Sol-Gel of Rice Husk Ash: Entrapment of Alkaliphilic Lipase from *Pseudomonas* sp. KLB1

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ABSTRACT

Entrapment in sol-gel from rice husk ash was established by immobilization of alkali lipase produced by *Pseudomonas sp*. KLB1 lipase. The enzyme was strongly immobilized by entrapment in neutral sol- gel of rice husk ash. It was optimally active at 60°C and pH 9. Its activity was constant in the medium pH 9 under temperature 70°C for 1 hr. The immobilized lipase was activated by Ca^{2+}, K^{+}, Na^{+}, EDTA and ascorbic acid but inhibited by Fe^{2+}, Mn^{2+}, Co^{2+}, and KI. Both Ca^{2+},and Na^{+} were mainly found in rice husk ash and xerogel. The effective concentration of $CaCl_2$ and NaCl on the enzyme activation were 60 mM and 20mM, respectively. According to reusability, it was applied into the hydrolysis of p-nitrophenyl palmitate by 12 times which lost 50% activity. The Km, and Vmax of p-NPP hydrolysis catalyzed by the entrapped enzyme were 7.43×10^{-2} mM, and 1.94×10^{-5} mM s^{-1}, respectively, whilst by the free enzyme were 21.69 mM, and 3.90×10^{-3} mM s^{-1}, respectively.
Key work: entrapment, lipase, rice husk ash, *Pseudomonas* sp. KLB1, sol-gel

INTRODUCTION

Rice husks are a residue produced in significant quantities in Thailand. In some region, most of them are used as a waste which cause pollution and disposal problems while they can be used as a fuel. In some factories such as “Meang Cheng Rice Mill” in Si Sa Ket province and “Pathum Rice Mill” in Pathum Thani province, the rice husks are burnt under controlled conditions to conserve electrical energy and to utilize the ash as a building material that be export product to Europe (personal communication).

Rice husk ash (RHA) is rich in silica ca. 60%. Soluble silicates from rice husk ash are widely used in glass, ceramics, and cement (as a major component), phamaceuticals, cosmetics and detergents industries as a bonding and adhesive agent (Laxamana, 1982). Due to the fact that rice husk ash contains high silica content, it can be used as an economically viable material for silica gel and powder production (Kamath and Proctor, 1998).

Silica gel is a rigid 3 dimensional network of colloidal silica. It is classified as aquagel (pores are filled with water), xerogel (water in the pores is removed by evaporation) and aerogel (solvent in the pores is removed by supercritical extraction). The xerogels are mainly used as catalytic substrates, ultrafiltration and chromatography column packing materials because of their high porosity and surface area (Brinker and Scherer, 1990).

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In the present experiment, the immobilization techniques of entrapment by lipase from *Pseudomonas* sp. KLB1 in Sol-gel of Rice husk ash (RHA) were studied. The three immobilization patterns, adsorption, covalent adsorption and entrapment of lipase onto rice husk ash and derivative were studied.

MATERIALS AND METHODS

Materials

1. Chemicals

Rice husk was a sample from local rice mill industry (Pathum Thani, Thailand). Polyvinyl Alcohol was purchased from Carlo Erba (Val de ruil, France). Bovine Serum Albumin and Coomassie blue G 250 came from Sigma (St. Louis, USA). Ingridients for bacterial growing came from Difco (USA.). Acetone came from Merck (Darmstadt, Germany). Commercial 95% Ethanol and Palm olein oil was purchased from local market. All other chemicals were analytical grade.

2. Lipase preparation

The lipase was from *Pseudomonas* sp. KLB1 which isolated from wastewater of palm oil refining industry (Bhumibhamon *et al*., 2002). The 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). Medium consists of palmoelin 2% (v/v), Protease peptone 0.1%, $(NH_4)_2SO_4$ 0.5% K_2HPO_4 0.5% and $MgSO_4.7H_2O$ 0.1% . The system conditions were controlled at pH 7.0, 37°C, 250 rpm, air flow rate 1 vvm for 21 hr. The pH was adjusted at 7.0 with 4 N NaOH and 1 N Phosphoric acid throughtout the operating time. The supernatant containing crude lipase was separated by centrifugation at 8,000 rpm at 4°C for 10 min and was then concentrated by lyophilization (VIRTIS SENTRY^{TM} Freezemobile 5SL, The VIRTIS Company, INC, New York.) for 24 hr. The concentrated supernatant were also analyzed for lipase activity and total protein.

3. Preparation of rice husk ash (RHA)

Rice husk was burnt to release moisture until it became to be a black carbon on hotplate. Then, it was burnt in furnace (NEY 2-525 Series II, USA) at 600°C for 8 h. After that, the temperature was raised to 900°C and held on for 1 hr. The rice husk ash was preserved in dissector.

4. Preparation of xerogel and gel for entrapment

The xerogel of rice husk ash was produced by following the methods of Kalapathy *et al*., (2000).

5. Determination of mineral content

The amount of Ca, Fe, Mg, Na, and K in the rice husk ash and xerogel were determined with UNICAM 929 Atomic Absorption Spectrophotometer described by Payá *et al*. (2001).

Methods

1. Entrapment

According to the procedure used to extract silica from rice husk ash, the enzyme was put to together with the filtrate adjusted to pH 7. The silica gels formed would entrap the enzyme while was aging for 18 hr. Ten ml of n-hexane was added to gels and then were broken to make as a slurry. The slurry was centrifuged at 2500 rpm for 15 min. The immobilized enzyme was washed with 30 ml of DDW for 2-3 times. The clear supernatant was discarded and determined the protein content. The entrapped gel was dried by lyophilization for 24 hr.

2. Effect of metal ions and some chemicals on the enzyme activation

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

3. Effect of the Ca^{2+} and Na^{+} concentration on the enzyme activation

The different concentration of $CaCl_2$ and NaCl (0-100mM) was added into the hydrolysis reaction of palm oil emulsion performed at 50°C, pH 9.0 for 1 hr. The activity retention was pursued.

4. Protein determination

Determination of protein in the supernatant was performed based on Bradford's method (Robert and Lata, 1989). One ml of sample was mixed with 4 ml of Coomassie blue solution (which prepared by dissolving 100 mg of coomassie blue G 250 in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid. The solution was diluted to 800 ml with distilled water). The mixture was left for 5 min (not over 1 h) at ambient temperature. After incubation, absorbance at 596 nm was measured on a VS-VCS Shimadzu UV-120, Japan spectrophotometer. The 1 ml of distilled water was used as blank. Bovine Serum Albumin was used as standard.

5. Lipase activity assay

5.1 Emulsion hydrolysis assay

Modification of method used by Yamada *et al.* (1962) was used to routinely determine lipase activity. Olive oil was replaced with palmolein as a substrate. The substrate was treated with 45% of 4% Polyvinyl alcohol, 45% of distilled water, and 10% of palmolein. The palmolein emulsion (4 ml) was hydrolyzed with 1.0 ml enzyme solution in 5.0 ml of 0.1 M Glycine-NaOH buffer pH 9.0 (excepted an experiment of pH optimum that done at pH 6.0-10.0) at 37 C, 250 rpm for 1 hr. The palmitic acid released was neutralized by titration with 0.01 N KOH. One unit of lipase activity was defined as the amount of enzyme required to release 1 mmol of palmitic acid per ml per hour.

5.2 p- Nitrophenyl palmitate (pNPP) hydrolysis assay

For reusability and kinetics study of both free and immobilized lipase, the *p*-Nitrophenyl palmitate (pNPP) was used as a substrate. This method employed by Manuela *et al.* (1995). The activity was determinated as content of p-Nitrophenol released from hydrolysis reaction. One unit of lipase activity was defined as the amount of enzyme requried to release 1 µg of p-nitrophenol per ml per hour.

6. The degree of immobilization

The availability activities of the immobilized *Pseudomonas* sp. KLB1 Lipase (IML) were estimated as the activity retention (eq.1). Bear in mind, this method was compared with the degree of immobilization that was calculated by measuring the transformed protein in the supernatant as equation 2.

Activity retention on IML (%) =

$$\frac{\text{IML activity}}{\text{Free lipase activity}} \times 100 \qquad \text{..........(1)}$$

Degree of immobilization(%) =

$$\frac{(\text{Free lipase protein } \Phi \text{Residual protein})}{\text{Free lipase protein}} \times 100 \qquad \text{..........(2)}$$

7. Reusability of the immobilized lipase

The immobilized enzyme obtained was reused for 20 times hydrolysis reaction of the pNPP. After each cycle, the enzyme was washed with 10 ml of n-hexane 3-4 times, and dried under vacuum atmosphere before used again. The relative activity (eq. 3) was evaluated in the stability of enzyme.

Relative activity (%) =

$$\frac{\text{Enzyme activity of cycle}}{\text{Initial activity}} \times 100 \qquad \text{...........(3)}$$

8. Physicochemical and kinetics properties of immobilized lipase

8.1 Effect of pH and temperature on the enzyme activity

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.

8.2 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,and10 at 40, 50, and 60°C for 1 hr before determining activity. The control was operated at 50°C and pH 9.

8.3 Kinetics properties; Km, and Vmax *p*-Nitrophenylpalmitate (pNPP) substrates

The hydrolysis of *p*-Nitrophenylpalmitate (0.1 – 0.7 mM) was catalyzed by 1047 U/g of the immobilized enzyme and 298.31 U/g at 50°C, pH 9.0 and 250 rpm for 1 hr. The initial velocity was evaluated Km and Vmax were read from Lineweaver-Burk Plots.

RESULTS AND DISCUSSION

1. Entrapment

The *Pseudomonas* sp. KLB1 lipase was entrapped in a sol-gel matrix of rice husk ash. The extract formed gel at pH 7, 37°C for 18 hr. The load of enzyme (0 to 719 Unit) was investigated until excess protein content carried out in the supernatant. The results is showed on Figure 1.

From figure 1, the maximum enzyme content entrapped in sol-gel matrix was less than 719 unit per 100 cm^3 of sol-gel rice husk ash (10 g). At this content, the degree of immobilization was 97.82% and the residual protein in the supernatant was 0.0096 mg/g. The immobilized lipase obtained expressed dramagically higher activity (1449.09 U/g).

According to the final activity retention of the enzyme immobilized by covalent adsorption and entrapment, the results indicated the entrapment carried out higher efficiency. Many applications of enzyme entrapment was performed on the gelatin optimized with the precusor as fucntional groups (Noureddini *et al.*, 2002; Hsu *et al.*, 2000) whereas this methods did not used.

2. Effect of metal ions and some chemicals on the enzyme activation

To explained that why activity of the immobilized enzyme was dramatically increased, the content of metal ion and other chemical agent which effect on the enzyme activity in the support were applied into the hydrolysis reaction of the

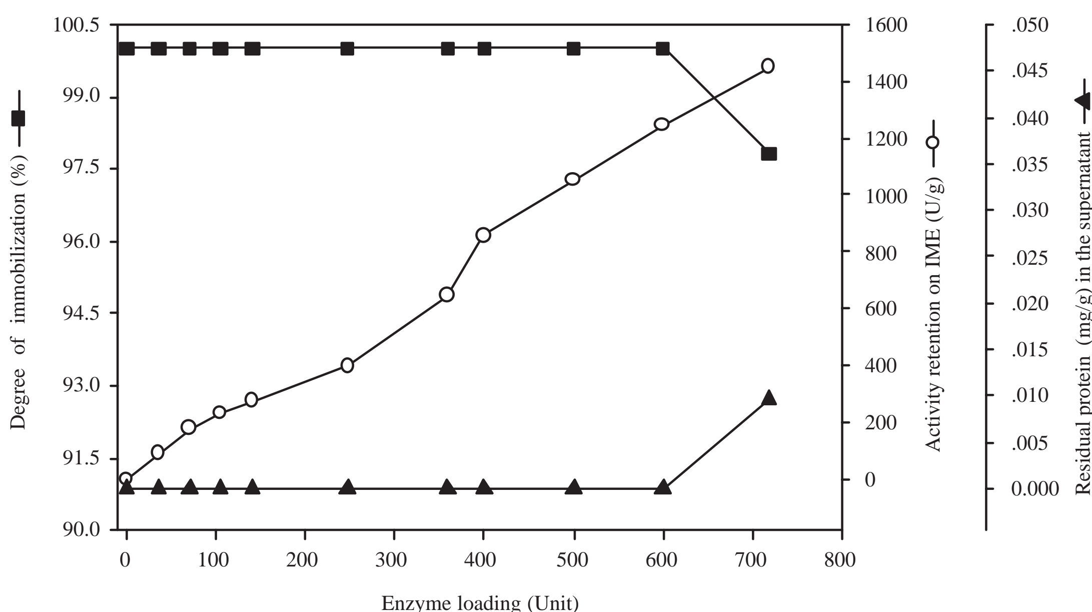


Figure 1 The profile of a degree of immobilization, activity retention on IME and residual protein in the supernatant during *Pseudomonas* sp. KLB1 lipase entrapment in sol-gel matrix from rice husk ash.

immobilized enzyme. The results was showed in Figure 2 .

The results showed that Ca^{2+}, K^{+}, Na^{+} ions and EDTA activated the lipase of the immobilized and the free enzyme whereas Mg^{2+}, Fe^{2+} and ascorbic acid inhibited enzyme activity. After Ca^{2+} and EDTA were added into the medium ,the activity was increased to 160.88 Unit/g and 160.22 Unit/g, respectively. While the activity of 133.20 Unit/g was found by using Na^{+} (Figure 2). Even though the nature function of these ions is not clear, many papers concluded that the lipase conformation was modified by ions (Sánchez *et al*., 1996).

Bear in mind, all of the lipases immobilized by entrapment played higher activity than soluble form. These were proposed that these ions which, either activation (Ca^{2+}, Na^{+}, and K^{+}) or deactivation (Mg^{2+}, Fe^{2+}). Therefore, the content of ions were investigated as showed on Table 1.

Three orders of the metal ions mainly consisting in the rice husk ash are Ca^{2+}, Na^{+} ,and K^{+}. The content of Ca^{2+} was the maximum (7.45%). After rice husk ash was modified to be xerogel (dried gel), the Ca^{2+} and Na^{+} were increased whilst the Mg^{2+} and Fe^{2+} were decreased by acidic

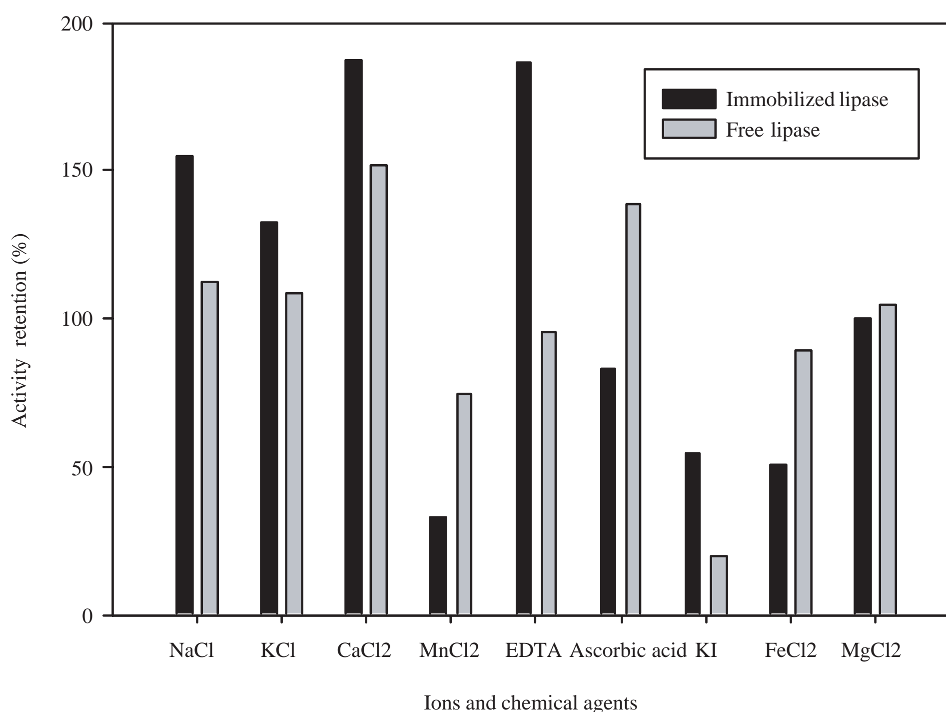


Figure 2 Effect of metal ions and other chemical agent on hydrolysis activity of the free and immobilized *Pseudomonas* sp. KLB1 lipase.

Table 1 The metal ions content consist of the rice husk ash and the xerogel.

Support	Metal ions content (mg/100 mg)*				
	Ca^{2+}	Mg^{2+}	K^{+}	Na^{+}	Fe^{2+}
Rice husk ash	7.45^{a}	0.2^{a}	1.58^{a}	2.37^{a}	0.068^{a}
Xerogel	16.7^{b}	0.003^{b}	1.07^{b}	12.67^{b}	0.0046^{b}

* In a column, means followed by the same letter are not significantly different at 5% by LSD.

purification process (Kalapathy *et al*., 2000).

These results hinted that the activity of the immobilized enzyme were activated by Ca^{2+} and Na^{+} in suports.

Calcium ion, especially, played a role as lipase-cofactor. It is used in Ca^{2+} binding processing which impact to position specificity on active site (Birute *et al.,*2002; Gao *et al.* , 2000 and Huan *et al*., 1999). Amada *et al*. (2001) studied the function of Ca^{2+} on lipase and reported that one is important factor for folding of 1,3 *Sn* lipase family. Moreover, the holo lipase required a single Ca^{2+} for its activity and the Apo lipase is probably converted to the holo-lipase if the system obtain enough Ca^{2+} concentration.

3. Effect of calcium and sodium ions concentrations on enzyme activation

According to the reports of Amada *et al.* (2001) and Sáchez *et al*. (1996) who demonstrated the effect of ions concentration (Ca^{2+} and Na^{+}) on the activity of lipases. Moreover, the above experiments show that both Ca^{2+} and K^{+} were effected on the lipase activation. Therefore, the optimization of Ca^{2+} and Na^{+} concentration was studied.

The different concentrations (0-100 mM) of $CaCl_2$ and NaCl were added into the hydrolysis of palm oil emulsion catalyzed by the soluble lipase and the immobilized lipase.

The immobilized enzyme showed rapid activation upto 343.97% when the $CaCl_2$ was raised to 20 mM, then decrease (Figure 3). This indicated the Ca^{2+} strength effect on the lipase activation. However, the effect of NaCl concentration, on the enzyme activity were slightly increased to 115.64% when the NaCl was raised to 60 mM (Figure 3) indicated that the NaCl was an unspecific effect of ionic strength on the enzyme reaction . This result was similar to the activation of silica –immobilized lipase by Na^{+} studied by S?chez *et al*. (1996).

4. Physicochemical properties of immobilized lipase

4.1 pH and temperature optimum

The immobilized lipase was carried out in different pH (7-10) and temperature (40°C–60°C) for 1 hr to catalyze the palm oil emulsion. Figure 4A, shows that the immobilized lipase displayed

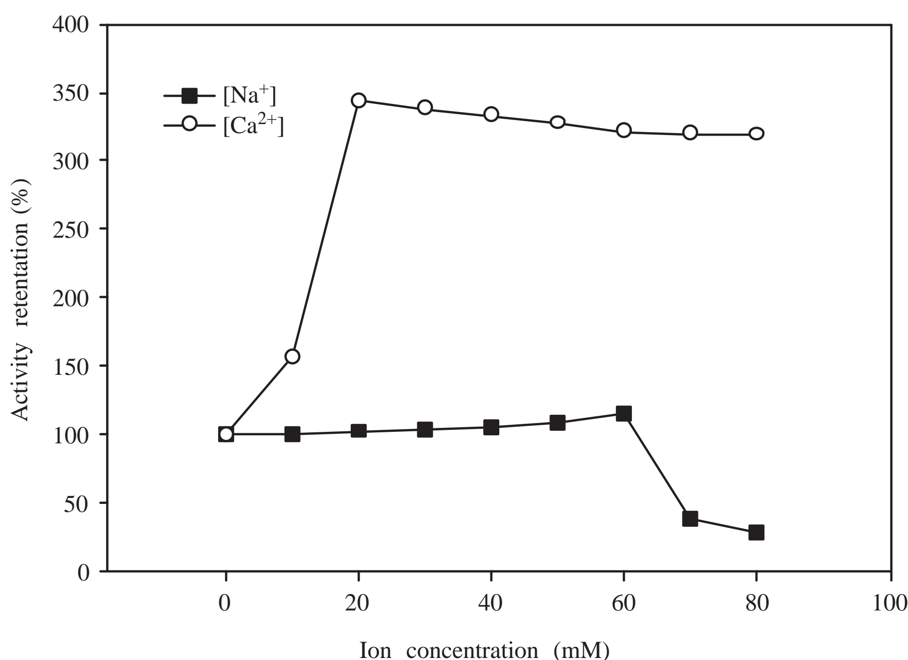
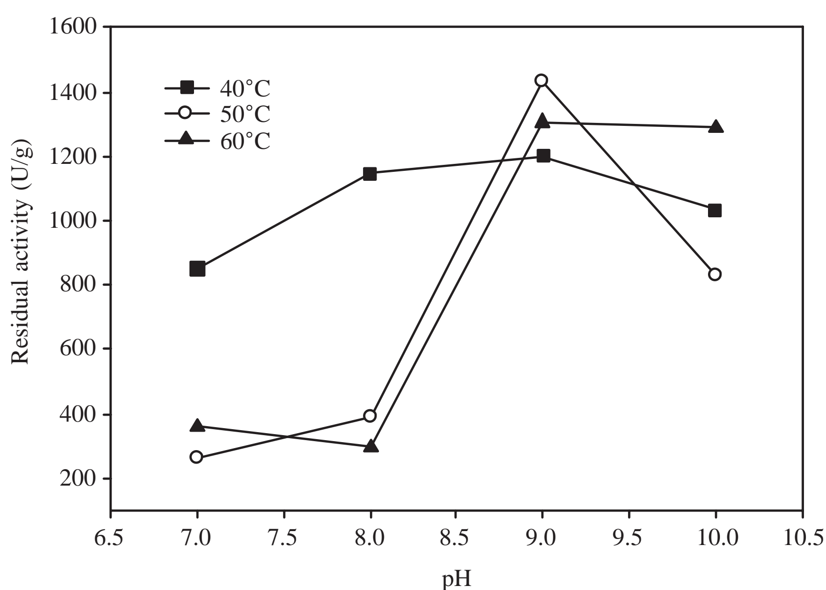


Figure 3 The activation of immobilized *Pseudomonas* sp. KLB1 lipase by different concentration of $CaCl_2$ and NaCl.

the optimum pH of 9.0 that was an alkaline environment. It played the same optimal pH as free lipase (Figure 4B). According to temperature, the immobilized enzyme catalyzed the reaction well under 50°C (pH 9.0) that offered the highest activity (1,432.80 U/g). However, its activity rapidly decreased if the pH was higher than 9.0 (figure 4A). This shows the same results as free lipase activity played in medium pH 9.0 and temperature at 50°C (Figure 4B).

4.2 pH and temperature stability

The effects of temperature and pH on the immobilized lipase and free lipase stability were shown in Figure 5. The maximum activity of the immobilized enzyme (109.87%) occurred in medium pH 9.0 under temperature at 70°C (Figure 5). With respect to pH, the results were found that the immobilized enzymes incubated under different temperature could be stable on pH 9.0. This is indicated that the stability of the immobilized enzyme was depended on pH more than temperature.



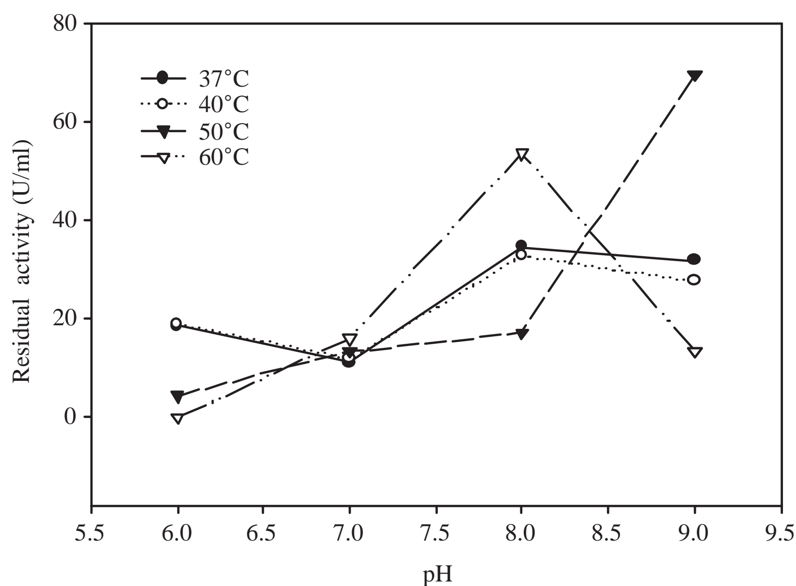


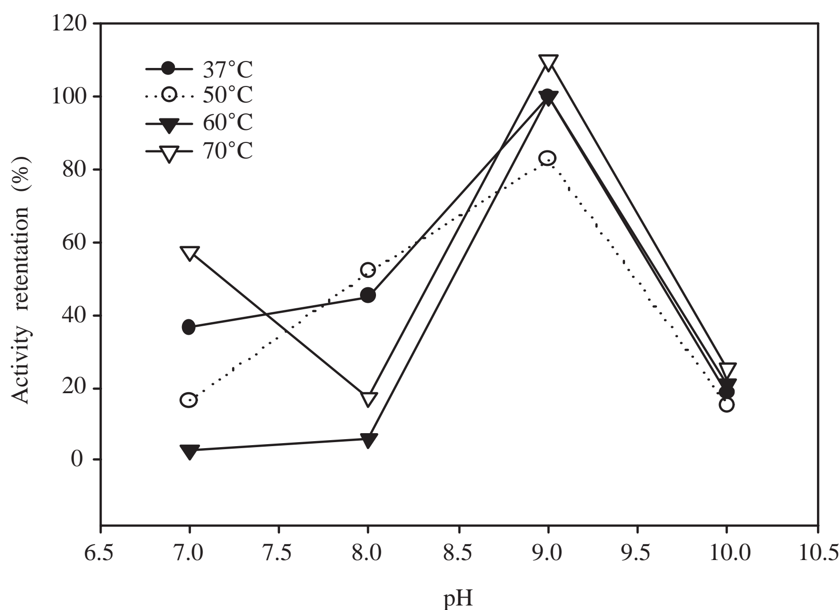
Figure 4 pH and temperature optimum of *Pseudomonas* sp. KLB1 lipase; entrapped in rice husk ash gel (a) and lipase (B).

When the pH and temperature stability of the immobilized enzyme was compared to the free enzyme, it was found that the immobilized lipase displayed 109.87% activity retention at pH 9.0, 60°C (Figure 5A) whereas the free form shows 76% activity retention at pH 10, 70 C and 73 .39% at pH 9.0, 60°C (Figure 5B). This results indicated that the tolerant capacity of the immobilized enzyme is more stable on temperature.

4.3 Reusability of the immobilized lipase

The reusability of *Pseudomonas* sp. KLB1 entrapped in gel of rice husk ash catalyzed the hydrolysis reaction of *p*-Nitrophenyl palmitate (pNPP) was shown in Figure 6.

Action stability in hydrolysis pNPP was established 20 times. One cycle of reaction time was 1 hr. The immobilized enzyme seemed to be quite stable in 1-4 cycle. Its activity was



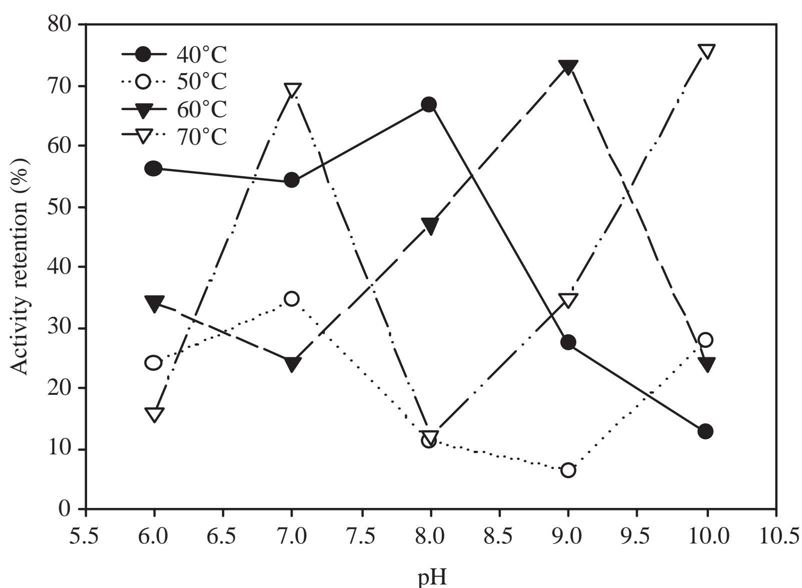


Figure 5 pH and temperature stability of entrapped *Pseudomonas* sp. KLB1 lipase in rice husk ash gel (A) and of free lipase (B).

continuously decreased after the cycle number 4. The activities were lose about 50% and completely lose at cycle of reaction No. 12 and No. 20, respectively. This results showed that the immobilized enzyme was stable enough to catalyzed the hydrolysis of pNPP for 20 times.

4.4 Kinetics properties

Km and Vmax of the immobilized *Pseudomonas* sp. KLB1 lipase that catalyzed *p*-Nitrophenylpalmitate (pNPP) were obtained from Lineweaver-Burk Plots (Figure 7A). The hydrolysis demonstrated that Km, and Vmax were 7.43×10^{-2} mM, and 1.94×10^{-5} mM.s^{-1}, respectively whereas these kinetics parameters of free lipase were 21.69 mM, and 3.90×10^{-3} mM.s^{-1} respectively (Figure 7B). The Km value of the immobilized *Pseudomonas* sp. KLB1 lipase was in the optimum range that use in the most industries (1×10^{-2} to 1×10^{2} mM), also (Fullbrook, 1996).

CONCLUSION

The lipase of *Pseudomonas* sp. KLB1 was immobilized by entrapment method in neutral sol-

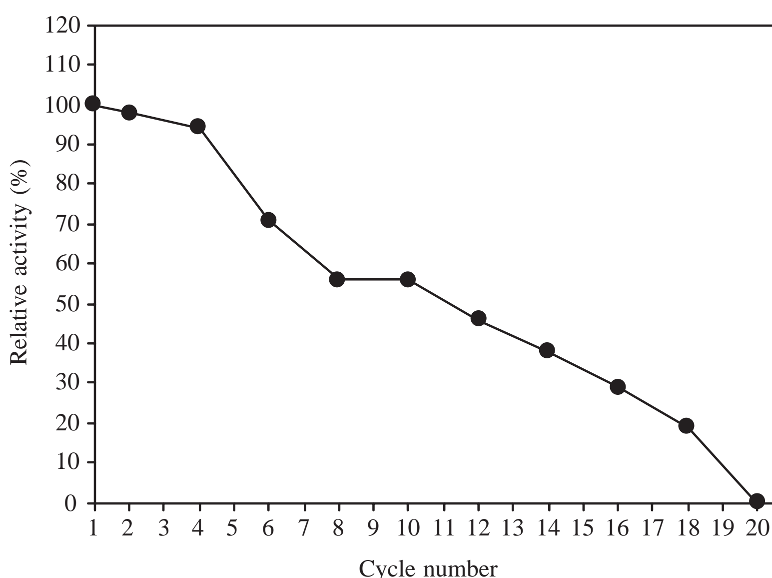


Figure 6 Reuse of sol-gel rice husk ash entrapped *Psuedomonas* sp. KLB1 lipase catalyzed the hydrolysis p-Nitrophenylpalmitate.

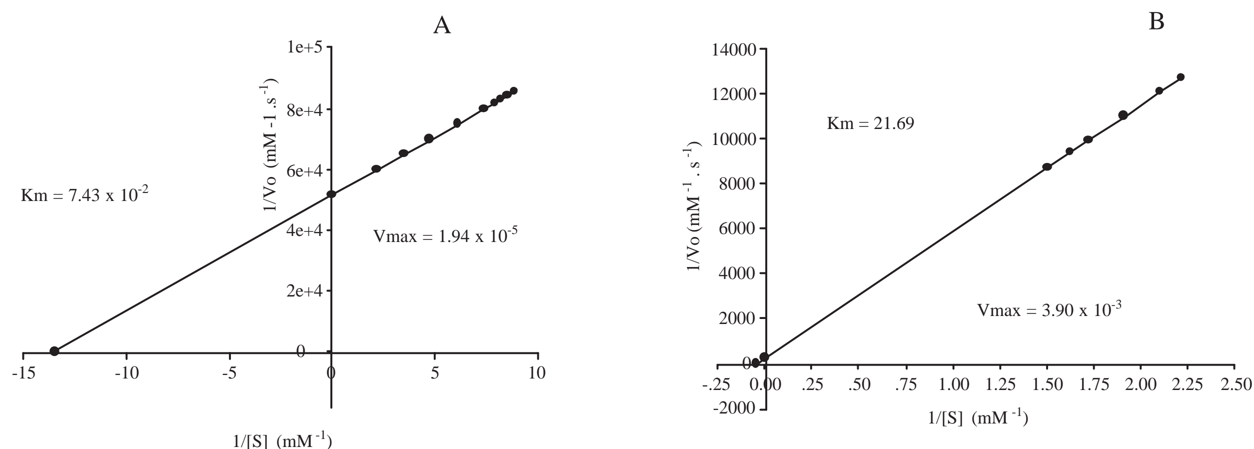


Figure 7 Lineweaver-Burk Plots of the hydrolysis of p-Nitrphenylpalmitate catalyzed by immobilized (A) and free *Pseudomona*s sp. KLB1 lipase (B).

gel of rice husk ash. It was optimally active at 60 °C and pH 9. Its activty was constant in the medium pH 9 under temperature 70°C for 1 hr. The immobilized lipase was activated by Ca^{2+}, K^+, Na^+, and EDTA but inhibited by Fe^{2+}, Mn^{2+}, Mg^{2+}, and ascorbic acid. The Ca^{2+},and Na^+ were also found mainly in rice husk ash and xerogel. The effective concentration of $CaCl_2$ and NaCl on the enzyme activation were 60 mM and 20mM, respectively. The shelf life of entrapped lipase in the hydrolysis of *p*-nitrophenyl palmitate was lose 50% at reaction cycle No. 12. The Km, and Vmax of p-NPP hydrolysis catalyzed by the entrapped enzyme were 7.43×10^{-2} mM, and 1.94×10^{-5} $mM.s^{-1}$, respectively whilst by the free enzyme were 21.69 mM, and 3.90×10^{-3} $mM.s^{-1}$, respectively.

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LITERATURE CITED

Amada, K., H. Kwon, M. Haruki, M. Morikawa, and S. Kanaya. 2001. Ca^{2+} induced folding of a family 1,3 lipase with repetitive Ca^{2+} binding mofits at the C-terminal. **FEBS Letters**. 509 : 17-21.

Bhumibhamon O, J. Jinda. 2003. Hydrolysis characteristics of alkaline lipase from *Pseudomonas* sp. KLB1, pp. 209-217. *In* **The P roceedings of 41st Kasetsart University Annual Conference**. Bangkok.

Bhumibhamon O, A. Koprasersak and S. Fungthong. 2002. Biotreatment of high fat and oil wastewater by lipase producing bacteria. **Kasetsart Journal (Nat. Sci.).** 36 : 261-267.

Birute, S. V. Bendikiene, B. Juodka, I. Bachmatova, and L. Marcinkevichiene. 2002. Characterization and physicochemical properties of a lipase from *Pseudomonas mendocina* 3121-1. **Biotechnol. Appl. Biochem**. 36 : 47-55.

Brinker, C.J. and G.W. Scherer. 1990. Applications, pp. 839-880. *In* **Sol-Gel Scince, The Physics and Chemistry of Sol-Gel Processing**. Academic Press, San Diego, CA.

Fullbrook, P.D. 1996. Practical applied kinetics, pp. 483-540. *In* T. Godfrey, and S. West, eds. **Industrial Enzymology**, 2nd ed. Stockholm Press. New York.

GaO, X-G, S. Cao, and K. Zhang. 2000. Production, properties and application to nonaqueous enzymatic catalysis of lipase from a newly isolated *Pseudomonas* strain. **Enzyme Microb Technol**. 27 : 74-82.

Huan, D., S. Gao, S. Han and S. Cao. 1999. Purification and characterization of a *Pseudomonas* sp. lipase and its properties in non-aqueous media. **Biotechnol. Appl. Biochem**. 30 : 251-256.

Kamath, S. R., and A. Proctor. 1998. Silica gel from rice hull ash: preparation and characterization. **Cereal Chemistry**. 75 : 484-487.

Kalapathy, U, A. Proctor and J. Shultz. 2000. A simple method for production of pure silica from rice hull ash. **Biores. Technol.** 73 : 257-262.

Laxamana, N.B. 1982. Binders from rice hull ash low-cost housing materials. Forbride-Dig. College: **Forest Products Research and Industries Development Commission**. 11: 27-30.

Monsan, P. 1977/78. Optimization of glutaraldehyde activation of a support for enzyme immobilization**. J. of Molecular Catalysis**. 3: 371-384.

Noureddini, H., X. Gao, S. Joshi and P.R. Wagner. 2002. Immobilization of *Pseudomonas cepacia* lipase by sol-gel entrapment and its application in the hydrolysis of soybean oil.

JAOCS. 79 : 33-40.

Payá, J, J. Monzó, M.V. Borrachcro, A. Mellado and L.M. Ordoñez. 2001. Determination of amprphous silica in rice husk ash by a rapid analytical method. **Cem. Concr. Res**. 31: 227-231.

Robert, L. D. and G. F. Lata. 1989. **Experiment One; Methods for Quantitative Protein Determination.** Oxford University Press. 346-347.

Sánchez, E.M., J.F. Bello, M.G. Roig, F.J.Burguillo, J.M.Moreno and J.V.Sinisterra. 1996.Kinetic and enantioselective behavior of the lipase from *Candida cylindracea*: A comparative study between the soluble enzyme and the enzyme immobilized on agarose and silica gels. **Enzyme Microb. Tech.** 18 : 468-476.

Yamada K, Y. Ota, H. Machida. 1962. A modified method for lipase activity assay with emulsified olive oil as substrate. **Nippon Nogeikagaku Kaishi**. 36 : 860.