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# Hydrolysis of Olive Oil by Lipase, Immobilized on Hydrophobic Support

**JUNG, JU-YOUNG, HYUN SHIK YUN*, AND EUN-KI KIM**

*Department of Biological Engineering, Inha University, 253 Yonghyundong Namku, Inchon 402-751, Korea*

Two commercially available lipases, Lipase OF (non-specific lipase from *Candida rugosa*) and Lipolase 100T (1,3-specific lipase from *Aspergillus niger*), were immobilized on insoluble hydrophobic support HDPE (high density polyethylene) by the physical adsorption method. Hydrolysis performance was enhanced by mixing a non-specific Lipase OF and a 1,3-specific Lipolase 100T at a 2 : 1 ratio. The results also showed that the immobilized lipase maintained its activity at broader temperature (25~55°C) and pH (4~8) ranges than soluble lipases. In the presence of organic solvent (isooctane), the immobilized lipase retained most of its activity in upto 12 runs of hydrolysis experiment. However, without organic solvent in the reaction mixture, the immobilized lipase maintained most of its activity even after 20 runs of hydrolysis experiment.

Fatty acids are produced with glycerol when lipids are decomposed. After the distillation process, fatty acids are used for the manufacturing of soap, cosmetics, and food emulsifying agents (1, 4, 12). Currently applicable methods for the hydrolysis of lipids are the Colgate-Emery steam hydrolysis method and the enzyme hydrolysis method. Fatty acid synthesis by steam hydrolysis is energy-intensive (250°C, 50 atm) and requires a high capital investment. However, fatty acid synthesis by lipase uses milder conditions (30~40°C, 1 atm) and improves the quality of fatty acids produced (3, 13, 14).

For industrialization of the enzyme hydrolysis method, the cost of enzyme is important. Furthermore, the enzyme should be thermostable and able to hydrolyze various lipids independently of the type and the length of chain (7, 10). By immobilizing lipase, stability of the change in pH and temperature can be maintained (2, 18). Also, enzyme cost can be reduced since separation of the enzymes and products is easy and enzymes can be reused. Lipase can be easily adsorbed to the hydrophobic supports-polypropylene and polyethylene powders-and its activity can be maintained at almost 100% of original value after repeated use (2, 3, 13).

In a solvent-free system, which does not contain any organic solvents in the reaction mixture, hydrophobic lipids entangle with enzyme supports during the hydrolysis reaction. This requires an increase in the agitation speed during reaction and makes separation difficult after reaction. However, a solvent-free system is safer and does not require special facilities compared with the two-phase system that uses organic solvent (5, 9, 15, 16).

The objectives of this research are to study the effects of temperature, pH, and agitation speed on the hydrolysis of olive oil as well as to find a proper lipase and a support for immobilization. The effects of repeated use of immobilized lipases on the hydrolysis reactions in a two-phase system and a solvent-free system were also studied.

## MATERIALS AND METHODS

The lipases used in this study were Lipolase 100T, Lipolase 100L, Lipase OF, Lipase PL, Burcotase LP-30, and lipase from *Pseudomonas fluorescens*. Lipase OF was obtained from Meito Sangyo Co. Ltd., Japan and the other enzymes were obtained from NOVO Industry, Nordisk, Denmark. Olive oil (Sigma Chemical Co., St. Louis, MO) was used as a substrate. Cupric acetate (Sigma Chemical Co.) was used to determine the content of fatty acids. Other chemicals used in this study were reagent grade or higher. Polyethylene, polypropylene, homo-polymer, block-copolymer (Dongyang Nylon Co.), glass beads (Han-glass Co.), Amberite 200, and XAD-4 (Sigma Chemical Co.) were used as enzyme supports.

**Immobilization of Lipase**

Lipase was immobilized on various supports by physical adsorption methods (2, 3). High density polyethylene, polypropylene, homo-polymer, and block-polymer were

*Corresponding author
Phone: 82-32-860-7517. Fax: 82-32-875-0827.
E-mail: hyunshik@dragon.inha.ac.kr.
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used as hydrophobic supports; and glass bead, Amberite 200, and XAD-4 were used as hydrophilic supports. Binding efficiency and hydrolysis were measured for these supports. The immobilization procedures were as follows. The hydrophobic group of lipases were immobilized on the hydrophobic support by physical adsorption. Lipase (0.15 g) was dissolved in 25 mM phosphate buffer solution (100 ml, pH 7.0) and then the support prewetted with ethanol was added. After two hours of adsorption in a shaking incubator (35°C, 250 rpm), immobilized enzymes were filtered and dried before hydrolysis. The amount of enzymes immobilized on the supports were estimated by measuring the concentration of enzyme in the solution before and after immobilization by the Bradford protein assay method. Scanning electron micrographs of the surface of the support particles were taken with a Hitachi SEM X650 scanning electron microscope using a 70,000× magnification to verify the immobilization of the enzymes on the support.

**Assay**

The amount of free fatty acids were measured by modified cupric acetate assay (11) and the titration method (9). In the titration method, the reaction was ceased by adding 20 ml of acetone/ethanol (1 : 1, v/v). Two to three drops of phenolphthalein were added and then the solution was titrated with 0.1 N KOH. The hydrolysis (%) of the lipid was calculated by the following equation (11).

$$\text{hydrolysis (\%)} = \frac{\mu\text{mol fatty acid liberated}}{(\text{saponification value})\,(1000/56.1)\,(\text{g oil})} \times 100$$

**Hydrolysis of Olive Oil by Immobilized Lipase in Two-phase System**

In the two-phase system, the bottom phase is water and the top phase is organic solvent. To investigate the effects of organic solvent on the hydrolysis, chloroform, acetonitrile, ethanol, benzene, hexane, and isooctane were used. The organic solvent that showed the highest reactivity was selected by comparing hydrolysis (%). In the two-phase system, the amount of free fatty acid was measured by the modified cupric acetate method (11). The composition of reactants was: 15 ml of enzyme solution, 5 ml of organic solvent, and 5 g of olive oil in the soluble enzyme experiment and 2 g of immobilized enzyme, 5 ml of buffer solution, 10 ml of organic solvent, and 5 g of olive oil in the immobilized enzyme experiment. Various buffer solutions were prepared to study the effect of pH on the hydrolysis reaction. Phosphate buffer solution was used for pH 6, 7, and 8. Succinate buffer solution was used for pH 4 and 5. Glycine-NaOH buffer solution was used for pH 9 and 10. After hydrolysis reaction, the immobilized enzymes were recovered by No. 5 Wattman filter paper and dried using a vacuum pump. The enzyme reuse experiments were performed every 24 h.

**Hydrolysis of Olive Oil by Immobilized Lipase in Solvent-free System**

Buffer solution, which is used to maintain pH during reaction, affects the activity of the enzyme by its concentration. To examine the effect of concentration of the buffer solution, hydrolysis reaction was carried out in the various concentrations of buffer solutions and in distilled water only. In this experiment, organic solvent was not used and the volume of organic solvent was replaced by an equal amount of distilled water. In the solvent-free system, titration methods were used to measure the amount of free fatty acids since the olive oil became entangled with enzymes or enzyme supports during the hydrolysis reaction and this made the sampling for cupric acetate assay difficult. The effects of agitation speed and temperature were examined in the range of 0~700 rpm and 25~75°C, respectively. In the solvent-free system, a vacuum pump was used longer than in the two-phase (water and organic solvent) system since the separation of immobilized enzymes and reactants was not easy without an organic solvent. The residual fatty acids remained in the support were extracted with organic solvent.

## RESULTS AND DISCUSSION

Various lipases were reacted with olive oil at 35°C (Fig. 1). Lipase OF (from *Candida rugosa*) showed much higher hydrolysis (%) than other lipases tested. The activity of Lipolase 100 T was the highest at 55°C (Fig. 2). It decreased significantly at temperatures higher than 55°C. Lipase from *P. fluorescens*, however, showed highest activity at 55°C and was more stable at higher

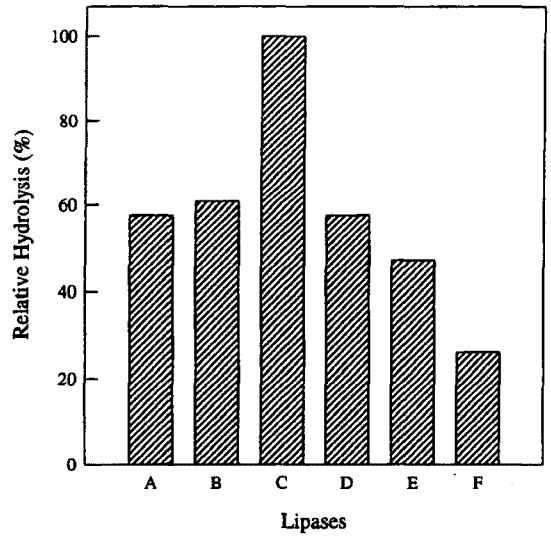


**Fig. 1.** Hydrolysis of olive oil by various lipases.
A, *P. fluorescens*; B, lipolase 100L; C, lipase OF; D, lipolase 100T; E, burcotase LP-20; F, lipolase-PL.

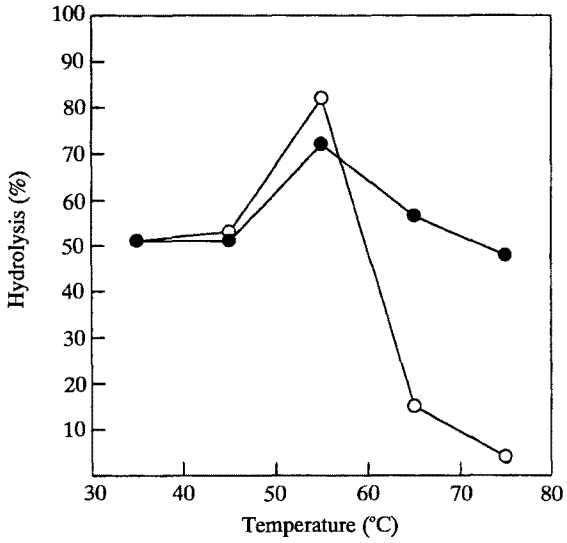


**Fig. 2.** Hydrolysis of olive oil by *Pseudomonas fluorescens* lipase and Lipolase 100T at various temperatures.
●, *P. fluorescens* lipase; ○, Lipolase 100T.

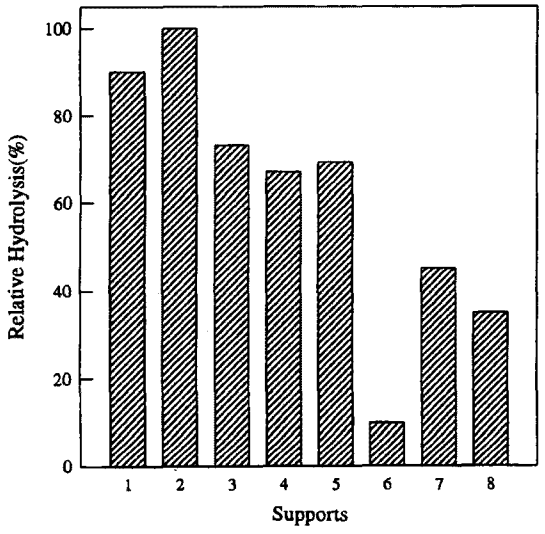


**Fig. 3.** Hydrolysis of olive oil by lipase immobilized on various supports.
1, soluble lipase; 2, HDPE; 3, blockcopolymer; 4, homopolymer; 5, polypropylene; 6, glass bead; 7, AM 200; 8, XAD4.

temperatures.

Immobilization of enzymes on a solid carrier is important for repeated use and enhanced recovery. Among the enzyme supports tested, a hydrophobic support, HDPE showed the highest hydrolysis (%) (Fig. 3). The amount of adsorbed enzymes was estimated by measuring the absorbance (595 nm) of enzyme solution using the Bradford method before and after enzyme immobilization. On one gram of HDPE, 11.5 mg of lipase

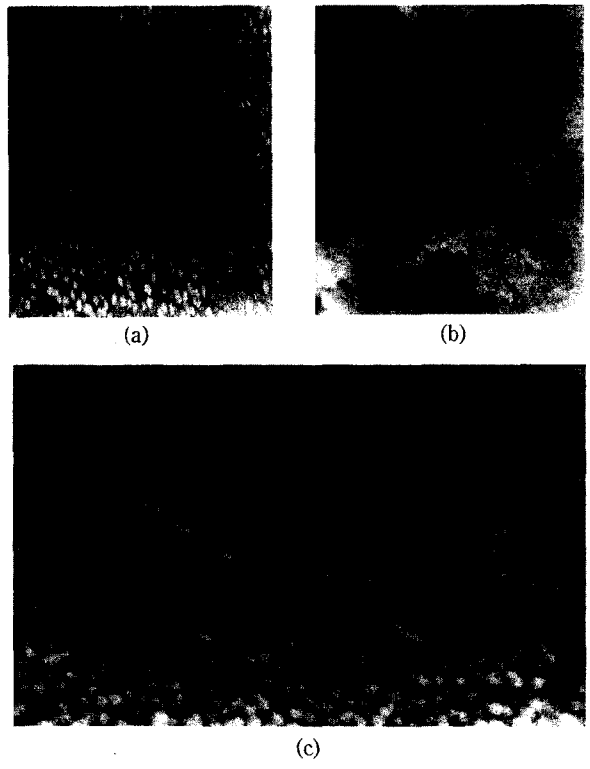


**Fig. 4.** SEM photograms of support and immobilized lipase (original magnification 70,000×).
(a), before ethanol prewetting; (b), after ethanol prewetting; (c), loaded with enzyme.

was adsorbed and the activity was 250 Units (One Unit was defined as the amount of fatty acid liberated (μmol) per one minute.). The lipase immobilized on the HDPE surface was verified by observing the surface of the support by SEM before and after immobilization. The scanning electron micrographs of the surface before and after ethanol treatment and after immobilization are shown in Fig. 4. When enzyme supports were treated with ethanol before immobilization, the impurities on the surface of the support could be removed and resulted in an increase in the binding efficiency (percentage of bound protein over total protein added).

When 1,3-specific lipase, Lipolase 100T (from *Aspergillus niger*) and non-specific lipase, Lipase OF (from *Candida rugosa*) were used together, the hydrolysis (%) was higher than when either enzyme was used alone (Fig. 5). This phenomenon was observed with both soluble lipase and immobilized lipase experiments. Furthermore, the enzyme cost could be lowered by using an enzyme mixture since the cost of Lipolase 100T is much higher than that of Lipase OF. For immobilized lipase, maximum hydrolysis was obtained with a 2 : 1 mixture of Lipase OF and Lipolase 100T. Other immobilized enzyme ex-

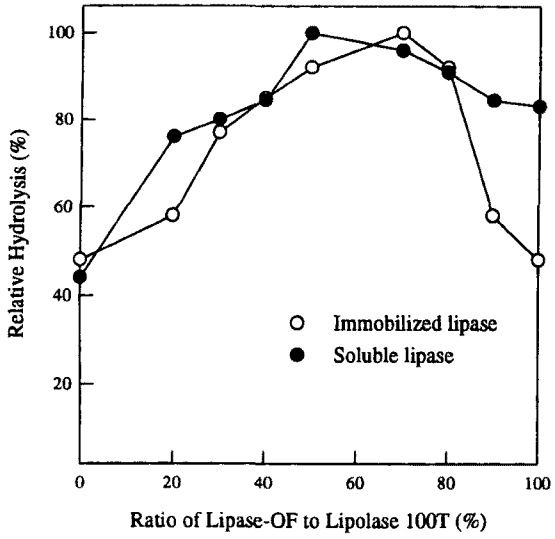


**Fig. 5.** Hydrolysis by various ratios of Lipase OF to Lipolase 100T concentration.

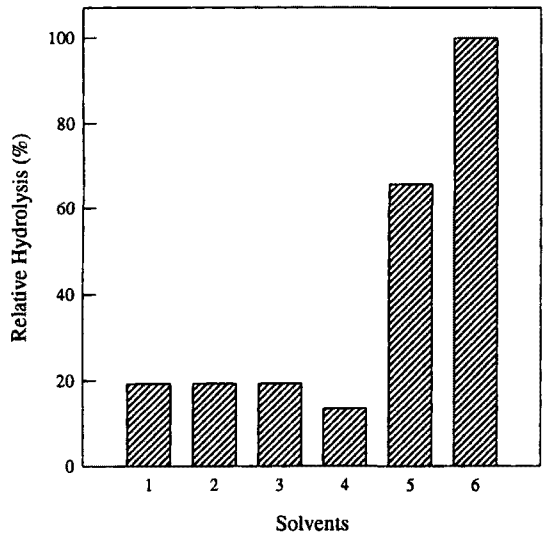


**Fig. 6.** Hydrolysis of olive oil with various solvents.
1, chloroform; 2, acetonitrile; 3, ethanol; 4, benzene; 5, hexane; 6, isooctane.

periments were carried out using the mixture.

**Hydrolysis of Olive Oil by Immobilized Lipase in Two-phase System**

In the two-phase system, the bottom phase is water and the top phase is organic solvent. Olive oil is dissolved in the solvent phase. Lipase immobilized on HDPE participates between the two phases. As a result, the organic solvent improves the contact of enzymes and substrates during the reaction and the separation of the products and enzymes, since fatty acids locate in the top phase and glycerol locates in the bottom phase after the hydrolysis reaction (7, 16). As shown in Fig. 6, isooctane (2,2,4-trimethylpentene) showed the highest hydrolysis (%) among the organic solvents tested. In the two-phase system the enzymes remain in a relatively water-rich phase that includes a low concentration of organic solvent (6) and some polar solvents are found to decrease the stability of enzymes (17). As a result, the low water solubility (0.064 (w/w)) and non-polarity of isooctane resulted in the highest hydrolysis in two-phase system using the organic solvents tested. The effect of pH on the hydrolysis of olive oil is shown in Fig. 7. The optimum pH of Lipase OF was pH 7. However, for both soluble and immobilized lipases, the optimum pH of the enzyme mixture (2 : 1 mixture of Lipase OF and Lipolase 100T) was 8. The immobilized enzyme has the same pH optimum as the soluble lipase (Fig. 7). Immobilized lipase showed a greater stability at lower pH values. However, the hydrolysis of soluble lipase decreased significantly at a pH higher than 8. In the presence of organic solvent, the increased stability of immobilized enzymes is probably due to the fact that immobilization fixes the enzyme conformation and increases the hydrophilicity (8). The hydrolysis rate increased as the agitation speed increased up to 250 rpm (Fig. 8). The hydrolysis of olive oil is considered to take place at the interface between the organic and aqueous. Hence the reaction rate is a function of the interfacial area, which is dependent on the agitation speed. Agitation speeds higher than 250 rpm did not increase the hydrolysis rate, which implies that the interfacial area is filled with enzyme molecules at that agitation speed. The ability to retain activity in consecutive reactions is an important factor to determine the practical utility of the enzyme. The lipase

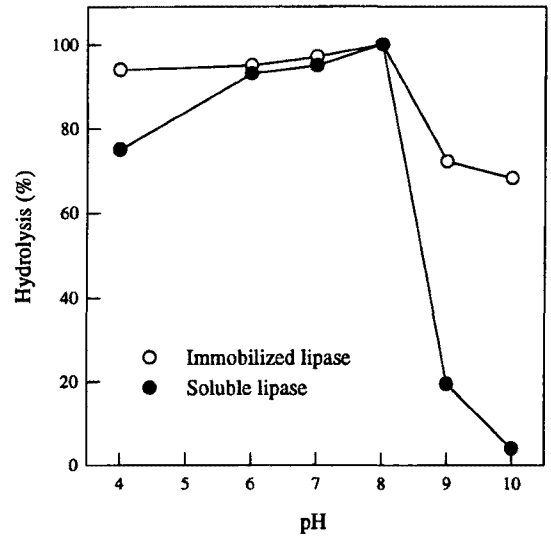


**Fig. 7.** Effect of pH on hydrolysis of olive oil.

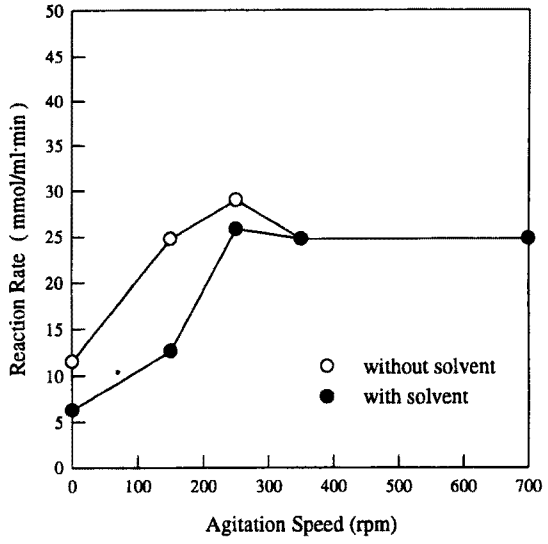


**Fig. 8.** Effect of agitation speed on the hydrolysis rate of olive oil.

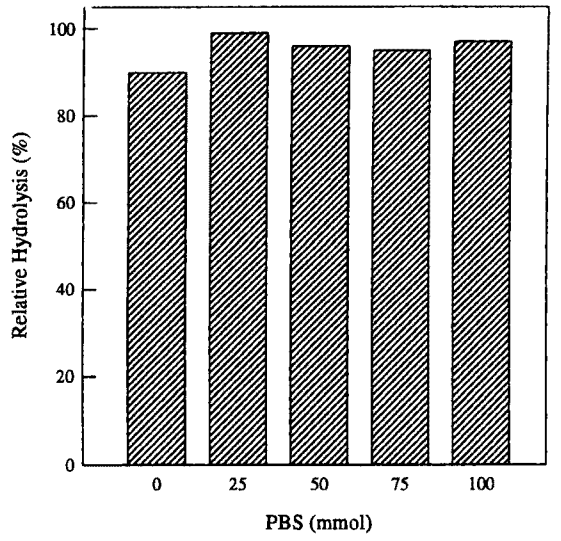


**Fig. 10.** Hydrolysis of olive oil with various phosphate buffer solution concentrations.

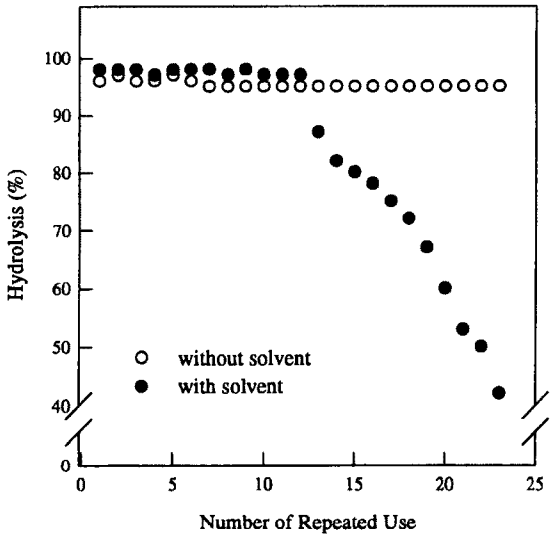


**Fig. 9.** Effect of repeated use on hydrolysis of olive oil in two-phase system and solvent-free system.

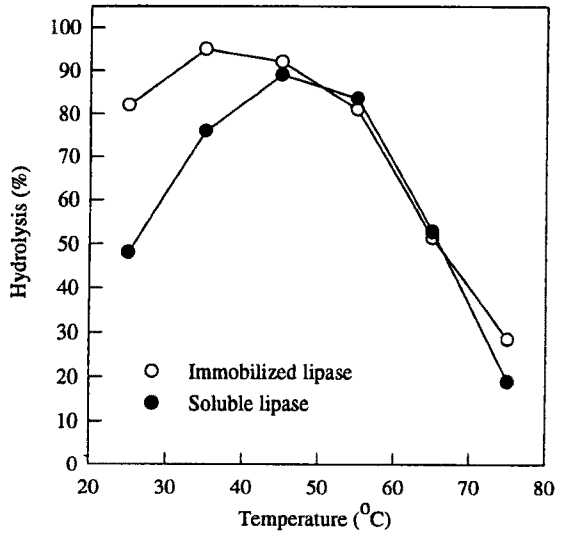


**Fig. 11.** Effect of temperature on hydrolysis of olive oil.

immobilized on HDPE maintained most of its activity through up to 12 runs of repeated hydrolysis reactions. The activity decreased to 60% of the original value after 20 runs of the experiment (Fig. 9).

**Hydrolysis of Olive Oil by Immobilized Lipase in Solvent-free System**

As shown in Fig. 10, the concentration of phosphate buffer solution did not significantly affect the activity of the immobilized enzyme in the solvent-free system. The replacement of the buffer solution by distilled water also did not influence the activity of the enzymes. Later experiments in a solvent-free system were carried out using distilled water. When fatty acids are purified after hydrolysis, the salts in the buffer solution have to be removed. By using distilled water instead of the buffer solution in the hydrolysis reaction, this step could be omitted. The effect of temperature on hydrolysis is shown in Fig. 11. The immobilized lipase showed the highest hydrolysis at 35°C whereas the soluble lipase showed the highest performance at 45°C. As shown in the figure, the immobilized lipase maintained its activity at broader temperature ranges (25~55°C) than the soluble lipase. The effect of agitation speed showed similar trend

with the two-phase system (Fig. 8). The highest reaction rate was observed at 250 rpm. Further increases in agitation speed did not affect the reaction rate. Probably this is because there is more contact between oil and enzymes in the solution phase as the agitation speed increases up to 250 rpm. In the solvent-free system the separation of fatty acids after reaction is difficult as they are combined with the supports. However, the decrease in enzyme activity could be avoided. Fig. 9 shows the effect of repeated use on olive oil hydrolysis by the immobilized lipase. The immobilized lipase in the solvent-free system maintained most of its activity even after 23 runs of the experiment.

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