Continuous Production of Fatty Acids from Palm Olein by Immobilized Lipase in a Two-Phase System

Aran H-Kittikun*, Poonsuk Prasertsan, and Chatchai Sungpud

Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112, Thailand

ABSTRACT: Commercial lipases were tested for the ability to hydrolyze palm olein in isooctane in a two-phase system. Lipase OF (from *Candida rugosa*) showed the highest specific activity of 209 U/mg protein where 1 U is the amount of lipase enzyme required to produce 1 µmol of fatty acid (as palmitic acid) per minute. The enzyme was adsorbed completely on Accurel EP100 (particle size <200 µm) with 20.5% activity retained. The soluble and the immobilized lipase OF showed optimal activity at the same pH and temperature (pH 6.5–7.5 and 35°C). However, the immobilized lipase had a wider range of pH and higher temperature stability. Continuous hydrolysis of palm olein was performed in a packed-bed reactor with 656 U of immobilized enzyme. The substrate (20% palm olein in isooctane) and Tris/maleate buffer were fed concurrently at the flow rates of 0.08 and 0.04 mL/min, respectively. The system gave a degree of hydrolysis (DH) of 90–100% for up to 250 h. A more stable system allowing for more than 300 h operation at DH > 95% was achieved by mixing the immobilized enzyme with 1000–1500 µm Accurel EP100 to increase the system porosity and continuous feeding of the aqueous phase recycling from the product mixture. A similar result was also obtained using 1007 U of the immobilized enzyme and 60% palm olein in isooctane fed at 0.06 mL/min.

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KEY WORDS: Accurel, *Candida rugosa,* fatty acids, immobilized lipase, lipase, palm olein, two-phase system.

Industrial production of fatty acids from fats and oils is generally achieved by a fat-splitting process. The fats are hydrolyzed by countercurrent treatment with steam at high temperature and pressure (250°C and 60 bar). Besides fatty acids, undesirable compounds such as ketones and hydrocarbons are produced under these drastic conditions (1). Enzymatic hydrolysis of fats using lipases offers an alternative method for fatty acid production due to its mild reaction conditions. However, one of the major problems in hydrolysis by lipases is that the catalytic activity of the enzyme occurs in a heterogeneous system containing water and insoluble substrate. The biphasic process, or the organic two-phase system in which the fat or oil is dissolved in an organic solvent and hydrolyzed by lipases, has been investigated by many scientists (2–7). Use of an organic solvent, such as isooctane or cyclohexane, in the reaction medium increased the rate of hydrolysis of triacylglycerols by lipase (8–10). The cost of the lipases necessary for catalysis is very high and therefore the application in industry is still in its infancy. This situation would change if the price of lipase were reduced or the quality requirement of free fatty acid increased. The use of immobilized lipase for continuous production of free fatty acids not only improves the quality of the final product but may also reduce the cost of the enzyme, and is friendly to the environment.

In this study various lipases were used to hydrolyze palm olein in an organic solvent two-phase system. The continuous production of fatty acids in a packed-bed reactor was also studied.

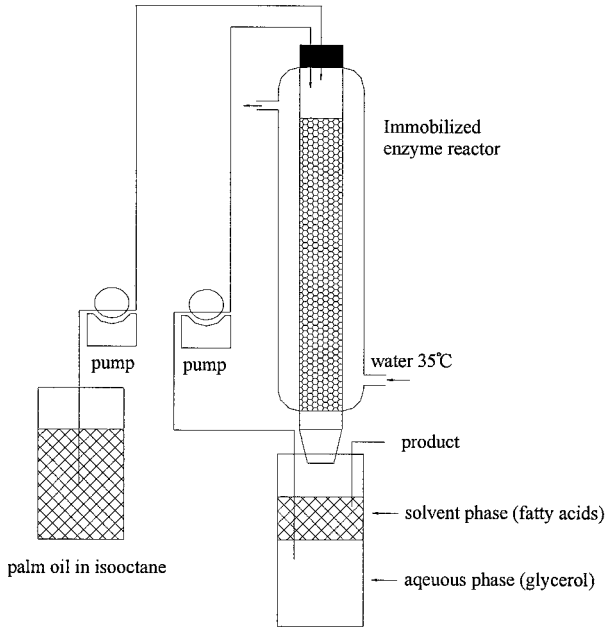
*To whom correspondence should be addressed.
E-mail: haran@ratree.psu.ac.th

EXPERIMENTAL PROCEDURES

Materials. Lipase PS *(Burkholderia cepacia),* lipase L *(Candida lipolytica),* lipase FAP-15 *(Rhizopus oryzae),* and lipase AK *(Pseudomonas fluorescens)* were kindly supplied by Amano Pharmaceutical Co. (Nagoya, Japan). Lipase OF-360 *(C. rugosa)* was a gift from Meito Sangyo Co. (Osaka, Japan). All lipases were used as received with no further purification (11). Microporous polypropylene powder, Accurel EP100 (particle size <200 µm) provided by Membrana GmbH (D-63785 Obernburg, Germany), was used as a support to immobilize lipase. The palm olein was obtained from commercial cooking oil in Thailand. Other chemicals used were standard analytical grade.

Immobilization of lipase. Pretreatment of Accurel EP100 (200 mg) was done by adding 1.2 mL of absolute ethanol for a few minutes. Then the required amount of lipase dissolved in 20 mL of 50 mM Tris/maleate buffer pH 7.0 was added to the support and shaken at 200 rpm at 4°C for 24 h. The immobilized lipase was collected by filtration, washed twice with the buffer (40 mL) followed by distilled water (20 mL), and dried under vacuum in a desiccator at room temperature until no further weight loss occurred.

Hydrolytic activity. The lipase activity was assayed in a two-phase system according to Lee and Rhee (12). The reaction mixture consisted of 1.0 mL of 10% (wt/vol) palm olein dissolved in isooctane, 0.5 mL buffer, and 0.2 mL of enzyme solution or 2.0 mg of immobilized lipase. The reaction mixture was incubated in a shaker at 250 rpm and 35°C for 30



SCHEME 1

min. The reaction was stopped by adding 0.3 mL of 6.0 M HCl. The free fatty acids (FFA) dissolved in the isooctane layer were determined by the cupric acetate method as described by Kwon and Rhee (13).

One unit of enzyme activity (U) is defined as the amount of enzyme required to produce 1 µmol of FFA (as palmitic acid) per minute. The degree of hydrolysis (DH) of the lipid is expressed as the percentage of FFA liberated. When DH = 100%, all the oil has been hydrolyzed.

The effects of pH and temperature on the hydrolytic activity and stability (24 h) of the native and immobilized enzyme were studied over the pH range 4.5–8.0 and at temperatures from 30–60°C. Duplicate runs were carried out for each experiment.

Experimental apparatus for continuous hydrolysis. A schematic diagram of the packed-bed column reactor (PBR) containing immobilized lipase and used to hydrolyze palm olein is shown in Scheme 1. The reactor consisted of a glass cylinder with a working volume of 6.0 mL (inside diameter 0.62 cm, height 20 cm). The immobilized enzyme was packed into this column. The temperature of the reactor was controlled by running water into the jacket at 35°C. The substrate and aqueous phase were fed concurrently into the PBR with a volumetric ratio of 2:1. The substrate consisted of palm olein dissolved in isooctane, and the initial aqueous phase was 100 mM Tris/maleate buffer (250 mL). Later it was circulated from the aqueous phase of the product. Fatty acids and glycerol produced were determined at the outlet by a cupric acetate method (13) and a chromogenic method (14), respectively.

RESULTS AND DISCUSSION

Hydrolytic activity of different lipases. Five commercial lipases from *C. rugosa* (lipase OF), *C. lipolytica* (lipase L), *B. cepacia* (lipase PS), *P. fluorescens* (lipase AK), and *R. oryzae* (lipase FAP-15) were compared for their ability to hydrolyze palm olein in organic solvent in a two-phase system. The results (shown in Table 1) indicated that lipase OF showed the highest specific activity (209 U/mg protein). When using different lipases at the same activity levels (10 U) to hydrolyze palm olein in a two-phase system for 24 h, lipase OF showed the highest specific activity and achieved nearly complete hydrolysis. Lipase from *C. rugosa* can randomly hydrolyze all the ester bonds of triacylglycerols with the ability to liberate all types of acyl chains regardless of their position in the triacylglycerol (15). Therefore, lipase OF was selected for further studies.

Effect of pH and buffer on activity and stability. The effect of various buffers on the hydrolytic activity of soluble lipase OF is shown in Figure 1A. The enzyme showed the highest activity between pH 6.5–7.0 and the Tris/maleate buffer allowed higher relative activity than the other buffers. Immobilized lipase OF showed optimal activity at pH 7.0. The effect of buffer components on the enzyme activity depended on the buffering capacity, type of charge, and strength. The buffer components may alter the composition or electrostatic condition of the interface, which could affect the direction of pH shift, the state of enzyme hydration, or the active conformation of the enzyme (16).

TABLE 1
Activity of Different Lipases in Palm Olein Hydrolysis in a Two-Phase System

Enzyme origin	Commercial code	Activity[a] (U mg^{-1} powder)	Specific activity (U mg^{-1} protein)	Degree of hydrolysis[b] (%)
Candida rugosa	OF	49	209	97
Burkholderia cepacia	PS	12	155	75
Pseudomonas fluorescens	AK	17	122	45
C. lipolytica	L	7	124	43
Rhizopus oryzae	FAP-15	57	35	60

[a]Activity of lipase determined by the cupric-acetate two-phase emulsion method (12). Protein levels determined by the Lowry method (11).
[b]The reaction mixture contained 10% palm olein in isooctane (1.0 mL), 100 mM Tris/maleate buffer (pH 7.0, 0.5 mL), and enzyme (10 U, 0.2 mL). Incubation time: 24 h. 1 U is defined as the amount of lipase required to produce 1 µmol of fatty acid (as palmitic acid) per minute.

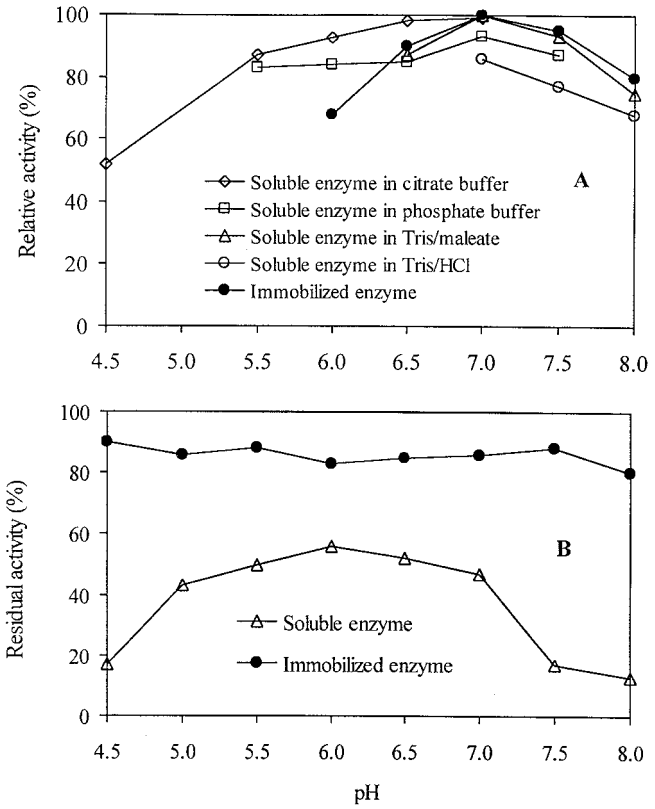


FIG. 1. Effect of pH on the activity (A) and stability (B) of soluble and immobilized lipase OF from *Candida rugosa* (Meito Sangyo Co., Osaka, Japan).

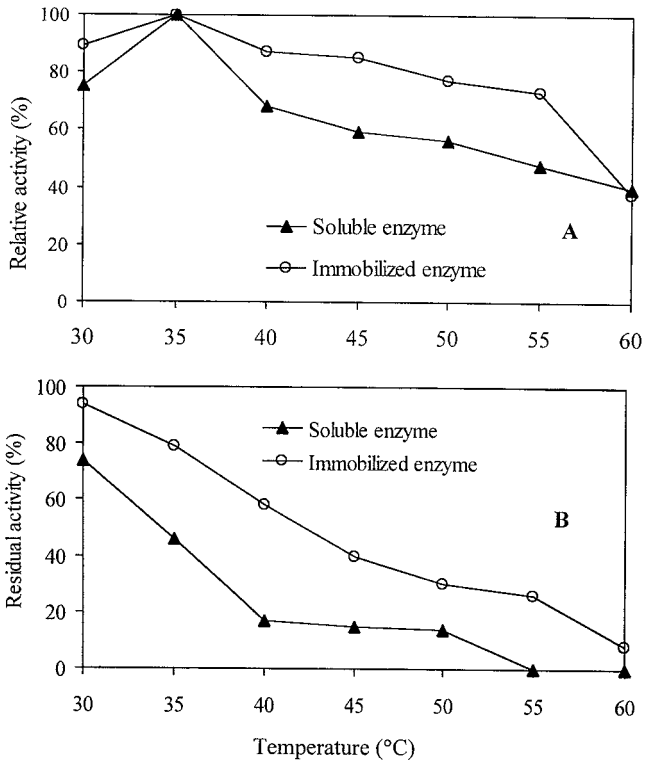


FIG. 2. Effect of temperature on the activity (A) and stability (B) of soluble and immobilized lipase OF. For enzyme information see Figure 1.

Lipase OF immobilized on Accurel EP100 was stable over a wide range of pH values, from 4.5–8.0 (Fig. 1B). The microenviroment in the microporous support most likely prevented a pH shift because it had a nonpolar nature, and the diffusion of buffer from macroenviroment to microenviroment was difficult (17). It was noted that the residual activity of the immobilized lipase was higher than the residual activity of the soluble lipase.

Effect of temperature on activity and stability. The maximal activity of soluble and immobilized enzymes was observed at 35°C (Fig. 2A). To investigate thermal stability, the enzyme was incubated at various temperatures for 24 h. Immobilized lipase OF had higher thermal stability than soluble lipase (Fig. 2B), which confirmed the report of Virto *et al.* (15).

Continuous hydrolysis of palm olein. The continuous production of fatty acids from palm olein was performed with immobilized lipase OF in the PBR. First, only the immobilized enzyme on Accurel EP100 (<200 µm) was packed into the column, with a total enzyme activity of 656.8 U (0.8 g) being employed. The substrate, consisting of 20% palm olein in isooctane, was fed at a flow rate of 0.08 mL/min. Palm olein was hydrolyzed to a DH in the range of 90–100%, a fatty acid production rate of 3 mmol/h, and a mean glycerol concentration of 25 mg/mL for 250 h (Fig. 3A). After that the DH decreased drastically owing to column clogging. In order to solve this problem, the immobilized lipase OF was mixed with the larger size of Accurel EP100 (1000–1500 µm) in a 2:1 ratio before filling a column so as to increase the system porosity. The substrate (20% palm olein in isooctane) was fed at a flow rate of 0.08 mL/min concurrently with Tris/maleate buffer, which later was recycled from the aqueous phase of the product mixture. The palm olein was hydrolyzed to a DH > 95% for more than 300 h (data not shown). When the substrate concentration was increased to 40% palm olein in isooctane and fed at the flow rate 0.08 mL/min, the reactor could be operated continuously with a DH > 95%, at a fatty acid production rate of 6.5 mmol/h. Glycerol accumulated in high concentration in the aqueous phase (Fig. 3B).

When the palm olein concentration in isooctane was increased to 60% and fed at a flow rate of 0.06 mL/min the hydrolysis of the oil was incomplete. Only 83% DH was observed at the start of the experiment, and this decreased to 40% after 120 h (Fig. 4A). This was related to the high concentration of the substrate over the capacity of the immobilized enzyme. Therefore, the concentration of the immobilized enzyme was increased to 1007 U (0.8 g). The process could then be operated more than 260 h with complete hydrolysis of palm olein (DH 100%), a fatty acid production rate of 7.8 mmol/h, and a glycerol concentration that increased to 420 mg/mL at 396 h. Since the glycerol concentration in the circulated buffer was high, new buffer was used from that time on. The process operated well to 500 h, with a DH of 70% at that time (Fig. 4B). The results in Figures 3 and 4 show that the enzyme and substrate concentrations have a profound effect on the DH. If

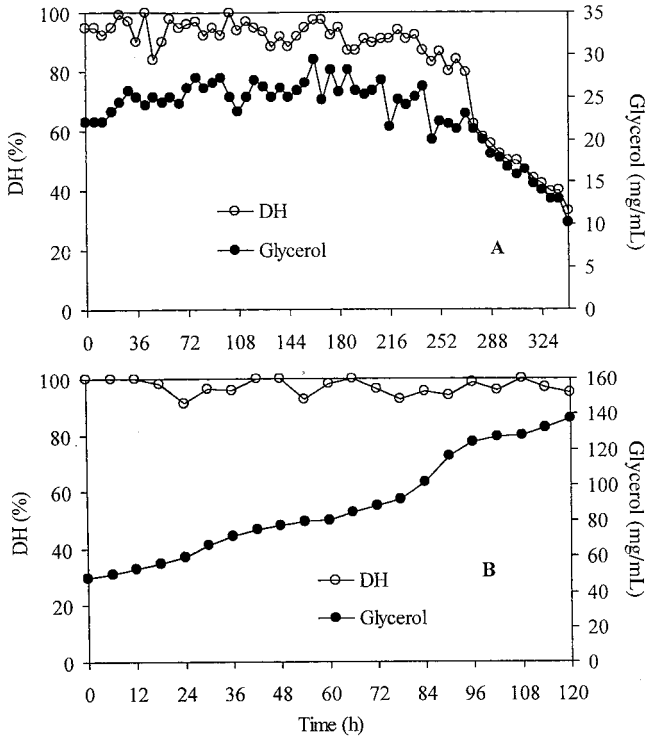


FIG. 3. Continuous hydrolysis of palm olein in a two-phase system with immobilized lipase OF. (A) Immobilized lipase 0.8 g (656 U); substrate (20% palm olein); flow rate 0.08 mL/min; and no recycling of buffer; and (B) immobilized lipase 0.8 g (656 U) mixed with Accurel (Membrana GmbH, Obernburg, Germany) size 1000–1500 µm; substrate (40% palm olein); flow rate 0.08 mL/min; and recycling of buffer. DH, degree of hydrolysis; for enzyme information see Figure 1. 1 U is defined as the amount of lipase required to produce 1 µmol of fatty acid (as palmitic acid) per minute.

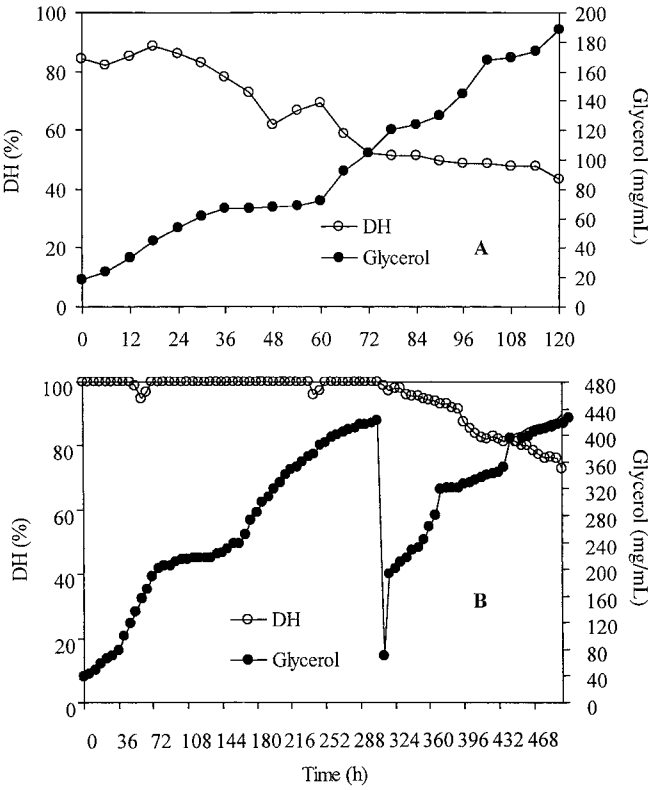


FIG. 4. Continuous hydrolysis of 60% palm olein in a two-phase system with immobilized lipase OF. (A) 656 U and (B) 1007 U. (Immobilized lipase, 0.8 g, mixed with Accurel size 1000–1500 µm; substrate flow rate 0.06 mL/min; recycling of buffer). For laboratory suppliers see Figures 1 and 3 and for abbreviation see Figure 3.

the PBR was properly optimized in terms of immobilization, substrate flow rate, and phase mixing, it is probable that the productivity could be improved.

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