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APPENDICES

APPENDIX A

Properties of enzyme carrier

Table A-1. Details of seven types of hydrophobic support for immobilization of *Candida rugosa* lipase

Type of support	Polymer base	Functional group	Spec. surface area (m^2/g)	Pore diameter	Particle size (mm)
Amberlite XAD 2	styrene DVB	-	300	90 Å	-
Amberlite XAD 4	Polystyrene DVB	-	750	100 Å	490-690
Amberlite XAD 7	Aliphatic ester	-	$\geq$ 380	450 Å	560-710
Amberlite XAD 16	Polystyrene DVB	-	$\geq$ 800	150 Å	560-710
Amberlite XAD 761	Formophenolic	Phenol	150-250	600 Å	560-760
Sepabeads EC-BU	Acrylic	Butyl	-	30-40 nm	150-300
Sepabeads EC-OD	Acrylic	Octadecyl	-	30-40 nm	150-300

APPENDIX B

Hydrolysis assays

Preparation of solutions for hydrolysis assays

1 *Tris* buffer solution (Tris HCl)

1 M Tris buffer, pH 8.0

Tris base	121	g
Distilled water	800	ml

Tris base was dissolved and pH was adjusted to 8 with HCl. Then, solution was adjusted to 1L with distilled water. Tris buffer solution was later steriled at 121 °C, pressure15 psi for 15 min. The buffer solution was kept at 4 °C

50 mM Tris buffer, pH 8.0

1 M Tris buffer, pH 8.0	25	ml
Distilled water	475	ml

buffer solution was kept at 4 °C

2 *p*-nitrophenyl palmitate solution

p-nitrophenyl palmitate	50	mg
Absolute ethanol	10	ml

p-nitrophenyl palmitate was dissolved with absolute ethanol. Then, the solution was mixed well and kept in the brown bottle. (This solution was prepared before use)

APPENDIX C

Protein determination

1. Preparation of solutions for protein assays

The assay reagent is prepared by diluting 1 volume of the dye stock with 4 volumes of distilled H_2O. Then solution was filtered by filter paper, Whatman No. 1. The solution should appear brown, and have a pH of 1.1. It is stable for 4 weeks in a brown bottle at 4°C.

2. Standard curve of BSA

Protein standards should be prepared in the same buffer as the samples to be assayed. A convenient standard curve can be made using bovine serum albumin (BSA) with concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/ml. The method is as follows;

1. Prepare stock bovine serum albumin with concentration 20 mg/ml.
2. 20 mg/ml BSA was diluted with distilled water as 0.1-0.6 mg/ml (Table C-1)

Table C-1 Composition for standard BSA

BSA (mg)	Reagent volume (µl)	
	stock of BSA	dH_2O
0	-	1000
0.1	5	995
0.2	10	990
0.3	15	985
0.4	20	980
0.5	25	975
0.6	30	970

2. Pipet 5 µl of each standard from stock solution was into 96 wells microplate. Protein solutions are normally assayed in duplicate.

3. Add 300 µl of diluted dye reagent to each well and incubated at room temperature for 5 minutes.

4. The product was measured by an increase in the absorbance at 595 nm with micro plate reader.

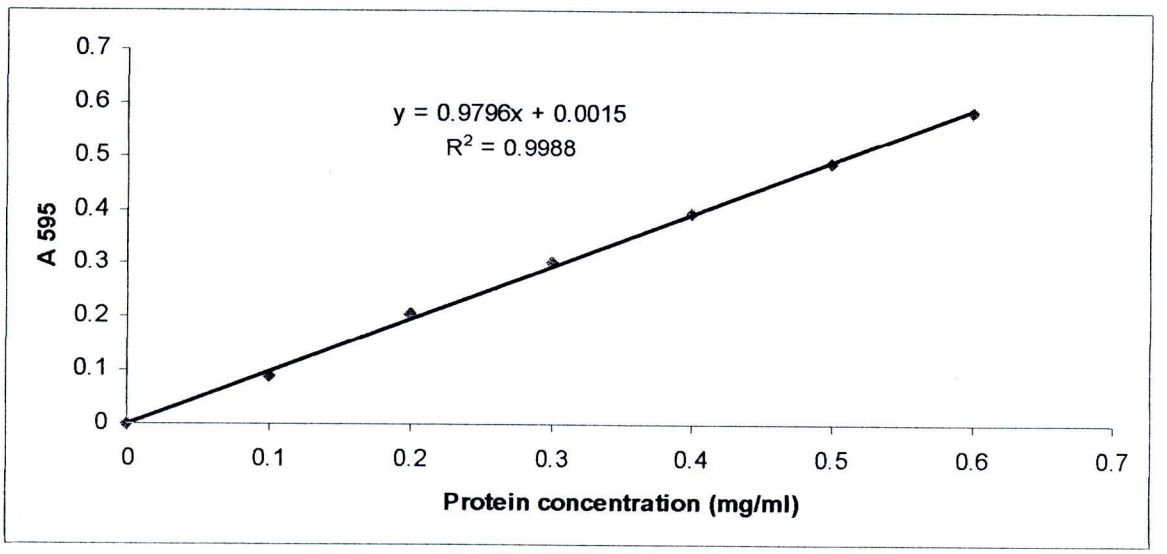

Figure C-1. Calibration curve for protein determination by Bradford's method

3. Calculation of total protein

The absorbance value at 595 nm was calculated by:

$$Y = aX + b$$

Where

Value X axis = Standard protein concentration (mg/ml)

Value Y axis = Absorbance at 595 nm

The amount of bound protein on the support was calculated from the difference between the amount of protein introduced into the reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization. Amount of bound enzyme onto support (mg/g) was calculated from the following formula:

$$p = \frac{C_i V_i - (C_f V_f + C_w V_w)}{m_s}$$

p = Amount of bound enzyme onto support (mg/g)

C_i = Initial protein concentration (mg/ml)

C_f = Protein concentration of filtrate (mg/ml)

C_w = Protein concentration of washing solution (mg/ml)

V_i = Initial volume of enzyme solution (ml)

V_f = Volume of filtrate (ml)

V_w = Volume of washing solution (ml)

m_s = Weight of the support (g)

APPENDIX D

Calculation of the lipase activity

1. Calculation of enzyme immobilization

The efficiency of immobilization was evaluated in terms of lipase activity, specific activity, protein loading and activity yields as follows:

$$A_{410} = \varepsilon_{410} bc \qquad \text{Equation D 1.1}$$

Where

A_{410} = Absorbance at 410 nm

ε_{410} = Molar extinction coefficient of *p*-nitrophenol at 410 nm

= 15,000 $M^{-1} cm^{-1}$

b = 1 cm

c = Concentration of *p*-nitrophenyl palmitate

One unit (1 U) was defined as that amount of enzyme that liberated 1 µmol of *p*NPP per minute under the test conditions. Lipase activity was calculated from

$$\text{Lipase activity (U/g-support)} = \frac{\text{Activity of immobilized lipase}}{\text{Amount of immobilized lipase}}$$

$$\text{Specific activity (U/mg-protein)} = \frac{\text{Activity of immobilized lipase}}{\text{Amount of protein loading}}$$

$$\text{Protein loading yield (\%)} = \frac{\text{Amount of protein loading}}{\text{Amount of protein introduced}} \times 100$$

$$\text{Activity yield (\%)} = \frac{\text{Specific activity of immobilized lipase}}{\text{Specific activity of free lipase}} \times 100$$

(Chang *et al.*, 2007)

2. Calculation of thermal stability

Thermal stability was calculated according to equation (1) and (2)

(Santos *et al.*, 2008)

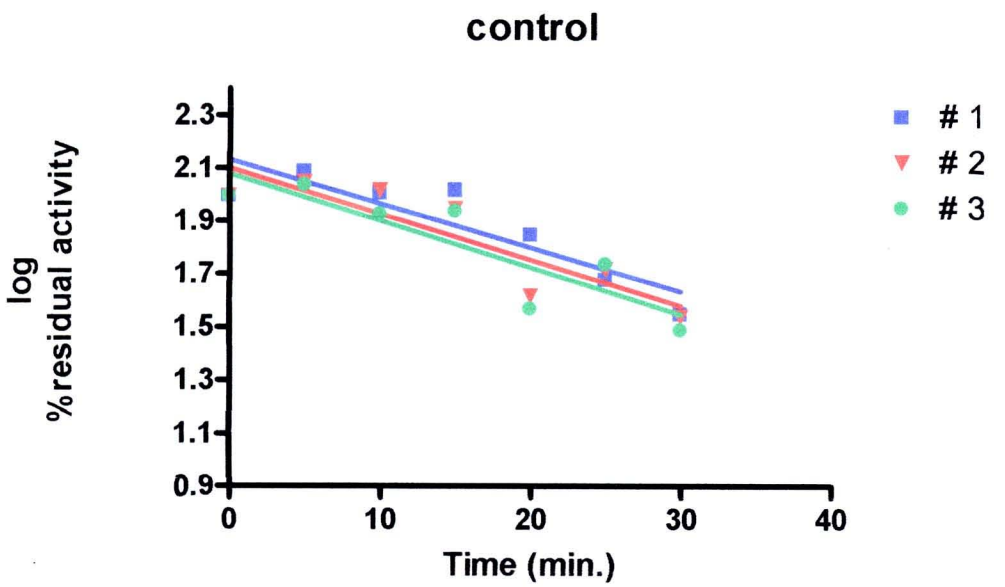

Figure D-1 Thermostability of untreated immobilized enzyme

$$A_{in} = A_{in0} \exp(-k_d t) \quad \text{.....(1)}$$

$$t_{1/2} = \frac{\ln 2}{K_d} \quad \text{.....(2)}$$

where

A_{in} = the hydrolytic activity at given time

A_{in0} = the initial hydrolytic activity at given time

K_d = thermal deactivation constant

t = the incubation time

Since, $\text{slope} = \frac{K_d}{2.3}$ (From Fig D-1)

So, $t_{1/2} = \frac{0.693}{2.3 \times \text{slope}}$

untreated immobilized enzyme

APPENDIX E

Properties of plant oil

1. Calculation of oil content

In oil extraction, the thimbles were weighed before and after addition of the ground plant seed including the flask before and after extraction.

Calculation method was as follows;

$$\% \text{ Oil content} = \frac{(W_4 - W_3)}{(W_2 - W_1)} \times 100 \quad \text{w/w of seed}$$

W_1 = weight of thimble before paked (g)

W_2 = weight of thimble include plant seed (g)

W_3 = flask (500 ml) + boiling chip (g)

W_4 = flask (500 ml) + boiling chip (g) + oil

2. Calculation of each fatty acid composition in oil

Fatty acid composition of plants oil (Table E-1) was determined by GC analysis. The chromatogram of fatty acid methyl ester as shows in Figure E-1: The percentage of each fatty acid in oil was calculated as follows:

$$\text{Percentage of fatty acid (\%)} = \frac{\text{Peak area of fatty acid}}{\text{Total peak area of fatty acids}} \times 100$$

The example of fatty acid composition in palm oil

$$\text{Palmitic acid (\%)} = \frac{155596}{1075+3021+155596+3156+367686+39777+723745+40218+1692+1673} \times 100$$

$$= 11.63\ \%$$

Figure E-1. Chromatogram of methyl ester from transesterification of the 1g of palm oil with 10% (w/w of oil) sodium hydroxide at 55oC, 350 rpm at 24 hr.

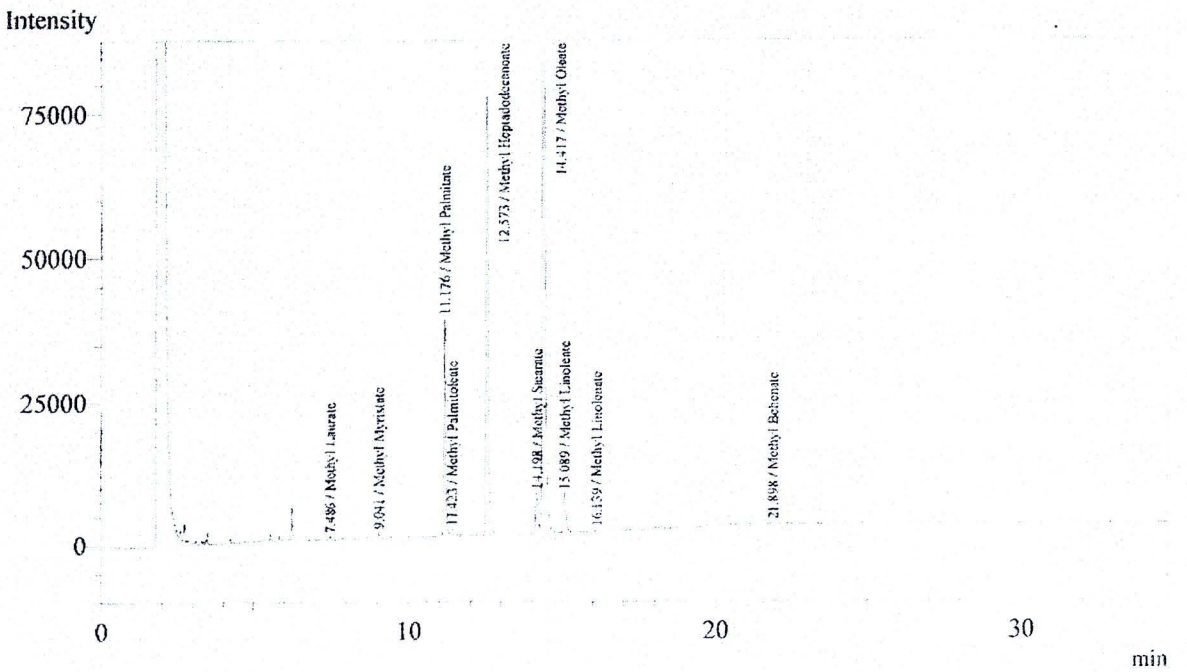

Table E-1. Retention time, peak area and peak height of standard fatty acid methyl ester, used in the calculation of fatty acid composition in oil.

Peak	Compound name	RT	Area	Height
1	Methyl Laurate	7.486	1075	477
2	Methyl Myristate	9.041	3021	962
3	Methyl Palmitate	11.176	155596	37879
4	Methyl Palmitoleate	11.423	3156	916
5	Methyl Standard	12.573	367686	76057
6	Methyl Stearate	14.198	39777	7310
7	Methyl Oleate	14.417	723745	127619
8	Methyl Linoleate	15.089	40218	7002
9	Methyl Linolenate	16.139	1692	289
10	Methyl Arachidate	21.898	1673	217

2. Molecular weight of palm oil

Triglyceride (TAG) is the major composition in oil. Therefore, the molecular weight of triglyceride represents the molecular weight of oil. To calculate the molecular weight of TAG (i.e. molecular weight of oil), equation E-1 was use.

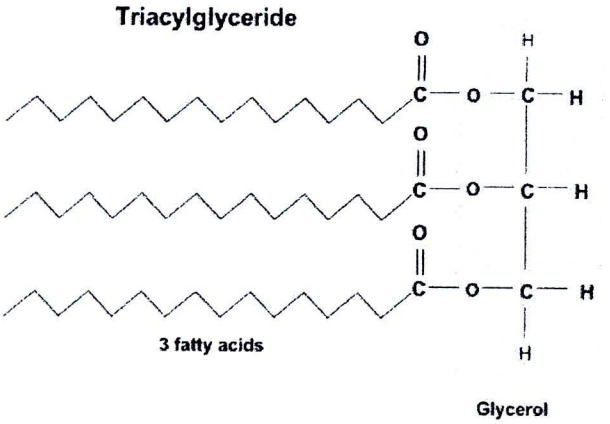

Figure E-2 Molecular structure of triglyceride

$$MW_{TG} = 3R_{Aver} + 173 \quad \text{equation E-1}$$

$$R_{Aver} = \frac{(\%FA_n \times MW_n)}{100}$$

Where,

MW_{TG} = Molecular weight of triglyceride = Molecular weight of palm oil

R_{Aver} = Mass of three fatty acid esterified with glycerol (minus molecular weight of COOH)

$\%FA_n$ = Percentage of each fatty acid in oil

MW_n = Molecular weight of three fatty acid – COOH

= MW_{FA} – 45 (from main structure of triglyceride) (Fig E-2)

Table E-2 Fatty acid composition of palm oil

Common name	Abbreviation	% Fatty acid
Lauric acid ($C_{12}H_{24}O_2$)	12 : 0	0.59
Myristic acid ($C_{14}H_{28}O_2$)	14 : 0	0.96
Palmitic acid ($C_{16}H_{32}O_2$)	16 : 0	38.67
Palmitoleic acid ($C_{16}H_{30}O_2$)	16 : 1	0.11
Stearic acid ($C_{18}H_{36}O_2$)	18 : 0	3.32
Oleic acid ($C_{18}H_{34}O_2$)	18 : 1	45.45
Linoleic acid ($C_{18}H_{32}O_2$)	18 : 2	10.87
Linolenic acid ($C_{18}H_{30}O_2$)	18 : 3	0.20
Arachidic acid ($C_{20}H_{40}O_2$)	20 : 0	0.23
Behenic acid ($C_{22}H_{44}O_2$)	22 : 0	0.02

The example of triglyceride in used palm oil calculation

$$R_{Ave} = \left(\frac{0.59}{100}\times 155\right)+\left(\frac{0.96}{100}\times 183\right)+\left(\frac{38.67}{100}\times 211\right)+\left(\frac{0.11}{100}\times 209\right)+\left(\frac{3.32}{100}\times 239\right)$$
$$+\left(\frac{45.45}{100}\times 237\right)+\left(\frac{10.87}{100}\times 235\right)+\left(\frac{0.20}{100}\times 233\right)+\left(\frac{0.23}{100}\times 267\right)+\left(\frac{0.02}{100}\times 295\right)$$

$$= 0.915 + 1.757 + 81.594 + 0.221 + 7.935 + 107.717 + 25.545 + 0.466 + 0.614 + 0.059$$

$$= 226.823$$

$$MW_{TG} = (3 \times 226.823) + 173$$

$$= 853.469$$

Therefore molecular weight of palm oil is equal to 853.47

Table E-3. Molecular weight of non-edible and waste plant oil

Common name		MW
Papaya	*Carica papaya* Linn.	871.18
Physic nut	*Jatropha curcas* L.	870.41
Pomelo	*Citrus maxima* (Burm.) Merr.	860.27
Pumpkin	*Cucurbita moschata* Duchesne	867.38
Rambutan	*Nephelium lappaceum* L.	899.13
Rubber	*Dipterocarpus alatus Roxb.ex G.Don*	872.15
Tangerine	*Citrus reticulate Blan co*	901.66
White silk cotton	*Ceiba pentandra* (L.)Gaertn.	865.65
Wild almond	*Irvingia malayana* Oliv. ex A.W. Benn.	748.51

3. Calculation SN,IV, CN and η values

Calculation IV of plant seed oils were calculated from their fatty acid methyl ester compositions as Equation E-2, SN were calculated from the equation E-3 and CN of fatty acid methyl ester were calculated from Equation E-4. Also, the mass fraction was used in preference to the mole fraction to conform to the mass unit that is implicit in the units for viscosity used in this study. With these two modifications, Equation E-5 was used to predict the viscosity of biodiesel fuels based on their fatty acid composition

$$IV = \sum(254 \times D \times A_i) / MW_i, \quad \text{Equation E-2}$$

$$SN = \sum(560 \times A_i) / MW_i, \quad \text{Equation E-3}$$

$$CN = 46.3 + 5458 / SN - 0.225 \times IV \quad \text{Equation E-4}$$

$$\ln \eta_m = \sum Z_i \ln \eta_i \quad \text{Equation E-5}$$

Where,

A_i = Percentage of each component in the chromatogram

MW_i = Molecular mass

D = Number of double bonds

Z_i = Mass fraction

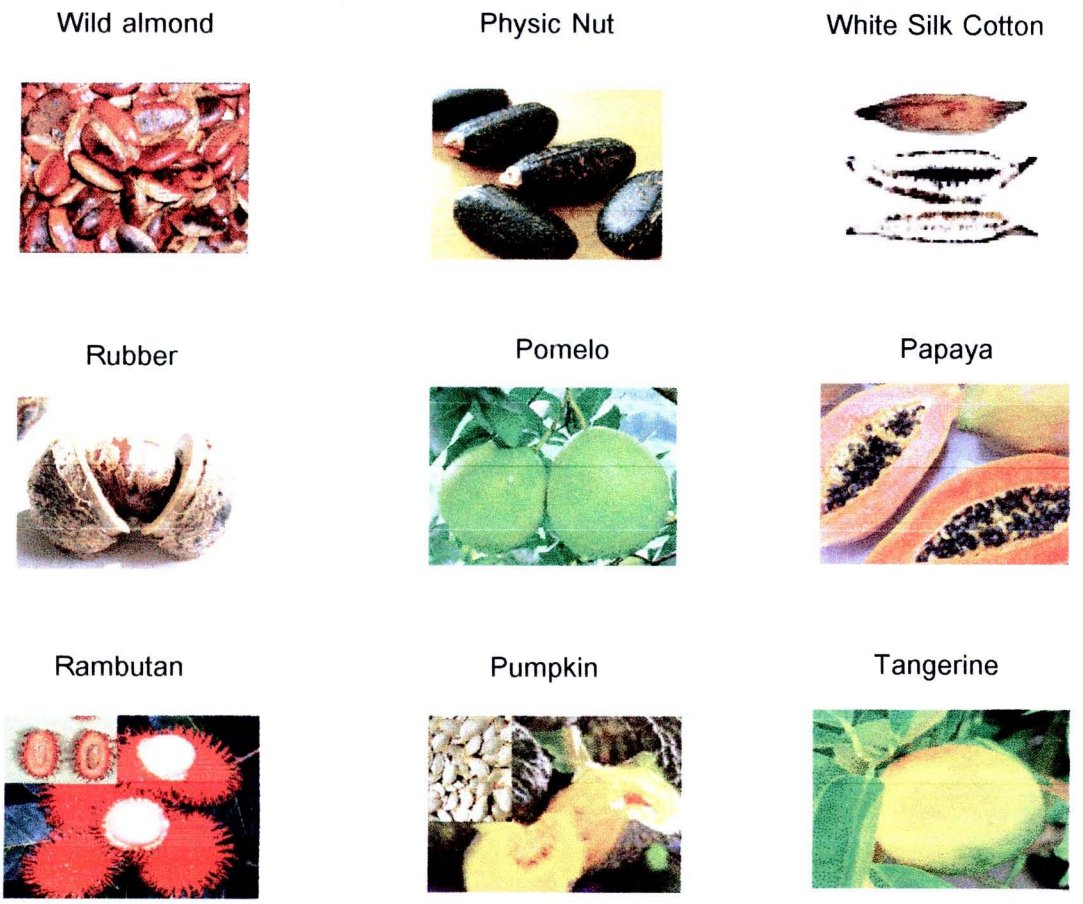

Figure E-3. Non-edible and waste plant oil

APPENDIX F

Calculation of transesterification reaction

1. Volume of methanol

The stoichiometry of this reaction requires 3 mol methanol per mol triglyceride to yield 3 mol fatty acid methyl ester or biodiesel and 1 mol glycerol. The biodiesel yield could be elevated by introducing an excess amount of methanol to shift the equilibrium to the right-hand side. So, the ratio of oil and methanol is 1:3 according to the equation in Fig D-3. The applied volume of methanol was determined by using the molecular weight of palm oil from section 4 equal to 853.47.

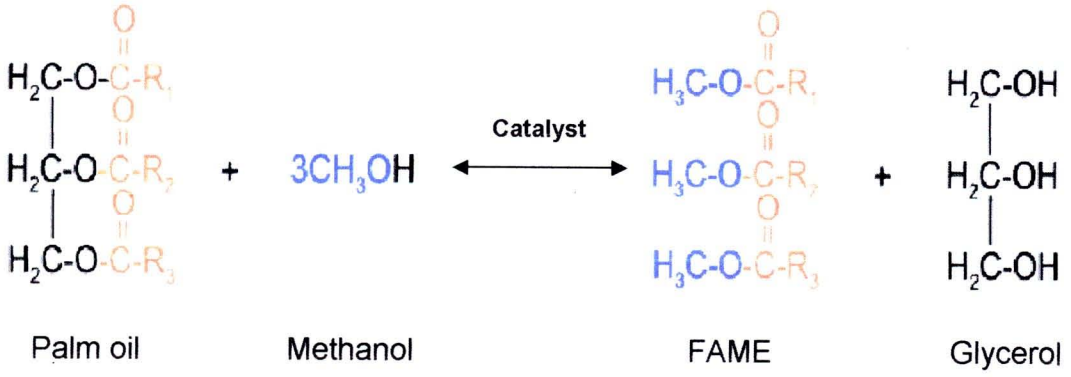

Figure F-1 Transesterification of palm oil and methanol

So, palm oil 1 g = 1/853.47

$\simeq 1.171 \times 10^{-3}$ mole

Thus, the volume of methanol $= 3 \times 1.171 \times 10^{-3}$

$= 3.513 \times 10^{-3}$ mole

Since molecular weight of methanol is equal to 32

So, methanol 3.513×10^{-3} mole $= 3.513 \times 10^{-3} \times 32 = 0.1124$ g

From $D = \frac{M}{V}$

D = Density of methanol (0.792)

M = Mass of methanol (0.1124 g)

V = Volume of methanol (ml)

$$V = \frac{0.1124}{0.792}$$

So, the volume of methanol is equal to 0.140 ml.

2. %conversion yield from HPLC analysis

All FAME were assumed by HPLC can be calculated as follows;

$$\% \text{ FAME} = \frac{\text{FAME}}{\{\text{FAME} + \text{FFA} + (\text{TAG} \times 3) + (1,3 \text{ DAG} \times 2) + (1,2 \text{ DAG} \times 2) + \text{MAG}\}} \times 100$$

FAME = Concentration of methyl ester

FFA = Concentration of free fatty acid

TAG = Concentration of triglyceride

DAG = Concentration of diglyceride

MAG = Concentration of monoglyceride

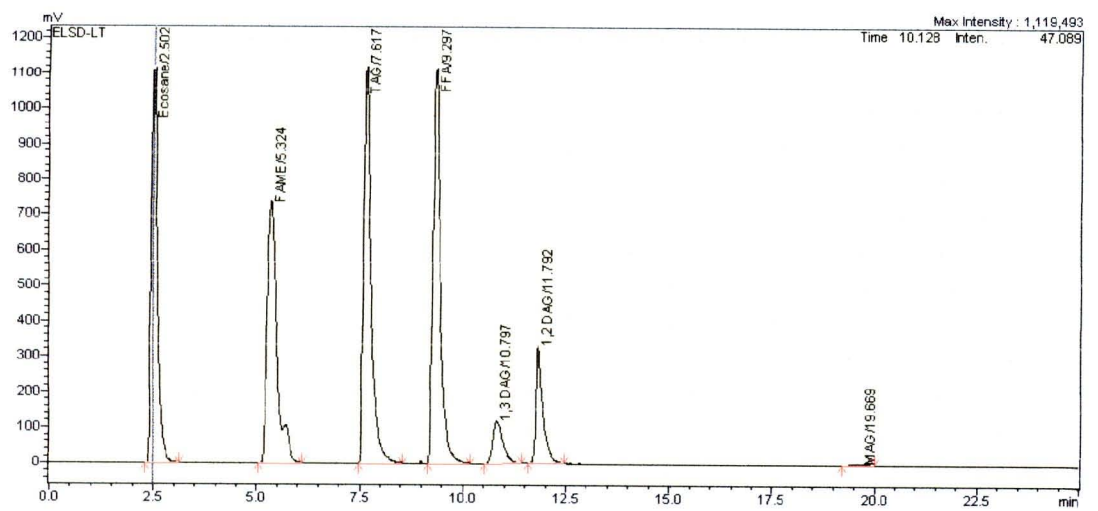

Figure F-2 Chromatogram of methyl ester from transesterification catalyzed by immobilized lipase and analyzed by high performance liquid chromatography

Where

Peak 1 = Eicosane

Peak 2 = Fatty acid methyl ester (FAME or Biodiesel)

Peak 3 = Triglyceride (TAG)

Peak 4 = Free fatty acid (FFA)

Peak 5 = 1,3 Diglyceride (1,3 DAG)

Peak 6 = 1,2 Diglyceride (1,2 DAG)

Peak 7 = Monoglyceride (MAG)

APPENDIX G

Property of commercial lipase

Property	Novozyme® 435	Lipozyme® RM IM
Microorganism	*Candida antarctica* fraction B	*Rhizomucor miehei*
Type of support	Macroporous resin	Acrylic resin
Specificity	Non-specific	1,3-specific
		

References: Martín. E. H. and Otero. C., 2007

BIOGRAPHY

Miss Kingkaew Piriyakananon was born on November 27, 1983 in Bangkok, Thailand. She graduated with the Bachelor Degree of Science from the Department of General Science, Faculty of Science, Kasetsart University in 2005. Later in 2006, she furthered her Master's Degree of Science in Biotechnology from Program in Biotechnology, Department of Biochemistry, Faculty of Science, Chulalongkorn University, at which she finished with the "very good" merit from her defense examination on the 2nd of April of 2009. During her postgraduate studies, she had presented her work at both national and international levels. In addition, she received 2 awards from oral presentation competition and poster presentation. Most importantly, part of her thesis was already published in *Biomass and Bioenergy* in the year 2008. The details are as follows:

AWARDS

Oral presentation

Second prize for the thesis oral research presentation at the national competition for postgraduate students: The CGI Award for Young Scientists 2008. 21-22 November 2008. organized at Chulabhorn Research Institute & Chulabhorn Graduate Institute. Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W., and Yongvanich, T. 2008. Optimal immobilization condition of lipase from *Candida rugosa* for biodiesel production.

Poster presentations

1. **Commendable prize** for Poster Presentation at the Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. 28-29 August 2008. Chulabhorn Research Institute Conference Center.

Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W., and Yongvanich, T. 2008.Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. Chulabhorn Research Institute, Bangkok, Thailand. 28-29 August, 2008.

2. **First prize** for Poster Presentation at the The Science Forum 2009.
Symposium 1: Energy and Environmental Research For Sustainable Development
Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W., and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. The Science Forum 2009. Faculty of Science, Chulalongkorn University, Bangkok, Thailand. 12 -13 March, 2009.

PUBLICATION

Winayanuwattikun, P., Kaewpiboon, C., Piriyakananon, K., Tantong, S. Thakernkarnkit, W., Chulalaksananukul, W. and Yongvanich, T. 2008.Potential Plant Oil Feedstock for Lipase-catalyzed Biodiesel Production in Thailand. 2008. *Biomass and Bioenergy* 32. 1279-1286.

PROCEEDINGS

Piriyakananon, K., Thakernkarnkit, W., Chulalaksananukul, W., Winayanuwattikun, P. and Yongvanich, T. 2008. Lipase catalyzed biodiesel production from non-edible and waste plant oils. The 7th International Symposium of High Temperature Air Combustion and Gasification (HiTACG 2008). Phuket Spa and Resort , Phuket , Thailand. 13-16 January, 2008

PRESENTATIONS

Poster presentations

1. Kaewpiboon, C., Piriyakananon, K., Chulalaksananukul, W. and Yongvanich, T. 2007. Comparative studies on biodiesel production from physic nut and coconut oils by different immobilized lipases. The 8th International Conference on Agricultural, Food and Biological Engineering & Post Harvest/Production Technology. Sofitel Raja Orchid Hotel, Khon Kaen, Thailand. 21 – 24 January, 2007.

2. Piriyakananon, K., Thakernkarnkit, W., Chulalaksananukul, W., Winayanuwattikun, P. and Yongvanich, T. 2008. Lipase catalyzed biodiesel production from non-edible and waste plant oils. The 7th International Symposium of High Temperature Air Combustion and Gasification (HiTACG 2008). Phuket Spa and Resort , Phuket , Thailand. 13-16 January, 2008.

3. Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W. and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. Chulabhorn Research Institute, Bangkok, Thailand. 28-29 August, 2008.

4. Winayanuwattikun, P., Piriyakananon, K., Chulalaksananukul, W. and Yongvanich, T. Lipase-mediated biodiesel production using various acyl acceptors. Third Annual Symposium Protein Society of Thailand: Frontiers in Protein Research. Chulabhorn Research Institute, Bangkok, Thailand. 28-29 August, 2008.

5. Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W. and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. 13th Biological Science Graduated Congress. National University of Singapore, Singapore. 15-17 December, 2008.

6. Piriyakananon, K., Winayanuwattikun, P., Chulalaksananukul, W. and Yongvanich, T. 2008. Optimal immobilization of lipase from *Candida rugosa* on hydrophobic supports for the production of biodiesel. The Science Forum 2009, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. 12 -13 March, 2009.