CHAPTER III

MATERIALS & METHODS

3.1 Materials and chemicals

Purified palm oil used in this study was bought from local supermarket (Bangkok, Thailand). Palm fatty acid was kindly provided by Songchai Thamphimukwattana, Buraphamunkong Co. Ltd.,. Lipase from *Candida rugosa* was purchased from Sigma Chemical Co. Ltd., (St. Louis, USA). 95% ethanol was used as a reactant in this work. All chemical reagent used in this work were analytical grade, and were purchased from commercial sources in Thailand.

3.2 Lipase Immobilization

3.2.1 Immobilization of *C. rugosa* lipase on $CaCO_3$

The enzyme immobilization was made onto $CaCO_3$ according to Rosu et al. (1997) with a slight modification. A support powder (0.5 g) was added to 3 ml of enzymatic solution (containing 120 U). The mixture was incubated 1 h at 4 °C under mild agitation. Afterwards, 10 ml of chilled acetone was added, and the suspension was filtered through a Buchner funnel, The immobilized lipase was washed two times with another 10 ml aliquot of chilled acetone, dried in a vacuum desiccator at room temperature for 6 h and stored at 4 °C until use.

3.2.2 Entrapment of *C. rugosa* lipase immobilized on $CaCO_3$ in calcium alginate bead

C. rugosa lipase which was pre-immobilized by adsorption on $CaCO_3$ was encapsulated in calcium alginate beads. The formation of calcium alginate bead was modified from the previous work (Hertzberg et al., 1992) by the following procedure:

1% sodium alginate solution (w/v) and 0.1 M calcium chloride solution (10 ml) prepared in 0.01 M phosphate buffer (pH 7.0). 700 mg of enzyme immobilized on $CaCO_3$ was dispersed uniformly in 1% sodium alginate solution. The sodium alginate solution containing the enzyme was injected through a syringe 0.1 M calcium chloride solution from a constant distance. The beads were allowed to harden in calcium chloride solution for a half of hour. The beads were kept in organic solvent for 1 h.
Prestudying of the controlled parameters of immobilized *C. rugosa* lipase on hydrolysis activity were including;

- The effect of lipase quantity
- The effect of adsorption time
- The effect of enzyme/$CaCO_3$ ratio
- The effect of bead diameter
- The effect of alginate concentration
- The effect of temperature

3.3 Enzymatic transesterification and esterification reaction

The transesterification and esterification reactions were carried out in 50 ml erlenmayer flask containing mixture of palm oil and ethanol in a molar ratio of 1:9 (or the molar ratio of palm fatty acid to ethanol 1:3) and 5% lipase (based on weight of oil) in 3 ml 0.01 M phosphate buffer (or 700 mg immobilized lipase) under shaking at 250 rpm.
The considerable variables on the ethyl ester production were including;

- The effect of shaking speed
- The effect of molar ratio of ethanol to reactants
- The effect of mass ratio of substrate mixture
- The effect of addition time of palm fatty acid
- The ethyl ester production by using immobilized *C. rugosa* lipase
- Repeated use of the immobilized *C. rugosa* lipase

3.4 Analytical methods

3.4.1 Enzyme activity assay

Titrimetric method

The hydrolytic activities of enzymes in various preparation were assayed titrimetrically using olive oil emulsion method (Yadav et al., 2005). The substrate was prepared by mixing 50 ml of olive oil with 50 ml of arabic gum solution (7% w/v). The reaction mixture consisting of 20 ml of emulsion, 2 ml of 0.1 M sodium phosphate buffer, pH 7.0 and free enzyme 1 ml (or immobilized lipase 200 mg), was incubated for 12 h at 37°C. The liberated fatty acid was titrated with 0.05 N potassium hydroxide solution using phenolphthalein as an indicator and consumed amount was marked V_1. The consumed amount in the control experiment (lipase was not added into the reaction mixtures.) was marked V_0.

One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of free fatty acids per minute under the assay conditions. The specific activity was expressed as U/mg-protein.

Lipase activity (U) calculation formula : $$\frac{(V_1 - V_0) x N_{KOH} x10}{t x10^{-6} x1000}$$

3.4.2 Protein Assay

Protein concentration was estimated by the method of Bradford using Bio-Rad protein dye reagent concentrate and bovine serum albumin as a standard (Coomassie Plus-The Better, Bradford™ Assay Kit).

3.4.3 Ethyl ester analysis

The ethyl ester (EEs) and the fatty acid ethyl esters (FAEEs) analysis was carried out using GC (Shimadzu 2010 model) with a flame ionization detector (FID) in which one microliter of the sample was injected into column. The GC consists of a capillary column (DB-WAX, Carbowax 20 M, 30 m, 0.32 mm ID, 0.25 μm). The injector, detector, and column temperatures were at 250, 260 and 200 °C respectively.

Pressure was 64.1 kPa and linear velocity was 25 cm/sec. The carrier gas was helium (He) and the make-up gas was nitrogen (N_2). The sample was prepared by adding 0.1 ml of FAMEs to 4.9 ml of n-hexane. The ethyl ester yield was estimated from the ratio of the quantity of FAMEs to that of the reactants (purified palm oil or palm fatty acids):

$$\% \text{ yield of methyl ester} = \frac{W_{ME}}{W_F} \times 100 \qquad (3.1)$$

where W_{ME} and W_F are weights of ethyl ester (g) and the feed reactant (g), purified palm oil or palm fatty acid, respectively.