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Isolation and Characterization of *Pseudomonas* sp. KLB1 Lipase from High Fat Wastewater

Orapin Bhumibhamon, Jantana Jinda and Suptawee Fungthong

ABSTRACT

Six isolates of lipase-producing micro-organisms were screened from two types of wastewater. The bacterial isolates were KLB1, KLB2, KLB3 and isolated yeasts were KLY1, KLY2 and KLY3. The isolate showed highest lipase activity was KLB1 which later was identified as *Pseudomonas* sp. Lipase of *Pseudomonas* sp. KLB1 was found to be an inducible enzyme with palmolein. The model for lipase production was growth associate pattern. As regards to the physicochemical properties, the *Pseudomonas* sp. KLB1 lipase had maximal activity at 50°C and pH 9. For its stability, even though this enzyme showed the maximal stability at pH 7.0 and 37°C, its stability was increased when incubated at pH 8.0-10 and 37, 50, 60, and 70°C. The residue activity was 76% and 76.23% at pH 10, 70°C and pH 9, 37°C. However, the lipase showed two pH stability ranges that possible indicated two types of lipases were formed in *Pseudomonas* sp. KLB1. The lipase was activated by Ca^{2+}, K^{+},and Na^{+}, $(NH_4)_2S_2O_3$ and ascorbic acid but inhibited by Zn^{2+}, Mn^{2+}, Co^{2+}, KI and EDTA. The enzyme is also more specific on the medium and long chain triacylglycerol of vegetable oil than of animal fat. The Km and Vmax of tributyrin hydrolysis were 110.9 mM, and 2.45 mM s^{-1}, respectively whereas these kinetic parameters from palmplein hydrolysis were 1,188.8 mM, and 5.25 mM s^{-1}, respectively.

Key words: characterization, isolation, lipase, *Pseudomonas* sp. KLB1

INTRODUCTION

Lipases (Triacylglycerol acylhydrolases, EC 3.1.1.3) are enzymes which play a role as catalysts for the triacylglycerol hydrolysis reaction in the high water activity content system and for the transesterification reaction in the low water activity content system. They are widely applied in variety of industries, for example, pharmaceutical, cosmetic, food, detergent, paper, and environment (Ashok *et al.,* 1999 and Rohit *et al.,* 2001). Extracellular lipases have been produced from microorganisms, such as fungi, yeast, and bacteria beside from plants, and animals. Commercial lipases from *Pseudomonas* genus are produced from *Burkholderia cepacia* (*Pseudomonas cepacia*), *Pseudomonas alcaligenes, Pseudomonas mendocina* (Karl-Erich and Reetz ,1998) Chigusa *et al.* (1996) isolated lipase-producin yeasts from Soya bean oil refining plant while Cappe *et al.* (1994) isolated *Acinetobacter* sp. which could produce lipase from the activated sludge waste treatment plant. These lipases express different physicochemical properties that depend on metal ions, substrate, pH and temperature (Birute *et al*., 2002 ;Xiu-Gong *et al.*, 2000 and Huan *et al.*, 1999). In the present study, the isolation of lipase producing microorganisms and produced lipase

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characterization were investigated.

MATERIALS AND METHODS

Isolation and screening of lipase-producing microorganisms

1. Enrichment of lipase-producing microorganisms

Double layer technique was performed for screening and isolation of lipase-producing microorganisms (Jacques and Fred, 1996). Two loops of wastewaters from palm oil refining plant and fish canning factory were put into 5 ml of sterilized distilled water. Then 0.1 ml of the suspensions were transfered to 5 ml of the selective medium which composed of 0.5% $(NH_4)_2SO_4$, 0.05% K_2HPO_4, 0.03% $MgSO_4.7H_2O$, 2 ml of Olive oil, and pH 7.0. They were then incubated on shaker incubator with controlled temperature at 50°C, 100 rpm until getting turbidity broth. This step was done rountenely in order to maintain the microorganisms.

The microbial suspensions were diluted and 0.2 ml of them was poured into the melted selective medium with added 2% agar. The spread plate technique was used in this step. The solid medium was covered with the selective medium that was composed of 1.5% agar and 0.5%(v/v) olive oil after being cool. They were incubated at 30-35°C for 24 h. A colony of microorganism which had clear zone around suggested that it can produce an extracellular lipase, this step was repeated until get a single colony (3 times) after which it was picked up to grow on nutrient agar slant.

3. Screening of effective the lipase-producing microorganisms

The lipase-producing efficiency of the isolated microorganisms were evaluated with diffusion technique described by Cappe *et al*.,1994. Two hundred microliter of isolated microorganisms suspension were spotted on the emulsion tributyrin agar then incubated at 30°C for 24-48 h. The diameter ratio of clear zone and colony was measured.

The strains which showed the diameter ratio of at least 3 isolates were studied for their lipase-producing capacity in liquid medium. The 5% of selected strains inoculum were added to the 250 ml of Erlenmeyer flask containing 50 ml of the selected medium with 0.5%(w/v) $CaCO_3$ and pH 7.0, then were grown in the shaker incubator at 100 rpm, 30°C for 48 h. The cell was separated by centrifugation at 8000 rpm and 4°C for 10 min. The supernatants were collected and used to determine the lipase activity. The isolation expressed the highest activity was selected, for further studied.

4. Identification of lipase-producing microorganism

The isolation selected was identified by Department of medical Science, Ministry of Public Health (2001).

5. Study lipase production model

The lipase was produced by growing 5% of *Pseudomonas sp.* KLB1 in 2.0 Liter fermentor (NEW BRUNSWICK SCIENTIFIC, Model Bio Flo 2000 Ediso NJ USA) which contained 1.5 Liter of lipase producing medium with and without 1%(v/v) of palmoelin as carbon and energy sources. The lipase producing was carried out at 37°C, 250 rpm, air flow rate 1 vvm for 72 h. The pH was controlled at 7.0 with 4 N NaOH and 1 N Phosphoric acid throughout the operating time. The cultures were collected every hour. The lipase activity of the culture broth supernatants (obtained by centrifugation at 8000 rpm at 4°C for 10 min) was evaluated and the growth curve of cell was studied by total plate count on the palmolein emulsion agar and incubated at 35°C for 24 h.

5. Lipase assay

The method used by Yamada *et al.* (1962) was modified and used to determine lipase activity. Olive oil was replaced with palmolein as substrate. The substrate was treated with 45% of 4%Polyvinyl alcohol, 45% of distilled water, and 10% of

palmolein.One ml of crude lipase was poured into the tube containing 4 ml of substrate and 5 ml of 0.1 M glycine-NaOH buffer pH 9.0. The reaction was carried out at 37°C, 250 rpm for 1 hr. One unit of lipase activity was defined as the amount of enzyme required to release 1 μmol of palmitic acid per ml per hour. The palmitic acid content was measured by titration with 0.01 N KOH.

For the experiments of pH optimum, the lipase activities were assayed at pH 6.0-10.0.

Physicochemical and kinetics properties of lipase

1. Lipase preparation

The lipase production was performed by growing 5% of *Pseudomonas* sp. KLB1 inoculum in 2.0 Litre fermenter (NEW BRUNSWICK SCIENTIFIC, Model Bio Flo 2000 Ediso NJ USA) containing lipase producing mediun with 1% (v/v) of palmolein. The operation was controlled at pH 7.0, 37°C, 250 rpm, air flow rate 1 vvm for 24 hr. The pH was adjusted at 7.0 with 4 N NaOH and 1 N Phosphoric acid throughtout the operatng time. The supernatant containing crude lipase was separated by centrifugation at 8,000 rpm at 4°C for 10 min. The obtained supernatant were also analyzed for lipase activity. The supernatant containing crude lipase was separated by centrifugation at 8,000 rpm, 4°C for 10 min and was, then, preserved as lyophilized form for next using.

The lyophilized lipase was dissolved with buffer solution in the same volume of supernatant before lyophilization.

2. pH and temperature optimum

The enzyme activity assay was carried out at 37, 40, 50 and 60°C in 0.1 M potassium phosphate buffer pH 6, 7, and 8 and 0.1 M glycine-NaOH buffer pH 9 and 10.

3. pH and temperature stability

The enzyme was incubated in 0.1 M potassium phosphate buffer pH 6, 8, 8.5 and 0.1 M glycine-NaOH buffer pH 9, 9.5, and 10 at 40, 50, and 60°C for 1 hr before determining activity. The controls were operated in buffer pH 76, 7,8,9,and 10 at 370 C.

4. Effect of metal ions and some chemicals on lipase activity

The 1 mM chloride salt of Ca^{2+}, Zn^{2+}, K^{+}, Mn^{2+}, Na^{+}, and Co^{2+} were poured into the enzyme dissolved in glycine-NaOH buffer pH 9.0 and incubated at ambient temperature for 4 hr before its activity was determined. The oxidizing agent (KI, $(NH_4)_2S_2O_3$), reducing agent (ascorbic acid) and chelating agent (EDTA) was also studied.

5. Substrate specificity

The four groups of oil used as substrate were (1) tributyrin (short chain length fatty acid, lower 8 carbon atoms), (2) soybean oil, olive oil and palmolein (medium and long chain length fatty acid, 8-12 and higher 12 carbon atoms, respectively) (3) fish oil (polyunsaturated fatty acid) and (4) butter fat (saturated triacylglycerol).

6. Kinetics properties

The kinetics properties of Km and Vmax were carried out by using tributyrin and palmolein as a substrate

6.1 Tributyrin

The hydrolysis of tributyrin emulsion (0.2 – 1.5 M) was catalyzed by 30.54 U/ml of the enzyme at 37°C, 250 rpm for 1 hr. The initial velocity was evaluated Km and Vmax were read from Lineweaver-Burk Plots (Piszkiewiez, 1977).

6.2 Palmolein

The palmolein emulsion was prepared and hydrolyzed following the procedure. The average molecular weight of palmolein was deterimined from below equation which used by White *et al*.(1973).

$$\text{average molecular weight} = \frac{(3 \times 56 \times 1000)}{\text{Saponification number}}$$

RESULTS AND DISCUSSION

Isolation of lipase-producing microorganisms

Six isolates grown in the selective medium

were found to produce lipase which were identified as bacteria: KLB1, KLB2, KLB3 and yeasts: KLY1, KLY2 and KLY3. Their growth showed that they can use olive oil as a carbon source and showed the lipase-producing feasibility.

Evaluation of the lipase-producing efficiency, based on the clear zone around colony, indicated that all of them could produce lipase enzyme. The result of KLB1 was outstanding (Figure1). When their produced lipases were applied on the emulsion tributyrin agar and incubated at 30°C for 24 hr. Their abilities of lipase producing were evaluated with the diameter ratio of clear zone and colony (Table 1).

The diameter ratio of clear zone and colony of KLB2 is the highest (5.00) whereas of KLB1 and KLY2 are 4.00 and 3.00, respectively (Table 1). According to their lipase activity produced in the liquid medium by shaking flask, the lipase produced by KLB1 showed the higher activity than KLY1 (6.59 Unit/ml) and KLB2 (Figure 2). The KLB1 lipase activity was 7.11 Unit/ml whereas the KLB2 lipase was 4.67 Unit/ml. This results were compared with that of lipase-producing microorganisms (KUL8 and KUL39) which were selected in the previous study (Bhumibhamon *et al*., 2002) and found that the KLB1 expressed the higher activity than other (Figure 2).

It was found that the lipase enzyme of the KLB1 is more specific with the olive oil in the liquid medium than the tributyrin in the emulsion



Figure 1 The clear zone displayed by lipase around the KLB1 colony on the emulsion tributyrin agar at 30°C for 24 h.

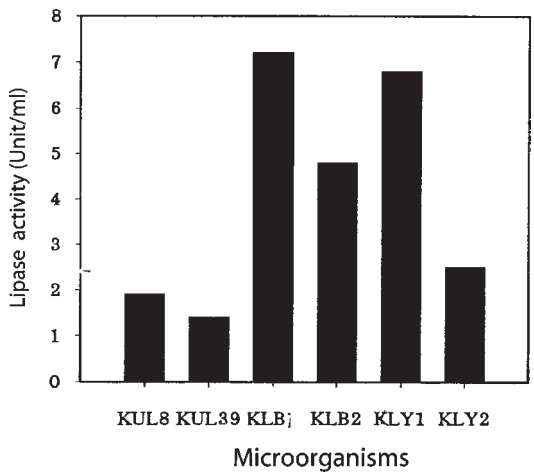


Figure 2 The lipase activity of the selected microorganisms produced in the liquid medium.

Table 1 The diameter ratio of clear zone and colony of the isolated microorganisms.

Microorganisms	Diameter ratio of clear zone and colony	Wastewater sources
KLB1	4.00	Palm refining plant
KLB2	5.00	Palm refining plant
KLB3	1.22	Fish canning plant
KLY1	2.00	Fish canning plant
KLY2	3.00	Fish canning plant
KLY3	1.67	Fish canning plant

agar. The lipase from *Pseudomonas aeruginosa* YS-7 was reported to be more specific on oleic acid than triolein (Shabtai and Daya-Mishne, 1992).

Lipase production model

The KLB1 strain secreted lipase when cultured in the medium with palmolein as carbon source. According to the growth and activity curve of KLB1 strain, the activity started to be detected in the early log phase (4 h) and was found throughout the incubation time. The relation of the lipase production and KLB1 growth obeyed to the *Growth Associated* pattern. The activity showed maximum value of 72.1 U/ml at final log phase about 25 hr of culture but did not exhibit in the medium without palmolein (Figure 3).

The results show that the lipase of KLB1 strain is a member of inducible enzymes. These type of lipase were also found in *Pseudomonas aeroginosa* YS-7 (Shabtai and Daya-Mishne, 1992), and *Acenitobactor calcoceticus* BD413 (Cordenons *et al*., 1996).

With respect to the lipase activity produced, the KLB1 was identified and found that it is a member in *Pseudomonas* genus by Department of Medical Science, Ministry of Public Health (2001). Therefore, the KLB1 was classified as *Pseudomonas* sp. KLB1 and the physicochemical and kinetics properties of this enzyme were further studied.

Physicochemical and kinetics properties of lipase

1. pH and temperature optimum

The extracellular lipase produced from *Pseudomonas sp*. KLB1 was optimum at high pH and temperature. Its pH and temperature optimum were 9 and 50°C, respectively. It expressed the highest activity (69.87 U/ml) followed by the activity at pH 8 and 60°C (52 U/ml) (Figure 4). The lowest activity for almost all pH was at 40°C . The results confirmed that the lipase from *Pseudomonas sp*. KLB1 was classified as thermophilic alkaline lipase group, which was also found in lipase from *Pseudomonas mendocina* (pH 9.5 and 52°C) (Birute *et al*, 2002) and from *Pseudomonas* sp. (pH 7.0-9.0 and 45-60°C) (Huan *et al.*, 1999).

2. pH and temperature stability

The activity of lipase incubated at pH 7 and 37°C for 1 hr showed the highest activity (100%). However, the residue activities of enzyme showed the highest tolerant at high temperature (60, and

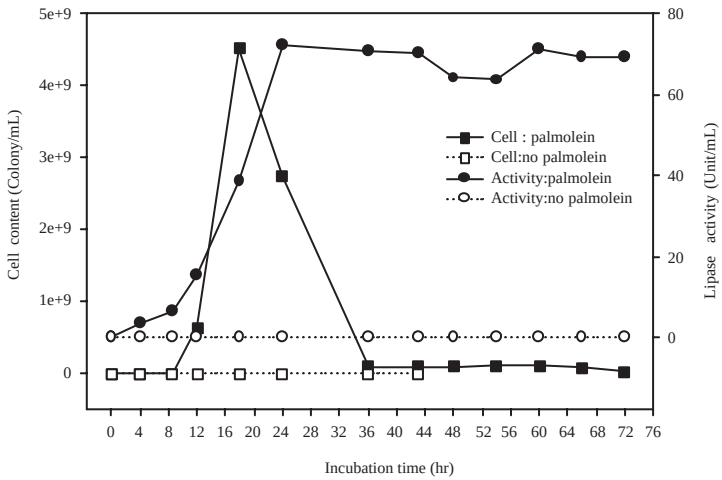


Figure 3 Growth and lipase activity of KLB1 strain cultured in the medium with and without 1% of palmolein for 72 h.

70°C) were 76.23 and 76% in buffer pH 7 and 10, respectively. (Figure 5). Generally, the lipases from bacteria were stable up to 70°C (Kulkarni and Gadre, 1999). Figure 5, the results show that activities of all lipases incubated in buffer pH 7-8 were decreased by higher temperature. These were opposite results of lipases incubated in alkaline buffer (higher than 8) showed increasing of residue activities. These show that the enzyme activity was activated in alkaline pH. When the residue activity of lipases incubated in the same pH of buffer at variety of temperatures and the ones incubated at the temperature in variety of pH were compared, these were found that the activity of lipase incubated in the same pH buffer at different temperature were less decreased others. In this case, the enzyme activity was deactivated by upon exposure to more pH than temperature.

Although the activity of resulted enzyme was decreased by higher temperature, it could

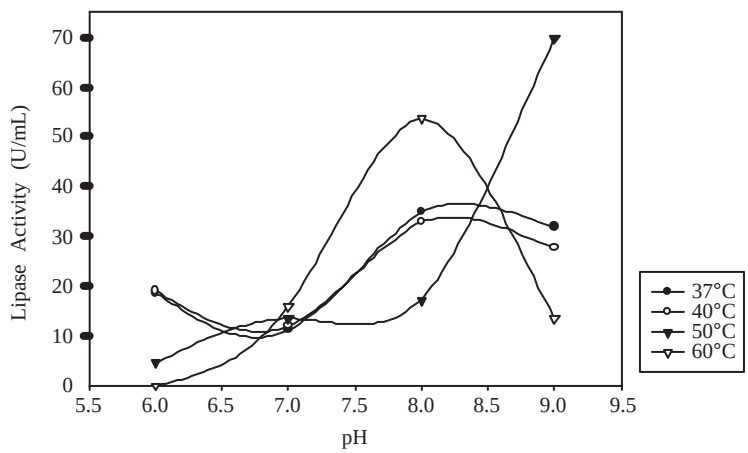


Figure 4 Effects of pH and temperature on *Pseudomonas* sp. KLB1 lipase activity.

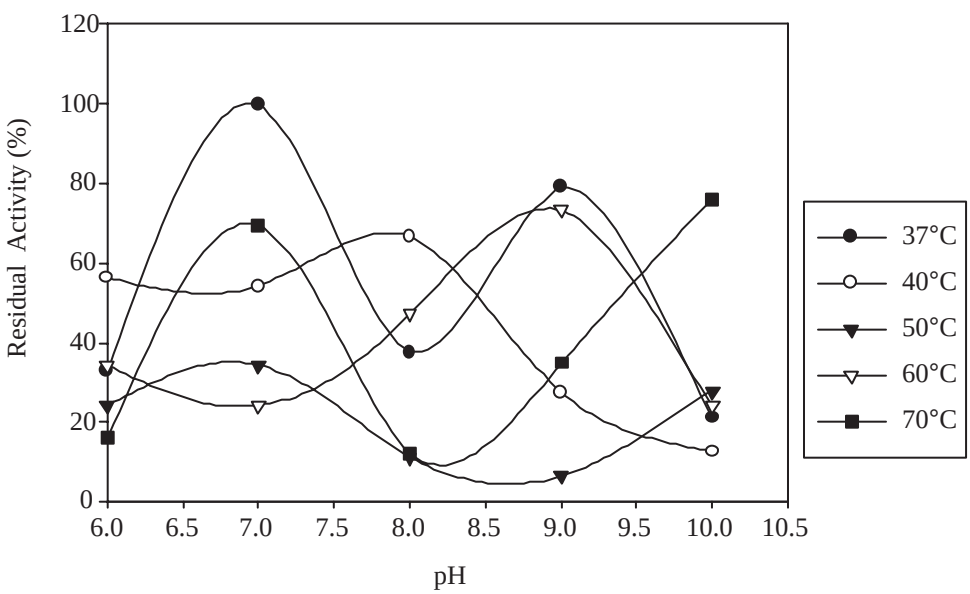


Figure 5 Effects of pH and temperature on the *Pseudomonas* sp. KLB1 lipase stability.

contain more 75% of residue activity in buffer pH 10 at 70°C. Klump *et al*. (1992) and Jaeger *et al*. (1994) described that thermal stability of enzyme is related to the content of hydrophobic amino acids in protein.

However, the results showed that this enzyme is stable in 2 pH and temperature ranges throughout pH 7-10 at 37-70°C. Hence, it is probably 2 types of lipases with different properties are found in *Pseudomonas* KLB1. In the same case, *Geotrichum candidum* lipase and *Candida rugosa* lipase were reported to have 2 isoenzymes (A and B) (Hernáiz *et al.*, 1999; Tomas and Otto, 1995) This will be studied further.

3. Effect of metal ions and some chemicals on lipase activity

The results indicated that Ca^{2+}, K^{+}, Na^{+} ions, ascorbic acid, and $(NH_4)_2S_2O_3$ activated the lipase activity by different degree (Table 2). These ions have been reported to play a role as lipase-cofactor (Huan *et al*., 1999 and Xiu-Gong *et al*. , 2000). Calcium ion , especially, is used in Ca^{2+} binding processing which impact to position specificity on active site (Birute *et al.*,2002). However, the enzyme was inhibited by Zn^{2+}, Mn^{2+}, Co^{2+}, KI and EDTA (Table 2)

4. Substrate specificity

The *Pseudomonas sp.* KLB1 lipase most efficiently catalysis the hydrolysis oftributyrin (288.89%) followed by fish oil, olive oil, soybean oil, and palmolein (Table 3). This showed that this enzyme specific on more medium and long chain fatty acid vegetable oil and polyunsaturated fatty acid (PUFA) oil than butterfat. Although, these fat structures are complex, vegetable oils are consisting of more unsaturated fatty acids which can be easily hydrolyzed (Rohit *et al*., 2001).

5. Kinetics parameters; Km, and Vmax

Km and Vmax of the *Pseudomonas* sp. KLB1 lipase that catalyzed tributyrin and palmolein hydrolysis obtaine from Lineweaver-Burk Plots (Figure 6 and 7). The tributyrin (monoene triacylglycerol) hydrolysis demonstrated that Km, and Vmax were 1,188.8 mM, and 5.25 $mM.s^{-1}$, respectively (Figure 6). Moreover, the polyene triacylglycerol (palmolein) showed Km, and Vmax

Table 2 Effect of metal ions on hydrolysis activity of *Pseudomonas* sp. KLB1 lipase.

Effector	Relative activity (%)
Non effector	100.00
Metal salts; $CoCl_2$	75.84
NaCl	112.12
$MnCl_2$	74.77
$ZnCl_2$	89.04
KCl	108.82
$CaCl_2$	151.59
Oxidizing agents;	
KI	
19.79	
$(NH_4)_2S_2O_3$	145.09
Reducing agents	
Ascorbic acid	138.50
Chelating agent	
EDTA	95.63

Table 3 Effect of substrate on hydrolysis activity of *Pseudomonas sp*. KLB1 lipase.

Substrate	Relative activity (%)*
Palm oil	100
Soybean oil	116.67
Olive oil	155.56
Fish oil	188.89
Butter fat	33.33
Tributyrin	288.89

• The data were average from triplicate experiments.

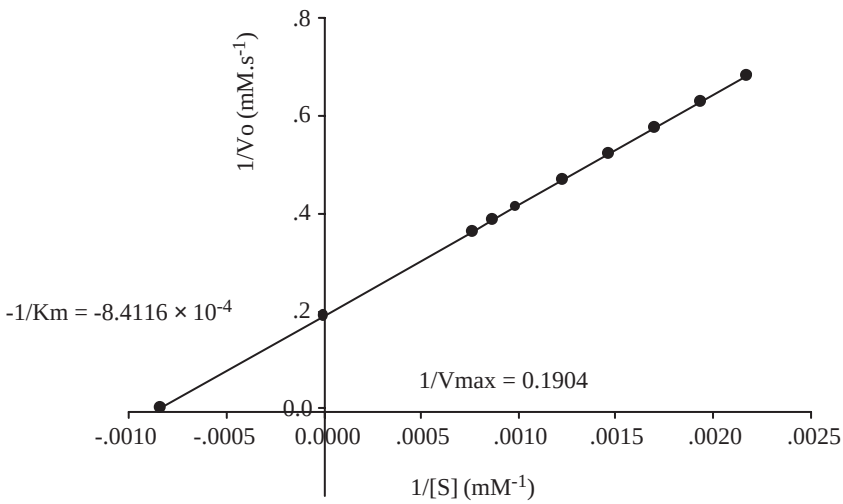


Figure 6 Lineweaver-Burk Plots of Tributyrin concentration for *Pseudomonas* sp. KLB1 Lipase.

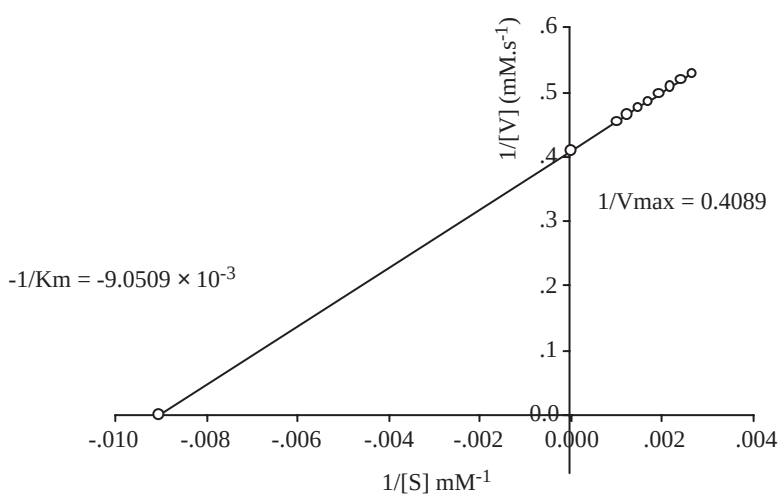


Figure 7 Lineweaver-Burk Plots of Palmolein concentration for *Pseudomonas* sp. KLB1 lipase.

of 110.9 mM, and 2.45 mM.s^{-1}, respectively (Figure 7). These Km values showed that *Pseudomonas sp*. KLB1 lipase prefered polyene triacylglycerol than monoene triacylglycerol. According to these results, the enzyme is specific on large structure triacylglycerol.

CONCLUSION

The KLB1 was isolated from refining palm oil plant wastewater. It produced lipase enzyme and was later identified as *Pseudomonas* sp. According to the pattern of the lipase producing and growth curve, it indicates to be a *Growth Associate* model. The enzyme was an inducible one. The characteristics of lipase showed a good activity at alkaline ranges with the optimal hydrolysis activity at pH 9 and temperature 50°C. However, it showed two ranges of pH stability were pH6.0-7.0 and 9-10 at 37, 50 and 70°C and most tolerant on pH 10 at 70°C that possible indicated two types of lipase. Although, this enzyme showed the most pH and temperature tolerant at pH 7 and 37°C, it could contain of ca. 76% residue activities at pH 9, 37°C and pH 10, 70°C, respectively. This enzyme activity was stimulated with chloride salts of Ca^{2+}, K^{+},and Na^{+}, $(NH_4)_2S_2O_3$ and ascorbic acid. Moreover, it was found to be specific not only on palmolein but also on olive oil, fish oil, and soybean oil. The enzyme had a higher affinity for palmolein (Km 110.9 mM) than for tributyrin (Km 1,188.8 mM) and had more hydrolysis efficiency for palmolein (Vmax 2.45 mM.s^{-1}) than for tributyrin (Vmax 5.25 mM.s^{-1}). These results indicated that this lipase more specifically hydrolyzed to the long chain fatty acid triacylglycerol than the short chain fatty acid triacylglycerol.

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