Diesel Fuel as a Solvent for the Lipase-Catalyzed Alcoholysis of Triglycerides and Phosphatidylcholine

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ABSTRACT: The abilities of three commercially available lipases—Lipozyme IM 20, Amano PS-30, and Amano CE—to synthesize fatty acid ethyl esters via the alcoholysis of soy triglycerides (TG) and phosphatidylcholine (PC) in grade No. 2 diesel fuel were investigated. All three enzyme preparations were active in water-saturated diesel fuel, synthesizing fatty acid esters from both TG and PC. Response surface methodology, based on a Modified Central Composite design, was employed to examine the coordinate effects of lipid, water, and ethanol concentrations on enzyme activities and to identify conditions yielding maximum alcoholysis. For all three enzymes, optimal activities toward TG occurred at added water concentrations of less than 0.3 M. With PC as substrate, optimal enzyme activities occurred at added water concentrations as much as tenfold greater than this, and the amount of water required for maximum activity was proportional to the substrate concentration. For both substrates the enzyme activities were generally reduced as ethanol concentrations rose. The exceptions to this were the Lipozyme–TG combination, where activity increased with increasing ethanol concentrations, and the PS-30–PC combination, where activity was roughly constant across the range of water and ethanol concentrations examined. Hydrolytic activities of the enzymes in aqueous reactions were poor predictors of transesterification activity in organic solvent: the aqueous hydrolytic activities of CE and PS-30 toward TG were roughly comparable, and 25 to 50 times greater than that of IM 20. However, in the alcoholysis of TG in diesel fuel the order of activities was PS-30 > IM 20 > CE. The activities of Lipozyme and CE toward PC were similar to one another, and PS-30 was considerably less active on this substrate. CE lipase was more active toward PC than toward TG. Lipozyme displayed comparable activity toward the two substrates. PS-30 was considerably more active on TG than on PC. Degrees of conversion were consistent with the transesterification of only one fatty acid of TG, and slightly greater than one fatty acid for PC. Preliminary studies indicated that for CE and PS-30, but not for Lipozyme IM 20, the degree of conversion of TG, but not PC, could be significantly increased by the further provision of ethanol. Expansion of this work could lead to a method for the production of simple fatty acid esters, which are suitable as diesel fuels, from multicomponent agricultural materials containing TG and PC.
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"Biodiesel" is the term applied to ester-based fuel oxygenates derived from biological sources and intended for use in compression-ignition engines (1). Among the attractive features of the use of biodiesel as a fuel are the facts that (i) it is domestically produced, offering the possibility of reducing petroleum imports, (ii) it is plant-, not petroleum-derived, and as such its combustion does not increase current net atmospheric levels of CO_2, a "greenhouse" gas, (iii) it is biodegradable, and (iv) relative to conventional diesel fuel its combustion products have reduced levels of particulates, carbon monoxide and, under some conditions, nitrogen oxides (1). As a consequence, there is considerable interest in exploring and developing the use of biodiesel as a fuel (1–5).

Although triglycerides (TG) can fuel diesel engines, their relatively high viscosities and other problems have led to the investigation of various derivatives. Chief among these are fatty acid esters, which are currently the favored compounds for biodiesel. Methyl esters derived by chemical transesterification with methanol (alcoholysis) from various vegetable oils or from waste animal fats and greases have received the most attention. However, due to the relatively high costs of vegetable oil, methyl esters produced from it cannot compete economically with petroleum diesel unless granted protection from the considerable tax levies applied to the latter. As tax relief is a controversial subject, there is a need to explore alternate feedstocks for the production of biodiesel.

The processing of oilseeds for the production of edible vegetable oil generates by-product streams containing TG, phospholipids, and free fatty acids (6). In many cases these streams are of considerably lower value than the finished oil. In prior work (7) we have investigated the possibilities of increasing the value and utilization of these by-products by using them as sources of free fatty acids, which are widely used in a variety of applications. These earlier studies employed lipolytic enzymes as catalysts to hydrolyze the TG and phospholipid components of soybean soapstock. Enzymatic catalysis was employed because phospholipids are not suitable for hydrolysis by the high-temperature, high-pressure technologies used in contemporary industrial fat splitting. We have now expanded these studies to explore the enzymatic alcoholysis of these compounds. Successful development of a scheme for ester synthesis from low-value lipids could address the economic barriers to a wider adoption of biodiesel fuels.

The use of lipophilic organic liquids as solvents for the conduct of enzyme-catalyzed reactions has gained considerable attention since its description 15 years ago (8–10). For many types of reactions this approach offers advantages over water-based reactions. Among these are enhanced catalyst stability, increased substrate/product solubility, decreased side reactions, an absence of microbial contamination, and the ability to conduct reactions which are thermodynamically unfavorable in aqueous systems. Accordingly, there is vigorous research in this area, often employing lipases (triacylglycerol acylhydrolase, E.C. 3.1.1.3) as catalysts (11,12). In much of this work the solvents have been hexane or isooctane, due largely to the fact that solvents in this polarity range often support the highest enzyme activities. However, it is clear that enzymes display activity in various solvents with a range of polarities (13–17). It has been shown that lipases are able to catalyze the alcoholysis of triglycerides in both aqueous (18,19) and nonaqueous systems (10, 20–24). However, we are unaware of any investigation of the use of diesel fuel, a heterogenous mixture of liquid olefins, aromatics, and normal, cyclo- and branched paraffins, or any other common fuel, as a solvent for enzymatic catalysis. Such liquids could be useful for the synthesis of biodiesel from glycerides as the ester products would be soluble in the solvent, whereas other products (e.g., glycerol, glycerophosphorylcholine) and the catalyst itself would be insoluble. This might greatly simplify product recovery. Therefore we have conducted an initial investigation of the enzymatic alcoholysis of TG and a phospholipid in diesel fuel. Ethanol was used as the co-reactant alcohol as it also can be derived from renewable resources and because fatty acid ethyl esters are acceptable biodiesel fuels.

EXPERIMENTAL PROCEDURES

Chemicals. L-α-Phosphatidylcholine (PC, >99%, from soybeans) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Ethyl linoleate was from Sigma Chemical Co. (St. Louis, MO). Food-grade soybean oil, obtained locally, was used directly as a source of soy TG. Lipozyme IM 20, consisting of *Rhizomucor miehei* lipase immobilized on a Duolite resin, was the product of Novo Nordisk BioChem (Franklinton, NC). Lipases CE (from *Humicola lanuginosa*) and PS-30 (*Pseudomonas* sp.), supplied as dry powders, were obtained from Amano Enzyme U.S.A. Co., Ltd. (Troy, VA). Enzymes were lyophilized overnight and stored over calcium sulfate at 4°C prior to use. Ethanol (U.S.P., 200 proof, anhydrous) was produced by the Warner-Graham Co. (Cockeysville, MD). Hexane and isopropanol (Burdick and Jackson Brand) were purchased from Baxter (Muskegon, MI). Base 11 diesel fuel (an additive-free diesel fuel; Mobil Oil Corp., Edison, NJ) was provided by Dr. Lloyd Nelson of ERRC (Wyndmoor, PA). Grade No. 2 diesel fuel, obtained from local automotive fuel dealers, was saturated with distilled, deionized water by overnight shaking at room temperature (diesel/water, 5:1, vol/vol) prior to use.

Determination of lipolytic activity. A pH-stat method employing a continuous titrating pH meter (Radiometer, Copenhagen, Denmark) was used to determine the lipolytic activity of each lipase in an aqueous reaction system using emulsified soybean oil as the substrate (7,25). Incubations were conducted at 25°C. Enzymes were assayed at pH 8, the optimal pH for all.

Determination of ester synthesis. A Modified Central Composite experimental design (26) was employed to study

TABLE 1
Settings for the Variable Factors Examined

Substrate	Factor	Enzyme[a]	Minimum		Midpoint		Maximum
Soy triglyceride	Substrate	All	0.19 mL	0.70	1.17	1.57	1.80
	Water	IM 20 (35 mg)	0 μL	2.5	5	10	15
		CE (50 mg)	2.5 μL	6	13	20	25
		PS-30 (35 mg)	0 μL	6	15	24	30
	Ethanol	All	45 μL	100	195	290	350
Soy PC	Substrate	All	0.20 g	0.58	1.05	1.41	1.67
	Water	IM 20 (35 mg)	5 μL	15	90	135	200
		CE (50 mg)	5 μL	30	140	250	350
		PS-30 (35 mg)	450 μL	600	750	900	1050
	Ethanol	IM 20, CE	100 μL	240	450	660	800
		PS-30	50 μL	100	150	200	250

[a]Lipase IM 20 from Novo Nordisk (Franklinton, NC); lipases CE and PS-30 from Amano (Troy, VA).

the effects of the concentrations of water, lipid substrate, and ethanol on the enzymatic alcoholysis of either TG or PC. For each enzyme, the appropriate concentration ranges of these variables (Table 1) were established by preliminary experiments which identified the portion of variable space beyond which enzymatic activity declined. The upper concentration limit of PC was dictated by the fact that more concentrated solutions were extremely viscous, which restricted proper mixing and prevented accurate sampling. The TG concentration range was chosen to be equimolar to the PC range. The amounts of enzyme employed were chosen to yield between 25 and 40% esterification of TG within a 6 to 28 h incubation. Reaction times were PS-30, 6 h; CE, 20 h; Lipozyme, 28 h. The same amounts of enzyme were used in studies of the esterification of PC, sometimes necessitating longer incubation periods (CE, 17 h; Lipozyme, 48 h; PS-30, 49.5 h) to achieve substantial degrees of conversion.

Alcoholysis reaction mixtures (approximately 5 mL) contained water, ethanol, catalyst, and either TG or PC in water-saturated diesel fuel. Reactions were made by first dissolving the lipid substrate in diesel fuel, dispensing 5 mL to 20 × 150 mm screw-cap tubes, and adding desired amounts of water, ethanol, and enzyme. Reactions were conducted at 42°C, with orbital shaking at 350 rpm. When time course studies were conducted with TG as the substrate, 50-μL samples were removed from each reaction tube at predetermined incubation times and their ester contents were determined. When PC was the substrate, several identical reactions were incubated, with a whole tube being prepared for analysis at each sampling time. Time course studies were conducted in duplicate for each enzyme–substrate combination. The average variation of the degree of esterification in each reaction tube from the mean for the replicate pairs was 2.5% for the TG substrate and 0.6% for the PC substrate.

Following incubation, the reactions were diluted with hexane, filtered over Millipore Brand Millex FX13 membranes (0.5 μm, Sigma Chemical Co.), and their ethyl ester contents were determined by high-performance liquid chromatography (HPLC) using a Hewlett-Packard (Valley Forge, PA) 1050 Chromatography System. Samples containing PC were analyzed with a 3 × 100 mm Lichrosorb DIOL column (Chrompack Inc., Raritan, NJ) eluted isocratically with 0.1% isopropanol in hexane at a flow rate of 0.5 mL/min. When TG was the substrate the determination was conducted using a 3 × 100 mm Lichrosorb Si 60-5 column eluted with gradients of isopropanol and water in hexane/0.6% glacial acetic acid (7). Analyte peaks, which were baseline-resolved under these HPLC conditions, were detected with a mass-based detector (ELSD IIA; Alltech, Deerfield, IL) operating at a nitrogen flow rate of 3.5 L/min and a nebulizer temperature of 60°C for TG and 2.4 L/min, 40°C for PC. Fatty acid ethyl ester was quantitated by reference to a response curve generated using pure ethyl linoleate. Ester yields are expressed as percentages of theoretical maximum, calculated on the basis of three available fatty acids in TG and two in PC. Neither hydrolysis of the substrates nor nonenzymatic esterification was observed during these investigations.

RESULTS AND DISCUSSION

Initial studies demonstrated that Amano CE, Amano PS-30, and Novo Nordisk Lipozyme IM 20 were able to synthesize fatty acid esters by alcoholysis of glycerides in commercial diesel fuel. Additive-free diesel fuel gave a generally comparable performance. It was not used here because the fuel-grade material is more readily available.

To facilitate comparison of these enzymes, their hydrolytic activities toward emulsified soybean oil were determined. Amano PS-30 and CE exhibited roughly comparable activities, with Lipozyme IM 20 being about one-thirtieth as active (Table 2).

Statistical experimental design concepts were used to determine the coordinate dependence of the alcoholysis activities of these enzymes in diesel fuel on the concentrations of water, lipid, and ethanol. The estimated regression equations resulting from these studies are of the form shown in Equation 1:

$$\text{Predicted esterification (\%)} = X_0 + X_1\,(\text{lipid}) + X_2\,(\text{water}) + X_3\,(\text{ethanol}) + X_4\,(\text{lipid})(\text{water}) + X_5\,(\text{lipid})(\text{ethanol}) + X_6\,(\text{water})(\text{ethanol}) + X_7\,(\text{lipid})^2 + X_8\,(\text{water})^2 + X_9\,(\text{ethanol})^2 \quad [1]$$

where the coefficients X_0, X_1, X_2 etc., which are unique for each enzyme-substrate pair, are listed in Table 3 (concentrations expressed in units of molarity). These coefficients have units of % (for X_0), % Molarity (for X_1 through X_3) and % Molarity^2 (for X_4 through X_9). The coefficients of determination, R^2, for these calculations indicate that the derived models fit the data well, accounting for between 64 and 80% of the total variability of the data (Table 3). The R^2 values also indicate that the enzymes were most sensitive to variations in the water concentration and the interaction of water and ethanol concentrations (Table 3).

Equation 1 and Table 3 were employed to construct response surfaces displaying the predicted degrees of alcoholysis of TG and PC over the range of water and lipid concentrations studied here. Representative surfaces are shown in Figures 1 and 2.

Just as in the hydrolysis of soybean oil in aqueous reactions (Table 2), Amano PS-30 was the most active of the enzymes studied here in the esterification of TG in diesel fuel. A six-hour incubation with this enzyme resulted in degrees of esterification not achieved with Lipozyme IM 20 or Amano CE until after 20 h of incubation (Fig. 1). It is also notable that despite the substantially lower activity of Lipozyme IM 20 in the aqueous hydrolytic reaction (Table 2), the amount

TABLE 2
Aqueous Hydrolytic Activities of Selected Lipases[a]

Enzyme	pH	Activity (U/mg)[b]
Lipozyme IM 20	8.0	0.37
Amano PS-30	8.0	17.8
Amano CE	8.0	8.81

[a]See Table 1 for company sources.
[b]U = μmole fatty acid released per minute, using emulsified soybean oil as substrate.

TABLE 3
Coefficients of the Generic Equation (Eq. 1) Relating Esterification Activity to Reactant Concentrations[a]

Enzyme	Substrate	X_0	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	R^2
Lipozyme IM 20	TG	37.57	−0.1447	−34.50	0.0581	−0.0001	1.160	−2533	0.0002	0.0647	−0.0001	0.6375
CE	TG	48.18	−0.0846	−76.00	−0.0035	−0.0001	0.5647	−554.3	0.0001	−0.0190	0	0.7075
PS-30	TG	5.844	0.0254	124.8	0.0492	−0.0002	0.3300	−703.3	0	−0.0803	0	0.7235
Lipozyme IM 20	PC	32.29	0.0090	3.589	−0.0100	0.0004	0.1045	−9.962	0.0001	−0.0059	0	0.9332
CE	PC	13.30	0.1095	16.58	−0.0030	0.0007	0.0703	−5.552	0.0001	−0.0055	0	0.9008
PS-30	PC	52.566	−137.2	−4.876	−33.78	1.345	52.50	2.651	202.8	0.2539	0.3822	0.8180

[a]See Table 1 for company sources. TG, triglyceride; PC, phosphatidylcholine.

of this enzyme and the duration of incubation required to obtain significant esterification of TG in diesel fuel was not vastly different than for CE and PS-30 (Fig. 1).

Enzymes require water in order to be active in organic solvents, but an excess of water causes inactivation (27,28). In the alcoholysis of TG the enzymes studied displayed a marked sensitivity to water that was largely independent of the concentrations of lipid and ethanol (Fig. 1). PS-30 was the most resistant to this effect, but even its maximum activity occurred at or below an added water concentration only 150 mM over and above that necessary to saturate the solvent (13.5 µL per 5 mL reaction mixture).

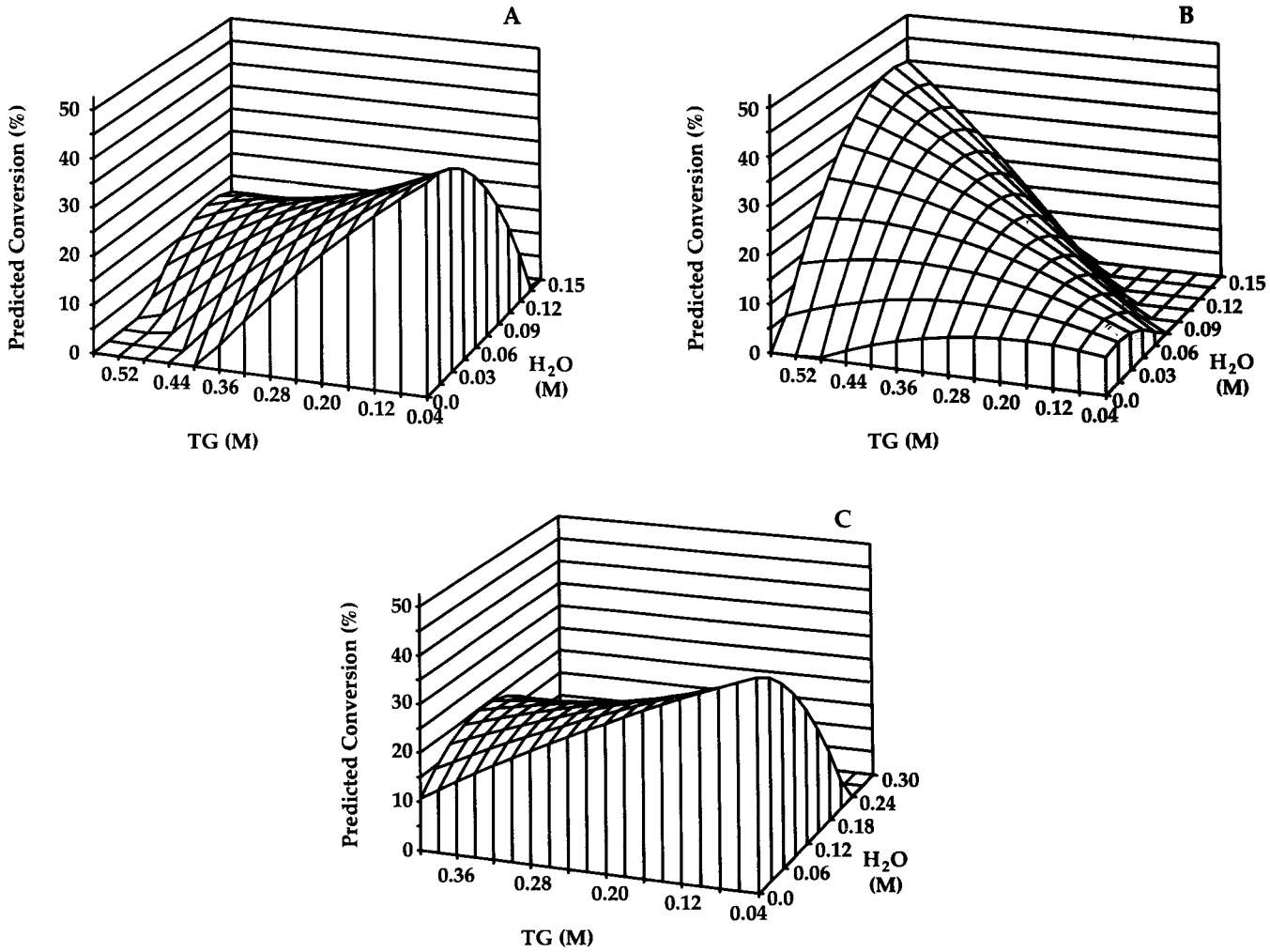

FIG. 1. Predicted degrees of ethanolysis of soybean triglycerides (TG), as a fraction of maximum theoretical conversion, by three commercial lipase preparations at 42°C. Results are derived from Equation 1 and Table 3. (A) Lipozyme IM 20 lipase (35 mg), ethanol: 330 mM, 28-h incubation; (B) as (A) but ethanol 925 mM; (C) CE lipase (50 mg), ethanol: 330 mM, 20-h incubation; and (D) Amano PS-30 lipase (35 mg), ethanol: 330 mM, 6-h incubation. Lipases from Amano (Troy, VA).

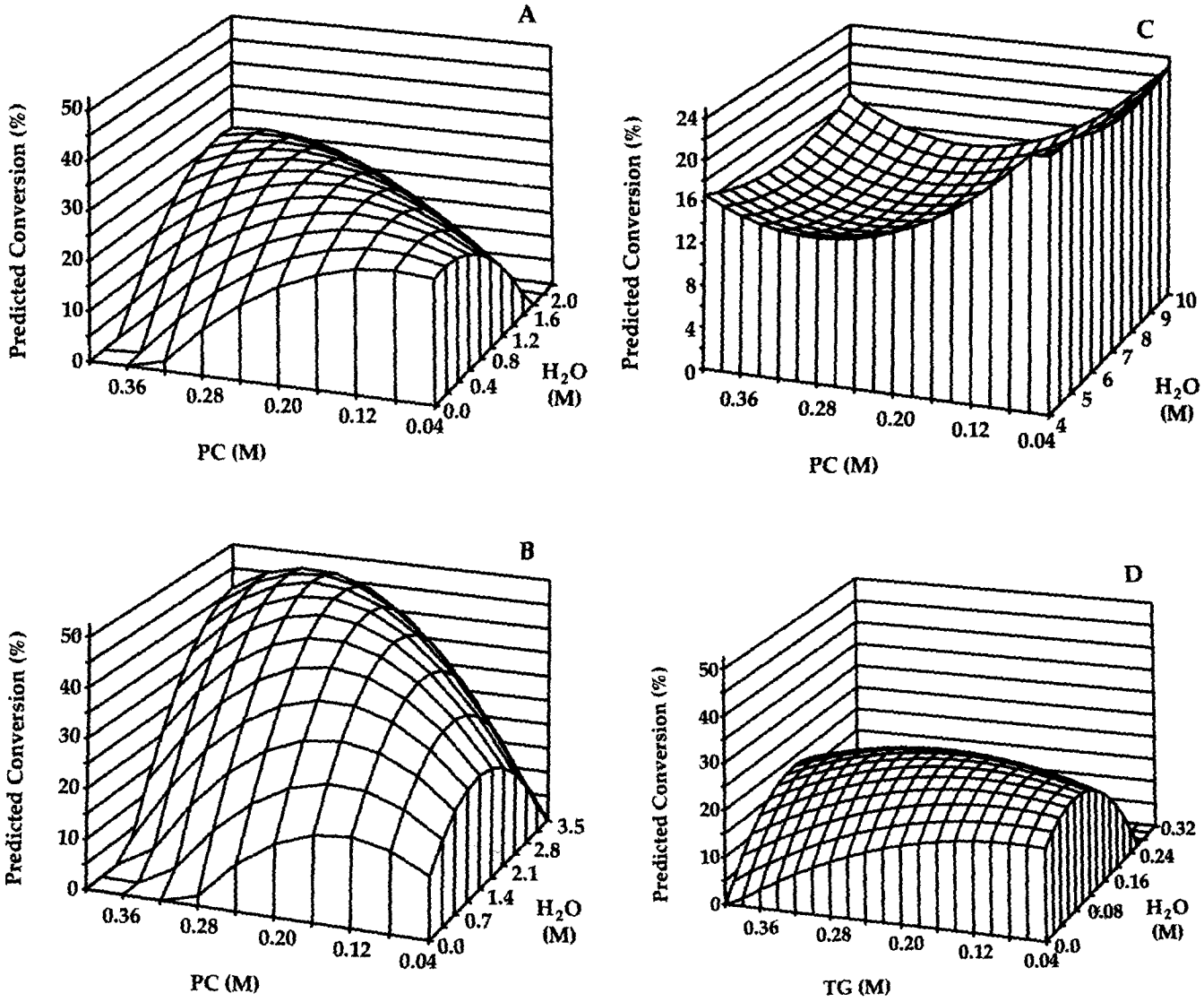

FIG. 2. Predicted degrees of ethanolysis of soybean phosphatidylcholine (PC), as a fraction of maximum theoretical conversion, by commercial lipase preparations at 42°C. Results are derived from Equation 1 and Table 3. (A) Lipozyme IM 20 (Novo Nordisk, Franklinton, NC) lipase (35 mg), ethanol: 750 mM, 48-h incubation; (B) Amano CE lipase (50 mg), ethanol: 750 mM, 17-h incubation; (C) Amano PS-30 lipase (35mg), ethanol 301 mM, 49.5-h incubation. See Figure 1 for other company source.

In general, the alcoholysis of TG and PC was only slightly affected by variations in ethanol concentration, with minor to moderate reductions predicted by Equation 1 and Table 3 at elevated ethanol concentrations. An exception to this generalization occurred in the alcoholysis of TG by Lipozyme IM 20. At low alcohol concentrations (e.g., 0.33 M), predicted esterification by this enzyme was greatest at the lowest levels of TG and declined at higher levels (Fig. 1A). In contrast, at higher ethanol concentrations (e.g., 0.9 M), the activity increased as the TG concentration increased, reaching a maximum at the highest TG level examined (Fig. 1B).

Because the response surface methods and incubation times used here were designed to identify optimal reaction conditions, not maximum yields, the fact that the extents of predicted esterification reached only 10 to 30% in these studies says nothing about the maximum yields that might be achievable.

All three enzymes were able to transesterify PC in diesel fuel (Fig. 2). The activity of CE generally rose as the PC concentration increased, and at optimum was roughly twice the optimal activity toward TG (Figs. 1C and 2B). For Lipozyme, roughly twice as long an incubation was required with PC to obtain extents of reaction comparable to those for TG. Unlike the situation with TG (Fig. 1B), Lipozyme did not exhibit a stimulation of activity toward PC at high ethanol concentrations (data not shown). The tolerance of PS-30 to water in the presence of PC was quite notable, with maximum activity occurring throughout the range of concentrations examined (Fig. 2C). Optimal water concentrations were five- to tenfold greater than those for Lipozyme and CE. However, the relative activity of PS-30 toward phospholipids was low—with 40- to 50-h incubations required to achieve degrees of hydrolysis barely half of those achieved in a 6-h incubation when TG was the substrate (Fig. 1D vs. Fig. 2C).

In the ethanolysis of PC the enzymes were 10- to 50-fold more tolerant of water than they had been in the ethanolysis of TG. Maximum activities occurred at water concentrations between 1 and 10 M (Fig. 2). Furthermore, the amounts of water necessary for maximum activity generally increased as PC concentrations increased (Fig. 2). Similar behavior was observed in earlier studies of the hydrolytic activities of lipases toward TG and PC in organic solvents (29). We observed that PC increased the solubility of water in organic solvents, probably as a result of its well-known emulsifying activity. Evidently this interaction also modulates the availability of water to the enzymes.

With PC as substrate, both Lipozyme IM 20 and Amano CE displayed greatest activity at the lower ethanol concentrations examined, with slight to moderate reductions at higher ethanol concentrations (Figs. 2A and 2B). PS-30 activity was roughly constant across the range of ethanol concentrations examined (Fig. 2C). Lipozyme required longer incubations than CE lipase to achieve significant levels of esterification (48 vs. 17 h). However, as seen with TG as substrate, this is a relatively small difference compared to that in the activities of these enzymes in aqueous systems (Table 2). Evidently Lipozyme retains its activity better in diesel fuel than do the other enzymes studied here.

Time course studies were conducted at TG and water concentrations predicted by Equation 1 and Table 3 to yield high enzyme activities (Fig. 3). The levels of activity agreed with those predicted by Equation 1 and Table 3. However, complete transesterification was not achieved. Despite more than 45 h of incubation, esterification stopped at between 20 and 25% of the maximum theoretical value for all three enzymes. PS-30 was the most active enzyme, achieving 20% esterification within the first four hours of incubation. However, it exhibited only slight additional activity beyond that time. Qualitatively similar, though quantitatively much lower, activity was shown by the CE. Both the PS-30 and CE are reported by their manufacturer to be positionally nonspecific. Their failure to achieve complete conversion here is at least partly due to the fact that the ethanol concentrations, chosen because they were the ones giving highest enzyme activity, were insufficient to support the ethanolysis of more than approximately half the fatty acids in the substrate. In the case of Lipozyme IM 20, although sufficient ethanol was present to support the alcoholysis of more than 85% of the fatty acids present in the TG substrate, only 25% esterification was achieved. *Rhizomucor miehei* lipase, the catalytic component of Lipozyme IM 20, is known to hydrolyze only the primary ester positions of glycerides (30). This reduces the maximum potential ester yield by this enzyme to 67% of the total fatty acids present, still greater than that achieved here. Further additions of ethanol increased the degrees of TG conversion by PS-30 and CE to 48 and 88%, respectively. However, this approach did not increase transesterification by IM 20.

The time courses of PC alcoholysis by Lipozyme IM 20 and Amano CE are shown in Figure 4. (Due to its low activity on PC, a time course was not run for PS-30.) As with TG transesterification, the activities correspond to those predicted by Equation 1 and Table 3. Of the two enzymes, CE was the most active, achieving 50% conversion in 30 h. This corresponds to alcoholysis of one fatty acid per PC molecule, and may represent an initial transesterification of the *sn*-1 position of the substrate. Further ethanol additions did not increase the degree of esterification by either enzyme. It is notable that the CE preparation was more active on PC

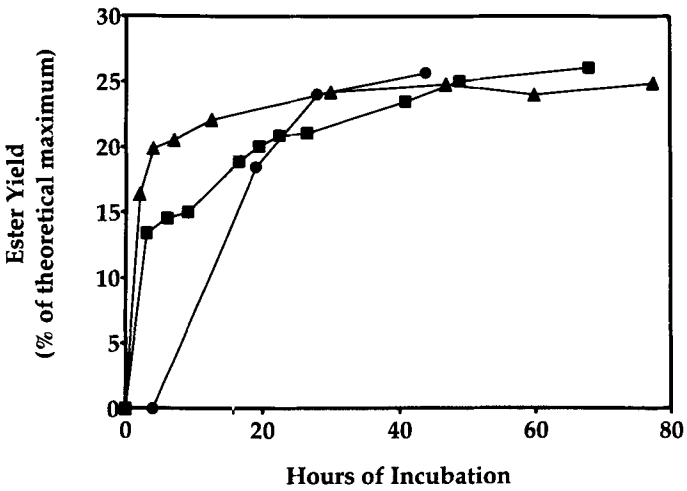

FIG. 3. Progress curves of the enzymatic ethanolysis of soybean triglycerides (TG) in diesel fuel under reaction conditions predicted by Equation 1 and Table 3 to yield high enzyme activities. (●): Lipozyme IM 20 (35 mg), TG: 0.36 M, water: 0.060 M, ethanol: 0.927 M; (■): CE lipase (50 mg), TG: 0.095 M, water: 0, ethanol: 0.129 M; (▲): PS-30 lipase (35 mg), TG: 0.29 M, water: 0.12 M, ethanol: 0.33 M. Data are the averages of duplicate determinations. See Figures 1 and 2 for company sources.

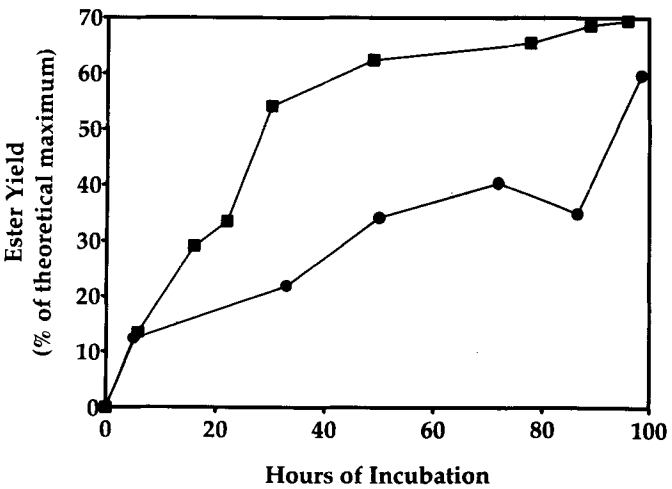

FIG. 4. Progress curves of the enzymatic ethanolysis of soybean phosphatidylcholine (PC) in diesel fuel under conditions predicted by Equation 1 and Table 3 to yield high enzyme activities. (●): Lipozyme IM 20 (35 mg), PC: 0.374 M, water: 1.85 M, ethanol: 0.749 M; (■) Lipase CE (50 mg), PC: 0.272 M, water: 3.57 M, ethanol: 0.311 M. Data are the averages of duplicate determinations. See Figures 1 and 2 for company sources.

than on TG, achieving greater conversion in a comparable amount of time despite the fact that the initial PC concentration was nearly fourfold greater than that for TG (Figs. 3 and 4).

The alcoholysis of PC by Lipozyme IM 20 proceeded more slowly than that by CE (Fig. 4). This is probably due to the fact that the IM 20 reaction contained 30% less enzyme (mass basis) and a 38% higher substrate concentration, suggesting that here the activity of Lipozyme is closer to that of CE than was seen when comparing their hydrolytic activities in aqueous reactions (Table 2). Because Lipozyme is *sn*-1,3-regiospecific, one would expect a maximum transesterification of 50% of the fatty acid content of PC. The fact that a slightly higher yield than this is achieved (Fig. 4) indicates either a relaxation of enzyme specificity in these reactions or the occurrence of acyl migration in the lysophosphatidylcholine generated by a first transesterification event. The maximum activities of Lipozyme toward PC and TG are roughly comparable, but the enzyme is able to achieve a more complete alcoholysis of the former substrate (Figs. 3 and 4).

Using lipases as catalysts for the ethanolysis of sunflower oil, Mittelbach (20) achieved degrees of esterification much greater than seen in our studies. This difference could be due to Mittelbach's use of amounts of alcohol in excess of those theoretically necessary for complete ethanolysis (20). Such an approach was not possible in our work because of enzyme inhibition by the amounts of alcohol needed to match the higher substrate concentrations used here. Shaw *et al.* (23) reported high degrees of alcoholysis in a solvent-free system. These authors noted that the reaction proceeded much slower in hexane, an observation which is analogous to our results. These previous results (20,23) are not strictly comparable to those reported here, particularly because they employed significantly less substrate and more catalyst than we used. However, they do suggest some approaches that may prove useful in increasing the extent of alcoholysis.

This work establishes the feasibility of using diesel fuel as a solvent for the enzymatic synthesis of ethyl esters from TG and phospholipids. This suggests the possibility of applying this reaction to the synthesis of biodiesel from low-value materials, such as soapstock, which are rich in these lipids and in related compounds. The feasibility of such a procedure remains to be explored.

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