CHAPTER III
EXPERIMENTAL

3.1 Materials

Fresh Rice bran was purchased from Manathanya Phanich Co., Ltd. Rice bran oil was a product from Thai Edible Oil Co., Ltd. Phenolphthalene, potassium hydrogen phthalate, dipotassium hydrogen phosphate, and standard buffer pH 4, 7, 10 were obtained from Ajax Chemicals, USA. Ammonium sulphate was obtained from Ajax Chemicals and ground by mortar before use. Adipic acid, potassium dihydrogen phosphate, isopropyl ether, and 1,4-butanediol were purchased from Fluka. Acetone, and tetrahydrofuran were bought from J.T. Baker Hexane, absolute ethanol, calcium chloride, and sodium hydroxide were purchased from Lab Scans, Carlo Erba, Merck, and Akzo Nobel, respectively. Adipic acid was recrystallized, while diisopropyl ether and 1,4-butanediol were distilled before use.

3.2 Equipment

3.2.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

FTIR spectra were obtained from VECTOR 22 Bruker Spectrometer equipped with deuterated triglycine sulphate (DTGS) detector. An absorbance mode was used with 16 scans and 4 cm^{-1} resolution.

3.2.2 Ultraviolet-Visible Spectrophotometer (UV-VIS)

Rice bran lipase solution and its concentration were qualitatively and quantitatively analyze by a Lambda-16 UV-VIS spectrophotometer, Perkin-Elmer.

3.2.3 Gel Permeation Chromatography (GPC)

GPC chromatograms were performed by Waters GPC 600E with RI (Waters 410) and UV detectors (Water 486). The separating columns were HR 0.5 and HT 3 for the range of molecular weight 0-1,000 and 500-30,000, respectively. The difference of refractive index was used for determination the molecular weight of polyester.

3.2.4 High Speed Refrigerated Centrifuge

A high speed refrigerated centrifuge (Sorval Super T 21) was used for separation the rice bran particles and rice bran lipase at 4°C, 10,000 rpm and 15,000 rpm for 10 and 15 minutes, respectively.

3.2.5 Lyophilizer

The rice bran lipase solution was lyophilized by Flexi-Dry FTS systems from STONE RIDGE, New York, USA.

3.2.6 Nuclear Magnetic Resonance (NMR)

FT-NMR spectra were obtained from JEOL (JNM-AL300) to qualitative measure the concentration of adipic acid, 1,4-butanediol and the produced polyester.

3.3 Methodology

3.3.1 Preparation of Rice Bran

Fresh rice bran was sieved to exclude broken grains, rice hull, and insects by using 35 mesh size sieving machine.

3.3.2 Specific Activity of Rice Bran Lipase Solution

Rice bran was suspended and stirred in two solvents i.e., 0.05 M phosphate buffer pH 7 and 10 mM calcium chloride varying ratios of rice bran to solvent from 1:3 to 1:6 for 1, 2, 3, 4, 5, 6, 7 and 8 hours in ice bath. After extraction, the obtained rice bran was re-extracted for three times.

The rice bran lipase solution was filtered to remove rice bran particles. The filtrate was centrifuged at 4°C, 10,000 rpm for 10 minutes. After centrifugation, the supernatant was collected to measure the activity and amount of protein. The amount of protein can be determined by equation (3.1)

$$\text{amount of protein (mg/mL)} = \frac{A_{RBL}}{E^{1\%}_{280}} \qquad (3.1)$$

where A_{RBL} is absorbance at 280 nm of rice bran lipase solution

$E^{1\%}_{280}$ molar extinction coefficient of rice bran lipase

Rice bran lipase activity was measured by titration technique as follows. Rice bran oil solution was prepared by adding 1 mL of rice bran oil, 1 mL of 10 mM calcium chloride, 3 mL of distilled water, and 4 mL of 0.05 M phosphate buffer pH 7 in 250 ml flask. The solution was heated to 30°C in shaking bath for 15 minutes and the prepared rice bran lipase solution was added. The mixture was shaken at 30°C for 1 hour, followed by adding acetone:ethanol (1:1) for 20 mL to terminate the hydrolytic reaction. The

solution was centrifuged at 10,000 rpm for 10 minutes. The fatty acid product was determined by titration of the aqueous phase with 0.05 N sodium hydroxide using phenolphthalene as an indicator. The changing of pH was also confirmed by pH meter. The procedures were repeated for the control system without the addition of the rice bran lipase solution. These procedures were repeated for three times. The specific activity was evaluated from the average values of the fatty acid in the micromole (μmole) unit per 1 minute per mg protein, as shown in eq. (3.2)

$$\text{Specific activity (mU/mg)} = \frac{\text{activity (mU/mL)}}{\text{amount of protein (mg/mL)}} \qquad (3.2)$$

3.3.3 Purification of Rice Bran Lipase

The sieved rice bran (800 g) was suspended in 2.4 L of 10 mM calcium chloride and stirred for 3 hours in ice bath. The rice bran lipase solution was filtered to remove rice bran particles. The filtered solution was centrifuged at 4°C, 10,000 rpm for 10 minutes. The supernatant was collected and kept in the ice bath. Ammonium sulphate powder was gradually added to make the concentration to 60% saturation and the turbid solution was left overnight. The solution was centrifuged at 4°C, 15,000 rpm for 15 minutes to obtain a brown slurry. A small amount of 1 mM calcium chloride was added to dissolve the product. Ammonium sulphate was removed by dialysis in 1.5 L of 1 mM calcium chloride overnight in ice bath. The solution was centrifuged at 4°C, 15,000 rpm for 15 minutes. The supernatant was solidified by liquid nitrogen and dried by lyophilizer machine. The obtained rice bran lipase solid was stored at 4°C until use.

3.3.4 Specific Activity of Rice Bran Lipase Solid

Rice bran lipase solution was prepared by dissolving 3 mg rice bran lipase in 3 mL distilled water to obtain the concentration of 1 mg/mL. The activity was measured as the same procedures mentioned in 3.3.2.

3.3.5 Purification of Monomers and Solvents

Adipic acid was purified by recrystallization in water and acetone, respectively. The purified adipic acid was dried in the oven at 120°C for 2 hours and kept in dessicator until use. 1,4-butanediol was purified by vacuum distillation after treatment with anhydrous sodium sulphate overnight. Isopropyl ether was stored with sodium hydroxide overnight followed by fractional distillation.

3.3.6 Rice Bran Lipase-Catalyzed Esterification and Polyesterification

An equimolar amount of adipic acid and 1,4-butanediol (22.49 mmol), as monomers. Rice bran lipase (3 g) was added into diisopropyl ether followed by adipic acid. The mixture was allowed suspending in the solvent at 35°C for a certain time. When the reaction time reached 1, 3, 5, and 7 days, the suspension was filtered to separate rice bran lipase from the solution. Rice bran lipase was washed thoroughly with THF. The THF phase was collected and evaporated to give a viscous solution of the crude product. For the filtrate solution, the solvent was removed to obtain a viscous solution of the crude product. The crude product from each phase was combined and extracted by hexane. The hexane solution was concentrated to achieve the product, which was characterized by FT-IR and GPC. The similar condition was operated by using diphenyl ether as a solvent.