PROPERTIES OF IMMOBILIZED LIPASE FROM *Rhizopus delemar* ON POLYPROPYLENE MEMBRANE

(SIFAT-SIFAT ENZIM LIPASE AMOBIL DARI Rhizopus delemar DALAM MEMBRAN POLIPROPILEN)

Retno Indrati[1], Djagal W. Marseno[1], Yoshiyuki Ohta[2]

ABSTRACT

Properties of *Rhizopus delemar lipase* immobilized on hydrophobic polypropylene membrane were studied by physical adsorption using various concentration of enzyme loading. The result shows that free fatty acid liberation was affected by the enzyme loading, which increased with the increasing enzyme loading. The maximum enzyme adsorption was achieved at 1.2 mg/cm^2 membrane. The initial velocity of hydrolysis reaction seems not to be affected by the amount of enzyme bound. The immobilization efficiency was very high reaching more than 60 % at enzyme loading of 0.3 mg/cm^2, although a suppression of efficiency was detected at higher loading. The immobilized lipase could hydrolyze 97 % olive oil after 72 h using 1 mg/cm2 of initial enzyme loading. The kinetic parameter indicated that the affinity of the enzyme to substrate was very low (K_m = 183 mg/ml). The immobilized enzyme was very stable during storage at 4 °C with proximate half life 65 days. In addition, it maintains ability for subsequent reuses. The membrane can be regenerated by washing for fresh enzyme immobilization.

Key words: lipase, *Rhizopus delemar*, immobilization, polypropylene, membrane

INTRODUCTION

The properties of an immobilized enzyme were largely depended on the types of support used. According to Germeiner (1992) the support should possess a well-developed internal structure, a large surface area provided by high porosity, and reasonable pore size distribution. Kang and Rhee (1989) reported that the support used for immobilization should possess mechanical strength, resistance against microorganisms, thermal stability, chemical durability, low cost, hydrophobicity, regenerability, and high immobilizing capacity of the enzymes. However, there was no substance that fulfils all of these requirements. Brady *et al* (1988) and Malcata *et al* (1992) reported that hydrophobic supports (in form of microporous powders, membranes, or fibers) possessed the best performance, as little of the lipase's activity was lost upon immobilization. Lipolytic activity of lipase was usually retained better on hydrophobic porous supports than on hydrophilic ones (Brady *et al* 1988). According to Al-Duri and his co-workers (1995), porosity and composition of the hydrophobic support was more important than the surface area. On the other hand, Rucka *et al* (1990) reported that those did not influence the lipase activity.

Lipases were activated by interface between two phases, oil and water. This led the polymeric - microporous membranes to be selected as efficient carriers for lipase immobilization. The advantages of this carrier included high effective diffusivity, high chemical stability, high surface area, ease of scale-up, stable interface, and elimination of emulsification problems (Malcata *et al* 1992, Rucka *et al* 1990).

From those advantages of hydrophobic membranes as enzyme carriers, we are interested in experiments with the membranes attempting to find efficient carriers for lipase. In the previous paper, we reported the immobilization of lipase on hydrophobic polyethylene membranes (Indrati *et al* 1999), where membrane's thickness and enzyme loading affected the efficiency of immobilization. A higher enzyme loading suppressed the efficiency. This paper reports the use of polypropylene membrane as support for lipase from *Rhizopus delemar* and the properties of the immobilized enzyme were discussed.

MATERIALS AND METHODS

Enzyme and membrane

Lipase D from *Rhizopus delemar* was supplied by Amano Pharmaceuticals (Nagoya, Japan). Polypropylene membranes were purchased from Millipore (pore size 5.0 μm).

Immobilization of lipase

Lipase was immobilized by adsorption on the membrane surface (4.34 cm^2) at 4 °C for 24 h. The amount of enzyme adsorbed was varied and calculated from the difference between the concentrations before and after the

[1] *Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta, Indonesia.*
[2] *Laboratory of Microbial Biochemistry, Faculty of Applied Biological Science, Hiroshima University, Higashi Hiroshima, Japan*

enzyme immobilization. The efficiency of lipase immobilization was calculated by the following equation:

$$\text{Immobilization efficiency (\%)} = \frac{E_1}{E_o V_o} \times 100$$

Where E_o was the lipase activity of the original lipase solution (U/ml), V_o was its volume (ml); E_i was the lipase activity of the immobilized enzyme (U). The enzyme activity was calculated as free fatty acid liberation by the method of Marseno *et al* (1998). One unit of enzyme activity (U) is defined as the amount of enzyme converting 1 μ mole free fatty acid per minute under the assay conditions.

Enzyme reaction

The reaction was carried out at 30 °C for 2 h (unless otherwise stated) with shaking in a 100 ml Erlenmeyer flask containing immobilized lipase (4.34 cm^2) corresponding to 0.034 to 2.204 mg/cm^2 of enzyme immobilized initially, 5 ml of 60 % (v/v) olive oil in isooctane, and 5 ml of 0.1 M phosphate buffer pH 7.0. In some cases, other reaction conditions were varied. Free fatty acid released was measured according to the method of Marseno *et al* (1998). The degree of hydrolysis of the oil is calculated by the following equation:

$$\text{Degree of hydrolysis (\%)} = \frac{\text{μmol fatty acid liberated}}{\text{(SV) (1000/56.1) (g oil)}} \text{ X } 100$$

SV was the saponification value of olive oil, which is 192.

Repeated batch hydrolysis

The experimental conditions were similar to those described above, with 1 mg enzyme immobilized initially per cm^2 membrane. After 6 or 15 h for each run, the hydrolysis degree was determined, and the immobilized lipase preparations were washed with water and re-used with fresh substrate.

Storage stability

Storage stability was estimated from measurement of immobilized enzyme activities (1 mg enzyme loading per cm^2 membrane) that were stored at 4 °C. The activity measurements were continued over a period of 40 days.

Membrane regeneration

Membrane status was monitored by cleaning the used membranes and reused them for immobilization. Membrane cleaning was done with 98% ethanol, 0.1 M sodium hydroxide, 1 M NaCl, and phosphate buffer 0.1 M pH 7.0. After cleaning, a fresh lipase solution was immobilized on the membrane.

Kinetic Analysis

Kinetic parameters were calculated using Michaelis - Menten equation for enzyme reaction kinetics at variation of olive oil concentrations in isooctane. Immobilized enzymes used were 4.34 cm^2 and initial enzyme loading was 1 mg/cm^2.

Protein Analysis

Protein amount was measured by the method of Lowry *et al* (1951) which was modified by Peterson (1977) using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Activity of immobilized lipase

Fig. 1 shows the effect of enzyme loading on the activity of immobilized enzyme. The enzyme adsorbed onto membrane was gradually increased as more lipase was loaded onto the membrane as indicated by the higher enzyme activity (calculated from μ mole free fatty acids liberation). These free fatty acids liberation were linearly increased up to 1.2 mg/ cm^2 of enzyme loading. At higher enzyme concentration, the production rate declined probably because of saturated adsorption of enzyme on membrane. This pattern was similar with other reported data on lipase immobilization (Hoq *et al* 1985, Guit *et al* 1991, Kang and Rhee 1989). The polypropylene membrane studied had better enzyme adsorption capacity compared to hydrophobic polyethylene membranes (Indrati *et al* 1999). At the same enzyme loading polypropylene membrane adsorbed approximately 10 times higher with maximum protein adsorption was 1.2 mg/cm^2, whereas for polyethylene membranes the maximum protein adsorption was 0.3 and 0.1 mg/cm^2 for thick and thin membranes, respectively (Indrati *et al* 1999).

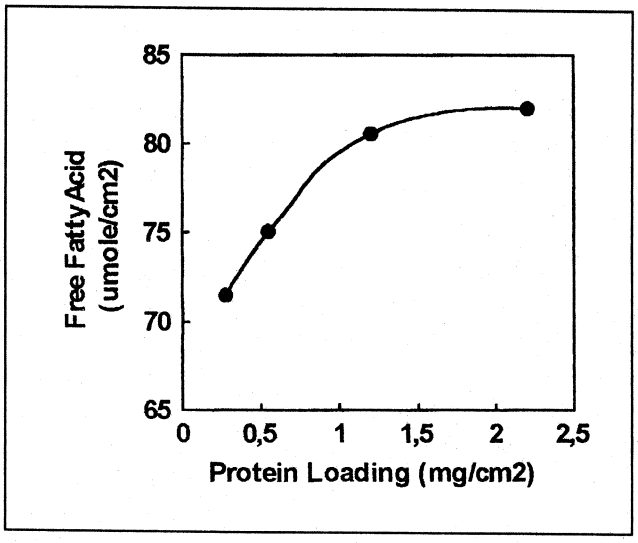

Figure 1. Effect of various enzyme loadings on hydrolysis of olive oil by immobilized lipase on polypropylene membrane. Reaction times 20 min.

Fig.2 shows that the efficiency of immobilization declined as enzyme loading increased. This is a typical phenomenon of immobilized enzyme (Bosley and Peilow 1997) and was the same to that immobilized on polyethylene membrane (Indrati *et al* 1999). At low loading, all enzymes might be adsorbed onto membrane and most of them located at the surface of the membrane, so that mass transfer could maintain well, resulting an efficiency near to 100 % at the enzyme loading of 0.14 mg/cm^2 or more than 60 % at 0.3 mg/cm^2. At higher loading, mass transfer limitation might have pronounced, as shown by the efficiency fell to less than 20 % at enzyme loading of more than 1 mg/ cm^2 and 9.6 % at 2.2 mg/cm^2. These efficiency values of polypropylene membrane were higher than that of polyethylene, which was less than 15 % although at low enzyme loading (Indrati *et al* 1999).

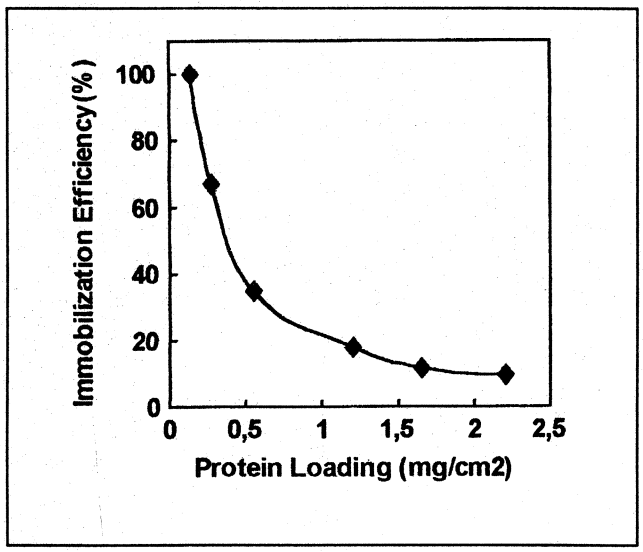

Figure 2. Effect of various enzyme loadings on efficiency of immobilization of lipase on polypropylene membranes. Reaction times 10 min.

Free fatty acids liberation with immobilized lipase on polypropylene membrane as a function of reaction time at various enzyme loadings was shown in Fig. 3. At the initial rate of hydrolysis reaction, the amount of free fatty acids released was linearly proportional to the enzyme bound; both at low and high concentration. At the initial rate of hydrolysis reaction, the amount of enzyme loading slightly affected the liberation rate. However, at prolonged time of hydrolysis the liberation rate increased with the increased of the enzyme loading. Furthermore, at this prolonged hydrolysis time the liberation rate decreased compared with that of the initial rate (data not shown). This might caused by a limitation of substrate transfer, especially at high loading or prolonged hydrolysis.

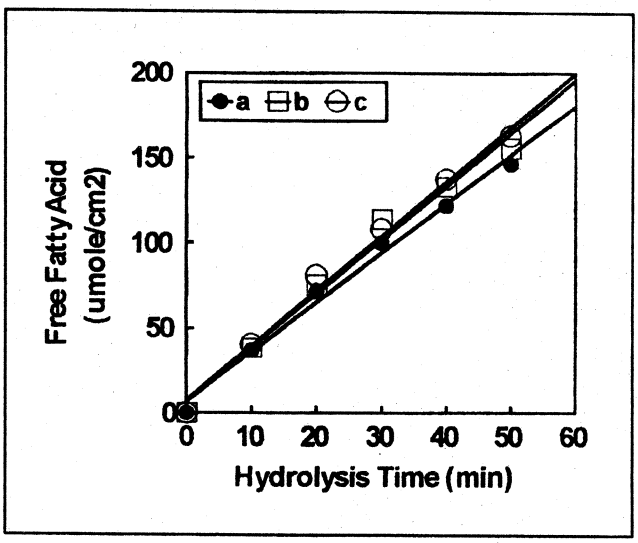

Figure 3. Hydrolysis of olive oil by immobilized lipase on polypropylene membrane at various enzyme loadings: a. 230; b. 460; c. 922 µg/cm^2.

The data show that the enzyme loading of 1 mg/cm^2 was the best in giving higher free fatty acids liberation and efficiency value, and this concentration was then used for the next experiments.

Degree of hydrolysis

Fig. 4 shows the degree of hydrolysis of olive oil at various times. Olive oil was approximately 68 % hydrolyzed in 24 h, and increased to 97 % in 72 h. This graph indicated that there was mass transfer limitation for diffusion of substrate through membrane pores at prolonged time of hydrolysis, and took almost 3 days to reach nearly complete hydrolysis. These results were similar to the previous report using thin polyethylene membranes (65 % for 24 h of hydrolysis) (Indrati *et al* 1999).

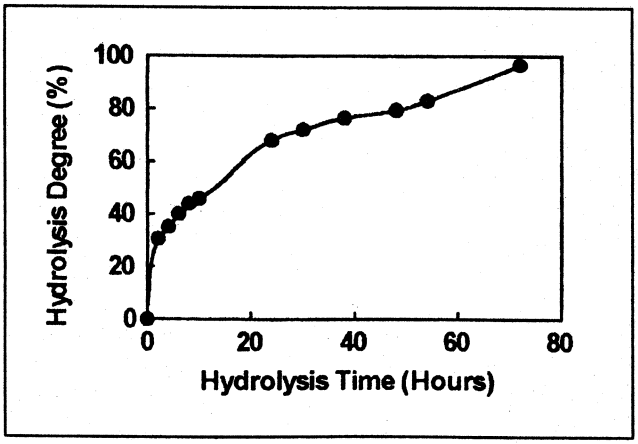

Figure 4. Degree of hydrolysis of olive oil by lipase immobilized on polypropylene membrane on various times of hydrolysis. Enzyme loading 1 mg/cm2. Membrane area 17.4 cm2

Enzyme kinetics

Fig. 5 shows the Lineweaver-Burk plot for olive oil hydrolysis at different concentration in isooctane. The data indicated that the hydrolysis reaction of immobilized lipase fully obeys the Michaelis-Menten kinetics, where the K_m value was 0.183 g/ml and V_{max} was 33.3 U. This high K_m value indicated that the affinity of the immobilized enzyme to substrate was low.

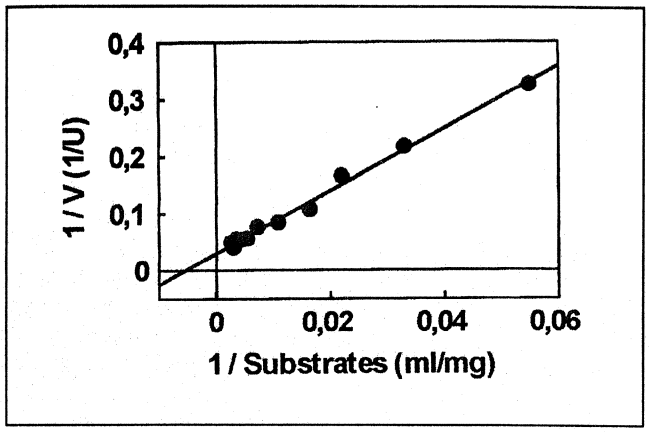

Figure 5. Lineweaver - Burk plot of lipase immobilized on polypropylene membrane. V (reaction rate) was expressed as activity unit (U).

Reusability

The immobilized lipases were used for hydrolysis of olive oil for 6 and 15 h. Immobilized lipase on polypropylene membrane displayed good stability for up to 10 subsequent reuses (Fig.6). The free fatty acids release decreased to 57 and 60 % in the second use for 6 h and 15 h reaction, respectively. After that subsequent reuses caused a slightly decrease of free fatty acids liberation. For 15 h of hydrolysis, enzyme inactivation occurred fast, where after 5 subsequent reuses or more, the free fatty acids liberation was the same with that of 6 h of hydrolysis. From those data, the 6 h of hydrolysis gives more advantages.

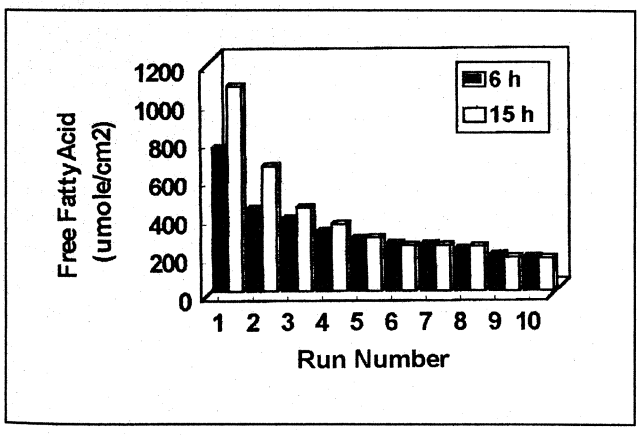

Figure 6. Reusability of polypropylene membrane-immobilized lipases on hydrolysis of olive oil at 30 °C for 6 and 15 h. Enzyme loading 1 mg/cm2. Membrane area 4.34 cm2.

Fig. 6 also shows that after 3 subsequent reuses, the hydrolysis rate was almost constant with slopes of -22.04 and -27.50 for 6 h and 15 h of hydrolysis, respectively. The free fatty acids release at the 4th reuses was 311 and 350 μ mole/cm^2 for 6 and 15 h of hydrolysis, respectively, or 42 and 33 % of the first uses.

Regeneration

Regeneration test of the membranes was carried out to evaluate whether the membrane could be used repeatedly for enzyme immobilization. As shown in Fig. 7 and 8, the membranes were regenerable. Lipase immobilized on both new and regenerated membrane had the same patterns of free fatty acids liberation (Fig. 7). In addition, the release was still high even after 5 subsequent washing (Fig. 8). These indicated that the membrane was strong enough during successively washing with ethanol, NaOH, and NaCl.

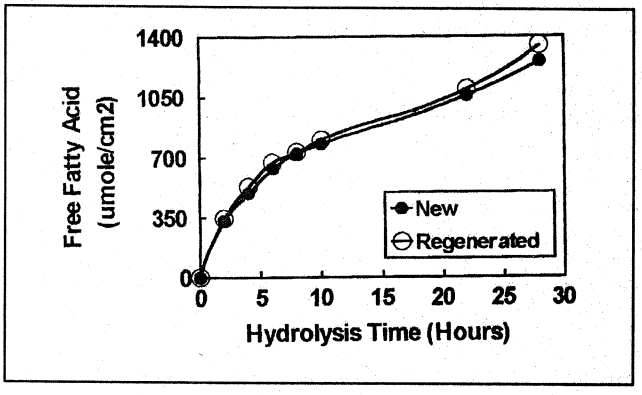

Figure 7. Pattern of free fatty acids release during hydrolysis of olive oil by lipase immobilized on a new and regenerated polypropylene membrane. Membrane area 4.34 cm2. Enzyme loading 1 mg/cm2.

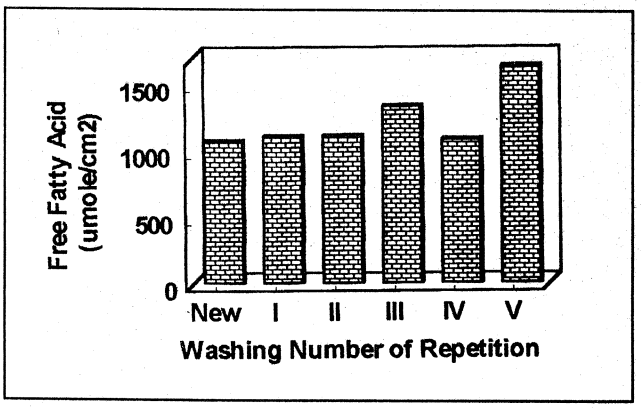

Figure 8. Washing and repeated use of polypropylene membrane for lipase immobilization used for olive oil hydrolysis. Five subsequent washings and repeated uses were carried out, compared to the new membrane. Hydrolysis times 22 h. Enzyme loading 1 mg/cm2. Membrane area 4.34 cm2.

Effect of storage on lipase stability

Fig. 9 shows that the immobilized enzyme on polypropylene membrane was stable during storage in a closed bottle at 4 °C, similar to that free enzyme. After 20 days, the remaining activity for immobilized enzyme was 88 %, and decreased to 68 % after 37 days. The half-life of the immobilized enzyme was calculated from the graph to be 65 days (slope -0.78), while free enzyme was 80 days (slope -0.61). From the hydrolysis degree, reusability, membrane regeneration and storage stability, the polypropylene membrane was a good choise as carrier for lipase immobilization.

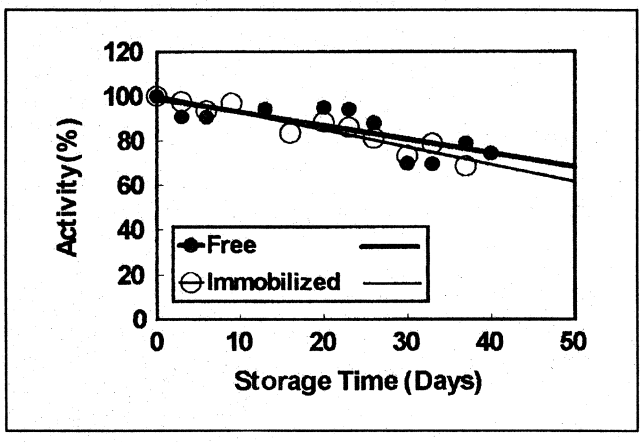

Figure 9. Storage stability of immobilized lipase on polypropylene membrane at 4 °C compared to free enzyme. Enzyme loading: 1 mg/cm2. Membrane area 4.34 cm2. Hydrolysis times 2 h.

CONCLUSION

Lipase adsorbed on polypropylene membrane was found to effectively catalyze olive oil hydrolysis, which after 72 h it hydrolyzed approximately 97 % of oil. The immobilization efficiency was very high reached more than 60 % at enzyme loading of 0.3 mg/cm^2. At high loading, a suppression of efficiency was detected. The maximum enzyme adsorption was 1.2 mg/cm^2 membrane. This immobilized enzyme was very stable on storage (half life 65 d) as well as on repeated use. The membrane was also regenerable so it could be used again for fresh lipase immobilization. The K_m value of immobilized lipase on polypropylene for olive oil hydrolysis was 0.183 g/ml, while the V_{max} was 33.3 U.

ACKNOWLEDGEMENTS

We wish to thank the National Research Council (DRN) of Republic of Indonesia for Research Grant through Riset Unggulan Terpadu VI (RUT-VI) Project. The support from the Laboratory of Microbial Biochemistry, Faculty of Applied Biological Science, Hiroshima University-Japan, is gratefully acknowledged.

REFERENCES

Al-Duri, B., Robinson, E., McNerlan, S., and Bailie, P. 1995. Hydrolysis of edible oils by lipases immobilized on hydrophobic supports: Effects of internal support structure. JAOCS, 72: 1351-1359.

Bosley, J.A. and Peilow, A.D. 1997. Immobilization of lipases on porous polypropylene: Reduction in esterification efficiency at low loading. JAOCS, 74: 107-111.

Brady, C., Metcalfe, L., Slaboszewski, D., and Frank, D. 1988. Lipase immobilized on a hydrophobic, microporous support for the hydrolysis of fats. JAOCS, 65: 917-921.

Gemeiner, P. (Ed). 1992. Enzyme engineering: Immobilized biosystems. Ellis Horwood, New York.

Indrati, R., Marseno, D.W., and Ohta, Y. 1999. Immobilization of lipase from *Rhizopus delemar* on polyethylene membrane. Ind. Food Nutr. Prog. 6: 19-24.

Kang, S.T. and Rhee, J.S. 1989. Characteristics of immobilized lipase-catalyzed hydrolysis of olive oil of high concentration in reverse phase system. Biotechnol. Bioeng. 33: 1469-1476.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Malcata, F.X., Reyes, H.R., Garcia, H.S., Hill, C.G.Jr., and Amundson, C.H. 1992. Kinetics and mechanisms of reaction catalysed by immobilized lipases. Enzyme Microb. Technol. 14: 426-446.

Marseno, D.W., Indrati, R., and Ohta, Y. 1998. A simplified method for determination of free fatty acids for soluble and immobilized lipase assay. Ind. Food Nutr. Prog., 5 : 79-83.

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry *et al*, which is more generally applicable. Anal. Biochem. 83:346-356.

Rucka, M., Turkiewicz, B., and Zuk, J.S. 1990. Polymeric membranes for lipase immobilization. JAOCS, 67: 887-889.