Large-Scale Immobilization of Lipase from *Pseudomonas fluorescens* Biotype I and an Application for Sardine Oil Hydrolysis

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ABSTRACT: Large-scale production of thermostable lipase from *Pseudomonas fluorescens* biotype I was carried out in a fermenter with an antifoaming agent and physical deforming treatments. After cultivation, heat treatment was applied to kill the bacteria and to inactivate other enzymes. Large-scale immobilization of the lipase to a macroporous weak-anion exchange resin was performed with a lipase solution that had an ionic strength of less than 0.1 and an ethanol concentration of 50%. Almost all eicosapentaenoic acid and docosahexaenoic acid were liberated continuously from sardine oil by the immobilized lipase in a countercurrent fluidized-bed reactor. The cost of enzyme used in the reactor has been compared with a process in which soluble lipase from *Candida rugosa* was used. *JAOCS 72,* 1281–1285 (1995).

KEY WORDS: Antifoam, cost of enzyme, docosahexaenoic acid, eicosapentaenoic acid, immobilized lipase, lipolysis, *Pseudomonas fluorescens* biotype I, sardine oil, scale-up.

Lipase attacks ester bonds that bind hydrophobic and hydrophilic residues in amphiphilic lipids. There are many possibilities for application of lipase in the oleochemical industry such as ester synthesis, lipolysis, transesterification, and optical resolution of racemic compounds. These lipase processes are carried out at ambient temperature and atmospheric pressure. They are suitable for use in small-scale factories. We used immobilized lipases for enzymatic refining of rice bran oil high in free fatty acid (FFA) (1,2), synthesis of triacylglycerols from polyunsaturated fatty acid (PUFA) (3), and continuous lipolysis in a loop reactor (4). The running cost, energy cost, and the capital investment required for setting up the process should not be too high, and the process can be assessed from the standpoints of enzyme cost and the value added by the process. Lowering the cost of immobilized lipase is an important factor for industrial application of such enzymatic processes.

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Many carriers for lipase immobilization are hydrophobic in nature (5–7). To immobilize high-density lipase on hydrophobic carriers is difficult because of the lipase's water-soluble nature. In previous reports on lipase immobilization, purified enzymes were used (8–10). However, the cost of immobilized pure lipase is too high. Adsorption of lipase on hydrophobic polymer supports is promoted by pretreatment of the support with ethanol to increase the hydrophilicity or water wettability (5,6). Montero *et al.* (5) immobilized lipase from *Candida rugosa* on a polypropylene support that had been pretreated with ethanol. An enzyme loading of 1,500 units per gram of support, which had the maximum activity yield (about 7%), was achieved.

We reported previously on the lipase from *Pseudomonas fluorescens* biotype I, which hydrolyzed oil at more than 90% at 50–70°C (11). We also described functional immobilization of the thermostable lipase so that product inhibition of lipolysis was eliminated (12). With the immobilized lipase, lipolysis was performed in a continuous countercurrent fluidized-bed reactor for more than three months (13). This paper describes an industrially applicable procedure for preparing the functional immobilized lipase and for using it in the hydrolysis of oils that contain PUFA.

EXPERIMENTAL PROCEDURES

Materials. The supports used were Dowex MWA-1 and Dowex 66 (The Dow Chemical Co., Midland, MI), which are macroporous weakly alkaline anion exchange resins; Dowex 66 is mechanically stronger than Dowex MWA-1. Immobilized lipase of Enzylon PF was obtained from Rakuto Kasei Co. Ltd. (Ootsu, Japan). More than 2,500 units of lipase from *Pseudomonas fluorescens* biotype I (11) was adsorbed on 1 g of Dowex MWA-1. Adecanol LG109 was obtained from Asahi Electro-Chemical Co., Ltd. (Tokyo, Japan), sardine oil from Yoneyama Reagent Co. (Osaka, Japan), olive oil from Tsukishima Yakuhin Co. (Tokyo, Japan), tung oil from Nakarai Chemicals Ltd. (Kyoto, Japan), linseed oil from Kanto Chemical Co. Inc. (Tokyo, Japan), and castor oil from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Enzyme assay methods. Lipase activity was measured with

an olive oil (12.5%) emulsion as described previously (11). Five mL of olive oil emulsion, 4 mL of 0.1 M phosphate buffer at pH 7, and 1 mL of lipase solution were incubated at 60°C. To stop the reaction, 20 mL acetone/ethanol (1:1) mixture was added, followed by titration with 0.05 N sodium hydroxide solution. Residual activity of immobilized lipase was obtained as follows. The Enzylon PF lipases (new and used) were washed with hexane three times, once with a mixture of hexane and ethanol (1:1), and twice with ethanol. After that, the enzymes were ground, and both activities were measured by the nonemulsion method (13). The reaction mixture was composed of 1 g olive oil and 0.2 mL of enzyme solution dissolved in 0.1 M phosphate buffer at pH 7. The mixture was incubated at 60°C with shaking. The reaction was stopped by the addition of 10 mL chloroform/methanol mixture (2:1). One unit of lipase was defined as the amount of enzyme that liberates 1 μmole of fatty acid per min. Protein was assayed by the method of Lowry *et al.* (14) with bovine serum albumin as the standard.

Batch hydrolysis of oil. The reaction mixture was composed of 1 g oil, 1 g water, and 1 g of immobilized lipase, in a 50-mL Erlenmeyer flask. It was incubated with shaking at 60°C. Each flask was plugged with a silicone-rubber stopper to prevent evaporation of the reaction mixture. The hydrolysis ratio was obtained from the acid value and the saponification value of the oil in the reaction mixture (11,12).

Immobilization ratio. Lipase activity in the washing water was measured to obtain adsorbed lipase activity, and the immobilization ratio was calculated as follows: Immobilization ratio (%) = 100 × (applied lipase activity – lipase activity in the washing water)/applied lipase activity.

Fatty acid fraction from the hydrolysate. In a 100-mL Buchner funnel, 10 mL hexane, 10 mL water, 10 mL methanol, and 0.1 mL of hydrolysate were mixed, and 0.5 M ethanolic KOH was added until the mixture became red with phenolphthalein. The mixture was settled to separate the water phase and the organic phase. The water phase was collected, and the organic phase was extracted again with 50 mL water. Both water phases were combined, and 0.5 M HCl was added slowly until nearly pH 1.8. The fatty acid fraction was extracted with 4 mL hexane. The purity of this fraction was more than 98% fatty acid. Each sample was saponified to avoid triacylglycerol contamination in the capillary column. The saponified sample was methylated by boron trifluoride methanol complex (Merck 801663; Merck, Darmstadt, Germany). Fatty acid compositions were determined by gas chromatography with a capillary column. The column was a fused-silica WCOT with stationary phase CP-Sil-88 (Chrompack, Middelburg, The Netherlands). The content of the components was determined by high-performance liquid chromatography with gel-permeation liquid chromatography columns as described previously (1).

Countercurrent fluidized-bed reactor. In the reaction compartment, 64.4 g of Enzylon PF was placed as shown in Figure 1. Sardine oil and water were provided in the reaction compartment. Pulse agitation was provided from the bottom

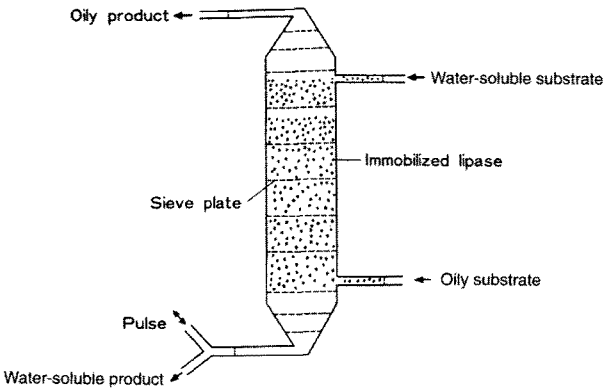

FIG. 1. Schematic diagram of countercurrent fluidized-bed reactor.

of the reactor by the pump (Nihon Feeder Industrial Co., Osaka, Japan). The pulse volume was 15 mL and provided 15 times per minute. The substrates and the product were saturated with nitrogen gas to avoid oxidation. The reaction compartment and separation compartment in the reactor were maintained at 50°C. The pipes to supply sardine oil and to collect oil product were heated with a ribbon heater to avoid choking. The speeds for providing sardine oil and collecting oil product were 5 mL/h. The speeds for water and water-soluble product were almost half of the oil speed. The total volume of the reactor was 421 mL. The net volume without the volume of immobilized lipase and sieve plates was about 200 mL. The mean residence time of oil was 40 h and that of water was 80 h.

Peroxide value. The peroxide value (meq/kg) was measured by the standard oil and fat assay methods of the Japan Oil Chemists' Society (2.4.12-86) as described previously (1). The hydrolyzate was extracted with hexane and dried with sodium sulfate. Then, the hexane was evaporated, and the peroxide value was determined.

Glycerol determination. The glycerol concentration was determined in the water phase of the reactor with KIO_4 (15). One mL of sample and 0.4 mL of 0.0025 M KIO_4 were reacted for 5 min. Then, 0.1 mL of 0.5 M sodium arsenate was added. After 10 min, 9 mL of chromotropic acid reagent was added (110 mg of chromotropic acid disodium salt in 10 mL water with 120 mL of 50% H_2SO_4 added). The tubes were placed in boiling water bath for 30 min. The cooled tubes were adjusted to a volume of 25 mL with water, and the absorbance at 570 nm was measured.

RESULTS

Conditions for high-density enzyme immobilization (16). Table 1 shows that immobilization of lipase directly from the culture supernatant on to Dowex 66 was poor. However, addition of 50% of a polar solvent, such as ethanol or isopropy alcohol, to the supernatant, increased the specific activity o the applied lipase remarkably, and the immobilization rati rose to 96–97%, probably due to sedimenting of most pro teins except the lipase and the increased hydrophilicity o

TABLE 1
Effect of Organic Solvent on Immobilization[a]

Additive	Supernatant activity (unit/mL)	Specific activity (unit/mg)	Immobilization ratio (%)	Hydrolysis ratio[b] (%)
Water	300	28.5	53.4	79.4
Methanol	275	25.8	92.7	89.8
Ethanol	325	30.1	96.3	90.9
Isopropanol	325	30.9	96.9	89.3
Acetone	275	28.2	97.8	74.7

[a]Additive solvent (50%) was added to a lipase solution and centrifuged at 5000 rpm (4500 × *g*) for 20 min. Lipase activity and protein content in the supernatant were measured, and the supernatant was used for immobilization of lipase on Dowex 66 (The Dow Chemical Co., Midland, MI). The supernatant (40 g) and Dowex 66 (10 g) were shaken at 8°C for one night. After that, 25% glutaraldehyde solution (1.5 g) was added and shaken at 8°C for 10 min. Then, 10% sodium bisulfite solution (2 g) was added and shaken at 8°C for 10 min. The resultant carrier was thoroughly cleaned with water and dried on a glass filter with suction.
[b]The reaction mixture, composed of 1 g of the prepared immobilized lipase, 1 g olive oil, and 1 g water, was incubated at 60°C for 48 h.

TABLE 3
Effect of Lipase Amount on Immobilization[a]

Enzyme amount (unit)	Immobilization ratio (%)	Hydrolysis ratio 4 h[b] (%)	Hydrolysis ratio 48 h[b] (%)	Hydrolysis ratio 48 h[c] (%)
900	99.4	70.5	84.3	
1,000	95.0		93.9	91.1
1,800	99.4	73.2	93.6	
2,000	93.7		92.2	88.1
3,600	98.9	83.3	93.7	
4,000	88.7		95.5	83.5
7,200	91.6	83.2	94.1	
8,000	89.0		94.4	89.5
12,000	79.6		93.0	89.9
14,400	54.9	87.0	93.0	
16,000	64.0		95.5	93.7

[a]Immobilization was carried out with a lipase solution containing 50% ethanol. Immobilized carrier was Dowex MWA-1. See Table 1 for company source. Other conditions were the same as shown in Table 1.
[b]Substrate concentration was 50%.
[c]Substrate concentration was 75%.

water wettability of the polystyrene support. Table 2 shows that the immobilization ratio was more than 97% when the lipase solution had an ionic strength of less than 0.1, indicating that electrostatic interactions had an important effect on the binding of the immobilized lipase. Table 3 shows that, when the amount of lipase increased to more than 3,600 units per gram of support, it could not improve its lipolysis ability (hydrolysis ratio at 4 h) any further, indicating that immobilization at that density made the lipase oversaturated on the support. The hydrolysis ratio in a reaction mixture with a high substrate concentration (75%) and this saturated immobilized enzyme remained high (90%), which indicates that this type of immobilization has a function that eliminates lipolysis product inhibition as reported previously (12).

Large-scale immobilization of lipase. Pseudomonas fluorescens biotype I (11) was grown in a medium composed of peanut oil 2%, monobasic potassium phosphate 0.3%, potassium nitrate 0.5%, magnesium sulfate 0.133%, sodium borate decahydrate 0.05%, polypepton 0.1%, calcium carbonate 0.03%, and Adecanol LG109 1.5%. The growth was carried out in a working volume of 1,300 L at 30°C with an agitator at 135–180 rpm and an aeration rate at 0.87 vvm in a 2,000-L fermenter. Internal pressure with the aeration was maintained at 1.25 atm during the first 14 h, and then the pressure was increased to 2.0 atm to prevent foaming. The initial agitation speed was at 135 rpm and then, after 22 h of cultivation time, it was increased from 157 to 180 rpm to break the foam mechanically. After 33 h of cultivation, 320 units of lipase per mL was produced as measured by the olive oil emulsion method. The broth was then heated to 60°C with agitation at 157 rpm for 30 min to kill the bacteria and to inactivate other enzymes. A lipase activity of 325 units per mL was observed in the culture filtrate. The filtrate was concentrated and desalted by hollow-fiber ultrafiltration (Labo Module AHL 1010; Asahi Kasei Co. Ltd., Tokyo, Japan), and the concentrated culture broth (3,250 units/mL) was used as the lipase solution. The lipase solution (120 L) and ethanol (120 L) were mixed with stirring for 1 h and filtered again to obtain 200 L of filtrate that contained 1,500 unit of lipase per mL. The filtrate (140 L) and Dowex MWA-1 (40 kg, 5,250 units/g) were mixed for 16 h at 8°C with shaking. Then, glutaraldehyde solution (25%, 4 L) was added and shaken for 10 min at 8°C to make Shiff base. The mixture was shaken with 20% sodium bisulfite solution (6 L) at 8°C for 10 min to reduce the Shiff base and the excess amount of glutaraldehyde. The resultant carrier was thoroughly cleaned with water. The immobilization ratio was 85.7%. Consequently, about 5,000 units of lipase adsorbed per gram of Dowex MWA-1.

TABLE 2
Effect of Ionic Strength on Immobilization[a]

Ionic strength	Immobilization ratio (%)	Hydrolysis ratio (%)
0	99.1	87.5
0.025	99.1	84.8
0.05	97.4	85.4
0.075	97.3	89.3
0.1	97.0	82.6
0.25	91.5	81.2
0.5	83.8	81.9

[a]Immobilization was carried out with a lipase solution containing 50% ethanol and various concentrations of NaCl solution. The lipase concentration was 293 units per g of Dowex 66. Other conditions were as shown in Table 1. See Table 1 for company source.

Lipolysis of PUFA oils by Enzylon PF (17,18). The immobilized lipase of Enzylon PF, prepared as mentioned above, was used for the lipolysis. Enzylon PF showed the following hydrolysis ratios of olive oil, sardine oil, tung oil, linseed oil, and castor oil: 94, 92, 81, 89, and 72%. The lowest activity was with castor oil, possibly due to the high content of ricinoleic acid, which converts partly to estolide. The high final hydrolysis ratios of sardine oil were obtained at 50 and 60°C. The temperature chosen to carry out the sardine oil hydrolysis was 50°C to prevent thermal decomposition of the PUFA

TABLE 4
Fatty Acid Composition (%) of Fraction from Sardine Oil Hydrolysate

Fatty acid	Methanolysis	Enzylon PF[a] Reaction time (h) (batch reaction) 1	3	5	15	48	Continuous reaction
		Hydrolysis ratio (%)					
		41	54	66	80	93	81
$C_{14:0}$	3.0	4.4	3.1	3.2	3.1	2.8	3.1
$C_{16:0}$	17.4	25.4	19.1	14.9	16.8	16.2	18.0
$C_{16:1}$	6.9	10.0	7.0	6.6	6.9	6.8	7.3
$C_{18:0}$	4.6	5.1	4.8	4.4	4.1	4.4	5.4
$C_{18:1}$	28.6	33.6	28.3	27.8	27.3	29.3	30.7
$C_{18:2}$	7.3	9.0	8.0	7.9	7.9	8.0	8.2
$C_{20:1}$	4.6	4.4	4.7	4.5	4.7	4.7	4.9
$C_{20:5}$	6.5	1.8	3.8	5.8	5.9	6.6	5.3
$C_{22:6}$	9.1	2.3	7.3	9.8	9.2	9.3	7.9

[a]Batch reaction was carried out in a reaction mixture composed of 1 g sardine oil, 1 g water, and 1 g of Enzylon PF (Rakuto Kasei Co. Ltd., Ootsu, Japan). Reaction temperature was 50°C. Other conditions are described in the Experimental Procedures section.

and because of good continuous operating conditions, especially good fluidity of the oil substrate.

The ability of lipolysis to liberate eicosapentaenoic acid (EPA, $C_{20:5}$) and docosahexaenoic acid (DHA, $C_{22:6}$) from sardine oil was determined and shown in Table 4. The immobilized lipase hydrolyzed EPA and DHA from sardine oil at a similar level as methanolysis with a hydrolysis ratio higher than 66%. This suggests that almost all EPA and DHA were liberated by the immobilized lipase.

Continuous hydrolysis of sardine oil was carried out in a countercurrent fluidized-bed reactor shown in Figure 1. In the countercurrent reactor, the viscous oil often was suspended in the water-soluble product, and water was carried into the oil product. Therefore, it was difficult to maintain a constant rate of water feeding and a stationary state of glycerol level (Table 5). The oxidation of PUFA was avoided by saturating the system with nitrogen, especially during continuous hydrolysis. The peroxide values of the oil products were kept at around 20 meq/kg, but sometimes they increased to 60–70 meq/kg. In spite of the high peroxide values observed, the hydrolysis ratios were maintained at 81–82% (Table 5). These results were different from our previous report on enzymatic refining (1). The lipase from *Rhizomucor miehei* was suddenly inactivated by contacting oil with a high peroxide value. The composition of the product was 10% triacylglycerols, 8% diacylglycerols, and 82% fatty acids. Almost all of the EPA and DHA was also liberated in the continuous reactor (Table 4). The initial activity of immobilized lipase obtained from the nonemulsion method was 212 units/g of Enzylon PF powder. The residual activity after use for 1472 h was 89.5 units/g of used Enzylon PF powder. This inactivation may be due to the use of hexane miscella (mixture 7:3 of sardine oil and hexane) during the operation around 840–1136 h. During this period, the hydrolysis ratios were decreased to 70–75%. However, the hydrolysis ratios recovered again to 81% by using isooctane miscella (1136–1376 h). Hydrolysis ratios without organic solvents, during 1376–1472 h, were 74–75%, which is rather low compared to our previous experiments (13).

TABLE 5
Continuous Sardine Oil Hydrolysis in a Countercurrent Fluidized-Bed Reactor

Reaction time (h)	94.5	112	160	360	546	713
Hydrolysis ratio (%)	75	82	80	81	81	81
Glycerol (mg/mL)	159	121	107	208	314	130
Peroxide value (meq/kg)	15	68	23	18	70	54

DISCUSSION

Antifoaming agents, such as silicone oil, had no effect on defoaming of the lipase fermentation by *P. fluorescens* biotype I. Physical defoaming processes, such as high internal pressure and vigorous mechanical agitation, were necessary in addition to chemical antifoamer, such as Adecanol LG109.

The lipase from *P. fluorescens* biotype I was thermostable (11). Heating at 60°C for 30 min after cultivation of the thermostable lipase provided complete killing of the *Pseudomonas* cells and a higher filtration speed. The resultant lipase was not inactivated (19).

Addition of polar solvent to the lipase solution can achieve a high immobilization ratio of up to 8,000 units of lipase per gram of carrier. The saturated amount of lipase on the carrier was 3,600 units per gram, because above that amount it did not improve lipolytic activity. *Pseudomonas mephitica* var. *lipolytica* produced lipase in the cell-bound fraction when it was grown in a medium with ammonium sulfate. The maximum cell-bound lipase production after 12 h of cultivation was about 1,200 unit per gram of dry cells (20).

The countercurrent fluidized-bed reactor in our previous work (12) was agitated with a stirring blade, but immobilized lipase was destroyed by the stirring blade. Air also gathered under the sieve plates and stopped the flow. The air had to be eliminated for continuous operation. The productivity [kg of fatty acid/(day × unit of enzyme)] of the reactor for olive oil hydrolysis was 5.15×10^{-4}. The countercurrent reactor in this paper was improved by using pulse agitation. Immobilized lipase was not mechanically destroyed, and no air gathered under the sieve plate. Productivity for sardine oil hydrolysis was 5.44×10^{-4}. However, in the countercurrent reactor, the viscous oil was often suspended in the water-soluble product and water was carried into the oil product. These phenomena were slightly improved by using 70% isooctane miscella. However, to completely resolve these problems, a novel loop reactor, connected to the immobilized lipase column, and an oil/water separator, such as shown in our previous paper (4), has to be devised.

Hydrolysis of tallow and lard (200–300 tons per month)

by *Candida rugosa* lipase is now used industrially at Miyoshi Oil & Fat (Kobe, Japan). They utilize soluble enzyme in a concentration of 0.01–0.02%, and the price for lipase is 30,000–50,000 yen/kg. Therefore, the enzyme cost is 3–10 yen/kg of fatty acid (21). However, the lipase from *C. rugosa* hardly liberates EPA and DHA (22). The enzyme cost in this experiment was 8,000/(1.36 × 200) = 30 yen/kg of fatty acid, where 1.36 is the productivity expressed as kg of fatty acid/(day × kg of Enzylon PF), 8,000 (yen/kg of Enzylon PF) is the price of the immobilized lipase, and 200 (days) is the life span of the immobilized lipase. The immobilized lipase could readily liberate DHA and EPA from sardine oil. The PUFA are fractionated from the oily product and can be converted to soap by simply adding alkali. Producing soap from fish oil is an interesting application of this reactor because fish oil has a therapeutic value in the treatment of psoriasis, a hyperproliferative, inflammatory skin disorder that is characterized by elevated LTB_4 (23–25).

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