Continuous Lipolysis Reactor with a Loop Connecting an Immobilized Lipase Column and an Oil–Water Separator[1]

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ABSTRACT: A continuous lipolysis system is described that consists of a loop connecting a fixed-bed reactor with the immobilized lipase from *Pseudomonas fluorescens* biotype I and an oil–water separator. In addition to continuous lipolysis, good continuous separation of oil product and water-soluble product and continuous concentration of glycerol can be integrated in this simple reactor.
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KEY WORDS: Concentration of product, continuous lipolysis, immobilized lipase, integrating bioreactor, lipase, lipolysis, loop reactor, oil–water separation, *Pseudomonas* sp.

Lipolysis provides the fatty acids and glycerol for the production of important oleochemicals such as soap, detergents, cosmetic products, food additives, paints, and explosives. In the present chemical lipolysis process, a continuous, uncatalyzed countercurrent system operates at high temperature and high pressure. The process requires high capital investment and energy consumption, causes degradation of polyunsaturated fatty acids, and pollutes the earth's environment. In contrast, enzymatic lipolysis occurs at ambient temperature and atmospheric pressure with no degradation of reactants, and the resultant products are of high quality.

Many lipolysis reactors with immobilized lipase have been described (1–10). However, no process applicable to commercial production has been reported because of two fundamental problems. Firstly, oil and water are not readily mixed, but this process must occur efficiently under ambient conditions for lipolysis to take place. Secondly, the lipase should be immobilized because of the cost and the advantages for continuous operation. We previously reported continuous lipolysis with immobilized lipase in a countercurrent reactor (7). Continuous lipolysis, continuous separation of oil and water-soluble product, and continuous concentration of the glycerol fraction could be integrated in the countercurrent method. However, the separation of oil and water was not adequate because the viscous oil often was suspended in the water-soluble product, and water was carried into the oil product. Loop reactors previously used for lipolysis (1,2,5,9) could not integrate the functions of continuous lipolysis, continuous separation, and continuous concentration of glycerol. Here we describe a novel loop reactor in which an immobilized lipase column and an oil–water separator are connected, allowing continuous lipolysis, good separation of oil and water, and concentration of glycerol.

EXPERIMENTAL PROCEDURES

Materials. Lipase from *Pseudomonas fluorescens* biotype I (11), immobilized on Dowex MWA-1 (12) (Enzylon PF), was obtained from Rakuto Kasei Co. Ltd. (Ootsu, Japan). More than 2,500 units of lipase, measured at 60°C by the olive oil emulsion method (12), was adsorbed on 1 g of the immobilized lipase. Rice bran oil was obtained from Boso Oil & Fat Co. Ltd. (Tokyo, Japan).

Assay methods. Residual lipase activity was measured with nonemulsion substrate containing 2 g of olive oil, 0.2 mL of 0.1 M phosphate buffer at pH 7, and immobilized lipase previously ground and passed through 0.149 mm mesh. To stop the reaction, 10 mL chloroform and methanol mixture (2:1) was added. To titrate the mixture, 0.05 N ethanolic sodium hydroxide solution was used. The hydrolysis ratio was expressed as (100 × acid value/saponification value) as described previously (7). Glycerol was measured in a colorimeter as described previously (13).

Repeated batch loop reactor. The fixed-bed reactor shown in Figure 1 was packed with 18 g of Enzylon PF. The volume of the packed-bed reactor was about 50 mL. The oil and water in the fixed-bed reactor should not channel separately. To achieve mixed paths of oil and water, a well-mixed emulsion of water and rice bran oil was passed through the bed. The fixed-bed reactor, oil–water separator, and pump were maintained in a room at 35°C. The path of the reactant was heated with a ribbon heater to prevent low-melting point materials from solidifying. The oil–water separator was a cylindrical beaker (55 mm diameter × 120 mm height) packed with fluoride rubber tubing (Nippon Rikagaku Kikai Co. Ltd., Tokyo,

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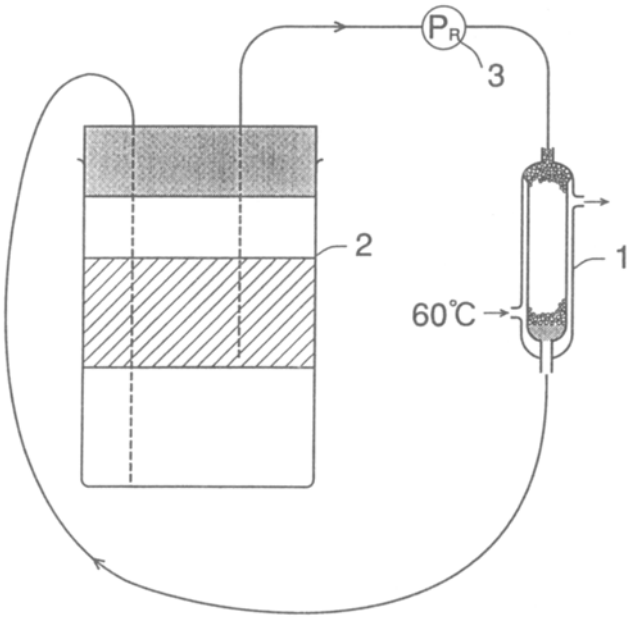

FIG. 1. Repeated-batch reactor for hydrolysis of oil. 1, Fixed-bed reactor at 60°C; 2, oil–water separator; 3, pump for circulation of the reactant.

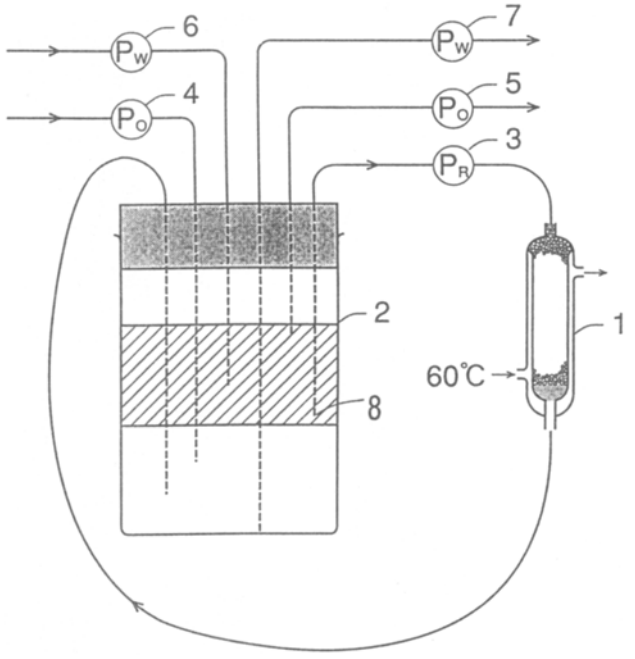

FIG. 2. Continuous reactor for hydrolysis of oil. 1, Fixed-bed reactor at 60°C; 2, oil–water separator; 3, pump for circulation of the reactant; 4, pump for oil substrate; 5, pump for oil product; 6, pump for water; 7, pump for water-soluble product; 8, inlet for the reactant in the loop.

Japan). The reaction mixture was passed through the fixed-bed reactor and sent to the bottom of the oil–water separator. The oily substrate (rice bran oil) and water were collected from the inlet in the loop. The inlet was located in the bottom of the upper layer or just above the interface of oil and water. The substrate was provided to the fixed-bed reactor by the pump. A liquid pump for recirculation of the reactant was operated at 5 mL/min, which was considered adequate to eliminate external diffusion of the substrate because the hydrolysis ratio of the oil did not increase at a circulation rate of 6 mL/min. The total flow volume in the fixed-bed reactor, pump, and paths of the loop was about 35 mL, obtained from reducing volume of the reaction mixture, and the mean residence time was 7 min. The reactant volume in the upper layer of the oil–water separator was about 60 mL, and the mean residence time in the separator was 12 min. Every 24 h, samples (or products) from the upper and lower layers of the oil–water separator were taken with a Pasteur pipette, and fresh rice bran oil and water were added.

Continuous-loop reactor. The oil–water separator shown in Figure 2 was equipped with inlets for the oil and water, and outlets for the oil and water-soluble products. The inlet for the oil substrate and outlet for the oil product were located as far apart as possible to prevent mixing of product with substrate. High-performance liquid chromatography pumps (Model 576; GL Science, Tokyo, Japan) were used for oil substrate, oil products, and circulation of the reactant. Peristaltic pumps (Microperpex 2132; LKB, Bromma, Sweden) were used for water and water-soluble product. The oil substrate in this experiment was rice bran oil miscella. The miscella contained 30 w/w% isooctane and 70 w/w% rice bran oil. The pumping rates for oil substrate and oil product were 0.375 g/h; those for water and water-soluble product were 0.096 g/h. To reduce the time to reach the stationary state, the initial water phase in the oil–water separator contained 4.6 mM glycerol/mL. Other operational conditions were the same as described above.

RESULTS

Table 1 shows that the hydrolysis ratios after a reaction time of 104 h were saturated at about 90%, indicating that initiation of the reactor in Figure 1 had finished after about 100 h. After 121 h, repeated batch operation with a Pasteur pipette was begun. Repeated batch lipolysis and separation of oily product and water-soluble product could be achieved in this simple reactor. Separation of the products was easily accomplished by removal from the upper and lower layers in the oil–water separator. The sampling position in each layer for the Pasteur pipette did not affect the hydrolysis ratio or glycerol concentration. However, the circulation rate in the loop significantly affected the hydrolysis ratio. After a reaction time of 288 h, the loop was plugged with crystals of hydrolysates, such as palmitic acid, despite heating of the loop. After a reaction time of 313 h, the oil substrate was changed to 70% rice bran oil miscella in isooctane, which resulted in the plugged material being dissolved. The glycerol concentration was increased up to 4.6 mM/mL (42.4 w/w%) after a reaction time of 649 h, suggesting that repeated batch concentration of the glycerol fraction also was achieved in this reactor. The residual activity of the immobilized lipase after the above experiment was about 90%.

TABLE 1
Repeated-Batch Hydrolysis of Rice Bran Oil

Reaction time (h)	Hydrolysis ratio (%)	Glycerol (mM/mL)
	Initiation[a]	
17	40.7	0.6
25	67.4	—
48	78.6	0.75
104	90.1	—
121	89.5	1.15
	Hydrolysis of rice bran oil[b]	
148	90.6	—
192	91.3	1.5
217	89.2	—
288	80.6	—
313	80.0	2.15
	Hydrolysis of rice bran oil miscella[c]	
385	89.2	—
457	88.3	2.9
529	86.8	—
625	89.4	—
649	88.7	4.6

[a]The reactants contained 100 g of rice bran oil and 100 g of water in the loop and were circulated.
[b]Every 24 h, 12 g of oil product and 6 g of water-soluble product were removed with a Pasteur pipette, and 12 g of rice bran oil and 6 g of water were added.
[c]Isooctane was added to the oil reactant to 30%. Every 24 h, 17.1 g of oil product and 6 g of water-soluble product were removed, and 17.1 g of rice bran oil miscella and 6 g of water were added.

TABLE 2
Continuous Hydrolysis of Rice Bran Oil Miscella

Reaction time (h)	315	362	391	420	444	464
Hydrolysis ratio (%)	83.8	83.0	77.1	83.4	84.5	84.2
Glycerol (mM/mL)	3.57	3.68	—	3.36	3.47	3.36
Reaction mixture	OW[a]	OW	W[b]	OW	OW	OW
Reaction time (h)	512	535	579	627	730	
Hydrolysis ratio (%)	86.0	82.4	86.0	78.8	82.1	—
Glycerol (mM/mL)	3.36	3.36	—	—	—	—
Reaction mixture	OW	OW	OW	O[c]	OW	—

[a]The inlet for the reactant in the loop was located just above the interface of the oil–water separator, and the reaction mixture contained oil and water-soluble reactants.
[b]The inlet in the loop was in the upper layer, and the reaction mixture contained only oil reactant.
[c]The inlet in the loop was in the lower layer, and the reaction mixture contained only water-soluble reactant.

Continuous operation of the loop reactor, shown in Figure 2, showed that the stationary phase of this process was achieved after a reaction time of 315 h (Table 2). The hydrolysis ratios were 84–85%, and the glycerol concentrations were 3.4 mM/mL. The mean residence time of the oil substrate in the oil–water separator was 160 h, and that of the water-soluble reactant was 625 h. The inlet for the reactant in the loop (8 in Fig. 2) should be located just above the interface of the oil and water because these substrates are provided to the fixed-bed reactor. When only oil substrate or water-soluble substrate was provided to the reactor, hydrolysis ratios decreased.

DISCUSSION

The loop reactor described here could achieve continuous lipolysis, good continuous separation of oily product and water-soluble product, and continuous concentration of the glycerol fraction. The productivities (kg of fatty acid/day × unit of enzyme) of the reactors were as follows. Productivity of the repeated batch reactor = $0.885 \times 0.714 \times 10^{-3} \times 24 \times 0.7/(18 \times 10^{-3} \times 2500) = 2.36 \times 10^{-4}$. Productivity of the continuous reactor = $0.839 \times 0.375 \times 10^{-3} \times 24 \times 0.7/(18 \times 10^{-3} \times 2{,}500) = 1.17 \times 10^{-4}$. The values of 0.885 and 0.839 are hydrolysis ratios, 0.714 and 0.375 are the production rates, 24 is hours/day, 0.7 is the concentration of oil in the miscella, 18 is the weight of used immobilized lipase, and 2,500 is adsorbed lipase unit per 1 g of the immobilized lipase. The fixed-bed reactor in the continuous reactor was channeling, and the productivity was half of the repeated batch reactor. The productivity of the countercurrent fluidized-bed reactor previously reported was 5.14×10^{-4} (7). The productivity of the loop reactor was not better than the fluidized-bed reactor, but the loop reactor was simple, manipulation was easy, and oil–water separation was good. We will optimize the operational conditions of the loop in future studies.

The enzyme cost (yen/kg of fatty acid) of the repeated batch reactor was $8000/(0.59 \times 200) = 68$ yen, where 0.59 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 used, and 200 is the life span of the immobilized lipase. The immobilized lipase could readily produce eicosapentaenoic acid and docosahexaenoic acid from sardine oil (14). The production of these polyunsaturated fatty acids in high quality is a useful process despite the high enzyme cost because the value of these products is high.

Small or middle-size factories often are used to make soap by alkaline saponification. The salting out process to eliminate glycerol in a large reaction vessel after the saponification takes 10–14 d. Fatty acid produced by the saponification is yellowish, and the glycerol is isomerized, resulting in considerable requirements for wastewater treatment. In contrast, the enzymatic lipolysis described here can be carried out at ambient temperature and pressure in a simple reactor. The fractionated fatty acid is of fine grade and can be converted to soap by simply adding alkali. The glycerol by-product is also of fine grade and concentrated. The running cost, energy cost, and the capital investment required for setting up the process should not be high, and the process can be assessed from the standpoints of enzyme cost and the value added by the process. If the price of oil used for lipolysis costs 60 yen/kg, fatty acid costs 200 yen/kg, glycerol costs 250 yen/kg, the price increase caused by the enzymatic lipolysis is 165 yen/kg because about 1 kg of fatty acid and 0.1 kg of glycerol are produced from 1 kg of oil. Generally, enzymatic lipolysis could be industrialized if the enzyme cost is reduced to less

than 5–10% of the value added or estimated price increase. A similar estimation was done for enzymatic refining of free fatty acid rice bran oil (15).

Ordinary waste water treatment cannot reduce oil content to less than 30 mg/L in wastewater with more than 1% oil. Nonpolar organic solvents, such as isooctane, could be used to extract the waste oil and the miscella containing the waste oil could be recycled for the lipolysis. The production of soap from waste oil is a possible application for enzymatic lipolysis. However, the use of living cells for wastewater treatment is an interesting approach, if they can be cultivated without contamination, because the cells or catalysts can reproduce themselves.

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