CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

All reagents used in this work were of analytical grade unless otherwise specified.

Proteins	**Manufacturer**
Candida rugosa lipase	Sigma, St. Louis, USA
Bovine Serum Albumin	Sigma, St. Louis, USA

Solvents	**Manufacturer**
Acetone	Merck, Germany
Ethanol	Merck, Germany
n-Hexane	Merck, Germany

Substrates	**Manufacturer**
Oleic acid (>80%)	Merck, Germany
1-Butanol (>99.5%)	Merck, Germany

Chemicals	**Manufacturer**
Sodium Hydroxide (NaOH)	Merck, Germany

Equipments	Manufacturer
Autotitrator (902 Titrando)	Metrohm, USA
Blast freezer (Model IM101S)	Sagi, Italy
Centrifuge (Allegra ^{TM} 25R)	Beckman Coulter, USA
Fourier Transform-Infrared Spectrometer (FT-IR) 1725X	Perkin Elmer, England
Freeze Drier (FD-550)	Eyela, Japan
Scanning Electron Microscope – Energy Dispersive X-Ray	Philips, USA
Waterbath Shaker (Model 903)	Protech, Malaysia
Vacuum Pump (DOA-P504-BN)	Gast, Germany
X-Ray Diffractometer	Bruker AXS, Germany
Micropipette	Nichipet, Japan

Computer Software	Supplier
SigmaPlot	Systat Software Inc. UK
GraphPad Prism	Graphpad Software Inc, California
ChemDraw Professional	Perkin Elmer

Polymer/Support	Manufacturer
Carboxymethyl cellulose	Sigma Chemical Co, USA
Polyvinyl alcohol	Sigma Chemical Co, USA

3.2 Methods

3.2.1 Protein Loading Assay

The amount of protein loading before and after immobilization was determined by the Bradford Coomassie Brilliant Blue Assay procedure (Bradford, 1976). In this method, the Bradford reagent mixture containing 25 mg of Coomassie Brilliant Blue G-250 was dissolved in 12.5 mL of ethanol (95%), and added with 25 mL of phosphoric acid (85%), and diluted to 250 mL with distilled water. In addition, 1 mg of Bovine Serum Albumin (BSA) was dissolved with 5.0 mL of distilled water by respective ratios (APPENDIX A) and measured at wavelength of 595 nm. The readings were used in constructing a standard curve (APPENDIX B). Then, lipase solution (before and after immobilization) and distilled water were then added to respective ratios, and measured at the same wavelength. The amounts of enzyme absorbed onto the support were expressed as a percent of ester conversion (APPENDIX E).

3.2.2 Partial Purification of Lipases

The commercial native lipase from *Candida rugosa* (CRL) was partially purified using water extraction prior to immobilization. The purification was carried out by adding 5.0 g of native CRL into 100 mL of distilled water. The mixture was stirred for 30 minutes and then centrifuged (Allegra TM 25R, Beckam Coulter, USA) at 0 ^{o}C for 15 minutes at 10,000 rpm. The undissolved solid suspension from the lipase solution was discarded after centrifugation while the supernatant was collected prior to immobilization.

3.2.3 Entrapment of Lipase in Polymeric Thin Film

The film-forming solutions containing CMC, PVA or a mixture of both were prepared by dissolving the polymers (0.4 g of each CMC, PVA and 0.2g of PVA: 0.2g of CMC blend) in constantly stirred 30 mL distilled water until completely dissolved. 1 mL of partially purified lipase solution, containing 50 mg of CRL was then added to each of the polymer solutions. The mixtures were continuously stirred for about 45 minutes before they were carefully poured into Teflon Petri-dish and dried at temperatures between 30-35 ^{o}C for 24 h. The thin films of entrapped lipase were collected and cut into regular sections of 3 mm^2 and kept at 2-8 ^{o}C, prior to use, based on Roberto et al (2005) method with some modifications.

3.2.4 Characterizations of Polymeric Thin Films

The CMC, PVA and PVA:CMC thin films with and without entrapped CRL were analyzed by using X-Ray Diffractometer (XRD), Scanning Electron Microscopy (SEM) equipped with Energy Dispersive X-Ray (EDX) and Fourier-Transform Infrared (FTIR) spectrometer to analyse the possible interactions between the immobilization matrix (polymer) and the biocatalyst.

3.2.4.1 Analysis using Scanning Electron Microscope and Energy Dispersive X-Ray (SEM/EDX)

In the characterization of samples using SEM (Phenom, G2 Pro), samples were mounted onto carbon tape and sputter-coated with gold before being scanned. The samples were then scanned at 15 kV at 1000x magnifications. Elemental analyses of samples were subsequently conducted using an EDX equipped with the SEM unit.

3.2.4.2 Analysis using X-Ray Diffractometer (XRD)

The samples were first mounted on glass slides and scanned between 2-65 °C with 2θ/min at 0.003° per analysis. The XRD patterns of polymer thin films were obtained after analysis using X-ray diffractometer (XRD D8 Advance, Germany) which was operated at 40 kV and 40 mA, with 1-D fast detector (Lynax-Eye) and CuKα radiation at a wavelength of λ= 1.54 Å. (Zaidan et al., 2010).

3.2.4.3 Analysis using Fourier Transform Infrared (FTIR)

In the analysis using FTIR, the samples were placed in horizontal attenuated total reflectance (HATR), consisting of Germanium crystal at a controlled temperature (20 °C). All spectra were measured and subtracted with a background spectrum of air, at each scanning of the samples. The spectrum was recorded as transmittance value at each frequency point data conducted for 6 samples in triplicates.

3.2.5 Lipase Activity Assay

The catalytic activities of native and immobilized *Candida rugosa* lipases were studied for the esterification reaction between 1-butanol and oleic acid in n-hexane, in triplicates. Equimolar (5.0 mmol) of oleic acid and n-butanol and 5 mL of n-hexane were mixed in screw cap vials. Native (50 mg) or immobilized CRL containing an equal amount of protein as in the native CRL were then added to the reaction mixture. The mixtures were incubated in waterbath shaker (Model 903, Protech Malaysia) at 200 rpm, for 6 hours at 37 ^{o}C. Control reactions without the presence of catalyst were also separately incubated. After the incubation period, 10 mL of acetone:ethanol was added into the vials. The remaining fatty acids in the mixtures were then titrated with 0.15M NaOH using autotitrator (902 Titrando, Metrohm, USA). The lipases activities

were expressed in terms of a specific activity (APPENDIX C), relative activity (APPENDIX D) and percentage conversion of ester (APPENDIX E).

3.2.6 Stability of Native and Immobilized Lipases

3.2.6.1 Storage Stability

Native (50 mg) and immobilized CRL with an equal amount of protein as in 50 mg of native CRL were separately stored at -20 ^{o}C, 0 ^{o}C, 4 ^{o}C and room temperature (27 ^{o}C), for a period of 60 days before their activities were assayed in triplicates following the procedures outlined in Section 3.2.4. The lipases activities were expressed in terms of a specific activity (APPENDIX C), relative activity (APPENDIX D) and percentage conversion of ester (APPENDIX E).

3.2.6.2 Reusability (Operational Stability)

The operational stabilities of the immobilized CRL were done by repeatedly utilizing the lipases in the esterification reaction system for nine times. After each reaction cycle, the reaction mixture was treated with NaOH to determine the remaining fatty acid in the mixture. The lipases activities were expressed in terms of a specific activity (APPENDIX C), relative activity (APPENDIX D) and percentage conversion of ester (APPENDIX E). Meanwhile the immobilized CRL were collected, rinsed with n-hexane and used in the subsequent reaction cycle.

3.2.7 Kinetic Studies on Native and Immobilized Lipases

In this study, native and immobilized CRL with equivalent amounts of protein as in 50 mg of native CRL (1.08 mg protein) were used. In the first set of experiments, the concentration of 1-butanol was varied from 2.0 mmol to 10.0 mmol while the concentration of oleic acid was kept constant at 2.0 mmol. In the subsequent set of

experiments, the concentration of oleic acid was varied from 2.0 mmol to 10.0 mmol while the concentration of 1-butanol was kept constant at 2.0mmol. The reactions were intubated at 37 ^{o}C with continuous agitation at 200rpm in waterbath shaker (Model 903, Protech, Malaysia). The reaction were terminated by addition of acetone/athanol (1:1 v/v, 10mL) and conversion of ester (%) was determined by titration the remaining free fatty acid in the reaction mixture with 0.15M NaOH using an autotitrator (902 Titrando, Metrohm, USA) to end point of pH 10. The initial reaction rates (V_o) were estimated from the slope of butyl oleate formation (mmol/L/mg of protein) plotted against the time (min). the value for kinetic constants, V_{max} and K_m were determined by plotting the Lineweaver-Burk plot using nonlinear regression method through Graphpad software (Prism 5) based on data points obtained from the experiments.