CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Chemicals and reagents used were of analytical grade unless otherwise specified. These were directly used without further purification.

Enzyme and Protein	**Manufacturer**
Lipase type VII from *Candida rugosa*	Sigma-Aldrich, St. Louis, USA.
Bovine serum albumin (BSA)	Sigma-Aldrich, St. Louis, USA.

Supports for Immobilization	**Manufacturer**
Kaolin	Gift from Cho Chin Cin Pot Factory, Gopeng, Perak.

Substrates	**Manufacturer**
Nonanol (≥ 99.5%)	Merck, Germany
Hexanoic acid (98%)	Merck, Germany

Chemicals	**Manufacturer**
Coomassie Brilliant Blue G-250	Merck, Germany
Ethanol (95%)	Fisher Scientific, Switzerland
Phosphoric acid (85%)	Merck, Germany
Hexane (≥99%)	Merck, Germany
Acetone (99.5%)	Merck, Germany
Benzyltriethylammonium chloride	Merck, Germany
Silver nitrate (99%)	Merck, Germany
Sodium chloride	Merck, Germany
Sodium hydroxide (99%)	Merck, Germany
Methylene blue	Merck, Germany

Instruments

X-ray diffractometer (AXS D8 Advance)	Bruker, Germany
Fourier transform infrared spectrometer (Spectrum 400)	Perkin Elmer, UK
Scanning electron microscope (S-3400N)	Hitachi, Japan
Energy dispersive X-ray spectrometer	Hitachi, Japan
Surface area and porosity analyser (ASAP 2020)	Micromeritic, USA
Analytical sieve shaker (AS200)	Retsch, Germany
Centrifuge (Allegra® 25R)	Beckman Coulter, USA
Freeze drier (FD-550)	Eyela, Japan
Furnace (BWF 1100)	Carbolite, England
Overhead stirrer (RW 20 digital)	IKA, Germany
UV-vis spectrophotometer (Cary 50)	Varian, USA
Water bath shaker (Model 903)	Protech, Malaysia
Gas chromatography-mass spectrum (GCMS-QP2010 Plus)	Shimadzu, Japan

3.2 Methods

3.2.1 Design of Experiments

The experiments involved in this study were designed according to the following flow chart in Figure 3.1.

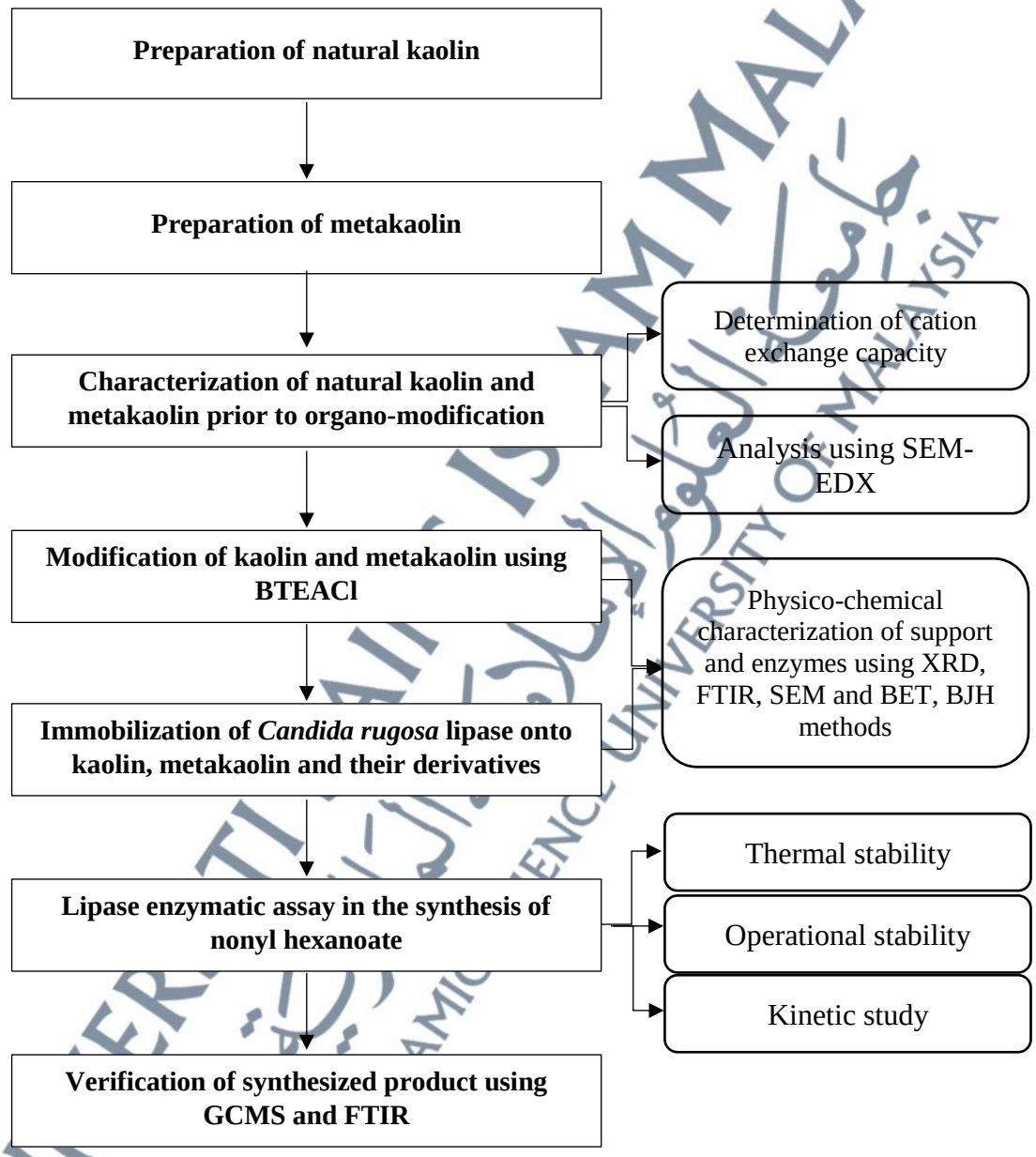

Figure 3.1: Experimental Flow Chart

3.2.2 Preparation of Natural Kaolin

Natural kaolin, which had been obtained in flaky form, was crushed and sieved at 100 mesh using an analytical sieve shaker (AS200, Retsch, Germany). Prior to usage, the powdered material was heated for 5 hours at 120 ^{o}C.

3.2.3 Preparation of Metakaolin

The thermal treatment on the natural kaolin was carried out in furnace (BWF 1100, Carbolite, England). Natural kaolin (10 g) was placed in a crucible and was thermally treated at a constant rate of 10 ^{o}C/min up to 650 ^{o}C. The sample was allowed to remain at the final temperature for 5 hrs. After that, the sample was left to cool and stored in an air-tight container placed in a desiccator for further use.

3.2.4 Determination of Cation Exchange Capacity (CEC) of Kaolin and Metakaolin

Prior to determination of CEC, 6 g of natural kaolin and metakaolin clays were separately homoionized in 1.0 M, at 300 ml sodium chloride. The solid clay particles were filtered and washed repeatedly with deionized water until free from chloride. This was done by testing the filtrate with silver nitrate. The clay particles were then dried at 60 ℃, ground and sieved to obtain ~100 mesh particle sizes.

Cation exchange capacities of clays were determined following the methylene blue-stain test. In this test, methylene blue dye solution was prepared by mixing 10 g of methylene blue with 1 L of distilled water, followed by 30 min of homogenization.

To determine the CEC, 30 g of clay sample was mixed with 30 ml of deionized water in a beaker. The suspension was allowed to mix for 5 minutes at 700 rpm. A burette was then filled with the previously prepared methylene blue solution, before 1 ml of it was added to the clay suspension. The mixture was allowed to mix for another

1 min. A drop of the mixture was taken and carefully placed onto a filter paper. Appearance of blue spot on the filter paper was observed.

The addition of 1 ml of methylene blue solution to the clay suspension and the subsequent procedures were repeated at every 1 min interval until a permanent halo of light blue dye was observed surrounding the darker blue spot on the filter paper (Figure 3.2), which marked the end of the test. The CEC of the clay sample, expressed as meq/100 g clay, was calculated according to Equation 3.1,

$$CEC = \frac{E \times V}{W} \times 100 \quad (3.1)$$

Whereby; E is milliequivalents of methylene blue per ml of distilled water, V is the volume (in ml) of methylene blue solution required to obtain a permanent halo light blue ring around the spot for the titration, and W is weight (in g) of dry clay.

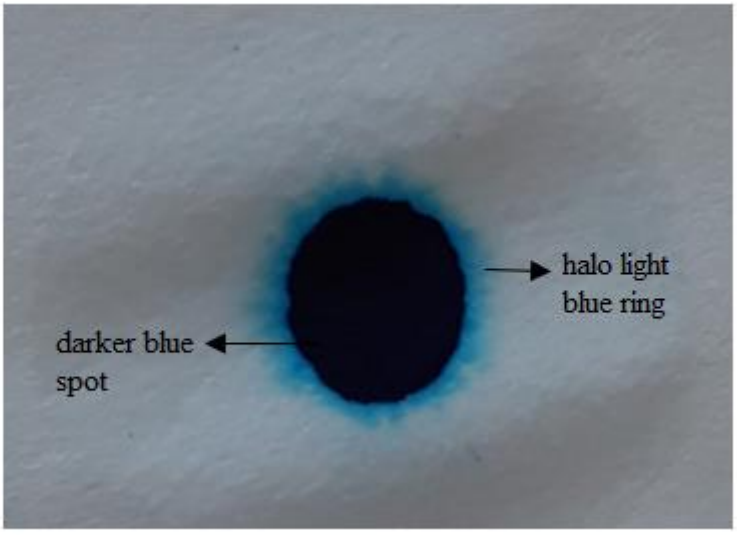

Figure 3.2: Halo Right Blue Ring Surrounding Blue Spot Observed on Filter Paper

3.2.5 Preparation of Organo-Modified Kaolin and Metakaolin

Modification of clay with benzyltriethylammonium chloride (BTEACl) was carried out with some modifications from the methods by Anggraini et al. (2014); Rostami et al. (2018); Ramos et al. (2014); Aroke and El-Nafaty (2014). BTEACl solution was prepared by dissolving BTEACl in 1 L deionized water. Amounts of BTEACl dissolved, relative to the CEC values of clays, are as shown in Table 3.1.

Table 3.1: Amounts of BTEACl Needed Relative to CEC of Clays Natural Kaolin (NK) and Metakaolin (N-MK)

Concentration of BTEACl Relative to CEC Amounts of Clay	**0.5x**	**1.0x**	**1.5x**	**2.0x**
[a]Amounts of BTEACl (g) per L Deionized Water in the Preparation of Organo-modified NK	0.057	0.114	0.171	0.228
[a]Amounts of BTEACl (g) per L Deionized Water in the Preparation of Organo-modified N-MK	0.034	0.068	0.103	0.137

[a] Amounts of BTEACl based on its molecular weight, 227.77 g/mol

Clay (20 g) were mixed with 200 mL of BTEACl solution, previously prepared. The mixture was stirred at 250 rpm for 24 h and heated at 45 ^{o}C for 10 hrs. The solid suspension were then separated by centrifugation at 3,000 rpm for 30 min and washed with distilled water for several times until no chloride was detected, with 0.1 M $AgNO_3$ solution. The solid suspension was then dried at 35 ^{o}C for 12 hrs, before stored in a desiccator prior to further analysis.

3.2.6 Partial Purification of Lipase Solution

Prior to the immobilization process, the commercial crude lipase from *Candida rugosa* was partially purified by water extraction. 1.5 g of crude commercial lipase was

dispersed in 15.0 mL of distilled water during purification. The mixture was mixed for 30 minutes before being centrifuged at 10,000 rpm for 15 minutes at 0 ℃. After centrifugation, the undissolved solid suspension was discarded, and the supernatant was recovered as partially purified lipase solution before immobilization. A small portion of the supernatant was lyophilized using freeze drier (FD-550, Eyela, Japan) and stored at -20 ℃, prior to use. The lyophilized supernatant was designated as free lipase.

3.2.7 Immobilization of Lipase

The immobilization of lipase was done by adding 2.0 g of clay to 15.0 mL of supernatant collected after centrifugation (refer to Section 3.2.6).

The mixture of supernatant containing lipase and clay was continuously agitated in a water bath shaker (Model 903, Protech, Malaysia) at 100 rpm for 1 h at room temperature. The lipase-loaded clay was separated from the remaining solution by filtration through Whatman No.1 filter paper and washed with distilled water. The immobilized lipase was then stored at -20 ^{o}C, before lyophilized using freeze drier (FD-550, Eyela, Japan) and stored at -20 ℃, prior to use.

Immobilization was expressed as percent of protein loaded onto the clay support and calculated based on the following equation:

$$\%\ Immobilization = \frac{ProteinA - ProteinB}{ProteinA} \times 100\% \qquad (3.2)$$

Protein A is the quantity of protein in the supernatant before it is immobilized, and Protein B is the amount of protein in the supernatant after it is immobilized.

3.2.8 Protein Assay

The Bradford (1976) Coomassie Brilliant blue assay method was used to determine the concentration of protein in this study. Bovine serum albumin (BSA) was used as standard protein, dissolved in deionized water to reach the concentration of 1 mg/ml.

The Bradford reagent was made by dissolving 25 mg of Coomassie Brilliant blue (G-250) in 12.5 mLof ethanol (95%), followed by the addition of 25 mL of phosphoric acid (85%). The mixture was then topped with distilled water to a total volume of 250 ml. Volumes of BSA solution, Bradford reagent and distilled water needed to prepare samples for spectrophotometric analysis is as shown in Appendix 1.

Absorbance for the standard protein solution of different concentrations were determined spectrophotometrically at the wavelength of 595 nm using UV-visible spectrophotometer (Cary 50, Varian, USA). A calibration curve (Appendix 2) was then constructed based on the results obtained and used as reference in the determination of protein amounts in solution.

3.2.9 Physico-Chemical Characterization of Supports and Lipases

3.2.9.1 Analysis Using X-ray Diffractometer (XRD)

The supports were characterised using an X-ray diffractometer (D8 Advance, Bruker AXS, Germany). It was run at a voltage of 40 kV and a current of 40 mA for the Cu-X ray tube. The powdered samples were first mounted on sample holders before being put into the sample chamber. For the determination of 2 at room temperature, the samples were tested between 10° and 80°. For data acquisition and analysis, X-ray diffractograms were acquired using DIFFRACplus software.

3.2.9.2 Analysis Using Fourier Transform Infrared (FTIR) Spectrometer

In this study, small quantity of powdered clay samples was individually spread on potassium bromide (KBr) cell and then placed at the light chamber of the spectrometer. Absorption spectrums were obtained from the scan within the range of 4000 - 500 cm^{-1} region. A reference spectrum detail of KBr, as blank, was subtracted automatically before the final spectrums of samples were obtained.

3.2.9.3 Analysis Using Scanning Electron Microscope

In the present work, analysis was done using scanning electron microscope, SEM (S-3400N, Hitachi, Japan). Samples were first sputter coated with approximately 40 - 60 nm of gold prior to imaging process. After that, the samples were adhered on a metal stub and examined under a microscope.

In addition, the natural kaolin and metakaolin were also analysed using SEM (S-3400N, Hitachi, Japan) coupled with energy dispersive X-ray (EDX) operated at accelerated voltage of 20 kV.

3.2.9.4. Analysis of Surface Area and Porosity Using Nitrogen Sorption Method

The surface areas, total pore volumes, and pore size distributions of the clay samples were determined by using surface area and porosity analyser (ASAP 2020, Micromeritics, UK). Prior to analysis, the samples were outgassed at 30 ℃ for 1 h, then at 40°C for 8 hours in a vacuum condition. Surface areas of the samples were determined according to the Brunauer-Emmet-Teller (BET) method, while the total pore volumes and pore size distributions were determined according to the Barrett Joyner and Halenda (BJH) method. All calculations were automatically done by the system, using the following equations.

BET equation:

$$\frac{1}{W\left[(P_0/P-1)\right]} = \frac{1}{W_m C} + \frac{C-1}{W_m C}\left(\frac{P}{P_0}\right) \quad (3.3)$$

Whereby; W is weight of gas adsorbed, P/P_0 is relative pressure, W_m is weight of monolayer adsorbate and C is the BET constant.

$$C = exp\left(\frac{E_1 - E_2}{RT}\right) \quad (3.4)$$

where E_1 is the heat of adsorption for the first layer, and E_2 is the heat of liquefaction or heat of vaporisation for the second and higher layers.

BJH equation:

$$V_{liq} = \frac{P_a . V_{ads} . V_m}{RT} \quad (3.5)$$

Where; V_{ads} is volume of gas adsorbed, V_{liq} is volume of liquid N_2 in pores of supports, V_m is molar volume of liquid adsorbate, P_a is ambient pressure, T is ambient temperature.

Physi-sorption isotherms and hysteresis loops obtained from the analysis were then classified according to the International Union of Pure and Applied Chemistry (1985).

3.2.10 Lipase Enzymatic Activity Assay

Lipase enzymatic activity assay was done by adding either 50 mg of free or immobilized lipases with equal amounts of protein (1.76 mg) contained in 50 mg of free

CRL (Table 3.2). The assay was conducted in reaction mixture which consisted of 2.0 mmol of nonanol and 2.0 mmol of hexanoic acid in 2.0 mL of hexane. All experiments were done in triplicates with the presence of subsequent control set without catalyst. The reaction mixtures were incubated at 30 ^{o}C for 5 hrs in a water bath shaker with continuous shaking at 150 rpm. (Model 903, Protech, Malaysia).

Table 3.2: Amounts of Immobilized Lipases Added to the Reaction Mixture

Immobilized Lipase	[a]Amount (mg)
CRL-NK	234.354
CRL-N-MK	224.203
CRL-0.5 NK	255.072
CRL-1.0 NK	269.938
CRL-1.5 NK	278.041
CRL-2.0 NK	300.854
CRL-0.5 MK	332.075
CRL-1.0 MK	162.812
CRL-1.5 MK	219.451
CRL-2.0 MK	118.678

[a] Amount of immobilized lipases refers to the mass of immobilized lipase with equivalent amount of protein as in 50 mg of lyophilized free CRL (1.76 mg protein)

After 5 hours of agitation, the reaction was stopped by adding 10 mL of acetone/ethanol (1:1 v/v) to the mixture. Titration with 0.15 M NaOH and phenolphthalein as an indicator was used to determine the residual free fatty acid in the reaction mixture.

Results were expressed in terms of percent conversion (%) as shown in Equation 3.6 and enzyme specific activity, according to Equation 3.7.

$$Conversion\ (\%) = \frac{Vcontrol - Vsample}{Vcontrol} \times 100\% \qquad (3.6)$$

$$\textbf{\textit{Specific activity}}\left(\frac{\frac{\boldsymbol{mmol}}{\boldsymbol{min}}}{\boldsymbol{mg}}\right) = \frac{(\boldsymbol{Vcontrol - Vsample})}{(\boldsymbol{t})(\boldsymbol{W})} \times \boldsymbol{M} \qquad (3.7)$$

Whereby, $V_{control}$ is titration volume of NaOH for control (ml)**,** V_{sample} **is t**itration volume of NaOH for sample (ml)**,** M is molarity of NaOH, t is reaction time (min), W is amount of protein (mg).

3.2.10.1 Screening of Immobilized Lipases

Immobilized lipases of equal amount of protein as in 50 mg of free CRL (Table 3.2) were screened following the lipase enzymatic activity assay procedures as outlined in Section 3.2.10. The immobilized lipases with highest percent conversion and specific activity were selected for further analysis.

3.2.10.2 Effect of Time Course

Lipase enzymatic activity assay as outlined in Section 3.2.11 was done by multiplying the amounts of enzymes, reactants and reaction medium by 20 folds. An aliquot (2 mL) of the reaction solution was taken at every 1 hour interval and analysed as outlined in Section 3.2.10.

3.2.11 Thermostability

Free and immobilised lipases were pre-incubated individually for 1 hour at various temperatures (30, 40, 50, 60, and 70 ℃). The lipase enzymatic activity assay was then performed and percent of conversion and specific activities were determined as outlined in Section 3.2.10.

Results of the assay were also expressed in terms of relative activities determined as shown in Equation 3.8:

$$\textbf{\textit{Relative activity}}\ (\%) = \frac{\boldsymbol{SA1}}{\boldsymbol{SA2}} \times \mathbf{100} \quad (3.8)$$

Whereby, SA1 is the specific activity at a specified pre-incubated temperature, while SA2 is the highest specific activity (obtained at optimal pre-incubated temperature).

3.2.12 Operational Stability

Reusability of the immobilized lipases were determined following the lipase enzymatic activity assay (Section 3.2.10), where immobilized lipases were filtered, washed and air dried after each reaction cycle. The immobilized lipases were then reused in the subsequent cycle of reactions following the procedures outlined in Section 3.2.10. Results obtained were expressed in terms of percent conversion, specific activity and relative activity according to Equation 3.6 - Equation 3.8.

3.2.13 Kinetic Study

Kinetic study was conducted on free and selected immobilized lipases. Lipase enzymatic activity assay was conducted in two sets of experiments. In one set of the experiment, amount of nonanol was varied between 2.0 to 10.0 mmol while the amount of hexanoic acid was fixed at 2.0 mmol. In another set of experiment, the amount of hexanoic acid was varied from 2.0 to 10.0 mmol while the amount of nonanol was kept constant at 2.0 mmol. Time course study was then conducted, together with time course plots for all reactions in both sets of experiments.

Initial reaction rates for each of the reactions above were then determined based on the slopes (mmol/L/mg of protein) of the time course plots constructed. These initial reaction rates were extrapolated against time (min).

Using enzyme kinetics module of the SigmaPlot® version 12.5 software (Systat inc., information obtained from the experiments were inserted and values of V_{max} and K_m were obtained. Kinetic model proposed by the software was then applied to simulate the enzymatic reactions in this study. The reaction rates simulated by the software was then compared with the reaction rates obtained from the experiment.

3.2.14 Verification of the Reaction Product

3.2.14.1 Isolation of Product from the Reaction Medium

Nonyl hexanoate was synthesized in a reaction system consisted of 2.242 g CRL-N-MK, 20.0 mmol of nonanol, 2.0 mmol of hexanoic acid and 20 mL of hexane. The reaction mixtures were incubated at 30°C for 5 hours in a water bath shaker with continuous shaking at 150 rpm. (Model 903, Protech, Malaysia). At the end of the reaction, enzyme was removed from the mixture through filtration while hexane was eliminated from the reaction mixture through rotary evaporation at 60℃. The isolated ester was then kept in a desiccator, prior to further verifications.

3.2.14.2 Verification of Product Using Fourier Transform-Infrared (FTIR) Spectrometer

Product of the reaction was identified using FTIR spectrometer (Spectrum 400, Perkin Elmer, UK). In this analysis, a small amount of the sample was spread evenly between two KBr plates, to create an even liquid film between the two plates. After that,

the plates were placed in a sample holder and an analysis spectrum was done. The absorption peaks for several functional groups is shown in Appendix 3.

3.2.14.3 Verification of Product Using Gas Chromatography-Mass Spectrometer (GC-MS)

Verification of the product was performed using GC-MS (GCMS-QP 2010 Plus, Shimadzu, Japan) instrument equipped with a 30 m, 0.25 mm ID, 0.25 μm thickness, non-polar column (TG-5MS VB-5 BPX 5). The carrier gas used was helium, set at a flow rate of 0.85 mL/min. The column temperature was set at 70 ℃, while the final temperature was set to 210 ℃. Before inserting the material into the instrument, an aliquot (0.2 L) was produced by dissolving 100 mg of it in 1 ml of hexane.