ratio of methanol was added to the enzymatic trans/-esterification reactions in Chapter 2 and 4. However, TAG molecules can consist of different combinations of fatty acids; such as saturated, unsaturated, and/or poly-unsaturated fatty acids with varying carbon chain lengths. In this dissertation, it was assumed that the TAG molecules had an average composition and properties to triolein; a triglyceride consisting of three identical units of oleic acid, an unsaturated fatty acid. Triolein has a molecular weight of 885.4 g/mol and a density of 0.91 g/mL. A sample calculation for determining the amount of methanol (MeOH) required to achieve a 3:1 stoichiometric molar ratio of methanol to TAG is shown below:

(1 mL oil) * (0.91 g /mL of oil) / (885.4 g /mol of oil) * (3 mol MeOH/ 1 mol oil) * (32.04 g / mol MeOH) / (0.7918 g / mL MeOH) * (0.001 mL / μL) = 124.8 μL MeOH.

To analyze the FAME content of the reaction at various times, a sample of 30 μL was extracted and diluted with 1 mL of isooctane. From this sample, 1 μL was injected into the GC via a 10 μL syringe. The resulting curve designated as FAME was shown in Figure 8. The area of the peaks was integrated by *OriginPro* and *TotalChrom* in Chapters 2 and 4, respectively. A sample calculation of FAME yield is shown below:

- Reaction
  - 1 mL oil = 0.9100 g
  - 500 U enzyme = (500 U) /(3000 U/g *T. lanuginosus*) = 0.1667 g
  - 4 mL isooctane = 2.7520 g
  - 125 μL MeOH = 0.0988 g
  - Total Reaction = 3.7608 g
- 30 μL Sample = 0.0357 g
- 1 mL isooctane = 0.7231 g
- GC area = 8.1829×10^{10}
- Calibration curve slope (Appendix A) = 1.09814×10^{-13}
- FAME conc. in sample = $(8.1829 \times 10^{10}) * (1.09814 \times 10^{-13}) = 8.99 \times 10^{-3}$ wt%
- FAME conc. in reaction = (Sample conc.)*(Isooctane + Sample) / (Sample) = (8.99×10^{-3} wt%)*(0.7231 g + 0.0357 g) / (0.0357 g) = 0.1910 wt%
- Total FAME mass in reaction = (Rxn conc.) *(Total reaction mass) = (0.1910 wt%)*(3.7608 g) = 0.7200 g
- FAME yield = (Total FAME mass) / (Initial oil mass) = (0.7200 g) / (0.9100 g) = 0.791 g/g

APPENDIX C

MANUFACTURER'S PROTOCOLS

The protocols used in Chapter 3 were conducted in accordance with the following manufacturer's protocols:

1. ZR Plant RNA MiniPrep Kit (Zymo Research, Cat. No. R2024)

Make sure guidelines are followed to ensure the RNA isolation procedure is performed in an RNase-free environment.

Buffer Preparation

Before starting, add 48 ml 100% ethanol (52 ml 95% ethanol) to the 12 ml **RNA Wash Buffer** concentrate (R2024).

Protocol[1]

1. Transfer a fresh or frozen finely minced plant sample into a **ZR BashingBead™ Lysis Tube** and add 800 µl **RNA Lysis Buffer** (≥4 volumes)[2] to the sample. *(e.g., 800 µl buffer will allow for processing of up to 400 µl lysate in Step 4).*
2. Secure in a bead beater fitted with a 2 ml tube holder assembly and process[3].
3. Centrifuge the **ZR BashingBead™ Lysis Tube** at ≥12,000 x *g* for 1 minute.
4. Transfer up to 400 µl of the supernatant from Step 3 into a **Zymo-Spin™ IIIC Column** in a **Collection Tube** and centrifuge at 8,000 x *g* for 30 seconds. *Save the flow-through!*
5. Add 0.8 volume ethanol (95-100%) to the flow-through in the **Collection Tube** and mix well *(e.g., 320 µl ethanol added to 400 µl flow-through).* *For small RNA recovery, use 2 volumes ethanol (95-100%).*
6. Transfer the mixture to a **Zymo-Spin™ IIC Column**[4] in a **Collection Tube** and centrifuge at ≥12,000 x *g* for 30 seconds[5]. Discard the flow-through.
7. Add 400 µl **RNA Prep Buffer** to the column. Centrifuge at ≥12,000 x *g* for 1 minute. Discard the flow-through and replace the **Zymo-Spin™ IIC Column** back into the **Collection Tube.**
8. Add 800 µl **RNA Wash Buffer** to the column. Centrifuge at ≥12,000 x *g* for 30 seconds. Discard the flow-through and replace the **Zymo-Spin™ IIC Column** back into the **Collection Tube**. Repeat the wash step with 400 µl **RNA Wash Buffer**.
9. Centrifuge the **Zymo-Spin™ IIC Column** at ≥12,000 x *g* for 2 minutes in the emptied **Collection Tube** to ensure complete removal of the wash buffer.
10. Carefully remove the **Zymo-Spin™ IIC Column** from the **Collection Tube** and place into a **DNase/RNase-Free Tube.** Add ≥25 µl **DNase/RNase-Free Water** directly to the column matrix and let stand for 1 minute.
11. Centrifuge at 10,000 x g for 30 seconds to elute the RNA from the column. RNA can be further treated for inhibitor removal[6] (proceed to Step 12), used immediately, or stored at ≤-70 °C.
12. Transfer the eluted RNA sample from Step 11 to the prepared[7] **Zymo-Spin™ IV-HRC Spin Filter** (see above) in a **DNase/RNase-Free Tube** and centrifuge at 8,000 x *g* for 1 minute. The filtered RNA is now suitable for downstream applications.

Notes:

[1] The kit is designed for efficient isolation of up to ~25µg total RNA and is compatible with RNAlater™.

[2] RNA Lysis Buffer volume may be adjusted to facilitate Step 4 (e.g. up to 150 mg pre-cut leaves of *Nicotiana sp.* in 1 ml RNA Lysis Buffer).

[3] Sample processing example: *Nicotiana sp.*, fresh leaves: 6 m/s, 30-60 seconds – the portable *Xpedition*™ Sample Processor, page 7. See manufacturer's a literature for operating information.

[4] Maximum loading volume for Zymo-Spin™ IIC and IIC Column is 800 µl. Column has to be reloaded to process volumes >800 µl.

[5] Optional DNase treatment: Following Step 6, samples can be in-column or in-tube DNase treated. See Appendices A and B, respectively.

[6] Sample specific step, for additional inhibitor removal.

[7] Zymo-Spin IV-HRC Spin Filter preparation:
A) Snap off the base.
B) Insert the filter into a Collection Tube.
C) Spin at exactly 8,000 x *g* for 30 seconds.
D) Wash the filter column twice by adding 400 µl DNase/RNase-Free Water.
E) Spin at exactly 8,000 x *g* for 3 minutes.

Appendix A

In-Column DNase Digestion

Fast-Spin column technology efficiently removes the majority of DNA during RNA purification and is satisfactory for most RNA-based applications. However, if necessary, complete removal of DNA can be achieved by performing a DNase I digestion.

The DNase digestion procedure can be performed using any source of RNase-free DNase I together with its 10X reaction buffer (*e.g.*, 100 U **RNase-free DNase I (1 U/µl) w/ 10X Reaction Buffer** – Zymo Research Cat. No. **E1007**). DNase I maintains activity in the **RNA Wash Buffer** provided in this kit.

1. Make the following DNase I cocktail (for each column to be treated):

RNase-Free DNase I	10 µl (1 U/µl)
10X Reaction Buffer	10 µl
RNA Wash Buffer	80 µl

2. Following Step 6 of the RNA isolation protocol[1], add 400 µl **RNA Wash Buffer** to the **Zymo-Spin™ IIC Column** in a **Collection Tube** and centrifuge at ≥12,000 x *g* for 1 minute. Discard the flow through.

3. Add 100 µl DNase I cocktail from Step 1 above directly to the matrix of the **Zymo-Spin™ IIC Column**. Keep the **Zymo-Spin™ IIC Column** in the **Collection Tube**.

4. Incubate the column at 25-37°C for ≥15 minutes[2], then centrifuge at ≥12,000 x *g* for 30 seconds.

5. Continue with Step 7 of the RNA isolation protocol[3].

Notes:

[1] See page 3, step 6.

[2] The temperature optimum for DNase I activity is at 37 °C. The optimal incubation time may vary.

[3] See page 3, step 7.

2. qScript cDNA Supermix Kit (Quanta Biosciences, Cat. No. 95048-100)

Description

qScript cDNA SuperMix provides a sensitive and easy-to-use solution for two-step RT-PCR. This 5X concentrated master mix provides all necessary components (except RNA template) for first-strand synthesis including: buffer, dNTPs, $MgCl_2$, primers, RNase inhibitor protein, qScript reverse transcriptase and stabilizers. qScript is a RNase H(+) derivative of MMLV reverse transcriptase, optimized for reliable cDNA synthesis over a wide dynamic range of input RNA. The unique blend of oligo (dT) and random primers in the qScript cDNA SuperMix works exceptionally well with a wide variety of targets. This blend is optimized for the production of targets < 1kb in length. qScript cDNA SuperMix produces excellent results in both real-time and conventional RT-PCR.

Components

qScript cDNA SuperMix	5X reaction buffer containing optimized concentrations of $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer and stabilizers.

Storage and Stability

qScript cDNA SuperMix is stable for 1 year when stored in a constant temperature freezer at -20°C. To extend the product's shelf-life, store the kit at -70°C. qScript cDNA SuperMix showed no loss in functional performance after 20 cycles of freezing on dry ice and thawing on ice. However, we recommend that the number of freeze-thaw cycles be kept to a minimum.

Reaction Assembly

Place components on ice. Mix, and then briefly centrifuge to collect contents to the bottom of the tube before using.

Component	Volume for 20-µL rxn.	Final Concentration
qScript cDNA SuperMix (5X)	4 µL	1X
RNA template	variable	(1 µg to 10 pg total RNA)
RNase/DNase-free water	variable	
Total Volume (µL)	20 µL	

Note: for smaller reaction volumes (i.e. 10-µL reactions), scale components proportionally.

Reaction Protocol

- Combine reagents in 0.2-mL micro-tubes or 96-well plate sitting on ice.
- After sealing each reaction, vortex gently to mix contents. Centrifuge briefly to collect components at the bottom of the reaction tube.
- Incubate:

  5 minutes at 25°C

  30 minutes at 42°C

  5 minutes at 85°C

  Hold at 4°C
- After completion of cDNA synthesis, use 1/5th to 1/10th of the first-strand reaction (2-4 µL) for PCR amplification. If desired, cDNA product can be diluted with 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA and stored at -20°C.

3. pCR8/GW/TOPO TA Cloning Kit (Invitrogen, Cat. No. 45-0642)

Introduction

This quick reference sheet is provided for experienced users of the TOPO® Cloning procedure. If you are performing the TOPO® Cloning procedure for the first time, we recommend that you follow the detailed protocols provided in the manual.

Note: The protocol for electrocompetent cells has been updated for improved cloning efficiency. For electroporation, follow the detailed protocols provided in the manual.

Step	Action
Produce PCR product	Produce PCR products using *Taq* polymerase and your own protocol. End the PCR reaction with a final 7–30 minute extension step.
Perform the TOPO® Cloning Reaction	1. Set up one of the following TOPO® Cloning reaction using the reagents in the order shown. (see table below) 2. Mix the reaction gently and incubate for 5 minutes at room temperature. 3. Place the reaction on ice and proceed to **Transform One Shot® Chemically Competent *E. coli*.**
Transform One Shot® Chemically Competent *E. coli*	1. For each transformation, thaw 1 vial of One Shot® *E. coli* cells on ice. 2. Add 2 µL of the TOPO® Cloning reaction into a vial of One Shot® chemically competent *E. coli* and mix gently. 3. Incubate on ice for 5–30 minutes. 4. Heat-shock the cells for 30 seconds at 42°C without shaking. Immediately transfer the tube to ice. 5. Add 250 µL of room temperature S.O.C. Medium. 6. Incubate at 37°C for 1 hour with shaking. 7. Spread 10-50 µL of bacterial culture on a prewarmed LB agar plate containing 100 µg/mL spectinomycin, and incubate overnight at 37°C.

Reagent	Volume
Fresh PCR product	0.5–4 µL
Salt Solution	1 µL
Water	to a final volume of 5 µL
TOPO® Vector	1 µL
Total volume	6 µL

Control reaction

We recommend using the Control PCR Template and the Control PCR Primers included with the kit to perform the control reaction. See the protocol on pages 19–20 for instructions.

4. Zyppy Plasmid Miniprep Kit (Zymo Research, Cat. No. D4020)

Protocol:

The following procedure is performed at room temperature.
Ensure that buffers have been prepared according to the instructions on page 3.

1. Add 600 µl of bacterial culture grown in LB medium to a 1.5 ml microcentrifuge tube.

   The Zyppy™ Plasmid Miniprep Kit may also be used with the classical centrifuge-based procedure for processing up to 3 ml of bacterial culture. The procedure should be modified as follows: 1A) Centrifuge 1.5 ml of bacterial culture for 30 seconds at maximum speed. 1B) Discard the supernatant. 1C) Repeat steps 1A and 1B as needed. 1D) Add 600 µl of TE or water to the bacterial cell pellet and resuspend completely.

2. Add 100 µl of **7X Lysis Buffer (Blue)**[1] and mix by inverting the tube 4-6 times. Proceed to step 3 within 2 minutes.

   After addition of 7X Lysis Buffer the solution should change from opaque to clear blue, indicating complete lysis.

3. Add 350 µl of cold **Neutralization Buffer (Yellow)** and mix thoroughly. *The sample will turn yellow when the neutralization is complete and a yellowish precipitate will form.* **Invert the sample an additional 2-3 times** to ensure complete neutralization.

4. Centrifuge at 11,000 – 16,000 x g for 2-4 minutes.

5. Transfer the supernatant (~900 µl) into the provided **Zymo-Spin™ IIN** column. Avoid disturbing the cell debris pellet.

6. Place the column into a **Collection Tube** and centrifuge for 15 seconds.

7. Discard the flow-through and place the column back into the same **Collection Tube**.

8. Add 200 µl of **Endo-Wash Buffer** to the column. Centrifuge for 30 seconds. *It is not necessary to empty the collection tube.*

9. Add 400 µl of **Zyppy™ Wash Buffer** to the column. Centrifuge for 1 minute.

10. Transfer the column into a clean 1.5 ml microcentrifuge tube then add 30 µl of **Zyppy™ Elution Buffer**[2] directly to the column matrix and let stand for one minute at room temperature.

11. Centrifuge for 30 seconds to elute the plasmid DNA.

Notes:

[1] Excessive lysis can result in denatured plasmid DNA. If processing a large number of samples, we recommend working with groups of ten or less at a time. Continue with the next set of ten samples after the first set has been neutralized and mixed thoroughly.

[2] The Zyppy™ Elution Buffer contains 10 mM Tris-HCl, pH 8.5 and 0.1 mM EDTA. If required, pure water (neutral pH) can also be used to elute the DNA.

5. Gateway LR Clonase II Enzyme Mix (Invitrogen, Cat. No. 11791-020)

Procedures

LR Reaction

LR Clonase™ II enzyme mix is supplied as a 5X solution. If you wish to scale the reaction volume, make sure the LR Clonase™ II enzyme mix is at a final concentration of 1X. For a positive control, use 100 ng (2 µl) of pENTR™-gus.

1. Add the following components to a 1.5 ml microcentrifuge tube at room temperature and mix:

Entry clone (50-150 ng)	1-7 µl
Destination vector (150 ng/µl)	1 µl
TE buffer, pH 8.0	to 8 µl

2. Thaw on ice the LR Clonase™ II enzyme mix for about 2 minutes. Vortex the LR Clonase™ II enzyme mix briefly twice (2 seconds each time).
3. To each sample (Step 1, above), add 2 µl of LR Clonase™ II enzyme mix to the reaction and mix well by vortexing briefly twice. Microcentrifuge briefly.
4. Return LR Clonase™ II enzyme mix to -20°C or -80°C storage.
5. Incubate reactions at 25°C for 1 hour.
6. Add 1 µl of the Proteinase K solution to each sample to terminate the reaction. Vortex briefly. Incubate samples at 37°C for 10 minutes.

Transformation

1. Transform 1 µl of each LR reaction into 50 µl of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells (Catalog no. C8540-03). Incubate on ice for 30 minutes. Heat-shock cells by incubating at 42°C for 30 seconds. Add 250 µl of S.O.C. Medium and incubate at 37°C for 1 hour with shaking. Plate 20 µl and 100 µl of each transformation onto selective plates. Note: Any competent cells with a transformation efficiency of >1.0 × 10^8 transformants/µg may be used.
2. Transform 1 µl of pUC19 DNA (10 ng/ml) into 50 µl of One Shot® OmniMAX™ 2 T1 Phage-Resistant Cells as described above. Plate 20 µl and 100 µl on LB plates containing 100 µg/ml ampicillin.

Expected Results

An efficient LR recombination reaction will produce >5000 colonies if the entire LR reaction is transformed and plated.

6. His-Spin Protein Miniprep Kit (Zymo Research, Cat. No. P2002)

Description

The **His-Spin Protein Miniprep™** provides researchers with a fast His-tagged protein purification technology. The simplified procedure is based on our innovative protein purification chemistry and custom designed fast spin columns. Up to 1 mg of His tagged protein can be purified in 5 minutes and eluted in as little as 100 ul of **His-Elution Buffer**. The purified protein can be used directly for enzymatic assays, protein biochemical analyses, SDS-PAGE and other applications. The product has been optimized for maximal protein purity: a single protein band is visible by Coomassie blue staining on SDS-PAGE gel (Figure 1.). The straightforward spin – wash – elute protocol dramatically simplifies protein purification: get results in minutes, not hours.

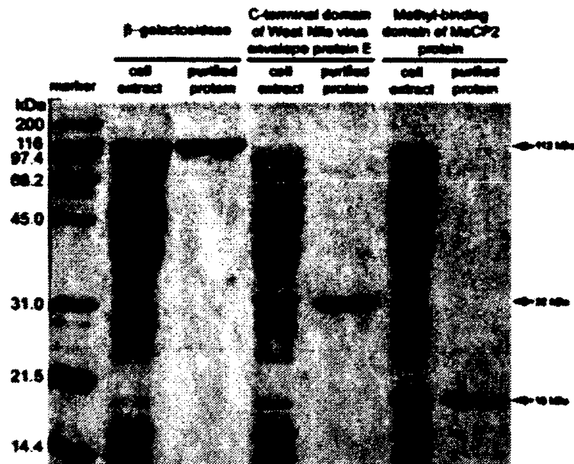


Figure 1. *E. coli* cell extracts, containing indicated proteins expressed as a N-terminal hexahistidine fusion, as well as the proteins purified using His-Spin Protein Miniprep™ were analyzed by SDS-PAGE on a 15% gel, and stained with Coomassie Blue®. The recombinant proteins were purposely expressed to a low level to demonstrate efficiency of the His-Spin Protein Miniprep™.

Protocol

Note: The procedure can conducted in cold or at room temperature. Use cold buffers and work on ice for sensitive proteins. Pay attention to centrifugation times: times listed include the time needed for acceleration. Centrifugal steps are carried out in standard tabletop microcentrifuge at maximum speed, usually corresponding to 13,000 to 15,000 g. Read the **Sample preparation** section below to make sure that the samples are in correct buffer before loading on the column.

1. Transfer 250 ul of **His-Affinity Gel** to the Zymo-Spin P1 column (make sure the resin is fully resuspended by shaking/vortexing the bottle before pipetting) and place the column into a collection tube.

   Use 1 ml pipette tip to transfer the His-Affinity Gel.: 200 ul-size (usually yellow) or smaller automatic pipette tips have small opening and may not be large enough for the affinity gel particles.

2. Centrifuge for 5-10 seconds.

   Ensure that the His-Affinity Gel is completely drained. Some older centrifuge models may require longer time of centrifugation. Do not over-dry the gel by long centrifugation times.

3. Add 150-300 ul of protein sample and resuspend the gel by shaking or tapping the column. Resuspend the gel a few more times during a two minute incubation period.

   It is important to allow the gel and your sample to interact for at least two minutes. If sample volume is larger than 200 ul, an additional 1-2 minutes binding time may be needed to improve yields of purified protein.

4. Centrifuge the column/collection tube 5-10 seconds. Discard the flow-through and place the column back in the collection tube.

5. Add 250 ul of **His-Wash Buffer** and resuspend the gel. Centrifuge 5-10 seconds.

6. Repeat the above wash step (step #5) one more time. Discard the collection tube.
7. Place the Zymo-Spin P1 column into a standard microcentrifuge tube. Add 150 ul of **His-Elution Buffer** to the column and resuspend the gel.

    Elution volumes can be between 100-200 ul. 150 ul of His-Elution Buffer elutes virtually all the column-bound protein. Smaller elution volumes are also possible and may yield more concentrated protein, but the elution efficiency may be compromised.

8. Centrifuge 5-10 seconds to elute the purified protein.

The eluate now contains the purified protein. The eluted protein is suitable for many applications. Use 1-10 ul for SDS-PAGE and Coomassie blue staining analysis. Store the purified protein at appropriate temperature.

Sample preparation

His-Binding Buffer is recommended for sample preparation. Cells expressing the polyhistidine-tagged protein may be directly resuspended in the **His-Binding Buffer** and lysed by standard methods including sonication, repeated freeze-thaw cycles, french press, etc. Other commercial protein extraction buffers (such as BugBuster® from EMD Biosciences or CelLytic from Sigma) are also compatible with the **His-Spin Protein Miniprep™** system and can be used after adjusting pH and imidazole concentration to values similar to those in the **His-Binding Buffer**.
Any cell extract or other complex protein mixtures can be used as a starting material as long as the proteins are soluble. pH value of the loaded sample should be between 7.5 and 8.0 . Too high or low pH can result in decreased protein yields and/or quality. The sample should not contain higher concentrations of imidazole or histidine (OK up to 10 mM) and should be completely devoid of metal-chelating agents, such as EDTA or EGTA, and strong reducing agents such as DTT. β-mercaptoethanol may be present up to 15 mM. If you are not sure what is in your sample, you can dilute the starting material with one volume of the **His-Binding Buffer** before proceeding with the purification process.

EXAMPLE PROCEDURE for protein purification from *E. coli* cell lysates: harvest 10 ml of grown culture and resuspend in 1 ml of **His-Binding Buffer**. Lyse the cells by sonication (or other methods) and centrifuge at ≥ 12,000 g at 4°C for 5 minutes to remove cell debris. Use 150 ul of the supernatant for protein purification.

Other Technical Considerations

1. **Starting material containing incompatible components such as EDTA, EGTA, DTT, > 15 mM β-mercaptoethanol, > 10 mM imidazole or histidine.**
   If your starting material contains these compounds, dilution with the **His-Binding Buffer** may help. Multiple loadings on the column will be necessary to load enough material. If the sample is in a different buffer, adjust the pH and imidazole and salt concentrations and carry out a test preparation. If the protein is still not bound, the sample needs to be dialyzed before use.

2. **Diluted starting material**
   If your starting material contains low levels of His-tagged protein and it requires more than 300 ul starting sample to purify enough protein, repeat steps #3 and #4 of the **Protocol** by loading 300 ul sample each time to mix with the **His-Affinity Gel**.

7. SDS-PAGE (BioRad)

1. **Prepare the gel:**
   i) Remove Gel from storage pouch and remove comb by placing both thumbs on the ridge of the comb and push upward in one smooth, continuous motion
   ii) Remove the tape: Pull gently to remove the green tape from the bottom of the cassette. If necessary, use the opening key or comb to help remove the tape at the corners
   iii) Rinse the wells: Use a syringe, wash bottle, or disposable transfer pipette to rinse the wells with running buffer (Reagent D). Straighten the sides of the wells, if necessary
2. **Preparing Mini-PROTEAN Tetra Cell assembly:**
   i. Set the electrode assembly to the open position on a clean, flat surface
   ii. Place the gel cassettes into the electrode assembly. Two cassettes are required to create a functioning assembly
      a) Place the first cassette with the short plate facing inward and so the gel rests at a 30° angle away from the center of the electrode assembly. Make sure the electrode assembly remains balanced and does not tip over
      b) Place the second gel or buffer dam on the other side of the electrode assembly, again by resting the gel on the supports. The gels rest at 30° angles, one on either side of the electrode assembly, tilting away from the center of the frame
   iii. Gently push both gels toward each other, making sure that they rest firmly and squarely against the green gasket that is built into the electrode assembly. Align the short plates to ensure the edge sits just below the notch at the top of the green gasket
   iv. While gently squeezing the gel cassettes (or cassette and buffer dam) against the green gaskets (maintaining constant pressure and with both gels in place), slide the green arms of the clamping frame one at a time over the gels, locking them into place
   v. The wing clamps of the electrode assembly lift each gel cassette up against the notch in the green gasket, forming a seal. Check again that the short plates sit just below the notch at the top of the green gasket.
   vi. Place the electrophoresis module into the tank and fill the buffer chambers with 1x running buffer (Reagent D):
      a) 200 ml in the inner buffer chamber
      b) 550 ml (1–2 gels) or 800 ml (3–4 gels, or >200 V) in the outer buffer chamber
3. <u>Prepare Sample</u>: Add 5.5 µL purified protein sample with 5.5 µL Laemmli sample buffer with β-mercaptoethanol (Reagent E), vortex & centrifuge
   i. Heat samples at 95 °C for 5 minutes in the Thermomixer
4. Load Wells using the Loading Guide (10-W)
   i. 15 µL Prestained SDS-PAGE Standard
   ii. 20 µL of sample
5. Running SDS-PAGE
   i. Place lid onto Tetra Cell and plug leads into power supply.
   ii. Turn on power supply to 200V and run for 25 – 45 minutes.
6. After electrophoresis is complete
   i. Turn off the power supply and disconnect the electrical leads

ii. Remove the lid from the tank and remove the gels from the cell. Pour off and discard the running buffer
iii. To open the cassette, align the arrow on the opening lever with the arrows marked on the cassette and insert the lever between the cassette plates at all four locations. Apply downward pressure to break each seal. Do not twist the lever
iv. Pull the two plates apart from the top of the cassette, and gently remove the gel

7. Perform Gel Staining Procedure

Gel Staining Procedure:

1. 1st Wash:
   i. Once SDS-PAGE is performed and is removed IAW SDS-PAGE Procedure (Step 7), wash the gel with 200 mL of ddH_2O for 5 minutes for a total of 3 times
2. Staining:
   i. Remove all water from the staining container and add 50 mL of Bio-Safe Coomassie Stain (enough to completely cover gel) and gently shake for 1 h.
   ii. Protein bands should be visible within 20 min and reach a maximum at 1 h
3. 2nd Wash:
   i. Rinse the gel with 200 mL of ddH_2O for 5 minutes for a total of 3 times, will remove background and allow for proper visualization of the bands.
   ii. Rinse gel in 200 mL of ddH_2O (covers gel) for at least 30 minutes. Can add a folded Kimwipe to assist in stain removal. Background SDS in the gel may cause background staining and interfere with band intensity while the gel is in the stain
   iii. Stained gels can be stored in water.
4. Take a picture of the gel with camera

Reagent Preparation Instructions:

A. **Running Buffer:** Dilute 100 mL 10x stock SDS-PAGE running buffer with 900 mL ddH_2O.
B. **Sample Buffer:** Add 950 μL Laemmli Sample Buffer to 50 μL β-mercaptoethanol.

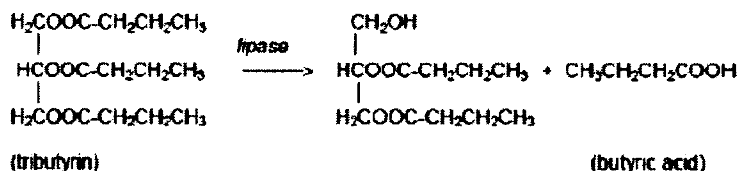
8. Lipase Activity Test – Tributyrin Assay (ChiralVison)

METHOD:

This method describes the procedure to determine the activity of enzyme in tributyrin units per gram enzyme (TBU/g).

PRINCIPLE

The method is based on the speed at which the enzyme hydrolyzes tributyrin at pH 7.5. The butyric acid which is formed is titrated with sodium hydroxide and the consumption of the latter recorded as a function of time.



(tributyrin) (butyric acid)

Analysis conditions:

Temperature:	**40°C ± 1°C**
pH:	**7.5**
Substrate:	**tributyrin (glycerol tributyrate)**
Reaction time:	**at least 3 minutes, up to 30 minutes. (Only the linear response is used to calculate the slope.)**

3. DEFINITION OF UNITS

1 TBU (lipase unit) is the amount of enzyme which releases 1 µmol titratable butyric acid per minute under the given standard conditions.

4. APPARATUS

pH-stat titration system, eg. Metrohm titrator, comprising:

- **Metrohm 665 Dosimat**
- **Metrohm 614 Impulsomat**
- **Tamson waterbath**
- **pH electrode**
- **magnetic stirrer**
- **double wall vessel (100 ml)**

4. REAGENTS AND SUBSTRATES

4.1 Chemicals

Sodium hydroxide (NaOH), (prepare 0.1 M solution), Sigma S5881
Glycerol tributyrate (tributyrin), e.g. Sigma-Aldrich 113026

Potassium dihydrogen phosphate p.a. (KH_2PO_4), (prepare 0.1 M solution) e.g. Sigma P2222

Buffer solution pH 7.0, e.g. Radiometer 943-112
Buffer solution pH 4.01. e.g. Radiometer 943-111

5. PROCEDURE
add to a 100 ml double walled reaction vessel (kept on 40 °C) equipped with a magnetic stirrer bar:

5 ml 100 mM Pi buffer pH 7.3 and 13 ml demi water
2 ml tributyrin

stir for 2 minutes
switch on titrator (use 0.1 M sodium hydroxide)
adjust pH to 7.5

if a blanc reaction runs, monitor the base consumption per minute, for 3 minutes.

add enzyme (~30 units maximum). ***Make sure to take a representative sample containing all particle sizes, specific activity may vary with particle size.***

wait until the first 100 µl base is added
reset and monitor the base consumption for 3 - 30 minutes, record each 0.5 minutes and calculate ml titrant / min

wash the vessel with ethanol, then water, dry with paper

6. CALCULATION

The measurements for the enzyme standards are used to plot a standard curve with enzyme activity as the x-axis and the associated mean slope (ml/min) as the y-axis. The data must be fitted to a straight line. The mean slope for the different dilutions of the samples is then used to read off the corresponding enzyme activity values from the standard curve. The activity of each sample is then calculated as follows:

$$\text{Sample activity (in TBU/g)} = \frac{\text{ml titrant/min} * \text{molarity of titrant} * 1000}{\text{sample weigth (g)}}$$

Unit definition
1 TBU unit = 1 µmol butyric acid released per minute / g enzyme

APPENDIX D

PH-STAT CALIBRATION

In this dissertation, lipase from *Thermomyces lanuginosus* (Sigma-Aldrich, Cat. No. L0777-50ML) was used as the activity standard for the tributyrin assay, as stated in Sections 3.2.20 and 3.3.6. The solution of lipase was diluted with 100 mM potassium phosphate buffer (pH 7.3) in order to obtain a concentration of 500 U/g. Various amounts were analyzed by the pH-stat in order to generate a calibration curve, as shown in Figure D1.

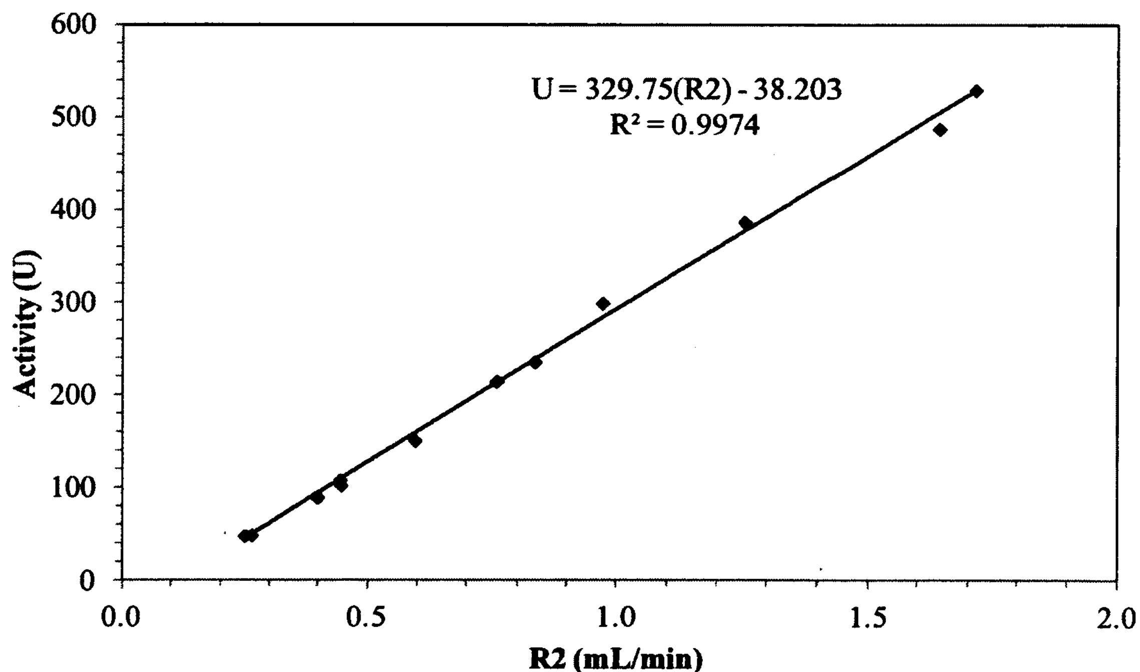


Figure D1. Lipase activity calibration curve.

APPENDIX E

SEQUENCING DATA

In this dissertation, the coding sequences (CDS) of lipases from Thermomyces lanuginosus (ATCC Strain 200065) were obtained from Genozymes Project database (http://genome.fungalgenomics.ca/), as shown below. The bolded nucleotides correspond to the start and stop codons at the beginning and end of the sequence, respectively. The underlined section of the sequences is the predicted signal peptide.

A. TL1: Thela2p4_000465 – 876 bp

ATGAGGAGCTCCCTTGTGCTGTTCTTCCTCTCTGCGTGGACGGCCTTGGCGCG
GCCTGTTCGACGAGCGGTTCCGCAAGATCTGCTCGACCAGTTTGAACTCTTTT
CACAATATTCGGCGGCCGCATACTGTGCGGCAAACAATCATGCTCCAGTGGG
CTCAGACGTAACGTGCTCGGAGAATGTCTGCCCTGAGGTAGATGCGGCGGAC
GCAACGTTTCTCTATTCTTTTGAAGATTCTGGATTAGGCGATGTTACCGGCCT
TCTCGCTCTCGACAACACGAATAAACTGATCGTCCTCTCTTTCCGCGGCTCTC
GTTCAGTAGAGAACTGGATCGCGAACCTCGCCGCCGACCTGACAGAAATATC
TGACATCTGCTCCGGCTGCGAGGGGCATGTCGGCTTCGTTACTTCTTGGAGGT
CTGTAGCCGACACTATAAGGGAGCAGGTGCAGAATGCCGTGAACGAGCATCC
CGATTACCGCGTGGTCTTTACCGGACATAGCTTGGGAGGCGCACTGGCAACT
ATTGCCGCAGCAGCTCTGCGAGGAAATGGATACAATATCGACGTGTTCTCAT
ATGGCGCGCCCCGCGTCGGTAACAGGGCATTTGCAGAATTCCTGACCGCACA
GACGGGCGGCACCCTGTATCGCATCACCCATACCAATGATATCGTCCCTAGA
CTCCCTCCTCGAGACTGGGGTTACAGCCACTCTAGCCCGGAGTACTGGGTCA
CGTCTGGTAACGACGTCCCAGTGACCGCAAACGACATCACCGTCGTGGAGGG
CATCGATTCCACCGACGGGAACAACCAGGGGAATATCCCAGACATCCCTTCG
CATCTATGGTATTTCGGTCCCATTTCAGAGTGTGAT**TAA**

B. TL2: Thela2p4_000466 – 876 bp

ATGAGGAGCTCCCTTGTGCTGTTCTTTGTCTCTGCGTGGACGGCCTTGGCCAG
TCCTATTCGTCGAGAGGTCTCGCAGGATCTGTTTAACCAGTTCAATCTCTTTG
CACAGTATTCTGCAGCCGCATACTGCGGAAAAAACAATGATGCCCCAGCTGG
TACAAACATTACGTGCACGGGAAATGCCTGCCCCGAGGTAGAGAAGGCGGAT
GCAACGTTTCTCTACTCGTTTGAAGACTCTGGAGTGGGCGATGTCACCGGCTT
CCTTGCTCTCGACAACACGAACAAATTGATCGTCCTCTCTTTCCGTGGCTCTC
GTTCCATAGAGAACTGGATCGGGAATCTTAACTTCGACTTGAAAGAAATAAA
TGACATTTGCTCCGGCTGCAGGGGACATGACGGCTTCACTTCGTCCTGGAGGT
CTGTAGCCGATACGTTAAGGCAGAAGGTGGAGGATGCTGTGAGGGAGCATCC
CGACTATCGCGTGGTGTTTACCGGACATAGCTTGGGTGGTGCATTGGCAACTG
TTGCCGGAGCAGACCTGCGTGGAAATGGGTATGATATCGACGTGTTTTCATAT
GGCGCCCCCCGAGTCGGAAACAGGGCTTTTGCAGAATTCCTGACCGTACAGA
CCGGCGGAACACTCTACCGCATTACCCACACCAATGATATTGTCCCTAGACTC
CCGCCGCGCGAATTCGGTTACAGCCATTCTAGCCCAGAGTACTGGATCAAAT
CTGGAACCCTTGTCCCCGTCACCCGAAACGATATCGTGAAGATAGAAGGCAT
CGATGCCACCGGCGGCAATAACCAGCCTAACATTCCGGATATCCCTGCGCAC
CTATGGTACTTCGGGTTAATTGGGACATGTCT**TTAG**

After cloning the lipase genes, they were inserted into pCR8/GW/TOPO vector and transformed into *E. coli*. The resulting colonies were separated and tested by restriction digest to determine proper size and ensure the transgene was inserted in the correct orientation. The successfully transformed vectors were then sent to UNH HCGS DNA Sequencing Core Facility.

Sample: TL2-B1-8

Forward (TL2-B1-8F)

Pairwise Alignment

Sequence 1: HCGS Sequence 2: Genozymes

Alignment score: 7862963 Identities: 1.0000000

Reverse (TL2-B1-8R)

Pairwise Alignment

Sequence 1: HCGS Sequence 2: Genozymes

Alignment score: 7862963 Identities: 0.9828179

Note: There were two mismatches towards the end of the reverse sample. However, this is not a concern since the signal was very weak and the forward sample did not indicate any mismatches at the corresponding positions.

Sample: TL2-B2-3

Forward (TL2-B2-3F)

Pairwise Alignment

Sequence 1: HCGS Sequence 2: Genozymes

Alignment score: 7862963 Identities: 1.0000000

Reverse (TL2-B2-3R)

Pairwise Alignment

Sequence 1: HCGS Sequence 2: Genozymes

Alignment score: 7862963 Identities: 1.0000000

Sample: TL2-B3-17

Forward (TL2-B3-17F)

Pairwise Alignment

Sequence 1: HCGS Sequence 2: Genozymes

Alignment score: 7862963 Identities: 0.9868735

Reverse (TL2-B3-17R)

Pairwise Alignment

Sequence 1: HCGS Sequence 2: Genozymes

Alignment score: 7862963 Identities: 0.9904535

Note: There were two mismatches that were confirmed with both the forward and reverse samples. One mismatch was changing an adenine (A) to a thymine (T), therefore changing the amino acid code from CTT to CTA, both of which are codons for leucine (L), so it will not affect the protein composition. The other mismatch changes a T to a cytosine (C), therefore changing the amino acid code from GGT to GGC, both of which are codons for glycine (G or Gly), so it will not affect the protein composition.