CHAPTER 3

RESULTS AND DISCUSSION

Part 1. Synthesis of Sugar Esters from Palm Oil and Palm Fatty Acid Distillates using Commercial Lipases

1. Physical and chemical properties of palm oil and PFAD

Compositions and properties of palm oil, crude palm fatty acid distillates (PFAD) and partially purified PFAD were analyzed before using as acyl donor for sugar esters (SE) synthesis. The results are shown in Table 13. Molecular weights of palm oil, crude PFAD and partially purified PFAD were 840.38, 287.54 and 257.25 g/mol as calculated from saponification value, respectively. The major composition in palm oil was 97.70% triacylglycerol (TAG). Kaewthong (2004) also showed the same result of 96.07% TAG. Crude PFAD was composted of 97.95% fatty acid (FA) and 2.05% TAG while partially purified PFAD contained 100% FA. Palmitic and oleic acids were the major FA in palm oil (38.28 and 46.19%, respectively) and crude PFAD (53.84 and 33.87%, respectively) while partially purified PFAD contained 94.50% palmitic acid (Table 14). The palmitic acid content in crude PFAD was increased since it was purified by winterization in hexane at 4°C. Similar result was reported that palmitic acid (~70%) was the major composition in PFAD (Mohd Suria Affandi, 1994).

2. Hydrolytic activity of commercial lipases

Hydrolytic activity of commercial lipases was analyzed by cupric acetate method using palm oil as substrate. The results are shown in Table 15. Lipase PS from *Pseudomonas cepacia* and lipase AY from *Candida rugosa* showed high specific activity of 180.71 and 155.40 U/mg protein, respectively. However, Kaewthong (2004) reported that the specific activity of lipase AY was higher than PS (241.91 and 189.63 U/mg protein, respectively). The lipase AK, lipase D and lipase M showed relative low specific activities (38.24, 17.69 and 20.62 U/mg protein). Three immobilized lipase, Novozym 435, Chirazyme L-1 and Lipozyme RM IM showed activity of 9.90, 10.15 and 25.40 U/mg immobilized enzyme, respectively.

Table 13. Compositions and properties of palm oil and PFAD.

Materials	Properties				
	Saponification value	Molecular weight (g/mole)	TAG (%)	DAG (%)	FA (%)
Palm oil	200.30	840.38	97.70	2.30	-
Crude PFAD	287.54	287.54	2.05	-[a]	97.95
Partially purified PFAD	257.25	257.25	-	-	100

[a]Not detectable

Table 14. Fatty acid compositions of palm oil and PFAD.

Fatty acid composition (%)	Fat materials[a]		
	Palm oil	Crude PFAD	Partially purified PFAD
Myristic acid (C14:0)	0.84 ± 0.05	1.08 ± 0.06	-
Palmitic acid (C16:0)	38.28 ± 0.56	53.84 ± 0.56	94.50 ± 0.55
Stearic acid (C18:0)	10.08 ± 0.35	4.26 ± 1.35	2.14 ± 0.16
Oleic acid (C18:1)	46.19 ± 0.95	33.87 ± 2.24	2.43 ± 0.31
Linoleic acid (C18:2)	4.30 ± 0.69	6.47 ± 0.76	-

[a]Mean±standard deviation from triplicate determination

3. Selection of support for lipase immobilization

Lipases were immobilized on different solid supports by physical adsorption. Accurel EP100 (<400 μm) displayed the best immobilized activity of all lipases (Table 16). Accurel was the hydrophobic microporous material, provided better performance for immobilized lipase. Kaewthong (2004) reported that the immobilized lipase PS on Accurel EP100 (<200 μm) displayed the best immobilized activity of 0.37 U/mg support. Moreover, Kimura *et al.* (1983) immobilized lipases on different inorganic and organic supports and found that the hydrophobic material e.g. polypropylene and Celgard 2500 showed high activity in hydrolysis of olive oil. It might be that the hydrophobic substrate as olive oil was attached on the surface of hydrophobic supporter so it increased the contact between enzyme and substrate. Therefore, the hydrophobic microporous polymeric material is superior adsorbents for lipase immobilization.

Table 15. Hydrolytic activity of commercial lipases (cupric acetate method).

Enzyme	Activity [a] (U/mg enzyme)	Protein [a] (mg/mg enzyme)	Specific activity [a] (U/mg protein)
Free enzyme			
Lipase AK	6.50 ± 0.05	0.17 ± 0.01	38.24
Lipase AY	35.42 ± 0.06	0.23 ± 0.005	155.40
Lipase D	15.04 ± 0.32	0.85 ± 0.02	17.69
Lipase M	3.30 ± 0.05	0.16 ± 0.009	20.62
Lipase PS	12.50 ± 0.10	0.07 ± 0.001	180.71
Immobilized enzyme			
Lipozyme RM IM	25.40 ± 0.05	ND	ND
Novozym 435	9.90 ± 0.07	ND	ND
Chirazyme L-1	10.15 ± 0.10	ND	ND

[a]Mean±standard deviation from triplicate determination

ND = not determined

Table 16. Hydrolytic activity of immobilized lipases on various supports.

Supporting materials	Immobilized activity (U/mg support)[a]				
	Lipase AK	Lipase AY	Lipase D	Lipase M	Lipase PS
Accurel EP 100	0.47±0.02[b]	0.40±0.09	0.36±0.02	0.42±0.01	0.48±0.06
Amberlite XAD-7	0.05±0.008	0.075±0.005	0.03±0.001	0.10±0.02	0.04±0.001
Cetlite 545	0.10±0.006	0.15±0.01	0.08±0.004	0.06±0.001	0.14±0.005
PVC (250 μm)	0.20±0.05	0.26±0.007	0.15±0.001	0.30±0.04	0.35±0.02
Syran (SIKUG 012)	0.16±0.06	0.08±0.004	0.09±0.002	0.12±0.006	0.14±0.006

[a]Activity of lipase was determined by cupric acetate method

[b]Mean±standard deviation from triplicate determination

4. Optimization of sugar esters synthesis from palm oil and palm fatty acid distillates

4.1 Selection of immobilized lipase

Immobilized lipase on Accurel EP100 (<400 μm) (IM-AY, IM-AK, IM-PS, IM-D and IM-M), Lipozyme RM IM, Chirazyme L-1 and Novozym 435 were used for SE synthesis in acetone using palmitic acid as acyl donor and glucose as acyl accepter. Only immobilized lipase B from *C. antarctica* (Chirazyme L-1 and Novozym 435) could yielded glucose ester (Figure 5). So Novozym 435 was selected to synthesize sugar esters using palm oil and PFAD as acyl donors. Many kinds of lipases were used for SE synthesis such as *Mucor miehei* lipase (MML), Porcine pancrease lipase (PPL), *Candida rugosa* lipase (CRL) and *C. antarctica* lipase B (CALB) (Degn, 2000).

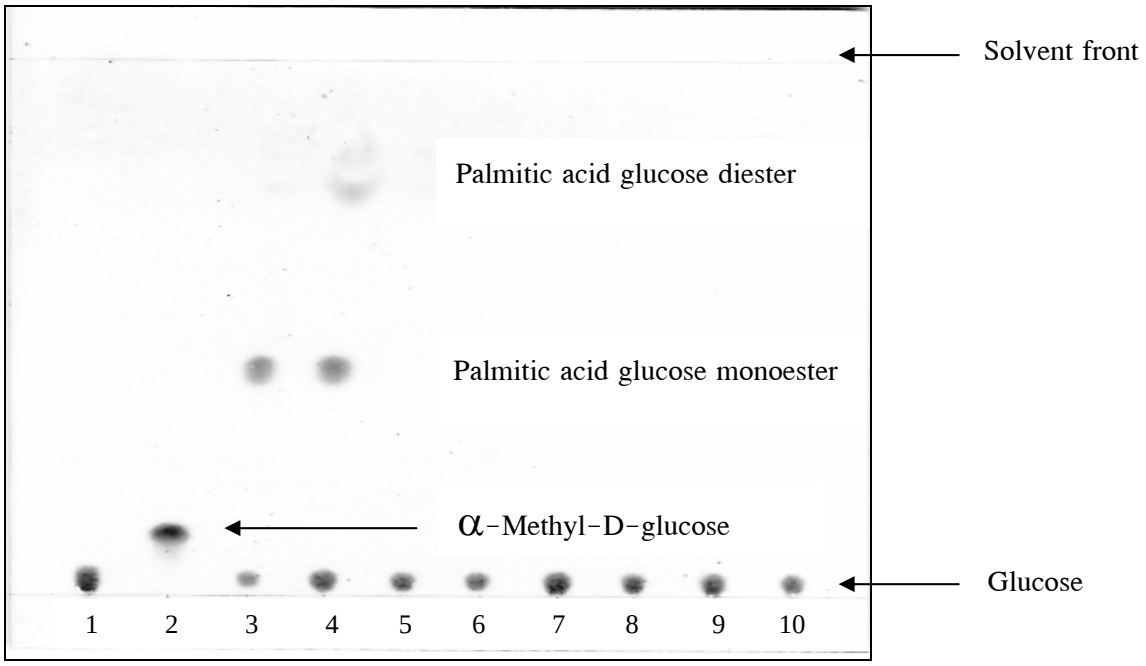


Figure 5. TLC of palmitic acid glucose ester catalyzed by various immobilized lipases.
(Lane 1: D-glucose, lane 2: α-Methyl-D-glucose (as standard), lane 3: Novozym 435, lane 4: Chirazyme L-1, lane 5: Lipozyme RM IM, lane 6: IM-AK, lane 7: IM-AY, lane 8: IM-D, lane 9 IM-D and lane 10: IM-PS)

Condition: Stationary phase: Silica gel plate (F_{254})
Mobile phase: Chloroform/methanol/formic acid (50:10:1 v/v/v)
Visualizing agent: 50% sulphuric acid in methanol, 110°C 10 min

Most reports have been used CALB for SE synthesis with high yield because CALB showed little or no interfacial activity and it showed relatively low hydrolysis activity of long chain fatty acid in TAG (Degn, 2000). For this reason, it may be better classified as esterase (Bornscheuer and Kazlauskas, 1999).

4.2 Effect of acyl donors

Palm oil, crude PFAD and partially purified PFAD were used as acyl donors for glucose ester synthesis in acetone catalyzed by Novozym 435. The glucose esters were obtained when both crude and partial PFAD were used as the substrate while palm oil could not be converted to glucose ester (Figure 6). Only few reports have shown the possibility of using TAG as acyl donor for SE synthesis. Ikeda *et al.* (1993) reported the synthesis of glucose ester from various TAG in anhydrous *tert*-butanol with phenylboronic acid as solubilizing agent catalyzed by *Pseudomonas* sp. lipoprotein lipase was achieved. The conversion yield of 54, 47, 42 and 41% were obtained with apricot seed, cotton seed, olive and corn oil. Though, TAG can be used as acyl donor in SE synthesis but the system is not compatible for food application due to the toxicity of organic solvent used.

Though, the major composition of crude PFAD was FA but the minor compositions was 2.05% TAG (Table 13). The TAG in crude PFAD was not converted to SE. Moreover, crude PFAD composted of various types of FA (myristic, palmitc, stearic, oleic and linoleic acid) as shown in Table 14. Trace amount of glucose oleate was observed while no product was obtained with linoleic acid (Figure 6). So, Oleic and linoleic acids were not good substrate for glucose ester synthesis in acetone catalyzed by Novozym 435. Arcos *et al.* (1998) reported the synthesis of glucose ester in acetone catalyzed by Novozym 435. They found that the high yield of glucose monoester (90-98%) was obtained with myristic, palmitic and stearic acids while only 50% was obtained with oleic acid. When the synthesis of glucose ester with various fatty acids in acetone catalyzed by Novozym 435, the high conversion yields were obtained with stearic, palmitic, myristic and lauric acids (92, 87, 78 and 70%, respectively) while oleic, caproic and caprylic acids yielded only 53, 45 and 51%, respectively (Cao *et al.*, 1997). Novozym 435 seems to have specificity toward medium to long chain saturated fatty acid (C_{12}-C_{18}).

Compared with crude PFAD, the partially purified PFAD composted of only FA (100%) with palmitic, stearic and oleic acids (94.50, 2.14 and 2.43%, respectively). Palmitic and stearic acids were good acyl donors for SE synthesis in acetone due to the specificity of Novozym 435. So this material was chosen as the source of FA for glucose ester synthesis in acetone catalyzed by Novozym 435.

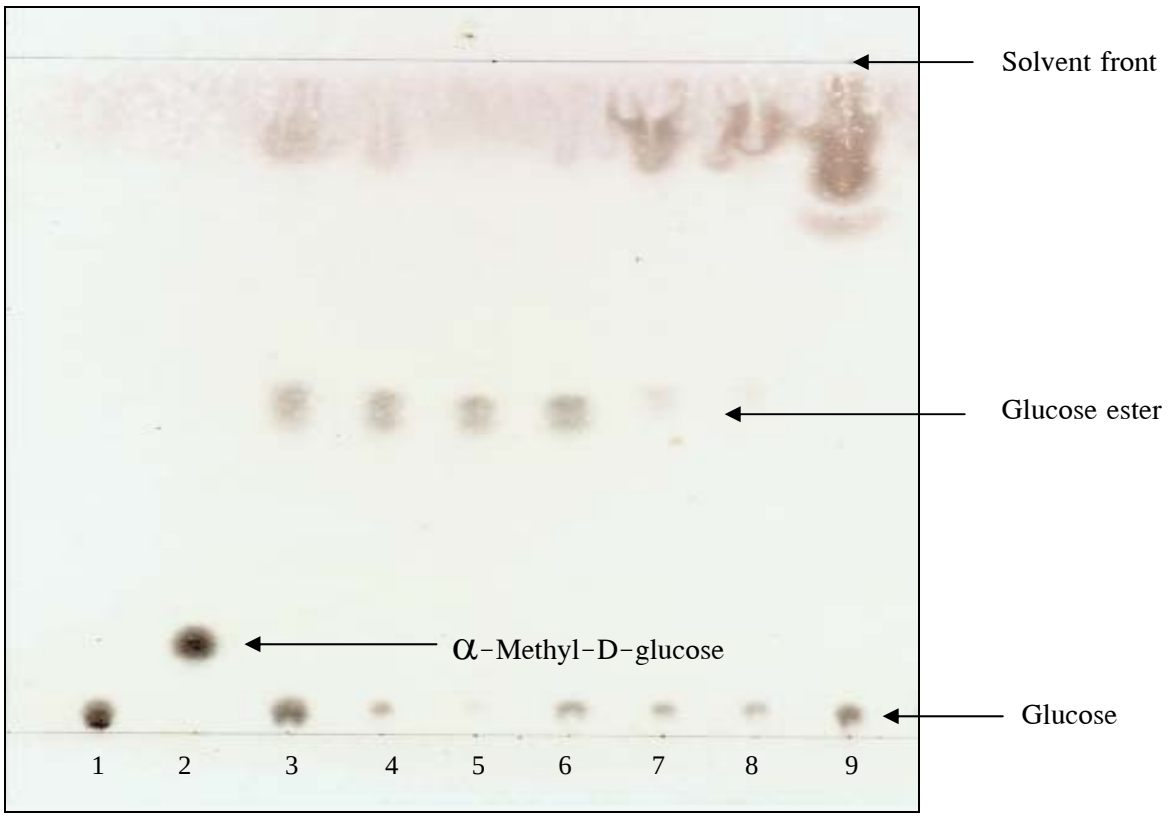


Figure 6. TLC of glucose esters catalyzed by Novozym 435.

(Lane 1: D-glucose, lane 2: α-methyl-D-glucose (as standard), lane 3: crude PFAD, lane 4: partially purified PFAD, lane 5: palmitic acid, lane 6: stearic acid, lane 7: oleic acid, lane 8: linoleic acid and lane 9: palm oil)

Condition: Stationary phase: Silica gel plate (F_{254})

Mobile phase: Chloroform/methanol/formic acid (50:10:1 v/v/v)

Visualizing agent: 50% sulphuric acid in methanol, 110°C 10 min

4.3 Effect of acyl acceptors

The effects of glucose, fructose, galactose, α-methyl-D-glucopyranoside, sorbitol and L-ascorbic acid on PFAD esters synthesis in acetone catalyzed by Novozym 435 were studied (Table 17). Galactose and sorbitol were poor acyl acceptors compared to glucose, while fructose was moderately converted to SE. In contrast, higher yield of fructose ester (17.0 mg/mL *tert*-butanol) compared to glucose ester (13.0 mg/mL *tert*-butanol) had been reported when *Mucor miehei* lipase was used as biocatalyst (Rakmi *et al*., 1997). These results indicated that the yield of a specific carbohydrate fatty acid ester could be controlled by the selection of a suitable acyl acceptor for the production process.

The synthesis of oleoyl ascorbate and palmityl ascorbate by Novozym 435 in 2-methyl-2-butanol showed the conversion yield of 16.8 and 19 g/L, respectively (Humeau *et al*., 1998; Song and Wei, 2002). The use of modified sugar as α-methyl-D-glucopyranoside showed the highest product yield (103% compared with glucose). It might be that this sugar showed moderately good solubility in acetone while only few amount of glucose (0.04 mg/mL) could be dissolved (Cao *et al*., 1996). Though, α-methyl-D-glucopyranoside could be converted to PFAD esters with high yield but this acyl acceptor was not good for food application due to its toxicity. So glucose seems to be the suitable acyl acceptor for PFAD esters synthesis.

Table 17. Yields of PFAD esters obtained in the presence of various acyl acceptors from the reaction catalyzed by Novozym 435.

Acyl acceptor	Relative conversion (%)
L-ascorbic acid	81
D(-)-Fructose	51
D(+)-Galactose	9
D(+)-Glucose	100
D-Sorbitol	24
α-Methyl-D-glucose	103

(The reaction mixture contained of 0.5 mmol acyl acceptor, 0.5 mmol PFAD, 1.0 g molecular sieves (4°A) and 1000 U lipase in 5.0 mL organic solvent with initial a_w of 0.11. The reaction was carried out on a shaker at 400 rpm, 45°C for 72 h.)

4.4 Effect of solvents

Acetone, methyl-*tert*-butyl ether (MTBE), *tert*-butanol and 2-methyl-2-butanol were used as organic media for the synthesis of PFAD glucose esters catalyzed by Novozym 435. The highest yield of PFAD glucose esters was obtained with acetone as solvent after 72 h of reaction (Figure 7). Similar result was reported for the synthesis of glucose palmitate with the highest conversion (88%) in acetone (Cao *et al.*, 1997). Although, sugar is only partially soluble in acetone but the reaction could be performed in heterogenous system or solid phase where most of the substrate is suspended in the reaction mixture (Cao *et al.*, 1997). Acetone has the advantage for SE synthesis that it can be easily removed from the reaction system for product recovery (Ducret *et al.*, 1995) and it is a solvent approved by the EU authority to be used in the manufacture of food and food additives (Soedjak and Spradlin, 1994).

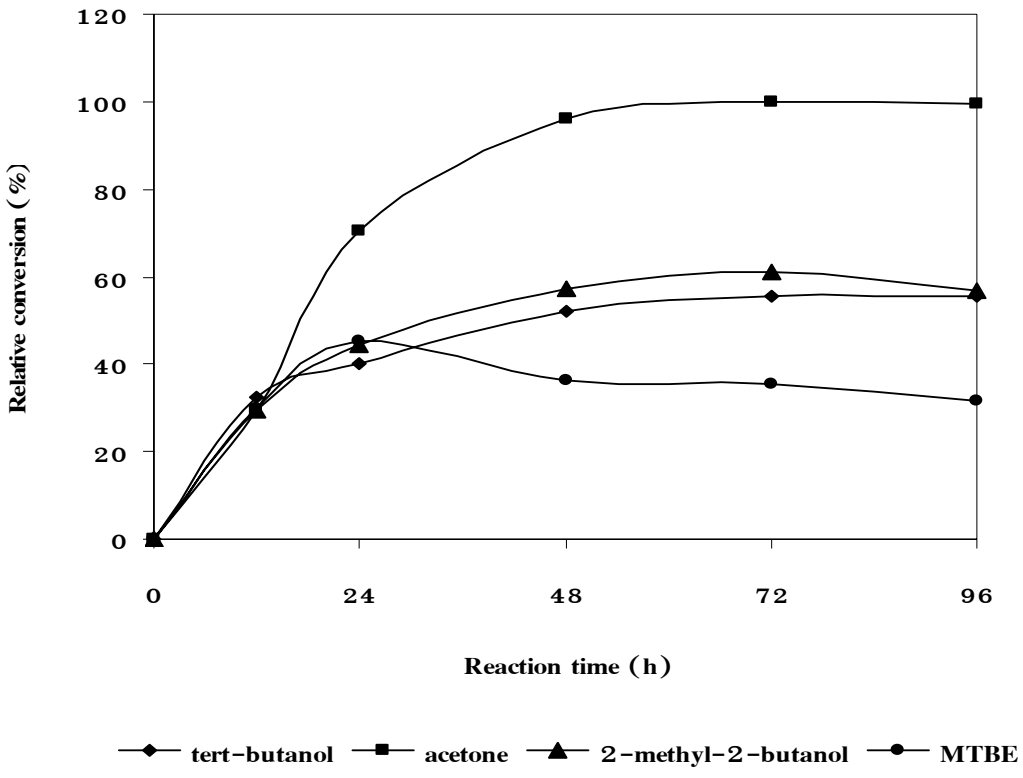


Figure 7. Acylation of glucose with partially purified PFAD by Novozym 435 in various organic solvents.

(The reaction mixture contained 0.5 mmol glucose, 0.5 mmol PFAD, 1.0 g molecular sieves (4°A) and 1000 U lipase in 5.0 mL organic solvent with initial a_w of 0.11. The reaction was carried out on a shaker at 400 rpm, 45°C.)

4.5 Effect of initial water activity (a_w)

The yield of the enzyme-catalyzed synthesis reaction in the organic medium significantly depended on the amount of water present in the reaction system (Chamouleau *et al*., 2001). The synthesis of PFAD glucose esters in acetone catalyzed by Novozym 435 with different water activities was compared. The reaction mixture with the initial a_w 0.07 showed high conversion which was not significantly different with the mixture with the a_w 0.11 ($p<0.05$). A sharp decrease in the glucose ester yield was found with a_w values higher than 0.23 (Figure 8). Humeau *et al*. (1998) reported the effect of initial a_w on ascorbyl palmitate synthesis in 2-methyl-2-butanol catalyzed by Novozym 435. The result showed that the production yield of ascorbyl palmtiate (5.7 g/L) was obtained with initial a_w 0.07 while only 1.0 g/L production yield was obtained with initial a_w 0.97. With higher water content, the equilibrium of the reaction is shifted to the hydrolysis of the ester, resulting in low yields of products.

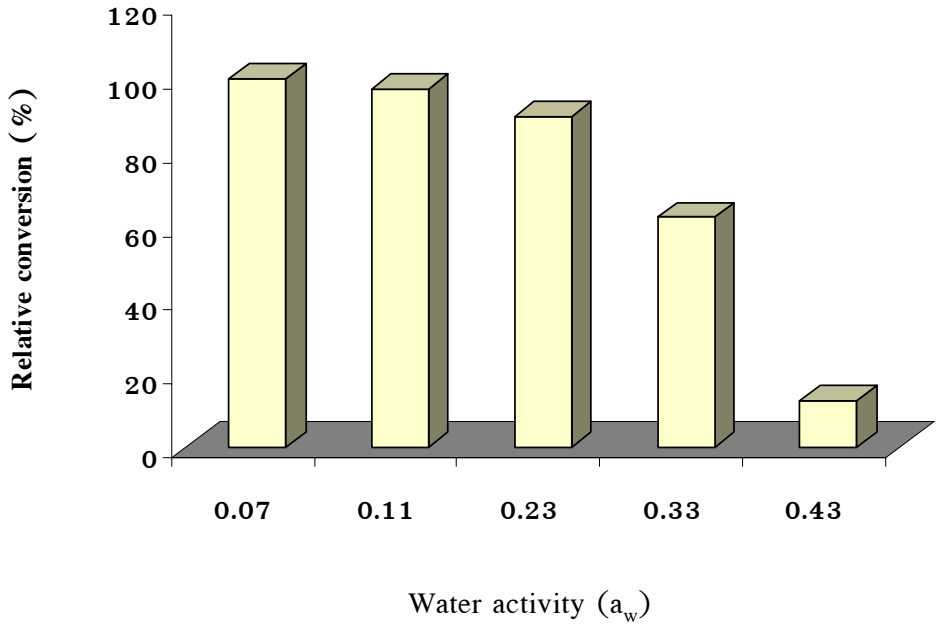


Figure 8. Effect of the initial water activity (a_w) of the reaction mixture on the yield of PFAD glucose esters synthesis catalyzed by Novozym 435.

(The reaction mixture contained of 0.5 mmol glucose, 0.5 mmol PFAD, 1.0 g molecular sieves (4°A) and 1000 U lipase in 5.0 mL acetone with initial a_w of (0.07-0.43). The reaction was carried out on a shaker at 400 rpm, 45°C for 72 h.)

4.6 Effect of molecular sieves

The effect of molecular sieves loading on production of PFAD glucose esters in acetone catalyzed by Novozym 435 with initial a_w 0.07 was studied. The highest product yield was obtained by the addition of 1.0 g of molecular sieves (4°A) while no products could be detected in the absence of molecular sieves in the reaction mixture. Adding of molecular sieves, more than 1.0 g did not increase the product yield. So the suitable molecular sieves loading, was 1.0 g in 5 mL acetone. The molecular sieves are added to the reaction medium to adsorb water produced during the reaction and to reduce the effect of competition of hydrolysis reaction (Degn, 2000). The effect of molecular sieves adding on the synthesis of L-ascorbyl oleate in 2-methyl-2-butanol catalyzed by immobilized lipase from *Candida* sp. was reported by Song and Wei (2002). They found that the conversion yield of 18.5 g/L of L-ascorbyl oleate was obtained in 10 h with adding of 50 g/L molecular sieves while only 14.0 g/L of the products was obtained without molecular sieves adding.

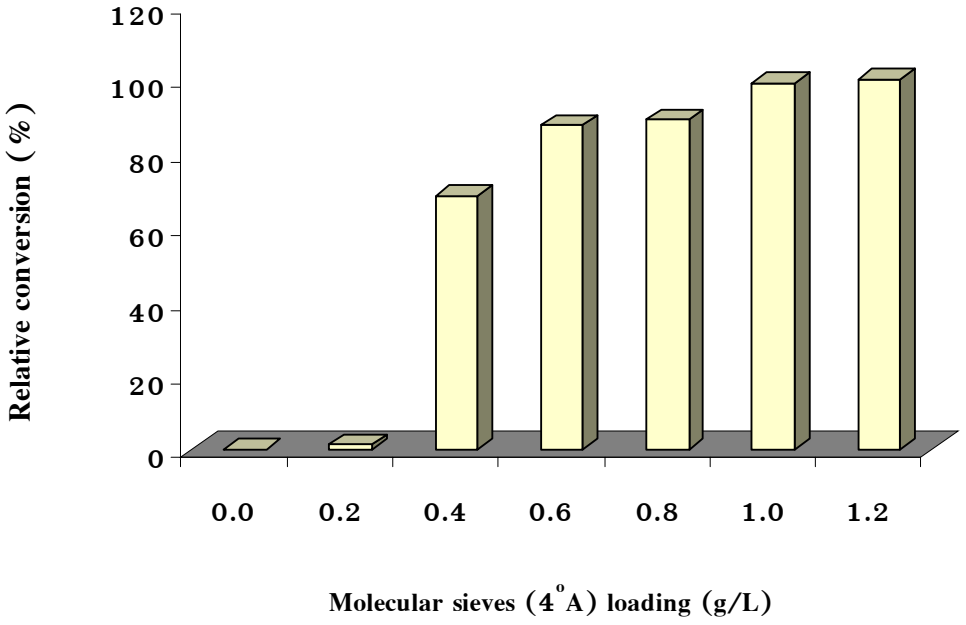


Figure 9. Effect of molecular sieves (4°A) loading on PFAD glucose esters synthesis catalyzed by Novozym 435.

(The reaction mixture contained of 0.5 mmol glucose, 0.5 mmol PFAD, 1000 U lipase in 5.0 mL acetone with initial a_w 0.07 and 0-1.2 g molecular sieves (4°A). The reaction was carried out on a shaker at 400 rpm, 45°C for 72 h.)

4.7 Effect of molar ratio of glucose to PFAD

To obtain the high conversion yield of PFAD glucose esters, the effect of molar ratio of glucose to PFAD (1:1 to 1:5 mol/mol) on glucose ester synthesis in acetone catalyzed by Novozym 435 with initial a_w 0.07 and 1.0 g molecular sieves was studied. The results showed that increasing the molar ratio from 1:1 to 1:5 did not significantly increase the productivity yield (Figure 9). So, the molar ratio of glucose to PFAD (1:1) was suitable for PFAD glucose esters synthesis. The effect of molar ratio on glucose palmitate synthesis in acetone catalyzed by Novozym 435 was also reported by Cao (*et al*. 1996). They concluded that increasing of the fatty acid concentration did not lead to a significant increase in productivity and conversion. The two fold excess of palmitic acid increased only 6% of conversion yield. Similar results was also reported by Sakaki *et al*. (2006) that only 3% of conversion yield increased when the molar ratio glucose to palmitic acid was varied from 1:1 to 1:5 of glucose palmitate production in 2-methyl-2-butanol catalyzed by CALB.

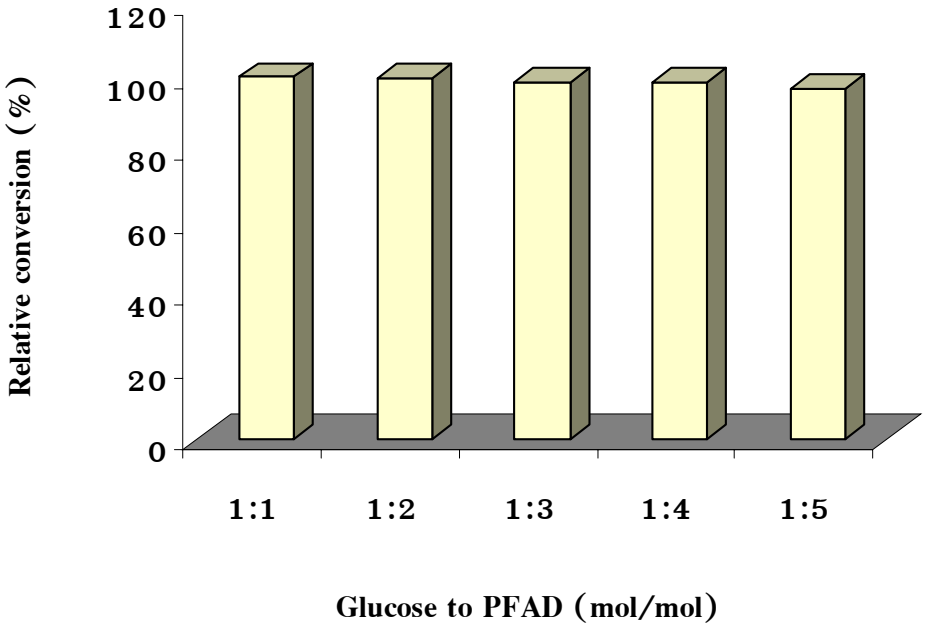


Figure 10. Effect of molar ratio of glucose to PFAD on PFAD glucose esters synthesis catalyzed by Novozym 435.

(The reaction mixture contained of 0.5 mmol glucose, 0.5-2.5 mmol PFAD, 1.0 g molecular sieves (4°A) and 1000 U lipase in 5.0 mL acetone with initial a_w of 0.07. The reaction was carried out on a shaker at 400 rpm, 45°C for 72 h.)

4.8 Effect of enzyme loading

The synthesis of PFAD glucose esters was further optimized by adjusting the amount of immobilized enzyme added to the reaction mixture. Raising the enzyme concentration from 25 mg (250 U) to 75 mg (750 U) showed the increase of product yield while further increase in enzyme concentration more than 750 U did not significantly increase the product yield (Figure 11). The optimal enzyme concentration was 750 U (75 mg Novozym 435 or 150 U/mL acetone) which resulted highest conversion yield.

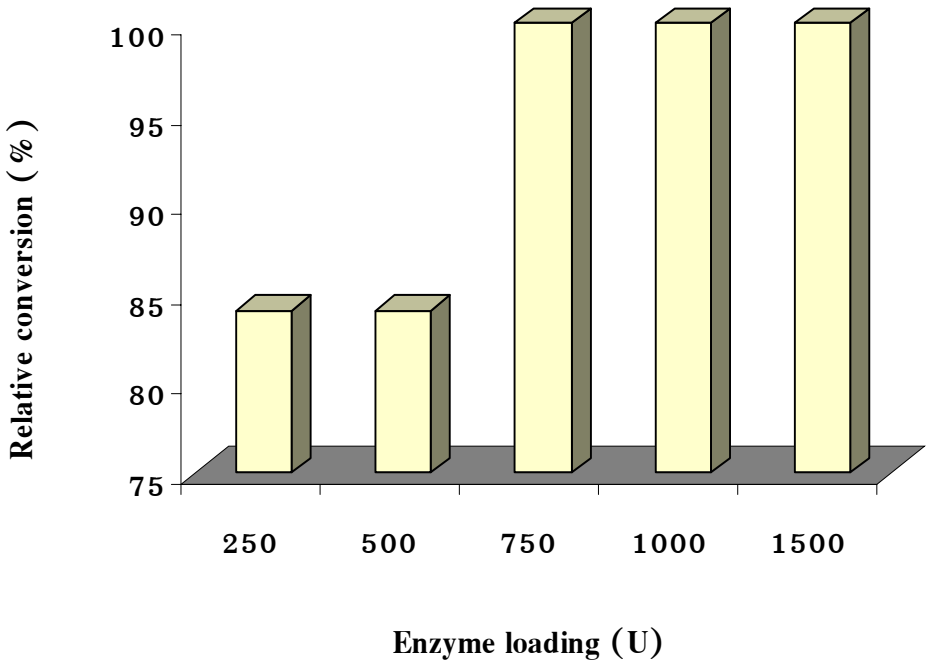


Figure 11. Effect of enzyme loading on PFAD glucose esters synthesis catalyzed by Novozym 435.

(The reaction mixture contained of 0.5 mmol glucose, 0.5 mmol PFAD, 1.0 g molecular sieves (4°A) and 250-1500 U of Novozym 435 in 5.0 mL acetone with initial a_w 0.07. The reaction was carried out on a shaker at 400 rpm, 45°C for 72 h.)

4.9 Effect of temperature

The reaction temperature has a significant impact on the product yield since the solubilization of the sugar and the reaction rate will be increased at higher temperatures (Degn *et al*., 1999). However, the enzyme can become inactivated in the organic solvents when the reaction temperature is increased. When the synthesis of PFAD glucose esters in acetone catalyzed by Novozym 435 was carried out in the range of 35-55°C, the highest yield of PFAD glucose esters (31.8 mg/mL acetone) was obtained at 40°C (Figure 12). Further increase of

the reaction temperature resulted in lower yields indicating an inactivation of the enzyme in the presence of acetone.

The effect of temperature on PFAD fructose ester synthesis in *tert*-butanol catalyzed by *M. miehei* lipase (MML) was reported by Rakmi *et al* (1997). The results showed that increase of temperatures from 40 to 55 °C did not significantly increase of product yield. The PFAD fructose ester yield of 17.0 and 17.5 mg/mL were obtained with 40 and 55°C, respectively. Furthermore, Arcos *et al*. (1998) reported the effect of temperature on glucose laurate synthesis in acetone catalyzed by Novozym 435. The highest yield of glucose monolaurate (95%) was obtained at 40°C while high temperature (60°C) decreased the conversion yield to 90%.

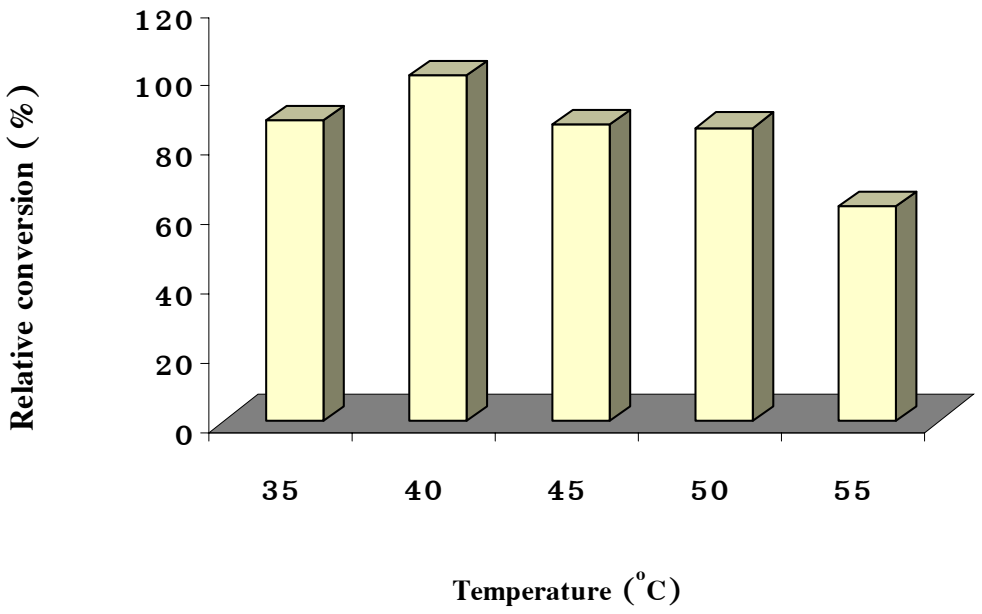


Figure 12. Effect of temperature on PFAD glucose esters synthesis catalyzed by Novozym 435.

(The reaction mixture contained 0.5 mmol glucose, 0.5 mmol PFAD, 1.0 g molecular sieves (4°A) and 750 U lipase in 5.0 mL acetone with initial a_w 0.07. The reaction was carried out on a shaker at 400 rpm, 35- 55°C for 72 h.)

4.10 Product identification

The synthesis of carbohydrate fatty acid esters from underivatized carbohydrate and free fatty acids by *C. antarctica* lipase B (Novozym 435) has been previously reported. The enzyme catalyzed specifically the acylation of the 6-position of glucose (Ljunger *et al*., 1994 and Arcos *et al*., 1998). Results of the analysis of the products obtained with PFAD glucose esters catalyzed by Novozym 435 in acetone after 72 h of reaction by GC are shown in Figure

13. The main peak with a retention time of 4.9 min corresponding to 6-*O*-palmityl-α-D-glucopyranoside, while the two minor peaks with 5.5 and 5.6 min retention time represented the stearic and oleic ester of glucose, respectively. The identity of 6-*O*-palmityl-α-D-glucopyranoside was confirmed by ^{1}H NMR and comparison with published data (Degn *et al.*, 1999) and by MALDI-TOF spectrometry where the expected mass of 6-*O*-palmityl-α-D-glucopyranoside was found (Appendix 12).

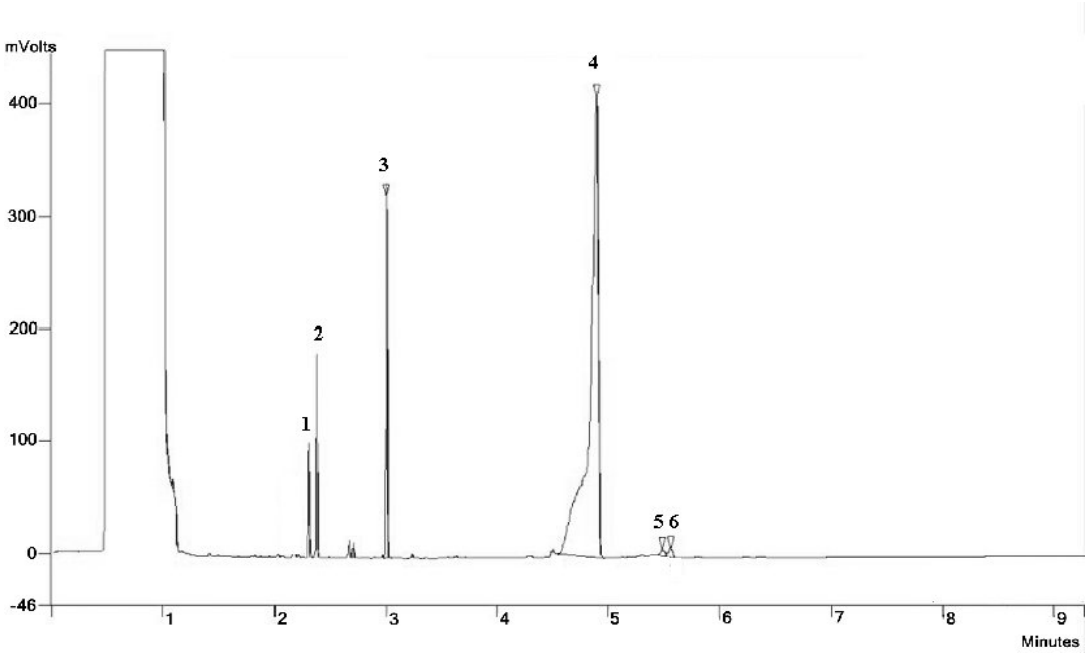


Figure 13. GC chromatogram of the reaction products obtained by incubation of PFAD and glucose with *C. antarctica* lipase. Peak 1: α-D-glucopyranose, peak 2: β-D-glucopyranose, peak 3: octyl-β-D-glucoside (internal standard), peak 4: palmityl ester of glucopyranose, peak 5: stearyl ester of glucopyranose and peak 6: oleyl ester of glucopyranose.

4.11 Time course of PFAD glucose esters synthesis

Production of PFAD glucose esters catalyzed by Novozym 435 was studied using optimum conditions and conversion was determined by GC. The optimum conditions were equi-molar of 50 mmol glucose and PFAD (as palmitic acid), 75 mg (750 U) Novozym 435, initial water activity of 0.07, and 1.0 g molecular sieves (4°A) in 5.0 mL acetone at 40°C. The conversion was rapidly increased in 48 h then was slightly increase from 48 to 72 h. The highest conversion yield of 76.3% or 31.8 mg/mL acetone was obtained at 72 h of reaction time. Cao *et al.* (1997) reported that the highest conversion yield (86%) was obtained in the synthesis of glucose palmitate from glucose and palmitic acid in acetone catalyzed by Novozym

435 at 60°C for 72 h. Though, the conversion yield (76.3%) obtained form this study is lower than the published reports but most of studies used free fatty acid to esterify with glucose. In our study, the use of PFAD which composts of 3 fatty acids (palmitic, stearic and oleic acids) might effect on the conversion yield.

Rakmi *et al.* (1997) reported the synthesis of PFAD glucose from PFAD in *tert*-butanol catalyzed by MML at 40°C. They found that the conversion yield of PFAD glucose esters (13 mg/mL *tert*-butanol) was obtained. However, in our study the conversion yield of PFAD glucose esters of 31.8 mg/mL was obtained.

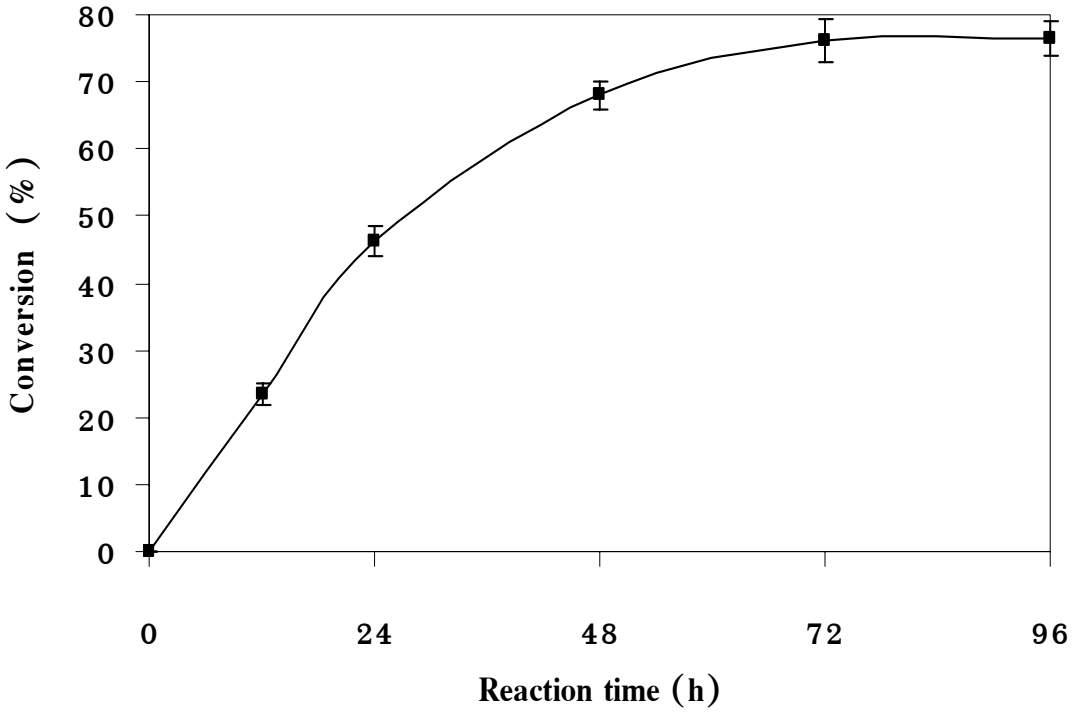


Figure 14. Time course of PFAD glucose esters synthesis catalyzed by Novozym 435 under optimal conditions.

(The reaction mixture contained 0.5 mmol glucose, 0.5 mmol PFAD, 1.0 g molecular sieves (4°A) and 750 U lipase in 5.0 mL acetone with initial a_w 0.07. The reaction was carried out on a shaker at 400 rpm, 40°C for 96 h.)

Part 2. Screening and Production of Lipase for Sugar Esters Synthesis

1. Screening of lipase for sugar esters synthesis

1.1 Screening of lipase producing bacteria

Three hundred bacterial strains (PSU-AH strains) were screened for extracellular lipase production in basal medium using 0.1% (w/v) Tween 80 as a carbon source at 45°C. Four strains of PSU-AH55, PSU-AH56, PSU-AH130, PSU-AH191, PSU-AH192 and LS produced lipase activity when they were cultivated in basal medium supplemented with 0.1% (w/v) Tween 80. Three *Actinomycetes* strains (ME162, ME168 and ME177) were cultivated in M65 medium using olive oil as a carbon source at 45°C. Only ME168 and 177 could produced lipase when were cultivated in M65 medium supplemented with 1.0% (w/v) olive oil. The ability of lipase production of all bacterial strains was also tested on agar plate which supplemented with 0.01% (w/v) of Rhodamine B as indicator. The strains appeared as orange-pink with clear zone under UV light were selected as lipase producers (Figure 15).

The hydrolytic activity of the cell free supernatants from 8 strains cultivated in appropriate medium was determined using *p*NPC$_8$ as the substrate at pH 7.5. When the PSU-AH strains were cultivated in basal medium using 0.1% (w/v) Tween 80 as a carbon source, PSU-AH130 and PSU-AH192 produced high lipase activity of 2.66 and 0.37 U/mL with specific lipase activity of 11.08 and 10.80 U/mg protein, respectively while other PSU-AH strains produced low activity (Table 18).

The *Actinomycetes* strain ME177 showed activity and specific activity of 0.65 U/mL and 1.42 U/mg protein, respectively while ME168 showed low activity (0.3 U/mL and 0.38 U/mg protein).

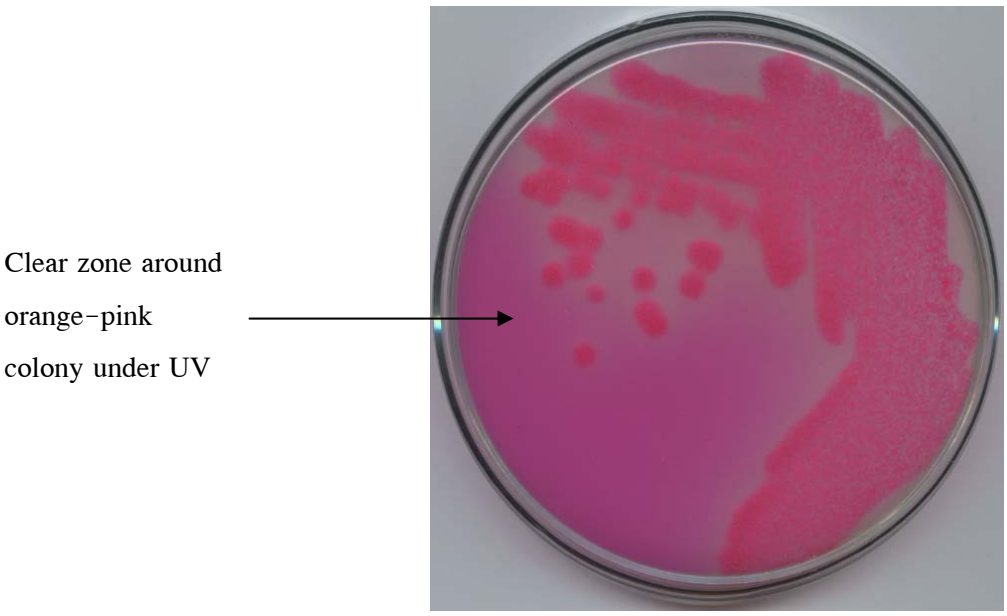


Figure 15. Bacterial colonies of PSU-AH130 on the agar plate of the basal medium supplemented with 0.01 % (w/v) of Rhodamine B.

Table 18. Hydrolytic activity of lipase from the selected strains.

Bacterial strains	Lipase activity[a] (U/mL)	Specific activity[b] (U/mg protein)
PSU-AH55	0.06 ± 0.004[c]	1.06 ± 0.008
PSU-AH56	0.10 ± 0.001	1.48 ± 0.006
PSU-AH130	2.66 ± 0.000	11.08 ± 0.021
PSU-AH191	0.35 ± 0.002	3.72 ± 0.010
PSU-AH192	0.37 ± 0.002	10.80 ± 0.04
LS	0.09 ± 0.003	2.58 ± 0.003
ME168	0.30 ± 0.001	0.38 ± 0.007
ME177	0.65 ± 0.001	1.42 ± 0.012

[a]Lipase activity was determined by using *p*NPC$_8$ as substrate at pH 7.5

[b]Protein concentration was determined by Bradford method (1976)

[c]Mean±standard deviation from triplicate determination

1.2 Screening of lipase for sugar esters synthesis

The large volume of lipase production was produced in 2 L flask containing of 300 mL suitable medium. Eight PSU-AH strains were cultivated in basal medium using Tween 80 as a carbon source for 48 h while 2 *Actinomycetes* strains were cultivated in M65 medium using olive oil as a carbon source for 96 h. After suitable cultivation time, the culture broths were centrifuged, concentrated and immobilized on celite by chilled acetone. The summary of production, concentration and immobilization steps from 8 strains are shown in Table 19. The hydrolytic activity of immobilized lipases was measured by cupric acetate method. Immobilized lipase from PSU-AH192 showed the highest activity (0.74 U/mg), while the cell free supernatant showed low lipase activity (0.37 U/mL). In contrast, cell free supernatant of PSU-AH130 showed highest lipase activity (3.01 U/mL) but the immobilized activity was moderately low (0.37 U/mg). It might be the effect of acetone which inactivated enzyme during the precipitation.

The ester synthetic activity of immobilized lipases from 8 bacterial strains was studied on synthesis using glucose as acyl acceptor and various vinyl esters as acyl donor in the solvent mixture of *tert*-butanol/pyridine (55:45 v/v), 100 U of immobilized enzyme, initial a_w 0.07 at 45°C for 72 h. Immobilized lipase from ME168 could produced relative high yield of glucose acetate, glucose butyrate and glucose caproate with conversion yield of 93, 66 and 56%, respectively (Figure 16 and Table 20). The commercial lipase, CALB (Novozym 435) was used as the control. This lipase showed high conversion yield of glucose ester of 90, 89 and 88% with vinyl acetate, butyrate and caproate, respectively.

Though the immobilized lipase from PSU-AH191 showed high glucose ester synthesis but the hydrolytic activity of immobilized enzyme was quite low (0.08 U/mg). Hence, the high amount of immobilized lipase (1,250 mg) was required for SE synthesis. Other immobilized lipase produced by PSU-AH55, PSU-AH56, PSU-AH130, PSU-AH192, LS and ME177 showed low synthetic activity, less than 15% of glucose caproate were obtained. So the immobilized lipase from ME168 was selected for glucose ester synthesis using vinyl ester as acyl donor.

Table 19. Summary of hydrolytic activity during immobilization of lipase from the selected bacteria.

Strains	Step					
	Culture broth		Concentrated culture broth			Immobilization
	Volume (mL)	Activity[a] (U/mL)	Volume (mL)	Activity[a] (U/mL)	Protein[b] (mg/mL)	Activity (U/mg)[c]
PSU-AH55	1,820	0.062	85	2.27	2.15	0.16
PSU-AH56	1,400	0.10	110	1.43	0.97	0.28
PSU-AH130	1,800	3.01	140	34.88	2.84	0.37
PSU-AH191	2,150	0.35	125	5.2	1.40	0.08
PSU-AH192	1,750	0.37	75	13.9	1.29	0.74
LS	1,900	0.11	80	3.6	1.22	0.14
ME168	1,500	0.29	100	4.0	10.3	0.16
ME177	1,800	0.65	180	18.2	12.8	0.26

[a]Lipase activity was determined by using *p*NPC$_8$ as substrate at pH 7.5

[b]Protein concentration was determined by Bradford method (1976)

[c]The activity of immobilized lipases was determined by cupric acetate method

Table 20. Summary of glucose ester synthesis from various fatty acid vinyl esters catalyzed by 8 immobilized bacterial lipases.

Immobilized lipase	Conversion (%)		
	Vinyl acetate	Vinyl butyrate	Vinyl caproate
CALB	90.21	89.56	88.72
PSU-AH55	19.29	10.07	8.54
PSU-AH56	52.40	19.58	7.50
PSU-AH130	32.70	18.42	13.32
PSU-AH191	76.79	40.40	34.63
PSU-AH192	48.60	23.30	12.32
LS	15.34	7.63	7.03
ME168	93.40	66.67	56.22
ME177	2.06	3.12	0.47

(The reaction mixture contained 0.3 mmol glucose, 0.3 mmol various fatty acid vinyl esters, 0.5 g molecular sieve (4°A) and 100 U lipase in 3.0 mL mixture of *tert*-butanol/pyridine (55:45 v/v) with initial a_w 0.07. The reaction was carried out at 400 rpm at 45°C for 72 h.)

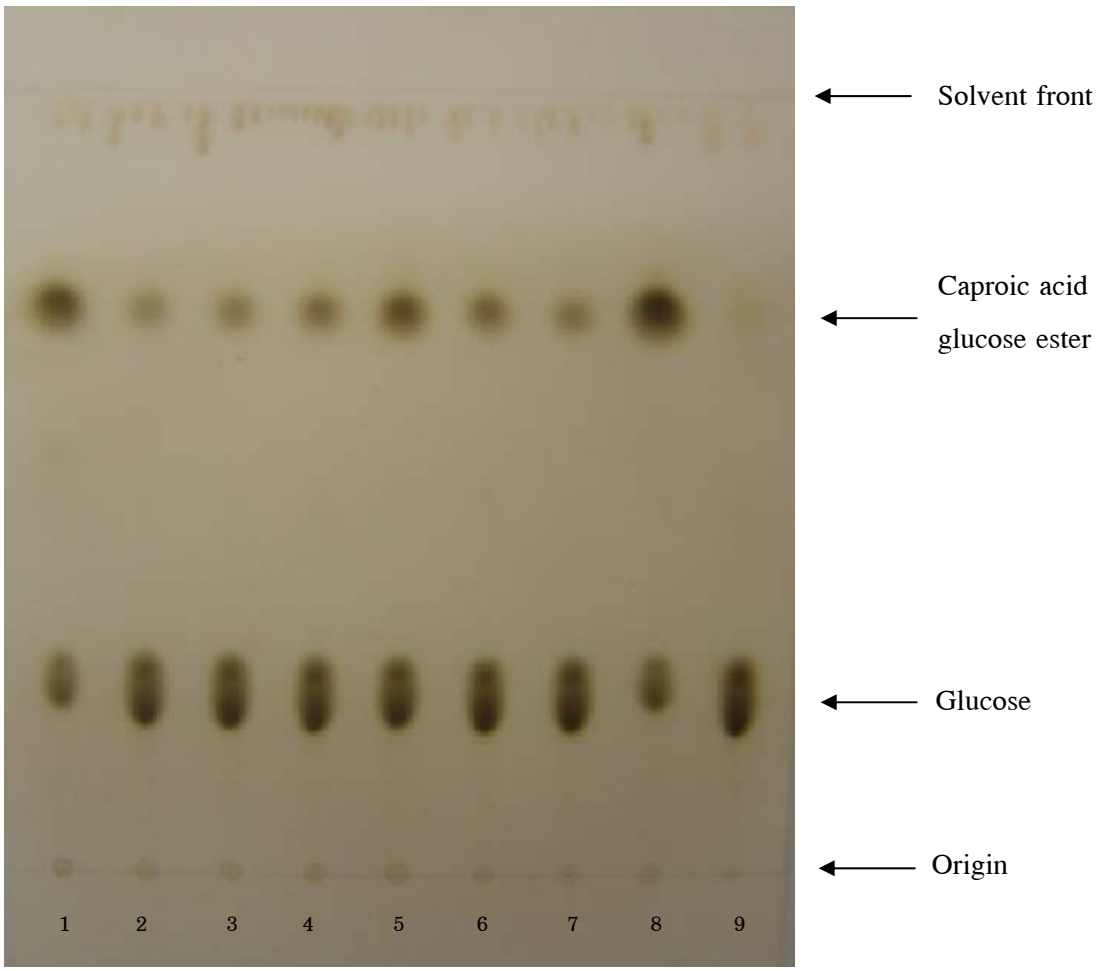


Figure 16. TLC chromatogram of caproic acid glucose ester catalyzed by various lipases. (Lane 1: CALB, lane 2: PSU-AH55, lane 3: PSU-AH56, lane 4: PSU-AH130, lane 5: PSU-AH191, lane 6: PSU-AH192, lane 7: LS, lane 8: ME168 and lane 9: ME177)

Condition: Stationary phase: Silica gel plate (F_{254})
Mobile phase: Ethanol/chloroform/acetic acid/water (3:1:1:1 v/v/v/v)
Visualizing agent: 50% sulphuric acid in methanol, 110°C 10 min

2. Identification of *Actinomycetes* ME 168

Identification of strain ME168 was performed on the basis of nucleotide sequence of bacterial 16S rDNA gene. The DNA sequence of ME168 showed 99.9% similarity with *Streptomyces thermocarboxydus* strain (1,465/1,466 bp) (Appendix 13). Therefore this strain was identified as *S. thermocarboxydus* ME168 (Figure 17).

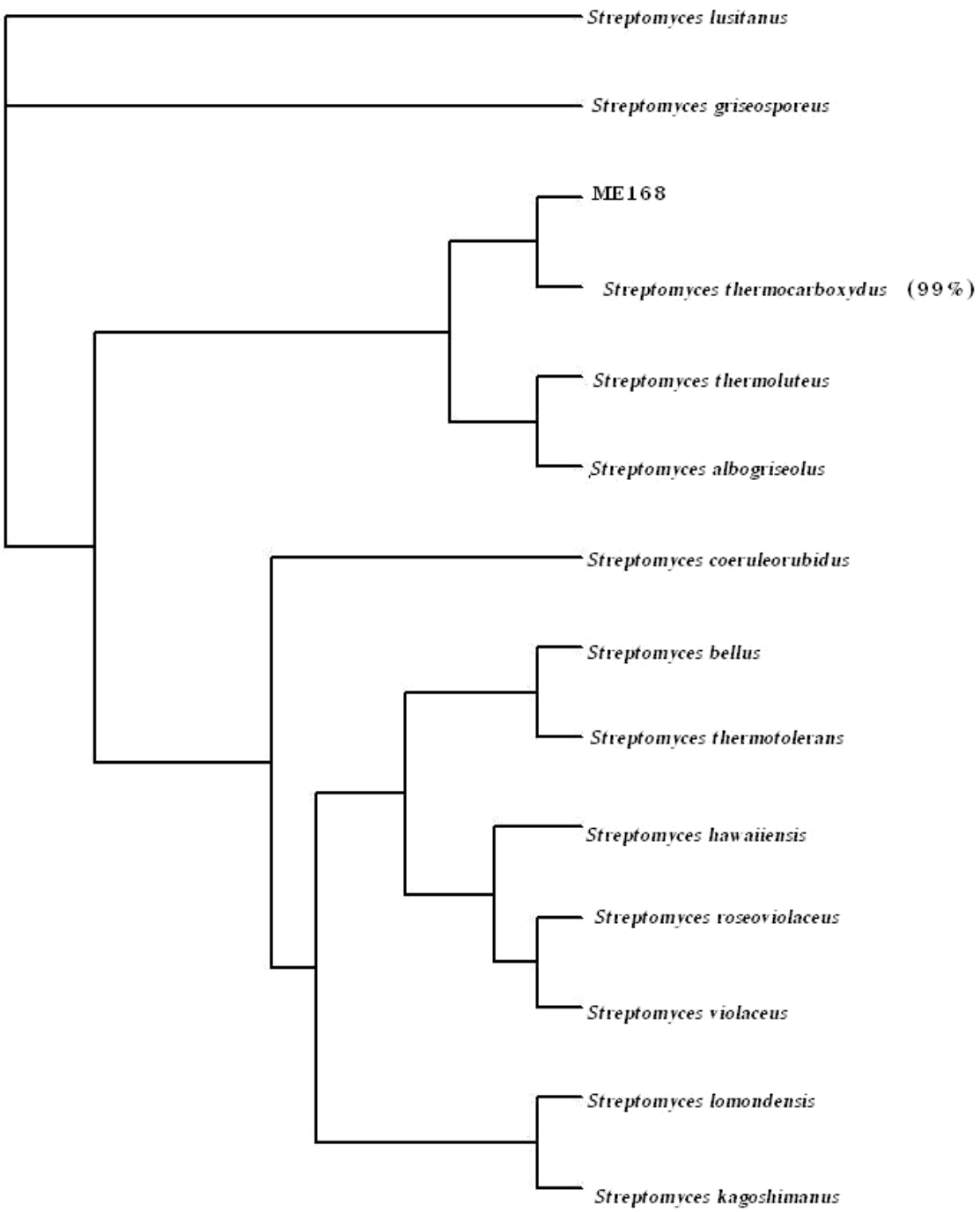


Figure 17. Phylogenetic tree of *Streptomyces thermocarboxydu*s ME168.

3. Optimization of sugar esters synthesis by immobilized lipase from *Streptomyces thermocarboxydus* ME168

Streptomyces thermocarboxydus ME168 produced extracellular lipase and showed good ability to synthesize glucose ester and fatty acid vinyl ester. So this strain was cultivated in large volume and the extracellular lipase was concentrated and immobilized on celite by co-precipitation with chilled acetone. The immobilized lipase was used for optimization of SE synthesis.

3.1 Effect of acyl acceptors

Various kinds of sugars (xylose, glucose, galactose, fructose, maltose and maltotriose) were used as acyl acceptors for the synthesis of SE using vinyl caproate as acyl donor in the solvent mixture of *tert*-butanol/pyridine (55:45 v/v) with initial a_w 0.07 catalyzed by immobilized lipase from *S. thermocarboxydus* ME168 (100 U). Glucose and fructose were converted to SE with more than 50% conversion while galactose and maltose showed low conversion yield (<10%) as shown in Table 21. So, glucose and fructose were the suitable acyl acceptors for SE synthesis catalyzed by immobilized lipase on celite from *S. thermocarboxydus* ME168.

The oligosaccharide, maltotriose was also investigated on SE synthesis but no product was obtained. Ferrer *et al.* (2000) reported the synthesis of the esters of leucrose (disaccharide) and maltotriose (trisaccharide) using vinyl laurate as acyl donor catalyzed by immobilized lipase on celite from *Thermomyces lanuginose*. They found that the yield of leucrose laurate (70%) was obtained in 8 h while maltotriose yielded only 21% of maltotriose laurate in 24 h. It seemed to be that when increase degree of polymerization of the sugar, the conversion yield dramatically decreased. This could be the result of low solubility of sugar in organic solvent when increasing degree of polymerization (Degn *et al.*, 1999).

Table 21. Effect of acyl acceptors on SE synthesis from vinyl caproate catalyzed by immobilized lipase of *Streptomyces thermocarboxydus* ME168.

Acyl acceptor	Conversion (%)[a]
D(+)-Xylose	32.9 ± 0.12
D(+)-Glucose	55.4 ± 0.26
D(+)-Galactose	4.2 ± 0.04
D(-)-Fructose	53.8 ± 0.21
Maltose	6.9 ± 0.01
Maltotriose	No reaction

[a]Mean±standard deviation from triplicate determination

(The reaction mixture contained 0.3 mmol sugars, 0.3 mmol vinyl caproate, 0.5 g molecular sieve (4°A) and 100 U lipase in 3.0 mL mixture of *tert*-butanol/pyridine (55:45 v/v) with initial a_w 0.07. The reaction was carried out at 400 rpm, 45°C for 72 h.)

3.2 Effect of acyl donors

The efficiency of different chain length of vinyl esters (C_2-C_{18}) on the formation of glucose ester in the solvent mixture of *tert*-butanol/pyridine (55:45 v/v) catalyzed by immobilized lipase from *S. thermocarboxydus* ME168 (100 U) was studied. The conversion yield of glucose ester decreased when the number of carbon atom in vinyl ester increased (Figure 19). The conversion yield of 93, 67, 55 and 5% were obtained when used vinyl acetate, vinyl butyrate, vinyl caproate and vinyl palmitate as acyl donor, respectively. Similar result was reported that synthesis of maltose ester by immobilized lipase on celite from *Thermomyces lanuginose* with the chain length of vinyl ester from C_{12}-C_{18} and the conversion yield of maltose ester decreased from 80 to 55 % (Ferrer *et al*., 2000).

Pedersen *et al*. (2002) reported the effect of acyl donor chain length on the synthesis of maltose ester catalyzed by CALB that the yield of maltose ester obtained from vinyl butyrate was 40.1% while vinyl laurate yielded only 3.6%. It might be that when the fatty acid chain length or the size of carbohydrate is increased, the steric effect on binding site of enzyme is occurred due to the reducing the probability of product formation (Pedesen *et al*., 2002).

The free fatty acid (palmitic acid) and PFAD were also used as acyl donors for glucose ester synthesis catalyzed by immobilized lipase from *S. thermocarboxydus* ME168. This immobilized lipase could synthesize glucose ester with very low yield from both palmtic acid and PFAD (data not shown). Though, vinyl acetate and vinyl butyrate can be converted to glucose

ester with relatively high conversion (%) but the glucose ester with short chain fatty acid are not good emulsifier. So the medium chain of vinyl caproate (C_6) seems to be the suitable acyl donor for glucose ester synthesis catalyzed by immobilized lipase from ME168.

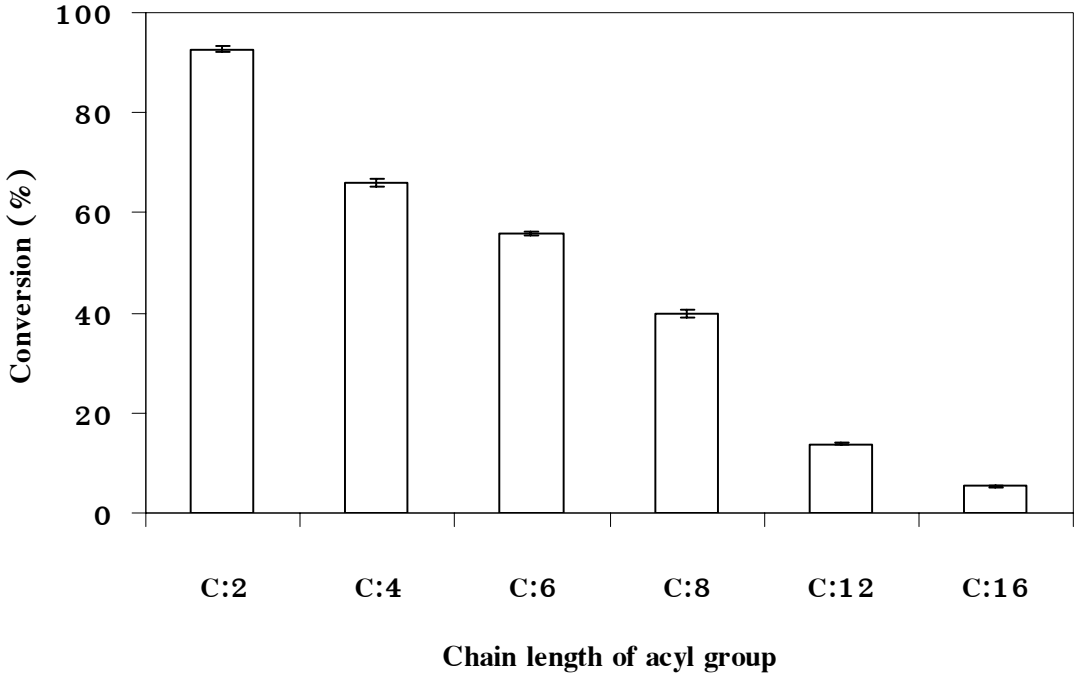


Figure 18. Effect of chain length of acyl donors on glucose ester synthesis catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168.

(The reaction mixture contained 0.3 mmol glucose, 0.3 mmol vinyl ester, 0.5 g molecular sieve (4°A) and 100 U lipase in 3.0 mL of mixture of *tert*-butanol/pyridine (55:45 % v/v) with initial a_w 0.07. The reaction was carried out at 400 rpm at 45°C for 72 h.)

3.3 Effect of organic solvents

The conversion yield of glucose to glucose caproate was investigated in various organic solvents (Table 22). Acetone and butanone showed very low conversion yield and no product was observed with hexane. In contrast, the highest conversion yield of 55% was obtained when the mixture of *tert*-butanol/pyridine (55:45 % v/v) was used. Similar result was also obtained by CALB (Degn and Zimmermann 2001).

The effect of maltose solubility on maltose laurate synthesis was reported that the solubility of maltose was 12 mM in *tert*-butanol and increased to 215 mM in the solvent mixture of *tert*-butanol/pyridine (9:11 v/v). When, more sugar was solubilized, the higher maltose laurate was formed. Though, increasing of the polarity of the solvent will increase the

solubility of the sugar but it will also increase the susceptibility of the enzyme denaturation (Pedersen *et al*., 2002).

Table 22. Effect of organic solvents on glucose ester synthesis from vinyl caproate catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168.

Organic solvent	Conversion (%)
Hexane	No reaction
Acetone	1.5 ± 0.03
Butanone	4.2 ± 0.18
tert-butanol/DMSO (95:5 %v/v)	16.3 ± 0.20
tert-butanol/pyridine (55:45 %v/v)	54.7 ± 0.72
tert-butanol/pyridine (2:1 %v/v)	43.3 ± 0.48

(The reaction mixture contained 0.3 mmol glucose, 0.3 mmol vinyl caproate, 0.5 g molecular sieve (4°A) and 100 U lipase in 3.0 mL various organic solvents with initial a_w 0.07. The reaction was carried out at 400 rpm at 45°C for 72 h.)

3.4 Effect of molar ratio

The effect of molar ratio of glucose to vinyl caproate (1:1 to 1:5 mol/mol) on the synthesis of glucose caproate catalyzed by immobilized lipase on celite from *S. thermocarboxydus* ME168 was studied. The results indicated that increasing the molar ratio from 1:1 to 1:5 did not increase the yield of glucose caproate (Figure 20). The two fold excess of vinyl caproate did not significantly increase the yield of glucose ester. The molar ratios 1:4 and 1:5 decreased the ester yield. It might be that high concentration of vinyl caproate could inactivate the immobilized enzyme. Glucose has only one primary hydroxyl group at C-1 position which will be esterified by fatty acid moiety. Though increasing of vinyl caproate concentration did not increase the ester yield because of no more primary hydroxyl group in glucose. So the suitable molar ratio of glucose to vinyl caproate on glucose caproate synthesis was 1:1 mol/mol.

In contrast, the disaccharides such as sucrose has 3 primary hydroxyl groups at C-1, C-1' and C-6' positions. So, 3 molecules of the fatty acids could be esterified with sucrose. The result obtained from Ferrer *et al*. (1999) indicated that increasing of molar ratio of sucrose to vinyl laurate (1:2 and 1:25) the productivity of sucrose monolaurate increased 2.5 fold when sucrose laurate was synthesized in solvent mixture of 2-methyl-2-butanol/DMSO (4:1 v/v) catalyzed by immobilized lipase on celite from *Thermomyces lanuginosa*.

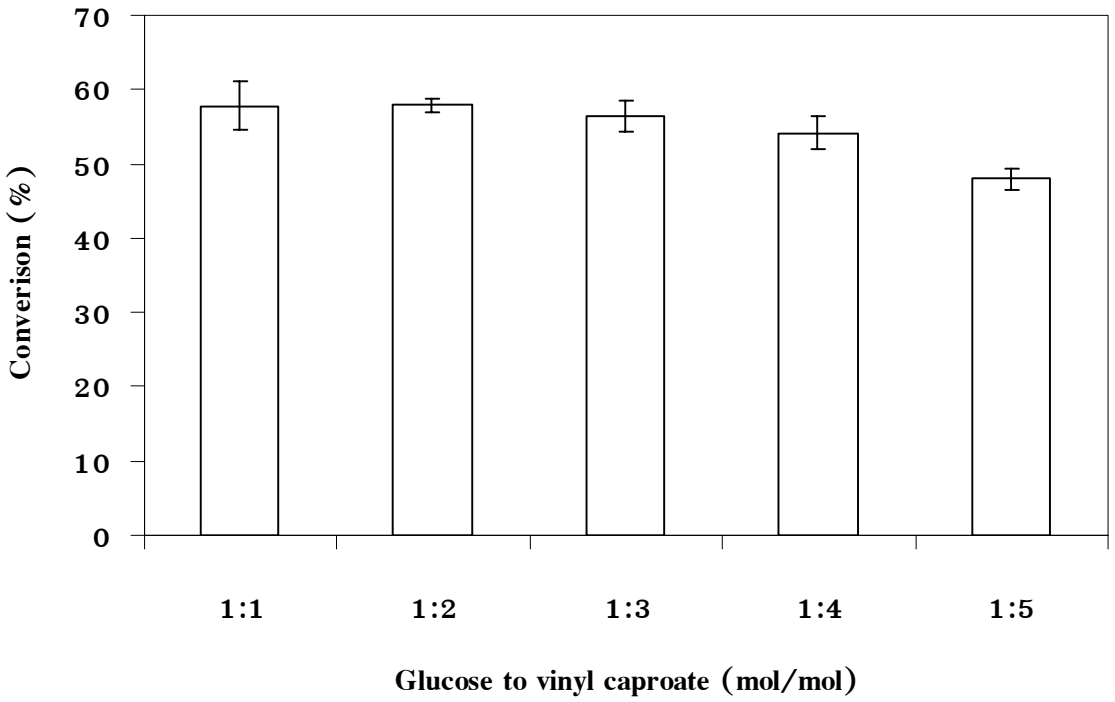


Figure 19. Effect of molar ratio of glucose to vinyl caproate on glucose ester synthesis catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168.

(The reaction mixture contained 0.3 mmol glucose, 0.3-1.5 mmol vinyl caproate, 0.5 g molecular sieve (4°A) and 100 U lipase in 3.0 mL of mixture of *tert*-butanol/pyridine (55:45 % v/v) with initial a_w 0.07. The reaction was carried out at 400 rpm at 45°C for 72 h.)

3.5 Effect of initial water activity

Synthesis of glucose caproate in *tert*-butanol/pyridine (55:45 % v/v) catalyzed by immobilized lipase from ME168 with different water activities (0.07-0.75) was compared. When increased a_w from 0.07 to 0.33, the ester yield was increased to 80.20% (Figure 20). With higher water activity than 0.33, the conversion yield was decreased and the lowest conversion of 50% was obtained at a_w of 0.75. The yield of the enzyme-catalyzed synthesis reaction in the organic medium is significantly depending on the amount of water present in the reaction system (Chamouleau *et al.*, 2001). Most reports show that the synthesis of SE is successful with low initial water activity (<0.23). Normally, the SE synthesis is the direct esterification reaction so only few amount of water to preserve the three dimension of enzyme is needed. In this study, the SE synthesis was carried out by tranesterification by the vinyl ester. So it might be quite different from the direct esterifacation.

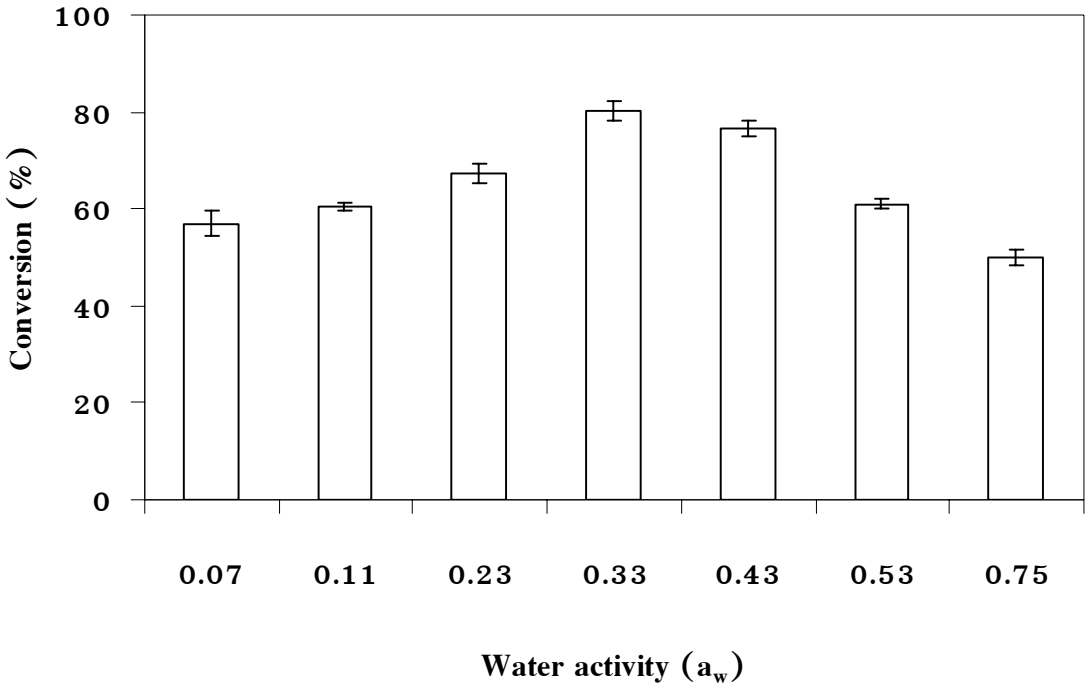


Figure 20. Effect of water activity on glucose ester synthesis catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168.

(The reaction mixture contained 0.3 mmol glucose, 0.3 mmol vinyl caproate, 0.5 g molecular sieve (4°A) and 100 U lipase in 3.0 mL of mixture of *tert*-butanol/pyridine (55:45 % v/v) with various initial a_w. The reaction was carried out at 400 rpm at 45°C for 72 h.)

3.6 Effect of temperature

Synthesis of glucose caproate was carried out at the temperature in the range of 25-60°C. The highest yield of glucose caprote was obtained at 50°C (Figure 21). The further increase in the reaction temperature resulted in lower yields indicating an inactivation of the enzyme in the presence of the organic solvent. Though, high temperature has a large effect on the product yield since the solubilization of the sugar and the reaction rate will be increased at higher temperatures (Degn *et al*., 1999).

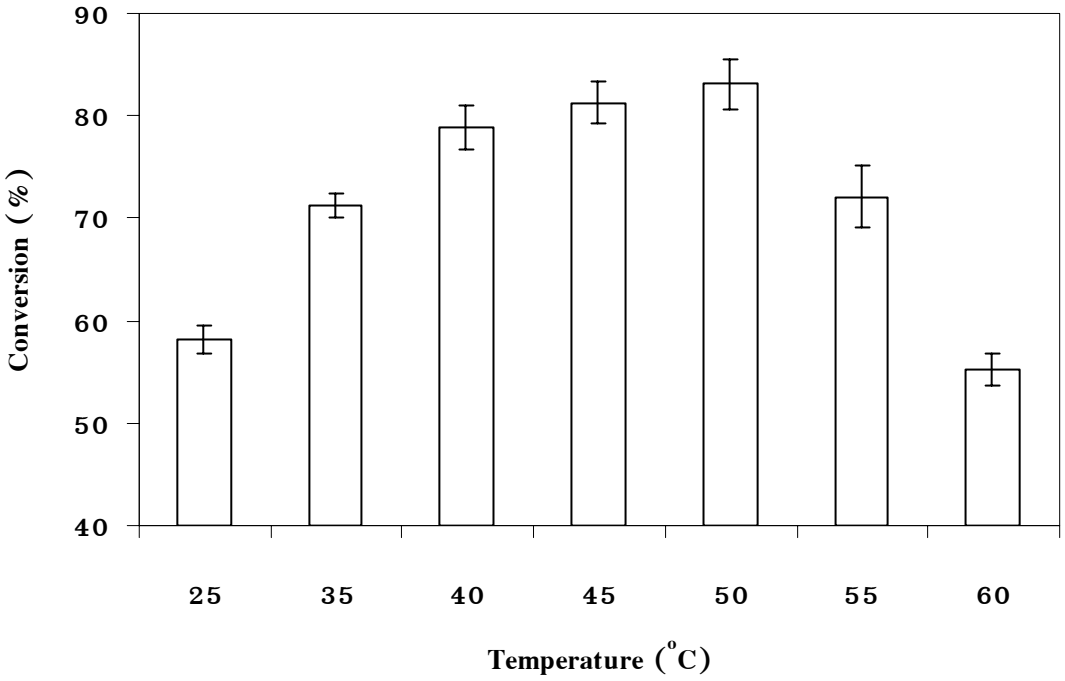


Figure 21. Effect of reaction temperature on glucose caproate synthesis catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168.

(The reaction mixture contained 30 mmol glucose, 30 mmol vinyl caproate, 0.5 g molecular sieve (4°A) and 100 U lipase in 3.0 mL of mixture of *tert*-butanol/pyridine (55:45 % v/v) with initial a_w 0.33. The reaction was carried out at 400 rpm at 25-60°C for 72 h.)

3.7 Time course of glucose caproate synthesis catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168

Production of glucose caproate catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168 was studied using optimum conditions and conversion was determined by GC. The optimum conditions were equi-molar of 0.3 mmol glucose and vinyl caproate, 100 U of immobilized lipase on celite, initial water activity of 0.33, and 0.5 g molecular sieve (4°A) in mixture of *tert*-butanol/pyridine (55:45 % v/v) at 50°C. The highest conversion of 82.00% (25.3 mg/mL solvent) was obtained at 72 h.

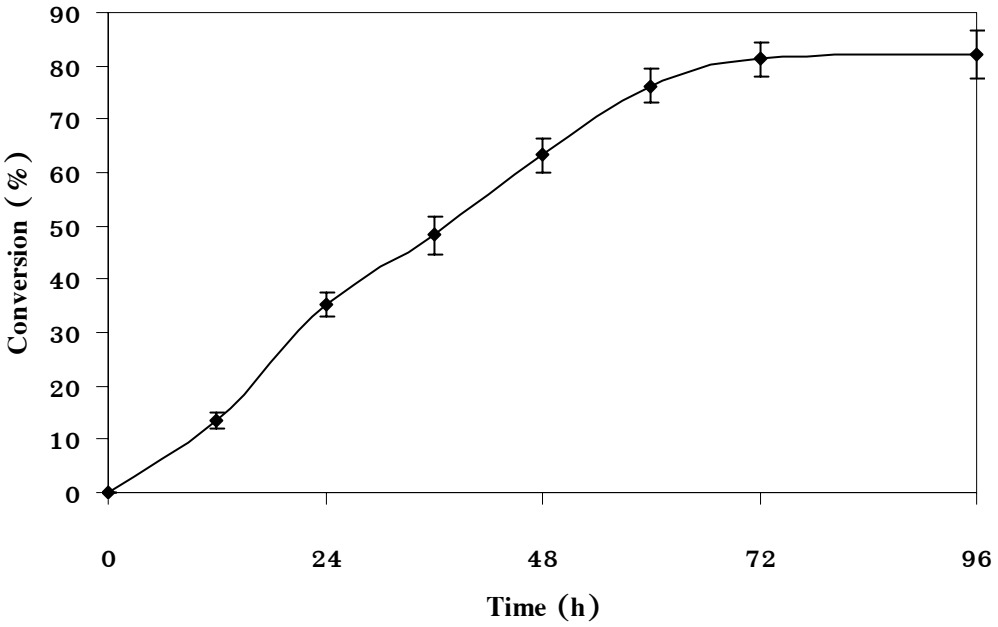


Figure 22. Time course of glucose caproate synthesis catalyzed by immobilized lipase from *Streptomyces thermocarboxydus* ME168.

(The reaction mixture contained 0.3 mmol glucose, 0.3 mmol vinyl caproate, 0.5 g molecular sieve (4°A) and 100 U lipase in 3 mL of mixture of *tert*-butanol/pyridine (55:45 % v/v) with initial a_w 0.33. The reaction was carried out at 400 rpm at 50°C.)

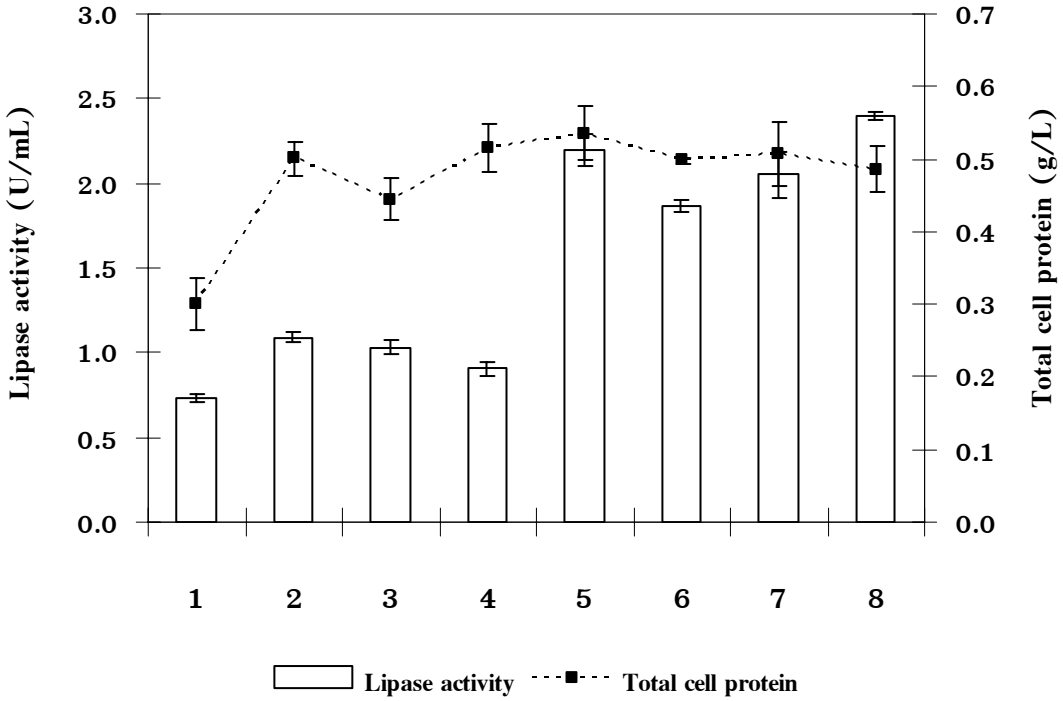
4. Optimization of lipase production by *Streptomyces thermocarboxydus* ME168

To obtain the maximum extracellular lipase production from *S. thermocarboxydus* ME168, the optimization conditions was studied based on modification of M65 medium which composted of 1.0% (w/v) malt extract, 0.4% (w/v) yeast extract and 0.4% (w/v) glucose supplemented with 1.0% (w/v) olive oil, initial pH 7.5. The cultivation conditions were shaking at 175 rpm at 45°C. The lipase activity and cell growth were determined after 120 h.

4.1 Effect of sugar

The effect of glucose, fructose, maltose, sucrose, sorbitol, mannitol and molasses (0.4% w/v) on lipase production from *S. thermocarboxydus* ME168 in M65 medium supplemented with 1.0% (w/v) olive oil was studied. The highest lipase activity (2.4 U/mL) and total cell protein (0.49 g/L) were obtained when cultivation in M65 medium supplemented with 0.4% (w/v) molasses (Figure 23). The control (no sugar adding) showed lowest lipase activity (0.70 U/mL). This result indicated that *S. thermocarboxydus* ME168 produced high lipase activity in M65 medium with the presence of carbon source (olive oil) combined with sugar. Similarly to the results obtained from Rathi *et al.* (2001) that when *Burkholderia cepacia* was cultivated in the medium using 1.0% (w/v) of mustard oil as a carbon source, this strain produced lipase activity only 20.0 U/mL when 1.0 % (w/v) glucose and mustard oil were used as combined carbon source, the highest lipase activity of 34.2 U/mL was obtained.

Sucrose, manntiol and sorbitol were also good sugars for lipase production from *S. thermocarboxydus* ME168 which showed lipase activity of 2.20, 2.05 and 1.86 U/mL, respectively. Molasses is the by product obtained from sugar factory. The cost of molasses is lower than sucrose. So this material was chosen as a suitable sugar for lipase production from *S. thermocarboxydus* ME168. When the concentration of molasses in M65 medium supplemented with olive oil was varied from 0 to 1.0% (w/v), *S. thermocarboxydus* ME168 produced the highest lipase activity (2.70 U/mL) in the medium with 0.8% (w/v) molasses with the total cell protein of 0.55 g/L (Figure 24). However, when adding molasses higher than 0.8% (w/v), *S. thermocarboxydus* did not produce higher lipase activity. In contrast, the total cell protein was increased from 0.55 g/L to 0.58 g/L. The lipase activity of *B. cepacia* in the medium using mustard oil as carbon source was also decreased when the concentration of glucose was increased from 1.0% to 2.5% (w/v) (Rathi *et al.*, 2001).



1) Control
2) Glucose
3) Fructose
4) Maltose
5) Sucrose
6) Sorbitol
7) Mannitol
8) Molasses

Figure 23. Effect of sugars on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 1.0% (w/v) olive oil after 120 h of incubation under shaking 175 rpm at 45°C)

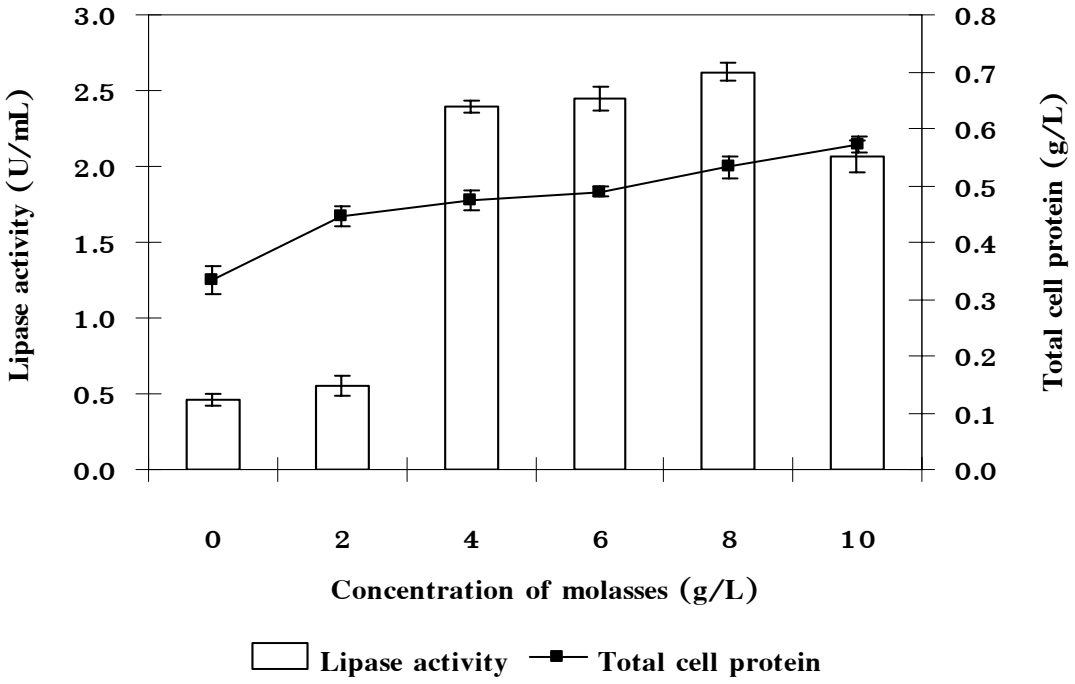


Figure 24. Effect of concentration of molasses on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 1.0% (w/v) olive oil after 120 h of incubation under shaking 175 rpm at 45°C)

4.2 Effect of malt extract

The M65 medium composted of 1.0% (w/v) malt extract. So, the effect of malt extract concentration (0-2.0% w/v) on lipase production from *S. thermocarboxydus* ME168 was studied with M65 medium supplemented with olive oil. When *S. thermocarboxydus* ME168 was cultivated in modified M65 medium containing 0.8% (w/v) of molasses and 1.0% (w/v) of olive as carbon source, it produced the highest lipase activity (2.70 U/mL) with total cell protein (0.53 g/L) at the concentration of malt extract 1.0% or 10 g/L (Figure 25). The suitable malt extract concentration for lipase production from *S. thermocarboxydus* ME168 in the modified M65 medium was 10 g/L.

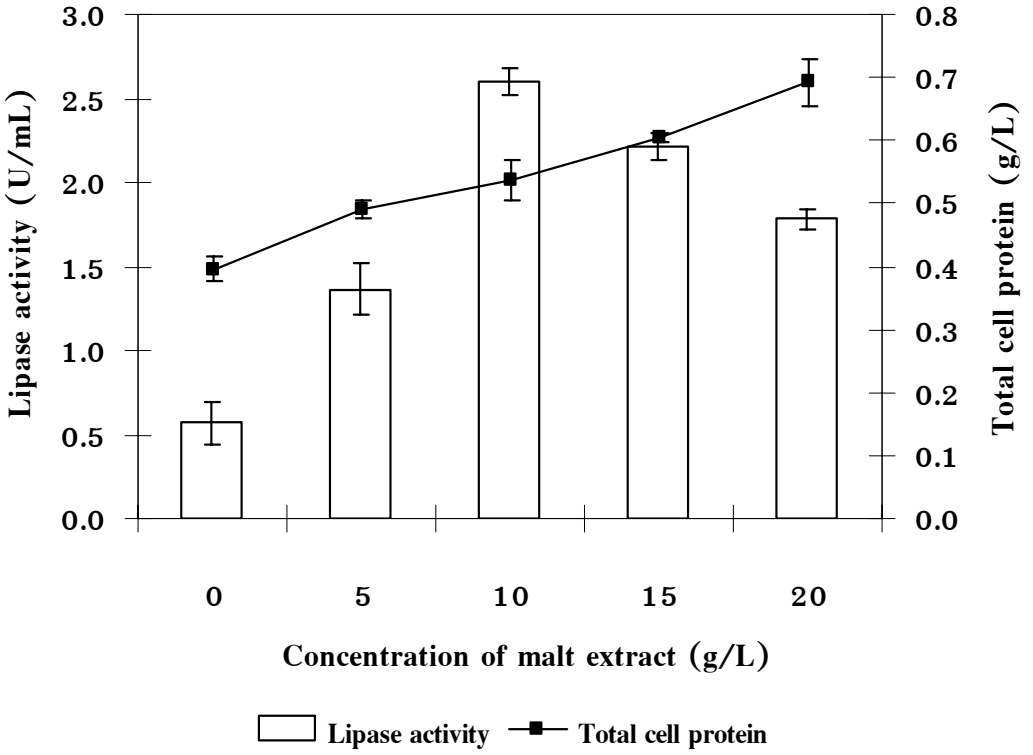


Figure 25. Effect of malt extract on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 0.8% (w/v) molasses and 1.0% (w/v) olive oil after 120 h of incubation under shaking 175 rpm at 45°C)

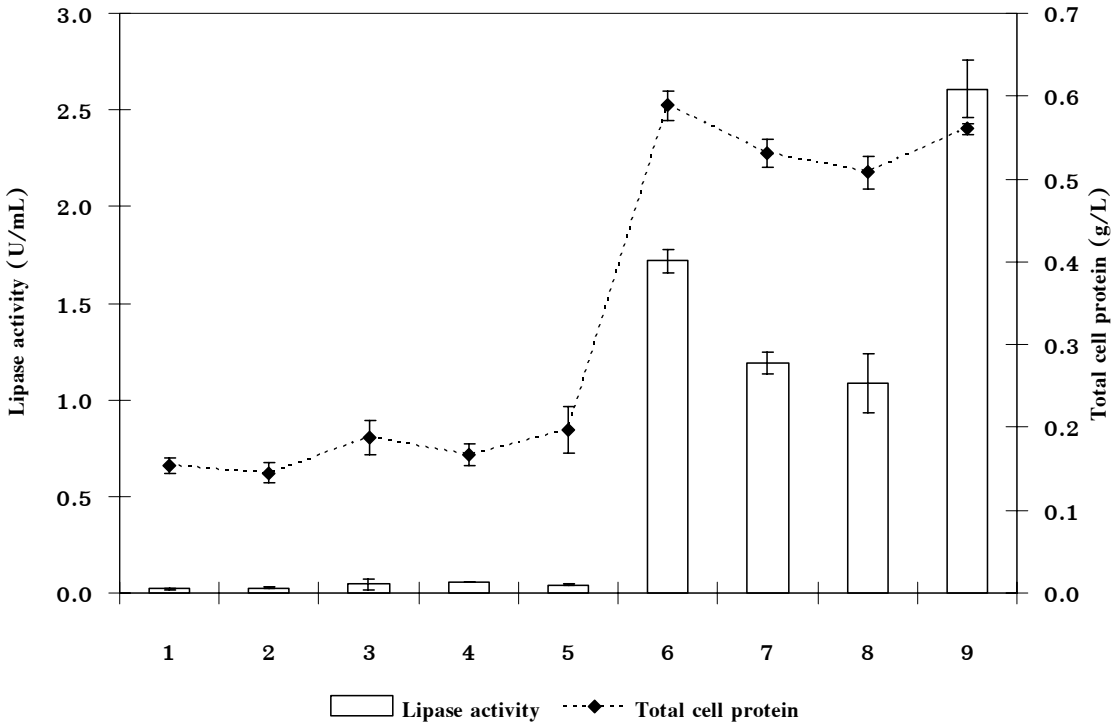
4.3 Effect of nitrogen sources

The effect of nitrogen sources (meat extract, soytone, tryptone, yeast extract, ammonium nitrate, ammonuium hydrogenphosphate, ammonium sulphate and urea) at the concentration of 0.4% (w/v) on lipase production from *S. thermocarboxydus* ME168 was studied. When *S. thermocarboxydus* ME168 was cultivated in the M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract and olive oil (1.0% w/v) using yeast extract as a nitrogen source, it produced highest lipase activity (2.5 U/mL) with total cell protein (0.54 g/L), respectively (Figure 26). However, when ME168 was cultivated in the medium with other organic nitrogen sources (beef extract, soytone and tryptone), it produced less lipase activity (1.50, 1.21 and 1.04 U/mL, respectively)

S. thermocarboxydus ME168 could not grow well in the presence of inorganic nitrogen. Less than 0.1 U/mL of lipase activity was obtained when it was cultivated in the medium with inorganic nitrogen (Figure 26). Kumar *et al.* (2005) reported that production of lipase from *Bacillus coagulans* BTS-3 in the medium using refined mustard oil as a carbon

source with various types of nitrogen sources. This strain produced the highest lipase activity with 0.67 U/mL in the medium with peptone (0.5% w/v) and yeast extract (0.5% w/v) as nitrogen source while only 0.12 and 0.43 U/mL were obtained when it was cultivated in the medium supplemented with urea and ammonium sulphate.

When the concentration of yeast extract in modified M65 medium was varied from 0 to 1.0% (w/v), *S. thermocarboxydus* ME168 produced the highest lipase activity (2.7 U/mL) with total cell protein (0.55 g/L) in the medium supplemented with yeast extract (0.4% w/v) as shown in Figure 27.



1) Control
2) Ammonium hydrogenphosphate
3) Ammonium nitrate
4) Ammonium sulphate
5) Urea
6) Beef extract
7) Soytone
8) Tryptone
9) Yeast extract

Figure 26. Effect of nitrogen sources on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract and 1.0% (w/v) olive oil after 120 h of incubation under shaking 175 rpm at 45°C)

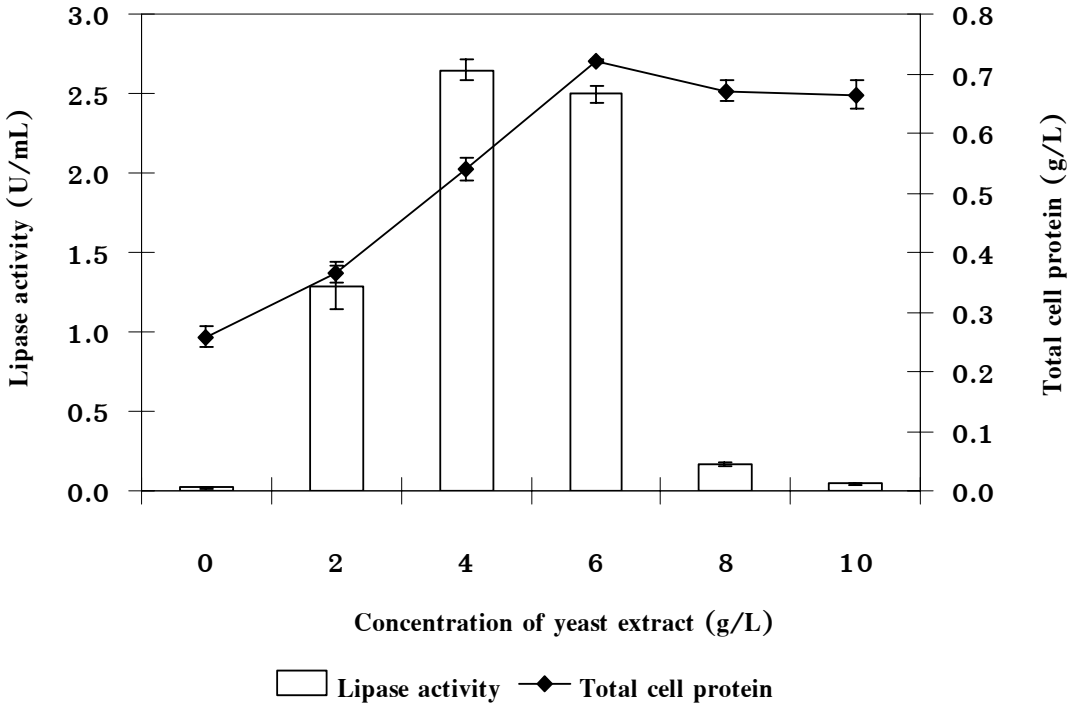
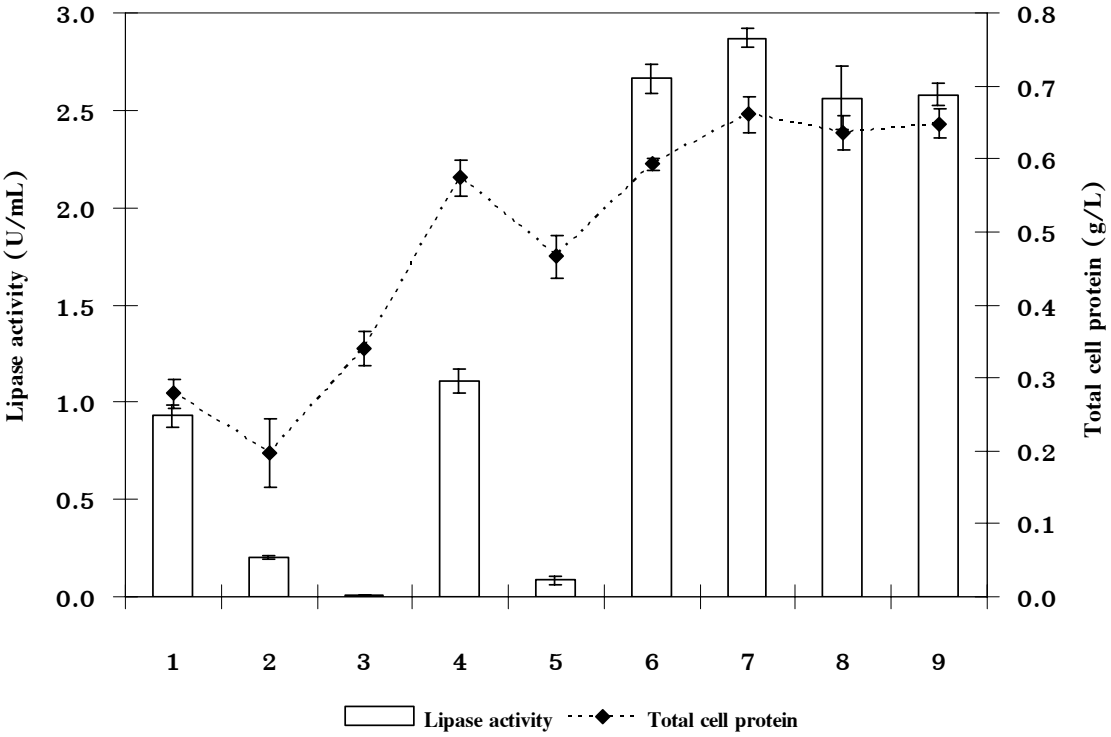


Figure 27. Effect of concentration of yeast extract on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract and 1.0% (w/v) olive oil after 120 h of incubation under shaking 175 rpm at 45°C)

4.4 Effect of oils

The effect of oils on lipase production from *S. thermocarboxydus* ME168 was studied with M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract and 1.0% (w/v) of Tween 20, Tween 80, tributyrin, coconut oil, lard, olive oil, palm oil, rice bran oil and soybean oil. Among the various oils used in this study, maximum lipase production (2.90 U/mL) with total cell protein (0.64 g/L) was achieved using palm oil as carbon source. Other vegetable oils, olive oil, soybean oil and rice bran oil were also good carbon source for lipase production (2.66, 2.58 and 2.56 U/mL, respectively).

When *S. thermocarboxydus* ME168 was cultivated in modified M65 medium supplemented with different kinds of TAG, it preferred plant oil to produce lipase. While no lipase activity was obtained when it was cultivated in tributyrin which contained short chain fatty acid. Normally, tributyrin is a good carbon source for esterase production due to its specificity on short chain fatty acid (Choi and Lee, 2001). Hence *S. thermocarboxydus* ME168 produced truly lipase enzyme.



1) Tween 20
2) Tween 80
3) Tributyrin
4) Coconut oil
5) Lard
6) Olive oil
7) Palm oil
8) Rice bran oil
9) Soybean oil

Figure 28. Effect of various kinds of oils on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract and 0.4% (w/v) yeast extract after 120 h of incubation under shaking 175 rpm at 45°C)

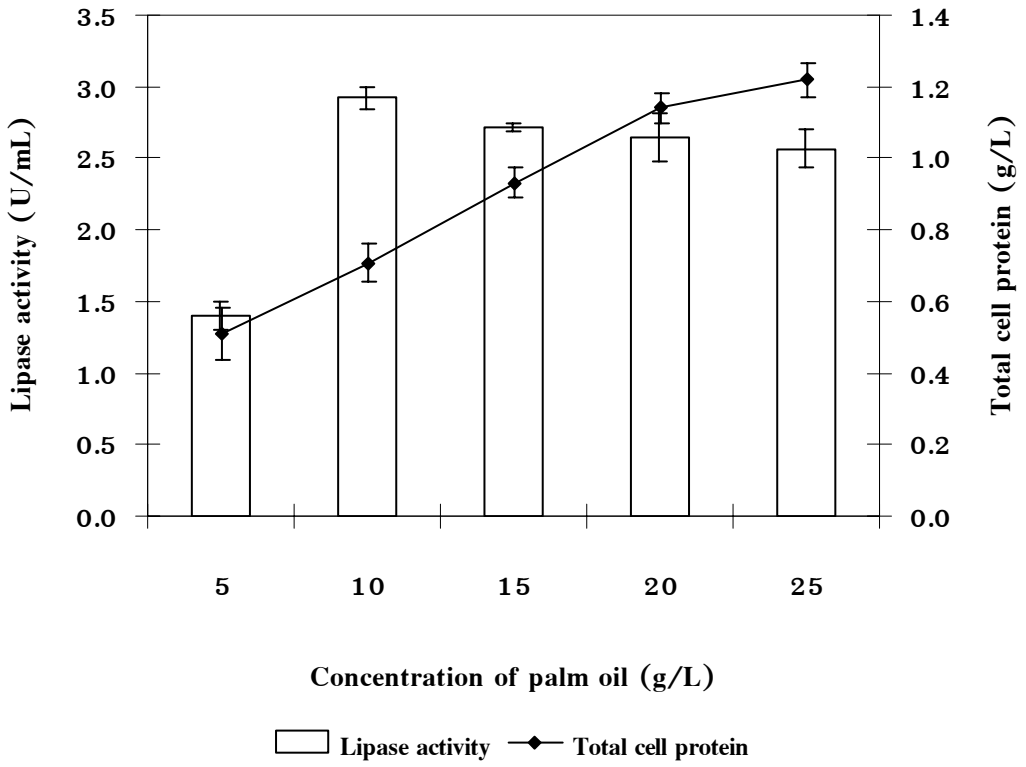
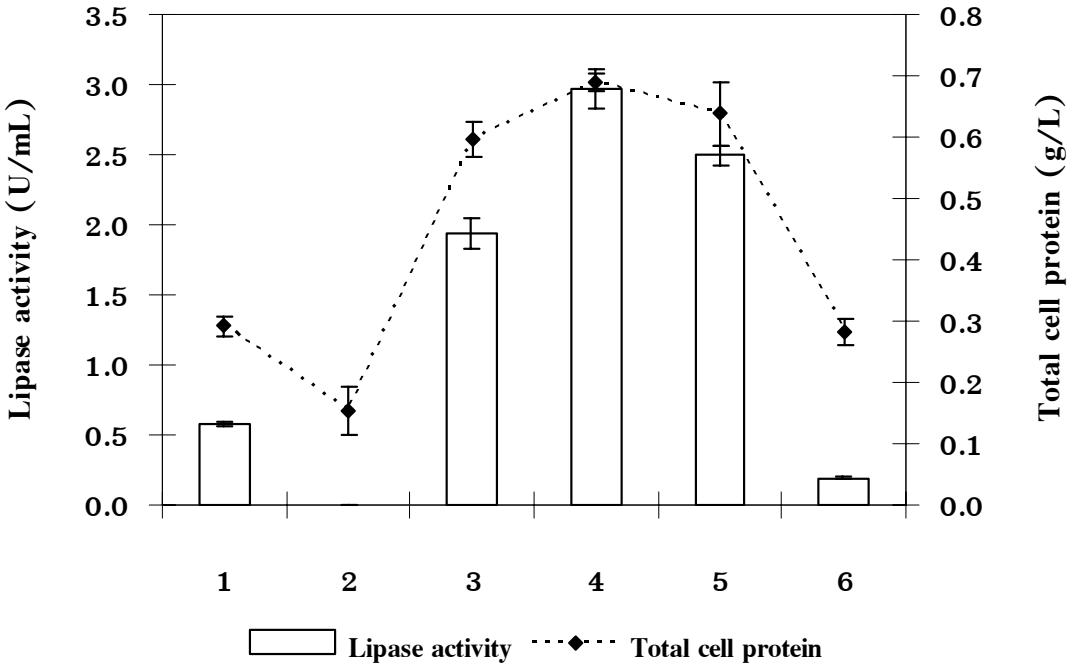


Figure 29. Effect of palm oil concentration on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract and 0.4% (w/v) yeast extract after 120 h of incubation under shaking 175 rpm at 45°C)

4.5 Effect of emulsifiers

The effect of surfactant, Tween 20, Tween 80, gum arabic, deoxycholic acid and polyvinyl alcohol (1.0% w/v) on lipase production from *S. thermocarboxydus* ME168 was studied. The results showed that when it was cultivated in modified M65 medium using 1.0% (w/v) gum arabic to emulsify palm oil, it produced the highest lipase activity (3.00 U/mL) with total cell protein 0.70 g/L. In the absence of emulsifier, *S. thermocarboxydus* ME168 could also produced lipase (0.55 U/mL) while no lipase activity obtained using deoxycholic acid as emulsifier (Figure 30).



1) Control
2) Deoxycholic acid
3) Polyvinyl alcohol
4) Gum arabic
5) Tween 20
6) Tween 80

Figure 30. Effect of emulsifiers on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract 0.4% (w/v) yeast extract and 1.0% (w/v) palm oil after 120 h of incubation under shaking 175 rpm at 45°C)

4.6 Effect of initial pH

The effect of initial pH on lipase production from *S. thermocarboxydus* ME168 was studied with modified M65 medium supplemented with 1.0% palm oil (Figure 31). The initial pH of 7.5 was suitable for lipase production. The highest lipase activity with 2.95 U/mL and total cell protein 0.73 g/L were obtained while no growth of *S. thermocarboxydus* ME168 was observed at the initial pH 4.0 and 5.0 and both growth and lipase production decreased dramatically in the medium with initial pH 9.0-11.0.

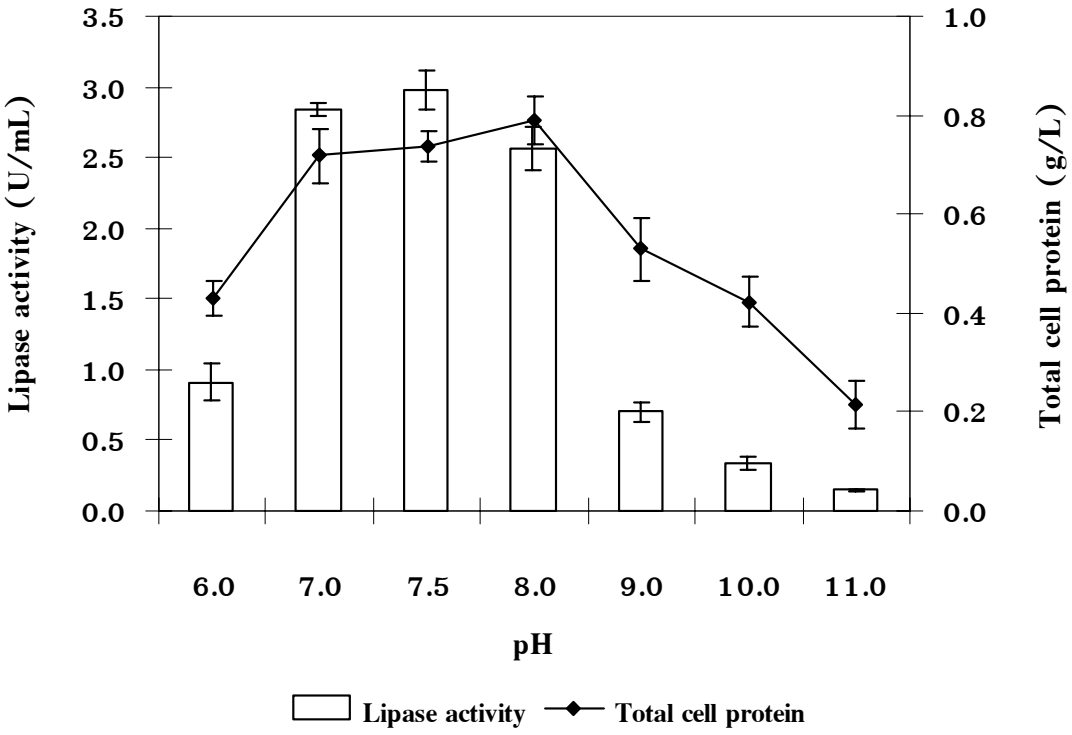


Figure 31. Effect of initial pH on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract 0.4% (w/v) yeast extract and 1.0% (w/v) palm oil after 120 h of incubation under shaking 175 rpm at 45°C)

4.7 Effect of temperature

The effect of cultivation temperature (30-55°C) on lipase production from *S. thermocarboxydus* ME168 was studied in modified M65 medium supplemented with 1.0% palm oil, pH 7.5 (Figure 31). The ME168 showed the highest lipase activity (3.08 U/mL) with total cell protein 0.92 g/L at 40°C. No growth was observed at 55°C. *S. thermocarboxydus* ME168 could grow and produced lipase at temperature above 45°C while the optimum temperature was 40°C. Hence this strain could be classified as thermotorerant *Streptomyces*.

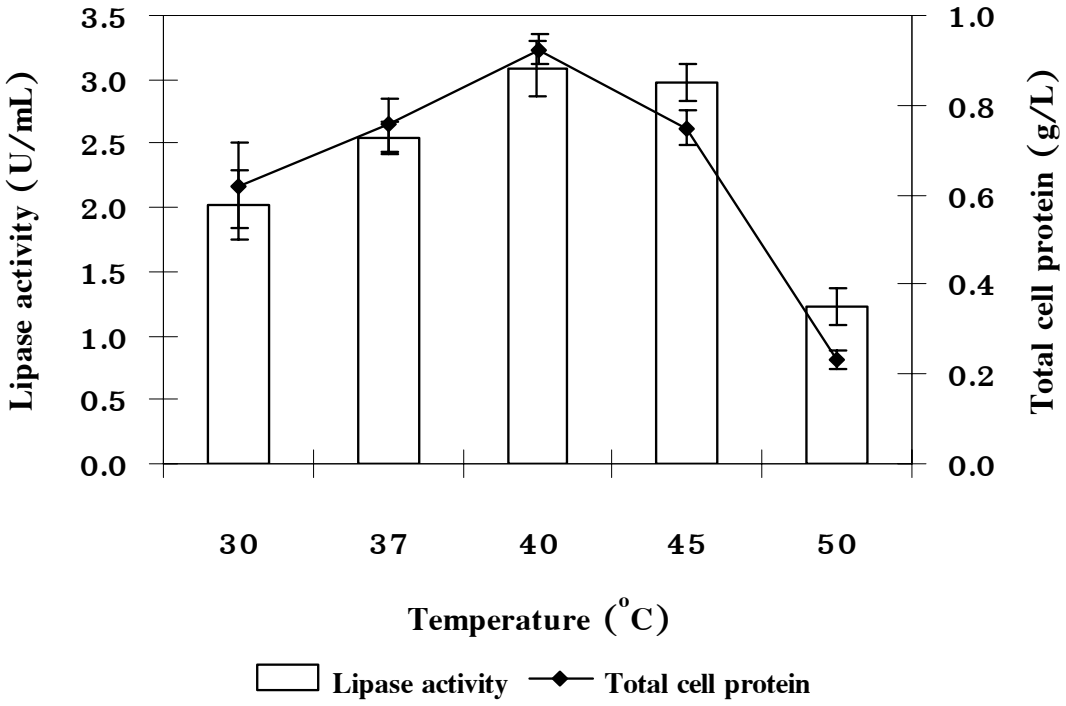


Figure 32. Effect of temperature on lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in M65 medium supplemented with 0.8% (w/v) molasses, 1.0% (w/v) malt extract 0.4% (w/v) yeast extract and 1.0% (w/v) palm oil, initial pH 7.5 after 120 h of incubation under shaking 175 rpm at 45°C)

4.8 Time course of lipase production from *Streptomyces thermocarboxydus* ME168

Production of lipase from *S. thermocarboxydus* ME168 was studied using M65 medium under optimal conditions. The pH, lipase activity and total cell protein were monitored. The modified M65 medium was composted of 0.8% (w/v) molasses, 0.4% (w/v) yeast extract, supplemented with 1.0% (w/v) palm oil and 1.0% gum arabic with initial pH 7.5 and the cultivation conditions were shaking at 175 rpm at 40°C for 144 h. The maximum lipase activity (3.01 U/mL) and total cell protein (0.98 g/L) were obtained at 120 h of cultivation time and the pH was dropped from 7.5 to 6.7 (Figure 32).

When *S. thermocarboxydus* ME168 was cultivated in modified M65 medium which composted of molasses and palm oil, this strain showed diauxic growth. The total cell protein was rapidly increased in 24 h then slightly increased until 48 h while no lipase activity was observed until 36 h. After 48 h, the total cell protein was rapidly increased again as well as lipase activity. The result indicated that *S. thermocarboxydus* ME168 used molasses to produce cell mass first then it turned to use fatty acid which obtained by lipase hydrolysis of palm oil.

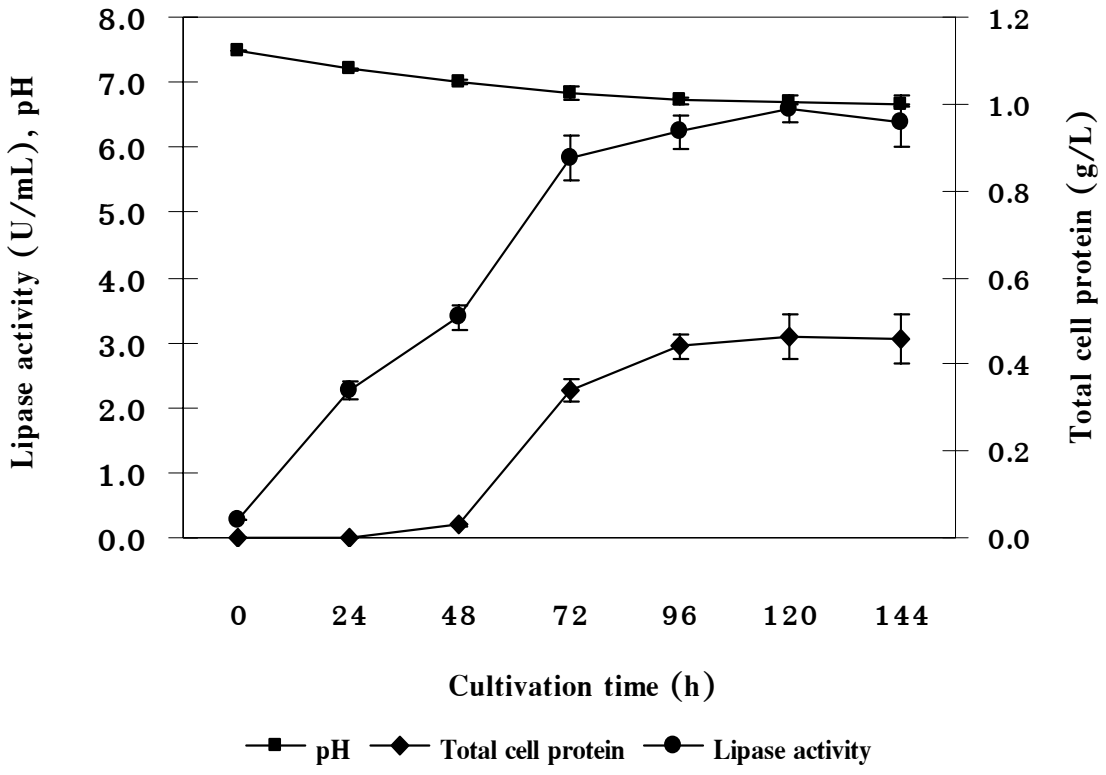


Figure 33. Time course of extracellular lipase production and cell growth of *Streptomyces thermocarboxydus* ME168.
(in modified M65 medium, initial pH 7.5 under shaking 175 rpm at 40°C.)

5. Purification and characterization of lipase from *Streptomyces thermocarboxydus* ME168

5.1 Purification of extracellular lipase

The extracellular lipase from *S. thermocarboxydus* ME168 was purified using three-step procedures (acetone precipitation, anion exchange and gel filtration chromatography) and the purification profile is summarized in Table 23. The concentrated supernatant was precipitated by chilled acetone (1.5 times).

The Resource Q was used as anion-exchange chromatography showed 2 peaks (Figure 34). The enzyme was eluted at 0.6 M NaCl during ion exchange chromatography step. The fractions contained lipase activity were pooled and concentrated by vacuum evaporation. It showed lipase activity and specific activity of 3.10 U/mL and 10.7 U/mg protein with the purity of 2.5 folds (Table 23). The concentrated enzyme was applied to the Superdex 200 gel filtration chromatography and showed 2 peaks (Figure 35). The lipase was eluted in the void volume. This suggested that the enzyme might be hydrophobic in native form because of its aggregated molecules (Lee *et al.*, 2001). The first peak showed lipase activity of 0.50 U/mL, specific activity of 41.4 U/mg protein with the purity of 9.6 folds and yield of 20.3% (Table 24). Forming of aggregates between lipase and lipophilic molecules such as lipopolysaccharides, fatty acids and glycerides were observed in many lipases like *Bacillus stearothermophilus* MC7 (Kambourove *et al.*, 2003), *B. thermoleovorans* ID-1 (Lee *et al.*, 2001) and *B. subtilis* 168 (Lesuisse *et al.*, 1993).

The molecular mass of purified lipase from *S. thermocarboxydus* ME168 was determined by SDS-PAGE under reducing condition and the single band of protein with a molecular mass of 21 kDa was estimated (Figure 36). This was similar to other *Streptomyces* lipases which were 27.5 and 29 kDa for *S. rimosus* (Abramic *et al.*, 1999) and *S. cinnamomeus*, repectively (Sommer *et al.*, 1997).

Table 23. Summary of purification steps for lipase from *Streptomyces thermocarboxydus* ME168.

Purification step	Volume (ml)	Total activity (U)[a]	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude supernatant	1650.0	1346.0	313.0	4.3	100.0	1.0
Concentration	185.0	1345.0	308.0	4.4	99.9	1.0
Acetone precipitation	120.0	1071.0	217.0	4.9	79.6	1.1
Resource Q	204.0	632.0	59.0	10.7	47.0	2.5
Superdex 200	570.0	273.0	6.6	41.4	20.3	9.6

[a]Activity was measured with *p*NPP as substrate at pH 8.5

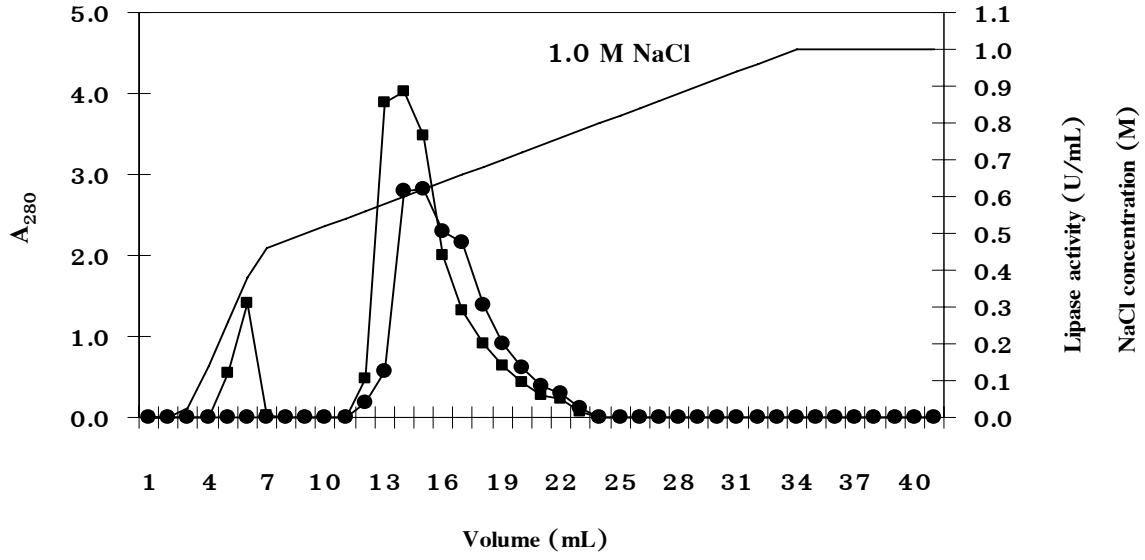


Figure 34. Purification profile of lipase from *Streptomyces thermocarboxydus* ME168 on anion exchange chromatography (Resource Q). (Absorbance at A_{280} (■), lipase activity (●) and NaCl concentration (—). Column was equilibrated with 0.1 M Tris-HCl buffer pH 7.5. The lipase was eluted with a gradient of NaCl in 0.1 M Tris-HCl pH 7.5 at a flow rate of 1.0 mL/min)

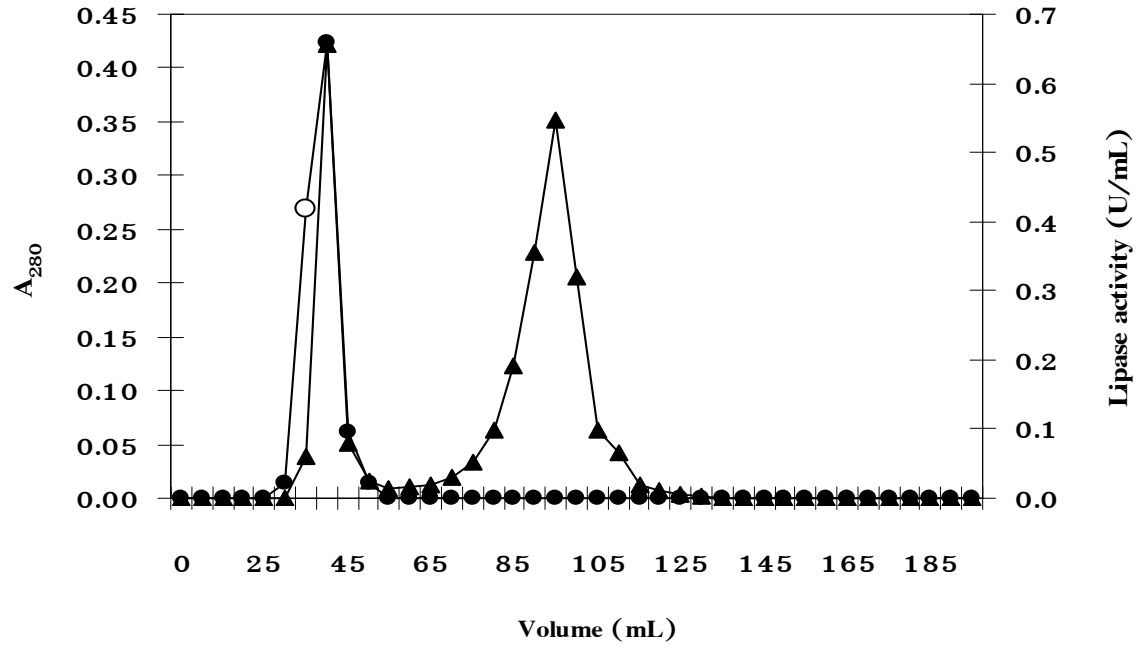


Figure 35. Purification profile of lipase from *Streptomyces thermocarboxydus* ME168 on gel filtration chromatography (Superdex 200). (Absorbance at A_{280} (■) and lipase activity (●). Column (1.6 x 70 cm) was equilibrated with 0.1 M Tris-HCl buffer pH 7.5 containing 0.15 M NaCl at a flow rate of 1.5 mL/min)

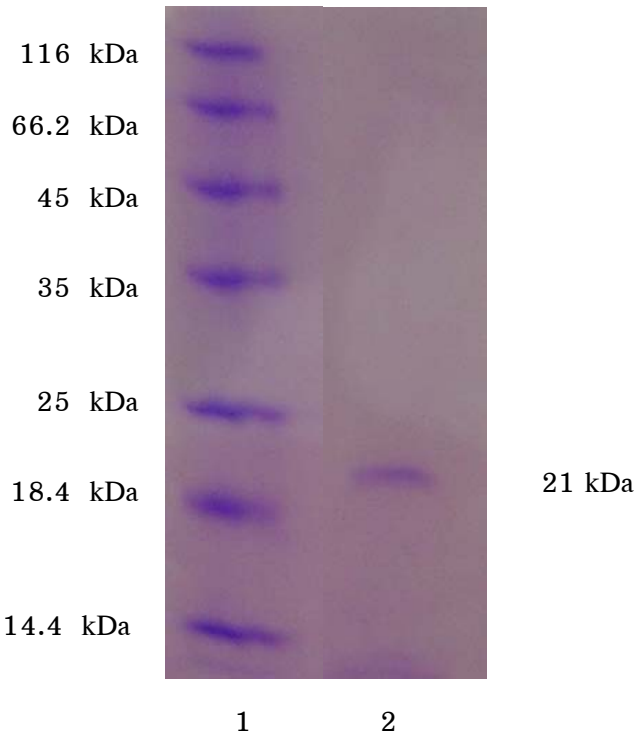


Figure 36. Sodium dodecyl sulphate polyacrylamide gel electrophoresis pattern of the purified lipase from *Steptomyces thermocarboxydus* ME168. (SDS-PAGE was conducted in 15% gel and the protein was stained with Coomassie brilliant blue R250. Lanes 1: protein markers and Lane 2: purified enzyme after gel filtration)

5.2 Characterization of lipase

5.2.1. Effect of pH and temperature

The purified lipase was active toward *p*NPP in a wide pH range (4.0-11.0). The optimum hydrolytic activity was achieved at pH 8.5 (Figure 37). The enzyme was not active at acidic pH (pH 4.0-5.5) and was dramatically decreased from pH 9.0 to 11.0. This lipase showed good stability for 3 h in the broad range of pH from 5.5 to 9.5 at 45°C. The purified lipase showed maximum activity toward *p*NPP at 50°C, pH 8.5 and at this temperature the enzyme was quite stable with the residual activity of 92% after 3 h (Figure 38).

The half life of the enzyme was more than 3 h at 65°C but after incubation at 80°C for 3 h, it was completely inactivated. The half life of the lipases from *S. rimosus* was 30 min at 65°C (Abramic *et al*., 1999) while the *B. stearothermophilus* P1 was 2 h at 65°C (Sinchaikul, *et al*., 2001), *B. stearothermophilus* L1 was 30 min at 62°C (Kim *et al*., 1998) and *B. thermocatenulatus* was 30 min at 60°C (Schmidt-Dannert *et al*., 1996). These results indicated that lipase from *S. thermocarboxydus* ME168 was more stable than the lipases from other thermophilic *Streptomyces* and *Bacillus* species. However, highly thermostable lipase from

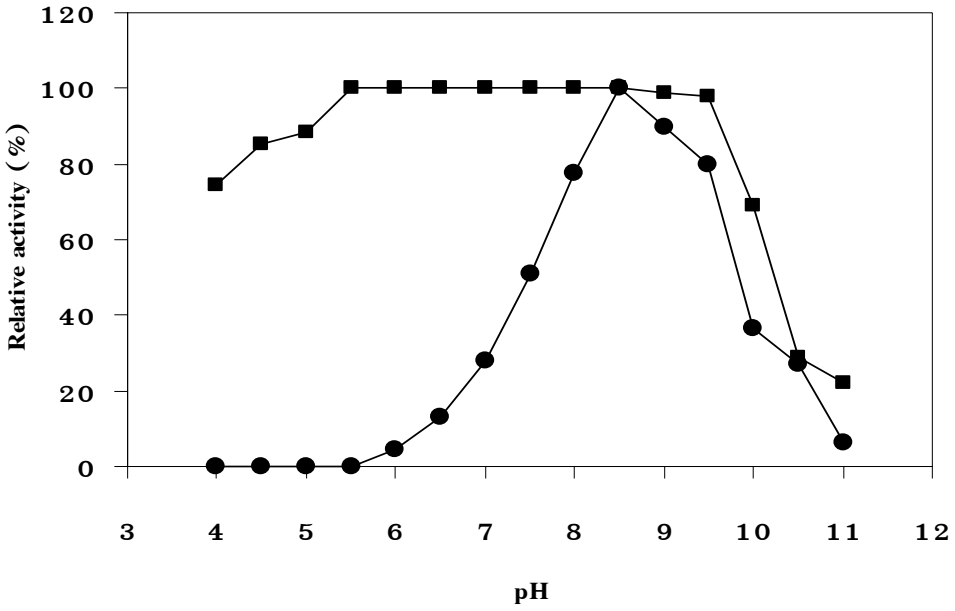


Figure 37. Effect of pH on activity (●) and stability (■) of lipase from *Streptomyce thermocarboxydus* ME168.

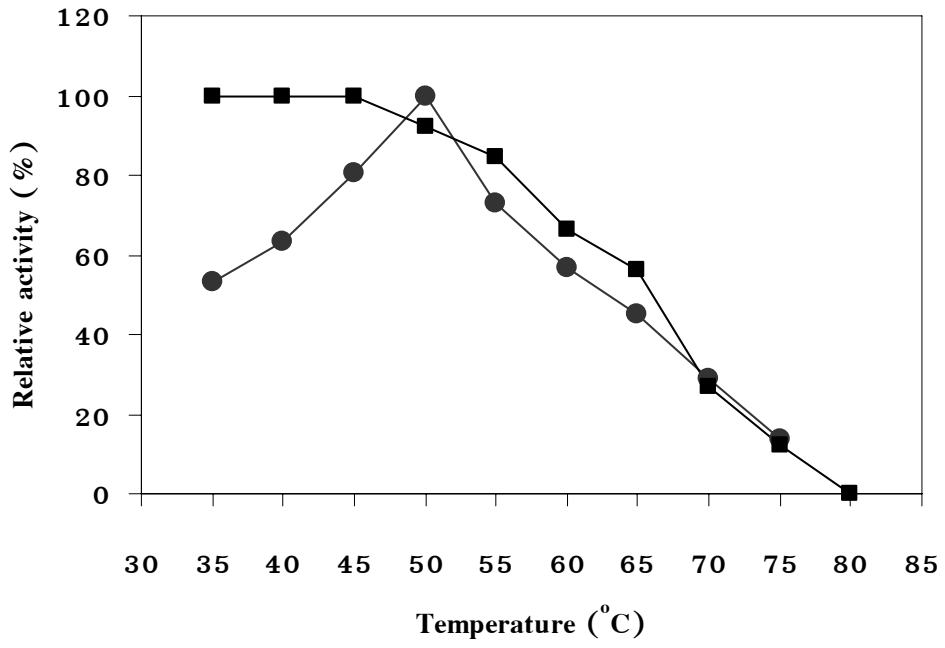


Figure 38. Effect of temperature on activity (●) and stability (■) of lipase from *Streptomyces thermocarboxydus* ME168.

Bacillus strain A30-1 (ATCC 53841) showed residual activity 100% after heating at 75°C for 30 min and the half life at 75°C was 8 h (Wang *et al*., 1995).

5.2.2 Kinetic parameters

The values of K_m and V_{max} of the purified lipase from *S. thermocarboxydus* ME168 using *p*NPP as substrate calculated from the Lineweaver-Burk plot were 0.28 mM and 1,428 U/mg, respectively (Figure 39). Most of industrially used enzymes have K_m in the range of 10^{-1} to 10^{-5} M when acting on biotechnologically important substrates (Fullbrook, 1996).

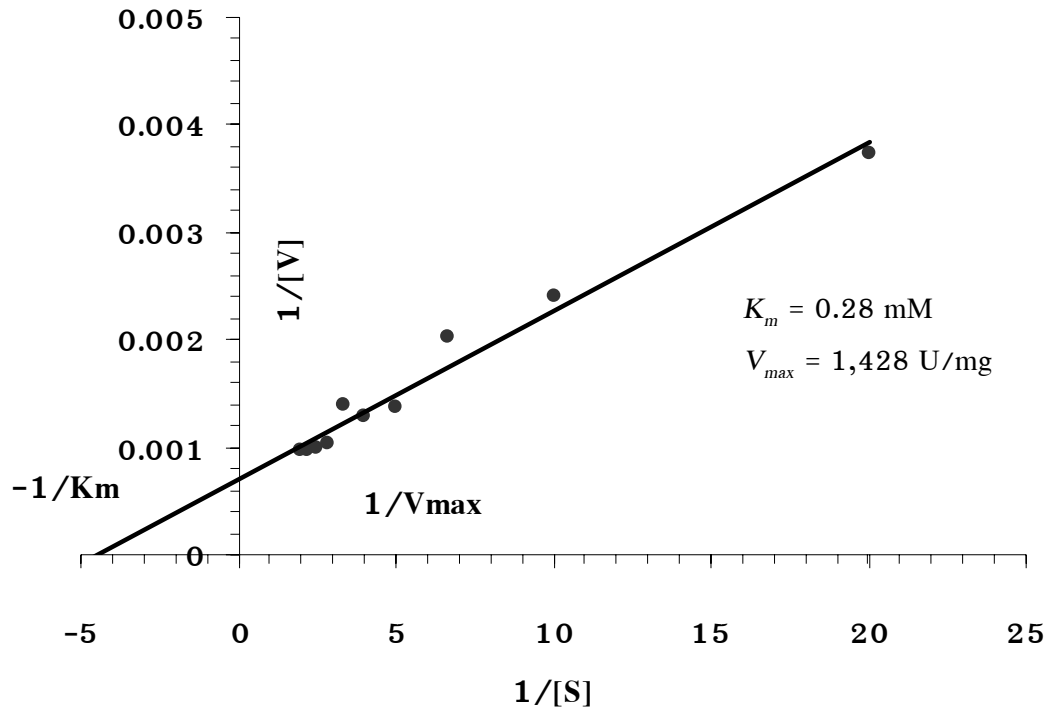


Figure 39. Lineweaver-Burk plot of the purified lipase from *Streptomyces thermocarboxydus* ME168 using *p*NPP as substrate.

5.2.3 Substrate specificity

The enzyme specificity was studied with *p*-nitrophenyl esters of various acyl chain lengths (C_2-C_{16}) (Figure 40). Among the substrates tested, the ester with long acyl chain, *p*NPP (C_{16}) was the best substrate for the lipase from *S. thermocarboxydus* ME168. While the esters with short acyl chian with *p*NPB (C_4) and *p*NPC (C_8) were also good substrates. However, very short chain ester, *p*NPA (C_2) was poor substrate. The similar specificity has been reported for *B. stearothermophilus* MC7 lipase which preferred long chain fatty acid than short chain fatty acid (Kambourova *et al*., 2003). Other reports showed that microbial lipases had preference for *p*NP esters with medium chain (C_8 and C_{10}) as substrates (Abramic *et al*., 1999; Castro-Ochoa *et al*., 2005 and Sin *et al*., 1998).

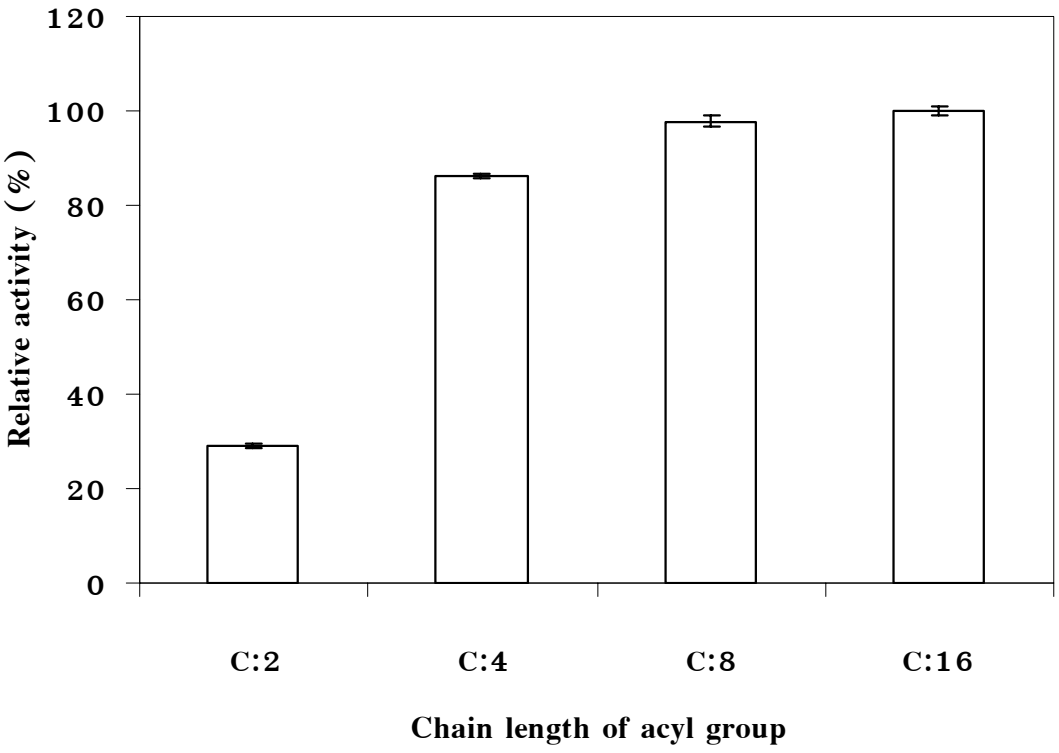


Figure 40. Hydrolytic activity on various *p*-nitrophenyl esters by the purified lipase from *Streptomyces thermocarboxydus* ME168.

5.2.4 Effect of organic solvents

The stability and activity of enzyme in organic solvents are important for the application in organic synthesis reaction (Snellman *et al.*, 2002). The stability of purified lipase from *S. thermocarboxydus* ME168 was studied in both water-miscible and immiscible organic solvents. This lipase was tolerant to some organic solvents (Table 24) e.g. dimethylsulfoxide (DMSO), methanol and dimethylformamide (DMF). However, acetone, *tert*-butanol, pyridine and acetronitrile significantly caused deactivation, less than 20% of initial activity was remained. While 95% ethanol resulted completely loss of the lipase activity. The extracellular lipase from *Acinetobacter sp.* RAG-1 (LipA) was completely inactivated by pyridine at a concentration of 30% (v/v) in 1 h (Snellman et al., 2002). 90 % ethanol and butanol were reported to inactivate lipase from *Aspergillus carneus* after 24 h incubation (Saxena *et al.*, 2003).

Table 24. Effect of organic solvents on the hydrolytic activity of the purified lipase from *Streptomyces thermocarboxydus* ME168.

Organic solvent	Relative activity (%)
Tris-HCl buffer pH 7.5 (Control)	100
Dimethylsulfoxide (DMSO)	54
Methanol	85
Dimethylformamide(DMF)	62
Acetronitrile	2
95% Ethanol	0
Acetone	18
Pyridine	1
tert-Butanol	2
2-Methyl-2-butanol	56
Hexane	45

5.2.5 Effect of metal ions and other reagents

Various metal ions and other reagents were studied for their effects on the activity of the purified lipase from *S. thermocarboxydus* ME168 at concentration 1.0 and 10.0 mM, 45°C for 60 min (Table 25). The ions of Na^{+}, K^{+} and Ca^{2+} did not affect on the lipase activity. In contrast, this lipase was sensitive to Zn^{2+} and Fe^{3+}. These metal ions could directly inhibit at the catalytic site of enzyme or form the complexes with fatty acids due to changing their solubility and behavior at the interface (Lee and Rhee, 1993). The chelating agent, ethylenediamine tetraacetic acid (EDTA) and sodium citrate reduced the enzyme activity to 51% and 89%, respectively.

The reducing agent, dithiothreitol (DTT) reduced lipase activity to 52 % indicated that the presence of disulfide bond in the molecule, which may stabilize the active conformation of lipase (Abramic *et al.*, 1999). In contrast, this enzyme was quite stable with oxidizing agents. It retained 82 and 99% activity with 10 mM ammonium persulfate and potassium iodide, respectively.

The serine inhibitor, PMSF showed slightly effect on the lipase activity at the concentration of 10 mM. Although all known lipases have serine in their active site but some lipases are resistant to inactivation by serine inhibitors e.g. lipase from *S. rimosus* (Abramic *et al.*, 1999), *Geobacillus* sp. *TW1* (Lee and Zhang, 2005) and *Mucor hiemalis* f. *hiemalis* (Hiol *et al.*, 1999). The reagent masking SH-group, *p*-hydroxymercuribenzoate (*p*HMB) did not strongly inhibit lipase activity suggested that free thiol group might be not essential for *S. thermocarboxydus* ME168 lipase activity as well as lipase from *S. rimosus* (Abramic *et al.*, 1999).

Table 25. Effect of metal ions and other compounds on the activity of the purified lipase from *Streptomyces thermocarboxydus* ME168.

Effecter molecule	Relative activity (%)[a]	
	1.0 mM	10.0 mM
Metal salts		
$CaCl_2$	102	105
KCl	100	104
NaCl	100	102
LiCl	100	97
$MgCl_2$	100	100
$ZnCl_2$	59	41
$FeCl_3$	75	64
$MnCl_2$	ND[b]	97
$AgNO_3$	ND	91
$CuSO_4$	ND	97
Reducing agents		
Dithiothreitol	66	52
Ascorbic acid	88	80
Oxidizing agents		
Ammonium persulfate	90	82
KI	ND	99
Chelating agents		
EDTA	67	51
Sodium citrate	102	89
Inhibitors		
PMSF	93	81
*p*HMB	99	76

[a]The activity was expressed as a percentage of the activity of untreated purified lipase

[b]ND: not determined

Part 3. Production of Lipase from *Burkholderia multivorans* PSU-AH130 and Application for Fatty Acid Methyl Esters Synthesis

1. Identification of the strain PSU-AH130

The strain PSU-AH130 was obtained from the stock culture collection of department of Industrial Biotechnology, Prince of Songkla University, Hat Yai, Thailand. The lipase produced by the strain PSU-AH130 could synthesize glucose ester from vinyl acetate, vinyl butyrate and vinyl caproate with relatively low conversion yields of 32.70, 18.42 and 13.32%, respectively (Table 21 and Figure 16). The immobilized lipase from PSU-AH130 was also checked ability for sugar esters (SE) synthesis from palm oil and PFAD but no product was obtained. In contrast, this immobilized lipase showed good ability to produce fatty acid methyl esters (FAME) from palm olein and methanol. Identification of the strain PSU-AH130 was performed on the basis of nucleotide sequence of bacterial 16S rDNA gene. The DNA sequence of this strain showed 99.5% similarity with *Burkholderia multivorans* strain (1,482/1,490 bp) (Appendix 14). Therefore this strain was identified as *Burkholderia multivorans* PSU-AH130 (Figure 41).

2. Production of lipase from *Burkholderia multivorans* PSU-AH130

Burkholderia multivorans PSU-AH130 showed high ability to produce extracellular lipase with the hydrolytic activity of 2.66 U/mL (Table 18) when it was cultivated in the basal medium supplemented 0.1% (w/v) Tween 80. Though, *B. multivorans* PSU30 produced high lipase when was cultivated in basal medium supplemented with palm oil but it affected on the ultrafiltration (UF) membrane. To avoid the fouling effect on UF membrane, Tween 80 was used as a carbon source for the large volume production. Production of lipase from *B. multivorans* PSU-AH130 was carried out in 2.0 L flasks containing 300 ml of basal medium supplemented with 0.1% (w/v) Tween 80. Time course of lipase production of *B. multivorans* PSU-AH130 is shown in Figure 42. It showed rapid growth in 12 h and the growth was constantly until 60 h. The initial pH of the basal medium was 7.5. During the growth of *B. multivorans* PSU-AH130 the pH gradually increased and the final pH was 8.6. The lipase activity was observed at 12 h of cultivation was maximum at 60 h of cultivation.

3. Immobilization of lipase from *Burkholderia multivorans* PSU-AH130

After 72 h of cultivation, the culture broth was centrifuged and concentrated by ultrafiltration. The lipase in the concentrated supernatant was immobilized on different solid supports by physical adsorption. Accurel EP100 (<400 μm) displayed the best immobilized

activity of all supporters with immobilized yield of 94.2 % and immobilized activity of 0.21 U/mg support, respectively (Table 26). Accurel was the hydrophobic micropourous material, provided better performance for immobilized lipase. Kaewthong (2004) reported that immobilization of lipase PS on Accurel EP100 displayed the best immobilized activity of 0.37 U/mg support. So Accurel EP100 was selected to immobilize lipase from *B. multivorans* PSU-AH130 in large scale and applied for fatty acid methyl esters (FAME) synthesis.

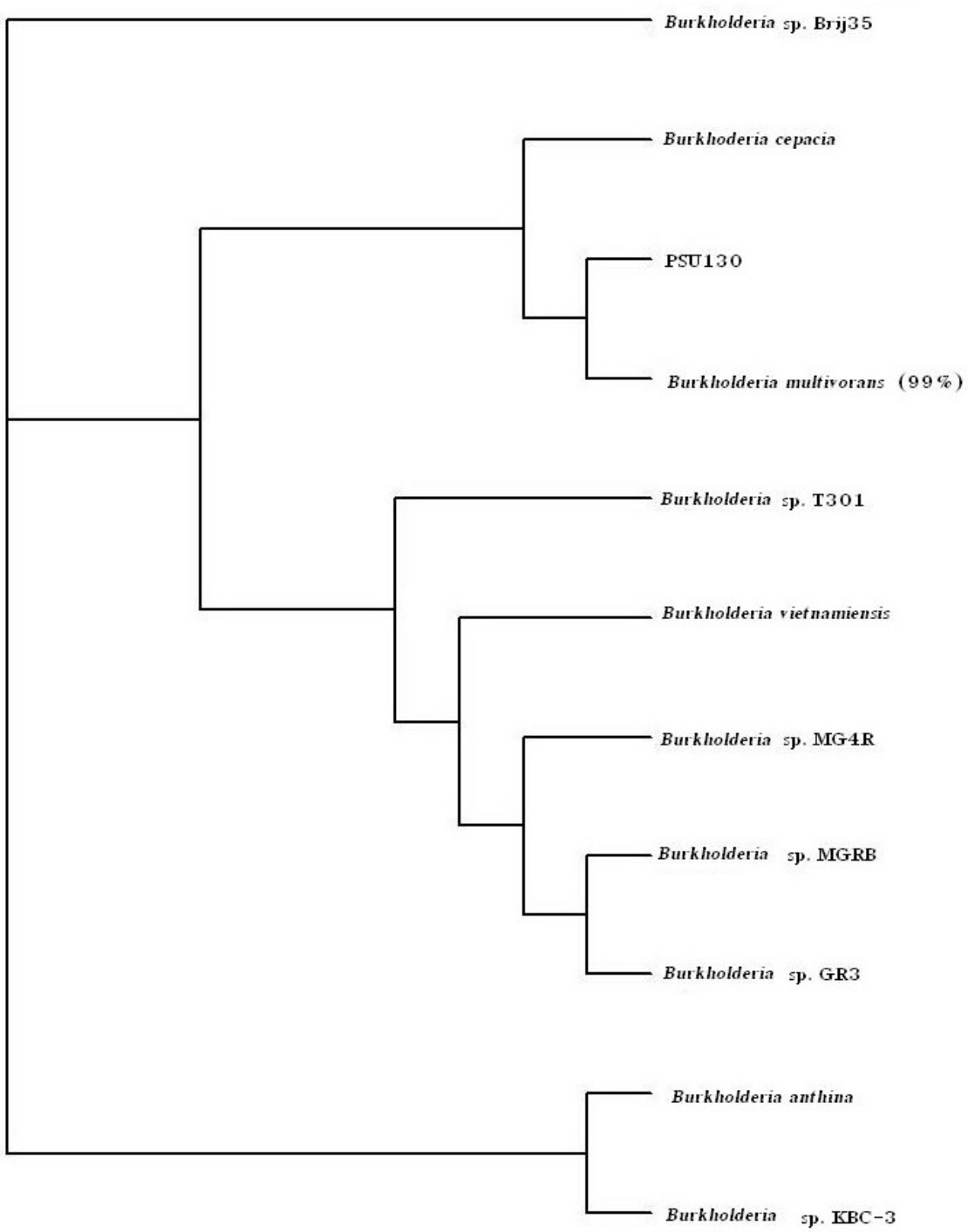


Figure 41. Phylogenetic tree of *Burkholderia multivorans* PSU-AH130.

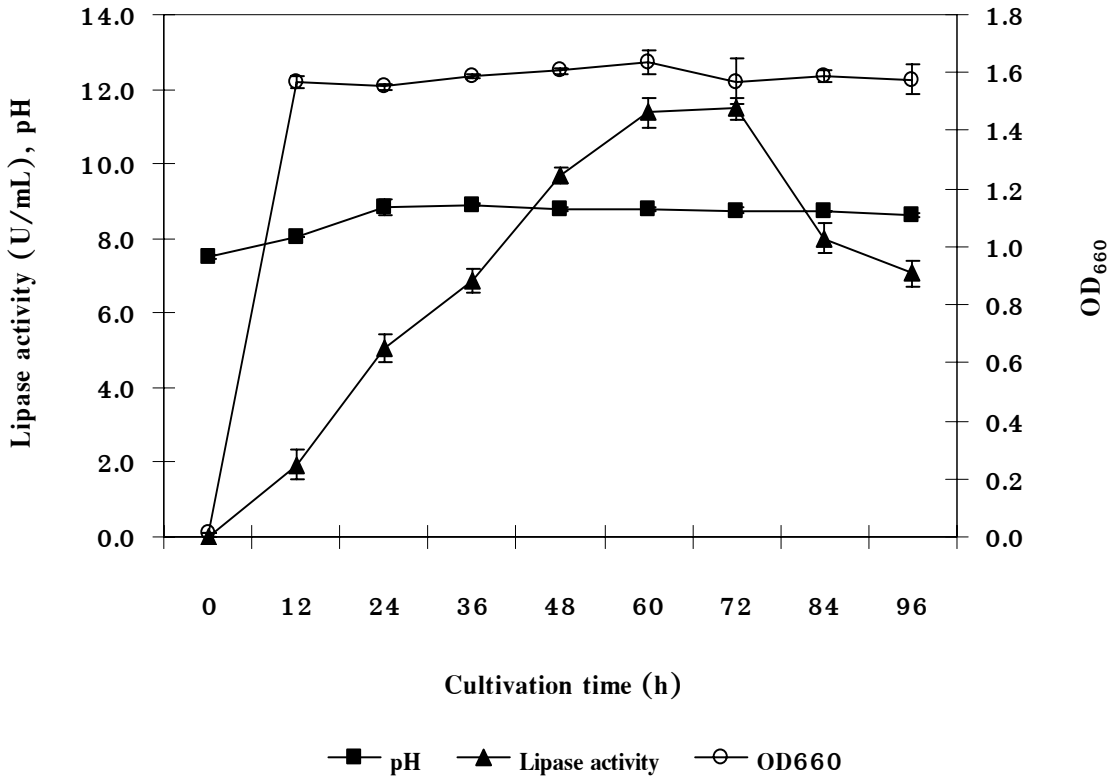


Figure 42. Time course of lipase production from *Burkhoderia multivorans* PSU-AH130 in the basal medium. (0.5% tryptone, 0.5% yeast extract, 0.2% K_2HPO_4, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.02% $MgSO_4.7H_2O$ and 0.02% $CaCl_2.2H_2O$) supplemented with 0.1% (w/v) Tween 80)

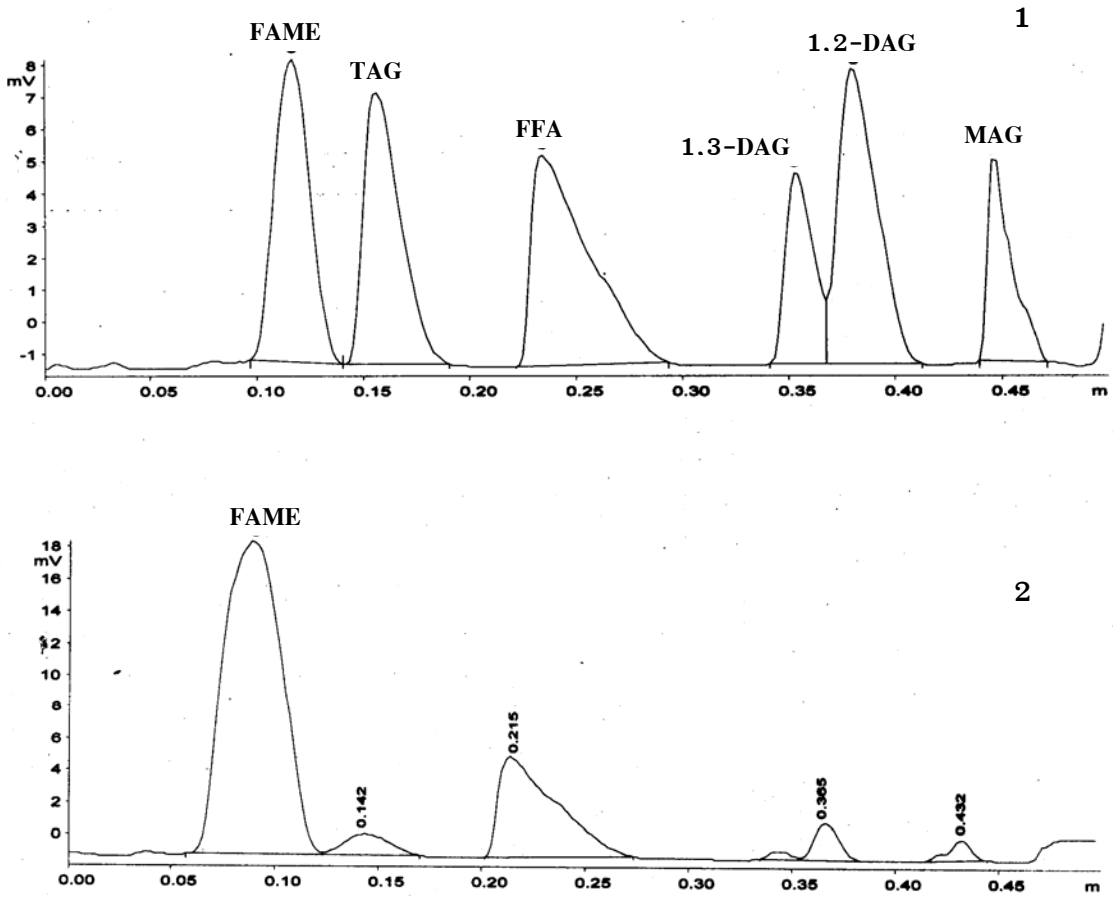
Table 26. Effect of supporters on immobilization of the lipase from *Burkholderia multivorans* PSU-AH130.

Supporting materials	Activity (U)[a]		Immobilized activity (U/mg)	Total immobilized activity (U)	Activity yield (%)
	Added	Unbound			
Accurel EP 100	484	28	0.21	423	87.4
Amberlite XAD-7	484	165	0.02	42	8.7
Cetlite 545	484	120	0.04	87	18.0
PVC (250 μm)	484	144	0.05	104	21.5
Syran (SIKUG 012)	484	82	0.03	90	19.4

[a]Activity of lipase was determined by cupric acetate method

4. Optimization of immobilized lipase for fatty acid methyl esters production

The lipase from *B. multivorans* PSU-AH130 was immobilized on Accurel EP100 and applied for FAME production followed the condition of Paripatanapairod (2003). The compositions of FAME as determined by TLC-FID compared with the standard are shown in Figure 43. After 60 h, the concentration of FAME (%) reached to 72.0% while TAG, DAG and MAG were less than 10% and FA was 20.0 %. This result indicated that the lipase from *B. multivorans* PSU-AH130 showed possibility to produce FAME by methanolysis of palm oil.



Condition:

Stationary phase:	CHROMAROD-SIII
Mobile phase:	Chloroform/diethyl ether/formic acid (50:20:0.7) for 15 min and benzene/hexane (50:50) for 35 min
Gas flow:	H_2 150 mL/min, air 700 mL/min
Scanning speed:	30 sec/Rod

Figure 43. TLC-FID chromatogram of standard compound (1) and reaction mixture of FAME catalyzed by immobilized lipase from *Burkholderia multivorans* PSU-AH130 (2).

4.1 Comparison of immobilized lipase from *Burkholderia multivorans* PSU-AH130 with other immobilized lipases

Different immobilized lipases on Accurel EP100 (<400 μm) or IM-PS, IM-D, IM-AK and IM-PSU-AH130 were studied on FAME production using palm olein as substrate. After 60 h, IM-PS, IM-AK and IM-PSU-AH130 yielded FAME more than 70% while IM-D gave only 20% FAME. Salis *et al.* (2005) reported synthesis of oleic acid butyl ester catalyzed by immobilized lipase PS on celite, Novozym 435 and Lipozyme RM IM from triolein and butanol. Immobilized lipase PS on celite showed the highest activity with 100% of oleic acid butyl ester in 6 h while Novozym 435 and Lipozyme RM IM showed 100% of the ester after 24 h. In our study, immobilized lipase PS on Accurel EP100 produced 76.2% FAME, while the immobilized lipase from *B. multivorans* PSU-AH130 on the same supporter could produce 78.0% FAME at 60h.

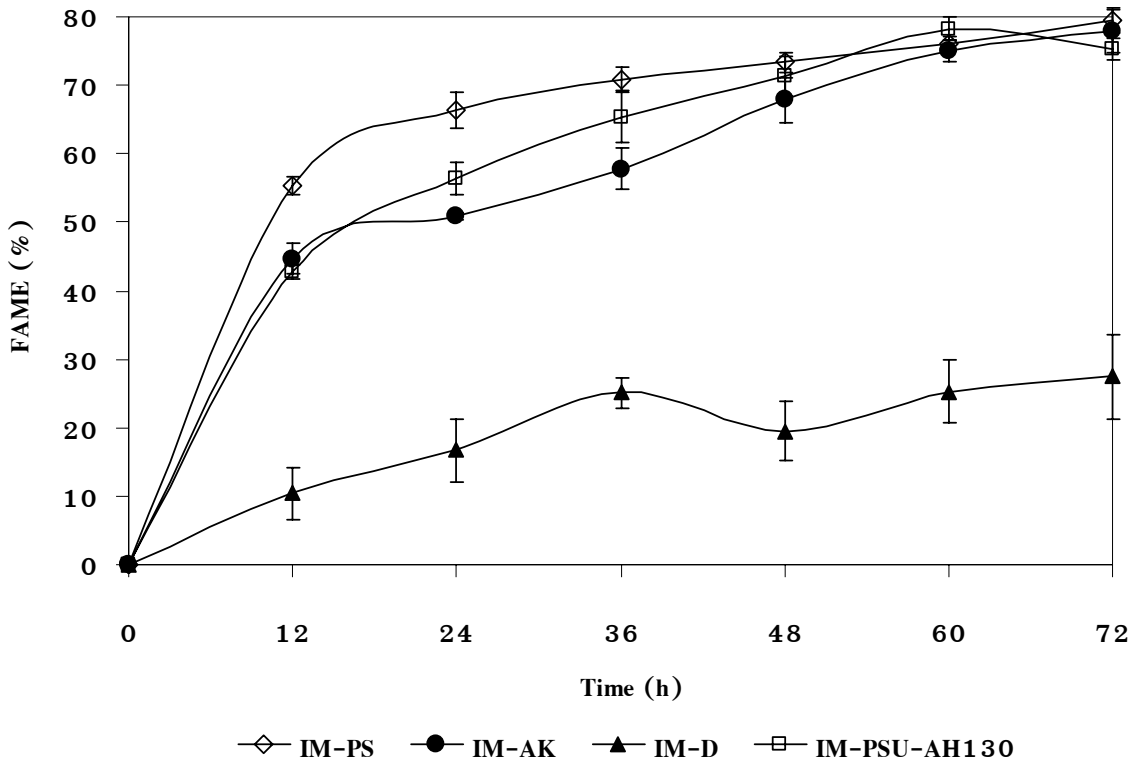


Figure 44. Effect of types of immobilized lipases on FAME synthesis from palm olein.

(The reaction mixture contained palm olein/methanol (1:3 mol/mol or 1.40:0.16 g), 0.32 mL of 50 mM phosphate buffer pH 7.0 and immobilized lipase (20 U). The reaction was carried out at 50°C and 400 rpm.)

4.2 Type of fat/oil

Effect of palm oil and palm stearin on FAME synthesis catalyzed by IM-PSU-AH130 was studied (Figure 45). Palm olein showed the significantly FAME content of 83.6 % higher than palm stearin (74.6%) at 60 h. This might be palm stearin was still solid at 50°C. Paripatanapairod (2003) reported that the FAME of 94% was achieved when used palm stearin as substrate catalyzed by IM-PS at 60°C for 24 h.

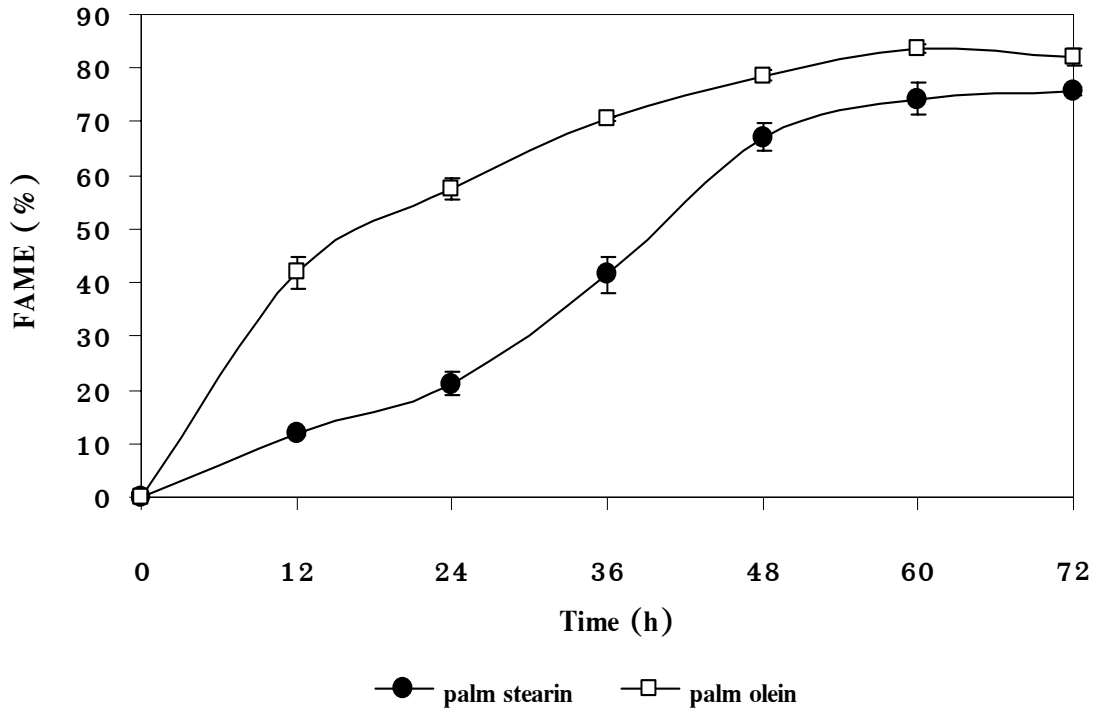


Figure 45. Effect of types of oils on FAME synthesis catalyzed by IM-PSU-AH130 at 50°C.

(The reaction mixture contained of palm olein or palm stearin/methanol (1:3 mol/mol), 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.)

4.3 Effect of water content

Effect of water content on FAME synthesis from palm olein catalyzed by IM-PSU-AH130 was studied in the reaction with the water content of 10-50% of substrate (w/w). The water content of 20% gave the highest of 83.2% FAME (Figure 46). Similar result was reported by Paripatanapairod (2003) that the FAME content of 92.4 % was achieved when used palm stearin as substrate with water content of 20% of substrate catalyzed by IM-PS at 60°C for 24 h.

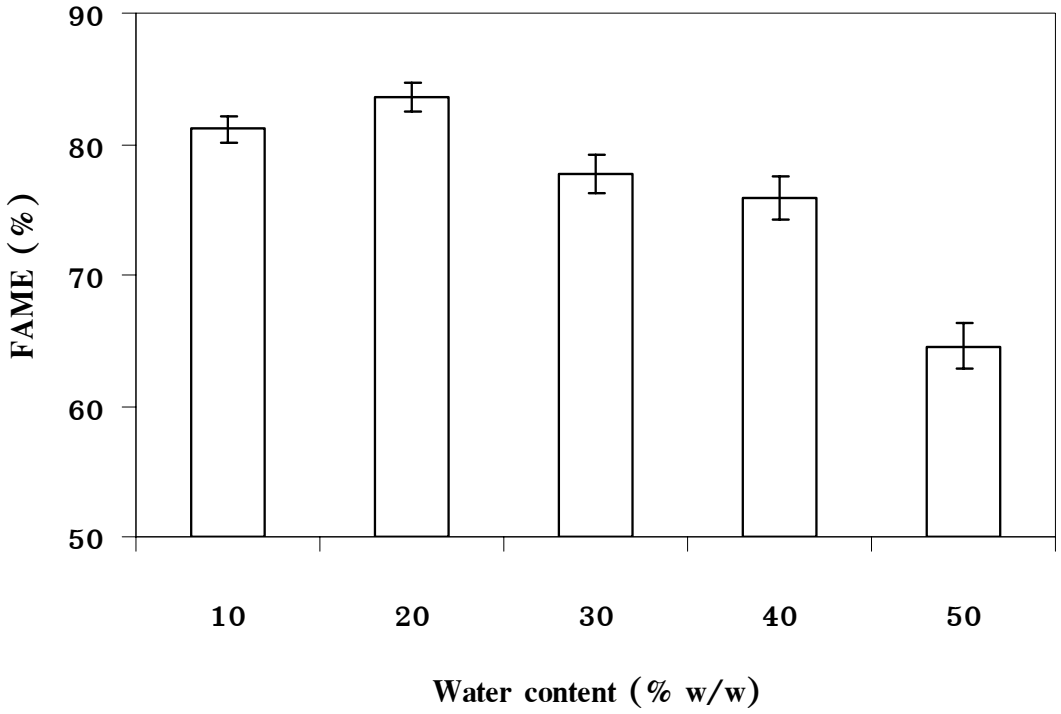


Figure 46. Effect of water content on FAME synthesis from palm olein catalyzed by IM-PSU-AH130.

(The reaction mixture contained of palm olein/methanol (1:3 mol/mol), 20% (w/w) 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 60 h.)

4.4 Effect of pH

Effect of pH on FAME synthesis from palm oil catalyzed by IM-PSU-AH130 was studied with the pH of the various buffers at the concentration of 50 mM; pH 5 (citrate buffer), pH 6-7 (phosphate buffer), pH 8-9 (Tris-HCl buffer) and pH 10 (NaOH-glycine buffer). The highest FAME (%) of 90.3 % was obtained with 50 mM phosphate buffer pH 6.0 (Figure 47). So, the initial pH of 6.0 was chosen for further studied.

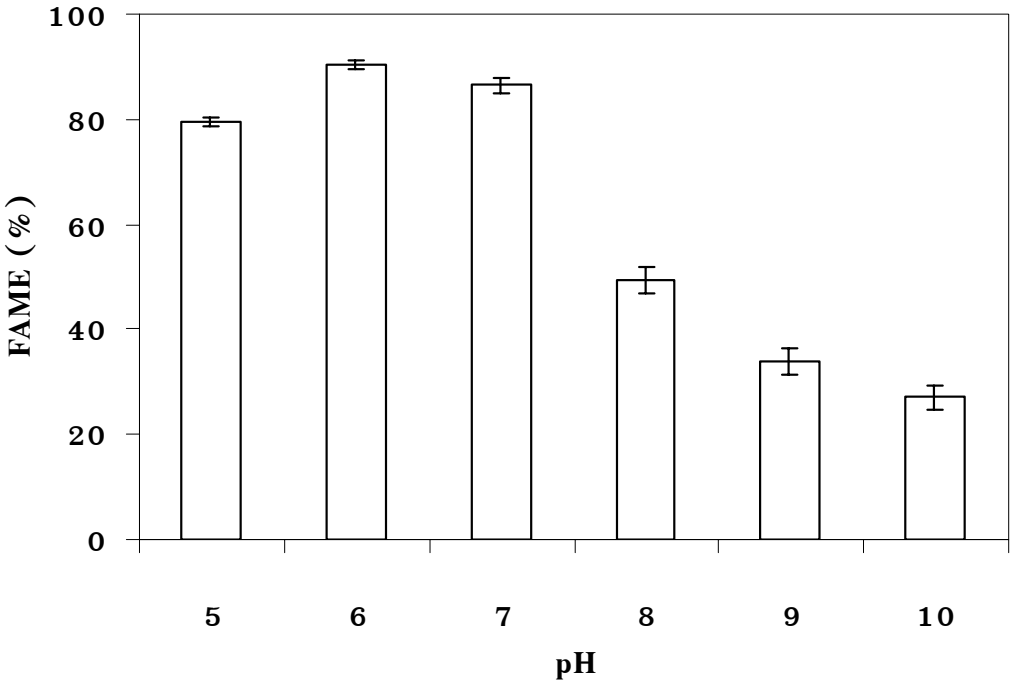


Figure 47. Effect of initial pH on FAME synthesis from palm olein catalyzed by IM-PSU-AH130.

(The reaction mixture contained of palm olein/methanol (1:3 mol/mol), 20% (w/w) of 50 mM various buffers pH 7.0 and 0.1 g immobilized lipase (20 U). The reaction was carried out at 50°C and 400 rpm for 60 h.)

4.5 Effect of molar ratio of palm olein to methanol

Effect of molar ratio of plam olein to methanol (1:1 to 1:6) on FAME synthesis catalyzed by IM-PSU-AH130 was studied. The highest FAME (%) of 93.3 % was obtained with molar ratio of palm olein to methanol of 1:4 mol/mol (Figure 48). Further increase of methanol concentration did not lead to an increase of the product. In contrast, FAME (%) was decreased when the molar ratio of methanol to palm olein was more than 4:1 mol/mol. This result indicated that high concentration of methanol might inactivate the enzyme activity. So the molar ratio of 1:4 was chosen for further studied.

Synthesis of butyl oleate catalyzed by immobilized lipase PS on celite using triolein and butanol as substrate was reported. The best ratios were 1:3 and 1:6 of triolein to butanol, the conversion yield of 100% was achieved in 4 h (Salis *et al*., 2005).

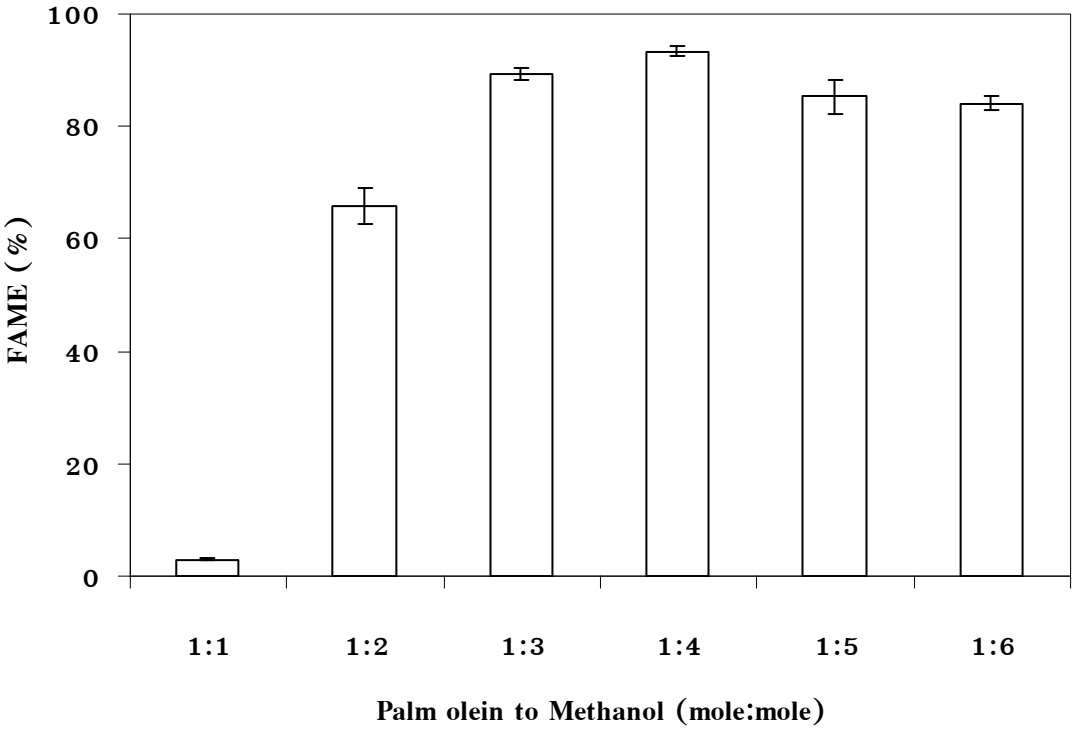


Figure 48. Effect of molar ratio of palm olein to methanol on FAME synthesis from palm olein catalyzed by IM-PSU-AH130.

(The reaction mixture contained of palm olein/methanol (1:3-1:6 mol/mol), 20% (w/w) of 50 mM phosphate buffers pH 6.0 and 0.1 g immobilized lipase (20 U). The reaction was carried out at 50°C and 400 rpm for 60 h.)

4.6 Effect of enzyme loading

Effect of enzyme loading on FAME synthesis from palm olein catalyzed by IM-PSU-AH130 was studied with the activity of enzyme (5-20 U). The highest FAME of 95.9 % was obtained when 15 U of enzyme was used after 60 h of reaction time (Figure 49). Further increase of enzyme loading did not increase the FAME yield.

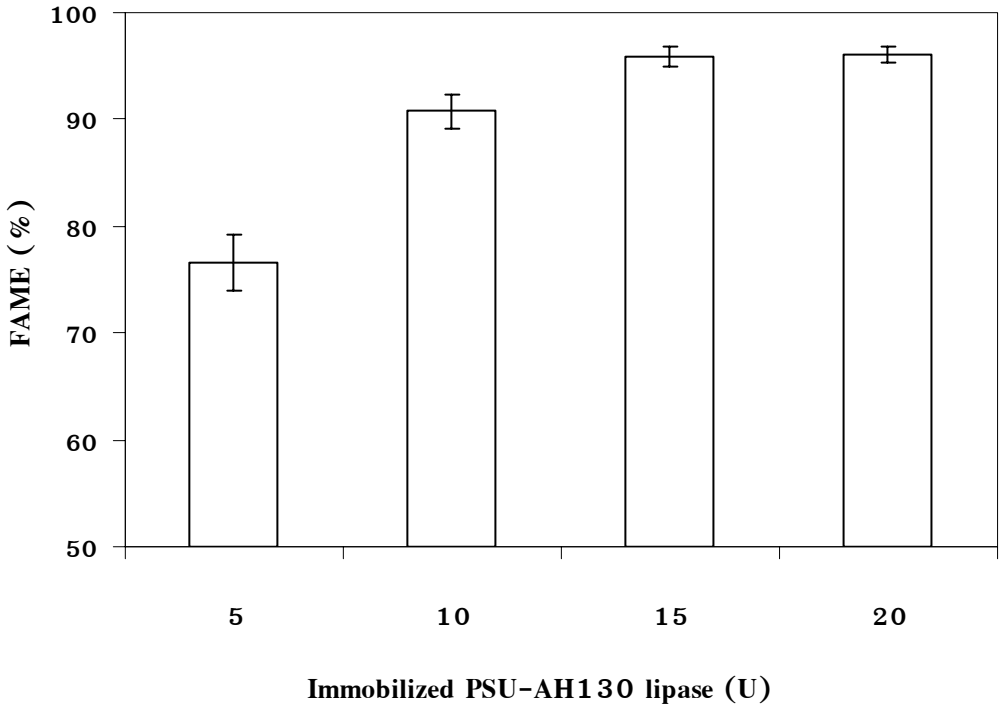


Figure 49. Effect of enzyme loading on FAME synthesis from palm olein catalyzed by IM-PSU-AH130 at 50°C for 60 h.

(The reaction mixture contained of palm olein/methanol (1:4 mol/mol), 20% (w/w) of 50 mM phosphate buffers pH 6.0 and immobilized lipase (5-20 U). The reaction was carried out at 50°C and 400 rpm for 60 h.)

4.7 Effect of temperature

Effect of temperature on FAME synthesis from palm olein catalyzed by IM-PSU-AH130 was studied at temperature between 40-60°C. The highest FAME of 95.34 % was obtained at 50°C after 60 h. Figure 50 shows the conversion of palm oil to FAME was influenced by temperature. Increasing of temperature from 40 to 50°C, the conversion yield was increased but at 55°C, the conversion yield decreased rapidly and no FAME was synthesized at 60°C.

The effect of temperature on the synthesis of propyl oleate from triolein catalyzed by immobilized lipase from *Pseudomonas fluorescens* showed that the highest conversion (100%) was obtained at 50 and 60°C. Whereas, the yield was dramatically decreased at the temperature above 60°C (Iso *et al.*, 2001). Reaction rate increases with increasing temperature but for synthesis of FAME does not continue indefinitely because too high temperature will inactive enzyme (Salis *et al.*, 2005).

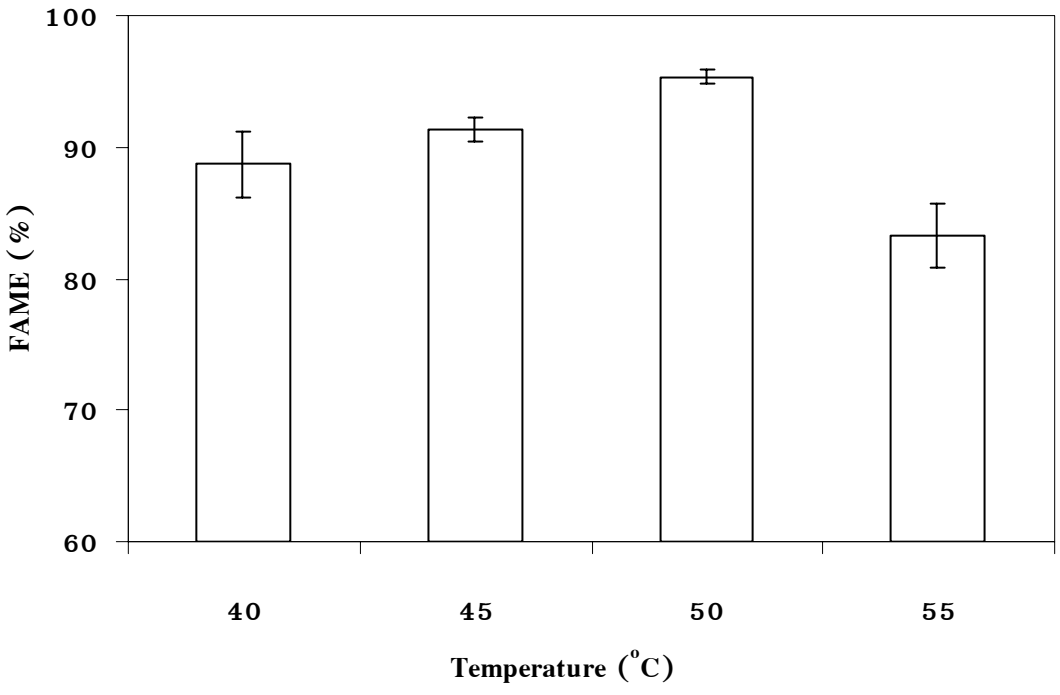


Figure 50. Effect of temperature on FAME synthesis from palm olein catalyzed by IM-PSU-AH130.

(The reaction mixture contained of palm olein/methanol (1:4 mol/mol), 20% (w/w) of 50 mM phosphate buffers pH 6.0 and immobilized lipase 15 U. The reaction was carried out at 40-55°C and 400 rpm for 60 h.)

4.8 Time course of fatty acid methyl esters production

After obtained the optimal conditions, the time course of FAME production from palm olein and methanol catalyzed by IM-PSU-AH130 was monitored by TLC-FID (Figure 51). The FAME content increased rapidly in 24 h then it was gradually increased until 48 h. The maximum FAME content of 94.28 was obtained at this time. At the end of reaction time (60 h), the compositions of FAME, TAG, FFA, DAG and MAG were 94.22, 0, 4.50, 1.28 and 0%, respectively.

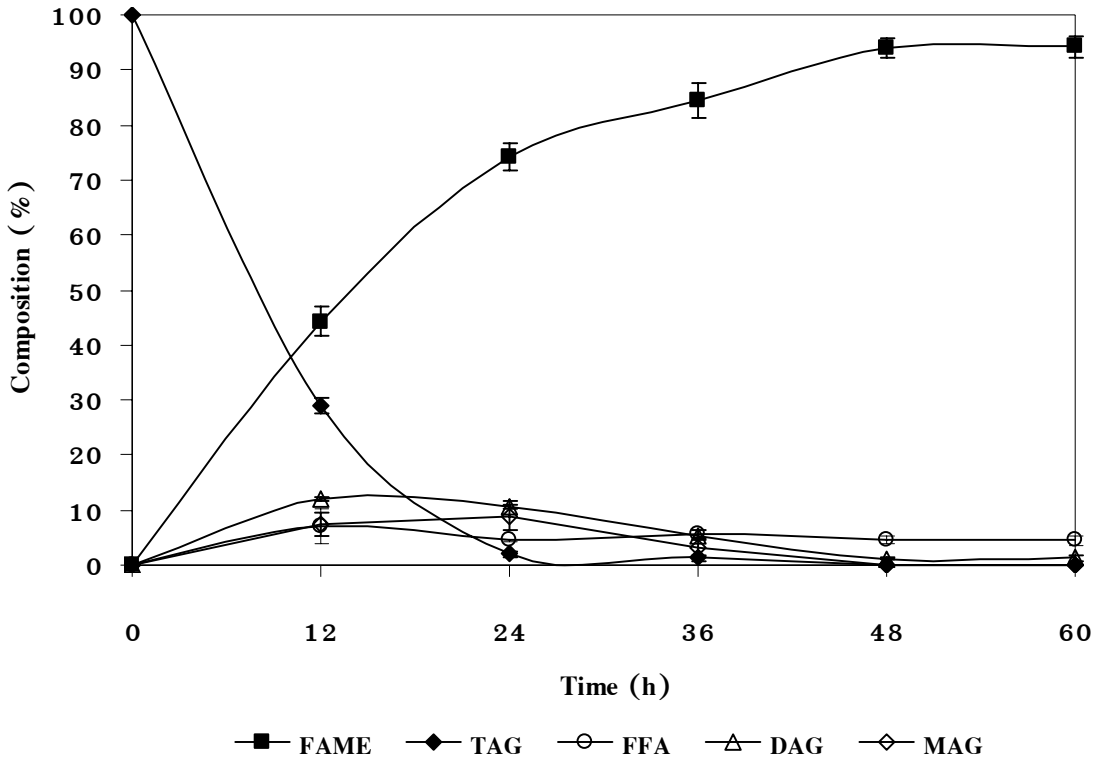


Figure 51. Time course of FAME synthesis from palm olein catalyzed by IM-PSU-AH130.

(The reaction mixture contained of palm olein/methanol (1:4 mol/mol), 20% (w/w) of 50 mM phosphate buffers pH 6.0 and immobilized lipase 15 U. The reaction was carried out at 50°C and 400 rpm for 60 h.)

5. Optimization of lipase production by *Burkholderia multivorans* PSU-AH130

In order to obtain the maximum lipase production by *B. multivorans* PSU-AH130, the effect of medium composition and environmental conditions were studied. *B. multivorans* PSU-AH130 was cultivated in the basal medium (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% $CaCl_2.2H_2O$) supplemented with 1.0% (v/v) palm oil as a carbon source, initial pH was 7.5, under shaking at 150 rpm at 45°C. The lipase activity and cell growth were monitored.

5.1 Effect of nitrogen sources

The effect of nitrogen sources on lipase production by *B. multivorans* PSU-AH130 was studied in the basal medium supplemented with 1.0% (w/v) palm oil as a carbon source. The effect of organic nitrogen sources (0.5% w/v); meat extract, yeast extract, soytone, tryptone, polypeptone and tryptone+yeast extract (as control) in the presence of 0.1% ammonium sulphate was studied. When *B. multivorans* PSU-AH130 was cultivated in the medium using tryptone as nitrogen source, the highest lipase activity (20.5 U/mL) was obtained while in the control experiment, the lipase production was 17.5 U/mL (Figure 52).

In the basal medium with out organic nitrogen source, only 0.39 U/mL of lipase activity was obtained in the presence ammonium sulphate (0.1% w/v). Production of extracellular lipase from *Burkholderia cepacia* in the medium with organic or inorganic nitrogen sources (0.2% w/v) did not significantly affect the lipase production. The highest lipase activity of 35 U/mL was obtained with corn steep liquor and the lowest of 32 U/mL was obtained with ammonium nitrate (Rathi *et al.*, 2001). The requirement of type of nitrogen source varied among microorganisms, some prefer inorganic form while others prefer organic nitrogen.

After obtained the suitable organic nitrogen source, the concentration of tryptone was varied from 0 to 1.0% (w/v). The highest lipase activity of 25.0 U/mL and total cell protein of 0.8 g/L were obtained with tryptone 0.2% w/v (Figure 53).

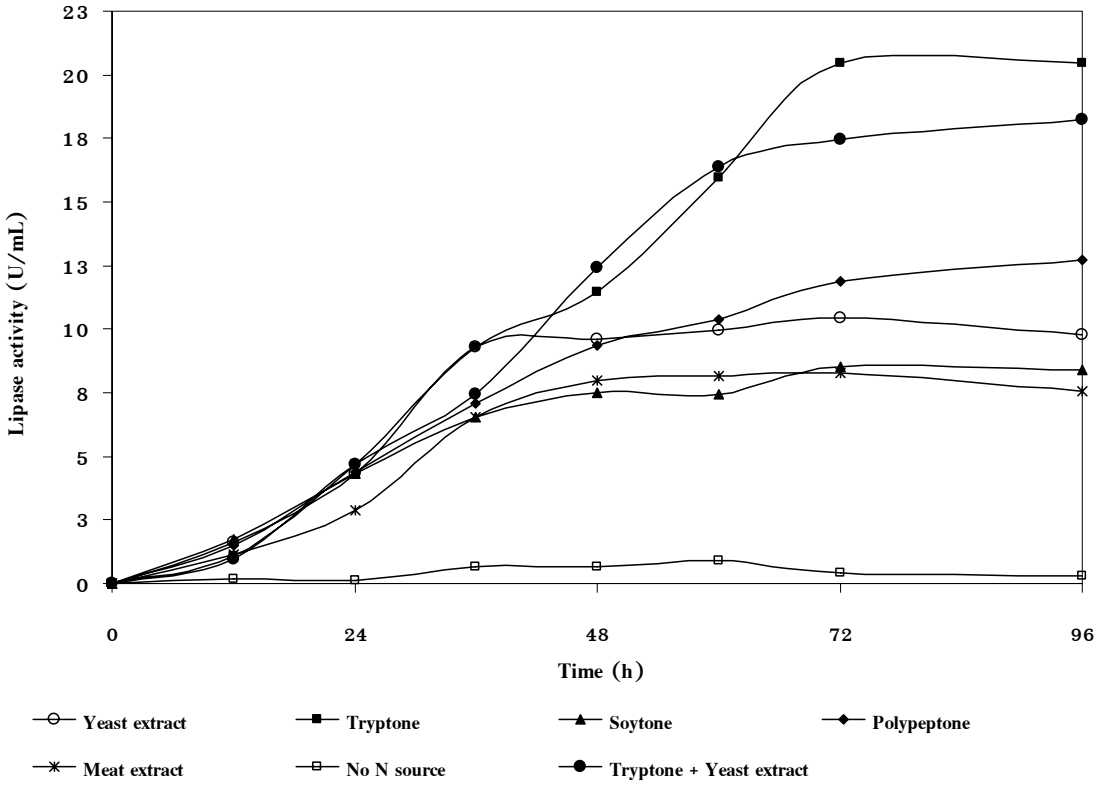


Figure 52. Effect of organic nitrogen sources (0.5% w/v) on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 1.0% (w/v) palm oil and 0.1% (w/v) ammonium sulphate after 72 h of incubation under shaking 150 rpm at 45°C)

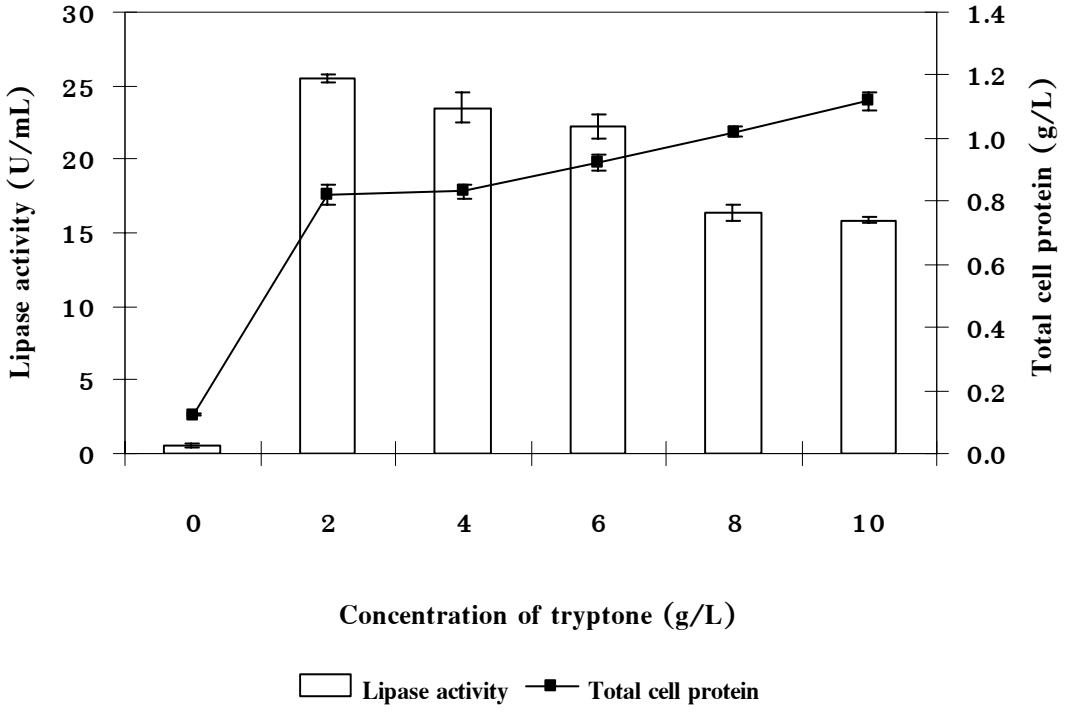
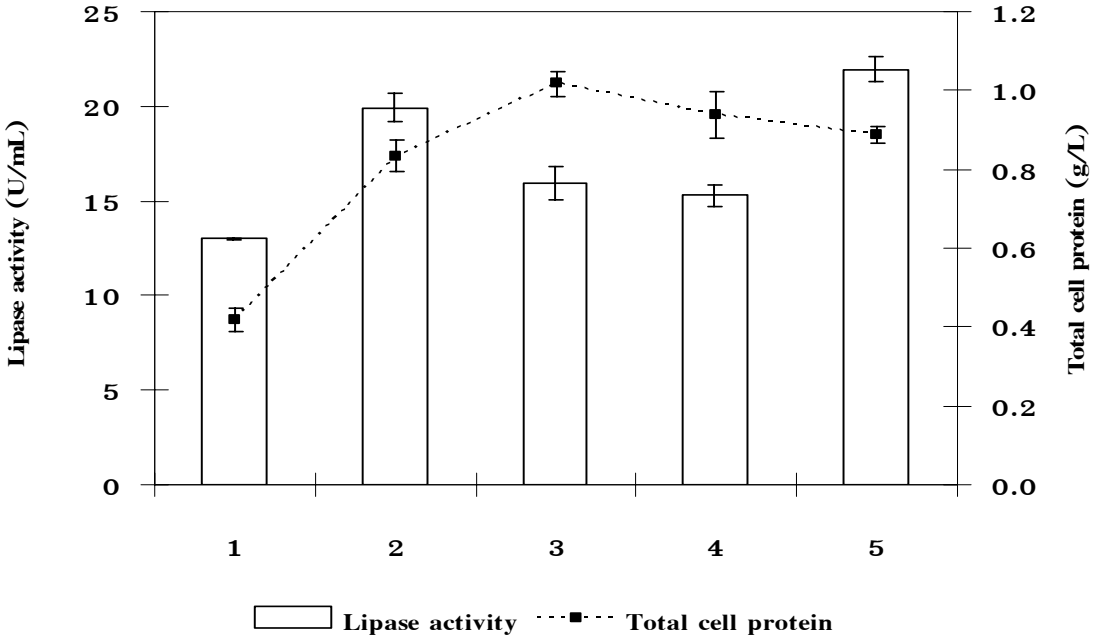


Figure 53. Effect of tryptone concentration on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 1.0% (w/v) palm oil and 0.1% (w/v) ammonium sulphate after 72 h of incubation under shaking 150 rpm at 45°C)

The effect of types of inorganic nitrogen sources in the presence of 0.2% (w/v) tryptone was also studied with ammonium chloride, ammonium hydrogenphosphate, ammonium nitrate and ammonium sulphate (0.1% w/v). Ammonium sulphate was the best inorganic nitrogen for lipase production from *B. multivorans* PSU-AH130. Lipase activity 23.0 U/mL and total cell protein of 0.85 g/L were obtained at 72 h whereas the maximum total cell protein of 1.0 g/L was obtained with ammonium hydrogenphosphate (Figure 54).

The concentration of ammonium sulphate was varied from 0 to 1.0% (w/v). At the concentration of 0.1% w/v, the highest lipase activity of 24.2 U/mL was obtained while the highest total cell protein of 0.95 g/L was obtained with 0.2% w/v of ammonium sulphate (Figure 55).



1) Control
2) Ammonium chloride
3) Ammonium hydrogenphosphate
4) Ammonium nitrate
5) Ammonium sulphate

Figure 54. Effect of inorganic nitrogen sources (0.1% w/v) on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 1.0% (w/v) palm oil, 0.2% (w/v) tryptone and 0.1% (w/v) ammonium sulphate after 72 h of incubation under shaking 150 rpm at 45°C)

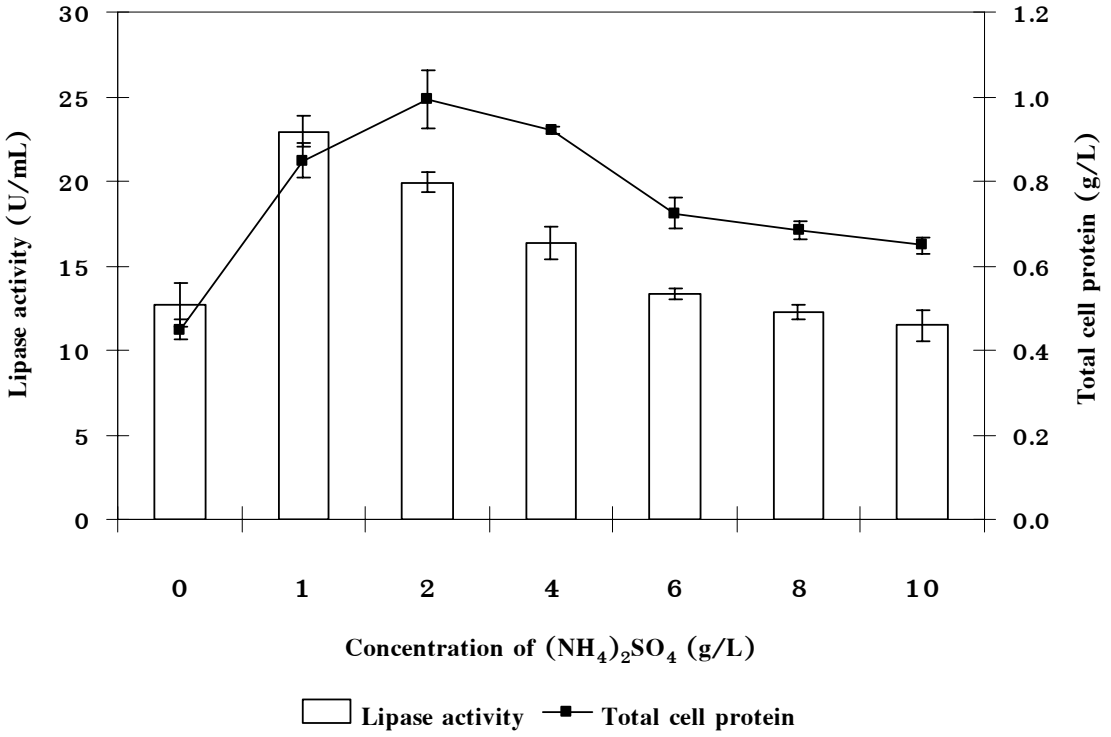
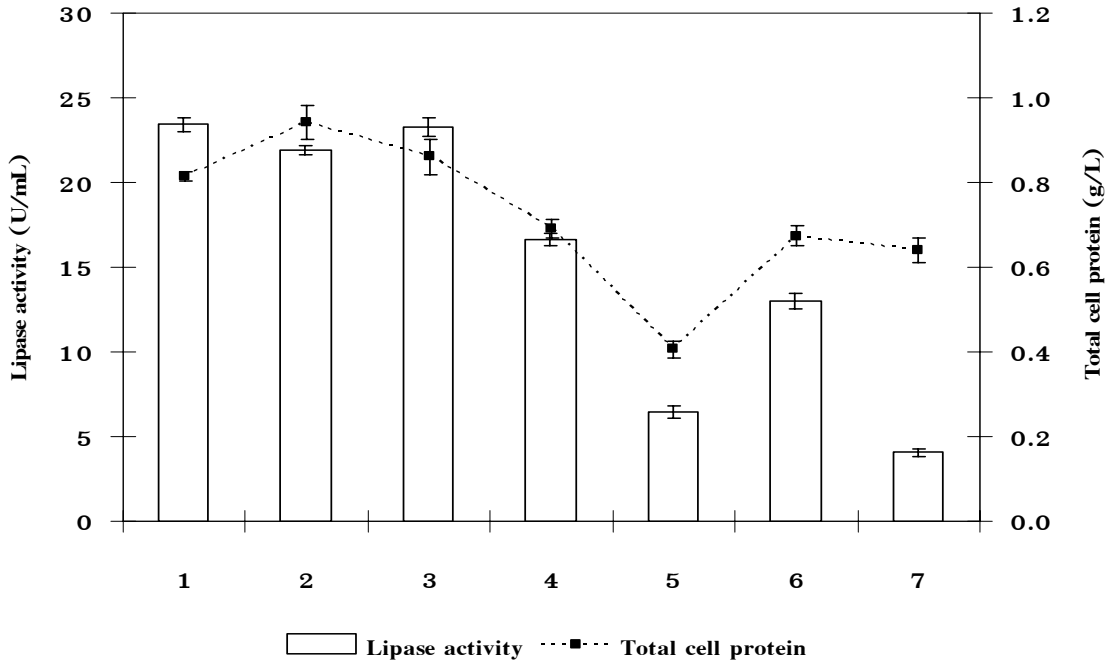


Figure 55. Effect of ammonium sulphate concentration on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 1.0% (w/v) palm oil and 0.2% (w/v) tryptone after 72 h of incubation under shaking 150 rpm at 45°C)

5.2 Effect of emulsifiers

The effect of emulsifier which was used to emulsify palm oil on lipase production by *B. multivorans* PSU-AH130 in the basal medium was studied with deoxycholic acid, gum arabic, polyvinyl alcohol, Triton X-100, Tween 20 and Tween 80 (1.0% w/v). The lipase activity of 23.4, 23.2 and 21.3 U/mL and total cell protein of 0.81, 0.86 and 0.94 were obtained in the medium with non-surfactant adding, gum arabic and deoxycholic acid at 72 h, respectively (Figure 56).

B. multivorans PSU-AH130 showed ability to use palm oil as carbon source with out adding of surfactant. The cell free culture broth of *B. multivorans* PSU-AH130 was also checked biosurfactant activity based on reducing of the surface tension of distilled water (72.0 mN/m) as shown in Figure 61. The 12 h cultivation broth showed surface tension (31.2 mN/m) whereas the basal medium showed (42.8 mN/m). This result indicated that *B. multivorans* PSU-AH130 produced biosurfactant during its growth. Hence no emulsifier needed for lipase production from *B. multivorans* PSU-AH130.



1) Non-surfactant
2) Deoxycholic acid
3) Gum arabic
4) Polyvinyl alcohol
5) Triton X-100
6) Tween 20
7) Tween 80

Figure 56. Effect of emulsifiers (1.0% w/v) on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 1.0% (w/v) palm oil, 0.2% (w/v) tryptone and 0.1% (w/v) ammonium sulphate after 72 h of incubation under shaking 150 rpm at 45°C)

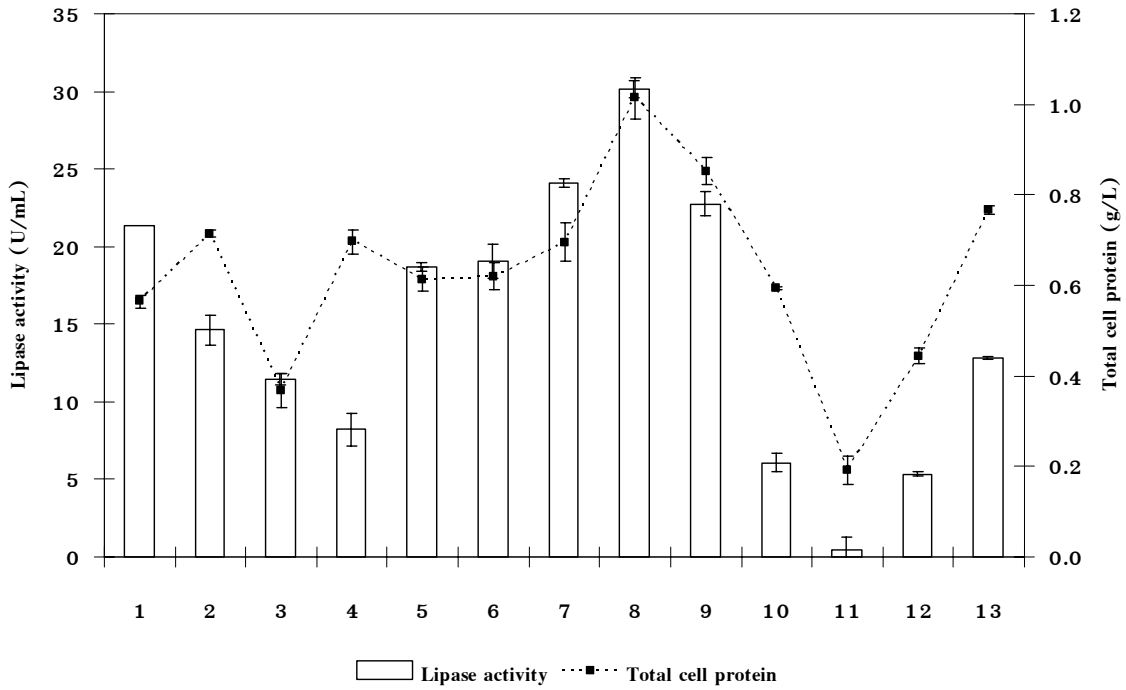
5.3 Effect of carbon sources

The effect of carbon sources on lipase production by *B. multivorans* PSU-AH130 in the basal medium was studied using palm oil, palm stearin, lard, tallow, sunflower oil, rice brane oil, soybean oil, olive oil, fish oil, coconut oil, tributyrin, Tween 20 and Tween 80 (1.0% w/v). The highest lipase activity (30.15 U/mL) and total cell protein (0.81 g/L) were obtained when olive oil was used as a carbon source (Figure 57). Other oils containing unsaturated long chain fatty acids (soybean, palm, sunflower, rice bran oils) were also good carbon source for lipase production. Palm stearin was moderately good carbon source with less than 15.0 U/mL of lipase activity was obtained but coconut oil was not a good carbon source for lipase production by *B. multivorans* PSU-AH130.

Lard and tallow was also not good carbon sources for lipase production by *B. multivorans* PSU-AH130. Whereas, TAG containing short chain fatty acid (tributyrin) was poor carbon source. The results indicated that *B. multivorans* PSU preferred to use TAG containing unsaturated long chain fatty acid as carbon source.

Lee *et al.* (1993) reported the production of lipase from *Pseudomonas fluorescens* S1K. The maximum specific activity of 7,359 U/mg was obtained in the medium with olive oil (0.1% w/v) as a carbon source. Rathi *et al.* (2001) reported that *B. cepacia* produced highest lipase activity with 34.2 U/mL in the medium with mustard oil while olive oil showed only 15.8 U/mL.

After obtaining the suitable carbon source source, the concentration of olive oil was varied from 0 to 3.0% (w/v). The highest lipase activity of 32.4 U/mL and total cell protein of 1.02 g/L were obtained when *B. multivorans* PSU-AH130 was grown in the basal medium with 1.5% of olive oil (Figure 58).



1) Palm oil
2) Palm stearin
3) Lard
4) Tallow
5) Sunflower oil
6) Rice bran oil
7) Soybean oil
8) Olive oil
9) Fish oil
10) Coconut oil
11) Tributyrin
12) Tween 20
13) Tween 80

Figure 57. Effect of carbon sources (1.0% w/v) on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 0.2% (w/v) tryptone and 0.1% (w/v) ammonium sulphate after 72 h of incubation under shaking 150 rpm at 45°C)

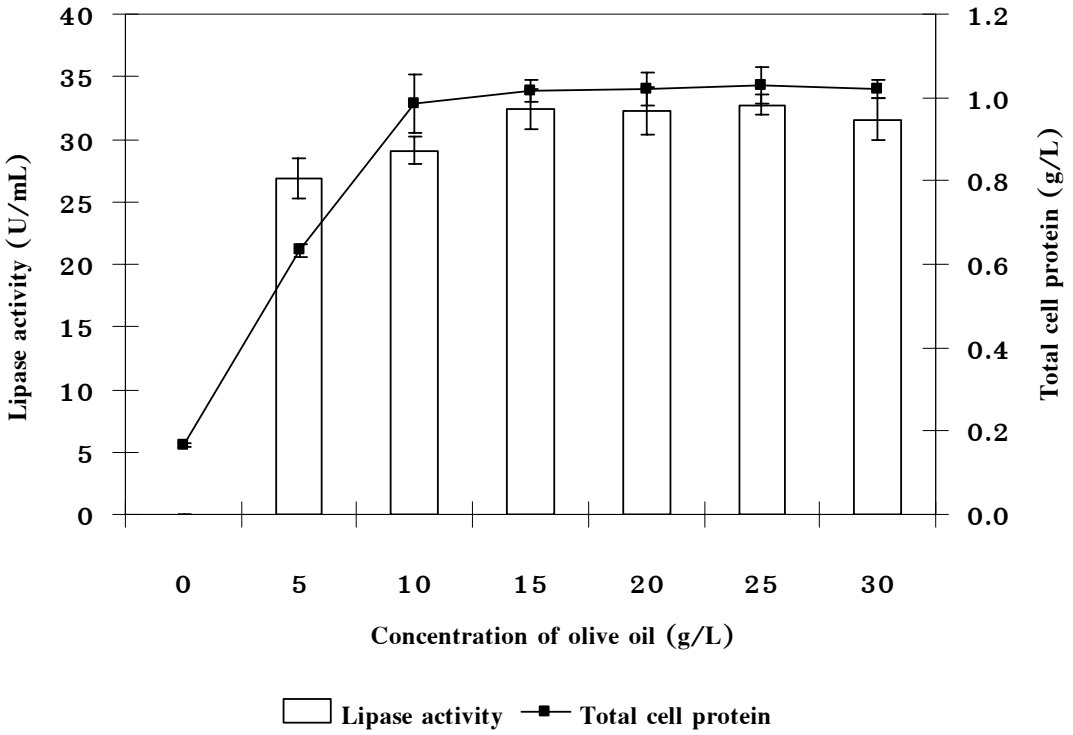


Figure 58. Effect of olive oil concentration on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 0.2% (w/v) tryptone and 0.1% (w/v) ammonium sulphate after 72 h of incubation under shaking 150 rpm at 45°C)

5.4 Effect of initial pH

The effect of initial pH (4.0-11) on lipase production by *B. multivorans* PSU-AH130 was studied in the basal medium supplemented with 1.5% olive oil. The initial pH 7.5-8.0 was the suitable initial pH, the lipase activity (30.5-31.5 U/mL) with total cell protein (~1.03 g/L) were obtained (Figure 59). While no growth of *B. multivorans* PSU-AH130 was observed in the medium with the initial pH 4.0.

The effect of initial pH (5-10) was studied on the production of lipase from *Pseudomonas* sp. G6. The maximum activity (27.5 U/mL) and dry cell weight (4.2 g/L) were obtained with optimum pH range 7.0-8.0 (Kanwar *et al*., 2002). Rathi *et al*. (2001) found that the highest lipase production (21.0 U/mL) was obtained when *B. cepacia* was cultivation in the medium supplemented with palm oil at the initial pH 7.0.

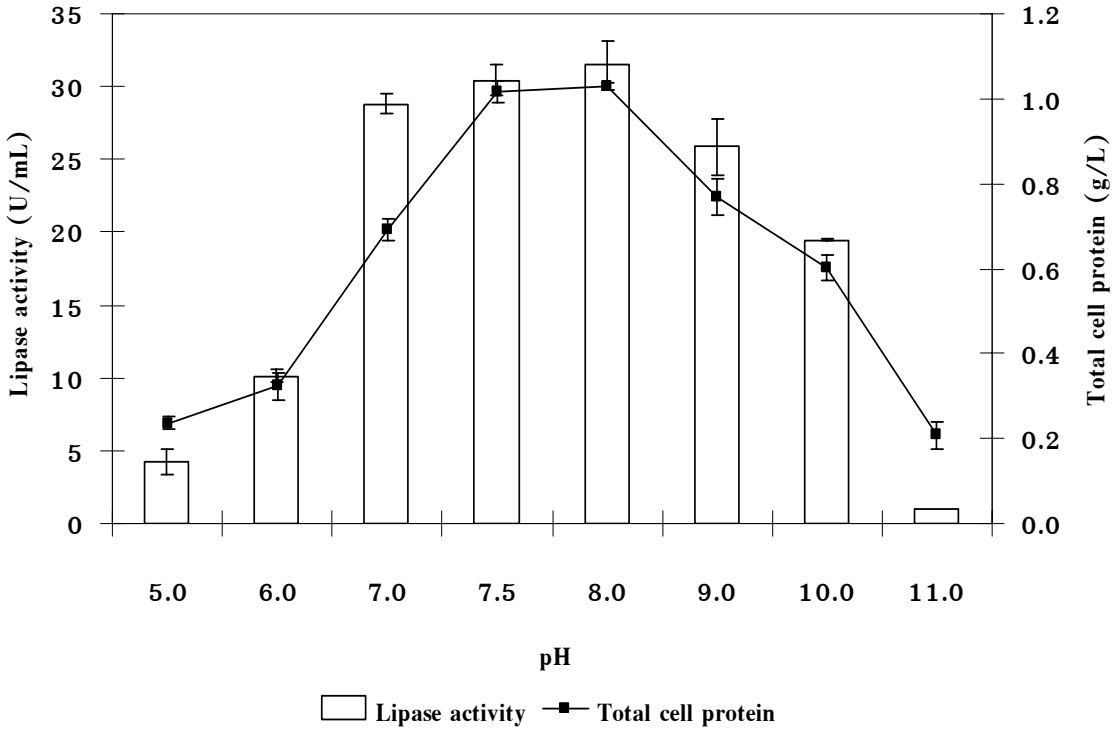


Figure 59. Effect of initial pH on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 0.2% (w/v) tryptone, 0.1% (w/v) ammonium sulphate and 1.5% (w/v) olive oil after 72 h of incubation under shaking 150 rpm at 45°C)

5.5 Effect of temperature

The effect of temperature on lipase production by *B. multivorans* PSU-AH130 was studied in the basal medium supplemented with 1.5% olive oil, pH 8.0, shaking rate 150 rpm at 30-50°C. The cultivation temperature of 37°C was the suitable temperature with the highest lipase activity (38.7 U/mL) and total cell protein (1.32 g/L) (Figure 60). While no growth of *B. multivorans* PSU-AH130 was observed at 50°C. The effect of temperature on cell growth and lipase production from *Pseudomonas* sp. G6 showed that the maximum activity of 25.0 U/mL and dry cell weight of 4.9 g/L were obtained at 35°C (Kanwar *et al.*, 2002) while the optimum temperature of lipase production from *B. cepacia* was 50°C with 24.3 U/mL of lipase activity (Rathi *et al.*, 2001).

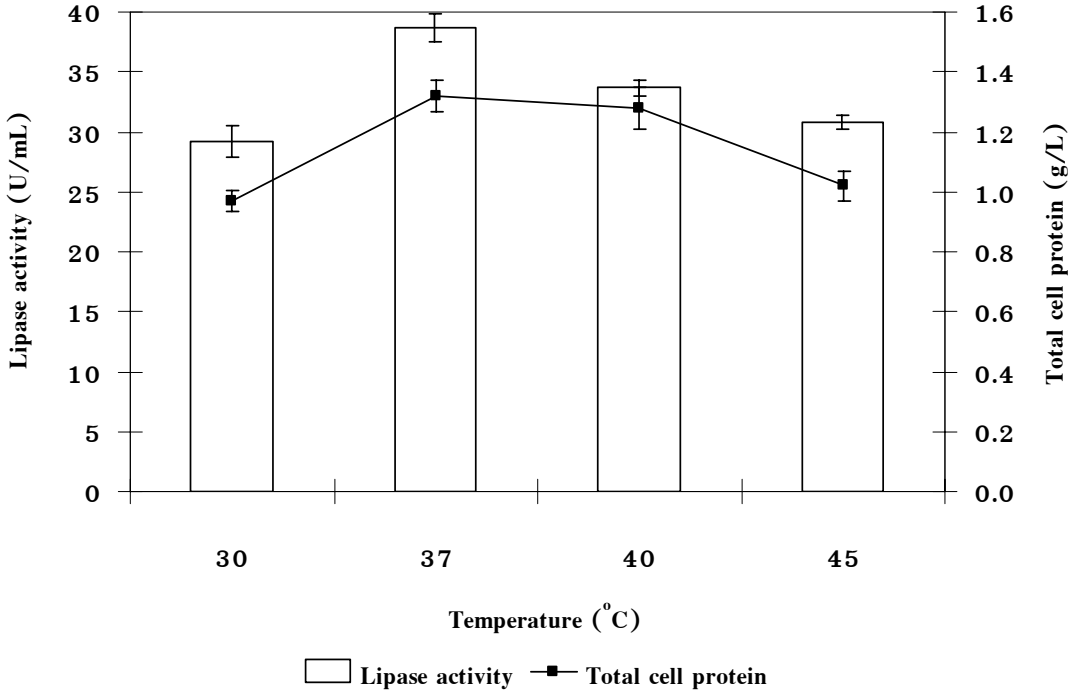


Figure 60. Effect of temperature on lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 0.2% (w/v) tryptone and 0.1% (w/v) ammonium sulphate and 1.5% (w/v) olive oil, initial pH 8.0 after 72 h of incubation under shaking at 150 rpm)

5.6 Time course of lipase production from *Burkholderia multivorans* PSU-AH130

Production of extracellular lipase by *B. multivorans* PSU-AH130 was studied using optimal conditions. The pH, lipase activity and total cell protein were monitored. The optimum conditions were modified the basal medium which composted of 0.2% tryptone, 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% $CaCl_2.2H_2O$ supplemented with 1.5% (w/v) olive oil, pH 8.0, shaking rate 150 rpm at 37°C. When *B. multivorans* PSU-AH130 was cultivated in modified the basal medium using olive oil as carbon source it showed rapidly growth in 36 h and then was gradually increased until 60 h. The growth was dropped after 72 h. The initial pH of the basal medium was decreased and the final pH was 6.3. The lipase activity was observed at 12 h of cultivation and increased until 60 h, then it was decreased as well as cell growth (OD_{660}). *B. multivorans* PSU-AH130 showed ability to use oil with out emulsifier adding. The result obtained from the surface tension measurement indicated that *B. multivorans* PSU-AH130 produce biosurfactant, the 12 h culture broth could reduce surface tension of distilled water from 72.0 mN/m to 31.2 mN/m. The

maximum lipase activity of 38.8 U/mL and total cell protein of 1.24 g/L were obtained at 72 h of cultivation (Figure 61).

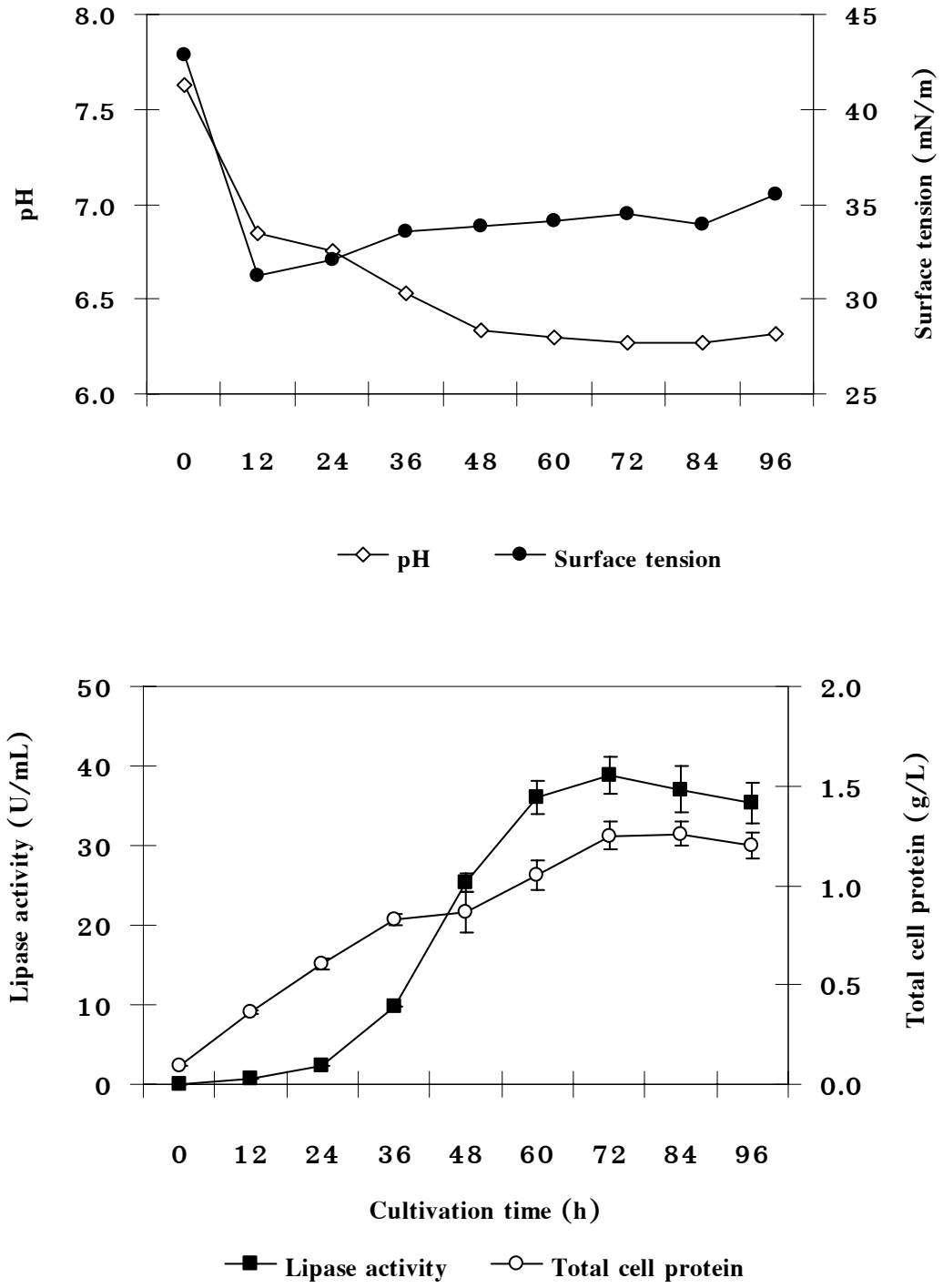


Figure 61. Time course of lipase production and cell growth of *Burkholderia multivorans* PSU-AH130. (in the basal medium with 0.2% (w/v) tryptone and 0.1% (w/v) ammonium sulphate and 1.5% (w/v) olive oil, initial pH 8.0, shaking (150 rpm) at 37°C)

6. Purification and characterization of lipase from *Burkholderia multivorans* PSU-AH130

6.1 Purification of extracellular lipase

The production of extracellular lipase from *B. multivorans* PSU-AH130 was carried out in the basal medium using Tween 80 as carbon source for 72 h. The cell free supernatant obtained after centrifugation the culture broth was purified by sequential three step methods which consisted of 70% ammonium sulphate precipitation, DEAE-Toyopearl ion-exchange chromatography and Sephadex G-150 gelfiltration chromatography. The purification profile was summarized in Table 28.

The lipase activity and specific lipase activity in the cell-free culture broth were 12.07 U/mL and 7.08 U/mg protein, respectively. The crude lipase was precipitated by 70% saturation of ammonium sulphate. The precipitate was reconstituted in a minimum volume of 20 mM Tris-HCl buffer, pH 7.5 and dialyzed against this buffer. The dialyzed enzyme showed lipase activity of 57.7 U/mL and specific activity of 13.25 U/mg protein.

The anion exchange chromatography of lipase using DEAE-Toyopearl showed four peaks (Figure 62). Enzyme was eluted at 0.58 M NaCl during ion exchange chromatography step. The fractions contained lipase activity were pooled and concentrated by ultrafiltration (Amicon Ultra-4, molecular weight cut off 30 kDa). It showed lipase activity and specific activity of 26.62 U/mL and 66.56 U/mg protein with the purity of 9.4 folds (Table 27). The concentrated enzyme was load to the Sephadex G-150 gel filtration column and showed 2 peaks (Figure 63). The second peak showed lipase activity of 11.18 U/mL and specific activity of 153.0 U/mg protein with the purity of 21.6 folds and the yield of 12.1%.

The extracellular lipase from *Pseudomonas aeruginosa* was purified with the steps of 80% ammonium sulphate precipitatiaon, Butyl-Toyopearl column and Toyopearl HW-55 column. The purity of 12.5 folds with the yield of 16.0% was obtained (Karadzic *et al.*, 2006). Single-step purification of extracellular lipase from *B. multivorans* using Accurel MP-1000 (< 1,500 μm) was reported that the crude enzyme was adsorbed on Accurel using butanol as solvent in buffer pH 9.0 then desorbed by 0.2% Triton X-100. The enzyme was finally precipitated with acetone (50% v/v). Overall enzyme yield of 66% with 3.0 folds purity was obtained (Gupta *et al.*, 2005).

Table 27. Summary of purification steps of extracellular lipase from *Burkholderia multivorans* PSU-AH130.

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification factor
Crude lipase	650	1108.0	7847.0	7.08	100	1
70% $(NH_4)_2SO_4$	80	348.2	4614.0	13.25	58.8	1.9
DEAE Toyopearl	85	34.0	2263.0	66.56	28.8	9.4
Sephadex G-150	85	6.2	948.6	153.0	12.1	21.6

[a] Activity was measured with *p*NPP as substrate at pH 8.5

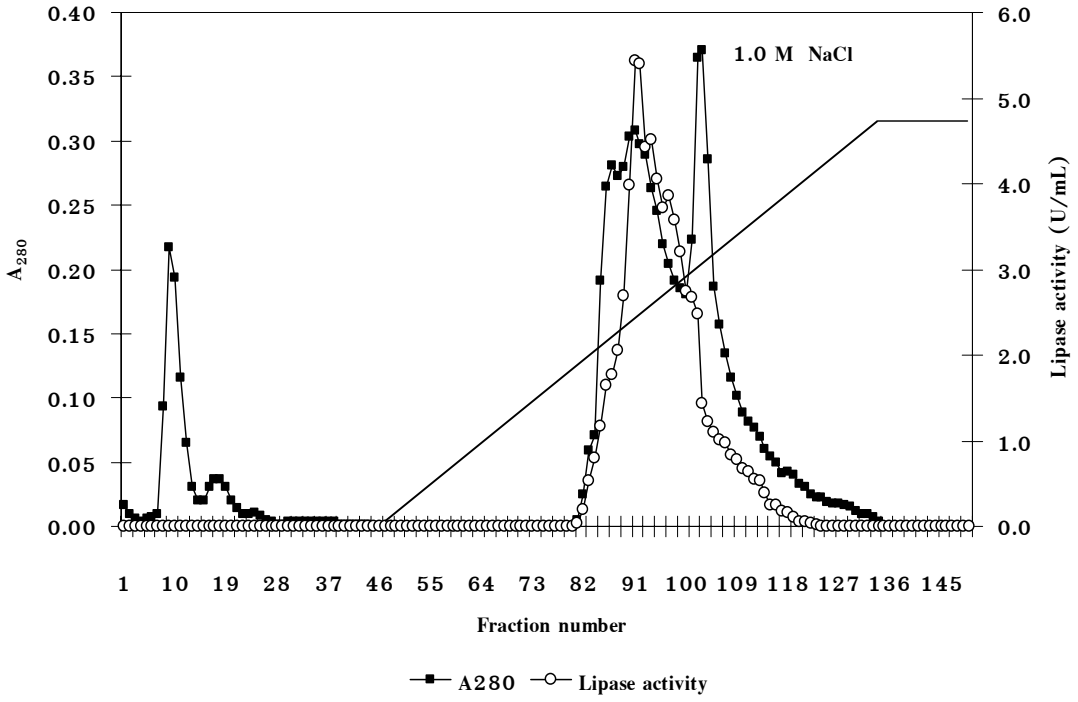


Figure 62. Purification profile of lipase from *Burkholderia multivorans* PSU-AH130 on DEAE-Toyopearl anion exchange chromatography.
(Absorbance at A_{280} (■), lipase activity (○) and NaCl concentration (—). Column (1 x 30 cm) was equilibrated with 50 mM Tris-HCl buffer pH 8.5. The lipase was eluted with a linear gradient of 1.0 M NaCl at a flow rate of 0.25 mL/min.)

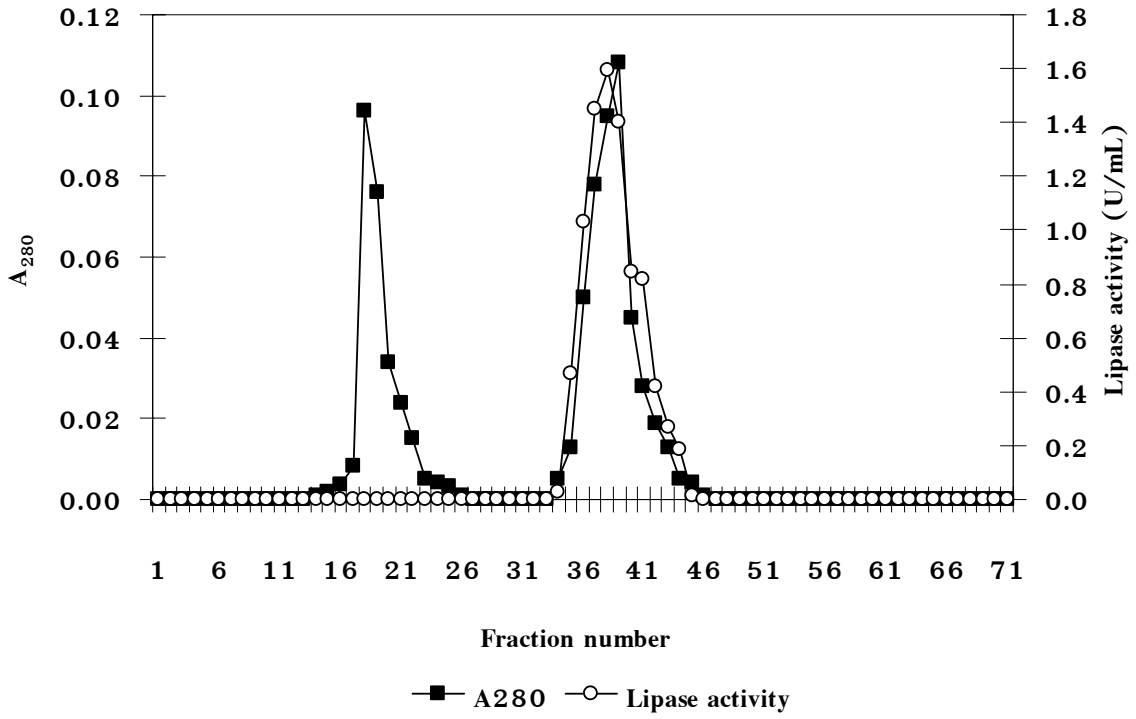


Figure 63. Purification profile of lipase from *Burkholderia multivorans* PSU-AH130 on Sephadex G-150 gel filtration chromatography.
(Absorbance at A_{280} (■) and lipase activity (○). Column (3 x 130 cm) was equilibrated with 50 mM Tris-HCl buffer pH 7.5 containing 0.15 M NaCl. The lipase was eluted with same buffer at a flow rate of 0.20 mL/min.)

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The molecular mass of purified lipase from *B. multivorans* PSU-AH130 was determined by SDS-PAGE. (Figure 65) showed the single band with a relative molecular weight of 57.8 kDa. The molecular masses of other extracellular lipases were reported, 54 kDa for *P. aeruginosa* (Karadzic *et al*., 2006), 30 kDa for *B. multivorans* (Gupta et al., 2005), 45 kDa for *Bacillus* sp. J33 (Nawani and Kaur, 2000), 60 kDa for *Antrodia cinnamomea* (Shu *et al*., 2006), 143 kDa for *P. psuedomallei* 12Sm (Kanwar *et al*., 2002) and 203 kDa for *B. coagulans* MTCC-6375 (Kanwar *et al*., 2006).

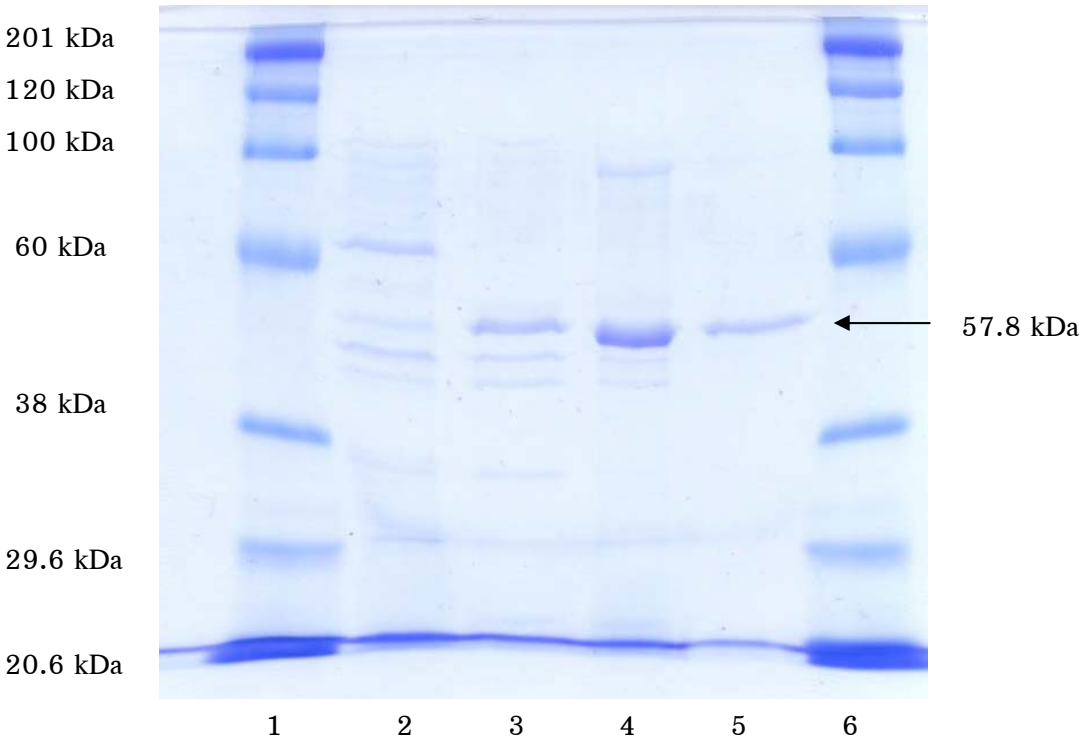

Figure 64. Sodium dodecyl sulfate polyacrylamide gel electrophoresis pattern of purified lipase. (SDS-PAGE was carried out in 15% gel and the protein was stained with coomassie blue R 250. Lane 1, protein markers; lane 2, crude enzyme; lane 3, ammonium sulphate precipitate; lane 4, DEAE-Toyopearl pool; lane 5, Sephadex G-150 pool; lane 6, protein markers)

6.2 Characterization of extracellular lipase from *Burkholderia multivorans* PSU-AH130

6.2.1 Effect of pH and temperature

The purified lipase from *B. multivorans* PSU-AH130 was assayed using *p*NPP as a substrate in a wide pH range (4.0-11.0). The optimum activity was achieved at pH 8.0 (Figure 65). The enzyme had low activity at acidic pH (pH 4.0-6.0) and basic pH (10.0-11.0). This lipase showed good stability for 2 h in the broad range of pH from 7.0 to 9.0 but the stability decreased rapidly at pH lower than 6.0 and above 9.0. The purified lipase showed optimum activity toward *p*NPP at pH 8.0 (Figure 65). The extracellular lipase from *Cryptococcus* sp. S-2 showed the optimum pH at 7.0 and stability at pH range of 5.0-9.0 at 40°C for 6h (Kamini *et al*., 2000). Moreover, Karadzic *et al*. (2006) reported that extracellular lipase from *Pseudomonas aeruginosa* had optimum pH 11.0 and stability between 4.0-11.5 for 3 h at 30°C.

The rate of a reaction approximately doubles for each 10°C increase in temperature. Assuming the enzyme is stable at elevated temperatures, the productivity of the reaction can be enhanced greatly by operating at a relatively high temperature. Consequently, thermal stability is a desirable characteristic of lipases (Janssen *et al*., 1994). The optimum temperature for lipase of *B. multivorans* PSU-AH130 was 55°C (Figure 66) and the enzyme was stable at this temperature with residual activity of 98% for 2 h. Half life of the enzyme was more than 2 h at 60°C but after incubation at 75°C for 2 h, it was completely inactivated. This lipase showed good thermostability compared with other mesophilic bacterial lipases e.g. alkaline lipase from *P. aeruginosa* showed half-life less than 10 min at 70°C (Karadzic *et al*., 2006), lipase from *Bacillus coagulans* MTCC-6375 was stable at 55°C for 20 min and lipase from *Cryptococcus* sp. S-2 retained activity about 71% at 60°C for 30 min.

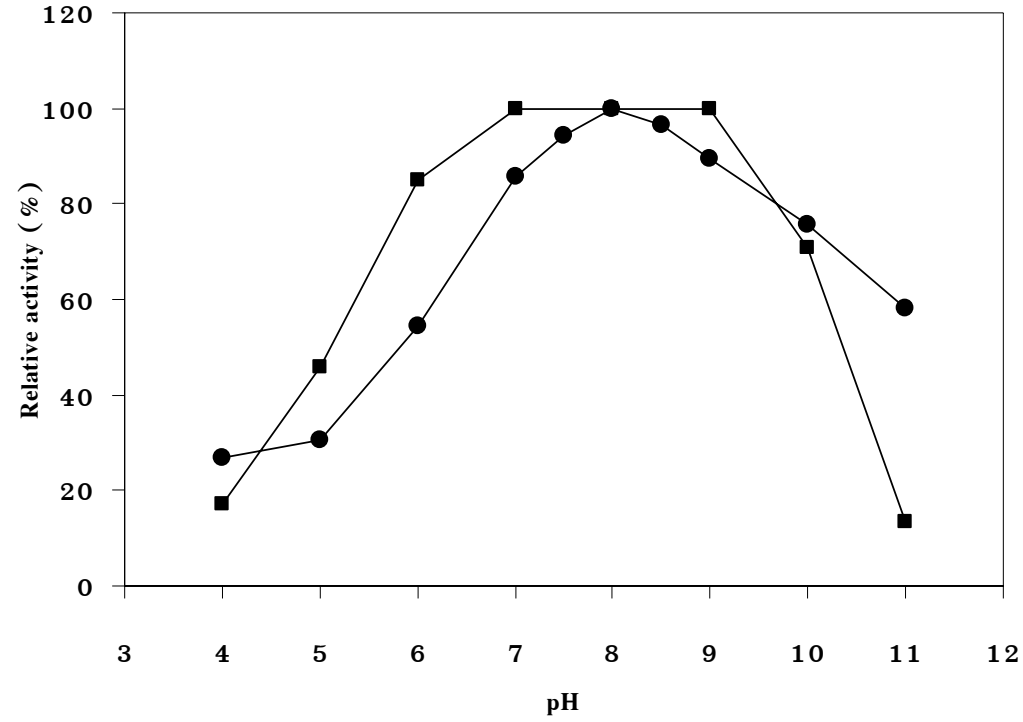

Figure 65. Effect of pH on activity (●) and stability (■) of lipase from *Burkholderia multivorans* PSU-AH130 lipase.

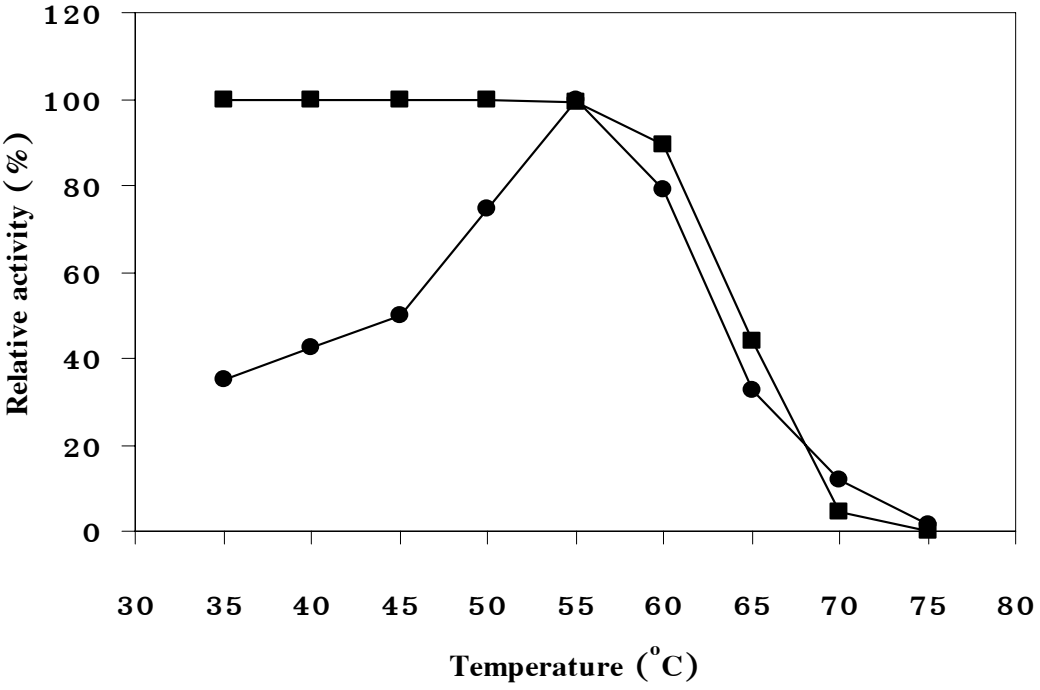

Figure 66. Effect of temperature on activity (●) and stability (■) of lipase from *Burkholderia multivorans* PSU-AH130 lipase.

6.2.2 Kinetic parameters

The values of K_m and V_{max} of the purified lipase from *B. multivorans* PSU-AH130 using pNPP as substrate calculated from the Lineweaver-Burk plot were 4.0 mM and 4,000 U/mg, respectively (Figure 67). Most of industrially used enzymes have *Km* in the range of 10^{-1} to 10^{-5} M when acting on biotechnologically important substrates (Fullbrook, 1996). Sharma *et al*. (2002) reported that the K_m and V_{max} of purified lipase from *Bacillus* sp. RSJ-1 toward *p*NPB were 2.2 mg/mL and 1,428 U/mL, respectively. The high value of V_{max} and the low value of K_m indicate that enzyme shows good activity toward the substrate used.

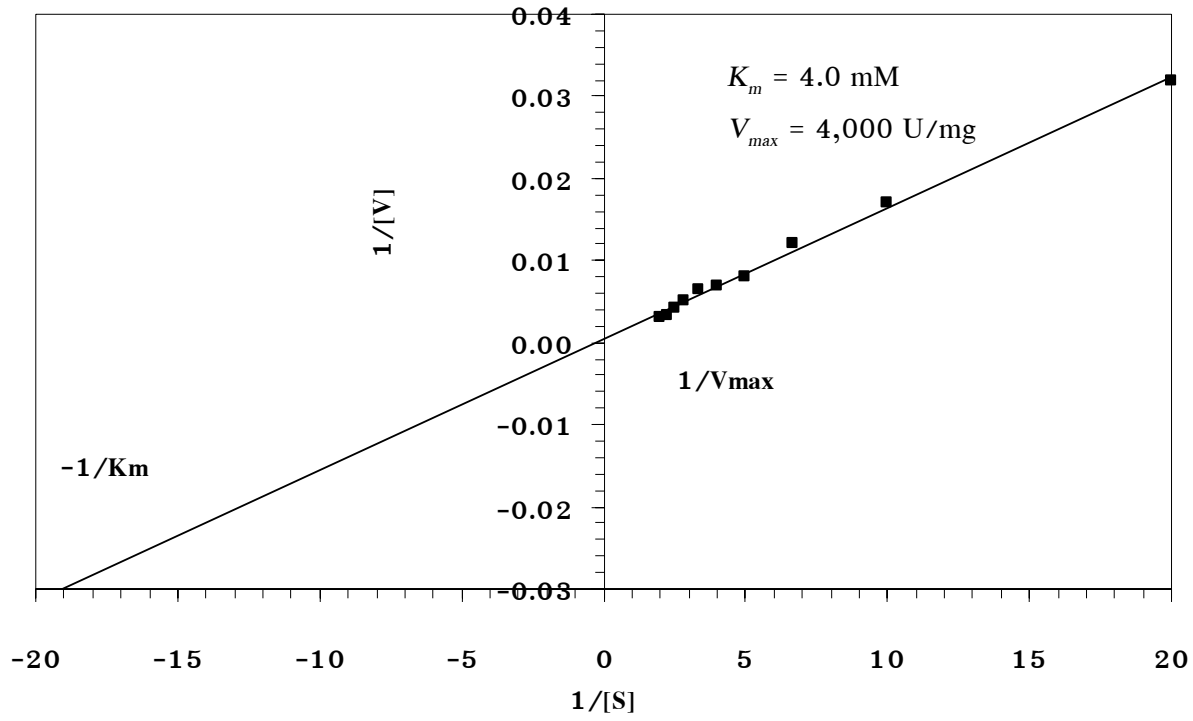

Figure 67. Lineweaver-Burk plot of the purified lipase from *Burkholderia multivorans* PSU-AH130 using *p*NPP as substrate.

6.2.3 Substrate specificity

The enzyme specificity was studied with *p*-nitrophenyl esters of various acyl chain lengths (C_2-C_{18}) (Figure 68). Among the substrates tested, *p*NPC (C_8) was the best substrate for the lipase from *B. multivorans* PSU-AH130, *p*NPB (C_4), *p*NPC (C_6) and *p*NPL (C_{12}) were also good substrates, whereas very short chain ester, *p*NPA (C_2) and long chain ester, *p*NPS (C_{18}) were poor substrates. So the *B. multivorans* PSU-AH130 lipase preferred medium chain fatty acid more than short and long chain fatty acid. The similar specificity has been reported for lipase from *Bacillus coaglulans* MTCC-6375 (Kanwar *et al*., 2006) and lipase from *B. thermoleovorans* CCR11 (Castro-Ochoa *et al*., 2005).

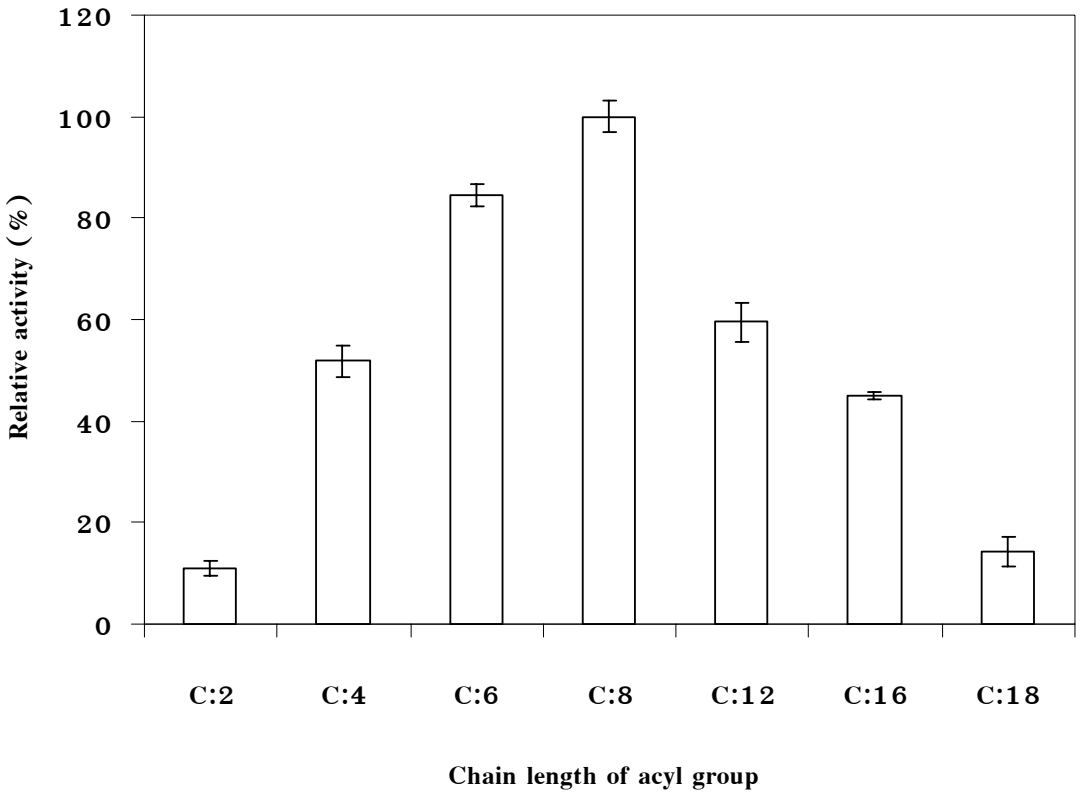

Figure 68. Effect of chain length of acyl group on the activity of the purified lipase from *Burkholderia multivorans* PSU-AH130.

6.3.4 Effect of metal ions and other reagents

Various metal ions and other reagents were studied for their effects on the activity of the purified lipase from *B. multivorans* PSU-AH130 at concentration 1.0 and 10.0 mM at 50°C for 90 min (Table 29). The ions of Ca^{2+} were found to stimulate lipase activity as well as the lipase produced from *Pseudomonas putida* 3SK (Lee and Rhee, 1993) and *P. aeruginosa* MB5001 (Chartrain *et al*., 1993) while the lipase from *P. aeruginosa* was inhibited by Ca^{2+} (Karadzic *et al*., 2006). The lipase from *B. multivorans* PSU-AH130 was sensitive to 10 mM of Ba^{2+}, Cu^{2+}, Fe^{3+}, Hg^{2+} and Mn^{2+} ,the remained lipase activity less than 50% was obtained. Whereas, strong effect of metal ion was observed with 10 mM Co^{2+} and Zn^{2+} and only 17 and 16 % activity were remained after 90 min at 50°C. Similar results were reported that lipase from *P. putida* 3SK was inhibited by Co^{2+} with 87% inhibition (Lee and Rhee, 1993), lipase from *P. aeruginosa* was inhibited by Zn^{2+} and Cu^{2+} with 85 and 92% inhibition, respectively (Karadzic *et al*., 2006) and lipase from *P. aeruginosa* MB5001 was strongly inhibited by Zn^{2+} with 94% inhibition (Chartrain *et al*., 1993).

The chelating agent, ethylenediamine tetraacetic acid (EDTA) and sodium citrate (10 mM) inhibited the enzyme activity, the remained activity were 6% and 12%, respectively. The result indicated that this lipase was a metalloenzyme. Similar to the results from Lee and Rhee (1993) that the lipase from *P. putida* 3SK was strongly inhibited by EDTA with only 8% remained activity. These results were contrast to the alkaline lipase from *P. aeruginosa* that 5 mM EDTA enhanced lipase activity with 30% higher than untreated enzyme (Karadzic *et al*., 2006). The reducing agent, β-mecaptoethanol and ascorbic acid did not affect on the lipase activity of *B. multivorans* PSU-AH130 lipase as well as the lipase A from *Acinetobacte*r sp. RAG-1 (Snellman *et al*., 2002). In contrast, this enzyme was inhibited with oxidizing agent (10 mM ammonium persulphate), only 4% remained activity was obtained (Table 29).

The serine inhibitor, PMSF did not effect on the lipase activity even if at the concentration of 10 mM. Although all known lipases have serine in their active site but some lipases are resistant to inactivation by serine inhibitors e.g. lipase from *Steptomyces rimosus* (Abramic *et al*., 1999), *Geobacillus* sp. *TW1* lipase (Lee and Zhang, 2005), *Mucor hiemalis* f. *hiemalis* (Hiol *et al*., 1999), *Acinetobacte*r sp. RAG-1 lipase A (Snellman *et al*., 2002) and *P. aeruginosa* lipase (Karadzic *et al*., 2006), whereas the lipase from *Aspergillus carneus* (Saxena *et al*., 2003) was completely inactivated by PMSF. The reagent masking SH-group, *p*-hydroxymercuribenzoate (*p*CMB) did not inhibit lipase activity, suggested that free thiol group might be not essential for *B. multivorans* PSU-AH130 lipase activity.

Table 28. Effect of metal ions and other reagents on the hydrolytic activity of the purified lipase from *Burkholderia multivorans* PSU-AH130.

Effecter molecule	Relative activity (%)[a]	
	1.0 mM	10.0 mM
Metal salts		
KCl	100	107
NaCl	100	102
$BaCl_2$	62	36
$CaCl_2$	111	123
$MgCl_2$	96	93
$CoCl_2$	29	17
$CuSO_4$	65	23
$FeSO_4$	57	48
$HgCl_2$	37	31
$MnCl_2$	29	26
$NiSO_4$	67	52
$ZnCl_2$	18	16
Reducing agents		
β-mecaptoethanol	95	92
Ascorbic acid	100	95
Oxidizing agents		
Ammonium persulfate	73	4
KI	106	103
Chelating agents		
EDTA	7	6
Sodium citrate	63	12
Inhibitors		
PMSF	99	98
*p*CMB	96	74

[a]The activity was expressed as a percentage of the activity of untreated purified lipase

6.3.5 Effect of organic solvents

The stability of purified lipase from *B. multivorans* PSU-AH130 was studied in both water-miscible and immiscible organic solvents. This lipase was tolerant to some organic solvents (Table 30) e.g. dimethylsulfoxide (DMSO), chloroform, hexane and iso-ocatne. However, ethanol, acetone, methanol, pyridine and dimethylformamide (DMF) caused significantly deactivation, less than 40% of initial activity was remained, while dioxane resulted completely loss of the lipase activity. The extracellular lipase from *Acinetobacter sp.* RAG-1 (LipA) was completely inactivated by pyridine at a concentration of 30% (v/v) in 1 h (Snellman *et al.*, 2002). The 90 % ethanol and butanol were reported to inactivate lipase from *Aspergillus carneus* (Saxena *et al.*, 2003) and lipase from *P. aeruginosa* (Karadzic *et al.*, 2006). Acetone and benzene at concentration 10% (v/v) inhibited the lipase activity from *Cryptococcus* sp. S-2 with remained activity of 9 and 0 %, respectively (Kamini *et al.*, 2000).

Table 29. Effect of various organic solvents on the hydrolytic activity of the purified lipase from *Burkholderia multivorans* PSU-AH130.

Organic solvent	Relative activity (%)
Tris-HCl buffer pH 7.5 (Control)	100
Dimethylsulfoxide (DMSO)	77
Methanol	21
Dimethylformamide(DMF)	39
Acetronitrile	42
Dioxane	0
95% Ethanol	33
Acetone	21
Pyridine	11
*t*ert-Butanol	51
2-Methyl-2-butanol	56
Chloroform	84
Hexane	90
iso-Octane	64