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APPENDICES

APPENDIX A

EXPERIMENTAL DATA FOR ANALYSIS

A-1 Experimental data of hydrolytic activity of *Candida rugosa* lipase (CRL)

Table A-1.1 The present of hydrolytic activity and ethyl ester yield at molar ratio of methanol to purified palm oil of 9:1, temperature 50 °C, mass ratio of lipase to purified palm oil and palm fatty acid were varied within the range of 1-10 % for reaction time 24 h

Mass ratio	Hydrolytic activity (U)	% Yield
1%	53.13	30.52
3%	81.21	55.00
5%	120.07	83.39
10%	122.67	84.27

Table A-1.2 The present of hydrolytic activity at mass ratio of lipase to purified palm oil 5% and adsorption time of *C. rugosa* lipase (CRL) were varied within the range of 30-180 minutes, using the olive oil as the substrate at pH 7.0, 37 °C

Adsorption time (minutes)	Activity yield (%)
0	0.00
30	61.52
60	88.50
90	86.81
120	87.77
150	85.12
180	87.30

Table A-1.3 The present of hydrolytic activity of *C. rugosa* lipase (CRL) at various enzyme/$CaCO_3$ ratio (w/w) between 1:1-1:4, using the olive oil as the substrate, pH 7.0, 37 °C, reaction time 12 h.

Enzyme/$CaCO_3$ ratio (w/w)	Specific activity (U/mg-protein)
1:1	15.25
1:2	17.25
1:3	14.98
1:4	11.11

Table A-1.4 The present of hydrolytic activity of immobilized *C. rugosa* lipase (CRL) at the various bead diameter between 1.7-4 mm and Na-alginate concentration between 1%-2%, using olive oil as the substrate, pH 7.0, 12 h.

Alginate Conc.	Hydrolytic activity (U)								
	CRLE			CRLAE with filtration step			CRLAE without filtration step		
	1.7 mm	2 mm	4 mm	1.7 mm	2 mm	4 mm	1.7 mm	2 mm	4 mm
1.0%	184.17	163.10	126.98	119.32	102.56	83.16	22.92	18.00	15.04
1.5%	155.32	140.00	102.00	101.94	85.32	64.67	19.21	16.00	12.00
2.0%	138.33	115.48	70.94	87.53	58.42	46.32	13.73	10.40	7.21

Table A-1.5 The present of hydrolytic activity of free and immobilized *C. rugosa* lipase carrier at the various temperature between 37 °C -60 °C, using olive oil as the substrate, pH 7.0, 12 h, 1% Na-alginate concentration and 1.7 mm bead diameter.

Temperature (°C)	Hydrolytic activity (U)			
	Free lipase	CRLA	CRLE	CRLAE
37	74.07	66.05	61.73	63.13
45	105.82	93.10	82.01	84.66
50	128.65	100.21	105.26	109.94
60	102.34	92.34	96.49	99.42

A-2 Experimental data of enzymatic transesterification reaction of purified palm oil and esterification reaction of palm fatty acid in batch system

Table A-2.1 The present of ethyl ester yield at the various shaking speed between 150-300 rpm, ethanol:purified palm oil molar ratio of 9:1, lipase:reactants mass ratio of 5 %, 50 °C, reaction time 24 h.

Time (h)	% Ethyl ester yield			
	Shaking speed (rpm)			
	150	200	250	300
0	0.00	0.00	0.00	0.00
6	15.64	25.71	32.71	35.71
8	21.00	31.40	37.40	43.40
10	29.63	36.59	42.59	52.59
12	35.13	41.65	47.65	59.65
20	43.43	58.48	67.48	70.48
24	43.43	58.48	67.48	69.48

Table A-1.5 The present of hydrolytic activity of free and immobilized *C. rugosa* lipase carrier at the various temperature between 37 °C -60 °C, using olive oil as the substrate, pH 7.0, 12 h, 1% Na-alginate concentration and 1.7 mm bead diameter.

Temperature (°C)	Hydrolytic activity (U)			
	Free lipase	CRLA	CRLE	CRLAE
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45	105.82	93.10	82.01	84.66
50	128.65	100.21	105.26	109.94
60	102.34	92.34	96.49	99.42

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Table A-2.1 The present of ethyl ester yield at the various shaking speed between 150-300 rpm, ethanol:purified palm oil molar ratio of 9:1, lipase:reactants mass ratio of 5 %, 50 °C, reaction time 24 h.

Time (h)	% Ethyl ester yield			
	Shaking speed (rpm)			
	150	200	250	300
0	0.00	0.00	0.00	0.00
6	15.64	25.71	32.71	35.71
8	21.00	31.40	37.40	43.40
10	29.63	36.59	42.59	52.59
12	35.13	41.65	47.65	59.65
20	43.43	58.48	67.48	70.48
24	43.43	58.48	67.48	69.48

Table A-2.2 The present of ethyl ester yield at the various range of molar ratio between 3:1-12:1 for both of purified palm oil and palm fatty acid, lipase:reactants mass ratio of 5 %, 250 rpm, 50 °C, reaction time 24 h.

Molar ratio	% Ethyl ester yield	
	purified palm oil	palm fatty acid
1:1	-	9.64
2:1	-	13.83
3:1	47.25	15.90
6:1	74.90	11.33
9:1	83.22	8.57
12:1	44.14	-
15:1	25.84	-

Table A-2.3 The present of ethyl ester yield at the various of mass ratio of mixed substrate at 90:10, 80:20, 70:30 and 50:50 of purified palm oil to palm fatty acid, lipase:reactants mass ratio of 5 %, 50 °C, 250 rpm, reaction time 24 h.

Mass ratio of mixed substrate	% Ethyl ester yield
100:0	77.60
90:10	11.75
80:20	14.24
70:30	19.48
50:50	6.07
0:100	12.60

Table A-2.4 The present of ethyl ester yield at the various of addition time of mixed substrate at 6 h, 12 h and 24 h, lipase:reactants mass ratio of 5 %, mass ratio of purified palm oil to palm fatty acid of 70:30, 50 °C, 250 rpm, reaction time continue to 48 h.

Addition time (h)	% Ethyl ester yield			
	Before addition of palm fatty acid	Std. Deviation	At total reaction time (48 h)	Std. Deviation
0	-	-	31.31	0.3520
6	34.89	3.3800	51.95	1.0525
12	47.99	2.2850	58.17	3.1604
24	83.43	1.1750	84.07	0.5699
48	-	-	86.34	4.2057

Table A-2.5 The present of ethyl ester yield by using the different techniques of immobilized lipase, lipase:reactants mass ratio of 5 %, mass ratio of purified palm oil to palm fatty acid of 70:30, 50 °C, 250 rpm, reaction time continue to 48 h.

Reaction time (h)	% Ethyl ester yield				
	Free lipase	$CaCO_3$	CRLA	CRLAE	CRLE
6	34.34	0.24	20.58	13.88	15.01
12	52.56	0.31	32.55	23.47	32.94
24	78.73	0.27	46.84	29.50	57.07
36	77.44	0.33	52.99	36.88	67.36
48	81.18	0.15	57.57	42.69	74.20

Table A-2.6 The present of ethyl ester yield by using the different types of immobilized lipase (CRLA, CRLE and CRLAE carrier), molar ratio of purified palm oil to ethanol was 1:9, immobilized lipase 700 mg (5% free lipase based on oil weight), mass ratio of purified palm oil to palm fatty acid of 70:30, reaction temperature 50 °C, addition time of palm fatty acid to the reaction at 24 h and total reaction time continue to 48 h, 250 rpm

Rx time (h)	% Ethyl ester yield								
	Cycle 1			Cycle 2			Cycle 3		
	CRLA	CRLE	CRLAE	CRLA	CRLE	CRLAE	CRLA	CRLE	CRLAE
6	20.58	15.01	13.88	13.03	12.12	8.95	7.85	10.16	5.04
12	32.55	32.94	23.47	15.21	25.79	10.98	9.12	13.14	8.21
24	46.84	57.07	29.50	20.89	37.10	16.04	11.13	18.84	11.01
36	52.99	67.36	36.88	25.88	48.99	18.97	14.07	25.94	12.00
48	57.57	74.20	42.69	31.23	59.12	23.13	16.11	32.02	13.05

Table A-2.7 The present of hydrolytic activity by using the different types of immobilized lipase (CRLA, CRLE and CRLAE carrier), molar ratio of purified palm oil to ethanol was 1:9, immobilized lipase 700 mg (5% free lipase based on oil weight), mass ratio of purified palm oil to palm fatty acid of 70:30, reaction temperature 50 °C, addition time of palm fatty acid to the reaction at 24 h and total reaction time continue to 48 h, 250 rpm

Rx time (h)	Hydrolytic activity (U)								
	Cycle 1			Cycle 2			Cycle 3		
	CRLA	CRLE	CRLAE	CRLA	CRLE	CRLAE	CRLA	CRLE	CRLAE
0	136.64	118.95	109.21	-	-	-	-	-	-
6	125.87	115.38	95.63	58.64	94.97	56.97	29.43	66.32	27.47
12	118.21	113.26	90.91	55.74	87.32	51.11	25.75	63.21	25.11
24	108.32	108.21	88.32	49.42	82.57	48.34	21.43	57.53	18.34
36	100.72	103.63	85.17	41.21	75.21	40.32	18.34	51.44	15.22
48	83.41	98.11	71.94	34.65	72.85	32.43	15.84	49.32	12.34

APPENDIX B

CALCULATION OF LIPASE ACTIVITY AND PERCENT YIELD OF ETHYL ESTER

B-1 Calculation of lipase activity

The hydrolysis activity of free and immobilized *C. rugosa* lipase were assayed titrimetrically using olive oil emulsion method (Yadav et al., 2005).
Lipase activity were calculated as follow;

$$\text{Enzyme activity (U)} = \frac{(V_1 - V_0) x N_{KOH} x 10}{t x 10^{-6} x 1000} \quad (\mu \text{ mole/min})$$

Where

V_1 = Volume of KOH used for sample titration (ml)

V_0 = Volume of KOH used for control titration (ml)

t = Assay reaction time (min)

B-2 Calculation of protein content

Bradford Assay (Coomassie Plus - The Better, Bradford™ Assay Kit)

1. Pipette 1.0 ml of each standard or unknown sample into appropriately labeled test tubes.

2. Add 1.0 ml of the Coomassie Plus Reagent to each tube and mix well.

3. Optional: For the most consistent results, incubate samples for 10 minutes at room temperature (RT).

4. With the spectrophotometer set to 595 nm, zero the instrument on a cuvette filled only with water. Subsequently, measure the absorbance of all the samples.

5. Subtract the average 595 nm measurement for the Blank replicates from the 595 nm measurements of all other individual standard and unknown sample replicates.

6. Prepare a standard curve by plotting the average Blank-corrected 595 nm measurement for each BSA standard vs. its concentration in mg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

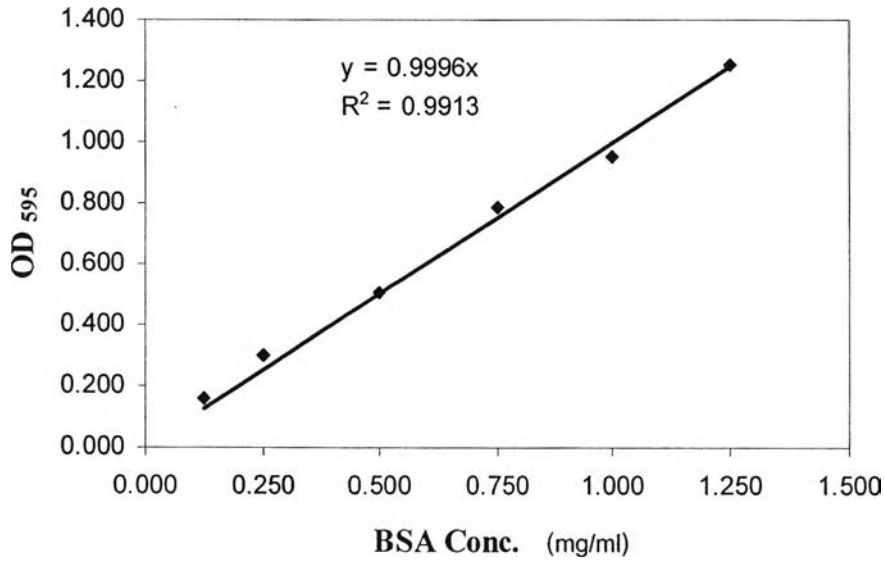


Figure B-2.1 The BSA standard curve

Protein concentration

$$\text{Protein concentration} = \frac{V_T \, x \, OD_{595} \, x \, V_0}{BSA \; std.\; slope \, x \, V_1} \quad \text{(mg/ml)}$$

Where

V_T = Total volume of assay condition (ml)

V_0 = Total volume of unknown sample (ml)

V_1 = Volume of unknown sample (ml)

Specific activity (U/mg protein)

$$\text{Specific activity} = \frac{\text{Activity of immobilized lipase}}{\text{Amount of protein loaded}}$$

Activity yield (%)

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

B-3 Calculation of molecular weight of palm fatty acids

The molecular weight of palm fatty acids is calculated from the weighted average of the molecular weight of the five key fatty acids: palmitic acid, oleic acid, stearic acid, linoleic acid, and linolenic acid. The compositions of palm fatty acids are shown in Table B-1.1

Table B-3.1 Composition and molecular weight of key components in of palm fatty acids.

Palm fatty acids	% Weight Fraction	Molecular weight
Palmitic acid	42.8	256.43
Oleic acid	40.5	282.47
Stearic acid	4.5	284.5
Linoleic acid	10.1	280.45
Linolenic acid	2.1	278.43

The data in Table B-1.1 can be used to compute the molecular weight of palm fatty acids as shown below:

1 mole of palm fatty acids

$$M_w = \text{Sum } (M_{Fa} \text{ x \% Weight fraction fatty acids}) \text{ ..... } \textbf{B-3.1}$$

Where

M_w = Molecular weight of fatty acids

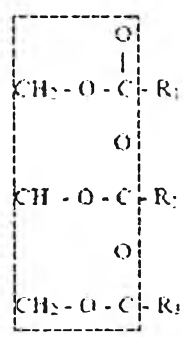
M_{Fa} = Molecular weight of each fatty acids

Such as (Data in the table B-1.1) apply in equation B-1.1

= (0.428 x 256.43) + (0.405 x 282.47) + (0.045x284.5) +
(0.101x 280.45) + (0.021x278.43)

= 271.13

B-4 Calculation molecular weight of purified palm oil



Triglyceride

R1, R2, R3: carbon chain of the fatty acids

Table B-4.1 Fatty acid composition in purified palm oil sample

Fatty acid	wt %
Lauric	0.10
Myristic	1.00
Palmitic	42.80
Stearic	4.50
Oleic	40.50
Linoleic	10.10
Linolenic	0.20

Molecular weight of triglyceride

$$Mw_{TG} = 3R_{aver} + 173 \qquad \text{..... B-4.1}$$

$$R_{aver} = \sum\left(\frac{\%Fa_n}{100} \times Mw_n\right) \qquad \text{..... B-4.2}$$

Where

Mw_{TG} = molecular weight of triglyceride

R_{aver} = average molecular weight of fatty acid

% Fa_n = percent of fatty acid in vegetable oil

Mw_n = molecular weight of fatty acid

Example Find molecular weight of palm oil

Such as (Data in the table B-2.1) apply in equation B-2.1 and B-2.2

$$R_{aver} = \left(\frac{0.1}{100} \times 200\right) + \left(\frac{1}{100} \times 228\right) + \left(\frac{42.8}{100} \times 256\right) + \left(\frac{4.5}{100} \times 284\right)$$
$$+ \left(\frac{40.5}{100} \times 282\right) + \left(\frac{10.1}{100} \times 280\right) + \left(\frac{0.2}{100} \times 278\right)$$

$= 267.08$

$3R_{aver} = 3 \times 267.08 = 801.23$

$Mw_{TG} = 801.23 + 173 = 974.23$

B-5 Calculation of reactants

Molar ratio of ethanol to reactants: $\frac{N_{EtOH}}{N_{reactant}}$

Volume of reactant: $\left(\frac{Mw_{reactant} \times N_{reactant}}{\rho_{reactant}}\right)+\left(\frac{Mw_{EtOH} \times N_{EtOH}}{\rho_{EtOH}}\right)=V_{reaction}$

Volume of ethanol: $V_{reaction}-V_{reactant}=V_{EtOH}$

B-6 Calculation of catalyst

Example Base on volume of purified palm oil is 5 g. The lipase to purified palm oil mass ratio of 5 %

Weight of catalyst = weight of purified palm oil x catalyst to purified palm oil mass ratio

= 5 x 5 /100

= 0.25 g

B-7 Calculation of the percent ethyl esters yield

The percent ethyl ester yield is defined as

$$\%\text{Yield of ethyl ester} = \frac{W_{EE}}{W_{Fa}} \times 100 \qquad \text{..... B-7.1}$$

$$W_{EE} = W_{EP}+W_{ES}+W_{EO}+W_{EL}$$

Where

W_{EE} = weight of ethyl ester (g)

W_{Fa} = weight of fatty acid (g)

W_{EP} = weight of ethyl palmitate (g)

W_{ES} = weight of ethyl stearate (g)

W_{EO} = weight of ethyl oleate (g)

W_{EL} = weight of ethyl linoleate (g)

Calculation weight of each ethyl ester

$$W_{EE} = \left(\frac{C \times V_{TD}}{V_S} \right) \times V_P \qquad \text{..... B-7.2}$$

Where

W_{EE} = weight of ethyl ester (g)

C = concentration of each ethyl ester from calibration curve (g/ml)

V_{TD} = total volume dilute (ml)

V_S = volume product dilute (ml)

V_P = total volume of product (ml)

The weight of ethyl esters for each fatty acid can be determined from GC data with corresponding calibration equation. Below are standard calibration curves for the key ethyl esters (Figure B-7.2-B-7.5). Then apply in the equation B-7.2

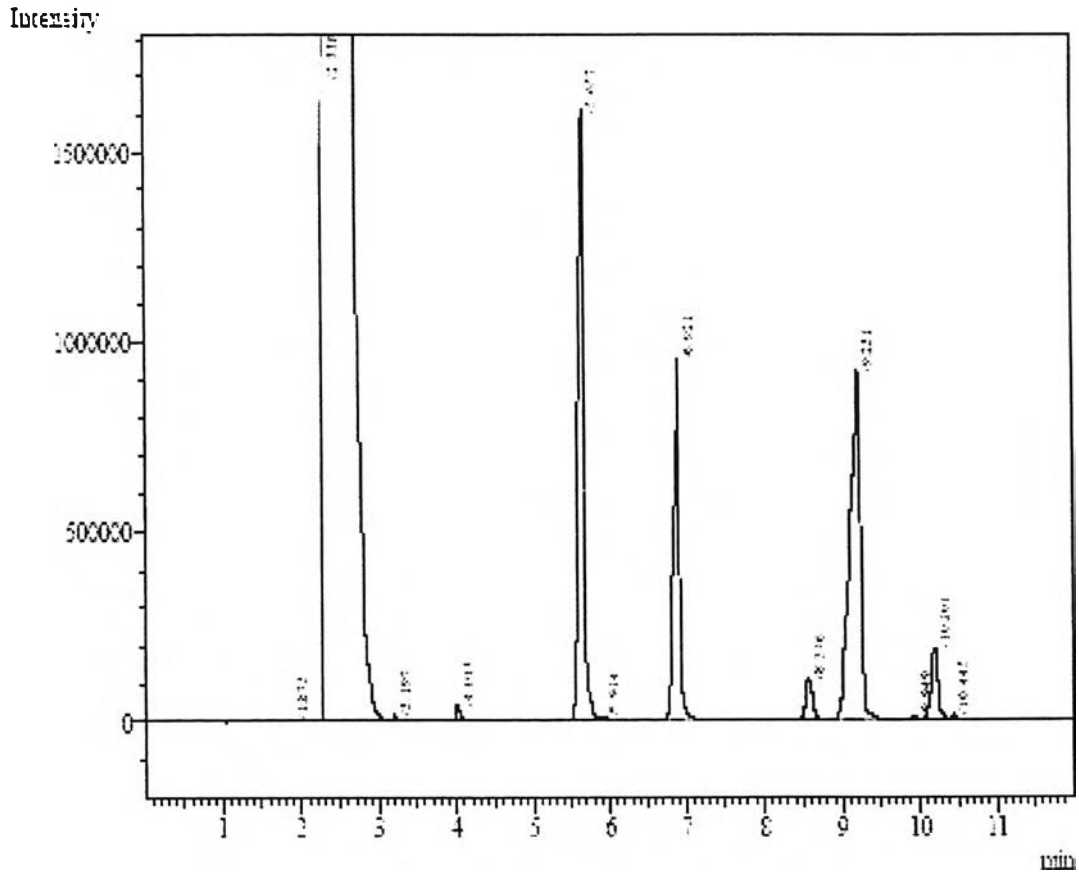


Figure B-7.1 Profile of component of each purified palm oil from GC. At mole fraction of ethanol and purified palm oil (9:1), mass fraction of lipase to oil (5 wt %)

Table B-7.1 Data of time and area in Figure B-7.1

Peak of saample	Retention time (min)	Area
Hexane	2.338	3619104354
Eethyl palmitate	5.657	7821533
Eethyl stearate	8.576	692129
Eethyl oleate	9.231	8531761
Eethyl linoleate	10.201	1142115

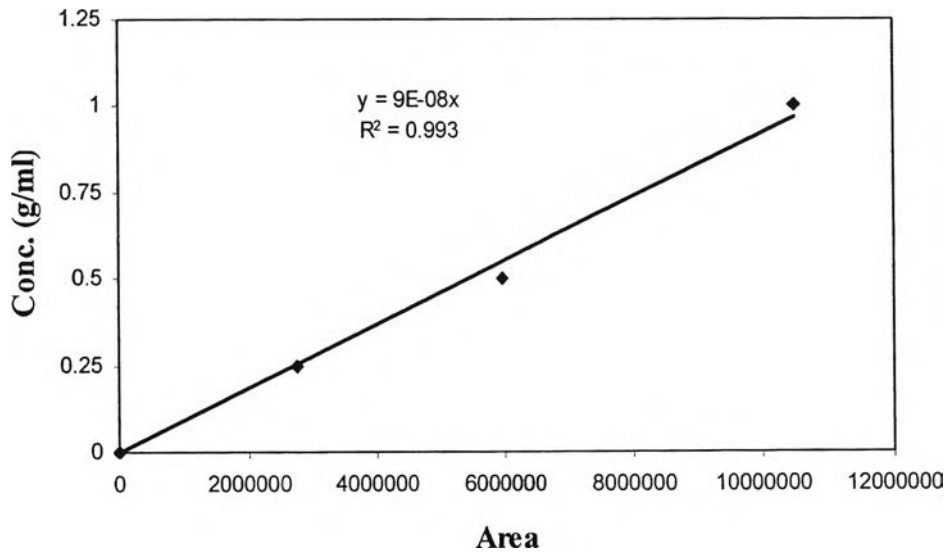


Figure B-7.2 Standard calibration curve for ethyl plamitate

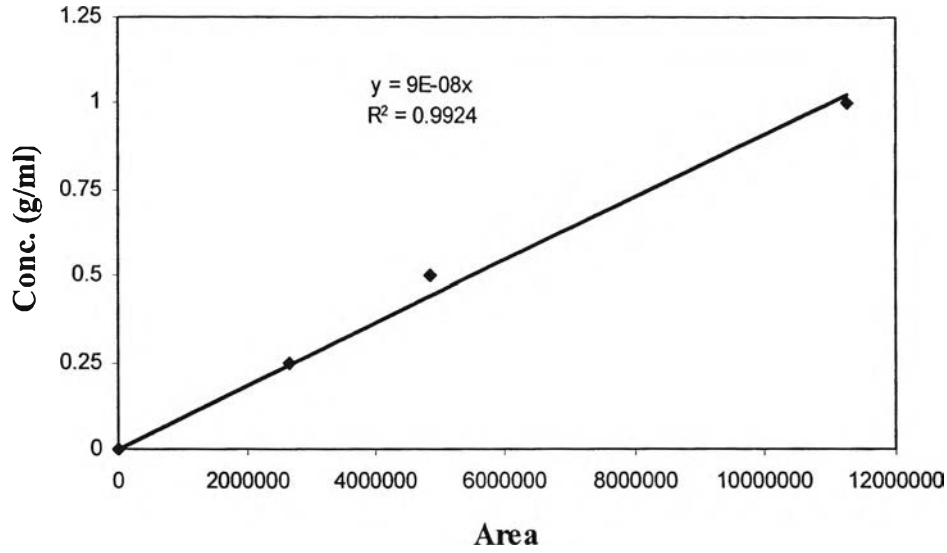


Figure B-7.3 Standard calibration curve for ethyl stearate

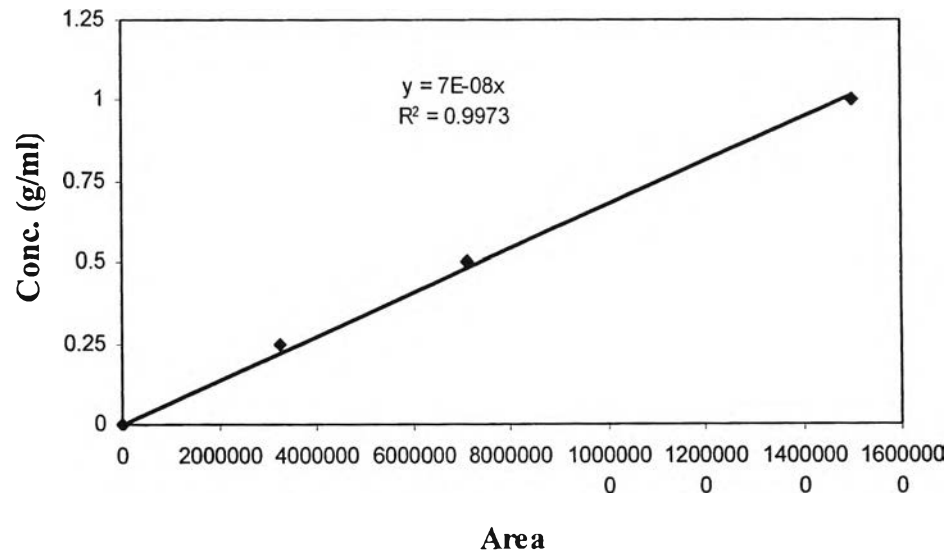


Figure B-7.4 Standard calibration curve for ethyl oleate

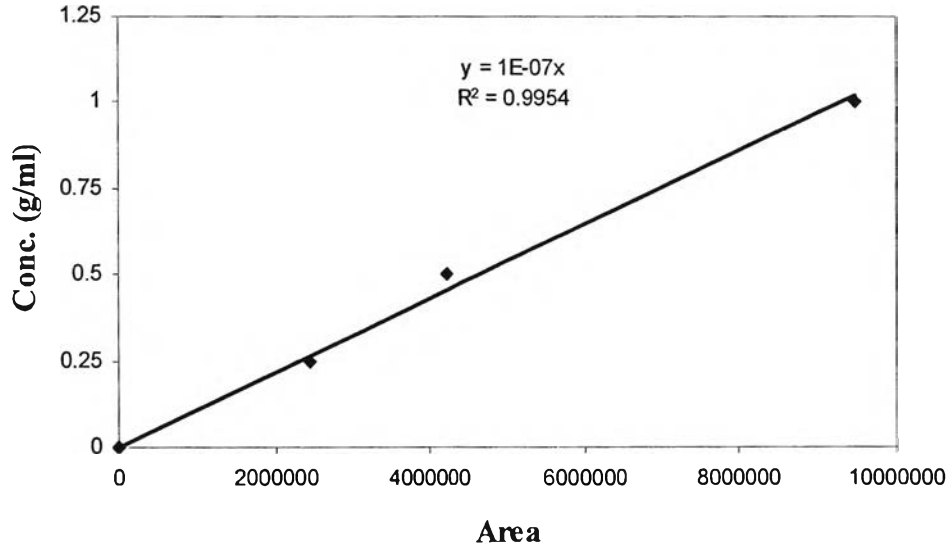


Figure B-7.5 Standard calibration curve for methyl linoleate

APPENDIX C

The 22nd Symposium of Malaysian Chemical Engineers (SOMChE) in conjunction with the 15th Regional Symposium on Chemical Engineering (RSCE)
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Immobilization of Lipase on $CaCO_3$ and Entrapment in Calcium Alginate Bead for Application in Biodiesel Production

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IMMOBILIZATION OF LIPASE ON $CaCO_3$ AND ENTRAPMENT IN CALCIUM ALGINATE BEAD FOR APPLICATION IN BIODIESEL PRODUCTION

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Keywords: alginate, biodiesel, calcium carbonate, immobilization, lipase

ABSTRACT

Lipase from *Candida rugosa* was immobilized by physical adsorption onto $CaCO_3$ followed by entrapment in calcium alginate bead for the application as biocatalyst in biodiesel production. The effects of bead diameter, sodium alginate concentration and operating temperature on lipase activity were examined. The maximum hydrolytic activity (330 U) of the immobilized lipase was obtained by using 1% (w/v) sodium alginate entrapment at the diameter beads of 1.7 mm. The optimal temperature for both free and immobilized lipase activities was 50 °C. The thermal stability of the immobilized lipase was higher than that of the free enzyme.

INTRODUCTION

According to the diminishing petroleum reserves and environmental consequences of exhaust gases from petroleum-fueled engines, biodiesel becomes more attractive and promising alternative energy sources. Biodiesel has proved to be eco-friendly far more than fossil fuels because biodiesel is non-toxic, biodegradable, and excellent replacement for petroleum diesel. Biodiesel is produced by esterification of fatty acids or transesterification of oils and fats with short chain alcohols. By using alkali or acid catalytic processes, high energy requirements, difficulties in the recovery of catalyst and glycerol and pollution from waste water are major disadvantages.

The biocatalyst such as lipase can eliminate the disadvantages of alkali and acid catalysts by producing product of very high purity and offers an environmentally attractive option. Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the breakdown of fats and oils with subsequent release of free fatty acids, diacylglycerols, monoacylglycerols, and

glycerol. Their specificity, regioselectivity and enantioselectivity allow them to catalyze reactions with reduced side products, lowered waste treatment costs and under conditions of mild temperature and pressure (Villeneue et al., 2000). However, the hurdle to use of lipase for biodiesel fuel production is the cost of biocatalyst. The use of immobilized enzymes could help to overcome this problem. Immobilizations of lipase can be achieved by adorption onto support matrices such as particles, fibres, by entrapping them in gel matrices or by covalent attachment (Carneiro-da-Cunha et al., 1999). The enzyme immobilization by adsorption onto a solid support (Van der Waals binding, ionic binding and covalent binding) or entrapment in biopolymer matrix such as calcium alginate remain the most simple and cost-effective. It was suggested that the immobilized lipase facilitates mass transfer by spreading the enzyme on a large surface area and by preventing the enzyme particles from aggregation (Wahlgren et al., 1991).

The $CaCO_3$ presented the advantages of being non-toxic and lacking of chemical reactivity. Furthermore, this support was selected as a suitable adsorbent leading to high dispersion of the crude *R. oryzae* lipase in the support and preserving the catalytic activity (Ghamgui et al., 2004). Once the enzyme has lost a significant amount of its initial activity; its desorption is sometimes possible by modification of the pH (Wahlgren et al., 1991), followed by binding of new active enzyme. However, ready desorption would also be a major drawback of this immobilization technique if it occurs during the catalyzed reaction. The other physical immobilization of a lipase is its inclusion in an insoluble polymer or entrapment in biopolymer matrix such as calcium alginate is attractive. The advantage of such an immobilization technique is that the enzyme does not chemically interact with the polymer; therefore, denaturation is usually avoided (Villeneue et al., 2000).

This study exploits the idea on developing a new enzyme carrier by adsorption of lipase on $CaCO_3$ followed by the entrapment in calcium alginate beads. The suitable conditions of the lipase immobilization are then investigated and the result was compared with that using free enzyme and immobilized enzyme in conventional calcium alginate beads.

MATERIALS AND METHODS

Enzyme and chemicals

Candida rugosa lipase (EC 3.1.1.3) was received as a gift sample from Amano Pharmaceuticals, Japan. Virgin olive oil was purchased from local market. Carbonate of calcium ($CaCO_3$) was from Univar. All substances are the analytical grade.

Immobilization of Candida rugosa lipase on $CaCO_3$ (CRL/$CaCO_3$)

The enzyme immobilization was made on to $CaCO_3$ according to Rosu et al. (1997) with a slight modification. A support powder (0.2 g) was added to 2 ml of enzymatic solution. 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 preparation of immobilized lipase was washed two times with another 10 ml aliquot of chilled acetone. dried in vacuum desiccator at room temperature for 1-2 h and stored at 4°C until use.

Entrapment of Candida rugosa lipase immobilized on $CaCO_3$ in calcium alginate beads (CRL/$CaCO_3$/Entrap)

Candida rugosa lipase which pre-immobilized on $CaCO_3$ by adsorption was entrapped in calcium alginate beads according to Hertzberg et al. (1991) by the following procedure: 1% sodium alginate solution (5 ml) and 0.1 M calcium chloride solution (10 ml) were prepared in 0.01 M sodium phosphate buffer (pH 7.0). 200 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 to 0.1 M calcium chloride solution from a constant distance. The beads were allowed to harden in calcium chloride solution for an hour.

Lipase hydrolysis activity

The hydrolysis activities of various enzyme preparations immobilized on $CaCO_3$ 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 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.

One unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 µmol of free fatty acids per min under the assay conditions.

Temperature activity profile

The optimum temperature for the free and the immobilized lipase forms were determined by measuring the hydrolytic activity using olive oil emulsion as substrate (Yadav et al., 2005) at different temperatures (35–60° C) and at pH 7.0.

RESULTS AND DISCUSSION

From preliminary study. the stability of *Candida rugosa* lipase immobilized on $CaCO_3$ in calcium alginate beads (CRL/$CaCO_3$/Entrap) is much better than the *Candida rugosa* lipase on $CaCO_3$ (CRL/$CaCO_3$) [data not shown]. In order to determine the suitable conditions of lipase immobilization for application in the biodiesel production. the influence of operating conditions on enzyme activity

was investigated using lipase-$CaCO_3$ immobilized in calcium alginate beads (CRL /$CaCO_3$/Entrap) in comparison to that using free enzyme and immobilized enzyme in calcium alginate bead (CRL/Entrap).

Effect of diameter of alginate bead and sodium alginate concentration

The effect of bead diameter and sodium alginate concentration on the activity of immobilized lipase were investigated by varying the diameter of bead at 1.7. 2 and 4 mm and the sodium alginate concentration in the range of 1-2 %. The diameter size is controlled by using the syringe. The hydrolysis activity is determined under the temperature at 37 ° C, pH 7.0. Fig. 1 shows the effect of bead diameter and sodium alginate concentration on the activity of *CRL/Entrap* and *CRL/$CaCO_3$/Entrap* carriers. The activity of *CRL/$CaCO_3$/Entrap* is significantly lower than that of *CRL/Entrap*. The lack of buffer solution in the bead was later proved to be the cause of the lower activity. For the *CRL/Entrap* carrier, the sodium alginate concentration and bead diameter for the maximum activity (184.17 U) were 1% w/v and 1.7 mm, respectively. It was found that the activity of immobilized lipase was decreased with the increase of bead diameter and sodium alginate concentration. The results indicated that the diffusional interference in hydrolytic reaction over the calcium alginate bead intruded to slow the rate of reaction.

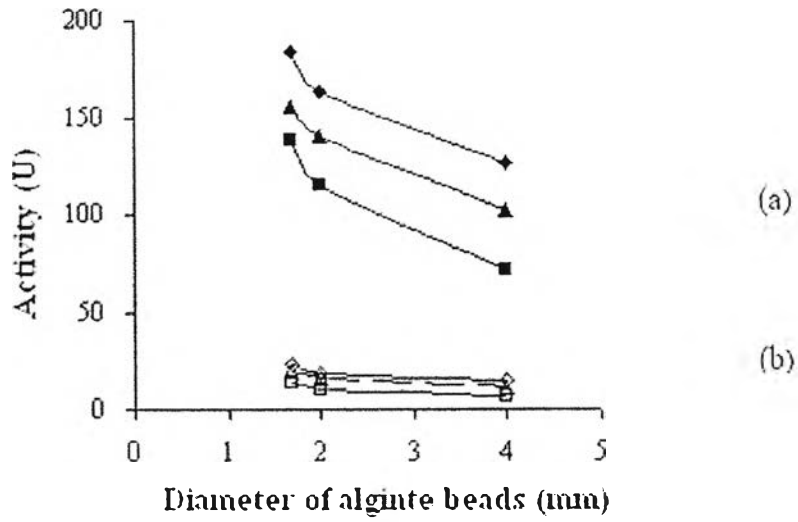

FIGURE 1. Effect of bead diameter at various alginate concentration on the hydrolytic activity of immobilized *Candida rugosa* lipase at pH 7.0, 37 °C and 12 h: (a) CRL/Entrap carrier using sodium alginate at concentration of 1%(◆), 1.5 (▲) and 2% w/v (■); (b) CRL/$CaCO_3$/Entrap carrier using sodium alginate at concentration of 1%(◇), 1.5 %(△) and 2.0 % w/v (□).

Effect of temperature on immobilized lipase activity

The effect of temperature on the activity of free enzyme and immobilized enzyme in *CRL/CaCO$_3$/Entrap* carrier were investigated by using olive oil as substrate at pH 7.0 in the temperature range of 37-60°C as shown in Fig. 2. The maximum activity of both free and immobilized enzymes appeared at 50° C. With the increase of the operating temperature up to 50°C, the activity of the free lipase was increased. However, above 50°C, the enzyme activity of the free enzyme was significantly decreased with the increasing temperature, while the enzyme activity of the immobilized enzymes slightly decreased. Therefore, the immobilization in CRL/Entrap and CRL/CaCO$_3$/Entrap carriers could provide good heat resistance.

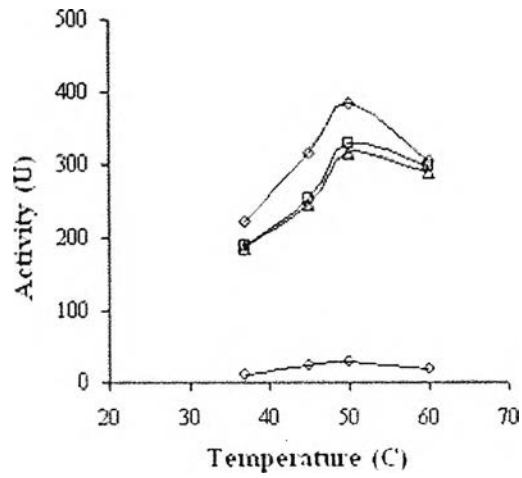

FIGURE 2. Effect of temperature on the hydrolytic activity of immobilized *Candida rugosa* lipase at pH 7.0 and 12 h by using (a) free enzyme (◇) and (b) immobilized enzyme in CRL/Entrap carrier (△); CRL/CaCO$_3$/Entrap carrier (□) and CRL/CaCO$_3$/Entrap carrier (with filtration step) (○).

The hydrolytic activity of enzyme in *CRL/Entrap* carrier was comparable to that of *CRL/CaCO$_3$/Entrap* carrier. The results from the carrier preparation with and without filtration step, also indicated that the water or buffer level in the carrier significantly affected lipase catalyzed activity. The hydrolytic activity of enzyme in *CRL/Entrap/Entrap* carrier, preparing without filtration step, was 330 U, which was more than 10 folds higher than that of the carrier prepared with filtration step (28 U).

CONCLUSION

A new enzyme carrier was developed by adsorption of lipase on CaCO$_3$ followed by the entrapment in calcium alginate beads. The suitable bead diameter and sodium alginate concentration was at 1.7 mm. and 1% w/v. respectively. The

optimum temperature was obtained at 50°C. With the different preparation method, it was found that the water or buffer levels in the carrier significantly affected the lipase catalyzed reaction. Moreover, the diffusional interference over the calcium alginate bead could intrude to slow the rate of reaction.

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VITA

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