CHAPTER III

EXPERIMENTAL METHOD

3.1 Materials

3.1.1 Chicken breast from CP Company was chosen to be a representative of meat to prepare used palm oil.

3.1.2 Refined palm oil from Morakot Company, Thailand was used as raw material in all experiment of this study. This oil was the 100 % refined palm olein from pericarp with light yellow color.

3.2 Chemicals

Chemicals for TLC analysis

Chemical	Description
Acetic acid	BDH Chemical, Analytical reagent, UN 2789, England
Ethyl acetate	BDH Chemical, Analytical reagent, UN 1173, England
Plate of TLC	Merck, Approx 95-97%, OB568395, Germany
Sulfuric acid	RIEDEL-DE-HAEN, Approx 95-97%, Germany

Chemicals for hydrolytic activity of immobilized lipase

Chemical	Description
Ethanol	Merck, Reagent grade, K34300183507, Germany
4-Nitrophenyl palmitate	Sigma-Aldrich, standard grade, SN 1492-30-4, USA
di-Potassium hydrogen phosphate anhydrous	Scharlau, Reagent grade, Spain
Potassium dihydrogen phosphate	Merck, Reagent grade, A377073218, Germany
Sodium carbonate anhydrous	Scharlau, Reagent grade, S00116, Spain

Immobilized Lipase

Chemical	Description
Lipozyme RM IM	Novozymes, LUX 00205, Denmark
Novozym 435	Novozymes, LC 200206, Denmark

Methyl Ester Standard

Chemical	Description
Diolein	Supelco, Approx 99%, SN 111-03-5, USA
Glycerin	Supelco, Approx 99%, SN 56-81-5, USA
Methyl Arachidate	Sigma-Aldrich, Approx 99%, SN 1120-28-1, USA
Methyl Behanate	Sigma-Aldrich, Approx 99%, SN 929-77-1, USA
Methyl Dodecanoate	Sigma-Aldrich, Approx 99%, SN 111-82-0, USA

Chemical	Description
Methyl Heptadecanoate	Sigma-Aldrich, Approx 99%, SN 1731-92-6, USA
Methyl Linoleate	Sigma-Aldrich, Approx 99%, SN 112-63-0, USA
Methyl Linolenate	Fluka, Approx 99%, SN 301-00-8, Switzerland
Methyl Myristate	Sigma-Aldrich, Approx 99 %, SN 124-10-7, USA
Methyl Oleate	Sigma-Aldrich, Approx 99 %, SN 112-62-9, USA
Methyl Palmitate	Sigma-Aldrich, Approx 99 %, SN 112-39-0, USA
Methyl Palmitoleate	Sigma-Aldrich, Approx 99 %, SN 1120-25-8, USA
Methyl Stearate	Sigma-Aldrich, Approx 99 %, SN 112-61-8, USA
Monoolein	Supelco, Approx 99 %, SN 2465-32-9, USA
Triolein	Supelco, Approx 99 %, SN 122-32-79, USA

PAHs Standard

Chemical	Description
Benzo[a]pyrene	Fluka, Approx 99 %, SN 200028, Switzerland
Napthalene	BDH Chemical, Approx 99 %, SN 29277, England

Solvent for GC

Chemical	Description
Hexane	Labscan, Analytical reagent, SN 9309-03, Thailand

Solvent for HPLC

Chemical	Description
Acetonitrile	Labscan, HPLC grade, SN C 2502 U, Thailand
Isopropanol	Labscan, HPLC grade, SN C 2519, Thailand
Methanol	Labscan, HPLC grade, SN C 2517 U, Thailand

Others

Chemical	Description
Refined Palm oil	Morakot, Refined Palm Olein from Pericarp, Thailand
Sodium hydroxide	Scharlau, Analytical reagent, SN S00425, Spain

3.3 Equipment

Equipment for FAME analysis

Equipment	Description
Gas Chromatography	Shimadzu, model: GC-2010, Japan
Gas Chromatography's column	J&W Scientific, model: DB-WAX, SN US5432531 H, length 30 m, i.d. 0.53 mm, film 0.25 μm, USA
High Performance Liquid Chromatography	Shimadzu, model: LC-20A, Japan
High Performance Liquid Chromatography's column	Alltech, model: Apollo Silica 5U, SN 605110712.1, length 250 mm, i.d.4.6 mm

Equipment for PAHs analysis

Equipment	Description
Gas chromatography/mass spectrometry's column	Hewlett-packard, HP-5MS series, CA, USA, 30 m, 0.25 mm., i.d. 0.25 μm film thickness
High performance liquid chromatography	Agilent, Agilent 1,100 Series, USA
High performance liquid chromatography's column	Hewlett Packard, ODS Hypersil, 250 x 4 mm, i.d. 5μm
Microplate	Inter Med, Nunc-Immuno Plate MaxiSorp, MicroPlate FLAT BOTTOM 96-wells
Rotator mixer	Bio – Active Co., Ltd.
Spectrophotometer	Becthai Bangkok Equiupment, Zenyth 200rt/25700, SN.257001027

Equipments for preparation of used frying oil

Equipment	Description
Electric pot	Classic Class, TOMEX DEEP FRYER 840 W ~ 230V, 50Hz, model: DF-243, Thailand
Thermometer	Temperature 0-200°C

Equipment for properties of oil

Equipment	Description
Gel documentation	Vilber Lourmat, France

Equipment for Transesterification reaction

Equipment	Description
Magnetic bar	Lio Lab Limited Partnership, Size 13 x 3 mm
Peristaltic Pump	Bioinstrument atto, Perista Pump, L-EQ 038
Vial	Size 10.0 ml
Water bath	Made in Thailand

Others

Equipment	Description
Assembled Vial Kit	Ligand Scientific CO., LTD, Clear vials, 12x32 mm, 8425 caps
Auto Pipette	Labnet International, Inc, BP 1000/100-1000 µl, SN.544061530
Auto Pipette	Labnet International, Inc, BP 200/20-200 µl, SN.544051295
Auto Pipette	Labnet International, Inc, BP 20/2-20 µl, SN.544031096

Equipment	Description
Auto Pipette	Labnet International, Inc BP 2/0.1-2 µl, SN.544010616
Crucible	made in Thailand
Furnace	Thermolyne furnatrol II series, sybron corporation
Hyper Clean Syringe Filters	Amani Co., Ltd, PTFE 0.45 µm, 17 mm
Microcentrifuge tube	Ligand Scientific Co., Ltd, size 1.5 ml
Viscometer (D2170)	BS/U-Tube Viscometer Model D2170, No. B53, viscometer constant is 0.01069 mm^2/S^2

3.4 Procedures

This study objected to utilization of used frying palm oil base on biodiesel production using lipase as a catalyst and determined the PAHs in used frying palm oil. The statistical analysis in all experiment was conducted using the Sigma Plot (Scientific Graphing Software, version 8.0). A paired t-test was applied to the result. Then, the overview of the experiment design shows in Fig. 3.1.

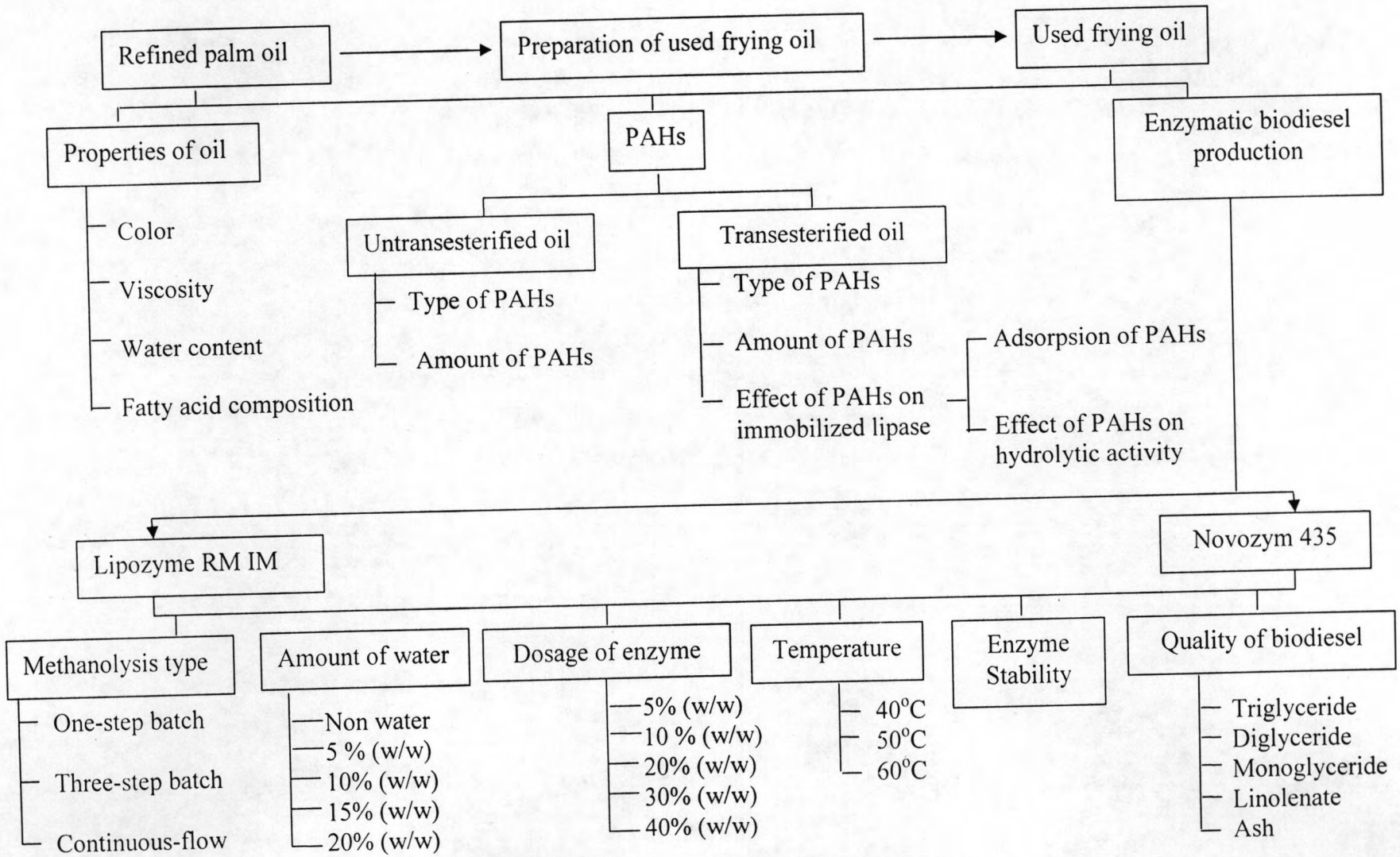

Fig. 3.1 Overview of the experimental design.

3.4.1 Preparation of used palm oil (frying palm oil)

An electrical deep fryer (Tomex Deep Fryer DF-243, Thailand) with a frying basket and 1.5 l capacity was used for frying of chicken breast (CP company, Thailand). Refined palm oil (0.7 l) (Morakot company) was heated to 170°C at the beginning. Then, chicken breast (100 g each) was fried for 15 min (the standard method shown in Appendix B1). After 15 min, the fried chicken breast was removed from the fryer and the oil sample (10.0 ml) was taken. This process was called as "one frying cycle" (Fig. 3.2). Then, the frying operation was carried out for a new chicken breast. The oil was subjected to 21 frying cycles. The volume of oil was not replenished to the original volume with fresh oil after any frying cycle. Used frying oil samples kept in glass vials were stored in a refrigerator (4°C) until use.

Fig. 3.2 One-chicken breast frying cycle

3.4.2 Determination of characteristics of palm oil

Color

The color of used frying palm oil was measured by comparison with the color of refined palm oil. The oil were photographed and converted to gray scale using gel documentation. The color intensity was compared using Bio-PROFIL program (Developed by Vilber Lourmat, France). The higher value of color intensity means the lighter color.

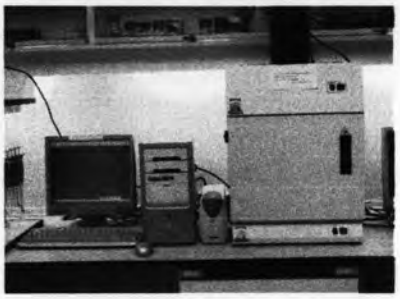

Fig. 3.3 Gel documentation

Viscosity

Oil viscosity was determined at room temperature using an ASTM D445 standard method using a viscometer, D2170. Viscometer constant of D2170 is 0.01069 mm^2/s^2, (cSt/s).

Used frying palm oil (10 ml) was drawn up into a reservoir and allowed to run through a capillary tube to another reservoir in the other limb of the U-tube. The time (efflux time) was taken for the level to fall between the marks is converted into cSt by multiplying the time by the viscometer constant (the calculation is described in Appendex A1).

Fig. 3.4 Viscometer D2170

Water content

Fig. 3.5 Karl-Fischer titration

The water content was determined before and after frying by Karl–Fischer titration, following using the AOAC standard method 991.02. This test was performed by Science Service Department, Bangkok, Thailand. Refined oil and the 21-cyc oil (8-10 g) were taken into Erlenmeyer flask containing a magnetic stirring bar. Methanol (200 ml) was added into flask and stirred magnetically for 15 min. The solids were settled and aliquoted to titration vessel containing pretitrated methanol. After that, the sample was titrated with Karl Fischer reagent (containing sulphur dioxide and iodine) and imidazole as base (B) to determine methanol (The calculation is described in Appendex A2). Methanol (MeOH) takes part in the reaction forming the methyl sulphite (equation 3.1). After that, methyl sulphite reacted with water in the equation 3.2

Equation 3.1

$$SO_2 + MeOH + B \rightleftharpoons MeSO_3^- + HB^+$$

Equation 3.2

$$MeSO_3^- + H_2O + I_2 + 2B \longrightarrow MeSO_4^- + 2HB+ + 2I^-$$

3.4.3 Determination of PAHs in refined oil and used frying palm oil

Extraction of PAHs

PAHs were extracted from used oil by liquid-liquid extraction using suitable solvent(s). Refined and used frying palm oil were dissolved in organic solvent such as acetronitrile (1:1 v/v) and cyclohexane (1:1 v/v) with dimethylformamide (DMF)-water (9:1 v/v) (Moret *et al.*, 2000). Then, the oil and organic solvent were completely mixed using rotator mixer at room temperature for 48 hr. The solvent phase containing PAHs was separated and characterized for type and amount of PAHs.

Identification of PAHs formed in used frying oil

Acetonitrile, PAHs extraction solvent, was throughly evaporated from the samples in a water bath at 100°C. The samples were resuspended in 500 µl of hexane and analyzed by gas chromatography (GC) coupled to mass spectrometry. The GC was equipped with a splitless injector (Pulse splitless mode). The GC temperature gradient was from 50°C (1 min) to 200°C at 25°C/min and 316°C at 8°C/min. The carrier gas was helium at constant flow rate of 1.5 ml/min. The capillary column used was an HP-5MS (Hewlett-Packard, CA, USA) (30 m, i.d. 0.25 mm., 0.25 µm film thickness). PAHs was done using ionization mode: electron-ionization (+EI).

Amount of PAHs in refined and used frying palm oil.

PAHs were quantified with a reverse phase high performance liquid chromatography (HPLC). HPLC analysis was performed with a Agilent 1,100 series (Algilent Technologies, Wilmington, DE, USA) equipped with a UV detector at a wavelength of 254 nm. Data collection and analysis were performed using Hewlett Packard Chemstation software. A Hewlett Packard ODS Hypersil (5μm film thickness, 250 x 4 mm) column was used. Acetonitrile was used as a mobile phase with flow rate of 1 ml/min and pressure of 45-46 bars at room temperature.

Adsorpsion of PAHs on immobilized lipase

During enzymatic transesterification reaction (section 4.4.3), PAHs in used oil could adsorbed onto immobilized lipase (Lipozyme RM IM and Novozym 435). Therefore, amount of PAHs adsorbed on immobilized lipase was analyzed using liquid-liquid extraction. Immobilized lipase (0.2 g) was mixed with 2.0 μmole of naphthalene or benzo[a]pyrene dissolved in acetonitrile (2 ml) using rotator mixer at room temperature for 48 hr. After that, the solvent phase containing PAHs was separated and analyzed for the amount of PAHs. Amount of PAHs adsorbed on the immobilized enzyme was calculated from:

PAHs adsorbed (μmole) =

PAHs added into the reaction (μmole)-PAHs remained in solvent phase (μmole)

PAHs on hydrolytic activities of immobilized lipase

Effect of PAHs on hydrolytic activity of Lipozyme RM IM and Novozym 435 were examined. PAHs (naphthalene and benzo[a]pyrene) (2.0 μmole) was dissolved in the mixture of methanol (8.0 ml), water (12 ml) and immobilized lipase (0.2g). The mixture was mixed together for 12, 24 and 48 hours at room temperature. Then, the immobilized lipase was separated by suction and divided into two parts. One part was kept for hydrolytic assay as described below, while the other was washed by hexane (2 ml, 10 min). After that, the hexane was removed from the reaction and evaporated them from the immobilized lipase.

Hydrolytic activity of immobilized lipase was assayed using 0.5% (w/v) *p*-nitrophenyl palmitate in ethanol as a substrate. The reaction mixture consisting of 1 ml of 0.05 M phosphate buffer pH 9 containing 0.2 g of immobilized lipase, was initiated by adding 1 ml of substrate and mixed for 5 min at 30°C. The reaction was terminated by adding 2ml of 0.5 Na_2CO_3 followed by centrifuging at 10,000 rpm for 10 min. The increase in the absorbance at 410 nm was produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-nitrophenylpalmitate. The *p*-nitrophenol was measured using spectrophotometer at 410 nm (Hung *et al.*, 2003). This experiment was stand in triplicate. The standard curve and the hydrolytic activity of immobilized lipase show in Appendex C4.

3.4.4 Transesterification of used frying palm oil

The analysis of fatty acid methyl ester or biodiesel.

FAME or biodiesel analysis was performed (as describe in Appendex D) using Gas Chromatograph (GC) (Shimadzu, GC 2010 series, Japan). Sample analysis was carried out on a DB-WAX fused silica capillary column (30m x 0.53 mm i.d., 0.25 μm film thickness, J&W Scientific, Folsom, CA, USA). Acquisition and processing of data were obtained using the GC- solution solftware version 2.30.00 SU6 (Shimadzu, Japan).

Sample (1 μl) was injected into GC column by an auto-sampler injector. The temperature program was set as followed: an isothermal period of 1 min at 70°C, then, the GC oven was heated at 20°C/min to 180°C, then at 3°C/min to 220°C and hold for 15 min. The temperature of injector and FID detector were set up for 250°C and 300°C, respectively.

Chemical transesterification

The chemical transesterification was carried out as a standard process using a condition previously (Fukuda *et al.*, 2001). Sodium hydroxide (1 %w/w) was mixed with methanol (5.0 ml) and oil (3.0 g). The reaction mixture was mixed together continuously for 24 hours at 50°C. The sample was taken to analyze the conversion of methyl ester (Samukawa *et al.*, 2000).

Thin layer chromatography as the analysis method of the conversion of triglyceride.

Fig. 3.6 Thin layer chromatography

The chemical conversion of triglyceride to methyl ester was determined using a thin layer chromatography (TLC). The oil sample was dissolved and mixed together in hexane (sample:hexane; 15μl:85μl). Then 1.0 μl of the sample was spoted onto the TLC plate (TLC-aluminium sheets 20 x 20 cm, Silica gel 60 F254; Merck, Darmstadt, Germany) developed by a solvent system containing hexane: ethyl acetate: acetic acid (90:10:2), and was stained by methanol: sulfuric acid (1:1). After that the plate was heated at 110°C for 30 minutes (Taichi *et at.*, 2000).

3.4.5 Enzymatic transesterification.

Immobilized lipase that used in all experiment was Lipozyme RM IM and Novozym 435. Oil reacted with methanol using immobilized lipase as a catalyst. The studies on important reaction parameters for the transesterification are described in section 3.4.5.2-3.4.5.5

3.4.5.2 Type of methanolysis

3.4.5.3 Amount of water in transesterification reaction

3.4.5.4 Dosages of immobilized lipase

3.4.5.5 Effect of temperature and methanol content on methanolysis.

3.4.5.1 The fixed parameter in the reaction

Enzymatic transesterification consisted of 3.0 g of palm oil (refined or used frying oil), methanol (final concentration of 3.0 mole) and 0.6 g immobilized lipase (Lipozyme RM IM or Novozym 435) as a catalyst.

3.4.5.2 Type of methanolysis

Methanolysis was carried out in 20 ml screw-capped vial using 20 % of immobilized lipase by weight of oil as a catalyst with two substrates, used frying palm oil and methanol. The reaction was stirred at all time at 300 rpm and the reaction temperature was kept constant at 40°C. The immobilized lipase (0.6 g) was preincubated in the 21-cyc oil for 30 min at room temperature. Three molar of methanol was 365 µl against 3.0 g of the 21-cycle oil (3.0g). The amount of water added to the reaction mixture (this reaction was called "Two phase reaction") was 0, 5, 10, 15 and 20 % by weight of oil. Methanol was added to the final concentrations of 3 moles (365 µl) to the reaction using three different methods: one-step batch methanolysis, three-step batch methanolysis and continuous-flow methanolysis.

One-step batch methanolysis

Three moles of methanol (365 µl) were added in the reaction mixture at one time, and the reaction was continued for 48 hours.

Three-step batch methanolysis

In this method, methanol was added to the reaction 3 times during the reaction. At the beginning of the methanolysis, 1/3 molar equivalent of methanol (122 µl) was added into the reaction mixture. The second 1/3 molar equivalent of methanol (122 µl) at the 8^{th} hour of reaction, and the third 1/3 molar equivalent of methanol was added at 16^{th} hour. The final concentration of methanol in the reaction was three moles. The reaction was continued for 48 hours.

Continuous-flow methanolysis

Three moles of methanol was continuously to added along the reaction mixture different flow rates of methanol, i.e. 0.018, 0.083 and 0.159 ml/min.

3.4.5.3 Amount of water in transesterification reaction.

Amount of water was optimized in each type of methanolysis. Water was added to the reaction mixture (0, 5, 10, 15 and 20% by

weight of oil). This reaction was called "two-phase reaction". Then, the reaction was continued for 48 hours.

3.4.5.4 Dosage of immobilized lipase

The amount of immobilized lipase was varied from 5% to 40% (% wt/wt of oil) in continuous-flow methanolysis with flow rate at 0.018 ml/min. The substrates in the reaction mixture were used frying palm oil and methanol at 40°C in 20.0 ml screw-capped vial with the stirring speed 300 rpm. The immobilized lipase was preincubated in the 21-cyc oil for 30 min at room temperature.

3.4.5.5 Effect of temperature and methanol content on methanolysis.

The effect of temperature and methanol content on methanolysis was determined as described:

1.) Methanol content in methanolysis was varied by varying the methanol to oil mole ratio (0.5:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1 and 9:1) while the reaction condition was carried out at 40°C and the condition as stared in

2.) The reaction time was varied to 40°C, 50°C and 60°C while the reaction conditions were in section 4.4.3.1.

3.4.5.6 Stabilities of immobilized lipase

Since there might be damages to the enzyme activity the reaction during the reaction due to reaction conditions, by product(s) formed, the stability of immobilized lipase was investigated. The immobilized lipase was filtered and consecutively reused after each reaction.

3.4.6 Determination of biodiesel physic properties.

The physical perperties of biodiesel were determined according to the following standard test methods;

3.4.6.1 Test method for methyl ester, triglyceride, diglyceride and monoglyceride contents.

The samples were analyzed with high-performance liquid chromatography (HPLC, Shimadzu LC-20A series, Japan) using evaporative light scattering (ELSD) detector. The operating condition of ELSD was as follows: drift tube temperature: 40°C, gas pressure 8 psig. Apollo Silica 5U (250 mm x 4.6 mm i.d.) column was used. The mobile phase was: solvent A (hexane: 2-propanol: ethyl acetate: formic acid (85: 10: 10: 10: 0.1 v/v) and solvent B (hexane: formic acid (100: 0.2 v/v) with flow rate at 1.5 ml/min. Data collection and analysis was performed using LC solution software (Shimadzu, Japan). An Eicosane was used as an internal standard. Sample was dissolved in hexane.

3.4.6.2 Test method for ash content

The ash represents as the amount of residual alkali catalyst present in the biodiesel as well as any other ash forming compounds that could contribute to injector deposits or fuel system fouling. Oil sample was weighed accurately (0.50 g) and placed in the crucible. The oil was burnt in furnace at 550°C until free from carbon (~3 hr). When ashing was completed, the crucible was allowed to cool in a descicator and the ash in the crucible was then weighted. The ash content could be calculated from:

$$\text{Ash content (\% wt)} = \frac{\text{weight of ash (g)} \times 100}{\text{weight of test portion sample (g)}}$$