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Development and Utilization of a Pair of Sol-Gel Entrapped Lipases for Biodiesel Production from High Free Fatty Acid Oils

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DEVELOPMENT AND UTILIZATION OF A PAIR OF SOL-GEL ENTRAPPED LIPASES FOR BIODIESEL PRODUCTION FROM HIGH FREE FATTY ACID OILS

by

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DEVELOPMENT AND UTILIZATION OF A PAIR OF SOL-GEL ENTRAPPED LIPASES FOR BIODIESEL PRODUCTION FROM HIGH FREE FATTY ACID OILS

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Biodiesel, which consists of fatty acid alkyl esters, is one of the most widely adopted and successful renewable fuels. Traditional physiochemical biodiesel production methods require high cost refined feedstocks, and so alternative methods of catalysis and feedstocks have been explored. This research investigated the use of polysiloxane entrapped lipases to catalyze the production of biodiesel from low cost feedstocks.

In this work, lipase from *Burkholderia cepacia* (lipase PS) and lipase B from *Candida antarctica* (CalB) were separately entrapped using sol-gel chemistry. Optimal reaction conditions for the esterification of free fatty acids by immobilized CalB with methanol were determined. Immobilized CalB and lipase PS were then used separately and in combination on a variety of model and real substrates, to assess their suitability as catalysts for biodiesel production, with yields of 97.2 wt.% fatty acid methyl esters (FAME) from a 50% free fatty acid (FFA) model substrate after a 24 h reaction time and 94.5 wt.% FAME from yellow grease. The stability of the pair of lipases over multiple uses was then examined, and the lipases were found to retain over 95% of their activity over 10 cycles of 6 h reactions.

Experiments were conducted to compare the efficacy of the lipases when ethanol replaced methanol as the acyl acceptor for the esterification and transesterification reactions, as biodiesel from ethanol has superior fuel characteristics and lower environmental impact. Reactions utilizing ethanol showed similar results to methanol reactions, but with slower reaction rates. The toxicity of methanol and ethanol to each lipase's activity was examined, and it was found that methanol caused a loss of over 90% of lipase activity to both the lipase PS and CalB when incubated for 6 h, while ethanol caused less than 50% activity loss in both cases.

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1 Chapter 1

Introduction

1.1 Overview of Biodiesel Production

The depletion of the world's reserves of fossil fuels is one of the largest challenges facing modern society. In addition to depleting limited petroleum reserves, the consumption of fossil fuels causes negative environmental effects as they produce greenhouse gases, especially carbon dioxide. To combat the exhaustion of these limited reserves of fossil fuels and to reduce the negative environmental impact their consumption causes, a wide range of alterative and renewable energy sources have been investigated. One of the most successful and widely implemented of these energy sources is biodiesel.(Agarwal, 2007)

Biodiesel is a liquid fuel that serves as a drop in replacement for traditional petroleum diesel. Its chemical and flow properties are similar enough those of petroleum diesel that most newer diesel engines can use biodiesel without modification. Molecularly, biodiesel consists of fatty acid alkyl esters (FAAE). Generally, the fatty acid chain portion of the molecule has 14 to 20 carbons and a varying degree of unsaturation. Length and degree of saturation of the fatty chain are dependent mainly upon the source of the oil from which the biodiesel was made. The alkyl group is most commonly a methyl group, although ethyl groups are also used and do not negatively alter the fuel properties of biodiesel.(Makareviciene and Janulis, 2003) The identity of the alkyl group is determined by the production method used.

Industrially, biodiesel is most commonly produced from oils derived from food crops, such as soybeans. These oils are refined so that they consist almost exclusively of triglycerides. These triglycerides are reacted with a short chain alcohol, in the presence of a catalyst, in a transesterification reaction. Each of the three fatty acid chains of the triglyceride has its ester linkage to the glycerol backbone replaced with an ester linkage to an alcohol molecule. The transesterification reaction yields three fatty acid alkyl ester molecules and one glycerol molecule from each triglyceride molecule. Glycerol, excess alcohol, and the catalyst are then separated from the FAAE via phase separation and water wash steps.

Most commonly, the transesterification reaction is catalyzed by a base, such as sodium or potassium hydroxide. The base catalyzed transesterification reaction has shown itself to be the most economical when used with processed triglycerides, as it can be done under methanol reflux conditions without high pressures, has low catalyst costs, and can yield very high conversions (99%). However, the base catalyzed process does have a few significant drawbacks. The base catalyst must be removed from the produced FAAE before they can be used as fuel, which requires washing with water and results in waste water. Additionally, the triglycerides to be reacted must be almost completely dry, as even low water levels result in hydrolysis of triglycerides rather than transesterification. Third, and most significantly, the triglycerides to be reacted must be free of free fatty acids (FFA).

The relatively high cost of processed vegetable oils has caused recent research to focus on using lower quality oils. These oils are waste products such as yellow and brown grease and oils from non-food crops such as algae. Commonly, these oils have

significant levels of FFA. The presence of over 0.5% by mass FFA in an oil being converted to biodiesel via base catalyzed transesterification is problematic. The FFA reacts with, and consumes, the base catalyst. Not only does this permanently remove the base catalyst from the system, but it also results in the formation of a soap molecule. Soap leads to severe processing issues, as its amphipathic nature impedes the separations typically used to isolate FAAE from glycerol and alcohol.(Canakci and Gerpen, 2001; Zhang and Jiang, 2008)

Because of the effect of FFA on the efficacy of the base catalyzed process, alternative methods for the production of biodiesel from high free fatty acid oils have been investigated. The most widely adopted method is the use of an acid catalyst. Acids, such as sulfuric acid, are able to catalyze the esterification of FFA to FAAE in the presence of a short chain alcohol, and with water as a byproduct. Most commonly, acid catalysts are used as a pretreatment to remove FFA from an oil sample, which is then subsequently transesterified via the base catalyzed process. Acid catalysts are capable of catalyzing the transesterification reaction, but the reaction is much slower than with the base catalyst. The two step process is unfavorable, as it increases processing costs and makes the whole process less economical. Additionally, the acid catalyzed step requires harsh conditions. High levels of acid are required, as well as high molar ratios of alcohol to fatty acid. Additionally, the acid catalyzed process requires the oil to be free of water, as water lowers the esterification of FFA to FAAE.

As the acid catalyzed process is less than ideal, other catalysts have been investigated. Heterogeneous inorganic acid and base catalysts have been the subject of significant research. Heterogeneous catalysts refer to a catalyst which is not in the same

phase as the reactants. This makes purification of the catalyst from the reactants and products much simpler and economical. It also allows for the catalyst to be recovered and reused, assuming it is stable.(Jothiramalingam and Wang, 2009) Heterogeneous acid and base catalysts suffer from significant problems, however. They can require very harsh conditions or high temperatures, and are much more expensive than simple acid or base catalysts. The ability to reuse the catalyst alleviates the cost concerns somewhat, but many catalysts suffer from loss of activity after repeated uses.(Sani et al., 2014) Lipases have been suggested and investigated as another alternative catalyst.

1.2 Lipases for Biodiesel Production

Lipases are a class of enzymes found in a wide range of organisms. When *in vivo*, lipases are responsible for the hydrolysis of triglycerides to fatty acids and glycerol. If they are extracted from their native environment, they can be used to catalyze a wide range of reactions. Lipases perform the hydrolysis of esters, the esterification of carboxylic acids, and the transesterification of esters with alcohols. These characteristics have led to lipases being investigated as catalysts for many different industrial processes. Their current applications include in transformations of oils to higher value oils, the synthesis of biodegradable polymers, several reactions in the textile industry, as a supplement in detergents to help with oil removal, modifications for food processing and taste enhancement, for resolving racemic mixtures of glycerides, and many other areas.(Hasan et al., 2006) Lipases have been further investigated as catalysts for biodiesel production.

The use of lipases instead of acid, base, or heterogeneous catalysts allows for a variety of advantages. Lipases do not require neutralization and extensive processing to remove them, as do acids and bases. Lipases are also capable of operating at very mild conditions, with low alcohol levels and only slightly above room temperature, unlike heterogeneous catalysts. Most significantly, they can perform both the esterification of FFA and the transesterification of triglycerides simultaneously and at mild conditions. The primary drawback of using lipases to make biodiesel is their high cost, which tends to be higher than even heterogeneous acid and base catalysts.(Tan et al., 2010; Yan et al., 2014)

To make enzymes economically feasible, much research has been done into immobilizing them. If enzymes are used without immobilization for biodiesel production, recovering and reusing them is very difficult and costly. Immobilizing lipases allows for them to behave as a heterogeneous catalyst, and be easily recovered and reused. In addition, immobilization can allow for the stability of lipases over multiple reaction cycles to be improved.(Zhang et al., 2012)

Three main methods of lipase immobilization for biodiesel production have been studied.(Tan et al., 2010; Yan et al., 2014) The first, and most widely studied, method is by adsorption. Adsorption relies upon weak forces to attach the lipase to the exterior of the immobilization medium. Van der Wall's interactions and hydrophobic interactions are the most commonly taken advantage of methods, but elucidating the exact mechanism of attachment can be problematic.(Jegannathan et al., 2008) Adsorption has the advantage of being very cheap to accomplish. The attachment of lipase to the immobilization carrier can be accomplished cheaply, and the carriers can commonly be

reused even if the lipase is lost. Adsorption has several key disadvantages, however. The most significant problem with adsorption is that as the attachment of lipase to the carrier is weak, lipase tends to be lost with repeated reuses. This significantly decreases the economic advantage of using adsorption. Additionally, as lipase only is able to attach to the exterior of the immobilization matrix, loading tends to be somewhat low.

Lipase PS, from *Burkhoderia cepacia*, was adsorbed onto a polypropylene matrix and used to catalyze the production of fatty acid ethyl esters (FAEE) from castor oil, with yields approaching 90% with a 6 h reaction time.(Baron et al., 2014) Adsorbed *Candida antarctica* lipase B (CalB) was used to catalyze the methanolysis of vegetable oil with a yield of over 98% of fatty acid methyl esters (FAME) after 48 h of reaction time, with 95% of lipase activity retained after 50 cycles of reactions.(Shimada et al., 1999) Another study immobilized lipase PS onto celite via adsorption found that it was able to achieve 98% conversion of jatropha oil to FAEE over 8 h of reaction, although the immobilized lipase was stable for only 4 cycles of reuse.(Shah and Gupta, 2007) Ognjanovic and coworkers also reported that adsorbed CalB was able to produce 95.6% FAME from sunflower oil, although they found the lipase to not be stable upon recovery and reuse and reported a complete loss of activity after 3 cycles.(Ognjanovic et al., 2009)

Lipases can also be attached to an immobilization matrix via covalent linkage. Here, a specific chemical reaction is used to chemically bond the lipase to the carrier molecule. Most commonly, glutaraldehyde chemistry is used. With this method, amine groups on the carrier molecule and on the lipase are linked by a glutaraldehyde. Glutaraldehyde has terminal aldehyde groups with react with amines to yield imine linkages. These linkages are much stronger than the interactions used in adsorption

chemistry, and greatly reduce the loss of lipase during repeated reuse. Covalent linkages have the drawback of requiring chemically active immobilization matrixes and more difficult attachment methodologies. Additionally, as the imine formation is random, the reaction can attach to an amine containing amino acid on the lipase at a location that reduces the activity of the lipase.

One study utilizing covalent attachment of lipase to carrier was conducted by Rodrigues and coworkers, and found that lipase from *Thermomyces lanuginosus* covalently attached to carrier was effective at performing the ethanolysis of soybean oil to FAEE, with yields of 100% after 10 h of reaction time.(Rodrigues et al., 2010) Another study showed that lipase from *Candida rugosa* could be effectively attached to chitin via glutaraldehyde chemistry with a high level of protein attachment, but only 27% retention of activity.(Gomes et al., 2004) Lipase PS covalently attached to a silica-PVA was used to produce biodiesel from babussa oil with yields of FAEE greater than 98% with 48 h of reaction time.(Freitas et al., 2009) Mendes and coworkers also covalently linked lipases from *Pseudomonas fluorescens* and *T. lanuginosus* separately to Toyopearl AAF-amino-650M resin to catalyze the transesterification of palm oil to FAEE, with yields of 100%.(Mendes et al., 2011)

The third main method of lipase immobilization for biodiesel production is entrapment. In entrapment, the lipase is dissolved in a liquid phase. Polymer precursors are then added around it, and polymerized via a catalyst. This entraps the lipase within the polymerized matrix. Entrapment allows for high retainment of lipase over repeated uses of the immobilized lipase. The entrapment can limit the mobility of the lipase, and so care must be taken in selecting an immobilization matrix.

While a range of polymers can be used for entrapment, one which has been shown to be effective for lipase entrapment is polysiloxanes. The lipase is entrapped within a polysiloxane matrix via sol-gel chemistry. In this method, the lipase is dissolved in water. The siloxane precursors are added, and then a catalyst is added. The catalyst causes the hydrolysis of the siloxane precursors to liberate an alcohol and generate a hydroxyl group on the silicon atom. The hydroxyl groups from multiple silicon atoms then react in a condensation reaction to create a siloxane bond. This reaction proceeds to form an interconnected, branched polymeric network. The exact nature of the precursors can be changed to give different characteristics to the matrix. For example, an isobutyl group can replace an oxygen linkage to the silicon atom in the precursor to yield a matrix with larger pore size and more hydrophobic characteristics.(Hench and West, 1990)

Sol-gel chemistry with polysiloxanes has been used previously for biodiesel production. Meunier and Legge used lipase NS44035 from Novozymes entrapped within a sol-gel matrix and supported on celite to achieve 100% use of methanol for the transesterification reaction of triolein to methyl oleate.(Meunier and Legge, 2012) Noureddini and coworkers utilized sol-gel entrapped lipase PS to convert soybean oil to FAME, with 65% yields achieved with 1 h of reaction time.(Noureddini et al., 2005)

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2 Chapter 2

Enzymatic Production of Biodiesel from High FFA Oils using Tandem Sol-Gel Immobilized Lipases

Abstract

Esterification and transesterification of high free fatty acid (FFA) oils to fatty acid alkyl ester (FAAE) by sol-gel entrapped lipase B from *Candida antarctica* (CalB) and lipase from *Burkholderia cepacia* were investigated. The optimal conditions for the esterification catalyzed by the immobilized CalB were determined to be 50 °C, 1.7:1 molar ratio of methanol to fatty acid, and 0.36 g of water per 8 g oil. The immobilized lipases were used together to yield 92 wt.% conversion of a model 50 wt.% FFA oil substrate and 91% conversion of yellow grease to FAAE in 6 h and over 97 wt.% conversion of the model 50% FFA oil substrate and 94% conversion of yellow grease to FAAE in 24 h. The pair of immobilized lipases was further examined for its stability and showed to retain over 95 % of its activity over 10 cycles of 6 h reuses.

2.1 Introduction

Biodiesel, which consists of fatty acid alkyl esters (FAAE), is one of the most successful and widely adopted renewable fuels. Transesterification of food quality vegetable oils via base catalysis for the production of FAAE is a well-established industrial process (Ma and Hanna, 1999). Due to high cost, other feedstocks have been widely investigated. These include tallow, yellow grease, brown grease, non-edible plant oils, and microalgae (Balat, 2011). However, base catalysis is problematic with these feedstocks, as they generally contain a significant level of free fatty acids (FFA). FFA react with the base catalyst and form soap, which lowers yield and complicates the downstream processing of the product (Ma and Hanna, 1999). Acid catalyzed esterification is frequently used as a pretreatment to remove FFA, followed by the base catalysis (Noureddini et al*.,* 2009). High molar ratios of methanol to FFA, as well as the difficulty of processing and neutralizing the acid catalyst, make this process less than ideal (Canakci and Van Gerpen, 2001). Heterogeneous inorganic acid and base catalysts have also been investigated, but their performance suffers from high catalyst cost and decay of activity over time (Yusuf et al., 2012).

Enzymatic conversion methods, using lipases, have been viewed as realistic alternatives to the conventional physiochemical transesterification methods (Tan et al., 2010; Ranganathan et al., 2008). Generally, they allow for more moderate reaction conditions and less costly downstream processing of the FAAE and glycerol. However, the high cost of the lipases makes the enzymatic processes economically unattractive. The higher enzyme cost may be offset by the immobilization of the lipase, which

increases lipase stability and allows for its recovery and reuse (Zhang et al., 2012). A variety of immobilization methods have been developed and used with varying degrees of success. Common techniques include physical adsorption where enzyme is bonded onto a solid carrier (Shah and Gupta, 2007; Shimada et al., 1999), covalent attachment of enzyme to a solid support carrier molecule (Freitas et al., 2009; Gomes et al., 2004), and physical entrapment of enzyme within a polymeric matrix (Noureddini et al., 1999, 2005). Lipase immobilized by entrapment tends to be more stable than physically adsorbed lipases, and avoids deactivation from covalent coupling agents. Sol-gel entrapped lipases have shown good conversion of soybean oil to FAAE, as well as stability after reuse (Meunier and Legge, 2012; Noureddini et al., 2005).

Lipases have been used as catalysts in the esterification of FFA as well as the transesterification and hydrolysis of triglycerides. While most lipases show some catalytic activity toward these reactions, many favor one over the others. In general, lipases from *Burkholderia cepacia* (lipase PS) and *Thermomyces lanuginosus* have been more successful in facilitating the transesterification of triglycerides to FAAE and glycerol (Tran et al., 2012; Dizge et al., 2009; Xie and Ma, 2010; Da Ros et al., 2010), *Candida antarctica* lipase B (CalB) and its commercially available immobilized form Novozyme 435 has proven to be more effective in esterifying FFA to FAAE (Xu et al., 2012; Adachi et al., 2013), and lipase from *Candida rugosa* has been shown to catalyze the hydrolysis of triglycerides to FFA and glycerol (Noureddini et al., 2003). Other research has shown the co-immobilization of lipases from *C. rugosa* and *Rhyzopus oryzae* exhibit excellent catalytic activity toward the transesterification of soybean oil triglycerides to FAAE and glycerol (Lee et al., 2013), and the simultaneous use of the

commercially available immobilized lipases from *C. antarctica* and *T. lanuginosus* has shown good activity toward the transesterification of waste grease with 21.7% initial FFA to FAAE and glycerol (Yan et al., 2012). A variety of other studies have shown lipases to be capable of producing FAAE from feedstocks with FFA levels too high for base catalysis to be used (Ngo et al., 2013; Meng et al., 2011; Adachi et al., 2013; Hsu et al., 2002; Vèras et al., 2011).

In this work, lipases from *C. antarctica* and *B. cepacia* were used to catalyze triglyceride substrates with high levels of FFA to FAAE. Lipases were immobilized separately by sol-gel entrapment prepared by polycondensation of tetramethoxysilane (TMOS) and *iso*-buthyltrimethoxysilane (*iso*-BTMS). The optimal conditions for the esterification reaction catalyzed by the immobilized CalB were determined using model high FFA oils. The combined immobilized lipases were used to catalyze the esterification and Transesterification of model triglyceride substrates with 0.0, 15, and 50% initial FFA, as well as yellow grease. The immobilized lipases were also examined for their stability and resistance to deactivation with model substrates and yellow grease.

2.2 Methods

2.2.1 Materials

Tetramethoxysilane (95%, TMOS), *iso*-butyltrimethoxysilane (97%, *iso*-BTMS), sodium fluoride (NaF), tricaprin (95%), technical grade oleic acid (90%), and lipase PS were purchased from Sigma-Aldrich (USA). Lyophilized CalB was generously donated by C-Lecta (Germany), all other lipases were donated from Amano Enzyme (Japan). Bis-(trimethylsilyl) trifluoroacetamide (derivative grade, BSTFA), hexane (GC grade),

ethanol (99.9%), methanol (99.9%), and pyridine (99.8%) were purchased from Fischer Scientific (USA). Refined soybean oil was purchased from a local retailer. Yellow grease was donated by Kruger Commodities (USA). The concentration of FFA in this sample was determined to be 16.0% FFA by weight.

2.2.2 Immobilization methodology

A specific amount of lipase (200 mg for CalB, 1 g for lipase PS) was weighed into a 125 mL flask, and 10 mL of distilled water was added. The mixture was stirred using a magnetic stirrer for 4 min at 150 RPM, until all lipase dissolved. To this solution, 1 mL of 1 M NaF, 1.83 g TMOS, and 8.38 g *iso*-BTMS were added. The flask was then stoppered, and allowed to stir at 150 RPM for 2 min after which the reaction had been completed and a gel had formed. The flask was removed from the stir plate and left for 24 h at room temperature. After 24 h, the stopper was removed, the remaining liquid was decanted from the flask and discarded, and the flask was placed in a 33 °C water bath to dry for 24 h. After drying, the solid gel in the flask was ground in a mortar and returned to the flask. The resulting powder was washed by adding 100 mL of water to the flask, and stirring at 500 RPM for 1 h. The immobilized enzyme powder was recovered via centrifugation at 2,500 g for 3 min. The immobilized enzyme was returned to a flask, and dried in a 33 °C water bath for 24 h. After drying, the powder was ground again in a mortar, and stored at 4 °C until use. Enzyme loading was determined to be 42 ± 1.7 mg enzyme per 1 g powder for CalB, and 195 ± 7.9 mg enzyme per 1 g powder for lipase PS.

2.2.3 Reaction setup

Reactions were performed in 50 mL flasks and stirred magnetically at 600 RPM. The desired amount of soybean oil, oleic acid, water, and methanol were added to the flask, and the flask was placed in a circulating water bath which was kept at uniform temperature (± 0.1 C) to allow the reaction to reach the desired reaction temperature. After 5 min, the designated amount of each enzyme was added and the reaction start time was recorded. The flask was kept sealed with a stopper at all times. Samples were taken at designated time points.

To determine the stability and reusability of the catalysts, the immobilized enzymes were separated and reused in a subsequent reaction with fresh substrates. The recovery method involved the addition of 30 ml distilled water to the reaction, vigorously shaking the mixture, and then centrifuging at 3,000 g for 3 min. After centrifugation, the mixture separated into 3 phases: an aqueous phase containing the water, glycerol, and methanol, an organic phase containing the FAME, FFA, mono-, di-, and triglycerides, and an enzyme matrix phase. To recover the immobilized lipases, the aqueous and organic phases were decanted. The recovered lipase was not treated further, and was reused in a subsequent reaction with fresh substrates.

2.2.4 Sampling and analysis

Samples were approximately 0.2 mL in volume and were collected in 10 mL glass vials. Samples were initially heated at 120 °C for 10 min to ensure enzyme denaturation. A Hewlett-Packard (HP) 6890 Series gas chromatograph (GC) was used for the chromatography work and HP Chemstation software was used for the data analysis. The

GC was equipped with a HP-5 column and a flame ionization detector. Samples were prepared by placing 0.035 mL of the denatured samples in 1.7 mL GC sample vials. Samples were then freeze dried to remove excess water and alcohol and derivatized with 0.1 ml of BSTFA by heating at 70 °C for 15 min. To the derivatized sample, 0.15 mL of 8 mg/ml tricaprin in pyridine was added as internal standard and hexane was added to bring the total volume of the samples to 1.2 mL. The samples were then analyzed by the GC. Sample volumes were 2 µl, the carrier gas was helium, and the GC was operated in constant flow mode with a flow of 12.0 mL/min. A split injector was used with a split ratio of 15:1 and a temperature of 350 °C. The FID detector was operated at 350 °C. The oven was initially held at 80 °C and then elevated to 180 °C at 15 °C/min, then to 250 °C at 5.0 °C/min, and finally to 325 °C at 8 °C/min. The oven was held at this temperature for 55 min, and then returned to 80 °C. Calibration of the GC method was carried out by analyzing standard solutions of mixed FAME, FFA, mono-, di-, and triglycerides. The standards were derivatized in the same fashion as the reaction samples. Standard curves were created for each species, and used to determine the composition of each sample. All experiments were performed in duplicate, except for recovery and reuse reactions, which were performed in triplicate.

2.3 Results and discussion

2.3.1 Lipase screening

Lipases are capable of catalyzing a variety of reactions, including hydrolysis and transesterification of triglycerides, and esterification of carboxylic acids. Previous work by Noureddini and co-workers (2005) investigated the catalytic activity of several lipases

for their transesterification activity of triglycerides. Among the tested lipases, lipase PS was found to be the most effective lipase in catalyzing the conversion of triglycerides to FAME. Lipase PS was then studied further in sol-gel immobilized form. Experimental results showed 67 mole% conversion of triglycerides to FAME in 1 h of reaction at the optimized reaction conditions, which were determined to be 35 °C, 1:7.5 oil/methanol molar ratio, 0.5 g water and 475 mg lipase. Results from this work are incorporated into the combined lipase experiments of the current study.

In this study, several lipases were screened for their catalytic activity in the esterification of FFA to FAME. In each reaction, 100 mg of free, powdered lipase was added to a mixture of 8 g oleic acid, 0.2 g water, and 1.36 g methanol (1.5:1 molar ratio of methanol to FFA), and reacted for 3 h. The resulting conversion of FFA to FAME as final wt.% FAME is presented in Table 2.1. Total weight percentage of FAME produced was used to determine the effectiveness of each lipase for the esterification of FFA. As shown in Table 2.1, among the tested lipases CalB was the most effective lipase in the esterification of the FFA and resulted in the esterification of about 56% of the initial FFA to FAME, while the next most effective lipase was lipase A which resulted in the conversion of 15.4% of FFA to FAME. This is consistent with the results found in the literature, where CalB is used to catalyze esterification of FFA. For example, 90% FAAE was produced from soybean oil hydrolysate (100% FFA) using CalB as the catalyst (Adachi et al., 2013) after 6 h.

Table 2.1 Lipases screened for esterification activity at 8 g oleic acid, 100 mg free enzyme, 1.36 g methanol, 0.2 g water, 40 °C, and 3 h.

Lipase	Source Organism	FAME Production
PS	*Burkholderia cepacia*	5.9 ± 1.7
F	*Rhizopus oryzae*	5.0 ± 2.3
G	*Penicillium camembertii*	1.8 ± 0.0
AY	*Candida rugosa*	1.4 ± 0.0
AK	*Pseudomonas fluorescens*	1.3 ± 0.2
A	*Aspergillus niger*	2.0 ± 0.3
M	*Mucor javanicus*	3.9 ± 1.1
R	*Penicillium roqueforti*	6.5 ± 0.8
CalB	*Candida antarctica*	62.7 ± 3.1
N	*Rhizopus niveus*	1.8 ± 0.4

2.3.2 FFA reaction optimization

Experiments were conducted in order to optimize the reaction conditions for the esterification of FFA catalyzed by free and immobilized CalB. The catalytic activity of the free and immobilized CalB were compared to examine how the immobilization impacted the enzyme's activity. Based on material balance, 1 g of immobilized enzyme was determined to be equivalent to 42 ± 1.7 mg of free enzyme.

Initially, water and methanol were kept at the same level as in section 2.3.1. The amount of enzyme was reduced to 30 mg free enzyme or an equivalent amount of immobilized enzyme, while the temperature was varied to find at what temperature the immobilized CalB most effectively produced FAME from FFA. The optimal temperature, at which the highest wt.% FAME was produced, was then used in a series of reactions in which only the water level was varied. Finally, the optimal temperature and

optimal water levels were used to conduct reactions at varied methanol levels, to determine the optimal molar ratio of methanol to FFA. These reactions yielded the temperature, water level, and molar ratio of methanol to FFA at which sol-gel entrapped CalB most effectively catalyzes the esterification of FFA to FAME.

2.3.2.1 Effect of temperature

Experiments were conducted to determine the optimal temperature for the free and the immobilized lipase CalB. In all reactions, 8 g oleic acid, 0.2 g water, 1.36 g methanol, and 30 mg free enzyme or the equivalent amount of immobilized enzyme, 0.71 g, were used. The temperature was varied from 38 to 58 °C in 4 °C increments, and the reactions were allowed to proceed for 3 h. The results are shown in Figure 2.1, as a plot of wt.% FAME produced against temperature of reaction. As shown in this figure, the activity of the free enzyme increased as the temperature was increased from 38 to 42 °C, after which it began to decline. The decrease in activity at higher temperatures was likely due to denaturation and resulting loss of activity. The results for the reactions with the immobilized lipase showed an increase in the formation of FAME as the temperature was increased from 38 to 50 °C. Further increases in the temperature from 50 to 58 °C did not result in a significant change in the formation of FAME. At 42 °C, the free lipase resulted in 59% FAME, while the immobilized lipase produced 68% FAME. The free enzyme activity, however, steadily decreased as temperature was increased, while the immobilized enzyme activity increased to 89% conversion at 50 °C. This was consistent with the work of other researchers who found that the sol-gel entrapped enzymes had greater stability at increased temperatures than the free enzyme (Noureddini et al., 2005;

Reetz, 1997). Previous investigations have attributed the increased activity of lipases from the sol-gel process to the lipophilic nature of the sol-gel environment, which causes a phenomenon that mimics a classical interfacial interaction. Additionally, it is likely that the hydrophobic environment of the sol-gel facilitates transport of the substrates to the active site of the biocatalyst. While CalB has shown to be unique and to not require interfacial activation, it still has its maximum activity at an oil and water interface (Martinelle et al., 1995). As such, its activity is likely aided by the sol-gel matrix in the same way as is described for other lipases (Reetz, 1997). The sol-gel entrapment also improves the temperature stability of the lipase via hydrogen bonding and other weaker interactions (Reetz, 1997).

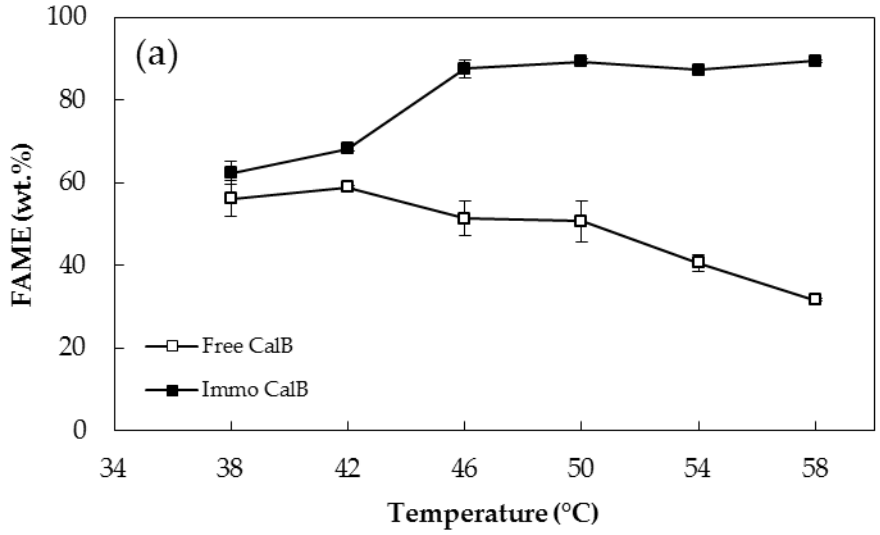

Figure 2.1 Effect of reaction temperature on FFA esterification to FAME at 30 mg free CalB or 0.71 g immobilized CalB, 8 g oleic acid, 1.36 g methanol, 0.2 g water, and 3 h.

2.3.2.2 Effect of water

Lipases, like other enzymes, utilize a catalytic triad to catalyze reactions (Martinelle et al., 1995) and the mechanism of reaction is dependent on the presence of a

small amount of water. The amount of water needed varies depending on the reaction medium and the immobilization support (Arroyo et al., 1999). Due to this, the amount of water present in the reaction is expected to be influential in the esterification activity. Reactions to determine the optimal water level were conducted as described in section 2.3.2.1 and at 50 °C. The water concentration was varied across the range of 0 to 1.8 g per 8 g of FFA. The results, as presented in Figure 2.1, show the formation of FAME as a function of the amount of water used in the reactions. As shown in this figure, the activity of the free CalB was dependent on the presence of water and the formation of FAME was increased from 11 wt.% at no added water to a maximum of 54 wt.% at 0.9 g water, and further addition of water did not have a significant effect on the formation of FAME. This data suggests that the activity of the free CalB is significantly affected in the presence of water, which is consistent with the existing literature (Martinelle et al., 1995). Contrary to the free CalB, the esterification reactions with the immobilized CalB showed the formation of 75% FAME with no added water and up to about 92% of FAME as the amount of water was increased to 0.36 g water per 8 g FFA. The production of FAME leveled off as the amount of the added water was increased further. The relatively high levels of FAME produced with the immobilized CalB, particularly at no added water, may be explained by the proposed lid structure of this lipase by Martinelle and co-workers (1995). The proposed hydrophobic helix adjacent to the core of the protein may interact with lipophilic R-groups in the sol-gel matrix, resulting in the fixation of favorable lipase conformation similar to the interfacial activation which occurs in the presence of water (Reetz, 1997). In addition, the sol-gel entrapment process likely traps a small amount of water on the exterior of the enzyme, which could lend additional

stability of the enzyme. The observed enhancement of the immobilized CalB activity was consistent with our previous study on the transesterification of soybean oil with sol-gel immobilized lipase PS (Noureddini et al., 2005) and also the work of Reetz (1997).

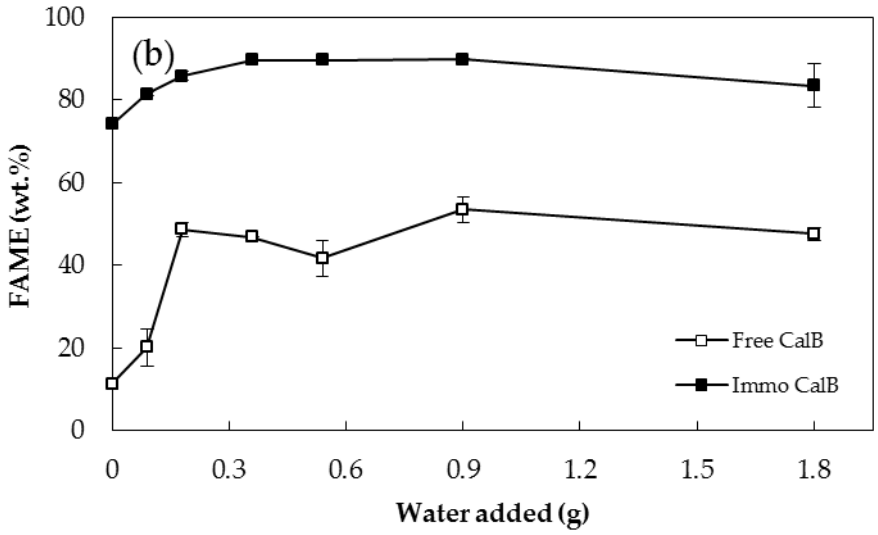

Figure 2.2 Effect of water level on FFA esterification to FAME at 30 mg free CalB or 0.71 g immobilized CalB, 8 g oleic acid, 1.36 g methanol, 50 °C, and 3 h.

2.3.2.3 Effect of methanol

To identify the optimal ratio of methanol to fatty acid, reactions were conducted as in section 2.3.2.1, with the optimized water level and temperature of 0.36 g of water per 8 g of FFA and 50 °C, respectively. The molar ratio of methanol to FFA was incrementally increased from 1:1 to 4.2:1. Results are presented in Figure 2.3 for the production of FAME as a function of the ratio of methanol to FFA. As is shown in this figure, there was an increase in the formation of FAME as the amount of methanol was increased for both the free and the immobilized lipases. The reaction with the immobilized CalB resulted in the highest formation of FAME (92 wt.%) at a methanol to FFA of 1.7:1, and further increases in the methanol concentration resulted in a decrease

in the formation of FAME. The formation of FAME with free CalB was increased from 27 wt.% to 58 wt.% as the molar ratio of methanol to FFA was increased from 1:1 to 4.2:1 and no decrease in FAME production was observed. This observation suggests that the decrease in the formation of FAME at the higher methanol concentrations with the immobilized lipase may not be due to losses in the activity of the lipase, as the free CalB appears to function well at these levels of methanol. The decrease in the formation of FAME with the immobilized CalB at higher methanol concentrations may be attributed to the faster diffusion of the smaller methanol molecules into the entrapment matrix which may impede the transport and consequently the availability of the FFA molecules at the imbedded reactive sites of CalB in the entrapment matrix. The free enzyme does not suffer from this impediment as the reactants form a homogeneous phase and diffusional limitation become insignificant. The optimal methanol to FFA ratio found in this study is similar to results found in the literature, where CalB has shown to require a slight molar excess of methanol to FFA to reach optimal conversion. One study by Brask and co-workers (2011) found a 1.5:1 methanol to FFA ratio to be superior in a batch esterification of palm oil FFA, while another study found the same optimal molar ratio of methanol to FFA, when converting soybean oil hydrolysate to FAME (Adachi et al., 2013).

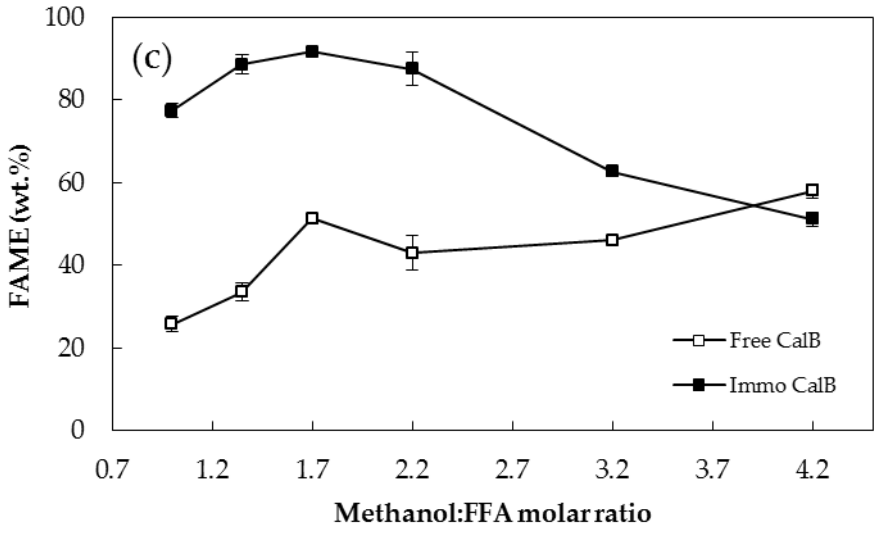

Figure 2.3 Effect of methanol:FFA molar ratio on FFA esterification to FAME at 30 mg free CalB or 0.71 g immobilized CalB, 8 g oleic acid, 0.36 g water, 50 °C, and 3 h.

2.3.3 Transesterification/esterification with immobilized lipases

Sol-gel entrapped lipase PS and CalB were tested separately on a model substrate that was 50% oleic acid and 50% refined soybean oil by weight to determine the catalytic activity of each lipase. Reactions were conducted using the optimal water, temperature, and methanol levels determined in section 2.3.2. The effect of variations in the conditions on sol-gel entrapped lipase PS was done in a previous study (Noureddini et al., 2005), and the optimal conditions were determined to be 0.5 g water per 10 g oil, a methanol to triglyceride molar ratio of 7.5:1, and a temperature of 35 °C, which are close to the optimal conditions determined for CalB. To maximize the esterification and transesterification conversions, the amount of the immobilized lipase added to each reaction was doubled from the optimization reactions. For lipase PS, 1.54 g of

immobilized lipase was used (equivalent to 300 mg free enzyme), and for CalB, 1.43 g of immobilized lipase was used (equivalent to 60 mg free enzyme). The formation of FAME and the consumption of FFA as functions of time are presented in Figure 2.4. As Figure 2.4 shows, CalB catalyzed the esterification of FFA to FAME as the wt.% of FFA was reduced from 50% to approximately 5% in 1 h, and the wt.% of FAME reached a plateau of 45% at the same time point. Tracking of the level of triglycerides with time confirmed that CalB mainly catalyzed the esterification of FFA to FAME and not the transesterification of triglycerides, as the wt.% of triglycerides in the reaction did not decrease after 24 h of reaction. The results for the reactions catalyzed by immobilized lipase PS, presented in Figure 2.4, show that the formation of FAME by immobilized lipase PS was initially slower than the reactions catalyzed by CalB, but after 3 h reaction surpassed the formation levels attained by CalB and reached 72% FAME after 6 h. Additionally, the decrease in the level of FFA was slower than the formation of FAME. This behavior is consistent with the catalytic activity of lipase PS which is known to catalyze the transesterification of triglycerides to FAME as well as the esterification of FFA to FAME (Noureddini et al., 2005). Tracking the levels of triglycerides in the reaction confirmed this as the wt.% of triglyceride was reduced to 6% from 50% after 6 h. The FAME produced by immobilized lipase PS is resulted from the esterification of FFA and the transesterification of triglycerides. The lack of transesterification activity of CalB for converting triglycerides to FAME conflicts with other studies, which report the use of CalB immobilized on various matrices to produce FAME from oils consisting primarily of triglycerides (Shimada et al., 1999; Ngo et al., 2012). The lack of consistency may be due to the method of immobilization used in this study which may

have caused an alteration or inhibition in the flexibility of the enzyme, and has rendered its active site unable to accept triglycerides. Additionally, the reaction conditions used in this study could have decreased the transesterification activity of CalB, relative to other studies.

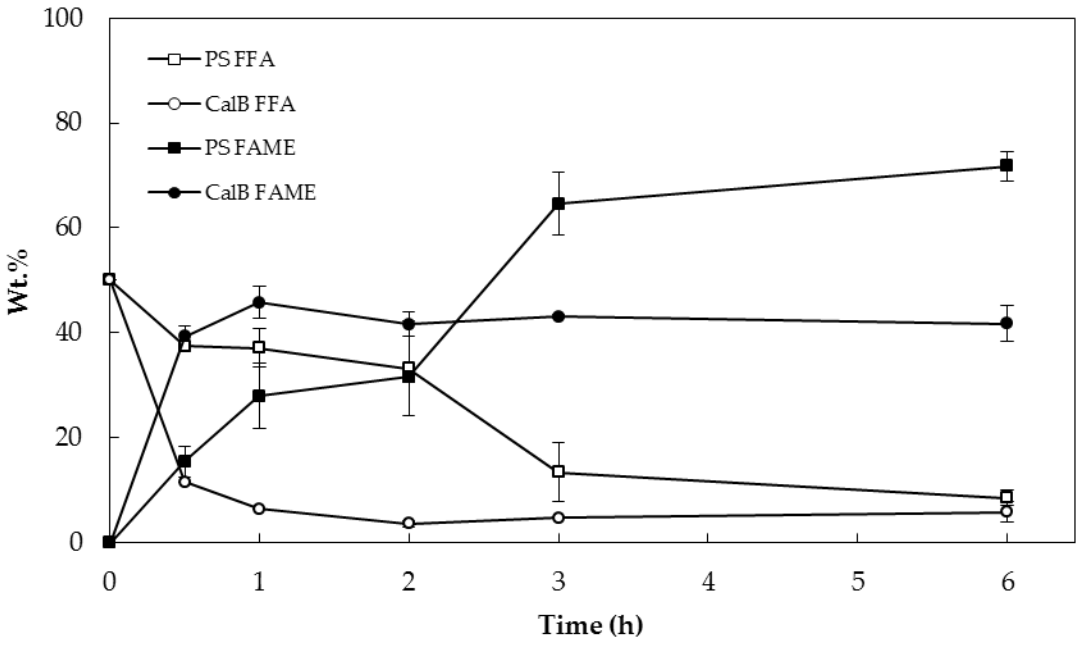

Figure 2.4 Esterification and transesterification of FFA and triglycerides to FAME using immobilized lipase PS and CalB separately at the optimized conditions on a 50% FFA substrate at 4 g oleic acid, 4 g soybean oil, 1.54 g immobilized lipase PS or 1.43 g immobilized CalB, 1.54 g methanol, 0.36 g water, and 50 °C.

2.3.4 Effect of combined lipases

The individual immobilized enzyme studies of section 2.3.3 suggested CalB as a more effective lipase in catalyzing the esterification of FFA to FAME, while lipase PS was viewed as a more effective lipase in catalyzing the transesterification of triglycerides to FAME. To maximize the overall conversion of high FFA triglyceride substrates to FAME, reactions were conducted using both immobilized lipases in the same reaction.

As in section 2.3.3, 1.54 g of immobilized lipase PS and 1.43 g of immobilized CalB were used. Reactions were otherwise conducted as described earlier in section 3.3. Initial FFA concentrations of 0, 15, and 50 wt.% were tested. Varied levels of FFA were tested to gauge the suitability of the combination of the two immobilized lipases as catalysts for FAME production from a variety of feedstocks. The formation of FAME and the variations in the concentration of FFA through 6 h of the reaction are shown in Figure 2.5, and the results after 24 h are presented in Table 2.2. As Figure 2.5 shows, the concentration of FFA in the reaction reaches a steady level of approximately 6% by weight after about 1 h, regardless of the initial FFA concentration. For the same reaction time of 1 h, the most FAME was formed with the 50% FFA substrate at about 81.2 ± 3.0% and slightly lower for the samples with lower initial FFA content. Comparing the results of the combined lipases with their individual catalytic activity clearly shows that the combined lipases are much more effective in converting high FFA substrates. For example, after 1 h reaction for the 50% FFA substrate, the concentration of FAME reached 45.8 ± 3.0% and 28.0 ± 6.0% with individual immobilized CalB and lipase PS, respectively (Figure 2.5), while the combination of the lipases resulted in the formation of 81.2 ± 3.0% FAME after the same time period. This suggests some degree of synergy when the lipases were combined and further implies to the inhibitory effects of FFA toward the transesterification activity of lipase PS. When used alone, lipase PS resulted in the formation of 28.0 ± 6.0% of FAME at 1 h, whereas, when was combined with CalB it accounted for about 37.8 ± 1.6% of the formed FAME with the remaining 43.4 ± 1.6% FAME formed through the esterification of FFA as the level of FFA was reduced from 50 to 6.6%. This hypothesis is further supported by examining the triglyceride levels at

different times. This examination revealed that nearly all of the initial triglycerides were removed after only 1 h in all cases when both CalB and lipase PS were present, while 5.9 ± 2.7% triglycerides remained unreacted after 6 h when only lipase PS was present in the reaction. The results after 24 h, shown in Table 2.2, show that the highest level of FAME was produced with the initial FFA of 50 wt.%. This could likely be due to the formation of much higher levels of glycerol with substrates containing little or no FFA, which could affect the equilibrium as well as the catalytic activity of the immobilized lipases by accumulating on the support structure. The pair of immobilized lipases was further used to catalyze the esterification and transesterification of yellow grease to FAME. Results as shown in Figure 2.5 revealed a trend similar to the model compound with 15% FFA.

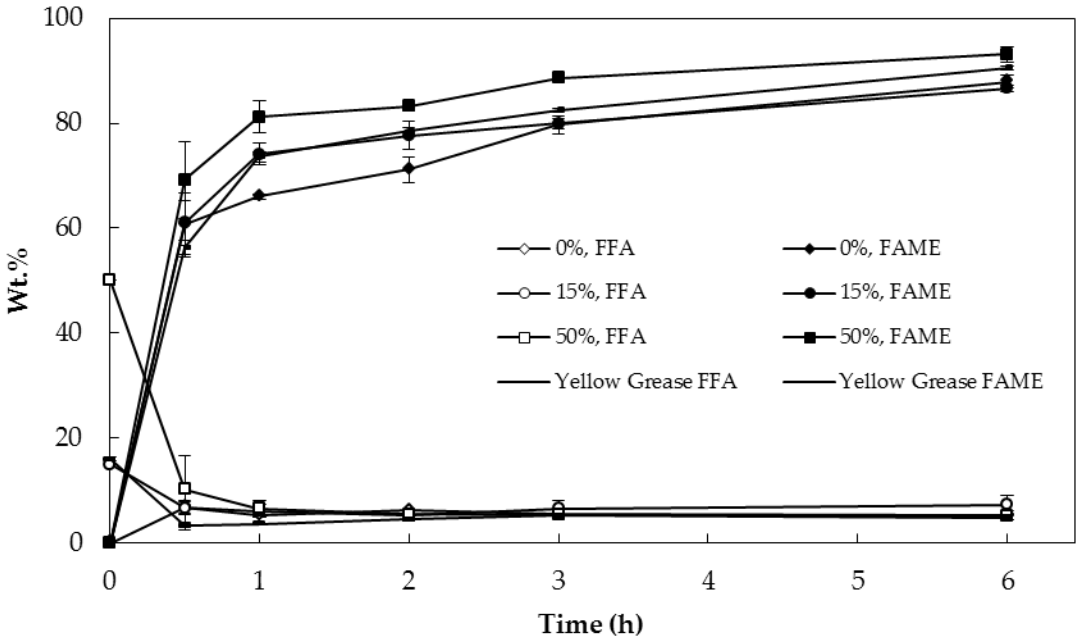

Figure 2.5 Esterification and transesterification of FFA and triglycerides to FAME using immobilized lipase PS and immobilized CalB together in a single reaction on substrates with varied initial FFA wt.% and yellow grease at 8 g combined soybean oil and oleic aci acid or yellow grease, 1.54 g immobilized lipase PS, 1.43 g immobilized CalB, 1.54 g methanol, 0.36 g water, and 50 °C.

Table 2.2 Conversion of FFA and triglycerides to FAME after 24 h at 8 g combined soybean oil and oleic acid or yellow grease, 1.54 g immobilized lipase PS, 1.43 g immobilized CalB, 1.54 g methanol, 0.36 g water, and 50 °C.

Oil Source	FFA% after 24 h	FAME% after 24 h
0% FFA	5.7 ± 0.6	93.0 ± 0.3
15% FFA	7.3 ± 1.1	91.1 ± 1.2
50% FFA	2.8 ± 0.3	97.2 ± 0.3
Yellow Grease	4.2 ± 0.2	94.5 ± 0.1

The examination of the combined use of the immobilized lipases was further tested at different lipase loadings. Reaction were conducted with a model substrate containing 50 wt.% FFA. All other conditions remained unchanged except that the mass

of the immobilized lipase was sequentially decreased by a factor of 2 from 1 to 1/2, 1/4, 1/8, and 1/16. The actual enzyme loading, labeled as 1, corresponded to 1.54 g of immobilized lipase PS and 1.43 g of immobilized CalB. The amount of loading was halved in the subsequent samples. For example enzyme loading labeled as 1/2 corresponded to 0.77 g of immobilized lipase PS and 0.72 g of immobilized CalB. The results are shown in Figure 2.6 and Figure 2.7, where FAME production and FFA consumption are presented as a function of time, respectively. As shown in this figure, enzyme loading had a significant effect on the kinetics of the reactions and the highest conversion which corresponded to the formation of 93.1 ± 2.0% FAME after 6 h resulted when the maximum amount of the immobilized lipases was used. As the enzyme loading was reduced in the reactions, the rate of formation of FAME and consumption of the FFA were also reduced. However, after 6 h reaction, the level of FFA was reduced to less than 10 wt.% in all cases except in the 1/16 loading. Nevertheless, the level of triglycerides which remained in the reaction was more than 40% for the 1/8 and 1/16 loadings levels, which suggests lipase PS as the main impediment in lower FAME formation. This behavior could be attributed to partial inhibition of lipase PS by FFA described earlier in this section.

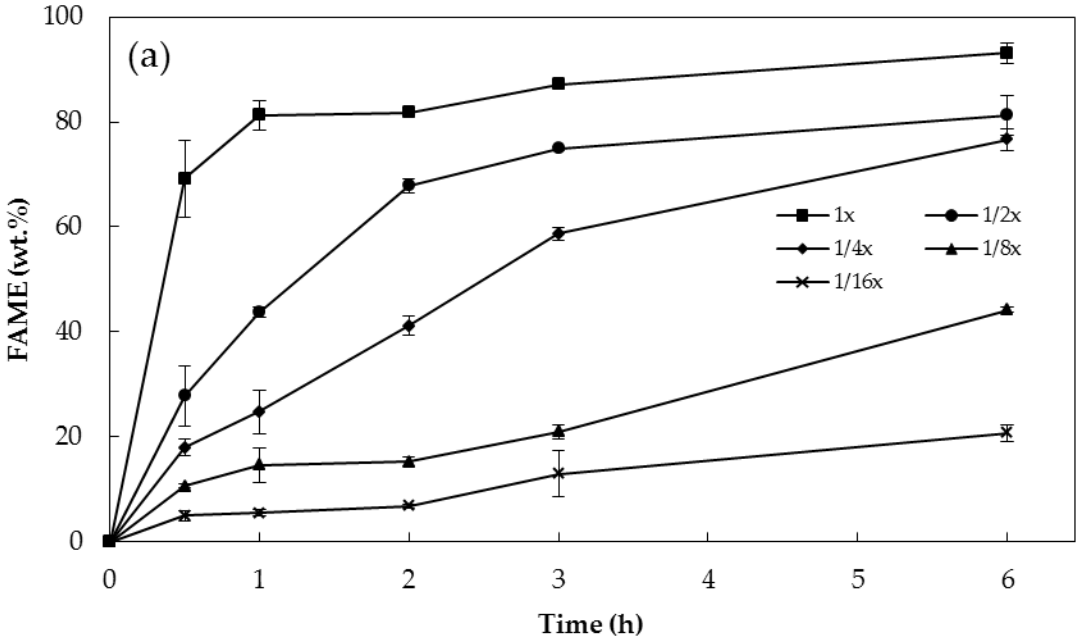

Figure 2.6 Effect of varied enzyme loading on esterification and transesterification of FFA and triglycerides to FAME with both immobilized lipase PS and immobilized CalB on a 50% FFA substrate : FAME production with time. All reactions at 4 g oleic acid, 4 g soybean oil, 1.54 g methanol, 0.36 g water, and 50 °C.

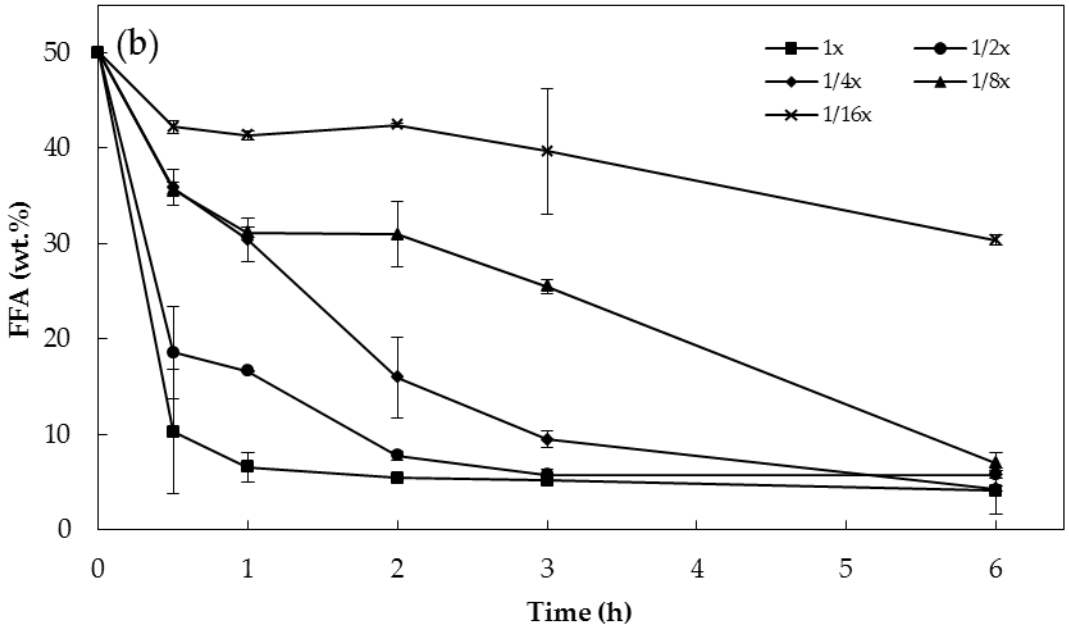

Figure 2.7 Effect of varied enzyme loading on esterification and transesterification of FFA and triglycerides to FAME with both immobilized lipase PS and immobilized CalB on a 50% FFA substrate. All reactions at 4 g oleic acid, 4 g soybean oil, 1.54 g methanol, 0.36 g water, and 50 °C.

2.3.5 Recovery and reuse of lipases

In order to make enzyme catalyzed esterification and transesterification of oil to biodiesel an economically viable process, the enzymes need to be recovered and reused. Significant obstacles are often encountered when trying to recover and reuse immobilized lipases, such as enzyme deactivation and/or leaching of the enzyme from the immobilization matrix (Ranganathan et at., 2008; Tan et al., 2010). To test the stability of the pair of sol-gel entrapped enzymes, reactions were conducted using the same conditions as in section 2.3.4, with 50% FFA and yellow grease as substrates. Reaction duration was 6 h and the recovery procedure was as described in section 2.2.3. The final wt.% of the FAME and FFA after each cycle are shown in Figure 2.8. As shown in this

figure, the result for the model 50% FFA and yellow grease followed similar trends. To gauge the loss in the activity of the immobilized lipases after each use, 100% activity was defined by the amount of FAME produced in cycle 1 for which the immobilized lipases were not subjected to the recovery and the washing steps. Based on this description, over 95% of the initial activity of the immobilized lipases was retained for both substrates after 10 cycles, while the level of unreacted triglycerides remained at 0.0 wt.%. The high level of activity of the immobilized lipases may be partially attributed to the water washing as it results in the removal of glycerol from the lipases and the sol-gel matrix sites which otherwise is believed to inhibit enzyme activity (Tan et al., 2010). Experiments were also performed to examine the potential synergy of a second solvent in the recovery process. Methanol, ethanol, and hexane were tested as co-solvents with water but the recovered immobilized lipases exhibited significant loss of activity which may have been caused by the use of the second solvent.

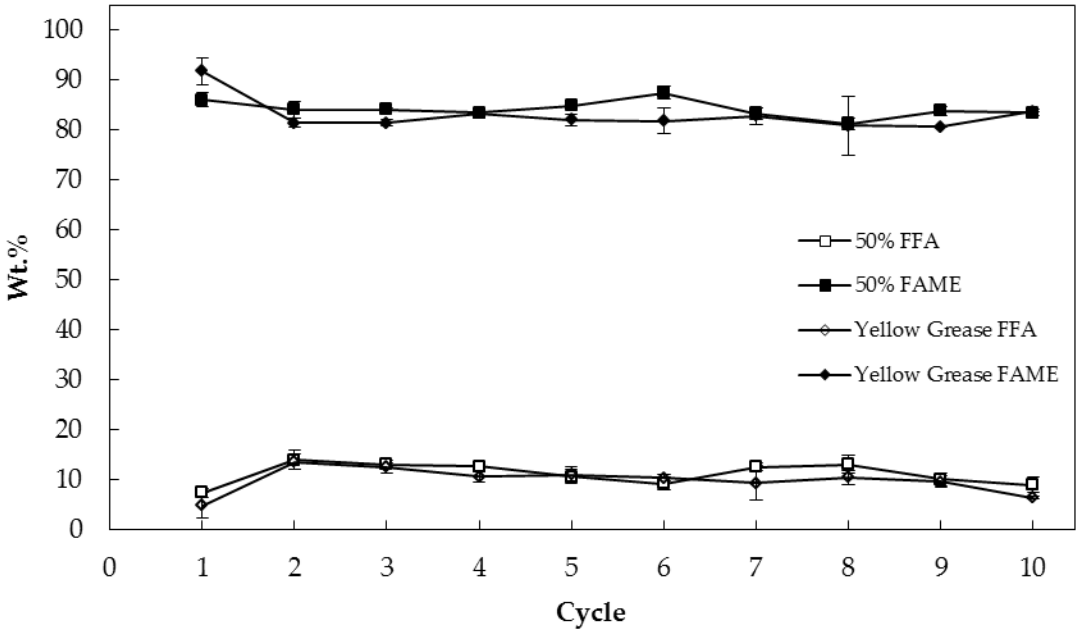

Figure 2.8 Reuse of both immobilized lipase PS and immobilized CalB in the esterification and transesterification of a 50% FFA substrate and yellow grease to FAME at 8 g combined soybean oil and oleic acid or yellow grease, 1.54 g immobilized lipase PS, 1.43 g immobilized CalB, 1.54 g methanol, 0.36 g water, 50 °C, and 6 h.

2.4 Conclusion

High FFA feedstocks such as yellow grease offer an attractive alternative to neat triglyceride resources for the production of biodiesel fuels. Many lower value triglyceride feedstocks contain high levels of FFA which makes their conversion to FAME problematic. In this study the immobilized enzyme transesterification and esterification of model high FFA substrates and yellow grease were investigated. Lipase PS and CalB were each immobilized in a sol-gel matrix and used individually and in combination to produce FAME. When used in combination, the pair of lipases yielded 93.0% FAME from a model 50% FFA oil and 90.6 wt.% FAME from yellow grease after 6 h. The pair of lipases showed only a small loss in activity after 10 reuses, with water washing and

centrifugation to recover the immobilized enzymes. These lipases, immobilized in sol-gel matrix, appear to have potential as industrial catalysts due to their stability, ease of recovery, and ability to handle high FFA feedstocks.

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3 Chapter 3

Ethanol as Acyl Acceptor for Sol-Gel Entrapped Lipase Catalyzed Biodiesel Production from High FFA Feedstocks

Abstract

The use of ethanol as the acyl acceptor for the transesterification and esterification of high free fatty acid (FFA) oils for biodiesel production was investigated. As catalyst, lipase B from *Candida antarctica* (CalB) and lipase from *Burkholderia cepacia* (lipase PS) were entrapped within a sol-gel matrix and used. Optimal conditions for the esterification of FFA to fatty acid ethyl ester (FAEE) by the entrapped CalB were determined, and found to be 50 °C, 1.35:1 molar ratio of ethanol to FFA, and 0.9 g of water per 8 g FFA. When used in combination at these optimized conditions, the lipases yielded 76.2 wt.% FAEE after 6h and 87.3 wt.% FAEE after 24 h from a model 50% FFA substrate. When used on yellow grease (16.0% FFA), 87.1 wt.% FAEE at 6 h and 89.7 wt.% FAEE after 24 h were produced. Further reactions were conducted, in which the pair of lipases were recovered and reused over 10 cycles and retained over 83% activity. Finally, experiments were conducted to show that ethanol caused a slower degradation of catalytic activity than methanol for both entrapped CalB and lipase PS.

3.1 Introduction

As the world's reserves of fossil fuels are consumed and greenhouse gases are emitted by their consumption at increasing rates, renewable and carbon neutral alternatives are being sought. One of the most prominent and extensively implemented is biodiesel, a drop in replacement for petroleum diesel fuel.(Ma and Hanna, 1999) Chemically, biodiesel consists of fatty acid alkyl esters (FAEE). Most commonly, it is produced by the base catalyzed transesterification of triglycerides with methanol. This reaction yields fatty acid methyl esters (FAME) and glycerol as products at high yields.(Meher et al., 2006) Triglyceride sources, however, must have low levels of free fatty acids (FFA) in order for the base catalyzed process to be feasible. Generally, low FFA oils are more expensive than high FFA oils, as they must undergo some processing and are often also used for food production. High FFA oils, or oils with FFA higher than 0.5% by weight, produce soap as a side product when reacted with a base catalyst, which lowers the yield from the transesterification reaction as well as making the downstream removal of glycerol problematic.(Canakci and Gerpen, 2001; Zhang and Jiang, 2008) To allow for biodiesel production from lower cost high FFA oils, alternative catalysts have been investigated. These catalysts include homogenous acid catalysts, lipases, and inorganic heterogeneous catalysts, although each these methods have significant drawbacks as well.(Lam et al., 2010)

While methanol is currently most commonly used as the acyl acceptor in the transesterification reaction to produce biodiesel, the use of ethanol is receiving increasing interest. Methanol is most widely made from synthesis gas, which is primarily made

from methane, a fossil fuel. Ethanol, on the other hand, is produced from fermentation of bio renewable resources, and is widely accepted as a "green" fuel. By utilizing ethanol as the acyl acceptor, biodiesel becomes a completely renewable fuel.(Al-zuhair, 2007) In addition to improved sustainability, FAEE have higher heating power and cetane number than FAME, better cold flow properties, and produce less CO and NO_x when burned.(Encinar et al., 2007; Makareviciene and Janulis, 2003; Mendow et al., 2011)

Lipase catalyzed production of biodiesel has several advantages over homogeneous and heterogeneous acid and base catalysts. They allow for more mild conditions and less complex processing of the product than physiochemical catalysts, as well as being tolerant of high FFA levels. Lipases are able to produce FAAE using either methanol or ethanol as the acyl acceptor. Lipases prefer ethanol, as has been reported by Kumari and coworkers.(Kumari et al., 2007) As the main issue with lipase catalyzed production of biodiesel is the cost of the lipases and their instability in the presence of short chain alcohols, immobilization of a variety of different lipases to allow for their recovery and reuse has been a very active area of research.(Chen and Wu, 2003; Narwal and Gupta, 2013; Villeneuve et al., 2000) Physical adsorption of lipases onto a solid carrier via hydrogen bonding, hydrophobic interactions, and weak interactions is the most widely studied method. Physical adsorption is favored because it is cheap and simple to perform, although lipase loss due to leaching off the support is a common concern.(Tan et al., 2010; Yadav and Jadhav, 2005) Watanabe and coworkers utilized a commercially available form of physically adsorbed *Candida antarctica* lipase B (CalB), Novozyme 435, and ethanol to yield 95% FAEE after 48 h of reaction.(Watanabe et al., 1999) Another study utilized lipase from *Burkholderia cepacia* (lipase PS) adsorbed onto

polypropylene powder and used to produce 90% FAEE from castor oil after 6 h.(Baron et al., 2014) Lipase PS was also adsorbed onto microcrystals and used to catalyze the ethanolysis of soybean oil to FAEE and showed a yield of almost 100% after 12 h, although the yield decreased to approximately 85% after 8 cycles and with a hexane wash to remove glycerol between each cycle.(Zheng et al., 2012) Lipase covalently bonded to a carrier molecule has also been studied for the ethanolysis reaction, although concerns exist about the nonspecific covalent linkage affecting the lipase's activity and the resulting size of the lipase bearing carrier.(Jegannathan et al., 2008) Lipase from *Thermomyces lanuginosus* was covalently linked to a carrier and used to catalyze the transesterification of soybean oil to FAEE with yields approaching 100% after 10 h, although its stability with repeated uses was not extensively studied.(Rodrigues et al., 2010) Mendes and coworkers used several different chemistries to covalently attach lipase from *T. lanuginosus* and *Pseudomonas fluorescens* to Toyopearl AF-amino-650M resin, and reported transesterification yields from palm oil approaching 100%.(Mendes et al., 2011) Entrapment of lipases within a polymeric matrix has also been studied, in which the lipase is not bonded to the immobilization carrier but is entrapped by polymerizing the matrix around it. Noureddini and coworkers used lipase PS entrapped within a sol-gel matrix via a polycondensation reaction to produce 63% FAEE from soybean oil after 1 h, with 53% conversion to FAEE after 10 cycles of reuse.(Noureddini et al., 2005)

In this study, CalB was entrapped within the same matrix as in Chapter 2. Conditions for the optimal esterification of FFA to FAEE by entrapped CalB were determined via a series of reactions. Lipase PS was separately entrapped by the same

procedure, and the two lipases were used individually and together to catalyze the production of FAEE from high FFA substrates. The stability of the catalytic esterification activity of the pair of lipases was tested over 10 cycles of recovery and reuse on both a 50% FFA model substrate and yellow grease, and then their resistance to deactivation by methanol and ethanol was examined.

3.2 Methods

3.2.1 Materials

Most materials were sourced from the same location as in section 2.2.1. Ethanol (100%) was purchased from Fischer Scientific (USA).

3.2.2 Immobilization methodology

The immobilization procedure followed the same procedure as described in section 2.2.2.

3.2.3 Reaction setup

Reactions and lipase recovery were conducted as in section 2.2.3, but with ethanol used in place of methanol. Additionally, all recovery procedures were heated to 50 °C before centrifugation.

3.2.4 Stability tests

Stability tests were performed in 50 ml Erlenmeyer flasks. To the flask, 1.1 g or 1.15 g of immobilized CalB or lipase PS, respectively, was added. Then, 15 ml of either ethanol or methanol was added, the mixture was heated to 50 °C, and then stirred for 6 h.

The mixture of alcohol and immobilized lipase was then centrifuged at 2,500 g for 3 min, and then the liquid phase was decanted off. The recovered immobilized lipase powder was dried for 12 h at 37 °C, ground with a mortar and pestle, and then used in an esterification or transesterification reaction as described in section 2.2.3.

3.2.5 Sampling and sample analysis

Sampling and GC analysis of sample composition was conducted as in section 2.2.4. Standards of FAEE were analyzed in addition to FFA, mono-, di-, and triglycerides.

3.3 Results and discussion

3.3.1 Optimization of reaction conditions

The work in chapter 2 and elsewhere in the literature has established that CalB is an effective lipase to perform the esterification of FFA to FAAE. It has also been established that entrapment within a sol-gel matrix of a lipase can lead to enhanced catalytic activity when compared to the free form of the same lipase under the same conditions.(Noureddini et al., 2005) With these two facts in mind, reactions were conducted to determine the optimal conditions for the esterification of FFA to FAEE by the entrapped CalB. As in section 2.2.2, a materials balance showed that 1 gram of immobilized CalB contained 42 ± 1.7 mg of free enzyme.

Initial conditions were selected based on the results from section 2.3.2, which utilized methanol in place of ethanol. Reactions were then conducted to determine at what temperature immobilized CalB most effectively catalyzed the esterification of FFA to FAEE. The optimal temperature was then used in subsequent reactions in which the

amount of water was varied, to determine the optimal water level. Finally, reactions were conducted at the optimal temperature and water levels to determine the optimal molar ratio of ethanol to FFA.

3.3.1.1 Temperature

Temperature is expected to be an influential factor on the activity of the immobilized CalB. Temperature has an effect on both the kinetics and the thermodynamics of all chemical reactions. Additionally, enzymes tend to have an optimal temperature, at which they are most active and above which they lose activity due to denaturation. For the determination of the optimal temperature for the esterification of FFA to FAEE by sol-gel entrapped CalB, a series of reactions were conducted in which the temperature was increased from 34 °C to 62 °C by 4 °C steps. All reactions had 8 g oleic acid, 0.36 g water, and 2.22 g ethanol, which corresponded to a 1.7:1 molar ratio of ethanol to FFA. Reactions were conducted for 3 h and mixed at 600 rpm, and then samples were analyzed via GC. In each reaction, 0.71 g of the immobilized lipase was used. The results are shown in Figure 3.1, and are reported as the resulting wt. % of FAAE. As can be seen, the yield of FAEE increased steadily from 34 °C to 50 °C until it reached 77%. At 50 °C, the amount of FAEE plateaued and stayed between 75 % and 80 % as the temperature was increased to 62 °C. Based on this, 50 °C was determined to be the optimal temperature for the esterification catalyzed by entrapped CalB.

In Figure 3.1, the temperature optimization using ethanol was compared to the temperature optimization done utilizing methanol. As can be seen, reactions utilizing

methanol as the acyl acceptor had more than a 20% higher yield at temperatures below 50 °C, and 10% at 50 °C and up. However, the methanol and ethanol reactions showed the same general trend in activity, with the optimal temperature occurring at 50 °C. This difference in activity, but same trend, is likely due primarily to methanol being a smaller molecule than ethanol, and thus reacting more quickly.

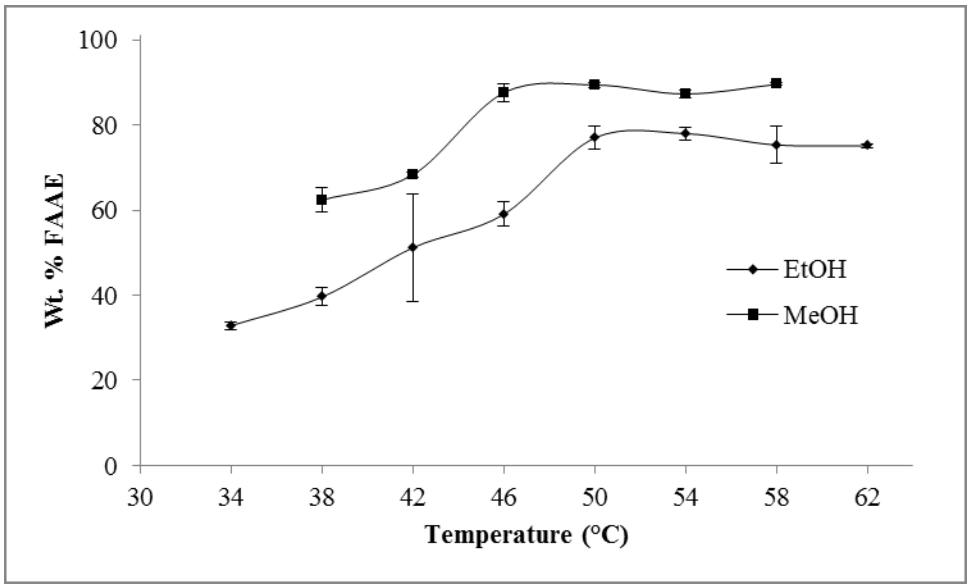

Figure 3.1 Effect of temperature on the esterification of FFA to FAEE with 0.71 g immobilized CalB, 8 g oleic acid, 2.22 g ethanol, 0.36 g water, and 3 h.

3.3.1.2 Water

As discussed in section 2.3.2.2, lipases utilize a catalytic triad at their active site to catalyze their reactions, and thus are highly dependent upon water for their activity.(Martinelle et al., 1995) Reactions were conducted to determine the optimal amount of water for the esterification catalyzed by CalB. Each reaction was conducted at the determined optimal temperature of 50 °C, and with 8 g oleic acid and 2.22 g ethanol. The amount of enzyme was kept at 0.71 g. Reactions were allowed to proceed for 3 h and were stirred at 600 rpm, and then sampled. The amount of water in each reaction

was varied, from 0 g to 3.6 g of water per 8 g oleic acid. The amounts of FAEE formed at each water level are shown in Figure 3.2. The yield of FAEE showed a gradual increase from 0 g water to 0.9 g water, after which it declined. These results show that the optimal amount of water in the reaction is 0.9 g per 8 g oleic acid, at which 83.8% FAEE was formed. The data also shows that while water is an important factor in enzyme activity, variation from the optimal water level did not result in large deviations from the optimal yield. The amount of FAEE formed stayed within a 10% range as long as at least 0.09 g of water per 8 g oleic acid is present. By comparing the results of the water optimization using methanol from section 2.3.3.3 to the results from the ethanol reactions, it can be seen that water has a similar impact on activity with either alcohol being used.

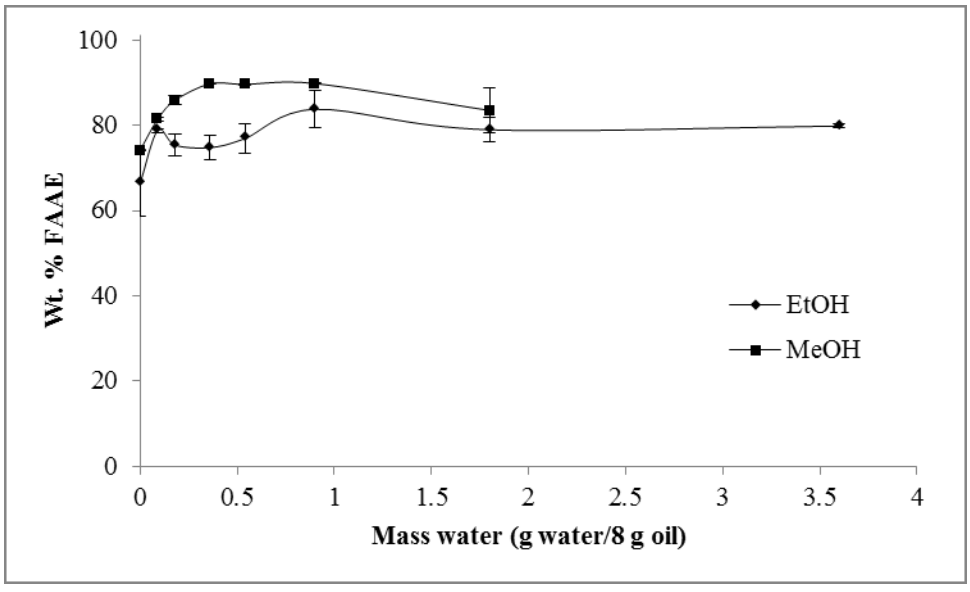

Figure 3.2 Effect of water on the esterification of FFA to FAEE with 0.71 g immobilized CalB, 8 g oleic acid, 2.22 g ethanol, 50 °C, and 3 h.

3.3.1.3 Ethanol

As the acyl acceptor in the esterification reaction catalyzed by CalB, the amount of ethanol will likely have a large effect on the resulting yield of FAEE. Increased levels of ethanol are expected to push the reaction equilibrium towards the products, resulting in higher yields. Reactions to determine the optimal ratio of ethanol to FFA were conducted by varying the molar ratio from 0.65 to 4.2. The temperature and water levels were held constant at 50 °C and 0.9 g, respectively, as these had been determined to be optimal. Again, 0.71 g of immobilized lipase was used. In Figure 3.3, the wt.% of FAEE present at the end of each reaction is plotted. As shown, the amount of FAEE produced increased as the molar ratio of ethanol to FFA was increased from 0.65 to 1.35. As the ratio was increased beyond 1.35, the amount of FAEE produced steadily decreased. A ratio of 1.35 was shown to be the optimal molar ratio. It is likely that the decrease in FAEE production at higher ethanol levels is due to slowing of the transport of FFA to the active site of the lipase, although it could also be caused by deactivation of the lipase.

Figure 3.3 shows both the optimization of the molar ratio of ethanol as well as the optimization of methanol from section 2.3.2.3. This shows that the alcohol level causes the same trend in activity, whether it is methanol or ethanol. The data in Figure 3.3 also supports the hypothesis that higher alcohol levels lead to decreased activity by slowing transport of FFA to the active site, and not due to lipase deactivation. While the methanol showed a higher optimal molar ratio of 1.7, the ethanol optimal ratio of 1.35 actually was a larger volume of alcohol. Ethanol levels of greater than 1.35 were likely

not favorable due to the slowed diffusion of FFA to the active site that their larger volume caused.

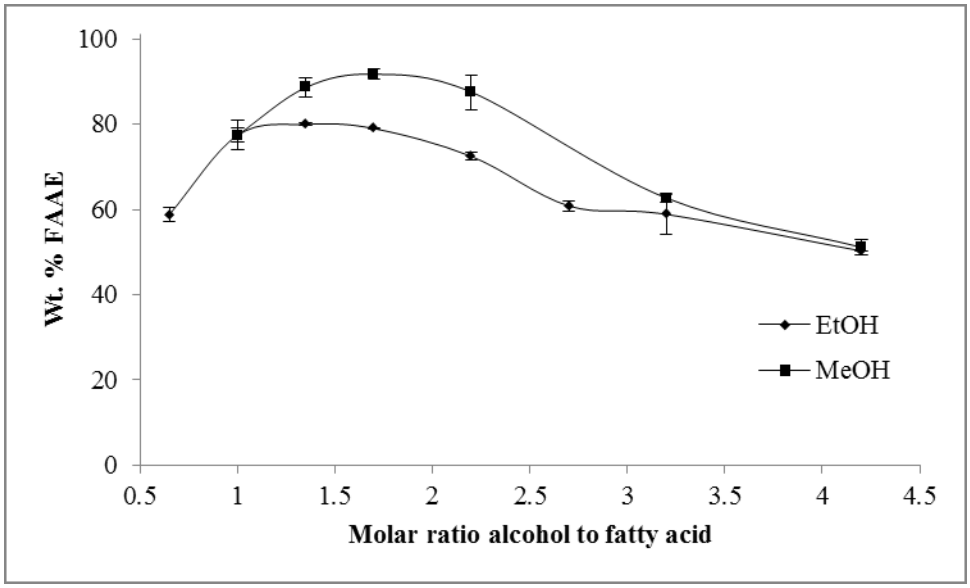

Figure 3.3 Effect of ethanol level on the esterification of FFA to FAEE with 0.71 g immobilized CalB, 8 g oleic acid, 0.9 g water, 50 °C, and 3 h.

3.3.2 Single enzyme reactions

Reactions were then conducted to determine the suitability of both the sol-gel entrapped CalB and sol-gel entrapped lipase PS as a catalyst for biodiesel production from high FFA substrates using ethanol. Each enzyme was used separately on a model substrate, which was 50% oleic acid and 50% refined soybean oil by weight. Since a previous study indicated that sol-gel entrapped lipase PS had its optimal activity at conditions similar to the conditions determined to be optimal for the esterification reaction catalyzed by CalB, the optimized CalB conditions were used.(Noureddini et al., 2005) Reactions catalyzed by CalB had 1.43 g of immobilized enzyme, which corresponded to 60 mg of free enzyme. Lipase PS reactions had 1.54 g of immobilized enzyme, which was equivalent to 200 mg of free enzyme. Each reaction had 4 g of oleic

acid, 4 g refined soybean oil, 0.9 g water, and 1.74 g ethanol, or a 1.35:1 molar ratio of ethanol to fatty acids. Reactions were conducted at 50 °C and stirred at 600 rpm, and samples were taken at 0.5, 1, 2, 3, 6, and 24 h.

The results for the reaction containing only immobilized CalB can be seen in Figure 3.4. As can be seen, almost all of the FFA present in the reaction was converted to FAEE within 0.5 h. After 1 h, FAEE reached 45.5 wt.% while FFA was decreased to 4.3%. These results show that the immobilized CalB is capable of quickly and effectively converting FFA to FAEE. The levels of FAEE, FFA, and triglycerides do not significantly change after 1 h, which suggests that the reaction reached equilibrium. The level of triglycerides remains at approximately 50% throughout the whole course of the reaction, which showed that the immobilized CalB is not capable of performing either the transesterification of triglycerides or the hydrolysis of triglycerides. This contrasts the majority of the literature on CalB, as other studies have reported CalB as capable of catalyzing the transesterification reaction(Ngo et al., 2012; Shimada et al., 1999) It is likely that the loss of transesterification activity for CalB is due to the method of immobilization, either due to the hydrophobic environment of the sol-gel matrix or due to the physical constrainment of the lipase by the matrix.

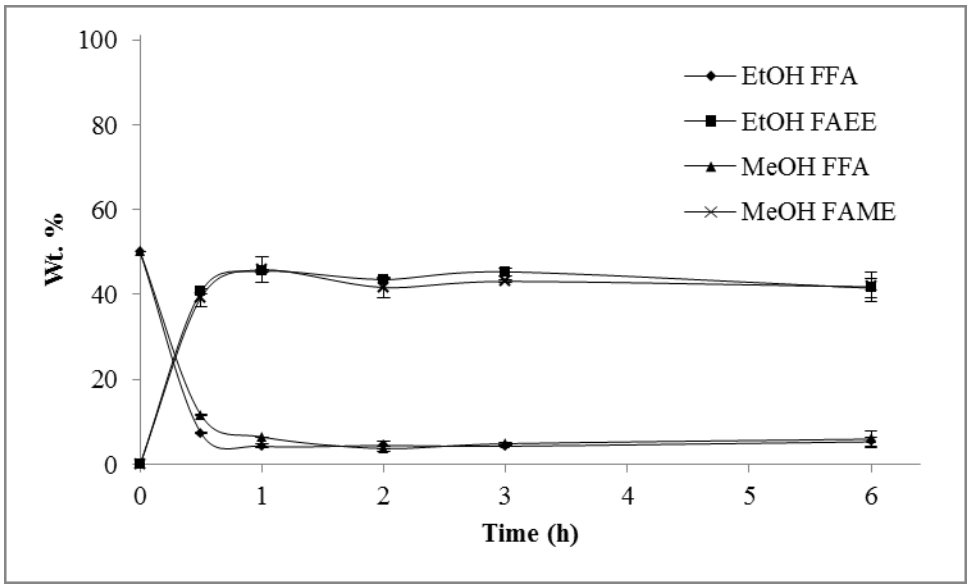

Figure 3.4 Esterification and transesterification of FFA and triglycerides to FAEE using immobilized CalB at the optimized conditions on a 50% FFA substrate at 4 g oleic acid, 4 g soybean oil, 1.54 g immobilized lipase PS or 1.43 g immobilized CalB, 1.74 g ethanol, 0.9 g water, and 50 °C.

In Figure 3.5, the results from the reaction containing only sol-gel entrapped lipase PS can be seen. Lipase PS showed a quick consumption of triglycerides, as they were reduced to 6.2% by weight after 2 h and were completely consumed after 24 h. The figure also shows a consistent removal of FFA from the reaction mixture, with their level reaching 8.0% after 6 h. The production of FAEE shown in the figure corresponds to the conversion of triglycerides to diglycerides and FAEE, diglycerides to monoglycerides and FAEE, monoglycerides to glycerol and FAEE, and FFA to FAEE and water. From this data, it is clear that lipase PS catalyzes both the transesterification of triglycerides and smaller glycerides as well as the esterification of FFA, although it appears to perform the transesterification more quickly under these conditions. This is confirmed by the level of FAEE reaching 79.3% after 6 h, which is more than can be accounted for by either the esterification or transesterification alone.

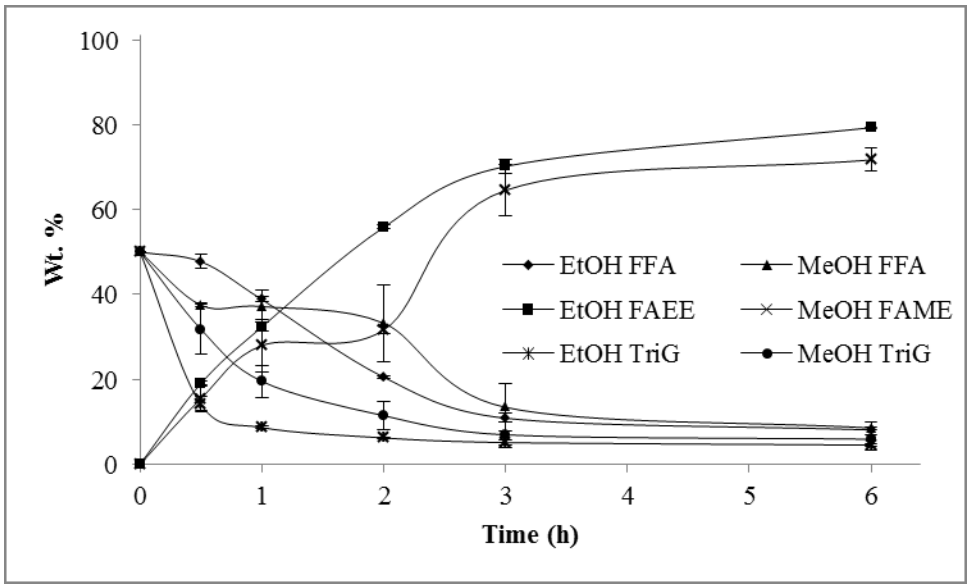

Figure 3.5 Esterification and transesterification of FFA and triglycerides to FAEE using immobilized lipase PS at the optimized conditions on a 50% FFA substrate at 4 g oleic acid, 4 g soybean oil, 1.54 g immobilized lipase PS or 1.43 g immobilized CalB, 1.74 g ethanol, 0.9 g water, and 50 °C.

In both Figure 3.4 and 3.5, the results from single enzyme ethanol reactions are compared to similar reactions that utilized methanol as the acyl acceptor. Figure 3.4 showed that CalB had the same effect on the 50% FFA substrate with either ethanol or methanol, at their respective optimized conditions. Figure 3.5 showed that lipase PS is more active when ethanol is used. Ethanol leads to triglycerides being consumed more quickly, as only 14.1% remained after 0.5 h while 31.8% remained after 0.5 h when methanol was used. Additionally, ethanol showed a higher yield of FAAE at every time point. It is unclear if this is due to the conditions used for the ethanol reactions being closer to the true optimal conditions for lipase PS than the methanol conditions, or if it is due to some other factor.

3.3.3 Combined enzyme reactions

As the immobilized CalB appeared to better catalyze the esterification of FFA to FAEE and immobilized PS appeared to more effectively catalyze the transesterification of glycerides to FAEE, both enzymes were used simultaneously to make FAEE. Reaction conditions were kept the same: 8 g total oil, 1.74 g ethanol or 1.35:1 molar ratio of ethanol to fatty acids, 0.9 g water, 50 °C, 600 rpm stirring, and sampling at 0.5, 1, 2, 3, 6, and 24 h. Additionally, in each reaction, 1.43 g of immobilized CalB and 1.54 g of immobilized PS were used. The reactions were performed on a total of four substrates: 0% FFA, 15% FFA, 50% FFA, and yellow grease (16.0% FFA) from a local supplier. This was to examine the efficacy of this pair of lipases as catalysts to convert substrates with a range of FFA to biodiesel, as well as their efficacy on a real world high FFA substrate. The results from the time course reactions are shown in Figure 3.6, where the wt.% of FAEE and FFA are displayed for each time point. As can be seen, the level of FFA reached an equilibrium point after approximately 1 h. For the initial FFA levels of 0 and 15%, this was around 7.5%. For the reaction with an initial FFA level of 50%, the equilibrium level of FFA was around 12%. The yellow grease sample had FFA levels that closely matched those of the 15% FFA model substrate. The FFA wt.% remained unchanged between 6 h and 24 h for all substrates. Each reaction showed consumption of triglycerides. After 6 h, all of the reactions had triglyceride wt.% of lower than 3%, and had all of their triglycerides consumed after 24 h. The 50% FFA substrate showed the highest production of FAEE after 0.5 h, with 55.8% formed. The 15% substrate was second fastest, with 47.7% formed, and the 0% FFA substrate was slowest, with 42.6%

FAEE formed. This is because the conversion of FFA to FAEE by CalB is a faster reaction than the transesterification of triglycerides to FAEE by lipase PS. When comparisons are made between the 50% FFA reaction in Figure 3.6, and the FAEE formation shown in Figures 3.4 and 3.5, it can be seen that the pair of lipases act in a synergistic fashion. For example, after 0.5 h only CalB yielded 40.7% FAEE, only PS yielded 19.1% FAEE, and the combined lipases yielded 55.8% FAEE. The same effect can be seen after 2 h, when only CalB produced 43.5%, only lipase PS produced 55.9%, and the pair of lipases yielded 76.2%.

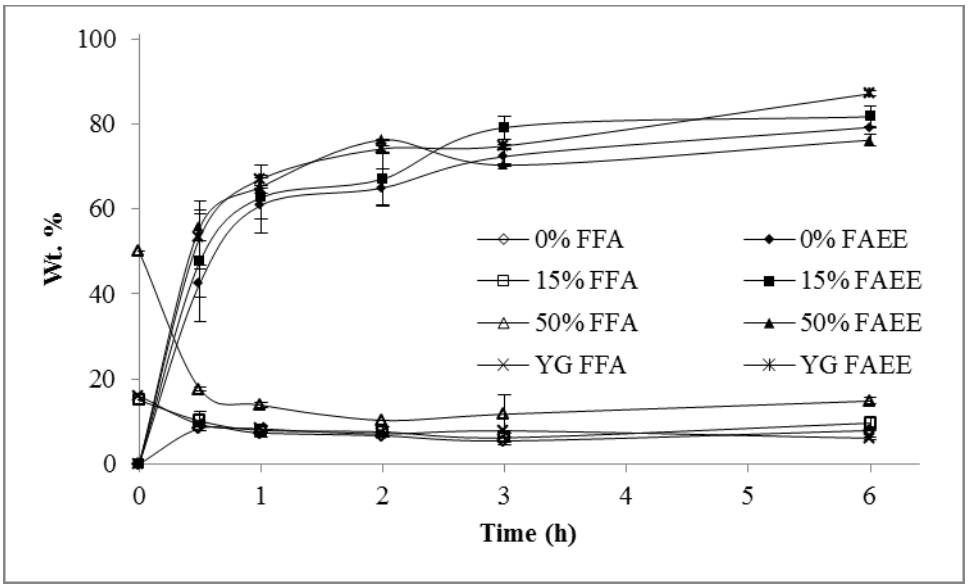

Figure 3.6 Esterification and transesterification of FFA and triglycerides to FAEE using immobilized lipase PS and immobilized CalB together in a single reaction on substrates with varied initial FFA wt.% and yellow grease at 8 g combined soybean oil and oleic acid or yellow grease, 1.54 g immobilized lipase PS, 1.43 g immobilized CalB, 1.74 g ethanol, 0.9 g water, and 50 °C.

Figure 3.7 shows the levels of FFA and FAAE from the combined enzyme reactions on the 50% FFA substrate with both ethanol and methanol as the acyl acceptor, and Figure 3.8 shows the same for reactions with yellow grease as the substrate. For both substrates, methanol yielded a higher level of FAAE than did ethanol. This can be

explained by a higher conversion of FFA to FAAE, as the methanol reactions had a lower FFA level at every time point taken. The difference is likely due to the lower initial molar amount of ethanol when compared to the molar amount of methanol. The lower initial amount of ethanol likely affected the equilibrium point,, and lead to lower yields of FAAE and consumption of FFA.

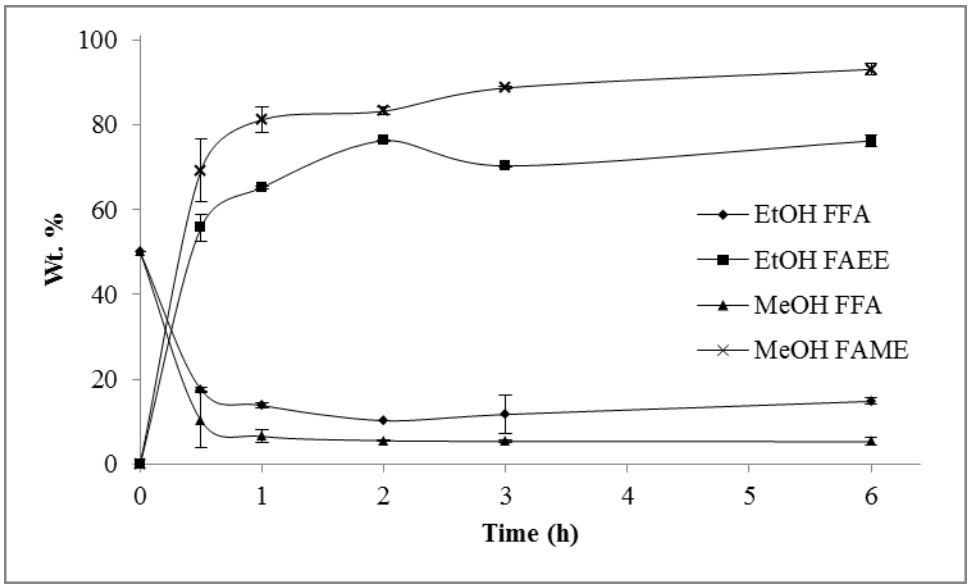

Figure 3.7 Comparison of methanol and ethanol as acyl acceptor with combined lipases and at respective optimized conditions on a 50% FFA model substrate.

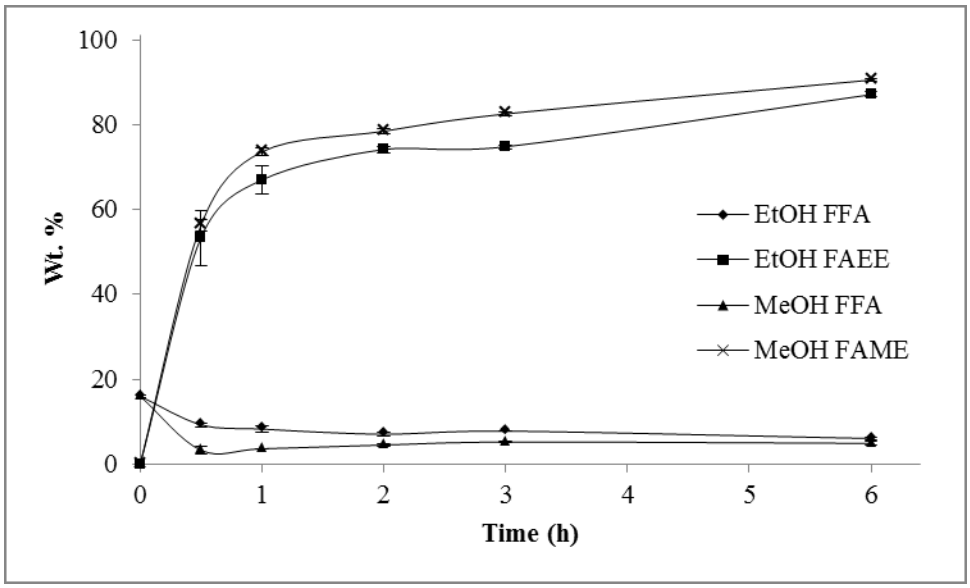

Figure 3.8 Comparison of methanol and ethanol as acyl acceptor with combined lipases and at respective optimized conditions on yellow grease.

The efficacy of the combined lipases was examined further by testing varied amounts of enzyme loading. Reactions were conducted on a 50% FFA substrate, with 8 g total oil, 1.74 g ethanol, 0.9 g water, 50 °C, 600 rpm stirring, and sampling at 0.5, 1, 2, 3, 6, and 24 h. The amount of enzyme was decreased by a factor of two over a series of reactions: that is, 1x had 1.43 g of immobilized CalB and 1.54 g of immobilized PS, 0.5x had 0.71 g CalB and 0.76 g PS, 0.25x had 0.36 g CalB and 0.37 g PS, and so on. The results, presented as wt.% FAEE formed, are shown in Figure 3.9. As can be seen, the amount of enzyme used had an effect on the yield of FAEE. As the amount of enzyme was decreased, the amount of FAEE formed at a given time also decreased. The consumption of triglycerides and of FFA followed the same trend as the production of FAEE, with a decrease in the amount of enzyme leading to a decrease in the consumption of triglycerides and FFA. This data shows that to maximize conversion to FAEE, high enzyme loading should be used.

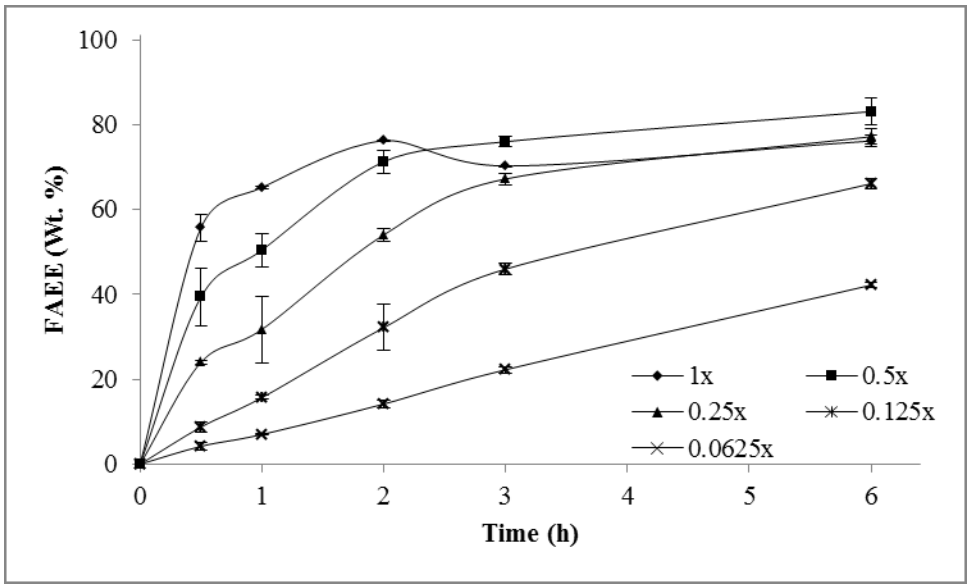

Figure 3.9 Effect of varied enzyme loading on esterification and transesterification of FFA and triglycerides to FAEE with both immobilized lipase PS and immobilized CalB on a 50% FFA substrate, with FAEE plotted against time, at 4 g oleic acid, 4 g soybean oil, 1.74 g ethanol, 0.9 g water, and 50 °C.

3.3.4 Enzyme recovery and reuse

In order for the pair of immobilized lipases to be effective catalysts for biodiesel production, they must be stable and active over repeated uses and be able to be recovered easily. Activity of lipases can be lost by several different processes, two of which are leaching of the lipase from the immobilization matrix and denaturation of the lipase leading to inactivity.(Ranganathan et al., 2008; Tan et al., 2010) To test the stability of the immobilized CalB and lipase PS, a series of reactions were conducted with the lipases recovered and reused in subsequent reactions. Both 50% FFA model substrate and yellow grease (16.0% FFA) were used as substrates, to ensure that nothing in the real substrate caused a loss in activity. Each reaction had 8 g total oil, 0.9 g water, 1.74 g ethanol, 600 rpm stirring, and was conducted at 50 °C. Reactions were allowed to

proceed for 3 h and were then sampled, as 3 h appeared to be the time at which most conversion to FAEE had been achieved in Figure 4. For the first reaction in each sequence, 1.43 g of immobilized CalB and 1.54 g of immobilized PS were used. After each reaction, water was added to the lipase, oil, and ethanol mixture, and the mixture was shaken and kept at the reaction temperature of 50 °C. After shaking, the mixture was centrifuged at 3000 g, and the lipases were recovered by decanting off the resulting aqueous and organic phases. The lipases were then directly used in the subsequent reaction. This procedure was completed for 10 cycles.

The results of the recovery and reuse reactions are shown in Figure 3.10, with the amount of FAEE formed after 3 h for each cycle reported. As shown, activity is maintained through 10 cycles with both 50% FFA substrate and yellow grease as the oil used. Both the yellow grease and 50% FFA substrate reactions yield approximately 70% FAEE for every cycle after the first. The drop in activity between cycles 1 and 2, in both cases, is likely due to imperfect removal of residual glycerol from the exterior of the immobilized lipases by the water wash step. Glycerol binding to the exterior of the enzyme or immobilization matrix has been reported as a cause of activity loss in reused lipases for biodiesel production.(Tan et al., 2010) The stability of the yield from cycle 2 through 10 suggests that the change in activity between cycles 1 and 2 is due to the change in reaction conditions between cycle 1 and cycle 2, and is not due to lipase leaching or denaturation. The stability of approximately 70% FAEE being produced on subsequent reactions suggests that no denaturation or leaching occurs, as the data in Figure 3.9 shows that a change in the amount of active lipase will lead to a measureable decrease in FAEE production.

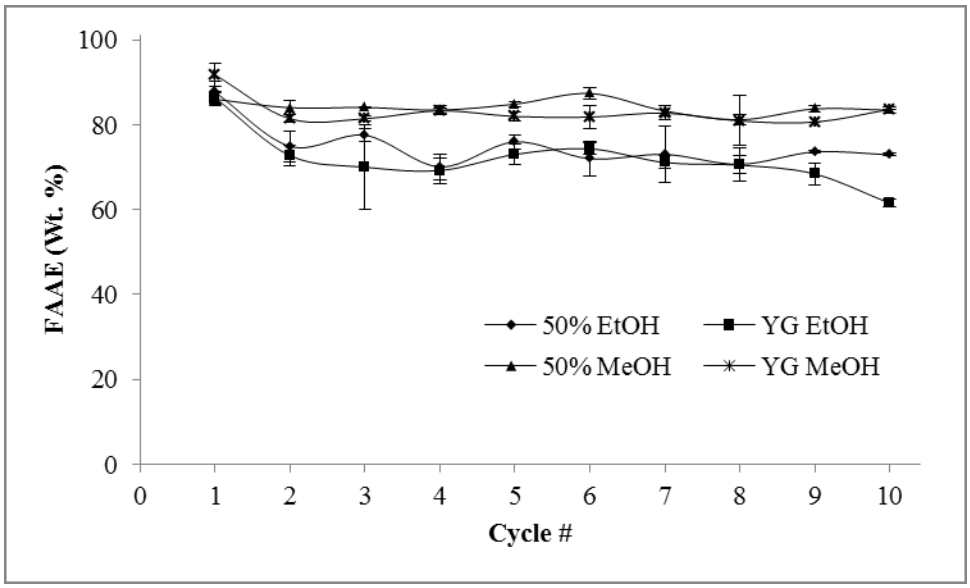

Figure 3.10 Reuse of both immobilized lipase PS and immobilized CalB in the esterification and transesterification of a 50% FFA substrate and yellow grease to FAEE at 8 g combined soybean oil and oleic acid or yellow grease, 1.54 g immobilized lipase PS, 1.43 g immobilized CalB, 1.74 g ethanol, 0.9 g water, 50 °C, and 6 h, and compared to the same reactions using methanol.

Figure 3.10 also shows data from similar recovery and reuse reactions which were performed in section 2.3.5. These reactions were 6 h reactions which used methanol rather than ethanol as the acyl acceptor. It was hypothesized that ethanol would be less deleterious to lipase activity over repeated uses, as lipase deactivation has been reported to be caused by contact with methanol droplets which are not dissolved in the oil phase. As ethanol is more hydrophobic, it was anticipated to be more soluble in the oil phase, leading to fewer undissolved droplets coming into contact with the immobilized lipase and deactivating it. Additionally, systems utilizing larger alcohols such as *tert*-butanol as part of the reaction medium for enzymatic biodiesel production have led to improved lipase stability.(Du et al., 2007) The data shown in Figure 3.10 does not support or contradict this, as neither the methanol nor the ethanol reactions show a significant downward trend in yield of FAAE that would indicate lipase deactivation.

3.3.5 Toxicity of alcohols on immobilized lipases

Further experimentation was done to investigate the difference in toxicity on immobilized CalB and lipase PS of ethanol and methanol. For these experiments, 1 g of immobilized lipase PS and immobilized CalB were separately incubated in 15 ml of pure methanol or ethanol at 600 rpm stirring at 50 °C. The length of time incubated was varied. The lipases were then recovered via centrifugation, dried, ground to powder form, and used in a reaction to assess their activity. For both CalB and lipase PS, 0.125x enzyme loading was used with 1.74 g ethanol, 0.9 g water, 50 °C, stirring at 600 rpm, and a sample taken after 3 h of reaction for analysis. For the CalB reactions, 8 g of oleic acid was used, while 8 g of soybean oil was used for the lipase PS reactions. Reactions were also conducted with untreated immobilized lipase PS and CalB, to provide a control.

The results from the conducted reactions are shown in Table 3.1. As shown, methanol and ethanol have varied effects on the stability of immobilized lipases. Immobilized CalB incubated in methanol for 6 h showed a loss of 91.1% of its esterification activity, while immobilized PS in methanol for 6 h showed a complete loss of transesterification activity. In contrast, 6 h of incubation in ethanol led to only a 39.5% loss in activity for CalB and a 49.7% loss in activity for lipase PS. This data shows that methanol is more toxic to the activity of both lipases than is ethanol. The improved stability of the lipases in ethanol as compared to methanol suggests that for long term lipase recovery and reuse, as would be encountered in any industrial use, ethanol as the acyl acceptor would be superior as it would lead to longer life of the lipases. Since the cost of lipases is a significant hurdle in the economical production of

biodiesel via enzymatic production, ethanol could prove to be a more suitable choice as the acyl acceptor.

Table 3.1 Treatments of each immobilized lipase to determine their stability in methanol and ethanol. Reactions to determine activity were conducted with 1/8x lipase loading (0.19 g lipase PS or 0.18 g CalB), 8 g soybean oil or oleic acid, 1.74 g ethanol, 0.9 g water, 50 °C, and 3 h.

Lipase	Treatment	FAEE wt.%		% Activity
		Avg.	St. Dev.	
CalB	None	38.4%	4.1%	**100.0%**
	6h MeOH	3.4%	0.2%	**8.9%**
	6h EtOH	23.2%	0.0%	**60.5%**
Lipase PS	None	47.6%	2.5%	**100.0%**
	6h MeOH	0.0%	0.0%	**0.0%**
	6h EtOH	24.0%	1.6%	**50.3%**

3.4 Conclusion

Biodiesel derived from fatty acids and ethanol has environmental and fuel characteristics more favorable than those of biodiesel from methanol, such as lowered emissions and better cold flow. The use of ethanol as the acyl acceptor has additional advantages over the use of methanol when lipases are the esterification and transesterification catalyst. In this study, the esterification and transesterification of both model and real high FFA substrates with ethanol by sol-gel entrapped CalB and lipase PS to FAEE was investigated. When used under optimized conditions and in combination, the pair of lipases produced 76.2% FAEE from a 50% FFA model substrate and 87.1% FAEE from yellow grease after 6 h. No significant loss in activity was noted over 10 cycles of reuse and recovery. Further experiments showed that 6 h in methanol resulted in almost complete deactivation of both immobilized lipases, while 6 h in ethanol caused

loss of less than half of their activities. Ethanol shows promise over methanol as the acyl acceptor for biodiesel production via sol-gel entrapped lipases over methanol, and the lipases show promise for future industrial applications due to their stability, ease of handling, and tolerance of high levels of FFA.

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4 Recommendations for Future Work

Investigation of the effects of varied immobilization monomers and alterations of their molar ratios could provide improved activity and stability, as well as offer insights as to how the sol-gel entrapment lead to improvements in activity when compared to the free lipases discussed in Chapter 2. By varying the hydrophobicity and pore size of the polymerized matrix, the activity of each lipase could be improved.

The behavior of the immobilization matrix could provide insights into how repeated uses affect its structure. Using isotopically labeled methanol, it could be seen if exchange between unreacted methoxy groups in the immobilization matrix and methanol in the reaction mixture occurs. The effect of the amount of time in the reaction mixture on the surface structure and on the pores in the matrix could also be investigated.

A series of experiments could be conducted to determine how influential transport effects are on the activity of the lipase. By conducting a series of reactions at low conversions and at a range of temperatures, the activation energy could be determined. By examining the activation energy, insights into the effect of transport to the reaction site could be determined.

Investigation of scale up of the use of the immobilized lipases is recommended, especially using yellow grease and other real world substrates. Modeling of a reactor system based upon the kinetic data in Chapters 2 and 3 will allow for an economic comparison between current methods of biodiesel production from high FFA substrates and the model utilizing the pair of sol-gel entrapped lipases.