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# Biocatalytic Production of Biodiesel from Vegetable Oils

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## 1. Introduction

Alcoholysis of vegetable oils is an important reaction to produce fatty acid alkyl esters which are excellent substitutes for diesel fuel and valuable intermediates in oleochemistry [1, 2]. Fatty acid methyl esters, a mixture of mono-alkyl esters are also known as biodiesel, obtained from both vegetable oils such as sunflower oil, canola oil, soyabean oil, jatropha oil, palm oil, rapeseed oil, peanut oil, cotton seed oil and animal fats such as beef tallow, and lard. Biodiesel can also be produced from other sources such as waste cooking oil, algae, and greases [3]. Biodiesel production has attracted considerable attention in the past two decades because of biodegradable, renewable, non-toxic, and environmentally friendly and socially responsible fuel [4]. Biodiesel can be produced by several methods: direct use or blending, microemulsion, thermal cracking (pyrolysis), and transesterification including acid-catalyzed processes, base-catalyzed processes, lipase-catalyzed processes, non-ionic base-catalyzed processes, and heterogeneously catalyzed processes [5, 6]. Among these methods, alkali catalyzed process including an alkali catalyst (usually NaOH, KOH, or sodium methoxide) has been accepted industrially due to its high conversion of triglycerides to methyl esters in a short reaction time and high reaction rates. In spite of these advantages of chemical transesterification process, it also possesses some disadvantages such as the need to eliminate the catalyst and salt from the biodiesel phase, to remove saponification products, the difficulty of recycling glycerol, and their energy-intensive nature, leading to development of alternative processes [7-9]. Alcoholysis is also carried out under acidic conditions, but this process requires higher reaction temperatures. In order to overcome these drawbacks, recently, enzymatic transesterification has attracted much attention for biodiesel production since it produces high purity product and provides an easy separation from the by-product glycerol. The use of enzymes (lipases) as catalysts in biodiesel production overcomes the problems inherent to alkali catalysts. It is reported that the enzymatic reactions are insensitive to free fatty acid (FFA) and water content

in the raw material [10]. So far, many attempts have been made to develop enzymatic process by using either extracellular or intracellular lipase as a biocatalyst [1, 11]. Lipases (EC 3.1.1.3), also defined as triacylglycerol acylhydrolases, catalyze the hydrolysis of ester bonds in long chain triacylglycerols (TAGs) to produce free fatty acids (FFAs) and glycerol. In general, the active site of lipases is formed by serine, aspartic (or glutamic) acid and histidine amino acid groups. Interfacial activation, which is unique to the class of lipases for its use in transesterification of fats and oils, takes place in presence of a substrate and lipase active site structure. Lipases are used in a wide range of fields due to their ability in utilizing all mono, di, and triglycerides as well as the FFA, low product inhibition, high activity and yield in non-aqueous media, low reaction time, temperature and alcohol resistance, but the high cost of enzyme remains a barrier for its industrial applications [10]. In order to decrease the cost of the process, the enzyme can be immobilized on a suitable carrier and reused many times. So far, many techniques and different carriers have been employed for immobilization of lipases to produce biodiesel. They have been successfully immobilized on porous kaolinite particle, biomass support particles, macroporous resin, gel-entrapped, celite, silica, and Eupergit C250L [12-16]. Several oils have been catalyzed with lipase enzymes until now. Lipase catalyzed production of biodiesel from soybean oil, sunflower oil, palm oil, kernel oil, coconut oil, rice bran oil, mixture of vegetable oils, grease and tallow oil, microbial oil, and waste oil containing vegetable oils have been reported in the past decades [17-25]. In this chapter, focus will be given toward enzymatic biodiesel production from various vegetable oils. *Thermomyces lanuginosus* lipase and *Candida antarctica* lipase A were immobilized on cotton cloth which is a low cost carrier. Transesterification of sunflower, canola, and waste cooking oil with methanol and ethanol was carried out by continuous operation system. The essential aim of this study was to investigate the production of biodiesel from vegetable oils by enzymatic transesterification with immobilized lipases on fibrous matrix by polyethyleneimine in a packed bed bioreactor at industrial scale.

## 2. Enzymatic transesterification

In transesterification reaction, one ester is converted into another ester. This conversion occurs as transfer of an acyl group. The acyl group transfer can take place between one ester and another ester (interesterification), an ester and an acid (acidolysis), or an ester and an alcohol (alcoholysis). In broad terms, the transesterification reaction between TAGs and alcohol to produce biodiesel is a sequence of three consecutive and reversible reactions, by which diacylglycerol (DAG) and monoacylglycerol (MAG) are formed as intermediates. Enzymatic synthesis of biodiesel has been usually performed at moderate temperature between 20 and 60 °C. When transesterification process is completed, the by-product glycerol (lower phase) is simply separated from the biofuel (upper phase) and neither neutralization nor deodorization of the product is necessary. However, an overdose of alcohol provides higher yield of biodiesel [26]. Biocatalysis has been considered a trend for sustainable synthesis technology due to biologic origin of the catalyst, selectivity and the possibility of reusing agro-industrial residues for biocatalyst production, which classifies the method as a green process [27]. Enzymatic catalysis has been applied for biodiesel which starts its industrial scale operation in China [28].

However, some factors such as substrate type, solvent type, alcohol type, water content of reaction medium, the reaction temperature, immobilization type and the lipase concentration influence the conversion of enzymatic transesterification reaction. In the literature, different lipases have been used upto now for biodiesel synthesis but it is hard to make any generalizations about the optimal reaction conditions. This is because, lipases obtained from different sources tend to respond differently to changes in the reaction medium [29-39]. Costs of chemical biodiesel production have still been lower than those of the enzymatic processes, however, if the pollution of natural environment is also taken into consideration, these costs are comparable. In the enzyme-catalyzed biodiesel production, the high enzyme cost significantly impacts the process profitability. The cost of commercial products for industrial use of enzymes is approximately 1, 000 \$/kg which is significantly higher than that of the alkali catalyst (0.62 \$/kg). Biodiesel fuel is expensive in comparison with petroleum-based fuel as 60-80% of the cost is associated with the feedstock oil [40]. Production of cheaper, robust lipase preparations and development of systems providing the long-term, iterative use of these biocatalysts can give rise to the replacement of chemical processes with enzymatic ones [28]. Currently, the high cost of biodiesel is the biggest obstacle to commercialization. The main reason is highly purified straight vegetable oil (SVO) used as a feedstock and this problem can be overcome by using used/waste vegetable oils that is much cheaper than SVO. Another obstacle in biodiesel production is the high food prices for oil. Both problems can be solved by using waste/used oil thereby gaining cost advantage. In addition, evaluation of the waste oil in terms of biodiesel can help to solve the problem of waste oil disposal. However, high free fatty acid (FFA) content of feedstock is the main problem encountered when using alkali catalyst. On the other hand, enzymatic transesterification does not have this limitation and hence can be used with waste/used oil. Moreover, almost all FFAs present in the waste/used oil can be converted to biodiesel in high yield using this approach [41].

## 2.1. Alcohol

Various types of acyl acceptors, alcohols, primary short-chain alcohols like methanol, ethanol, propanol, and butanol, as well as secondary alcohols like isopropanol and 2-butanol, straight and branched-chain, esters can be employed in transesterification using lipases as catalysts [42]. The prerequisites for selecting the alcohol for industrial-scale biodiesel production are that it must be cheap and in plentiful supply. Due to their price and availability, methanol and ethanol have been the most used alcohols for industrial biodiesel production. Currently, only methanol and ethanol, meet these two requirements. Ethanol is renewable and less toxic than methanol but methanol is preferred in biodiesel production because it is less expensive and more readily available in most countries than ethanol [30, 42]. However, these two alcohols are the stronger denaturing agents than longer aliphatic alcohols and inactivate enzymes. Besides, the rate of lipase-catalyzed transesterification reaction usually increases with the length of hydrocarbon chain of alcohol [30]. Meanwhile, the short alcohol chain causes lipase deactivation. It is believed that this is because the essential water layer around them which is essential for the optimum conformation of the enzyme is stripped off [43]. Most of the refined plant oils can be converted into fatty acid methyl esters to meet the specifications of biodiesel standard by stepwise alcohol addition to prevent an irreversible lipase inactivation [44]. Shimada et al. (1999) reported that the lipase from *Candida antarctica* (Novozym 435) in a

solvent-free system was deactivated irreversibly when the methanol concentration exceeded its solubility level. They found that stepwise addition of methanol prevented lipase deactivation. A three-step addition process converted 98.4% of the oil to its corresponding methyl esters in 48 h and the immobilized lipase was set to be reused for 50 batches [1]. Watanabe et al. (2000) investigated the influence of methanol using a two-step strategy on biodiesel synthesis. It has been shown that one-third of the alcohol adding at the beginning of the reaction caused slow conversion into biodiesel (10 h reaction time). After that, the rest of the alcohol was added in a single step and biodiesel conversion increased by the presence of the biodiesel since its solubility increased [21]. In another study [45] two lipases from *Pseudomonas fluorescens* and *Pseudomonas cepacia* (now *Burkholderia cepacia*) were used and they provided 58% and 37% conversion in the presence of 1:8 oil/methanol molar ratio in a solvent-free system, respectively. However, they have been shown to be completely inactive for another six lipases tested under these conditions. It is clear that the excess alcohol above and beyond the stoichiometric ratio increases the reaction rate, but too much alcohol may also deactivate the enzyme [30]. There are also some arguments against using excess alcohol in industrial-scale processes, such as higher energy consumption, larger equipment requirements, and the need to treat the unreacted alcohol. To prevent the alcohol deactivating the enzyme, many researchers have used organic solvents in the reaction medium to increase the solubility of the alcohol and reduce its concentration [12, 16, 18, 46].

## 2.2. Water content

The effect of water content is essential for enzymatic reactions due to formation of hydrogen bonds which are fundamental in the interactions for maintaining the conformation of the enzymes. Water has strong influence on the catalytic activity and stability of the lipase. Therefore, the transesterification yields depend on the size of interfacial area which can be increased by the addition of certain amounts of water as well as the availability of an oil-water interface. However, lipases increase the hydrolysis reaction in aqueous medium and excess water causes the decrease of the transesterification yield by promoting the hydrolysis reaction [42]. The ideal water content in the reaction medium varies greatly depending on the enzyme and the reaction medium, and so must be studied on a case-by-case basis. Water content in reaction mixture can be determined by either water activity or as weight percentage of feedstock oil. Water activity is the ratio of vapor pressure of a given system [38]. Optimum water content for the transesterification reaction is very important. The optimum water content in the reaction depends upon the lipase type and feedstock, immobilization technique and solvent type [47]. For example, Kaieda et al. 2001 found that the water concentrations that resulted in the best conversions were 8-20% for *Candida rugosa* lipase, 4-20% for *Pseudomonas fluorescens* lipase, and 1-2% for *Pseudomonas cepacia* lipase [35]. Deng et al. (2005) studied several immobilized commercially available lipases and reported that the conversion obtained from the transesterification reaction with all the other lipases (*Thermomyces lanuginosus*, *Rhizomucor miehei*, *Pseudomonas cepacia*, and *Pseudomonas fluorescens*) with the exception of *Candida antarctica* was higher when anhydrous ethanol was replaced with hydrous ethanol (4% water) [48]. It is also very important to take into consideration the amount of the water present in the reagents and even in the enzyme in order to design appropriate reaction medium. Studies of lipase reutilization at different water concentrations have to be carried out since water can

influence enzyme stability, making it crucially important for designing an economically feasible process [48]. Some authors have suggested that adding water into the enzymatic reaction medium can protect lipases against deactivation in the presence of short-chain alcohols [13, 19].

### 2.3. Organic solvent use

The use of organic solvents in enzymatic biodiesel synthesis improves mutual solubility of hydrophobic compounds (e.g. TAG and biodiesel), triglycerides and hydrophilic compounds (e.g. alcohols and glycerol). Organic solvents also protect enzymes for denaturation resulted high concentrations of alcohols [42]. Solvents also serve to reduce the viscosity of the reaction medium, enabling a higher diffusion rate to be achieved and reducing mass transfer problems. Therefore, a suitable solvent must be found, which both enhances the catalytic activity of the enzyme and keeps it stable. Thus, the presence of a solvent renders a high yield and reduces the enzyme inhibition by alcohol [47]. The most suitable non-polar hydrophobic organic solvents such as n-heptane, petroleum ether, isooctane, n-hexane and cyclohexane were used for enzymatic biodiesel synthesis and immobilized lipases showed high degree of efficiency in the presence of non-polar solvents. But when using hydrophobic solvents, glycerol is insoluble and remains in the reactor and it is adsorbed to the immobilized lipase. The polar hydrophilic organic solvents are much less useful in enzyme-catalyzed biodiesel production as they strongly interact with the essential water microlayer around the enzyme molecules influencing its native structure, thereby, leading to denaturation [42]. Recently, processes of transesterification, which is well known for its compatibility with lipases, have been also conducted in less conventional solvents, e.g. in supercritical gases like butane (C<sub>4</sub>H<sub>10</sub>) and carbon dioxide (CO<sub>2</sub>). CO<sub>2</sub> is also regarded as a green solvent owing to its low toxicity, non-flammability, and its environmentally good-natured character [30].

### 2.4. Biocatalysis type

Recently, lipases have been studied for biodiesel production as whole-cell immobilized lipases. Each type of biocatalyst has its strengths and weaknesses when it comes to reducing the contribution of the biocatalyst in the final cost of the biodiesel. Recent studies have been focusing on improving catalysis performance and stability of the enzyme with the aim to reduce the lipase cost in the biodiesel conversion process. Different approaches have been developed for application mode of lipases. Solid state fermentation, whole-cell biocatalyst and immobilized lipase in different supports are the main studied modes. The application of solid state fermentation was created for reducing cost in lipase production and could be used as a catalyst in batch and continuous operation. The solid state fermentation of agricultural residues permits for cost-efficient production and low-price when compared to commercial enzymes. Since solid state fermentation avoids the extraction, purification, and immobilization steps in enzyme production with satisfactory catalytic results in transesterification reaction [27].

#### 2.4.1. Free biocatalysis

Microbial lipases have gained wide industrial importance and they now share about 5% of the world enzyme market after proteases and carbohydrases. Lipases of microbial origin are more

stable than plant and animal lipases and are available in bulk at lower cost compared to lipases of other origin. Yeasts lipases are easy to handle and grow compared to bacterial lipases. Among the yeast lipases, *Candida rugosa* has gained good commercial importance. The most commonly used biocatalyst for biodiesel production are the microbial lipases that are produced by a number of fungal, bacterial, and yeast species [40]. Free enzymes are far cheaper than immobilized lipases. They can be purchased in an aqueous solution composed of the enzyme solution plus nothing more than a stabilizer to prevent enzyme denaturation (e.g. glycerol or sorbitol) and a preservative to inhibit microbial growth (e.g. benzoate) [49].

#### 2.4.2. Immobilized biocatalysis

Immobilization of lipases was carried out using entrapment, physical adsorption, ion exchange, and crosslinking. Carriers for lipase immobilization include polyurethane foam, silica, sephabeads, cellulosic nanofibers. Based on the criteria for selecting the immobilization technique and carrier dependings on the source of lipase, the type of reaction system (aqueous, organic solvent or two-phase system), and the bioreactor type (batch, stirred tank, membrane reactor, column and plug-flow) can be designed. The literature is replete with various lipase producing microorganisms, enzyme immobilization methods, and physical carriers. The challenge will be to select a carrier and immobilization technique that will allow maximum lipase activity, retention, and stability on the oil substrate. Among the immobilization method, adsorption technique is the simplest and most widely used technique for lipase immobilization. Adsorption method consists of bonding the lipase to the immobilization support surface through weak forces such as van der Waals or hydrophobic interactions. However, the main disadvantage of this technique is enzyme desorption from the support due to low bond strength between the enzyme and the support [40].

#### 2.4.3. Whole-cell biocatalysis

In recent years, whole-cell immobilized lipases have been studied for biodiesel production. This method is cheaper as it does not require the enzyme purification and isolation steps from fermentation broth. The efficiency of the transesterification process could be increased by using microbial cells that produce intra-cellular lipase as whole-cell biocatalysts [40, 46]. Filamentous fungi have been identified as robust whole-cell biocatalysts for biodiesel production: among these *Rhizopus* and *Aspergillus* have been most widely used [42]. There are several recent works reporting the utilization of bacteria, yeast and fungi as whole-cell biocatalysts in biodiesel process [27].

## 3. Experimental

### 3.1. Material

Commercial powder lipase from *Thermomyces lanuginosus* (fungal lipase, lyophilized, specific activity of 1400 U/mg solid) and *Candida antarctica* lipase A (specific activity of 2500 U/mg solid) were purchased from Codexis Inc. (Pasadane, CA). Sunflower oil and canola oil were supplied by a local company; waste cooking oil (WCO) was used following filtration to remove particles





### 3.2.2. Biodiesel production with immobilized lipase-catalyzed transesterification

Production of biodiesel by enzymatic catalyzed transesterification from various vegetable oils was studied in a packed bed reactor (Figure 2). A small piece of immobilized cotton cloth (1 g) was placed in the glass column reactor (1 cm diameter x 12 cm height) with a water jacket maintained at a constant temperature (30 °C). Substrate mixture (oil and alcohol) was continuously recirculated throughout the immobilized enzyme reactor with a peristaltic pump at a flow rate of 50 mL/min by adding of alcohol in three-steps. Immobilized cotton cloths were washed by *tert*-butanol before adding of alcohol to each reaction medium. Reaction was continued for 10 h. Samples were taken from the flask at appropriate time intervals and analyzed for fatty acid methyl esters (FAMES) and glyceride contents by high performance liquid chromatography (HPLC) [50].

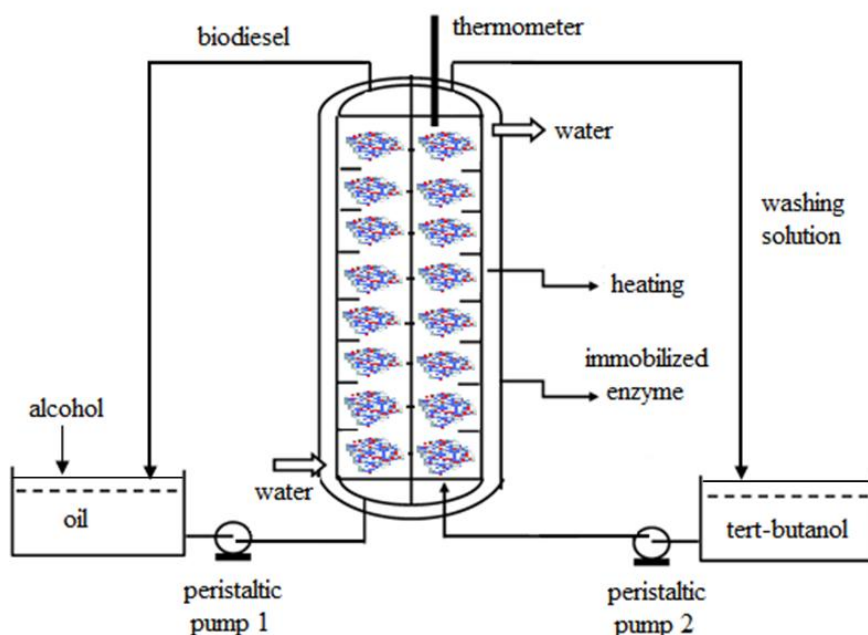


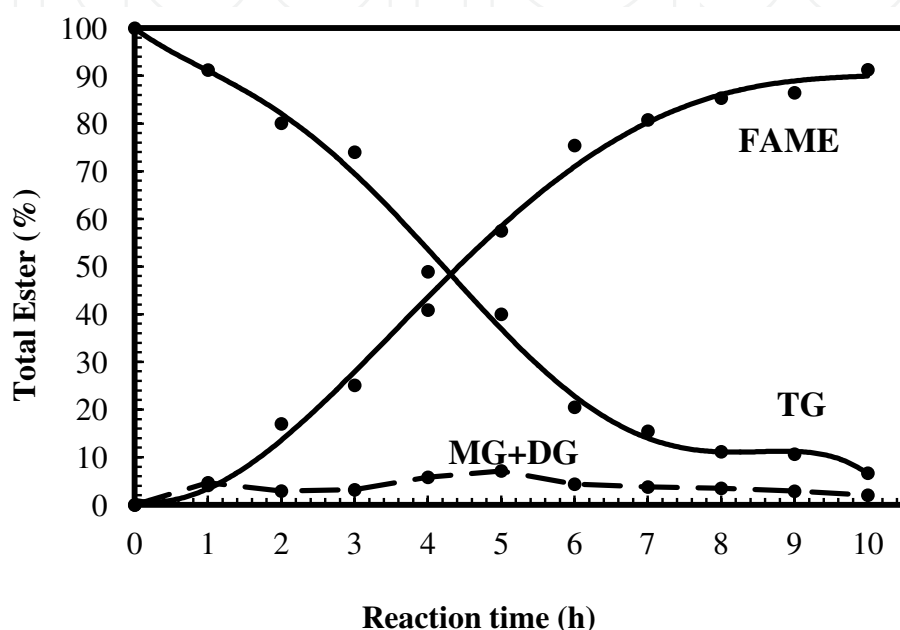
Figure 2. Schematic diagram of the enzymatic column reactor used in the transesterification reaction

## 4. Results and discussion

### 4.1. Lipase screening

Firstly, lipase screening was performed to find the lipase that has the best catalytic activity in the transesterification of sunflower oil. The most active lipase was then used in further transesterification studies. Two lipases, *Thermomyces lanuginosus* and *Candida antarctica* lipase A, were screened for their transesterification activity. The screening results for the tested lipases are presented in Figures 3 and 4. As can be seen from the figures, among the tested

lipases, *T. lanuginosus* lipase showed the highest activity in the transesterification reaction of sunflower oil with methanol at 30 °C. *C. antarctica* lipase A showed very little conversion in the transesterification reaction with both ethanol (44%) and methanol (28%). After 10 h of reaction with *T. lanuginosus* lipase, the product contained 91.3% of methyl esters, 2% monoglycerides and diglycerides, and 6.7% triglycerides (Figure 3). However, 28% methyl esters, 5% monoglycerides and diglycerides and 67% triglycerides were obtained with *C. antarctica* lipase A using methanol (Figure 4).

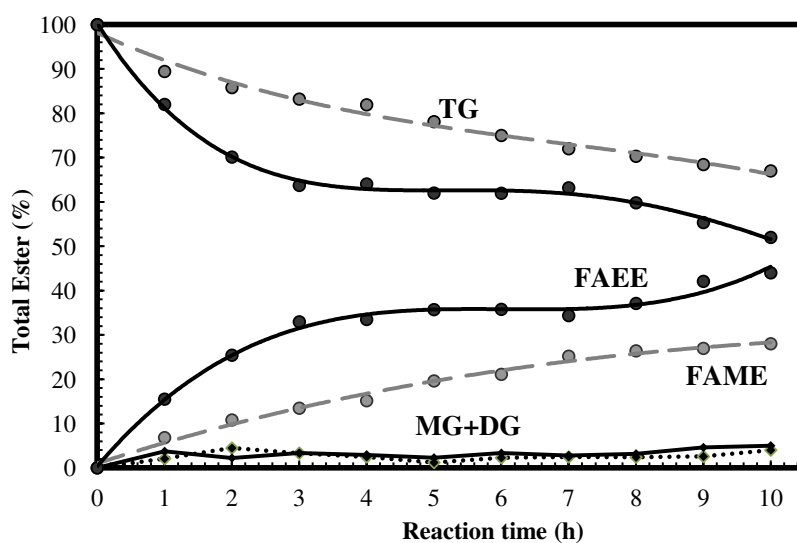


**Figure 3.** Immobilized *T. lanuginosus* lipase catalyzed transesterification of sunflower oil, 1:3 M ratio of methanol, 30 °C reaction temperature, 10 h reaction time (FAME: Fatty acid methyl ester; TG: triglyceride; DG: diglyceride; MG: monoglyceride)

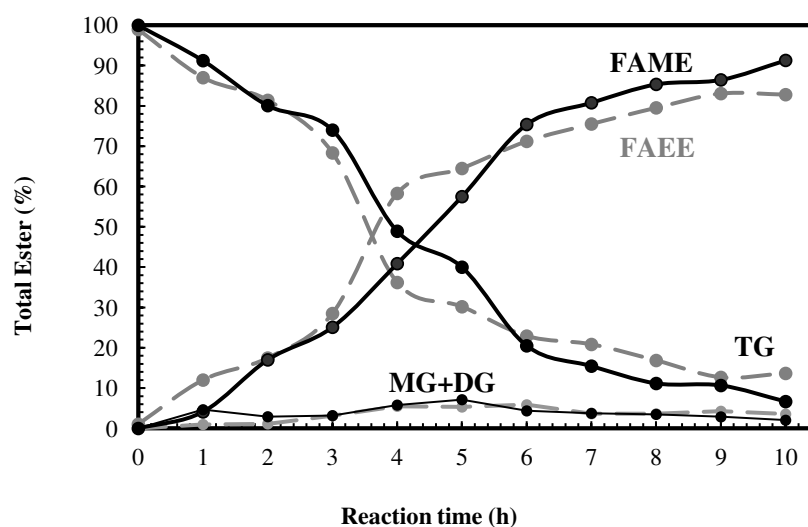
## 4.2. Effect of reaction parameters on transesterification

### 4.2.1. Effect of alcohol type

It is well known that excessive short-chain alcohols such as methanol might inactivate lipase seriously. However, at least three molar equivalents of methanol are required to complete conversion to its corresponding methyl esters of the oil [26, 30]. Experiments were performed to determine the yield of methyl or ethyl esters by varying the alcohol type using *T. lanuginosus* lipase. The reaction was carried out to avoid enzyme inactivation by adding methanol stepwise. The molar ratio of sunflower oil to alcohol was kept constant in the concentration of 1:3 for both methanol and ethanol. Results are summarized in Figure 5. As was expected, all alcohol types resulted in an increase in the yield of esters. However, the formation of esters reached a maximum level using methanol. The biodiesel conversions were about 91.3% of methyl esters and 82.8% of ethyl esters for methanol and ethanol, respectively.



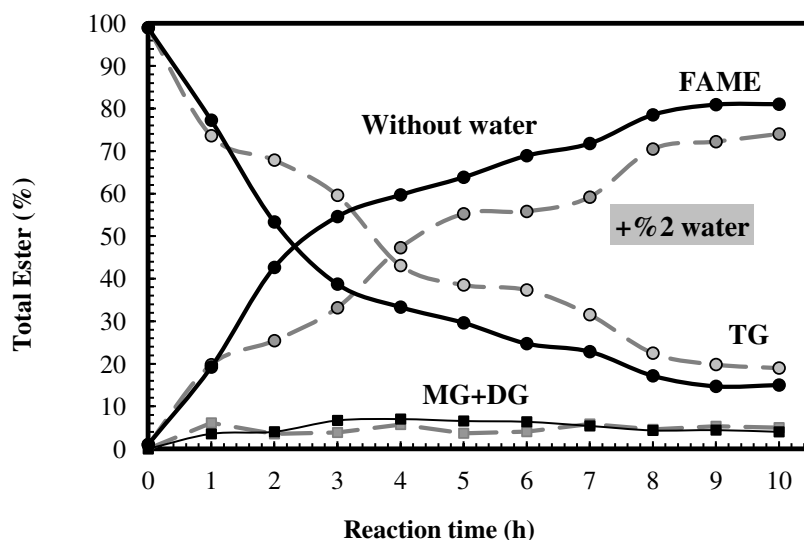
**Figure 4.** Immobilized *Candida antarctica* lipase A catalyzed transesterification of sunflower oil, 1:3 M ratio of methanol and ethanol, 30 °C reaction temperature, 10 h reaction time (FAME: Fatty acid methyl ester; FAEE: Fatty acid ethyl ester; TG: triglyceride; DG: diglyceride; MG: monoglyceride)



**Figure 5.** Effect of alcohol types on immobilized *T. lanuginosus* lipase catalyzed transesterification of sunflower oil, 1:3 M ratio of methanol and ethanol, 30 °C reaction temperature, 10 h reaction time (FAME: Fatty acid methyl ester; FAEE: Fatty acid ethyl ester; TG: triglyceride; DG: diglyceride; MG: monoglyceride)

#### 4.2.2. Effect of water concentration

The effect of water content was examined in the range of 0-2 g and at constant molar ratio of oil to methanol with sunflower oil. The reactions were carried out according to the reaction setup described earlier. The results presented in Figure 6 indicated that water was not required to activate the *T. lanuginosus* lipase. A maximum ester yield (81%) could be achieved at without water reaction conditions. Water concentration in reaction mixture is a characteristic and one



**Figure 6.** Effect of water content on immobilized *T. lanuginosus* lipase catalyzed transesterification of sunflower oil, 1:3 M ratio of methanol, 30 °C reaction temperature, 10 h reaction time (FAME: Fatty acid methyl ester; TG: triglyceride; DG: diglyceride; MG: monoglyceride)

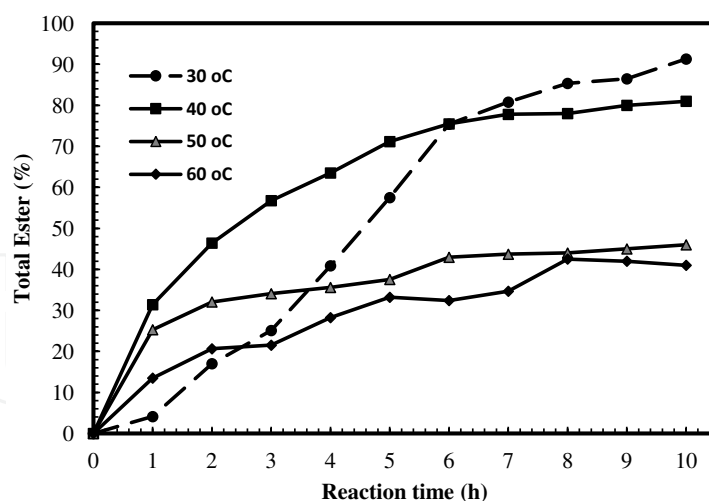
of the most important factors affecting lipase-catalyzed transesterification reaction rate and yield of biodiesel synthesis [12, 30, 32, 33]. Fukuda et al. [15] reported that the presence of excess water in the reaction mixture reduces the transesterification reaction rate.

#### 4.2.3. Effect of reaction temperature

Experiments were performed to determine the effect of temperature on catalytic activity of immobilized *T. lanuginosus* lipase in transesterification reaction. Temperatures in the range of 30-60 °C were examined with results shown in Figure 7. It was found that the enzyme lost its activity dramatically when temperature was increased above 40 °C. Optimal temperature observed for biodiesel production was 30 °C. Studies of biodiesel production performed with immobilized lipase under laboratory conditions have generally indicated use of temperatures between 30-40 °C [20, 26, 34-37]. On the other hand a number of studies have used temperatures between 40-50 °C [5, 9]. Biodiesel yields of enzymes have increased with increasing of temperature, but enzymes have denatured and decreased in efficiency in most temperatures [26].

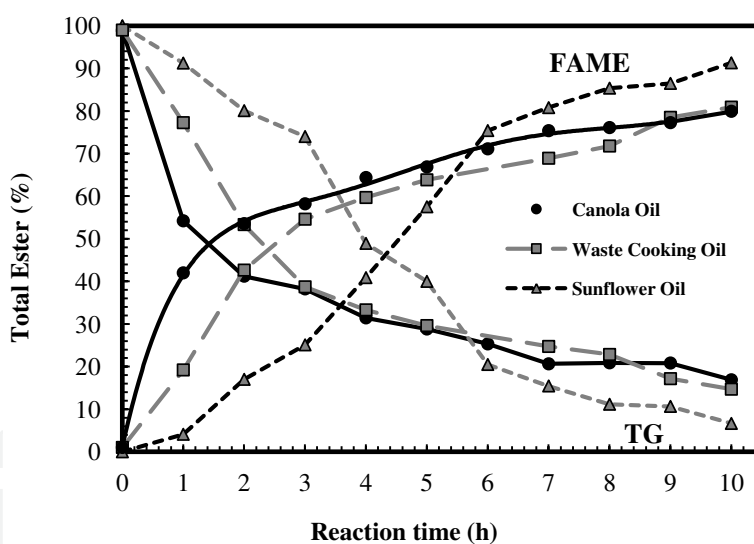
#### 4.2.4. Effect of oil type

The results depicted in Figure 8 shows that sunflower oil provided the highest methyl ester yield (91.3%) in reactions with methanol, among sunflower, canola, and waste cooking oil. However, the initial reaction rate was higher for canola oil and waste cooking oil than sunflower oil. Free fatty acids formed soaps with alkali salts when alkali-catalyzed process was used to produce biodiesel from waste cooking oils. Use of waste cooking oil in the production of biodiesel with immobilized lipase to cotton cloth has been effective enough in providing substantial methyl ester yield. Since hydrophilic feature of carrier used in immobi-



**Figure 7.** Effect of temperature on immobilized *T. lanuginosus* lipase catalyzed transesterification of sunflower oil, 1:3 M ratio of methanol, 10 h reaction time

lization process may adsorb the water on cotton cloth in the reaction medium. Fatty acid methyl ester yields from canola and waste cooking oil were 79.9% and 81%, respectively.

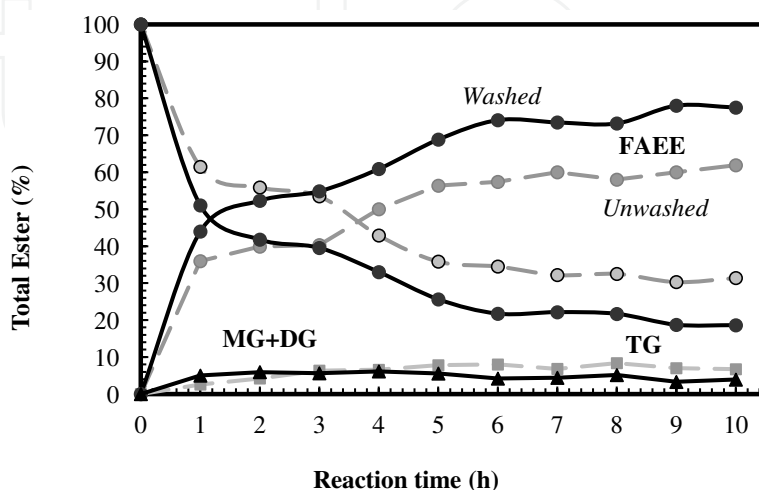


**Figure 8.** Effect of oil types on immobilized *T. lanuginosus* lipase catalyzed transesterification of sunflower oil, 1:3 M ratio of methanol, 30 °C reaction temperature, 10 h reaction time (FAME: Fatty acid methyl ester; TG: triglyceride)

#### 4.2.5. Effect of washing with *tert*-butanol

The effect of washing with *tert*-butanol of immobilized cotton cloths during transesterification reaction of sunflower oil and 1:3 molar ratio of oil to methanol is presented in Figure 9. It was shown that immobilized lipase decreased its activity from 91.3% to 77.5% when washed with *tert*-butanol during 10 repeated reactions at 30 °C, each lasting 10 h. However, immobilized lipase decreased its activity dramatically from 91.3% to 61.9% when unwashed with *tert*-

butanol. Activity of lipase increased by washing of immobilized cotton cloths with *tert*-butanol of before methanol addition, since the washing process removed hydrophilic glycerol that occurred during reaction, which couldn't be limited to the diffusion of substrate to lipase molecule. The methanol migrates from the reaction mixture to glycerol layer, and the lipase is inactivated by higher concentration of methanol in the glycerol layer [35].



**Figure 9.** Effect of washing with *tert*-butanol on immobilized *T. lanuginosus* lipase catalyzed transesterification of sunflower oil, 1:3 M ratio of methanol, 30 °C reaction temperature, 10 h reaction time, 10<sup>th</sup> repeated use (FAME: Fatty acid methyl ester; TG: triglyceride; DG: diglyceride; MG: monoglyceride)

## 5. Conclusions

The method of enzyme immobilization involving polyethyleneimine (PEI)-enzyme aggregate formation was developed and glutaraldehyde was used as a cross-linking agent between free amine groups increasing the enzyme stability. In the present study, a high biodiesel yield was obtained with *T. lanuginosus* lipase immobilized on cotton cloths. To avoid strong methanol inhibition, it was fed 3 steps into reaction medium and a high conversion (91.3%) was achieved. It was also found that glycerol has a significant inhibitory effect on the transesterification reaction. The immobilized enzyme was rinsed with *tert*-butanol to overcome this drawback. Additionally, immobilized lipase could be used at least 10 times (100 h) without any activity limitation. Since the immobilization method is very simple and cheap, it could also be used for the immobilization of other enzymes.

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