Repeated Use of Immobilized Lipase for Monoacylglycerol Production by Solid-Phase Glycerolysis of Olive Oil

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ABSTRACT: By using immobilized lipase for production of monoacylglycerol (MAG) by solid-phase glycerolysis of fats and oils, the enzyme could be recovered easily from the reaction mixture and recycled to reduce the cost of the catalyst. Several support materials ($CaCO_3$, $CaSO_4 \cdot 2H_2O$, $Ca_2P_2O_7$, and Celite) were screened for immobilization of *Pseudomonas* sp. lipase by adsorption and tested for solid-phase glycerolysis of olive oil. Immobilization made the reuse of enzyme feasible. $CaCO_3$ proved to be the best support: 90% MAG (wt% in the glycerol-free reaction mixture after 72 h of reaction time) was obtained until the fifth use, 80% after the seventh use, and 60% after the tenth use. The same support was found suitable for immobilization of two other bacterial lipases from *Chromobacterium viscosum* and *Pseudomonas pseudoalkali*.
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KEY WORDS: Bacterial lipases, $CaCO_3$, $CaSO_4 \cdot 2H_2O$, $Ca_2P_2O_7$, Celite, enzyme adsorption, glycerolysis, immobilized lipase, monoacylglycerol, reuse of immobilized enzyme.

Monoacylglycerols (MAG) of saturated or unsaturated fatty acids are the most commonly used surfactants in the food industry. Their emulsifying properties are employed also in preparation of cosmetics and pharmaceutical products, manufacture of alkyd resins, detergents, etc. (1).

They are produced industrially by transesterification of fats and oils with an excess of glycerol at high temperatures under alkaline catalysis. Depending on the glycerol excess and on the temperature, pressure and reaction time, equilibrium reaction mixtures of different compositions are obtained. The products contain 35–60% MAG, 35–50% diacylglycerols, 1–20% triacylglycerols,1–10% free fatty acids and their alkali metal salts, and 1–10% glycerol after *ca.* 1 h at 200–250°C (2). High-concentration MAG are obtained from the mixtures by molecular distillation. Use of the distilled product has increased with the desire to use purer and better products and because of their enhanced properties.

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The most serious drawbacks of the method are the low yields obtained and the high temperatures employed. The high temperatures lead to partial degradation of the products (formation of dark-colored by-products, burnt taste, peroxidation, and polymerization) and high energy consumption.

Recently, lipase-catalyzed glycerolysis and direct esterification for the biosynthesis of partial glycerides are increasingly being studied as possible alternatives to the classical method (3–7). The main reasons are the higher yields achieved and much milder reaction conditions, resulting in products of higher quality and less energy costs. Furthermore, an enzyme with positional, substrate, or stereo selectivity can yield special products that can be used as intermediates for the syntheses of tailored triglycerides, enantiomeric acylglycerols, etc.

Previous studies (8–12) have demonstrated that solid-phase glycerolysis of fats and oils, catalyzed by lipases, may be an advantageous approach for obtaining MAG. In this process, the yield of MAG is greatly influenced by the programmed reaction temperatures (8). The reaction is performed under a temperature program that depends on the type of substrate used. Initially, the reaction mixture consists of two liquid phases, a fluid fat and a glycerol phase in which the enzyme is suspended. The reaction occurs at the interface between the glycerol and the fat. The higher temperatures (25–50°C) employed for the initial stage ensure high reaction rates for all possible reactions in the system. If the process is continued at these temperatures, only a low yield of MAG can be obtained. For example, when olive oil was used, the MAG content in the equilibrium reaction mixture free of glycerol was *ca.* 25% (wt) at 25°C (Yamane, T., unpublished data). Further formation of MAG is achieved by lowering the temperature to an optimized value at which the MAG exceeds its solubility limit and begins to precipitate. The viscosity of the reaction mixture increases gradually with the accumulation of the solid MAG, and it becomes a thick paste, which eventually solidifies. The fractional crystallization of MAG is exploited to alter the equilibrium composition during glycerolysis and thus to reach a high yield of MAG (8–12).

The applications of lipases for MAG production, including solid-phase glycerolysis of fats, were comprehensively reviewed by Bornscheuer (7).

From a wide range of microbial lipases, which were screened for glycerolysis, bacterial lipases were found to be the most suitable for this process, but partial inactivation of the enzymes occurred during the reaction course (13).

In this work, enzymes were immobilized by adsorption onto $CaCO_3$, $CaSO_4 \cdot 2H_2O$, $Ca_2P_2O_7$, and Celite in an attempt to enhance the lipase stability, facilitate enzyme recovery from the reaction mixture, and permit reuse of the biocatalyst in the solid-phase glycerolysis reaction. The glycerolysis reaction of olive oil was chosen as standard reaction.

Adsorption equilibrium on support particles, reusability of immobilized lipases, and determination of the time course of glycerolysis during reuse of $CaCO_3$-immobilized bacterial lipases make up the outline of the present study.

EXPERIMENTAL PROCEDURES

Materials. Commercially available lipases (E.C. 3.1.1.3) were obtained from the following companies: *Pseudomonas* sp. KWI-56 lipase (PSL), culture supernatant solution (10600 U/mL hydrolytic activity, 7.3 mg/mL protein concentration) and pure (95 U/mg hydrolytic activity), from Kurita Water Industries Ltd., Tokyo, Japan; pure *Chromobacterium viscosum* lipase (CVL) (1183 U/mg hydrolytic activity), from Asahi Chemical Industry Co., Ltd., Tokyo; and *Pseudomonas pseudoalkali* lipase (PPL) (Liposam 1000 G, 1146 U/mg hydrolytic activity), from Showa Denko Co. Ltd., Tokyo. Support materials were purchased as follows: $CaCO_3$ (Softon 3200), from Shiraishi Calcium Co. Ltd., Osaka; $CaSO_4 \cdot 2H_2O$ (Type SF), from Mutsumi Chemicals Co. Ltd. Yokkaichi, Mie Pref.; $Ca_2P_2O_7$ (Type 504014 T), from Taihei Chemical Industries Co. Ltd. (Osaka, Japan); and diatomaceous earth (Celite, grade: Hyflo Super-Cel), from Johns-Manville, (Denver, CO).

Immobilization. The support powder (2.0 g) ($CaCO_3$, $CaSO_4 \cdot 2H_2O$, $Ca_2P_2O_7$, or Celite) was added to 5 mL lipase solution that contained approximately 10,000 U/mL enzyme (PSL culture supernatant solution, CVL or PPL) and stirred with a magnetic bar at 100 rpm for 1 h. Afterwards, 20 mL of chilled acetone was added, and the suspension was filtered through a Buchner funnel. The immobilized enzyme was washed on the filter paper with another 20-mL aliquot of chilled acetone and dried in a vacuum desiccator for 4 h.

Adsorption experiment. Pure PSL was used in this experiment. The support powder (0.100 g) was added to 0.250 mL enzyme solution (at the same support/solution ratio as for immobilization) in a 1.5-mL microcentrifuge tube and mixed for 1 h in an Iwaki Tuple Mixer Twin 3–28 at 25°C in an incubator. The suspension was centrifuged isothermally at 25°C for 10 min at 15000 rpm, and the clear supernatant was withdrawn. The quantity of adsorbed protein was obtained by measuring the optical density of enzyme solutions at 280 nm before and after immobilization.

Glycerolysis. In a typical experiment, 6000 U of immobilized lipase was added to a mixture of 5 g olive oil (low acidity, Nacalai Tesque Inc., Kyoto, Japan) and 2.6 g glycerol (99% purity) containing 3.1% moisture (approximately 4.8:1 molar ratio of glycerol/triolein) under stirring at 25°C. The initial water content of the glycerol phase was found to be optimal in the range from 2 to 3.5% with respect to the maximum MAG yield and minimum free fatty acid formation (8). After 1 h, the temperature was reduced to 10°C for 23 h, followed by cooling to 5°C. Reducing the reaction temperature to 10°C resulted in solidification of the reaction mixture owing to selective crystallization of the formed MAG. Thus, the reaction equilibrium was shifted toward MAG synthesis. The reaction was stopped after 72 h by dissolving the reaction mixture in acetone. When recovered enzyme was used for successive glycerolysis processes, the experiment was scaled down while keeping the relative amount of each component constant. The reactor was described in a previous article (8).

Recovery of product and immobilized enzyme. The immobilized enzyme was recovered from the acetone solution by filtration, washed with further acetone over filter paper, and dried under vacuum. The final product was obtained by evaporating the acetone from the filtrate in a rotary vacuum evaporator.

Analysis of the reaction mixture (during and after reaction). During the reaction course, samples of 3–5 mg were intermittently withdrawn from the reaction vessel and dissolved in 0.3 mL chloroform/methanol (1:1). The enzyme was separated by centrifugation at 15,000 rpm for 5 min. The removal of glycerol from the supernatant solution was achieved by extraction with water (0.1 mL). For an effective extraction of partial glycerides (soluble in water to some extent), two subsequent extractions of the water layer with 0.3 mL chloroform each were performed. The total chloroform extract (approximately 0.9 mL) was concentrated to dryness in a N_2 stream. Chloroform (0.2 mL) was added to the concentrate, and the solution was analyzed by thin-layer chromatography/flame-ionization detector (Iatroscan MK-5; Iatron Laboratories, Tokyo, Japan) with Chromarod S III quartz rods. One to 3 μL chloroform extract was applied to the rods, followed by development in benzene/chloroform/acetic acid (70:30:2) for 10 cm. The rods were dried and scanned under the following conditions: hydrogen flow rate, 160 mL/min, air flow rate, 2.0 L/min, 30 s/scan. Trioleylglycerol, 1,3-dioleylglycerol, 1,2-dioleylglycerol, 1-monooleylglycerol, and free oleic acid were effectively separated. Peak areas were calculated with an SIC Chromatocorder 12 integrator (System Instruments Co. Ltd., Tokyo, Japan). All results throughout this article were expressed as percentage of peak areas of the reaction mixture's components on a glycerol-free basis. They were regarded as weight percentages, and may vary slightly from the actual weight percent (14).

For analyzing the content of the final reaction mixture after the immobilized enzyme was removed, 0.5 mL of the acetone extract of the reaction mixture was evaporated, and the analysis was performed as described above.

Assay of lipase hydrolytic activity. Hydrolytic activity was measured by the olive oil emulsion method without addition of surfactants. For free enzyme, 1.5 mL olive oil was incubated with 4.0 mL phosphate buffer 0.1 M, pH 7, and 3.5 mL

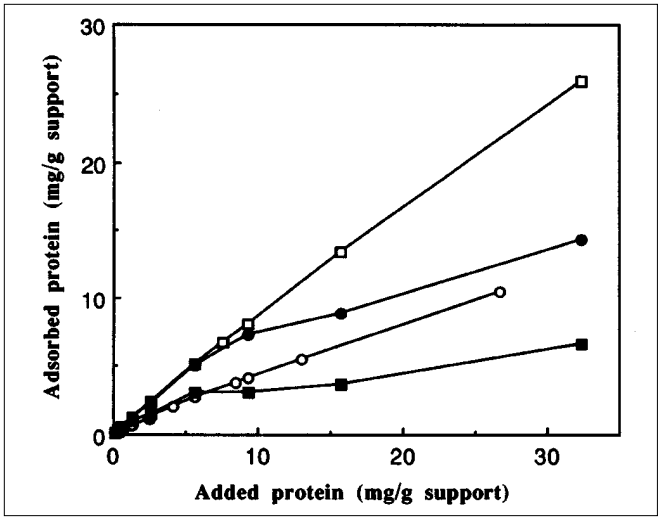

FIG. 1. Adsorption profiles of pure PSL (*Pseudomonas* sp. KWI-56 lipase (Kurita Water Industries Ltd., Tokyo, Japan) on several supports: $CaCO_3$, (□); $CaSO_4 \cdot 2H_2O$, (●); Celite, (○), and $Ca_2P_2O_7$, (■).

TABLE 1
Activity Yield of Immobilization of Crude PSL[a]

Support	Initial activity of immobilized enzyme (U/mg)	Amount of dry catalyst obtained (g)	Activity yield of immobilization[b] (%)
$CaCO_3$	14.8	2.07	57.8
Celite	15.2	2.06	59.1
$Ca_2P_2O_7$	15.0	2.00	56.6
$CaSO_4 \cdot 2H_2O$	13.6	2.05	52.6

[a]Initial activity of free enzyme solution (culture supernatant) was 1.06×10^4 U/mL; *Pseudomonas* sp. KWI-56 lipase (Kurita Water Industries Industries Ltd., Tokyo, Japan).
[b]Activity yield of immobilization was expressed as percentage of activity units of initial enzyme solution recovered in the dry catalyst. The filtrate after immobilization had no activity.

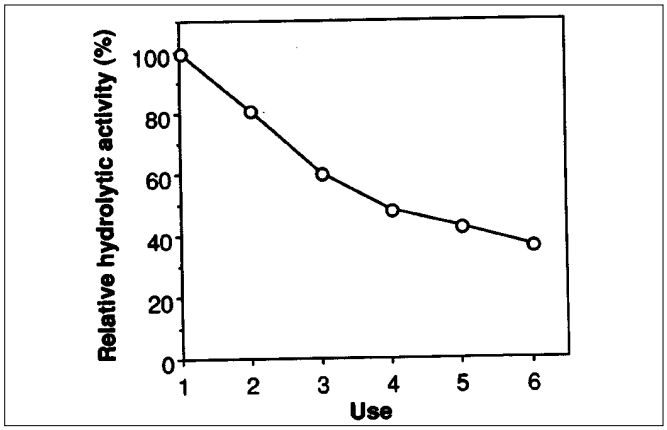

FIG. 2. Stability of $CaCO_3$-immobilized PSL during reuse. The specific hydrolytic activity of the recovered catalyst was determined after each use (72 h of reaction time) as described in the Experimental Procedures section. The relative hydrolytic activity was obtained by considering the initial specific activity of the catalyst as 100%. See Figure 1 for PSL information.

distilled water with stirring at 37°C for 10 min. One mL of enzyme solution was added to give a final volume of 10 mL. After 20 min, the reaction was stopped by adding 20 mL acetone/ethanol solution (1:1). The amount of free fatty acid was titrated with 0.05 N NaOH solution to pH 10. Blank samples were treated similarly.

For immobilized enzymes, 1 mL phosphate buffer was added to 0.5–2 mg immobilized enzyme weighed in a 1.5-mL microcentrifuge tube. Before starting the reaction, the suspension was mixed well in a touch mixer, and all of the suspension was withdrawn by a micropipet and added to the reaction mixture at the zero moment. The tube was washed with 0.5 mL distilled water, which was added to the olive oil–phosphate buffer assay emulsion immediately. Consequently, the initial mixture contained only 3 mL water to keep the final volume of 10 mL.

The activity of acetone filtrates after immobilization was assayed with and without evaporation of acetone.

Protein assay. Protein concentration in the culture supernatant (crude PSL) was determined by the Bradford method (Bio-Rad Protein Assay) (15).

Water content. Water concentration in glycerol was determined with a Karl-Fischer moisture meter (MKS-1; Kyoto Electronics Co. Ltd., Kyoto, Japan).

TABLE 2
Final Content of MAG for Subsequent Reuses of PSL Immobilized on Various Support Powders[a]

Support	Final content of MAG (%)[b] 1st use	2nd use	3rd use	4th use	5th use	6th use
$CaCO_3$	87.1	87.0	90.6	88.3	78.7	78.3
Celite	86.9	86.7	60.6	46.6	36.5	37.5
$Ca_2P_2O_7$	87.5	87.7	88.4	82.5	65.1	29.7
$CaSO_4 \cdot 2H_2O$	50.7	63.4	23.4	1.8	—	—

[a]For each run, the reaction was performed simultaneously for all supports in the reaction conditions described in the Experimental Procedures section. See Table 1 for PSL information.
[b]After 72 h of reaction time. The final monoacylglycerol (MAG) content was expressed as weight percentage of MAG in the final reaction mixture free of glycerol.

RESULTS AND DISCUSSION

Screening of supports for immobilization. In a preliminary experiment, performed to immobilize PSL by adsorption onto various supports (activated carbon, activated clay, kaolin, $CaCO_3$, and Celite) and to use them for obtaining MAG from palm oil by solid-phase glycerolysis, lipase immobilized on $CaCO_3$ powder and Celite exerted good activity. Approximately 70 and 60% MAG, respectively, were obtained (Yamane, T., unpublished data). A previous report on a stabilizing effect of the calcium ion on CVL activity in the continuous synthesis of glycerides (16) led to the idea that compounds containing calcium ion may be appropriate supports for immobilization of bacterial lipases. Therefore, $CaCO_3$, $CaSO_4 \cdot 2H_2O$, $Ca_2P_2O_7$, and Celite were chosen as supports for enzyme immobilization in this work.

Adsorption equilibrium of pure PSL on support particles. Figure 1 shows the enzyme adsorption profile on the support particles. $CaCO_3$ displayed the best capacity for adsorption; approximately 80% of the added protein was adsorbed, and in the studied range of protein concentration, the adsorption remained in the linear domain. A moderate adsorption capacity was demonstrated by $CaSO_4 \cdot 2H_2O$ and Celite. $Ca_2P_2O_7$ showed a poor capacity of adsorption, and its adsorption curve has an evident saturation tendency.

The support surface structure was investigated by scanning electron microscopy. $CaCO_3$ appeared as a powder with small crystalline particles to produce a large adsorption surface area. In contrast, the particles of $Ca_2P_2O_7$ were much larger with a small specific surface area, confirming the low adsorption. The typical honeycomb structure of Celite conferred a large surface area, but the adsorption interaction may be weaker than that of $CaCO_3$. $CaSO_4 \cdot 2H_2O$ had crystalline particles with not as large a surface area as $CaCO_3$ or Celite, but it may have stronger adsorption interactions.

Immobilization. The activity yield after immobilization on each of the four supports is shown in Table 1. Approximately 53 to 59% of the initial activity of the enzyme solution was

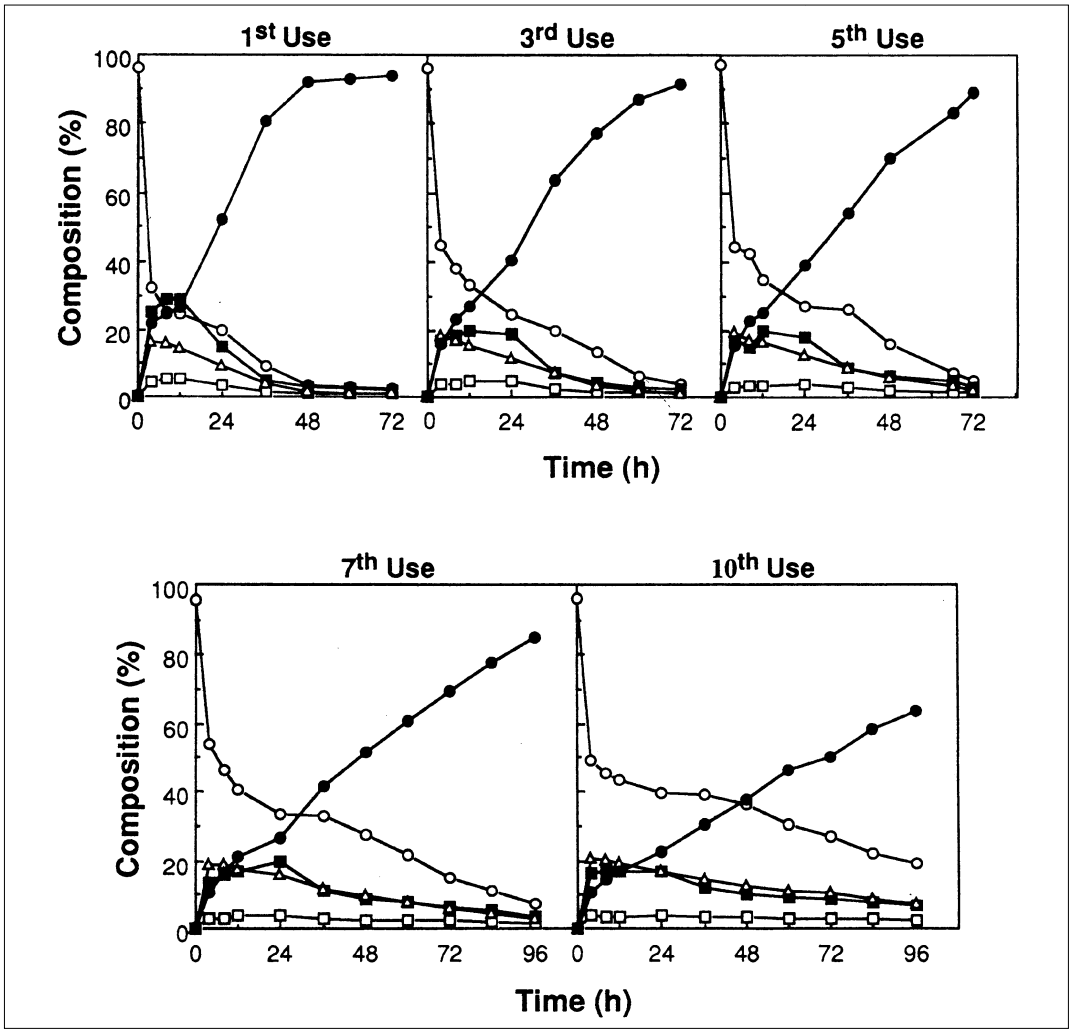

FIG. 3. Time course of glycerolysis catalyzed by $CaCO_3$-immobilized PSL during reuse: triacylglycerol, (○); 1,3-diacylglycerol, (■); 1,2-diacylglycerol, (△); 1-monoacylglycerol, (●); and free fatty acid, (□). See Figure 1 for PSL information.

found in the dry catalysts. Because the filtrate after immobilization had no enzymatic activity (enzyme was resistant to the denaturing effect of acetone), and the activity yield of immobilization is only slightly different for all supports, although their adsorption capacities are rather different (Fig. 1), it can be inferred that the amount of enzyme that was not adsorbed was precipitated by chilled acetone on the support particles' surface.

Reusability of immobilized enzyme. Immobilized lipase was used in the solid-phase glycerolysis of olive oil (Table 2). $CaCO_3$-immobilized lipase displayed good stability in this process. After six subsequent reuses, the content of MAG in the reaction mixture free of glycerol was 78.3% (wt), which established the effectiveness of immobilization and reusability of the immobilized enzyme. Immobilization on $CaCO_3$ resulted in a considerable enhancement of the enzyme stability: when the crude enzyme was used directly for glycerolysis, only 7% of its initial hydrolytic activity was found after 97 h of use (13), whereas the immobilized enzyme retained 40% of its activity after six reuses of 72 h each (Fig. 2). For Celite and $Ca_2P_2O_7$, recovered immobilized enzyme remained effective for one and three more reuses, respectively, but afterward, the final yield of MAG decreased rapidly, and the catalyst became unsuitable for further reuse. For $CaSO_4 \cdot 2H_2O$, enzyme inactivation occurred fast, with complete inactivation occurring by the fourth use. Even for the first use of the immobilized enzyme, only 51% MAG was achieved.

Time course of glycerolysis by $CaCO_3$-immobilized lipase. $CaCO_3$ was the best support for immobilization, and therefore the time course of the reaction was followed over 10 consecutive uses of the catalyst (Fig. 3). The free fatty acid content due to hydrolysis remained low throughout the glycerolysis and subsequent reuses. High positional specificity was achieved in this process. Only 1-MAG was obtained, the 2-MAG concentration being less than the sensitivity of the analysis method (less than 1%). The reaction rate of 1-MAG formation decreased gradually with the number of reuses. The yield of MAG after the tenth use was over 60%, proving that enzyme immobilization by adsorption may be a good industrial approach for obtaining an effective low-cost reusable catalyst.

Comparative MAG production courses for some $CaCO_3$-immobilized bacterial lipases. The time courses of MAG formation with three different bacterial lipases, PSL, CVL and PPL immobilized on $CaCO_3$, were examined (Fig. 4). For all of them, 90% MAG in the reaction mixture was obtained after the fifth use, and the catalysts showed a considerable stability and reusability as the result of immobilization. However, it is not yet clear how the substrate accesses the enzyme immobilized on the surface of the support particles in the solid state of the reaction mixture.

Further studies are necessary to ascertain the microscopic migration of the substrate, the effects of immobilization, acyl migration, enzyme specificity, and calcium ion to define the mechanism of solid-phase glycerolysis with immobilized enzymes.

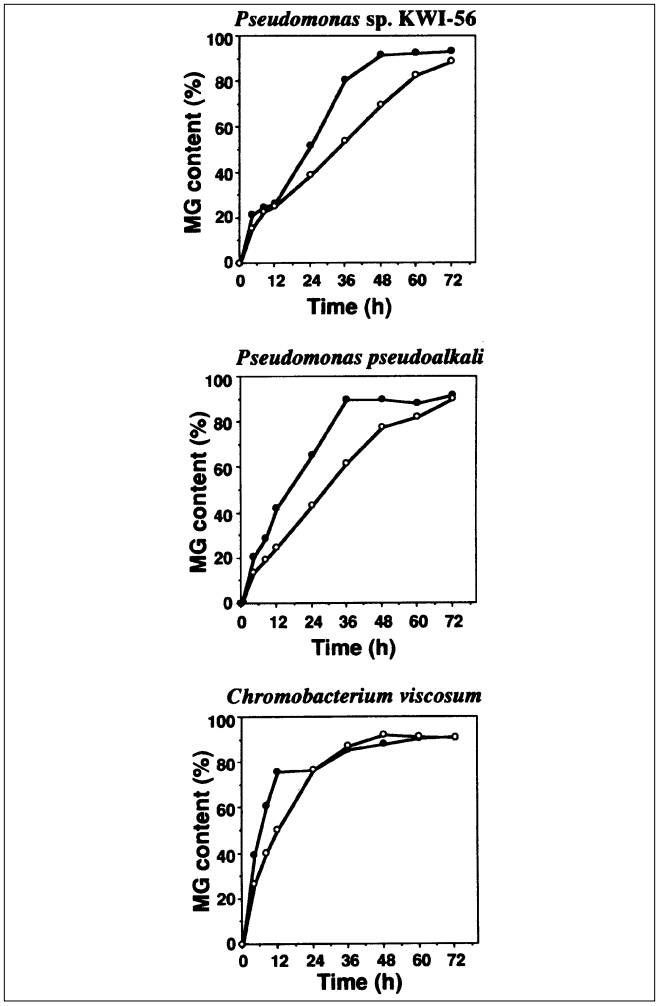

FIG. 4. Comparative reaction courses for three bacterial lipases immobilized on $CaCO_3$: first use, (●) and fifth use, (○), MG, monoacylglycerol.

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REFERENCES

1. Fuel Resources to Heat Stabilizers, in *Kirk-Othmer Encyclopedia of Chemical Technology*, 4th edn., Vol. 12, edited by M. Howe-Grant, John Wiley & Sons, Inc., New York, 1994, p. 692.
2. Starch and Other Polysaccarides to Surfactants, in *Ullman's Encyclopedia of Industrial Chemistry*, Fifth, Completely Revised Edition, Vol. A25, edited by B. Elvers, S. Hawkins, and W. Russey, VCH, Weinheim (Federal Republic of Germany), 1994, p. 792.
3. Pastor, E., C. Otero, and A. Ballesteros, Enzymatic Preparation of Mono- and Di-stearin by Glycerolysis of Ethyl Stearate and Direct Esterification of Glycerol in the Presence of a Lipase

from *Candida antarctica* (Novozym 435), *Biocat. Biotransformation 12*:147–157 (1995).
4. Kwon, S.J., J.J. Han, and J.S. Rhee, Production and *in situ* Separation of Mono- or Diacylglycerol Catalyzed by Lipases in *n*-Hexane, *Enzyme Microb. Technol. 17*:700–704 (1995).
5. Kuo, S.J., and K.L. Parkin, Acetylacylglycerol Formation by Lipase in Microaqueous Milieu—Effects of Acetyl Group Donor and Environmental Factors, *J. Agric. Food Chem. 43*:1778–1783 (1995).
6. Pastor, E., C. Otero, and A. Ballesteros, Synthesis of Mono- and Dioleylglycerols Using an Immobilized Lipase, *Appl. Biochem. Biotechnol. 50*:251–263 (1995).
7. Bornscheuer, U.T., Lipase-Catalyzed Syntheses of Monoacylglycerols, *Enzyme Microb. Technol. 17*:578–586 (1995).
8. McNeill, G.P., S. Shimizu, and T. Yamane, Solid-Phase Enzymatic Glycerolysis of Beef Tallow Resulting in a High Yield of Monoglyceride, *J. Am. Oil Chem. Soc. 67*:779–783 (1990).
9. McNeill, G.P., and T. Yamane, High-Yield Enzymatic Glycerolysis of Fats and Oils, *Ibid. 68*:1–5 (1991).
10. McNeill, G.P., and T. Yamane, Further Improvements in the Yield of Monoglycerides During Enzymatic Glycerolysis of Fats and Oils, *Ibid. 68*:6–10 (1991).
11. McNeil, G.P., D. Borowitz, and R.G. Berger, Selective Distribution of Saturated Fatty Acids into the Monoglycerides Fraction During Enzymatic Glycerolysis, *Ibid. 69*:1098–1103 (1992).
12. McNeil, G.P., and R.G. Berger, Enzymatic Glycerolysis of Palm Oil Fractions and a Palm Oil-Based Model Mixture: Relationship Between Fatty Acid Composition and Monoglyceride Yield, *Food Biotechnol. 7*:75–87 (1993).
13. Bornscheuer, U.T., and T. Yamane, Activity and Stability of the Lipase in the Solid-Phase Glycerolysis of Triolein, *Enzyme Microbiol. Technol. 16*:864–869 (1994).
14. Tatara, T., T. Fujii, T. Kawase, and M. Minagawa, Quantitative Determination of Tri-, Di-, Monooleins, and Free Oleic Acid by the Thin-Layer Chromatography–Flame Ionization Detector System Using Internal Standards and Boric Acid Impregnated Chromarods, *Lipids 18*:732–736 (1983).
15. Bradford, M.M., A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, *Anal. Biochem. 72*:248–254 (1976).
16. Hoq, M.M., T. Yamane, S. Shimizu, T. Funada, and S. Ishida, Continuous Synthesis of Glycerides by Lipases in a Microporous Membrane Bioreactor, *J. Am. Oil Chem. Soc. 61*:776–781 (1984).

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