CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

Autoclave	(Ta Chang Medical instrument, Taiwan)
Balance	(Sartorius, Germany)
Centrifuge tubes	(Oxygen scientific, U.S.A.)
Digital Balance	(Satorious, Germany)
Desiccator	(Sigma-Aldrich, U.S.A.)
Gas Chromatography	(Shimadzu, Japan)
Heater	(Chatcharee holding, Thailand)
High performance liquid chromatography	(Shimudzu, Japan)
Magnetic stirrer	(KIKA Labortechnik, Malaysia)
Magnetic bar	(Lio Lab Limited Partnership)
Microcentrifuge	(Global Medical Instrumentation, USA)
Microplate spectrometer	(BMG Labtech, Germany)
Microrefrigerated centrifuge: model 5417	(Eppendrof, UK)
pH meter	(Model 250, Denver Instrument)
Rotatory evaporator	(BÜCHI Labortechnik AG, Switzerland)
UV-VIS spectrophotometer	(Thermo scientific, UK)
Vacuum pump	(Scientific industries, USA)
Vortex	(Scientific industries,)
Whatman No.1	(Whatman, England)
Water bath	(T.S. Instrument, Thailand)

3.2 Chemicals

Acetone	(Carlo erba, Italy)
Acylglycerols standard (triolein, diolein and monoolein)	(Sigma, USA)
Amberlite XAD 2	(Rohm and Haas company, U.S.A.)
Amberlite XAD 4	(Rohm and Haas company, U.S.A.)
Amberlite XAD 7	(Rohm and Haas company, U.S.A.)

Amberlite XAD 16	(Rohm and Haas company, U.S.A)
Amberlite XAD 761	(Rohm and Haas company, U.S.A)
Bovine serum albumin, BSA	(Merck, Germany)
Bradford's reagent	(Biorad,U.S.A)
Butanol	(Carlo erba, Italy)
Candida rugosa lipase type VII	(Sigma,U.S.A.)
Di-potassium hydrogen phosphate	(Scharlau,Spain)
Eicosane	(Aldrich,Germany)
Ethanol	(Lab scan, Thailand)
Ethyl acetate	(Carlo erba, Italy)
Ethylene glycol	(Lab scan, Thailand)
Formic acid	(Lab scan, Thailand)
Glacial acetic acid	(Labscan, Thailand)
Hexane	(Labscan, Thailand)
Isopropanol: CH_3CHOH	(Lab scan,Thailand)
Lipozyme RM IM	(Novo Nordisk, Denmark)
Methanol	(Labscan, Thailand)
Novozym 435	(Novo Nordisk, Denmark)
Oleic acid	(Sigma, USA)
Oleic acid methyl ester (oleate)	(Sigma, USA)
Palm oil	(Morakot industry, Thailand)
Potassium di-hydrogen phosphate (KH_2PO_4)	(Merck, Germany)
p-nitrophenyl palmitate:	(Sigma, U.S.A.)
Sepabeads EC-BU	(Mitsubishi chemical corporation, Italy)
Sepabeads EC-OD	(Mitsubishi chemical corporation, Italy)
Silica gel plate (silica gel 60 F254)	(Merck, Germany)
Sodium carbonate	(Scharlau,Spain)
Sodium dodecyl sulfate, SDS	(Sigma, U.S.A.)
Sodium hydroxide	(Merck, Germany)
Sulfuric acid	(RIEDEL-DE-HAEN, Germany)
Tris (hydroxymethyl) aminomethane	(Scharlau,Spain)

Triton x-100	(Scharlau,Spain)
t-butanol	(Carlo erba, Italy)
Tween-80	(Lab scan, Thailand)

3.3 Data analysis

Statistical analysis program	(Graph Pad InStat3)
Graph analysis program	(Graph Pad Prism4)
GC data analysis program	(GC-solution solfware version 2.30.00 su6)
HPLC data analysis program	(LC solution software)

3.4 Research methodology

All experiments were performed at least in triplicates and the results were presented as mean values. The research methodology is as follows:

3.4.1 Immobilization of *Candida rugosa* lipase

3.4.1.1 Preparation of supports

Supports were prepared by suspending 1 g of support powder in 3 ml methanol. The suspension was kept stirred at 350 rpm at room temperature. After 30 min, methanol was removed from the reaction and supports were washed with 20 mM phosphate buffer pH 7.5 and kept stirred at 350 rpm at room temperature for 30 min 3 times. Then, the supports were separated into 2 groups, namely, one group was pretreated by drying at 45°C and the other was immediately used for immobilization.

3.4.1.2 Immobilization of enzyme

The enzyme solution was prepared by dissolving 200 mg crude *Candida rugosa* lipase in 3 ml 20 mM phosphate buffer solution, pH 7.5. The solution was centrifuged at 5,000 rpm, 4°C for 15 min to remove insoluble components. After centrifugation, supernatant was removed and the protein content of *Candida rugosa* lipase was determined by Bradford method. Protein content was compared with standard curve of BSA shown in Appendix C. Finally, the volume of lipase solution was calculated for 3 mg/ml of protein. The

supernatant was then brought in contact with 1 g of support. The lipase-support was stirred at 350 rpm for 6 hr at room temperature. After incubation, the solution was removed from immobilized enzyme and washed with 3 ml of 20 mM phosphate buffer pH 7.5 for 5 min until no enzyme was detected. The protein content in the solution was then measured.

The amount of adsorbed protein was calculated from the difference in protein content before and after adsorption. All solutions were quantitated for protein contents by Bradford method as described in section 3.4.5.2. Then, the immobilized lipases were dried at room temperature in desiccators and the enzyme was finally assayed for activities as described in section 3.4.5.1.

For industrial applications, enzymes have always been immobilized onto insoluble or solid supports, it is important to choose proper support for enzyme immobilization, since its interaction with enzyme may have remarkable influence on the stability and activity of the enzyme.

3.4.2 Support selection

In order to select a support that is suitable for hydrolytic activity, 7 types of supports were used as follows: Amberlite XAD 2, Amberlite XAD 4, Amberlite XAD 7, Amberlite XAD 16, Amberlite XAD 761, SepabeadsEC-OD and Sepabeads EC-BU.

3.4.3 Optimal condition for the immobilization

There are many factors affecting the activity recovery and reusability of enzymes in immobilization process. Some of the most important factors are the properties of the enzyme molecule, choice of support, concentration of enzyme, temperature and other. In addition , the factors studied were as follows: pH, ionic strength, protein loading, time of immobilization, temperature and adjuvant.

3.4.3.1 Effect of pH on immobilization

The effect of pH on the immobilization of lipase was studied at different pH values of buffer ranging from 4.0 to 10.0. The stock solution of buffer was prepared as 1 M buffer solution. All these buffer solutions were diluted to 20 mM before use which were used to dissolve crude lipase for immobilization method. The immobilized lipase was assayed by the method described in section 3.4.5. The result was expressed as immobilization efficiency.

3.4.3.2 Effect of ionic strength on immobilization

After the optimal pH was obtained, the following concentrations of buffer at that pH were prepared: 10, 50, 100, 250, 500, 750 and 1000 mM from the 1M stock solution to dissolve crude lipase. The immobilized lipase was assayed by the method described in section 3.4.5. The result was expressed as immobilization efficiency.

3.4.3.3 Effect of protein loading on immobilization

The effect of protein loading on the immobilization lipase was assayed by adjusting protein loading values such as 1, 3, 6, 8 and 10 mg/ml. Amount of proteins in lipase solution were prepared by dissolving commercial crude *Candida rugosa* lipase in the proper buffer solution from section 3.4.3.1 and ionic strength from section 3.4.3.2. The immobilized lipase was assayed by the method described in section 3.4.5. The result was expressed as immobilization efficiency.

3.4.3.4 Effect of time and temperature on immobilization

After the optimal pH, ionic strength and enzyme loading were obtained as described in section 3.4.3.1-3.4.3.3, the effect of time and temperature were investigated. The times of immobilization were performed by checking the residual activity of lipase solution as follows; 100 μl of lipase solution was taken for each time of immobilization for 10 hours at various temperatures namely, 10,

20, 25, 30, 40, 50 and 60 °C. The results were expressed as the percentage of the residual activity of the activity at room temperature (25 °C).

From the experiment described above, after the optimal period of time for the immobilization of was obtained, the effect of temperature on activity of immobilized lipase was examined by checking activity of immobilized lipase from each temperature of the immobilization. The assay methods for the free and immobilized lipase are described in section 3.4.5. The result was expressed as immobilization efficiency.

3.4.3.5 Effect of adjuvants on immobilization

After the optimal pH, ionic strength, enzyme loading, time and temperature were obtained as described in section 3.4.3.1-3.4.3.4, the effect of adjuvants was investigated.

3.4.3.5.1 Screening of adjuvant concentration

Adjuvants were divided into 2 groups namely, alcohol and detergents. The alcohol group consisted of methanol, ethanol, iso-propanol, n-butanol and t-butanol whereas the detergents were as follows: SDS, tween-80, ethylene glycol and triton X-100. In addition, the effect of type and concentration of adjuvants were also studied. The concentration of alcohols used were 0, 1, 2.5, 5, 10, 20 and 30 %(v/v) and those of detergents were 0, 0.1, 0.25, 0.5, 0.75, 1, 2.5, 5, 10 and 20 % (v/v). The experimental procedure to determine catalytic activity of enzyme solutions containing adjuvants were as follows: 200 µl soluble lipase from section 3.4.1.2 containing the corresponding amount of adjuvants were incubated at temperatures for the period of optimal times described in section 3.4.3.4 and was assayed according to the protocol described about hydrolysis of *p*NPP as described in section 3.4.5.1.

3.4.3.5.2 Screening of adjuvant types

When the proper concentration of each adjuvant from the method described above was obtained, the effect of the type of adjuvant on the immobilization of *Candida rugosa* lipase was then determined. The suitable concentrations of adjuvants were added in to the enzyme solution for 2 min before contact with support and expressed as immobilization efficiency of lipase. Transesterification was performed to confirm the activity of immobilized lipase .The yield of fatty acid methyl ester was determined by high performance liquid chromatography as described in section 3.4.11.3.

3.4.4 Transesterification and hydrolysis catalyzed by immobilized *Candida rugosa* lipase

After the optimal conditions for immobilization of *Candida rugosa* lipase were obtained as described section 3.4.3.1- 3.4.3.5, the activity of immobilized lipase was investigated. The lipase activities were determined by using immobilized lipase catalyzed transesterification and hydrolysis. The conditions for transesterification were as follows; 20 % (w/w of oil) enzyme, one to three mole ratio of oil to methanol, three addition steps of methanol at 0, 3, and 6 hours in 20 ml screw-capped vial. The reactions were carried out at 40 °C for 24 hours by magnetic stirrer. The conditions for hydrolysis were as follows; 20 % (w/w of oil) enzyme and 100 % (w/w of oil) water were mixed well in the reaction at 40 °C for 9 hours by magnetic stirrer. The yields of fatty acid methyl ester and fatty acid were determined by HPLC analysis as described in 3.4.11.3.

3.4.5 Enzyme activity and protein assay

3.4.5.1 Enzyme activity assay

Activity of the free and immobilized lipase was assayed using 0.5% (w/v) *p*-nitrophenyl palmitate (*p*NPP) in ethanol as substrate. The reaction mixture consisting of 0.25 ml of 50 mM Tris-HCl buffer, pH 8 containing immobilized lipase or 25 µl of free lipase was initiated by adding 0.25 ml of

substrate and mixed for 5 min at room temperature. The reaction was terminated by adding 0.5 ml 0f 0.25 M Na_2CO_3 followed by centrifugation at 14,000 rpm at 4°C for 5 min. The increase in the absorbance at 410 nm produced by the release of *p*-nitrophenol in the enzymatic hydrolysis of *p*-NPP was then measured.

One international unit (IU) of lipase was defined as the amount of enzyme needed to liberate 1μmol of *p*-nitrophenol per minute using *p*-nitrophenyl palmitate as substrate. Calculation for the unit of enzyme activity was described in Appendix D.

3.4.5.2 Protein assay

The amount of protein content before and after immobilization was determined by Bradford protein assay method. The reaction mixture consisted of 5 μl of sample containing 300 μl of Bradford reagent in 96 well plates and was incubated at room temperature for 5 min, and later measured for the absorbance at 595 nm. Standard curve was prepared to determine concentration of protein using bovine serum albumin (BSA) at the concentration of 0.1-0.6 mg/ml. The amount of protein bound to the enzyme carriers was determined as the difference between the initial and residual protein concentrations in the supernatants. The calculation method was shown in Appendix C.

3.4.5.3 Immobilization efficiency

The efficiency of immobilization was calculated in terms of lipase activity, specific activity, protein loading and activity yields. The calculation method was shown in Appendix D.

3.4.6 Screening of raw materials for feedstock

3.4.6.1 Extraction of seed oil samples

Prior to the extraction process, plant seeds were dried overnight at 100 °C in an oven to remove excess moisture. The dried seeds were then weighed and ground into fine particles of small size. The oil was then extracted using soxhlet extraction procedure. 15 g of plant seeds were packed in a thimble and the oil was extracted with 250 ml *n*-hexane for 6 hr. After extraction, the solvent was removed in a rotary evaporator at 60 °C, under moderate vacuum. The extracted oil was then measured to calculate the content of oil in the plant seeds. Yield was calculated on dry weight basis. The calculation method was shown in Appendix E.

3.4.6.2 Fatty acid composition analysis

3.4.6.2.1 Transesterification of plant oil by chemical catalysis

The direct transesterification of plant oils triglycerides was performed. One gram of oil was used in the transesterification experiments. Sodium hydroxide (final concentration of 10% w/w based on the mass of oil) was used as an alkaline catalyst. Methanol was employed in a 3:1 alcohol to oil molar ratio. The transesterification reactions were performed in 20 ml screw-capped vials, mechanically stirred and heated in a water bath for 24 hours at 55°C. After completion of reaction, samples were taken from the reaction and centrifuged at 12,000 rpm 30 min to separate upper ester layer and a lower glycerol layer. The upper layer was analyzed by gas chromatography to determine the yield of fatty acid alkyl esters as described in section 3.4.11.1. All transesterification tests were conducted in triplicates.

3.4.6.2.2 Characterization of oil

The quality of oil is expressed in terms of the physicochemical properties such as saponification value, iodine value, cetane number and viscosity (η) by equation shown in Appendix E.

3.4.7 Transesterification catalyzed by immobilized *Candida rugosa* lipase

Transesterification reactions were carried out in 20 ml screw-capped vial containing 1 g of palm oil and 20% by weight of the immobilized lipase and later added with 3:1 mole ratio of methanol using 3-steps addition mode of methanol at 0, 3 and 6 hours. The reactions were magnetically stirred in a water bath for 24 hours at 40°C. After completion of reaction, samples were taken from the reaction and analyzed by HPLC as described in section 3.4.11.2.

3.4.8 Optimization of the transesterification catalyzed by immobilized *Candida rugosa* lipase

After the optimal conditons for immobilization of enzyme were obtained, the optimized conditions for transesterification were determined as follows: addition mode of methanol, oil:methanol ratio, amount of enzyme, water content and reaction time and temperature.

3.4.8.1 Effect of addition mode of methanol

Starting mixture of 1 g palm oil and methanol was prepared in 20 ml screw-cap bottles containing 0.3 g immobilized lipase. Then, 3 moles of methanol (71.1 μl) were added using 1, 2, 3, 4, 5, 6 and 7 steps. The reactions were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.8.2 Effect of oil: methanol molar ratio

When the proper addition mode of methanol from the method described above was obtained, the effect of oil:methanol molar ratio on biodiesel production from palm oil was studied at different ratios of 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 and 1:9. In this study, 20% (w/w of oil) of the immobilized

lipase and 0.5 g palm oil were mixed and different ratios methanol were added into the reaction using the obtained optimal addition mode of methanol from section 3.4.8.1. Experiments were carried out for the reaction periods of 24 hours and stirred in a water bath at 40°C.

3.4.8.3 Effect of Enzyme loading

When the proper addition mode of methanol, oil:methanol molar ratio from the method described above were obtained, the effect of enzyme loading of 10%, 20% and 30% (w/w of oil) on biodiesel production from palm oil was studied. This experiment was studied by adding 0.5 g of oil with each amount of the immobilized lipase for transesterification. Then, methanol was added into the reaction using the addition mode of methanol, and ratio of oil and methanol obtained from section 3.4.8.1 and 3.4.8.2, respectively. The reactions were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.8.4 Effect of water content

When the proper addition mode of methanol, oil:methanol molar ratio and enzyme loading from the method described above were obtained , the effect of water content on lipase activity in transesterification reaction was examined at 0, 0.5, 1.0, 2.0, 5.0, 7.0 and 10.0 % (v/v) of the oil. The reaction mixtures were magnetically stirred in a water bath for 24 hours at 40°C.

3.4.8.5 Effect of reaction time and temperature

After the reaction mixtures were mixed using the optimized conditions from section 3.4.8.1-3.4.8.4, the reaction was incubated at different temperature 30, 40, 50, and 60 °C and stirred in a water bath for 48 hours to obtain the optimal time and temperature for the transesterification. Then, 100 µl of samples were taken from the reaction mixtures at 3, 6, 9, 12, 24, 36 and 48 hours.

3.4.9 Comparative studies of transesterification catalyzed by immobilized *Candida rugosa* lipase with Novozyme® 435 and Lipozyme® RM IM

After the optimal conditions for transesterification catalyzed by immobilized *Candida rugosa* lipase were obtained from section 3.4.8.1-3.4.8.5., lipase activities of immobilized *Candida rugosa* were tested. The selected non-edible and waste plant oils from section 3.4.6 were used as substrates for transesterification catalyzed by the optimized immobilized *Candida rugosa* lipase compared with Novozyme® 435 and Lipozyme® RM IM. Transesterification catalyzed by immobilized *Candida rugosa* lipase was carried out as follows; 0.5 g of oils were added with 30% (w/w of oil) of the immobilized *Candida rugosa* lipase with 1:3 mole ratio of methanol. The addition of methanol was performed at 0, 2, 4, 6, 8 and 10 hours and continuously stirred at 40°C for 12 hours. Transesterification catalyzed by commercial immobilized lipases were carried out as follows; one gram of oil was added with 20% (w/w of oil) of the Novozyme® 435 and Lipozyme® RM IM and later mixed with 1:3 mole ratio of methanol. The addition of methanol was performed at 0, 8 and 16 hours and continuously stirred with magnetic stirrer at 55°C for 24 hours. Samples were taken from the reaction mixture and later analyzed for the products by HPLC as described in 3.4.11.2.

3.4.10 Stability of immobilized lipase

3.4.10.1 Thermal stability

The thermal stability of immobilized enzyme was tested by incubating 2 mg of immobilized lipase at various temperatures from 30 to 80 °C in the temperature controlled heating block for 15 min. Then, the residual activities were determined to obtain proper temperature for the half life of the immobilized lipase. Then, the thermal stability of immobilized enzyme was tested by incubating 2 mg of immobilized lipase at that proper temperature as described above until none of the remained activities was detected. Then, the residual activities were determined as the percentage yield of activity compared to the activity at the optimum conditions and the half life time ($t_{1/2}$)

were calculated as shown in Appendix D. The results were expressed as the percentage of relative of the residual activity and half life time.

3.4.10.2 Repeated use of the immobilized *Candida rugosa* lipase

3.4.10.2.1 Repeated use on transesterification

The reusability of immobilized enzyme was tested by triplicate trials of reactions under optimum conditions. The optimum conditions determined in the present study were 2 g of palm oil, immobilized lipase content of 30% (w/w of oil), 1:3 oil:methanol ratio and water content of 5% (v/w of oil) and magnetically stirred in a water bath for 12 hours. In the present study, the immobilized lipase was rinsed with water, t-butanol and hexane after each batch reaction to remove glycerol and oil. The immobilized lipase was dried in desiccator at room temperature. The dried immobilized lipase was used in the next batch reaction composed of new substrates. The residual activity determined after complete reaction was expressed as relative conversion. The conversion achieved in the first batch was set to 100.

3.4.10.2.2 Repeated use on hydrolysis

The reusability of immobilized enzyme was tested by triplicate trials of reactions under optimum conditions. The optimum conditions in the present study were 3 g of palm oil, immobilized lipase content of 20% (w/w of oil) ,water content of 100% (v/w of oil) and magnetically stirred in a water bath at 40°C for 9 hours. In the present study, the immobilized lipase was rinsed with water after each batch reaction to remove glycerol and oil. The immobilized lipase was dried in desiccator at room temperature. The dried immobilized lipase was used in the next batch reaction with new substrates. The residual activity determined after complete reaction was expressed as relative conversion to the first batch set at 100.

3.4.11 Analysis of the fatty acid methyl ester (Biodiesel)

The biodiesel content in the reaction mixture was analyzed using a gas chromatography and high performance liquid chromatography.

3.4.11.1 Analysis of the fatty acid methyl ester by GC

3.4.11.1.1 Preparation of sample

Samples were taken from the reaction mixture at specified time and centrifuged at 12,000 rpm 30 min to obtain the upper layer. The 1 μl upper layer, 489 μl chloroform and internal standard 10 μl were precisely weighed into 1.5 ml vial.

3.4.11.1.2 Preparation of GC

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

Sample (1μl) was injected into GC column by an auto-sampler injector. The temperature program was set as follows: 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. The fatty acids of plants seed oil were identified in Appendix E.

3.4.11.2 Analysis of the fatty acid methyl ester by HPLC

3.4.11.2.1 Preparation of sample

Samples were taken from the reaction mixture at specified time and centrifuged at 12,000 rpm 30 min to obtain the upper layer. The 10 μl upper layer, 490 μl chloroform and internal standard 10 μl were precisely weighed into 1.5 ml vial.

3.4.11.2.2 Preparation of HPLC

Reaction products were analyzed by normal phase HPLC to separate and quantify the FAME, free fatty acid and acylglycerols. The LC-20A HPLC apparatus (Shimadzu Corp., Kyoto) was equipped with Apollo Silica 5U column (250 m x 4.6 mm x 5 μm) from Alltech (Deerfield, IL) and ELSD-LT Evaporative Light Scattering Detector (Shimadzu Corp., Kyoto). Two mobile phases were employed: phase A consisted of hexane, 2-propanol, ethyl acetate and formic acid (80:10:10:0.1 v/v) and phase B consisted of hexane and formic acid (100:0.05 v/v). The flow rate was 1.5 ml/min and the injection volume was 20 μl. The protocol employed for the mobile phase involved a linear elution gradient of 1% (v/v) phase A increasing to 98% (v/v) in 20 min. The final mixture (A:B, 98:2 v/v) was employed for 3 min. Next, the system was restored to its initial condition by passing the A:B, 1:99 (v/v) mixture through the column for 15 min.

3.4.11.3 Calculation of biodiesel

Biodiesel yield was calculated as the percentage of the actual amount of methyl ester detected in the reaction process divided by the theoretical quantity of methyl ester. Calculation of the biodiesel yield was described in Appendix F.