Enzyme, Medium, and Reaction Engineering to Design a Low-Cost, Selective Production Method for Mono- and Dioleoylglycerols

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ABSTRACT: The selective enzymic production of mono- and diolein (MO, DO) was optimized at high yields. A comparative study of the following distinct enzymic reactions was conducted: ethyl oleate glycerolysis, triolein (TO) glycerolysis, and direct esterification. Solvent-free systems were compared with media that contained different solvents. Native, modified (with polyethylene glycol), and immobilized lipases were used. Mechanical resistance, the support effect on enzyme and glycerol dispersion and on process reproducibility, and hydrophilicity of the support were considered in the process optimization. We report the use of an immobilized lipase on an inorganic support (Celite), which has high activities in both solid-phase glycerolysis (99% reaction conversion) and esterification (100% conversion). The optimum conditions for the distinct reactions were compared by considering their selectivities, conversions, yields, and cost of the substrates. We found less costly and more selective processes in the absence of solvents for glycerolysis of triolein and direct esterification. Although glycerolysis was the most interesting process to produce diolein, esterification was better for monoolein preparation with this biocatalyst. The esterification reaction yielded 93 wt% of MO, in the absence of either TO or oleic acid (OA), at low cost because of the 100% reaction conversion. Similar costs of the substrates (10.6 and 10.1 $/g) were necessary to obtain 67 and 80 wt% of DO in esterification and glycerolysis, respectively. The glycerolysis conversion was 96%. In esterification, the product mixture was impure, with a high amount of residual OA due to the low conversion (59%). The high activity of PSL-Celite in these solid-phase reactions has an advantage over the reactions with nonimmobilized lipases due to the ease of enzyme recovery. The absence of organic solvents reduces the need for solvent removal from the reaction mixtures.

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Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are enzymes which hydrolyze *in vivo* the long-chain aliphatic esters of triglycerides (1). Until this decade, microbial lipases were used mainly in the pharmaceutical industry for the production of digestive preparations. At present, specific lipases are used in oil modification and to prepare aromas and detergents (2). Lipases are used in the hydrolysis of oils for the production of soaps (3). In the future, wider use of lipases in industry is expected.

Mono- and diglycerides are nonionic surfactants used as food, cosmetic and pharmaceutical emulsifiers, and stabilizers. These are the largest single type of food-grade emulsifiers. They are consumed at an annual level of 85 million kg in the United States, which represents approximately 70% of the total emulsifiers used in food products. The directives of the World Health Organization (WHO) requires that these mixtures (EEC code 471) have at least 70% mono + diglyceride, and a minimum of 30% monoacylglycerol, and that the contents of both glycerol (G) and triglyceride be below 10%. Monoglycerides are emulsifiers with GRAS (Generally Recognized As Safe) status. The partially acylated glycerides are prepared in industry by chemical methods at high pressures and with toxic inorganic catalysts. They also can be obtained by means of enzymes under mild temperature and pressure conditions. Distinct enzymic methods have been described: (i) selective hydrolysis of triglycerides (4); (ii) glycerolysis of triglycerides (5); or (iii) direct esterification of G with variable acyl donors (6,7). The highest yields of monoglycerides (90 wt%) have been obtained by McNeill *et al.* (8–10) in a solid-phase glycerolysis of naturally occurring hard fats, with nonimmobilized lipases. Yamaguchi and Mase (11) obtained 90 wt% monoolein (MO) with 76% of the conversion by direct esterification. Lipases supported on macroporous resins proved only partially effective in these solid systems (8–10), and methods of removing or recovering the enzyme included in the solid mixture after completion of the reaction must be established to make these processes applicable in industry (10). Immobilization of enzymes has several advantages, besides their possible reuse and stabilization. In reactions of glycerol derivatives, Otero *et al.* (4) have demonstrated the effect of the hydrophilicity of the supports on the selectivity of these processes. The hydrophilicity has been defined by Reslow *et al.* (12) as L water in the matrix/L water in diisopropyl ether phase in contact with the solid. Also, hydrophobic supports facilitate enzyme contact with the apolar substrates in olive oil hydrolysis (13), fatty acid esterification

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(11), and interesterification reactions (14). The chemical modification of the enzyme molecule with polyethylene glycol (15) also may be used to change its solubility and accessibility to hydrophobic substrates.

In this paper, we optimized and compared distinct enzymic methods for the selective production of MO- and diolein (DO). Native, modified [with (PEG)], and immobilized lipases were used. We report the use of an immobilized lipase on an inorganic support (Celite) with high activity in solid-phase glycerolysis (99% reaction conversion) and in esterification (100% conversion), which also increased process reproducibility. The distinct reactions were compared by considering their selectivities, amount of the product, and cost of the substrates.

EXPERIMENTAL PROCEDURES

Materials. Tributyrin, oleic acid (OA, 99%), oleic acid ethyl ester (EO, 99%), triolein, (TO, 95%), 1-monooleoyl-*rac*-glycerol (MO, M7765), 1,3-diolein (DO, D3627), G, lipase type VII from *Candida rugosa*, and monomethoxypolyethylene glycol PEG (MW 5000) were ordered from Sigma Chemical Co. (St. Louis, MO); Celite for gas–liquid chromatography [30–80 mesh (0.18–0.59 mm)] from BDH (Poledorset, England); Duolite (A 568), a macroporous weakly basic anion exchange resin, was donated by Rohm and Haas France S.A. (Chauny, France); lipase PS (PSL) was supplied by Amano Pharmaceutical Co. (Nagoya, Japan); *n*-heptane and acetonitrile (ACN), high-performance liquid chromatography (HPLC) quality, were purchased from Scharlau (Barcelona, Spain). All solvents were previously dried with molecular sieves with an effective pore diameter of 4 Å from Sigma Chemical Co.

Immobilization of PSL on Celite. Lipase PS was immobilized by adsorption on Celite. Five grams of lipase powder was dissolved in 20 mL of 0.1 M phosphate buffer, pH 7.5. After stirring for 20 min at 0°C, the solution was centrifuged for 10 min at 11,950 × *g*. Celite (5 g) was added to the solubilized enzyme (less than 20% w/w), and the stirring continued for 1 h. Then, 5 mL of cold (–20°C) acetone was slowly added, with stirring. The immobilized enzyme (PSL-Celite) was filtered, washed with 2 × 10 mL acetone, dried 30 min *in vacuo*, and stored in a closed vial at 4°C.

Immobilization of PSL on Duolite. Lipase PS was adsorbed on the macroporous resin, Duolite. A saturated lipase solution was prepared by dissolving 5 g of PSL in 20 mL of phosphate buffer 0.1 M, pH 7.5. After centrifuging to eliminate the insoluble material, the enzyme solution was recirculated (at about 100 mL/h) for 1 h through the Duolite (5 g) placed in a column (i.d. 1.2 cm). The immobilized enzyme (PSL-Duolite) was then treated with acetone and vacuum-dried as described above for Celite.

Chemical modification with polyethylene glycol. Lipase PS was modified (PSL-PEG) by the procedures of Inada *et al.* (16). This method requires the modifier preparation, 2,4-*bis* (*o*-methoxypropyethylene glycol)-6-chloro-s-triazine, and subsequent binding to amino groups of the lipase. Two biocatalysts with distinct modification degrees were prepared: 17 and 25% of lipase amino groups, respectively, measured by trinitrobenzene sulfonate (17) disappearance after the covalent attachment of PEG.

Determination of hydrolytic activity. The hydrolytic activities of the different biocatalysts were evaluated by following the hydrolysis of tributyrin at 30°C in 1 mM Tris/HCl buffer, with 0.1 M NaCl and 0.1 M $CaCl_2$. The acid released was continuously titrated to pH 7.0 with a pH-stat from Radiometer (Copenhagen, Denmark), model TTT80.

Glycerolysis of EO in organic solvents. MO production from glycerol and ethyl oleate (transesterification process) was studied in acetonitrile and in *n*-heptane. The effect of different parameters on the reaction rate and product yields were studied with PSL lipase in native and immobilized forms: percentage of buffer solution added (0–30% vol/vol), molar ratio between substrates (EO/G = 1:1, 2:1, 3:1; and EO/G = 1:0.5 to 1:10), temperature (10–60°C) and reaction time (2–90 h).

The synthesis of MO was carried out by addition of the lipase to the reaction mixture (G + EO + 2 mL solvent), at constant temperature and 300 rpm. After the indicated time, the suspended lipase was separated by centrifugation at 2367 × *g* for 8 min. The supernatant was analyzed by HPLC. In the figures, the molar yields are calculated with respect to the less concentrated reactant, G or EO. Thus, 100% = % TO + % DO + % MO + % G (or % EO in Fig. 1B).

Glycerolysis of triolein in the absence of solvent. Glycerolysis of TO was carried out and analyzed in a similar manner to that of EO, but without addition of solvent. After the indicated time, 4 mL isopropanol was added to the reactor, which was shaken vigorously for 5 min for total solubilization of products. The biocatalyst was removed by filtration, and the filtrate was analyzed by HPLC. Distinct conditions (0–50% w/w of buffer added to glycerol, molar ratio TO/G = 1:2– 1:15, 3–15°C, 1–11 d of reaction time, and 25–200 mg of biocatalyst) were studied. In the figures, the yields are given as the molar percentages of the obtained reaction mixture, excluding the substrate in excess, where 100% = % TO + % DO + % MO.

Esterification between OA and G without solvent. OA, G, and the enzyme were placed into the reactor, which was thermostated and shaken at 100 rpm. Molecular sieve was added to mitigate water production during the biotransformation. The extraction and analysis of the product mixture were carried out in a manner similar to that used in the glycerolysis of TO. The yields are given in the figures as molar percentages of the obtained reaction mixture excluding the substrate in excess, where 100% = % TO + % DO + % MO + % OA. Different parameters (1–4 d of reaction time, temperatures from 3 to 60°C, and the ratio OA/G = 1:1–10:1) were varied to optimize the selective preparation of MO and DO.

Analysis of the reaction mixtures. The reaction products were analyzed by HPLC with a Spectra Physics isocratic pump (Fremont, CA), connected to a Spherisorb ODS-2 column (Sugelabor, S.A., Madrid, Spain), a refractive index detector (Shodex, Tokyo, Japan), and a Spectra Physics SP 4290

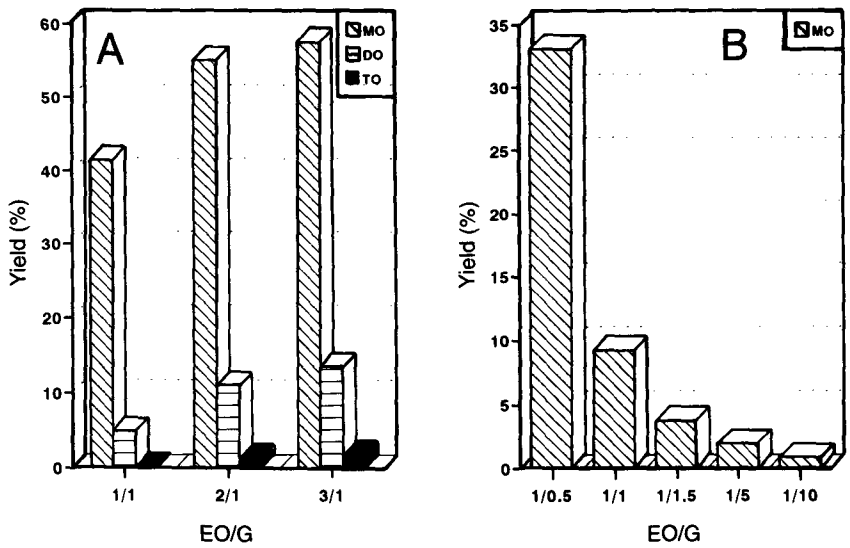

FIG. 1. Influence of the molar ratio of oleic acid ethyl ester (EO)/glycerol (G) in the glycerolysis of EO catalyzed by lipase PS (PSL)-Celite in acetonitrile. (PSL from Amano Pharmaceutical Co., Nagoya, Japan.) Conditions: A) 12.5 mg/mL PSL-Celite, 45 mg G, 2 mL acetonitrile (ACN), 0.5% vol/vol of buffer, 90 h and 30°C; B) 45 mg/mL PSL-Celite, 0.373 g EO, 2 mL ACN, 3 h and 30°C; MO, monoolein; DO, diolein; TO, triolein.

integrator. The separation of products was achieved with 1:1 (vol/vol) acetonitrile–acetone as mobile phase, at a flow of 2 mL/min. The temperature of the column and the detector was kept constant at 40°C. Calibration analysis was obtained with pure-grade OA, EO, MO, DO, and TO. Retention times were: 1.5 min for MO, 1.7 min for OA, 2.1 min for EO, 3.3 min for DO, and 14.7 min for TO. Three independent experiments were conducted for each reaction condition studied, and the data given in this work are the mean value of these three values.

RESULTS AND DISCUSSION

Immobilization of PSL. Immobilization allows reutilization of enzymes. Thus, it was interesting to find an immobilized lipase with high activity, especially in solid-phase reactions. PSL was adsorbed on distinct supports. Although the enzyme adsorption may be reversible in water, in low-water-content systems it is not. Thus, we selected this immobilization method based on simplicity and low cost. Considering the influence of water activity of the system on the bioprocesses, two distinct supports were studied: an inorganic matrix, Celite, with a low hydrophilicity (4), and a macroporous resin (Duolite). The percentage of protein adsorbed on the support was determined by the Peterson method (18). The degree of adsorption of PSL was 29 and 23% on Celite and Duolite, respectively. The activities of native and immobilized enzymes are PSL-PEG 25% modified (253); PSL-PEG 17% modified (248); PSL (240); PSL/Duolite (6.1); PSL/Celite (3.9), biocatalyst and hydrolytic activity (in parentheses), respectively where nkat/mg of lipase; conditions: tributyrin = 68 mM, Tris-HCl 1mM pH = 7.0, NaCL 0.1 M, $CaCL_2$ 0.1 M, 30°C. The hydrolytic activities per milligram of biocatalyst PSL-Celite and PSL-Duolite were 40 and 60 times lower than that of the native PSL, respectively. Some of this effect is due to the weight of the support and to diffusional limitations in the tributyrin reaction. However, the lipases modified with PEG had hydrolytic activities similar to native PSL in water.

Transesterification processes: (i) glycerolysis of EO in organic solvents. The glycerolysis of EO catalyzed by crude and purified *Candida rugosa* lipase was studied in ACN. This lipase is stable in ACN (19), but conversions higher than 6% were not obtained (not shown). PSL was a better lipase for this process. Thus, optimization of the transesterification was achieved through enzyme engineering [native (PSL), modified (PSL-PEG) and two distinct immobilized (PSL-Celite and PSL-Duolite) lipases] and medium engineering (polar and apolar solvents).

The pH dependence and influence of the water content. Enzyme activities in organic media have a pH dependence (2). Experiments (not shown) in acetonitrile indicated that, under the reaction conditions (0.5% vol/vol of buffer), the pH of the buffer has no great influence on the reaction rate, with pH 8.0 yielding slightly better results. This may be due to the small volume of buffer in the system. Thus, we selected phosphate buffer 0.1 M, pH 8.0 for all experiments.

The amount of water added to the reaction medium has a considerable influence on the product yields of transesterification and esterification processes (20). This is due to its effect both on the enzyme molecule and on the reaction equilibrium. Table 1 shows the results of the reaction between EO and G with distinct buffer contents, catalyzed by different derivatives of PSL. Independent of the biocatalyst used, the optimal water content for this reaction was 0.5% vol/vol in

TABLE 1
Glycerolysis of Oleic Acid Ethyl Ester (EO) in Two Distinct Solvents: Reaction Conditions and Molar Percentages of Glycerides in the Product Mixtures[a]

Biocatalyst	mg/mL	Time (h)	EO (M)	G (M)	% vol/vol Buffer	Temperature (°C)	% MO	% DO	% TO
Acetonitrile									
PSL	30	20	0.6	0.3	0	30	31.4	6.1	0
	30	20	0.6	0.3	0.5	30	38	6.9	0
	30	20	0.6	0.3	1	30	35.2	6.5	0
	30	20	0.6	0.3	5	30	34.4	4.9	0
	30	20	0.6	0.3	10	30	16	1.4	0
	30	20	0.6	0.3	30	30	0	0.9	0
PSL/Celite	30	20	0.6	0.3	0	30	34.5	7.5	0
	30	20	0.6	0.3	0.5	30	32.3	9.3	0
	30	20	0.6	0.3	5	30	15.1	6	0
	30	20	0.6	0.3	10	30	7.8	1.7	0
	30	20	0.6	0.3	30	30	3.7	1	0
PSL/Duolite	30	20	0.6	0.3	0	30	34.4	4.9	0
	30	20	0.6	0.3	0.5	30	34.4	6.1	0
	30	20	0.6	0.3	1	30	32.5	4.4	0
	30	20	0.6	0.3	5	30	20.9	1.8	0
	30	20	0.6	0.3	10	30	13.2	1.1	0
	30	20	0.6	0.3	30	30	0	0.5	0
PSL-PEG (25%)	10	20	0.6	0.3	0.5	30	30.3	12.3	2.4
PSL-PEG (17%)	5	20	0.6	0.3	0.5	30	27.4	8.9	1.2
Heptane									
PSL	30	22	0.6	0.3	0	30	1.2	0.1	0
	30	22	0.6	0.3	1	30	1.8	0.2	0
	30	22	0.6	0.3	3	30	1.7	0.2	0
	30	22	0.6	0.3	5	30	3.6	0.7	0
	30	22	0.6	0.3	7	30	1.3	0.1	0
	30	22	0.6	0.3	9	30	1	0	0

[a]G, glycerol; MO, 1-monooleoyl-*rac*-glycerol; DO, 1-3-diolein; TO, triolein. Celite (BDH, Poledorset, England); Duolite (Rohm and Haas France S.A., Chauny, France); PSL (Amano Pharmaceutical Co., Nagaoya, Japan); acetonitrile and *n*-heptane (Scharlau, Barcelona, Spain); the rest of the materials mentioned in Table 1 are from Sigma Chemical Co. (St. Louis, MO).

ACN. At higher water content, hydrolysis of the substrate (EO) was favored, and a continuous decay in the production rate of MO was found. The decay of the reaction rate with the enhancement of water content was similar for the various biocatalysts. The degrees of conversion were lower in *n*-heptane than in the polar solvent (Table 1). These findings can be explained by assuming that the partial glycerol solubilization in the polar solvent favors the formation of MO. Immobilized lipases were better biocatalysts than the native PSL because similar yields were obtained with the same biocatalyst amount (enzyme + support) at a given water content (i.e., 0.5% w/w). This seems to be due to the effect of the matrix, which favors the enzyme and glycerol dispersion in the reaction medium. It has been demonstrated that bioconversions with glycerol are greatly facilitated by prior adsorption of glycerol onto a solid support (6,21). The lipase modified with PEG was the best biocatalyst for the transesterification. Thus, one sixth of the amount of PSL-PEG (17%) was sufficient to obtain about 30% MO, which was nearly the same yield found with native or immobilized PSL after 20 h. Thus, the PEG chains linked to the protein enhance its contact with the apolar substrate. However, PSL-PEG produced TO and higher percentages of DO.

Effect of additives. Polyols are depressors of water activity, and their ability to reduce enzyme inactivation also has been demonstrated previously (22,23). In Table 2, we examined the reaction catalyzed by PSL and PSL-Celite in the presence of different amounts of PEG 5000. This polyol was added to the reaction mixture before the reaction was started by enzyme addition. The effect of this additive was small, but was greater in reactions with native than with supported lipase. The optimal content of this additive was 1:1 and 1:0.5 w/w of enzyme/PEG for native and immobilized lipases, respectively. The lower amount of this additive required with the immobilized lipase was also due to the low hydrophilicity of Celite (4). Nevertheless, PEG 5000 did not significantly increase the reaction yield, especially with PSL-Celite. These results do not justify the use of PEG 5000, considering the necessity of its removal from the final reaction mixtures. Considering the positive effect of Celite on water activity in the enzyme environment (pores of the support) and in the dispersion of reactants and enzyme in the viscous medium, the following experiments were conducted with PSL-Celite without additives.

Influence of the substrate concentrations. Two series of experiments were conducted to study the reaction conversion at different molar ratios EO/G. Results, shown in Figure 1A, were obtained for an excess of EO, and data of Figure 1B cor-

TABLE 2
Influence of the Additive Monomethoxypolyethylene Glycol (PEG) 5000 in the Glycerolysis of Oleic Acid Ethyl Ester (EO) in Acetonitrile[a,b]

Biocatalyst	Concentration (mg/mL)	Additive	Additive/enzyme (w/w)	% MO	% DO
PSL	20	PEG 5000	0.1:1	20.1	1.6
			0.5:1	20.8	3.7
			1:1	26.2	1.3
			3:1	23.7	3.7
PSL/Celite	45	PEG 5000	0:1	32.6	5.2
			0.5:1	35.8	4.3
			1:1	32.7	4.3
			3:1	27.4	5.3

[a]Conditions: EO = 0.494 M; G = 0.247 M, 2 h at 30°C.
[b]See Table 1 for other abbreviations and company sources.

respond to experiments with an excess of G. In both cases, the substrate in excess was increased while the other substrate was kept constant. The molar yields are expressed with respect to the less concentrated substrate. In Figure 1A, the conversion increased when higher EO was used, but in Figure 1B, a high G content decreased the reaction rate. This effect was probably due to the increased medium viscosity, making enzyme contact with EO more difficult. The highest ratio EO/G yielded a mixture of 58% MO, 14% DO, and 2% TO. In Figure 1, similar conversions were found with EO/G = 2:1 and 3:1. Thus, 100 mg MO was produced from 455 mg EO after 2 d. Considering the economics of the process, we selected the ratio 2:1 for process optimization in further experiments.

Effect of the temperature. Figure 2 shows the results obtained at various temperatures (0–60°C). An optimal temperature of 30°C was found for this reaction.

Time course of the reaction. The time course of the EO glycerolysis reaction was studied at the optimal conditions obtained in the preceding experiments (30°C, EO/G = 2:1, 0.5% vol/vol of buffer) and 12.5 mg/mL of PSL/Celite. Results are shown in Figure 3. Under these conditions, the process achieved equilibrium somewhere between 1 and 2 d of incubation (molar yields: 55% MO, 11% DO, and 1.5% TO).

Effect of enzyme concentration on conversion. To optimize the reaction at short times, the influence of the biocatalyst content on the reaction conversion was studied at the cited optimal conditions after 2 h. Data are shown in Figure 4. The yields increased with the lipase content in the range 0–260 mg. Similar yields were obtained either after a short time by increasing the biocatalyst amount (Fig. 4), or after long reaction times with a small enzyme amount (Fig. 3). However, when optimal conversions in Figures 3 and 4 were compared, we concluded that the addition of more biocatalyst to accelerate the reaction did not decrease the DO and TO percentages in the production mixture.

(ii) Glycerolysis of triolein in the absence of solvent. To improve the biocatalytic production of mono- and diglycerides, the glycerolysis of TO without solvent was also studied.

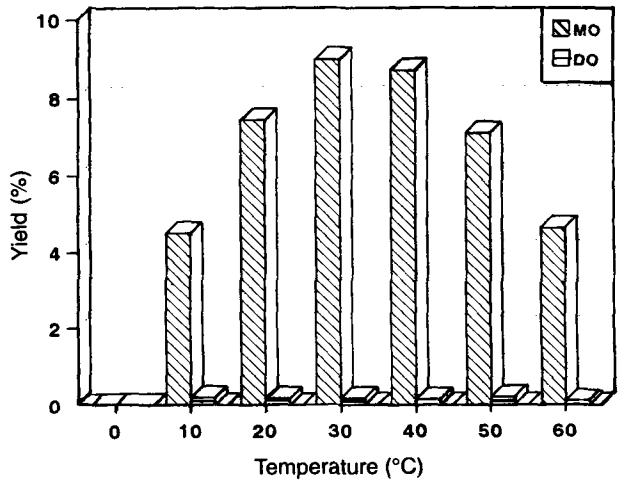

FIG. 2. Temperature dependence of EO glycerolysis in acetonitrile catalyzed by PSL-Celite. Conditions: 10 mg/mL biocatalyst, G = 0.25 M, EO/G = 2:1, 0.5% vol/vol of buffer, 30 min. Abbreviations and company source as in Figure 1.

Influence of water content. Glycerolysis of TO catalyzed by PSL-Celite was studied with various contents of buffer added to G (0–50% w/w) at 3°C. Table 3 shows the results obtained. The highest conversion was found with 2.5% w/w of buffer. High water content favored TO hydrolysis. A 2.5% w/w of buffer was selected to optimize this method.

Influence of TO/G molar ratio. To selectively prepare monoglycerides by this process, the G content was increased at a constant amount of TO (164 μmol). Figure 5A shows the results at 3°C and a relatively short reaction time (20 h). The reaction rate increased at higher G contents, and conversion of TO to DO and MO increased. Nearly total conversion of TO was found at TO/G = 1:15. At this short time, low excess of G (1:2 or 1:4) yielded similar percentages of MO and DO because only TO glycerolysis took place. When reaction rates were increased at a higher G excess, glycerolysis of DO also took place after 20 h. Thus, the MO percentage was twice that of the DO percentage at TO/G = 1:8. This was the optimal TO/G molar ratio for MO production. Considering the reac-

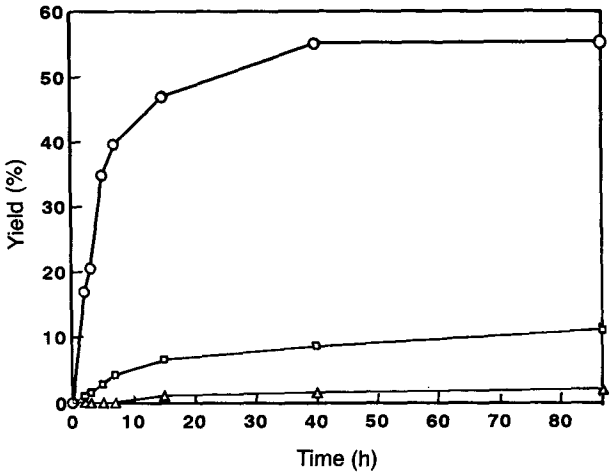

FIG. 3. The reaction course of EO glycerolysis in acetonitrile catalyzed by PSL-Celite. Conditions: 12.5 mg/mL biocatalyst, G = 0.25 M, EO/G = 2:1, 0.5% vol/vol of buffer, 30°C (○, monoolein; □, diolein; Δ, triolein). All data are mean values of three independent experiments. Abbreviations and company source as in Figure 1.

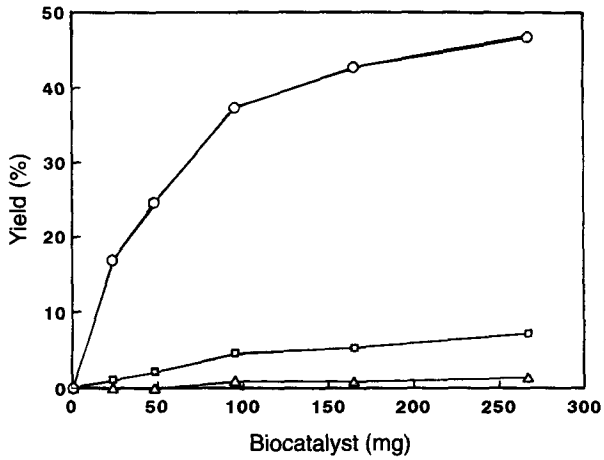

FIG. 4. Glycerolysis of EO in acetonitrile catalyzed by various amounts of PSL-Celite. Conditions: G = 0.25 M, EO/G = 2:1, 0.5% vol/vol of buffer, 2 h, 30°C (○, MO; □, DO; Δ, TO). Abbreviations and company source as in Figure 1.

tor dimensions and shape, it may be that the interfacial area between G and oleoylglycerols was constant at TO/G > 1:8, whereas the viscosity of the system increased when G was raised.

Temperature dependence. Chemical (24) and enzymic (8) processes of monoglyceride production are favored below a critical temperature at which the formed monoglyceride begins to precipitate. Here, results (not shown) demonstrated a negligible temperature dependence of the product yields in the range 3–15°C. In this range, crystallization of products in the reaction mixture was evident.

Effect of biocatalyst content. Figure 5B shows the effect of enzyme content in this reaction. The not-apparent reaction rate increased with an increase of biocatalyst content in the range of 25–200 mg of biocatalyst, and thus, conversion of TO into MO and DO was favored. Glycerolysis of DO also increased with higher enzyme concentrations, and the MO/DO molar ratio in the product mixture increased.

TABLE 3
Influence of the Water Content on Glycerolysis of Triolein (TO) in a Solvent-Free System[a,b]

% Water[c]	% MO	% DO	% TO	% OA
0	19	27	34	0
2.5	34	40	26	0
5	34	39	25	0
10	28	40	23	6
20	26	34	24	16
50	24	21	24	41

[a]Conditions: 50 mg of PSL-Celite, 145 mg of TO, 30 mg of glycerol (molar ratio TO/G = 1:2), 17 h at 3°C.
[b]OA, oleic acid. See Table 1 for other abbreviation and company sources.
[c]Water added to G w/w.

Reaction at longer times. The high stability of PSL-Celite under these conditions (fully active for months; Arcos, J.A., L. Robledo, and C. Otero, unpublished results) allowed us to study this reaction at long reaction times. *Pseudomonas cepacea* lipase supported on Celite also has long-term stability in a fatty acid/oil system (25). Two distinct conditions were studied to selectively obtain MO and DO. Figures 5C and D show the results for 50 and 100 mg of PSL/Celite, respectively. The stoichiometric amount of reactants (TO/G = 1:2) for MO synthesis was used in Figure 5D, whereas an excess of G was employed in Figure 5C to increase the maximum yield of MO. In the two figures, the process was very selective for MO production, which was optimal somewhere between 4 and 6 d in Figure 5C and after 4 d in Figure 5D. As expected, the highest yield was obtained for the conditions shown in Figure 5C due to the higher G content used. Under the conditions of Figure 5D, we managed to prepare DO in a molar yield similar to that of MO after a longer time (7 d). In this reaction, the same number of moles of DO and MO was initially produced by glycerolysis of TO, but the glycerolysis of DO also took place at longer times, which increased the yield of MO. Only when G was nearly consumed did MO start to yield G + DO. Production of DO at long times was probed to be reproducible. We have not found a complete explanation for this phenomenon, but it could be related to the tendency of the system to have a minimum G content. The fact that it happened only in processes with stoichiometric concentrations of reactants to obtain MO and after nearly total consumption of G, made more evident the trend of the system to produce G at that point. It never took place in the reactions of Figure 5C because G was in excess during all of the reaction time. The conditions for Figure 5C (or better TO/G=1:8 according to Figure 5A) can be used for selective production of MO, but the reaction conditions of Figure 5D were better for the preparation of DO. That different reaction temperatures were used in Figures 5C and 5D is not consid-

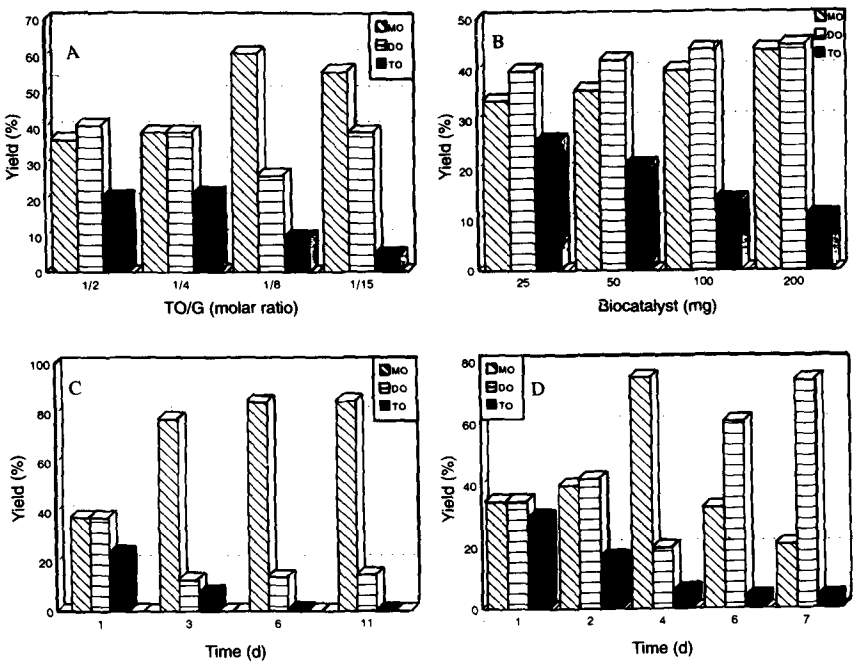

FIG. 5. Glycerolysis of triolein (TO) catalyzed by PSL-Celite in a solvent-free system: A) influence of the TO/G molar ratio, 50 mg enzyme, 145 mg TO, 20 h, 3°C; B) reaction with various amounts of biocatalyst, 145 mg TO, TO/G molar ratio = 1:2, 2.5 wt% of buffer added to G, 20 h, 3°C; C) yields at various times, 50 mg enzyme, 140 mg TO, TO/G molar ratio = 1:4, 2.5 wt% of buffer added to G, 3°C; D) yields at various times, 100 mg enzyme, 145 mg TO, TO/G molar ratio = 1:2, 2.5 wt% of buffer added to G, 10°C. Other abbreviations and company source as in Figure 1.

ered to be a significant contributing factor to differences in yield because our previous results (not shown) show that yield is not temperature-dependent in this temperature region.

Esterification without solvent. In the presence of a solvent, glycerolysis is a better process than direct esterification for the production of partially acylated glycerides (26). However, glycerolysis in the absence of solvent had not been compared with esterification. Thus, condensation between OA and G was also studied without the addition of any solvent.

Reaction at different OA/G ratios. In Figure 6A, the effect of the molar ratio of the substrates, G and OA, was studied. Considering the economical aspects of the process, it was more interesting to increase the G concentration. Thus, the experiments were conducted while keeping OA constant and increasing G. As opposed to glycerolysis of EO (Fig. 2) in acetonitrile, esterification without solvent was favored by high G contents. However, conversion decreased at OA/G ratios below 1:5, indicating the existence of high diffusional limitations when the excess of G was high. Esterification of the formed MO was fast, and a negligible amount of MO was obtained. The optimal OA/G molar ratio (1:5) selectively yielded DO (49%) with less than 9% of (MO + TO), in a relatively short reaction time (24 h).

Study at long reaction times. The reaction at optimal conditions of Figure 6A was studied at longer reaction times (1–4 d, Fig. 6B). Initially, the mixture of products was rich in DO because of the fast MO acylation. At longer times, glycerolysis of the formed DO competes with direct esterification. Thus, the fatty acid was totally consumed after 3 d, but the reaction did not achieve its equilibrium. Due to the G excess, the glycerolysis of DG was the most favorable process at these longer times. Thus, nearly all DO was converted to MO (97%) after 4 d. Results in Figure 6B demonstrate that esterification in the absence of solvent is a selective method to obtain MO and/or DO, depending on the reaction time.

The influence of temperature. The preceding experiments were performed at 3°C. Considering the high MO melting point, its production should be favored at low temperatures (8,10). In Figure 7, we studied the effect of temperature on esterification at the optimal conditions for DO production of Figure 6B (24 h). This plot demonstrated that process selectivity was higher at the lowest temperature studied. Thus, TO appeared in similar molar yields to MO and DO at 15°C. Negligible reaction was observed at 30–60°C. This effect was not due to enzyme inactivation (active for months at these temperatures; Arcos, J.A., L. Robledo, and C. Otero, unpublished data). It was most likely due to the positive effect of crystallization of the G esters below a given temperature.

Comparison among processes. We have compared three different methods to selectively produce partially acylated glycerides (glycerolysis of EO and TO, and esterification between OA and G). Each reaction was optimized independently by varying the enzyme (different biocatalysts), medium, and reaction engineering. A comparison of the opti-

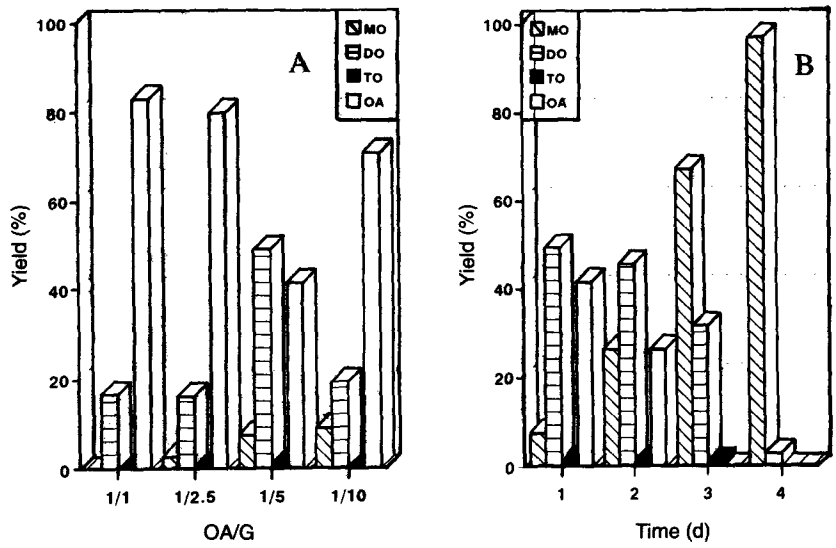

FIG. 6. Direct esterification between oleic acid (OA) and G in absence of solvent: A) effect of the OA/G molar ratio. Conditions: 75 mg PSL-Celite, 140 mg OA, 2.5 wt% of buffer added to G, 24 h, 3°C, molecular sieves; B) reaction at distinct times. Conditions: 75 mg PSL-Celite, 140 mg OA, OA/G molar ratio = 1:5, 2.5 wt% of buffer added to G, 3°C, molecular sieves. Other abbreviations and company source as in Figure 1.

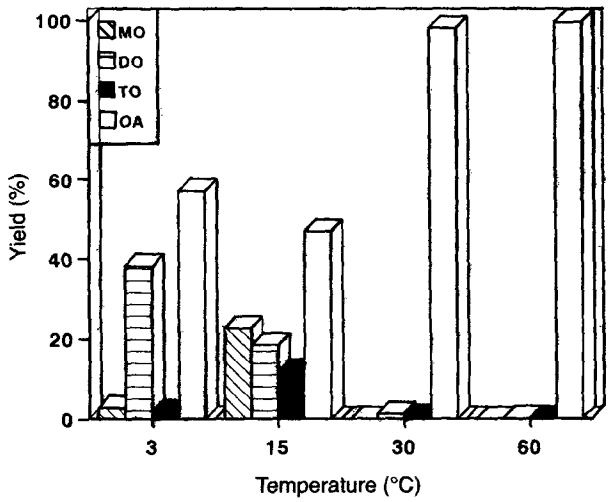

FIG. 7. Temperature effect in the esterification between oleic acid (OA) and G in absence of solvent. Conditions: 75 mg PSL-Celite, 140 mg OA, OA/G molar ratio = 1:5, 2.5 wt% of buffer added to G, 24 h, molecular sieves. Other abbreviations and company source as in Figure 1.

mal results of each process is shown in Table 4. The degree of conversion and the wt% of mono-, di-, and triglycerides in the final mixtures are compared in this table.

Mono- and diglycerides can be prepared by triglyceride hydrolysis. Although inexpensive, (naturally occurring triglycerides may be used), less than 40 wt% monoglyceride (corresponding to a molar yield of 33%) depending on the fatty acid, may be obtained after their total hydrolysis. Also, selectivity of the enzymic hydrolysis for MO and DO production is low (4). Consequently, hydrolytic processes were not considered in our study. The alcoholysis of triglycerides in organic solvents (27) has also been reported. It yielded 97% 2-monopalmitin (28), but required extraction of the solvent (methyl-*tert*-butyl ether) and the ethyl palmitate co-product at the end of the process.

Better reactions could be those that employ the formation of the ester linkage in low-water systems. Under these conditions, mono- and diglycerides are rather stable toward acyl migration. PSL was an effective lipase, and the supports (Celite and Duolite) were previously shown to be good matrices for hydrolysis of triglycerides (4,29). Celite should be more useful due to its low hydrophilicity and to the higher mechanical resistance of inorganic supports. The effect of Celite on the enzyme and G dispersion also has to be considered. Mixtures of the G derivatives found in process I with PSL-Celite (Table 4) satisfy the WHO requirements, and the highest conversions were 68% = 70 wt% MO + 24 wt% DO + 6 wt% TO. However, EO was used in excess, and a residue was present in the mixture of products. In organic solvents, glycerolysis of the fatty acid ethyl ester had been a better reaction than direct fatty acid esterification (26). In these media (e.g., process I in Table 4), the solvent acts like an inhibitor, due to reduction of the EO concentration at the interface. To mitigate this effect, G solubilization by *in situ* derivatization with phenylboronic acid has been used (30), but this requires elimination of the derivatizing agent. Alternatively, reactions of isopropylidene G, solubilized in *n*-heptane, have been used (31), but these were less selective than reactions studied here in ACN or in its absence.

We looked for the production of pure mono- and dioleoyl-

TABLE 4
Comparison of the Various Processes Studied, Considering Selectivity, Yield, and Cost[a]

Bioprocess	Solvent	Temp. (°C)	Time (d)	G (mg)	Donor/price[b] (mg/$)	MO (%/mg)[c]	DO (%/mg)[c]	TO (%/mg)[c]	OA (%/mg)[d]	Conv.	Cost[d] ($)
(**I**) EO + G	ACN	30	4	45	309:1.52	70:100	24:17	6:5	—	68	14.8
(**II**) TO + G	—	3	6	60	140:1.22	76:123	22:35	2:4	—	99	9.9
(**III**) TO + G	—	10	7	30	145:1.26	14:21	80:124	6:9	—	96	10.1
(**IV**) OA + G	—	3	1	228	140:1.05	6:9	67:99	2:4	25:38	59	10.6
(**V**) OA + G	—	3	4	228	140:1.05	93:167	7:9	—	—	100	6.4

[a]See Table 1 for other abbreviations and company sources; OA, oleic acid; ACN, acetonitrile.
[b](EO in **I**, TO in **II** and **III**, OA in **IV** and **V**)/(cost of these mg of donor).
[c]Weight of this glyceride/weight of total glycerides.
[d]Approximate cost of the substrates in dollars to obtain 1 g of the predominant glyceride product. The cost of G has been neglected, considering its low price.

glycerides. Table 4 shows the yields and cost of the substrates for the best processes. These were based on current catalogue, not bulk, prices and are used here only for comparative purposes to estimate the relative costs of the different reactions investigated. Also, unconverted substrates could be recovered and reused, which should reduce the differences in costs of processes **I–V**. We found less costly and more selective processes in the absence of solvents: glycerolysis of TO and direct esterification of OA (**II–V** in Table 4). PSL-Celite catalyzed 99% conversion in process **II**, and the MO (76 wt%) obtained was cheaper (9.9 $/g) but nearly as impure as that obtained at lower conversion (68%) in process **I**. However, esterification (process **V**) yielded 93 wt% of MO in the absence of either TO and OA and at lower cost (6.4 $/g), due to its 100% degree of conversion. Thus, esterification without solvent allowed us to obtain purer and more economical MO with this biocatalyst. This supported-lipase produced higher conversions and purer MO than those reported (90 wt% of MO for 76% conversion, and 74 wt% of MO for 90% conversion) by Yamaguchi and Mase (11). However, glycerolysis was the better method for selective DO preparation. Thus, similar costs of the substrates (10.6 and 10.1 $/g) were necessary to obtain 67 and 80 wt% of DO in esterification and glycerolysis, respectively (**III** and **IV** in Table 4). In the esterification (process **IV**), the product mixture contained a high amount of residual OA due to the low conversion of this process (59%). The high activity of PSL-Celite in the solid-phase reactions (low-temperature processes in Table 4) has an advantage over reactions with nonimmobilized lipases (8–10) due to the ease of enzyme recovery. Also, it produces yields similar to those reported by these authors for natural fats and oils.

Our comparative study showed that the physical state of substrates (pure or diluted) is a key factor in the production of glycerides. The number of EO molecules at the interface is much higher in solvent-free systems than in systems containing a solvent. Thus, an excess of the more expensive reactant (EO/G >1) is required to increase MO yield in the EO glycerolysis with ACN (**I** in Table 4). However, reactions in the absence of solvent require an excess of the cheapest substrate (G). Moreover, the more expensive reactant (TO or OA) was totally converted into products (**V**, Table 4). The alcohol produced in EO glycerolysis may restrict application of the product glycerides in certain foods. On the contrary, in the direct esterification process, water is produced instead of alcohol. In process **I**, an additional purification step is also necessary to eliminate the unreacted EO. Additionally, use of an organic solvent as the reaction medium may produce some restriction on use of the product in the food industry, and in any case, its elimination is required. ACN is not included in the EC directives for food additives. Consequently, reactions in the absence of solvent, such as those studied here, are more desirable. Reverse micellar systems (32) are not suitable because of the necessary surfactant elimination and restrictions of the G concentration into the droplets.

Although it is hard to solidify the reaction mixture while allowing lipase to be fully active (9), we demonstrated that PSL-Celite is a good biocatalyst for reactions in solid-phase systems. Thus, use of an immobilized lipase (PSL-Celite) with high activity in solid-phase reactions (glycerolysis and esterification) has been described in this paper. From these results and those reported for interesterification processes (33,34), diatomaceous earth (Hyflo Supercel and Celite) has been shown to be useful in biotransformations of fats and oils. Comparisons of the conversions, yields, and costs in this paper suggest that, while glycerolysis is the most interesting process to produce DO, esterification is better for MO preparation with this biocatalyst. Because these biotransformations produce higher yields than nonenzymic processes (40–60% monoglyceride), which require molecular distillation, thus increasing their manufacturing cost (35), they should be more interesting for industrial preparation of these pure emulsifiers and stabilizers.

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REFERENCES

1. Mattson, F.H., and L.W. Beck, The Digestion *in vitro* of Triglyceride by Pancreatic Lipase, *J. Biol. Chem. 214*:115–119 (1955).

2. Macrae, A.R., Extracellular Microbial Lipases, in *Microbial Enzyme and Biotechnology,* edited by W.M. Fogarty, Applied Science, London, 1983, pp. 225–250.
3. Gerhartz, W., Industrial Uses of Enzymes, in *Enzymes in Industry*, edited by W. Gerhartz, VCH Verlagsgesellschaft, Weinheim, Germany, 1990, pp. 89–91.
4. Otero, C., E. Pastor, V.M. Fernández, and A. Ballesteros, Influence of the Support on the Reaction Course of Tributyrin Hydrolysis Catalyzed by Soluble and Immobilized Lipases, *Appl. Biochem. Biotech. 23*:237–247 (1990).
5. Holmberg, K., B. Lassen, and M.B. Stark, Enzymatic Glycerolysis of a Triglyceride in Aqueous and Nonaqueous Microemulsions, *J. Am. Oil Chem. Soc. 66*:1796–1800 (1989).
6. Berger, M., and M.P. Schneider, Enzymatic Esterification of Glycerol. II. Lipase-Catalyzed Synthesis of Regioisomerically Pure 1(3) *rac*-Monoacylglycerols, *Ibid. 69*:961–965 (1992).
7. Pastor, E., A. Ballesteros, and C. Otero, Enzymatic Synthesis of Oleins in Organic Media, *Ann. N.Y. Acad. Sci. 750*:242–245 (1994).
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., S. Shimizu, 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. Yamaguchi, S., and T. Mase, High-Yield Synthesis of Monoglyceride by Mono- and Diacylglycerol Lipase from *Penicillium camembertii* U-150, *J. Fermentation and Bioeng. 72*:162–167 (1991).
12. Reslow, M., P. Adlercreutz, and B. Mattiasson, On the Importance of the Support Material for Bioorganic Synthesis. Influence of Water Partition Between Solvent, Enzyme and Solid Support in Water-Poor Reaction Media, *Eur. J. Biochem. 172*:573–578 (1988).
13. Kimura, Y., A. Tanaka, K. Sonomoto, T. Nihira, and S. Fukui, Application of Immobilized Lipase to Hydrolysis of Triacylglyceride, *Eur. J. Appl. Microbiol. Biotechnol. 17*:107–112 (1983).
14. Cho, S.W., and J.S. Rhee, Immobilization of Lipase for Effective Interesterification of Fats and Oils in Organic Solvents, *Biotechnol. Bioeng. 41*:201–210 (1993).
15. Inada, Y., T. Yoshimoto, A. Matshushima, and Y. Saito, Engineering Physicochemical and Biological Properties of Proteins by Chemical Modification, *Trends Biotechnol. 4*:68–73 (1986).
16. Inada, Y., K. Takahashi, T. Yoshimoto, A. Ajima, A. Matsushima, and Y. Saito, Application of Polyethylene Glycol-Modified Enzymes in Biotechnological Processes: Organic Solvent-Soluble Enzymes, *Ibid. 4*:190–194 (1986).
17. Habeeb, A.F.S.A., Determination of Free Amino Groups in Proteins by Trinitrobenzenesulphonic Acid, *Anal. Biochem. 14*:328–336 (1966).
18. Peterson, G.L., A Simplification of the Protein Assay of Lowry *et al.* Which Is More Generally Applicable, *Ibid.* 83:346–356 (1977).
19. Torres, C., and C. Otero, Activity Decay and Conformational Change of Lipase in Presence of Organic Solvents. A Fluorescence Study of *Candida rugosa* Lipase, *J. Mol. Catal. A: Chemical 97*:119–134 (1995).
20. Macrae, A.R., Interesterification of Fats and Oils, in *Biocatalysts in Organic Synthesis*, edited by J. Tramper, H.C. Van der Plas, and P. Lin Ko, Elsevier, Amsterdam, 1985, pp. 195–208.
21. Berger, M., K. Laumen, and M.P. Schneider, Enzymatic Esterification of Glycerol. I. Lipase-Catalyzed Synthesis of Regioisomerically Pure 1,3-*sn*-Diacylglycerols, *J. Am. Oil Chem. Soc. 69*:955–960 (1992).
22. Klibanov, A.M., Stabilization of Enzymes Against Thermal Inactivation, in *Advances in Applied Microbiology*, edited by A.I. Laskin, Academic Press, New York, 1983, vol. 19, pp. 1–28.
23. Ye, W.N., D. Combes, and P. Monsan, Influence of Additives on Thermostability of Glucose Oxidase, *Enzyme Microb. Technol. 10*:498–502 (1988).
24. Eckey, E.W., Directed Interesterification in Glycerides, *Industrial Engineering Chemistry 40*:1183 (1948).
25. Bornscheuer, U.T., and T. Yamane, Activity and Stability of Lipase in the Solid-Phase Glycerolysis of Triolein, *Enzyme Microb. Technol. 16*:864–869 (1994).
26. Pastor, E., C. Otero, and A. Ballesteros, Enzymatic Preparation of Mono- and Distearin by Glycerolysis of Ethyl Stearate and Direct Esterification of Glycerol in the Presence of a Lipase from *Candida antarctica* (Novozym 435), *Biocatalysis and Biotransformation 12*:147–157 (1995).
27. Mukesh,D., A.A. Banerji, R. Newadkar, and H.S. Bevinakatti, Mathematical Modelling of Enzymatic Butanolysis of Vegetable Oils, *Biocatalysis 8*:191–199 (1993).
28. Millqvist, A., P. Aldercreutz, and B. Mattiasson, Lipase-Catalyzed Alcoholysis of Triglycerides for the Preparation of 2-Monoglycerides, *Enz. Microb. Technol. 16*:1042–1047 (1994).
29. Kosugi, Y., and H. Suzuki, Functional Immobilization of Lipase Eliminating Lipolysis Product Inhibition, *Biotechnol. Bioeng. 40*:369–374 (1992).
30. Lang, S., A. Zieman, B. Steffen, A. Schlotterbeck, and F. Wagner, Lipase-Catalyzed Monoacylation of Polyols, in Abstracts 6th European Congress on Biotechnology, Firenze, vol. III, 1993, pp. WE149.
31. Akoh, C.C., Lipase-Catalyzed Synthesis of Partial Glyceride, *Biotechnol. Lett. 15*:949–954 (1993).
32. Holmberg, K., and E. Osterberg, Enzymatic Preparation of Monoglycerides in Microemulsion, *J. Am. Oil Chem. Soc. 65*:1544–1548 (1988).
33. Ison, A.P., P. Dunnil, and M.D. Lilly, Effect of Solvent Concentration on Enzyme Catalysed Interesterification of Fats, *Enzyme Microb. Technol. 10*:47–51 (1988).
34. Mojovic, L., S. Siler-Marinkovic, G. Kubic, B. Bugarski, and G. Vunjak-Novakovic, *Rhizopus arrhizus* Lipase-Catalyzed Interesterification of Palm Oil Midfraction in a Gas-Lift Reactor, *Ibid. 16*:159–162 (1994).
35. Lauridsen, J.B., Food Emulsifiers: Surface Activity, Edibility, Manufacture, Composition and Application, *J. Am. Oil Chem. Soc. 53*:400–407 (1976).

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