CHAPTER 2

MATERIALS AND METHODS

Materials

1. Microorganisms

Bacterial strains used in this study were obtained from the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Thailand and 3 strains of *Actinomycetes* were obtained from the Department of Microbiology and Bioprocess Technology, Institute of Biochemistry, University of Leipzig, Leipzig, Germany.

2. Lipases

Various commercial lipases used in this study, available in powder or immobilized forms, were obtained from various suppliers as shown in Table 12.

Table 12. Commercial lipase powder and immobilized lipases used in this work.

Lipases	Trade name	Supplier
Powder form		
Candida rugosa	Lipase AY	Amano, Japan
Pseudomonas cepacia	Lipase PS	Amano, Japan
Pseudomonas fluorescens	Lipase AK	Amano, Japan
Rhizopus delemar	Lipase D	Amano, Japan
Rhizomucor javanicus	Lipase M	Amano, Japan
Immobilized form		
Candida antarctica B	Novozym 435	Novo, Denmark
	Chirazyme L-1	Roche, Germany
Rhizomucor miehei	Lipozyme RM IM	Novo, Denmark

3. Supports

Celite 545 (200 μm) and Amberlite XAD-7 were obtained from Fluka, Switzerland. The polypropylene powder EP100 or Accurel (<400 μm) was obtained from Akzo Nobel Membrana, Germany. Syran (SIKUG 012) was obtained from Schott Engineering, Germany and Polyvinylchloride or PVC (250 μm) was obtained from Vinylthai, Thailand.

4. Raw materials

Palm oil, olive oil, sunflower oil, soybean oil, rice bran oil, lard, tallow and coconut oil were purchased from the local suppliers. Palm stearin was a gift from Chumporn Palm Oil Industry Public Co. Ltd., Thailand. Palm fatty acid distillate was obtained from the Department of Chemical Engineering, Faculty of Engineering, Prince of Songkla University, Hat Yai, Thailand.

5. Chemicals

All chemicals and solvents used were reagent grade and purchased from various suppliers as followings,

Chemical	Supplier
Acetic acid	Lab-Scan, Ireland
Acetic acid vinyl ester	TCI, Belgium
Acetone	Merck, Germany
Acetonitrile	Lab-Scan, Ireland
30% Acrylamide/bis-acrylamide (29:1) solution	Bio-Rad, USA
Ammonium chloride	Merck, Germany
Ammonium hydrogenphosphate	Carlo Erba, Italy
Ammonium nitrate	Fluka, Switzerland
Ammonium persulphate	Bio-Rad, USA
Ammonium sulphate	Merck, Germany
L-Ascorbic acid	Fluka, Switzerland
Benzene	Lab-Scan, Ireland
Butyric acid vinyl ester	TCI, Belgium
Blue dextran	Fluka, Switzerland
Bis(trimethylsilyl)trifluoroacetamide (BSTFA)	Macherey-Nagel, Germany
Bromophenol blue	Bio-Rad, USA

Chemical	Supplier
Calcium chloride	Fluka, Switzerland
Capric acid vinyl ester	TCI, Belgium
Caprylic acid vinyl ester	TCI, Belgium
Carproic acid vinyl ester	TCI, Belgium
Chloroform	Lab-Scan, Ireland
Citric acid	Fluka, Switzerland
Comassie Brilliant Blue G250	Fluka, Switzerland
Comassie Brilliant Blue R250	Bio-Rad, USA
Copper sulphate	Fluka, Switzerland
Cupric acetate	Fluka, Switzerland
Deoxycholic acid sodium salt	Fluka, Switzerland
DEAE Toyopearl	Tosoh, Japan
Diethyl ether	Lab-Scan, Ireland
Dimethylformamide	Univar, UK
Dimethylsulfoxide	Carlo Erba, Italy
Disodium hydrogenphosphate	Carlo Erba, Italy
Dithiothreitol	Bio-Rad, USA
Ethanol absolute	Lab-Scan, Ireland
Ethylene diamine tetraacetic acid (EDTA)	Fluka, Switzerland
Ferrous sulphate	Fluka, Switzerland
Ferric sulphate	Fluka, Switzerland
Formic acid	Carlo Erba, Italy
Fructose	Fluka, Switzerland
Gum arabic	Nacalai, Japan
D-Glucose	Fluka, Switzerland
D-Galactose	Fluka, Switzerland
Glycine	Bio-Rad, USA
Hydrochloric acid	J.T.Beker, USA
iso-Octane	Lab-Scan, Ireland
iso-Propanol	Lab-Scan, Ireland
Lauric acid vinyl ester	ICI, Belgium
Linoleic acid	Fluka, Switzerland
Lithium bromide	Fluka, Switzerland
Lithium chloride	Fluka, Switzerland

Chemical	Supplier
Magnesium chloride	Merck, Germany
Magnesium nitrate	Merck, Germany
Malt extract	Merck, Germany
Maltose	Sigma-Aldrich, Germany
Maltotriose	Sigma-Aldrich, Germany
2-Mercaptoethanol	Bio-Rad, USA
Methanol	BDH AnalaR, England
2-Methyl-2-butanol	Fluka, Switzerland
Methyl-α-D-glucopyranoside	Fluka, Switzerland
Methyl-*tert*-butyl ether	Fluka, Switzerland
Molecular sieves 4°A	Wako Pure, Japan
n-Hexane	J.T.Beker, USA
n-Heptane	J.T.Beker, USA
4-Nitrophenol	Fluka, Switzerland
4-Nitrophenyl acetate (*p*NPA)	Fluka, Switzerland
4-Nitrophenyl butyrate (*p*NPB)	Fluka, Switzerland
4-Nitrophenyl caprylate ($pNPC_8$)	Fluka, Switzerland
4-Nitrophenyl laurate (*p*NPL)	Fluka, Switzerland
4-Nitrophenyl plamitate (*p*NPP)	Sigma-Aldrich, Germany
4-Nitrophenyl stearate (*p*NPS)	Fluka, Switzerland
N,N,N,N'-Tetramethylethylenediamine (TEMED)	Bio-Rad, USA
Octyl-β-D-glucose	Sigma-Aldrich, Germany
Oleic acid	Fluka, Switzerland
Palmitic acid	Nacalai, Japan
Palmitic acid vinyl ester	TCI, Belgium
p-Chloromercuribenzoate (*p*CMB)	Sigma-Aldrich, Germany
p-Hydroxymercuribenzoate (*p*HMB)	Sigma-Aldrich, Gemany
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, Germany
85% Phosphoric acid	BDH AnalaR, England
Potassium acetate	Merck, Germany
Potassium carbonate	Merck, Germany
Potassium chloride	Merck, Germany
Potassium iodide	Merck, Germany
Protein markers	Bio-Rad, USA

Chemical	Supplier
Rhodamine B	Fluka, Switzerland
Resource Q 1 mL	Pharmacia, Sweden
Sephadex G-150	Pharmacia, Sweden
Silica gel 60	Merck, Germany
Silver nitrate	Merck, Germany
Superdex 200	Phamacia, Sweden
Sodium citrate	Wako Pure, Japan
Sodium chloride	Carlo Erba, Italy
Sodium dodecyl sulphate (SDS)	Bio-Rad, USA
Sodium hydroxide	Merck, Germany
Sodium sulphate anhydrous	Carlo Erba, Italy
D-Sorbitol	Fluka, Switzerland
Sulphuric acid	Lab Scan, Ireland
tert-Butanol	Fluka, Switzerland
Tributyrin	Fluka, Switzerland
Trimethylsilylimidazol (TMSI)	Macherey-Nagel, Germany
Tris(hydroxymethlyl)aminomethane	Carlo Erba, Italy
Triton X-100	Wako Pure, Japane
Tryptone	Merck, Germany
Tween 20	Labchem, Australia
Tween 80	Labchem, Australia
Xylose	Fluka, Germany
Zinc(II) chloride	Merck, Gemany

6. Instruments

Equipments	Series	Supplier
Chromarods	SM-III	Iatron Laboratories, Japan
Chromarods holder	SD-5	Iatron Laboratories, Japan
Developing tank	TD-150	Iatron Laboratories, Japan
Evaporator	SB- XL 651	Tokyo Rikakikai, Japan
Gas chromatography	XL	Perkin-Elmer, USA
	CP-3380	VARION, Germany
GC column 25 m x 0.25 mm	OPTIMA-5, OPTIMA-5MS (25 m x 0.25 mm)	Macherey-Nagel, Germany
Hot air oven	UM 200	Memmert, Germany
Mini Protein Dual Slab Cell	Model 1000-500	Bio-Rad, USA
^{1}H-NMR	250 MHZ	Bruker Avance, Germany
Mass spectrometry	MALDI-TOF	Shimadzu, Germany
Power supply	Model Power PAC-300	Bio-Rad, USA
TLC-FID (Iatroscan)	MK5	Iatron Laboratoties, Japan
Incubator	MIR-153	Sanyo, Japan
pH meter	420A	Orion Research Inc., USA
Shaker	GFL 3005	Germany
Silica gel 60 plate	F_{254}	Merck, Germany
Spectrophotometer	GENESYS^{TM} 10	Themo Electron Corporation, USA
Thermomixer	Comfort	Eppendorf, Germany
Ultrafiltration (UF)	Pellicon 2 Module	Millipore, USA

Analytical Methods

1. Hydrolytic activity of lipases

1.1 Free lipase

Hydrolytic activity of free lipase was determined by spectrophotometric method using *p*-nitrophenyl esters (*p*-nitrophenyl palmitate (*p*NPP) or *p*-nitrophenyl caprylate ($pNPC_8$)) as a substrate. The substrate solution composted of 1.0 mL of iso-propanol (propa-2-ol) containing 30 mg of *p*NPP or $pNPC_8$ mixed with 9 mL of 50 mM Tris-HCl buffer pH 7.5, containing Triton X-100 (0.4% w/v) and gum arabic (0.1% w/v). The 0.1 mL of lipase solution was mixed well with 0.9 mL of substrate mixture and measured the absorbance at 410 nm (Kademi *et al*., 2000). One unit of enzyme was the amount of enzyme liberating one μmol of *p*-nitrophenol/mL/min at 30°C. The molar adsorption coefficient of *p*-nitrophenol at pH 7.5 and 8.5 were 12,442 and 17,230 cm^2/mg, respectively (Appendix 1).

1.2 Immobilized lipase

Hydrolytic activity of free and immobilized lipases was assayed by the modified cupric acetate method (Lee and Rhee, 1993). The cupric acetate solution (5% w/v) was prepared and pH was adjusted to 6.1 by pyridine. For the lipase reaction in two-phase system, 0.5 mL of enzyme solution or 5.0 mg of immobilized enzyme, 1.0 mL of 50 mM Tris-HCl buffer pH 7.5 and 1.5 mL of 10% palm oil in iso-octane was mixed and incubated at 300 rpm at 45°C for 30 min. The enzyme reaction was stopped by adding 0.3 mL of 6 M HCl.

The upper iso-octane layer of 1.0 mL was taken out and mixed with 0.4 mL cupric acetate solution. Free fatty acid dissolved in iso-octane was determined by measuring the absorbance at 715 nm against the control which contained no free fatty acid. The lipase activity was determined by measuring the amount of fatty acid from the standard curves of palmitic acid (Appendix 2). One unit of enzyme activity was defined as the enzyme necessary to release 1 μmol of palmitic acid per minute at the specified condition.

2. Determination the compositions of glycerides, palm fatty acid distillates (PFAD), sugar esters (SE) and fatty acid methyl esters (FAME) by TLC-FID analysis

The compositions of palm oil and PFAD were determined using a thin layer chromatography equipped with flame ionization detector or TLC-FID (Iatroscan MK5, Iatron Laboratories Inc., Japan) (Rosu *et al*., 1998). One μL of palm oil or PFAD solution (diluted in chloroform at appropriate dilution) was spotted on to the Chromarods, which was then

developed in a solvent mixture of benzene/chloroform/acetic acid (50:30:0.5 v/v/v) until the solvent front reached 10 cm (approximately 35 min). The Chromarods were dried at 105°C for 5 min and scanned process was performed using hydrogen flow rate of 150 mL/min, an air flow rate 700 mL/min and a scanning speed was 30 sec/rod to produce a chromatogram. The compositions were calculated as % mole based on the peak area of each component (Appendix 3 and 4).

For the reaction mixture containing SE, Chromarods were developed in a solvent mixture of chloroform/methanol/formic acid (50:10:1 v/v/v) for 25 min. Then the Chromarods were dried at 105°C for 5 min and scanned with TLC-FID (Rosu *et al.*, 1998) (Appendix 5).

For the reaction mixture containing fatty acid methyl esters (FAME), Chromatrods were developed in two solvent systems (Paripatanapairod, 2003). First, development was carried out in a solvent mixture of *n*-hexane/diethyl ether/formic acid (50:20:0.7 v/v/v) for 15 min (solvent front = 8 cm). After that, Chromarods were subsequently developed in a second solvent system composed of benzene/hexane (1:1 v/v) for 33 min or until the solvent front reach to 10 cm. Then the Chromarod was dried at 105°C for 5 min and scanned with TLC-FID (Appendix 6).

3. Determination of fatty acid compositions by GC analysis

Fatty acid compositions of TAG and PFAD were determined by converting all fatty acids of TAG to the corresponding FAME followed by GC analysis (McNeill *et al.*, 1996). After evaporation of excess solvent, 10 mg of acylglycerol was methanolyzed with 0.5% NaOH (0.5% HCl in case of PFAD) in methanol (500 μL) and then incubated for 15 min at 60°C. The FAME was extracted with hexane (400 μL) for 1 min. The hexane layer was washed with 200 μL distilled water and dried over sodium sulphate anhydrous. Analysis was carried out with a PERKIN-ELMER AutoSystem XL Gas Chromatography (Perkin-Elmer Corporation Norwalk, CT, USA) on a OPTIMA-5 column (OPTIMA-5, 0.25 μm x 25 m, Macherey-Nagel, Germany) (Shimada *et al.*, 1994) with split ratio of 50:1. Helium was used as carrier gas with 1.65 mL/min flow rate. The temperature program used was 150°C (40°C/min, 0.5 min), 170°C (5°C/min), 195°C (10°C/min) and 215°C (9.5 min). Injector and detector temperatures were 250°C. Response factors were determined using a standard mixture of FAME (Appendix 7).

4. Determination of SE compositions by GC analysis

SE compositions were determined by converting to a trimethylsilyl compounds followed by GC analysis (Degn *et al.*, 1999). The sample was evaporated excess solvent using N_2 gas. Then pyridine (100 μL) containing 3.3 mg/mL of octyl-β-D-glucopyranoside (as internal standard), 75 μL BSTFA and 50 μL TMSI were added and the mixture was incubated at 70°C for 30 min. Pyridine was then removed by flushing with N_2 and 1 mL of *n*-heptane was added. Aliquot 0.5 μL was injected using splitless injection mode with gas chromatography equipped with an OPTIMA-5MS column (OPTIMA-5MS, 0.25 μm x 25 m, Macherey-Nagel, Germany) Helium was used as the carrier gas with 1.65 mL/min flow rate. The initial temperature was 90°C and then the temperature program used was 90 to 250°C (40°C/min) and from 250 to 310°C (15°C/min). Injector and detector temperatures were 250°C.

5. TLC separation of SE

Impregnated silica gel plates with fluorescent indicator (Silica gel 60, F_{254}) were prepared by drying at 105°C for 10 min and kept in a desiccator until used (Degn *et al.*, 1999). The reaction time of sugar ester synthesis was periodically taken and 1 volume of pyridine was added to dissolve sugar and its ester. The liquid sample of 20 μL was applied to the plate, dried at 105°C for 10 min and then developed in a solvent mixture of chloroform/methanol/formic acid (50:10:1 v/v/v) to separate sugar and its ester. The plate was dried as above conditions and sprayed with a visualizing agent (sulphuric acid/ethanol 1:1 v/v) followed by heating at 105°C for 15 min.

6. Purification of SE

After 72 h of reaction time, the immobilized lipase and molecular sieves were separated by filtration. The organic solvent was evaporated under reduced pressure and 5 volume of hexane was added to precipitate the product. The remaining sugar was removed by washing the precipitate several times with distilled water followed by drying in a desiccator for 24 h. The product 100 mg was dissolved in 10 mL of a mixture of chloroform/methanol/formic acid (50:10:1 v/v/v) and 2 g of silica gel 60 was added. The solvent was removed under reduced pressure and the silica gel was transferred to 10 mL disposable syringe. The remaining fatty acid was eluted with 15 mL of a solvent mixture of chloroform/methanol (95:5 v/v). The product was finally eluted with 15 mL of the solvent mixture of chloroform/methanol/formic acid (50:10:1 v/v/v) and the solvent was evaporated under reduced pressure (Ducret *et al.*, 1995). The product was checked the purity by GC analysis (Degn *et al.*, 1999).

7. Structure elucidation of SE by ^{1}H-NMR and mass spectrometry

The structure of purified product was elucidated by ^{1}H-NMR (250 MHZ, Bruker Avance spectrometry, Karlsruhe, Germany) in CD_3OD. The matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was carried out on an Axima QIT spectrometer (Shimadzu, Deutschland GmbH, Duisburg, Germany). The structure of purified product was compared with the published data (Degn *et al*., 1999).

8. Protein assay

Protein was measured using the method of Bradford (1976) with bovine serum albumin (BSA) as standard (Appendix 8). For the eluate from the chromatographic column, the protein concentration was determined by measuring the absorbance at 280 nm.

9. Cell growth

Cell growth of microorganism was measured by using spectrophotometer with the absorbance at 660 nm or measuring of total cell protein. The cell was hydrolyzed by 0.1 M NaOH at 95°C for 15 min and the cell protein was measured by Bradford method (Bradford, 1976).

10. Molecular weight of palm oil and PFAD

The molecular weight of palm oil and PFAD was calculated from the saponification value which determined by the method of AOAC (1999) (Appendix 9).

Experimental Methods

The experiments were done with triplicate and were designed in Completely Randomized Design (CRD). Analysis of Varian (ANOVA) and Duncan's Multiple Range Test (DMRT) were used for data analysis. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS for Windows:SPSS Inc.).

1. Purification of PFAD

Crude PFAD was purified by winterization at 4°C in *n*-hexane. The sample of 200 g was dissolved in 500 mL of *n*-hexane and set in the water bath at 45°C for 30 min. The un-dissolved impurity was filtered out and the organic phase was cooled to 4°C for 30 min. The precipitate was recovered by filtration, washed several times with chilled *n*-hexane, and dried in a desiccator until used. The composition of crude PFAD and partially purified PFAD were determined by TLC-FID and GC.

2. Hydrolysis activity of commercial lipase

The hydrolytic activity of eight commercial lipases (Lipase AK, Lipase AY, Lipase D, Lipase M, Lipase PS, Chirazyme L-1, Novozym 435 and Lipozyme RM IM) was determined using modified cupric acetate method (Lee and Rhee, 1993).

3. Selection of support for immobilized lipases

The commercial lipases (Lipase AK, Lipase AY, Lipase D, Lipase M and Lipase PS, 500 U) were dissolved in 10 mL of Tris-HCl buffer pH 7.5. One gram of various supporters including celite 545 (200 μm), Accurel EP 100 (<400 μm), Syran (SIKUG 012), PVC (250 μm) and Amberlite XAD-7 were added to the lipase solution and stirred using a magnetic stirrer for 1 h at 100 rpm at 30°C. The hydrophobic supporting materials (Accurel EP 100 and PVC) were pre-wetted with ethanol before addition to the enzyme solution. The solution was filtered through a Buchner funnel. The immobilized lipase was washed twice with the same buffer to remove unadsorbed soluble lipase, followed by drying in a desiccator for 2 days (Rosu *et al*., 1997). The hydrolytic activity of unadsorbed lipase and immobilized lipase was determined. The activity yield and immobilized yield were calculated using the following formula:

$$\text{Activity yield} = \frac{\text{Immobilized enzyme activity (U)} \times 100}{\text{Initial enzyme activity (U)}}$$

$$\text{Immobilized yield} = \frac{\text{Initial enzyme activity (U)} - \text{Unbounded enzyme activity (U)} \times 100}{\text{Initial enzyme activity (U)}}$$

4. Optimization of SE synthesis

The reaction mixture contained of palmitic acid 0.5 mmol and D-glucose 0.5 mmol in 5.0 mL acetone in capped vial. The initial water activity of reaction mixture and immobilized lipases were adjusted to 0.11 by incubating over saturated aqueous solution of LiCl for 3 day at 30°C. The reaction was started by adding of the immobilized lipase (150 U/mL solvent) in to the reaction mixture and 1 g of molecular sieves (activated by heating overnight at 150°C) was added to remove water formed in the reaction. The reaction mixture was shaken on a shaker with 400 rpm at 45°C. The reaction mixture 100 μL was periodically withdrawn and 100 μL of pyridine was added to dissolve sugar and the sugar ester. The compositions (sugar, SE and FA) of reaction mixture were determined by TLC and TLC-FID.

Several factors were varied to find the optimum reaction conditions.

- **Immobilized lipase** : Lipozyme RM IM, Novozym 435, Chirazyme L-1, Lipase AK, Lipase AY, Lipase D, Lipase M and Lipase PS.

- **Type of acyl donor** : Crude PFAD, partially purified PFAD and palm oil.

- **Solvent** : acetone, *n*-hexane, 2-methyl-2-butanol, methyl-*tert*-butyl ether (MTBE) and *tert*-butanol.

- **Effect of molecular sieve loading** : 0, 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 g

- **Effect of enzyme loading** : 250, 500, 750, 1,000 and 1,500 U

- **Initial water activity (a_w)** : 0.07, 0.11, 0.25, 0.33, 0.43 and 0.53 (Appendix 10).

- **Type of acyl acceptor** : D-glucose, D-fructose, D-sorbitol, D-galactose. L-ascorbic acid and methyl-α-D-glucopyranoside.

- **Molar ratio** : 1:1, 1:2, 1:3, 1:4 and 1:5 of acyl donor to acyl acceptor

- **Temperature** : 35, 40, 45, 50 and 55°C.

5. Time course of SE synthesis

Time course of SE synthesis was studied using the optimal conditions (section 4). The compositions of product were determined by TLC-FID and GC (Degn *et al*., 1999).

6. Production of thermostable lipase, purification, characterization and application for SE synthesis

6.1 Screening of thermostable lipase producing bacteria

Three hundred bacterial strains were obtained from the culture collection of Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Thailand and 3 strains of *Actinomycetes* were obtained from the Department of Microbiology and Bioprocess Technology, Institute of Biochemistry, University of Leipzig, Leipzig Germany. The bacterial strains were cultivated in basal medium with initial pH 7.5 using either 1.0% (w/v) palm oil or 0.1% (w/v) Tween 80 as carbon source under shaking speed of 175 rpm at 45°C for 48 h. The composition of basal was composted of 0.5% tryptone, 0.5% yeast extract, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.02% $MgSO_4.7H_2O$ and 0.02% $CaCl_2.2H_2O$.

The *Actinomycetes* strains were cultivated in M65 medium supplemented with 10% (v/v) of 10% olive oil (w/v) in 10% gum arabic solution (w/v) with initial pH 7.5, 175 rpm at 45°C for 96 h. The M65 medium was composted of 1.0% malt extract, 0.4% yeast

extract and 0.4% glucose. The basal medium and M65 agar were supplemented with 0.01% of Rhodamine B as indicator for detecting the production of lipase.

6.2 Selection of thermostable lipase for SE synthesis

The microorganisms produced lipase were cultivated in basal medium or M65 medium in 2 L flask contained of 300 mL medium supplemented with appropriate carbon source. After cultivation, the culture broths were centrifuged at 4,000 rpm (1,800 *g*) for 30 min. The cell free culture broths were concentrated by rotary vacuum evaporator at 45°C until 10 times concentrated supernatants were obtained. The concentrated supernatants were dialyzed against 50 mM Tris-HCl buffer pH 7.5 at 4°C, overnight. The supernatants were re-concentrated with evaporator as described above. For immobilization of lipase, the celite was added (25 mg per mg of protein) followed by the addition of chilled acetone (-20°C) 25% by volume and stirred by magnetic stirrer for 60 min at 4°C. Then 4 volumes of chilled acetone were added and stirred for 5 min. The precipitate was recovered by filtration and washed 3 times with 20 ml chilled acetone. The immobilized enzyme was dried in dessicator until used (Degn, 2000).

The synthesis of SE was carried out in a screw capped vial. The reaction mixture contained 0.3 mmol of caproic acid vinyl ester and glucose in 3.0 ml of *tert*-butanol/pyridine (55:45 v/v) and 100 U of immobilized lipases, which were separately incubated to equilibrium water activity (a_w) to 0.07 in LiBr saturated solution for 3 days. The reaction was started by addition of immobilized lipase to the substrate mixture. Molecular sieve 4°A (0.5 g) was added to absorb water produced during the reaction. The reaction was carried out by shaking at 400 rpm on the shaker at 45°C for 72 h. The products were qualitatively analyzed by TLC and quantitatively analyzed by GC (Degn *et al.*, 1999). The immobilized lipase produced highest SE was chosen for further study.

6.3 Optimization of SE synthesis catalyzed by selected immobilized lipase

The SE synthesis was optimized by the selected immobilized lipase. The reaction conditions were 0.3 mmol of caproic acid vinyl ester and D-glucose in 3.0 ml of *tert*-butanol/pyridine (55:45 v/v) and 100 U of immobilized lipases which were separately incubated to equilibrium water activity (a_w) to 0.07 in LiBr saturated solution for 3 days. The reaction was started by addition of immobilized lipase in to the substrate solution. Molecular sieve 4°A (0.5 g) was added to absorb water produced during the reaction. The reaction was carried out by shaking at 400 rpm at 45°C for 72 h in screw capped vial. The conversion yield was determined by GC (Degn *et al.*, 1999).

Several factors were varied as the following:

- **Type of acyl acceptor** : D-xylose, D-galactose, D-glucose, D-fructose, maltose and maltotriose.

- **Type of acyl donor** : acetic acid vinyl ester, butyric acid vinyl ester, caproic acid vinyl ester, capyric acid vinyl ester, lauric acid vinyl ester and palmitic acid vinyl ester.

- **Type of solvent** : acetone, hexane, butanone, *tert*-butanol/DMSO (95:5 v/v), *tert*-butanol/pyridine (55:45 v/v) and *tert*-butanol/pyridine (2:1 v/v).

- **Molar ratio** : 1:1, 1:2, 1:3, 1:4 and 1:5 of acyl donor to acyl acceptor (mol/mol).

- **Initial water activity** : 0.07, 0.11, 0.25, 0.33, 0.43, 0.53 and 0.75.

- **Temperature** : 35, 40, 45, 50 and 55°C.

6.4 Time course of SE synthesis catalyzed by selected immobilized lipase

Time course of sugar ester synthesis was studied using the optimal conditions (section 6.3). The compositions of product were determined by GC (Degn *et al*., 1999).

6.5 Identification of bacterial strain

The selected strain was identified based on its 16S rDNA sequence. The 16S rDNA was amplified by PCR using various universal primer sets and sequenced. The obtained sequences were BLAST searched against National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Nucleotide alignment and phylogenetic tree construction were accomplished with the TREEVIEW program.

6.6 Optimization lipase of production from selected *Actinomycetes* strain

To obtain the maximum lipase production from selected *Actinomycetes* strain. The optimization conditions were studied. The culture was grown in M65, pH 7.5, 175 rpm at 45°C. The 60 h old culture (10% v/v) was used as starter for lipase production. The selected strain was cultivated in M65 medium supplemented with 10% (v/v) of 10% olive oil (w/v) in 10% gum arabic solution (w/v) and initial pH was 7.5. The cultivation conditions were shaking at 175 rpm at 45°C. After 120 h, 2.0 mL of culture broth was taken, centrifuged at 10,000 rpm (8,000 *g*), 4°C for 15 min. The lipase activity of cell free culture broth was determined by using *p*NPP as substrate (Kademi *et al*., 2000) and the cell growth was determined by hydrolysis of microbial cell by 0.1 M NaOH at 95°C for 15 min, followed by measuring of total cell protein by Bradford method (Bradford, 1976). Several factors were studied to obtain the optimum condition for lipase production.

- Effect of sugars

The effect of sugar (glucose, fructose, maltose, sucrose, sorbitol, mannitol and molasses at the concentration of 0.4% w/v) on lipase production was studied using olive oil as carbon source and cultivation conditions as section 6.6. After obtained the suitable sugar, the concentration of suitable carbon source was studied by varying the concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1.0% (w/v), respectively. The suitable type and concentration of sugar were chosen for next studied.

- Effect of nitrogen sources

The effect of nitrogen source (beef extract, meat extract, soytone, tryptone, yeast extract, ammonium nitrate, ammonuium hydrogenphosphate, ammonium sulphate and urea) on lipase production was studied at the concentration of 0.4% (w/v). After obtained the suitable nitrogen source, the concentration of suitable nitrogen source was studied by varying the concentration of 0, 0.2, 0.4, 0.6, 0.8 and 1.0% (w/v), respectively. The suitable type and concentration of nitrogen source were chosen for next studied.

- Effect of malt extract concentration

Malt extract was the mineral source in M65 medium. To obtain the optimum condition of lipase production, the concentration of malt extract was studied at concentration of 0, 0.5, 1.0, 1.5 and 2.0% (w/v). The suitable concentration of malt extract was chosen for next study.

- Effect of oils

Various types of TAG and derivatives (Tween 20, Tween 80, tributyrin, coconut oil, lard, olive oil, palm oil, soybean oil and rice bran oil) were studied on lipase production at the concentration of 1.0% (w/v). After obtained the suitable TAG, the concentration of suitable oil was studied by varying the concentration of 0, 0.5, 1.0, 1.5, 2.0 and 2.5% (w/v), respectively. The suitable type and concentration of TAG source were chosen for next studied.

- Effect of emulsifiers

Various types of surfactants (Tween 20, Tween 80, gum arabic, deoxycholic acid, polyvinyl alcohol and triton X-100) were studied on lipase production at the concentration of 1.0% (w/v). The suitable type of surfactant was chosen for next study.

- Effect of initial pH

Effect of initial pH on lipase production was varied of 5.0, 6.0, 7.0, 7.5, 8.0, 9.0, 10.0 and 11.0 by 0.5 M HCl or 0.5 M NaOH. The suitable pH was chosen for next study.

- Effect of cultivation temperature

Effect of cultivation temperature on lipase production was varied at 30, 37, 40, 45, 50 and 55°C. The suitable temperature was chosen for next study.

6.7 Time course of lipase production and cell growth from selected *Actinomycetes*

Time course of lipase production from selected *Actinomycetes* was studied using optimal conditions (section 6.6). The lipase activity was determined by using *p*NPP as substrate (Kademi *et al*., 2000) and the cell growth was determined by hydrolysis of microbial cell by 0.1 M NaOH at 95°C for 15 min, followed by measuring of total cell protein by Bradford method (Bradford, 1976).

6.8 Purification and characterization of lipase from selected *Actinomycetes*

The selected *Actinomycetes* was grown in M65, pH 7.5, 175 rpm at 45°C and the 60 h old culture (10% v/v) was used as starter. For lipase production, the *Actinomycetes* strain ME168 was cultivation in 2 L flask containing 300 mL of M65 medium supplemented with 30 mL of 10% (w/v) olive oil in 10% (w/v) gum arabic solution, pH 7.5 with shaking at 175 rpm, 45°C. After 96-120 h of cultivation, the culture broth was centrifuged at 4,000 rpm (1,800 *g*) at 4°C for 30 min to obtain the cell free supernatant.

6.8.1 Purification of lipase

The purification method consisted of sequential concentration, precipitation, ion-exchange chromatography and gel-filtration chromatography. First, the cell free supernatant was 10 times concentrated by using rotary vacuum evaporator at 45°C. The concentrated supernatant was then precipitated by chilled acetone (60% v/v). The precipitate was centrifuged at 4,000 rpm (1,800 *g*) at 4°C to remove acetone and then dissolved in 100 mM Tris-HCl buffer pH 7.5 and dialyzed against this buffer for 12 h and stored at -20°C prior to chromatography.

The enzyme solution was applied on Resource Q 1 mL column equilibrated with 0.1 M Tris-HCl buffer pH 7.5. The column was washed with the same buffer to wash unbound proteins. The bound proteins were eluted with 40 ml NaCl gradient (0-1 M) in the same buffer with the flow rate of 1 mL/min. The fraction volume of 1.0 ml was collected. The active fractions were pooled, concentrated, dialyzed against 50 mM Tris-HCl buffer pH 7.5 and used for Superdex 200 gel filtration chromatography.

The fractions containing enzyme were applied to a Superdex 200 column (1.6 x 70 cm) previously equilibrated with 0.15 M NaCl in 0.1 M Tris-HCl buffer pH 7.5. Then the enzyme was eluted with the same buffer with the flow rate of 1.5 mL/min. The fraction volume

of 5.0 mL was collected and the active fractions were pulled and used for SDS-PAGE and enzyme characterization.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the Bio-Rad Mini Protein II DUAL SLAB cell according to the manufacturer's instructions. SDS-PAGE was performed on 15% polyacrylamide gel with 0.1 % sodium dodecyl sulfate (SDS) to establish the purity of protein. (Laemmli, 1970) (Appendix 11). SDS-PAGE was run along the standard; β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), restriction endonuclease *Bsp*98I (25 kDa), β-lactoglobulin (18.4 kDa) and lysozyme (14.4 kDa).

6.8.2 Characterization of purified lipase

- Effect of pH

The effect of pH on lipase activity was carried out at pH in the range of 4.0-11.0 using 50 mM different buffers; acetate buffer (pH 4.0-5.5), phosphate buffer (pH 6.0-6.5), Tris-HCl buffer (pH 7.0-8.5), glycine-NaOH buffer (pH 9.0-11.0). For pH stability study, one volume of purified lipase was mixed with three volumes of the above buffers and incubated from 0 to 180 min before assay.

- Effect of temperature

The effect of temperature on lipase activity was carried out by assay the enzyme activity at different temperatures in the range of 35-75°C at pH 8.5 using 50 mM Tris-HCl buffer. The thermostability of purified lipase was studied by incubation of the enzyme at different temperatures from 35 to 80°C for 180 min before assay.

- Kinetic parameters

The Michaelis-Menten kinetic parameters, K_m and V_{max} of purified lipase were carried out with increasing concentration of *p*NPP from 0.05 mM to 0.5 mM in 50 mM Tris-HCl buffer pH 8.5. The K_m and V_{max} were determined by Lineweaver-Burk plot.

- Substrate specificity

Since the *p*NPA and *p*NPB were not stable at pH above 8.5, all substrates specificity assays of the purified lipase was studied by hydrolysis of *p*-nitrophenyl esters of varying acyl chain lengths from C_2-C_{16} at pH 7.5 at 45°C. The molar adsorption coefficient of *p*NP was 12,442 cm^2/mg at pH 7.5.

- Effect of organic solvents

The effect of organic solvents on lipase activity was studied by incubation of 0.5 ml purified lipase in 0.5 mL of miscible or immiscible organic solvents (dimethyl sulfoxide, methanol, dimethylformamide, acetronitrile, ethanol, acetone, pyridine, *tert*-butanol, 2-methyl-2-butanol and hexane) at 45°C for 60 min before assay.

- Effect of metal ions, inhibitors and other compounds

The effect of various metal ions (KCl, NaCl, LiCl, $AgNO_3$, $CaCl_2$, $MgCl_2$, $ZnCl_2$, $MnCl_2$, $CuSO_4$ and $FeCl_3$) oxidizing and reducing agents (KI, ammonium persulphate, dithiothreitol and ascorbic acid), chelating agents (sodium citrate and EDTA) and inhibitors (PMSF and *p*HMB) on the enzyme activity was studied at a concentration of 1 mM and 10 mM at 45°C and pH 8.5.

7. Production of lipase from the strain PSU-AH130, purification, characterization and application for FAME production

7.1 Identification of the strain PSU-AH130

The strain PSU-AH130 was identified based on its 16S rDNA sequence. The 16S rDNA was amplified by PCR using various universal primer sets and sequenced. The obtained sequences were BLAST searched against National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/). Nucleotide alignment and phylogenetic tree construction were accomplished with the TREEVIEW program.

7.2 Production and immobilization of lipase from the strain PSU-AH130

Strain PSU-AH130 was grown in nutrient broth (NB), pH 7.5, 150 rpm at 45°C. The 24 h old culture was adjusted the turbidity (OD_{660}) to 1.0 before using as starter (10% v/v) for lipase production. The extracellular lipase from PSU-AH130 strain was produced in 2 L flask contained 300 mL basal liquid medium supplemented with 1.0% (w/v) Tween 80, pH 7.5 at 45°C for 48 h. The lipase activity was determined by spectrometric method using *p*NPP as substrate. The culture broth was centrifuged at 10,000 rpm (17,000 *g*) at 4°C for 20 min. The supernatant was 10 times concentrated by 30 kDa UF (Pellicon 2 Module, Millipore) to obtained concentrated lipase supernatant.

For immobilization of lipase, 2.0 g of supporters (Accurel EP 100 (<400 μm), celite 545 (200 μm), Syran (SIKUG 012), PVC (250 μm) and Amberlite XAD-7) were added to 20 mL of concentrated lipase supernatant (484 U). The mixture was stirred using magnetic stirrer at 30°C for 1 h then filtered, washed twice with 50 mL of 50 mM Tris-HCl buffer pH 7.5. The filtrate was collected and dried in dessicator for 48 h until used (Keawthong, 2004). The hydrolytic activity of unadsorbed lipase and immobilized lipase were determined. The activity yield and immobilized yield were also calculated using equation as section 3. The immobilized lipase which showed the highest activity yield and immobilized yield was used for further step.

7.3 Optimization of FAME production catalyzed by selected immobilized lipase

The modified method from Paripatanapairod (2003) was used for FAME production. The reaction mixture contained palm olein/methanol (1:3 mol/mol or 1.40 g:0.16 g), 0.32 mL of 50 mM phosphate buffer pH 7.0 and 0.1 g immobilized lipase (20 U). The reaction was carried out at 50°C and 400 rpm for 72 h. The sample (50 μL) was periodically taken and 50 μL chloroform was added. The compositions of products (FAME, TAG, FA, DAG and MAG) were analyzed by TLC-FID. Several factors were varied to find the optimum reaction conditions.

- **Type of immobilized lipase :** immobilized lipase PS (IM-PS), immobilized lipase AK (IM-AK), immobilized lipase AY (IM-AY) and immobilized lipase from PSU-AH130 (IM-PSU-AH130)
- **Type of oil :** palm oil and palm stearin
- **Water content :** 10, 20, 30, 40 and 50% (w/w)
- **pH :** 5, 6, 7, 8, 9 and 10
- **Molar ratio of palm olein to methanol :** 1:1, 1:2, 1:3, 1:4 and 1:5 (mol/mol)
- **Effect of enzyme loading :** 5, 10, 15 and 20% (w/w)
- **Temperature :** 40, 45, 50 and 55°C

7.4 Optimization of lipase production from the strain PSU-AH130

To obtain the maximum lipase production from the strain PSU-AH130, this strain was cultivated in basal medium and the physico-chemical conditions were optimized as section 6.6 and 6.7.

8. Purification and characterization of lipase from PSU-AH130

The purification method consisted of sequential concentration, precipitation, ion-exchange chromatography and gel-filtration chromatography. First, the cell free supernatant was precipitated by 70% saturation of ammonium sulphate at 4°C for 12 h. The precipitate was collected by centrifugation at 10,000 rpm (17,000 *g*) for 30 min and dissolved in 20 mM Tris-HCl buffer pH 7.5, dialyzed against the same buffer overnight and stored at -20°C prior to chromatography.

The enzyme solution (5.0 mL) was applied on DEAE Toyopearl column (1.5 x 25 cm), which equilibrated with 50 mM Tris-HCl buffer pH 8.5 at 4°C. The column was washed with same buffer to wash unbound proteins and bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl. The flow rate was adjusted to 0.25 mL/min and the fraction volume of 2.0 mL was collected. The active fractions were pooled, concentrated, dialyzed

against 20 mM Tris-HCl buffer pH 7.5 and used for Sephadex G-150 gel filtration chromatography.

The enzyme solution was concentrated by 10 kDa UF (Millipore) and then applied to the Sephadex G-150 column (3.0 x 130 cm) previously equilibrated with 0.15 M NaCl in 50 mM Tris-HCl buffer pH 7.5. The enzyme was eluted with the same buffer with flow rate of 0.2 mL/min. The fraction volume of 2.5 mL was collected and the active fractions were pooled, concentrated and dialyzed against 20 mM Tris-HCl buffer pH 7.5. The purified enzyme was concentrated by 10 kDa UF (Millipore) and used for SDS-PAGE and enzyme characterization as described in section 6.8.1 and 6.8.2. The SDS-PAGE was performed by 15% polyacrylamide gel and was run along the standard; myosin (201 kDa), β-galactosidase (120 kDa), bovine serum albumin (100 kDa), ovalbumin (60 kDa), carbonic anhydrase (38 kDa), Soybean trypsin inhibitor (29.7 kDa), lysozyme (20.7 kDa) and aprotinin (7.0 kDa).