Immobilized Lipase-Catalyzed Ethanolysis of Sunflower Oil

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ABSTRACT: The ethanolysis of sunflower oil catalyzed by an immobilized 1,3-specific porcine pancreatic lipase in a medium composed solely of substrates was investigated. The effects of the oil/ethanol molar ratio, temperature, amount of added water, and amount of enzyme used [respectively, 1:3, 45°C, 0% (vol/vol), and 0.5 g of lipase, i.e., 10% w/w of total substrate]. To investigate the reusability of the lipase, the four-step ethanolysis process was repeated by transferring the immobilized lipase to a substrate mixture. As a result, the percentage of conversion after the first usage decreased markedly.

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KEY WORDS: Biocatalysis, ethanolysis, immobilized lipase, sunflower oil.

Alcoholysis of vegetable oils is an important reaction that produces FA alkyl esters, which are valuable intermediates in oleochemistry. In particular, the methyl and ethyl esters of FA are excellent substitutes for diesel fuel (1–3). "Biodiesel" is the term applied to ester-based fuel oxygenates derived from fats and oils that are used in compression–ignition engines (4,5). Among the attractive features of the use of biodiesel are the facts that (i) it is plant-derived and, as such, its combustion does not contribute to atmospheric levels of CO_2, a "greenhouse" gas; (ii) it is domestically produced, offering the possibility of reducing petroleum imports; (iii) it is biodegradable; and (iv) compared with conventional diesel fuel, its combustion products have reduced levels of particulates, carbon monoxide, and, under some conditions, nitrogen oxides (4). As a consequence, there is considerable interest in expanding the use of biodiesel fuels (4).

For most applications, methyl esters are produced because methanol is the least costly as an absolute alcohol. For diesel fuel substitutes, however, it may be preferable to prepare ethyl esters because ethanol can be produced from biomass, and it is less toxic than methanol. Industrially, alcoholysis is usually carried out by heating vegetable oils in alcohols at 100 to 200°C in the presence of a catalyst. Because of the high energy cost of the conventional chemical process and the possible lower cost of enzymes, industrial application of lipase in the oleochemical industry has become more attractive (6,7).

The use of lipases as catalysts in the transformation of TAG has been widely investigated (8). To date, technical applications of lipases include modifying the FA compositions of TAG by interesterification (9); hydrolyzing TAG; directly synthesizing esters (10); and performing alcoholysis of vegetable oils in a solvent-free medium.

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In spite of the technical importance of FA esters (11,12), lipase-catalyzed transesterifications that involve high-M.W. FA only recently have been investigated (1,6). Lipase-catalyzed alcoholysis in a solvent-free medium may be important in industrial applications since such a system has the advantage of avoiding the problems of separation, toxicity, and flammability of organic solvents, thus lowering the cost of the final product and permitting recovery of the product without a solvent recovery step.

In the present study, an immobilized 1,3-specific lipase was utilized to prepare ethyl esters from sunflower oil (SFO) by alcoholysis. We show that this lipase in ethanol can convert SFO to the respective ethyl esters in high yield. Several reaction parameters that influence both the reaction rate and equilibrium composition were studied. The parameters studied were optimized for the ethanolysis of SFO in a medium composed solely of substrates.

EXPERIMENTAL PROCEDURES

Materials. A refined and edible grade of SFO with a FA composition of palmitic (7%), stearic (4%), oleic (25%), and linoleic (64%) acids was used. From that composition, an average molar mass of 876.4 was determined.

All reactions were catalyzed with 1,3-specific lipase from porcine pancreas, immobilized by ionic linkage on a macroporous anion exchange resin generously provided by Sigma Chemical Co. (St. Louis, MO). The resin had a macroporous structure that was appropriate for the immobilization of proteins. Absolute ethanol, butyl laurate, and chloroform were purchased from Merck (Darmstadt, Germany). FA alkyl esters and MAG, DAG, and TAG of the above-mentioned FA were obtained from Sigma. Silica gel 100 support (particle size 0.063–0.200 mm) was purchased from Fluka (Buchs, Switzerland).

Alcoholysis reaction. Stoichiometric amounts of the substrates (5.7 mmol of SFO and 17.1 mmol of absolute ethanol) were mixed together in a 50-mL capped glass vial, incubated for 10 min at 45°C and 250 rpm on a rotary shaker, and placed in an incubation chamber before adding 0.5 g of the immobilized porcine pancreas lipase. All reactions took place in capped glass vials. The progress of the reaction was followed by withdrawing 20 μL of the reaction medium at various time intervals and analyzing the reaction mixture by GC. Additional alcoholysis reactions were carried out by varying the substrate molar ratios, enzyme quantities, amounts of added water, and temperatures. As described by Berger *et al.* (13), a corresponding quantity (0.527

g, 7.7% w/w) of silica gel was mixed with the standard reaction medium to determine its effect on equilibrium yield during reuse of the same quantity of enzyme. The immobilized enzyme was recovered from the reaction medium by filtration.

Analysis of the reaction mixtures. The fatty alkyl esters and MAG, DAG, and TAG were analyzed with a Shimadzu model 6 AM gas chromatograph equipped with an FID, a compact low-thermal mass on-column capillary injector, and a Hewlett-Packard integrator. The gas chromatograph was fitted with a 12 m × 0.32 mm × 0.25 µm SGE BP × 5 column. Helium was used as the carrier gas at a flow rate of 30 mL/min. The detector temperature was 370°C. The column temperature was set to 50°C and increased at 50°C/min to 280°C, then at 10°C/min to 360°C, where it was held for 10 min. Twenty microliters of the reaction medium (the enzyme was first allowed to settle) was dissolved in 1 mL chloroform that contained butyl laurate as internal standard (1.72 g/L). Two microliters were injected directly into the gas chromatograph. For the determination of calibration curves, solutions of pure FA esters and MAG, DAG, and TAG were used. The amounts of each component were calculated as percentages of the initial amount of FA (14), and the sum of molar fractions of ethyl esters, MAG, DAG, and TAG was taken as 1.

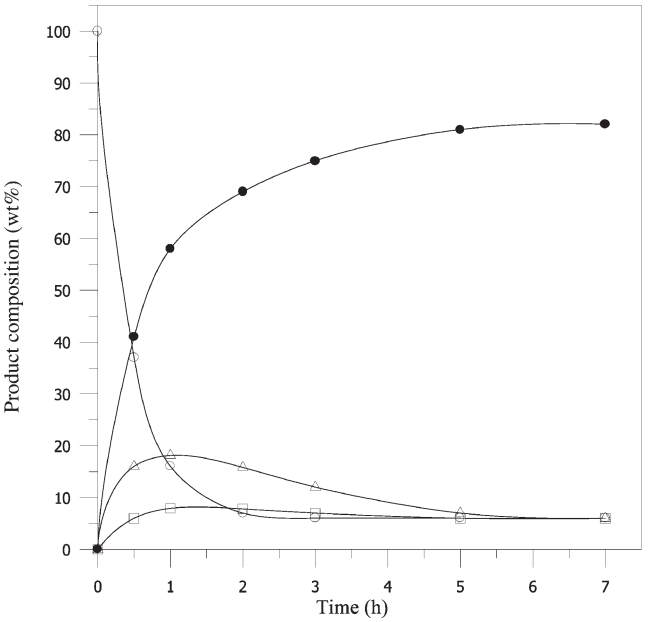

FIG. 1. Progress curve of the intermediates [ethyl esters (●), MAG (△), DAG (□), and TAG (○)] of sunflower oil (SFO) ethanolysis (substrate molar ratio 1:3, 45°C, and 500 mg of lipase).

RESULTS AND DISCUSSION

Time course of SFO alcoholysis. Alcoholysis is a transesterification reaction in which the ester bonds of TAG are cleaved by lipase to produce FA that subsequently react with the alcohol to form alkyl esters. To quantify the extent of alkyl ester synthesis in a solvent-free medium, the composition of the reaction medium was analyzed as a function of time. The TAG concentrations in the bulk rapidly decreased during the first hour (Fig. 1). MAG and DAG concentrations increased to a maximum of 18.1 and 7.9%, respectively, within 1 h. Alkyl esters appeared in the bulk and reached a maximum value of 81% within 5 h.

Substrate molar ratio. The aim was to obtain maximum SFO conversion with little or no residual ethanol. In this medium, composed solely of substrates, the optimal yield (81%) was obtained with stoichiometric amounts of reactants (an oil/ethanol molar ratio of 1:3) (Fig. 2). When an excess of ethanol was used, SFO conversion was always low, and the product mixture contained large quantities of residual ethanol—and sometimes MAG, DAG, and TAG. The relative ethyl ester yield decreased with an increase in the molar excess of ethanol within 7 h. For a 1:2 molar ratio, the ethyl ester yield at 7 h was 58%, whereas for 1:4.5 and 1:6 molar ratios, the yields at 7 h were 22 and 12%, respectively. The decreased activity and equilibrium yield at higher ethanol concentrations may reflect the ability of the excess ethanol to distort the essential water layer that stabilizes the immobilized enzyme (15). Consequently, a molar ratio of 1:3 was used in most subsequent trials.

Lipase quantity. As expected, an increase in the quantity of immobilized lipase increased the initial reaction rate and total alkyl ester yield during the first few hours (Fig. 3). Alkyl

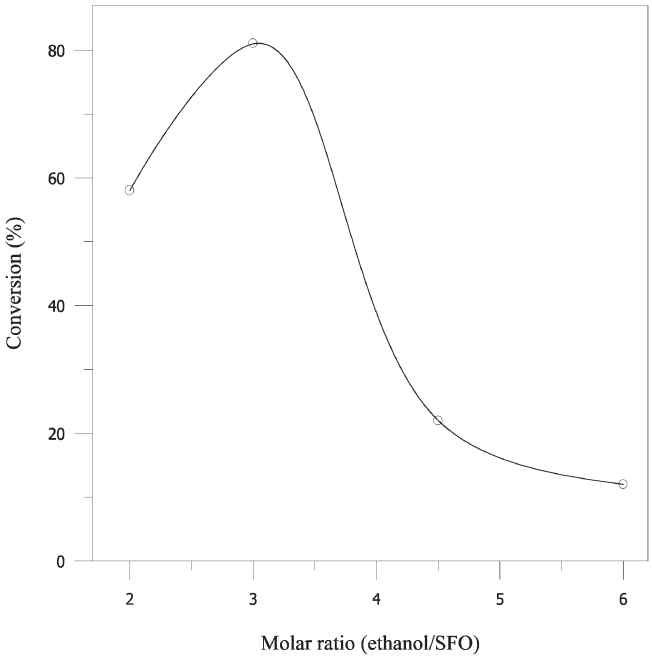

FIG. 2. Effect of substrate molar ratio on SFO ethanolysis at equilibrium after a covered batch reaction (5.432 mL of SFO, 45°C, and 500 mg lipase). For abbreviations, see Figure 1.

ester yield varied as a function of time with 3, 5, 7, and 10% (w/w of total substrate) immobilized lipase at a substrate molar ratio of 1:3. Under these conditions, the yield of alkyl esters was 50% within 30 min when the most lipase (500 mg) was used, whereas with 400, 300, and 200 mg, the yields were 30, 19, and 11%, respectively.

Added water. The importance of controlling the water content in lipase-catalyzed esterifications has been emphasized.

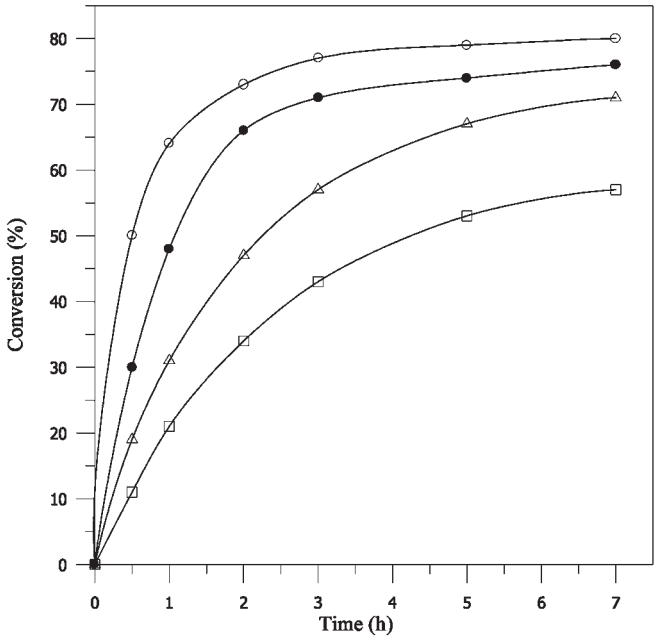

FIG. 3. Effect of lipase quantity on SFO ethanolysis (substrate molar ratio 1:3, 45°C, and 0% added water): 200 (□), 300 (△), 400 (●), or 500 mg (○). For abbreviations, see Figure 1.

To better understand the effect of water addition on SFO ethanolysis at 45°C when the substrate molar ratio was 1:3 at a lipase level of 500 mg, the composition of the reaction medium was analyzed as a function of time (Fig. 4). When the reaction was completed with 0.5% (vol/vol%) added water, a yield of about 14% alkyl esters was reached in 1 h, but an increase in time did not result in a further increase in alkyl ester formation. With 0.125% (vol/vol) added water, a yield of about 54% alkyl esters was reached in 1 h, and only about 74% was reached in 7 h. Interestingly, the reaction proceeded nearly identically with 0 and 0.125% added water. The water present in the lipase preparation (*ca.* 10% w/w of lipase) was essentially sufficient to give nearly 81% conversion of SFO in 7 h (Fig. 1). Additional water increases did not result in further improvements in SFO conversion. Thus, in comparison with the result obtained when working with an organic solvent (1), an opposite effect on SFO ethanolysis was obtained with added water when working in a solvent-free medium.

Temperature. The effect of temperature on the time course of SFO ethanolysis also was determined (Fig. 5). In comparison with a 35°C reaction temperature, at 45 and 55°C little difference was observed and the reaction proceeded nearly identically, with yields of about 53 and 58% ethyl esters reached within 2 h; the same conversion yield was observed at 4 h. These results agreed with Mittelbach (1), according to whom the optimal temperature for SFO alcoholysis catalyzed by various microbial lipases in an organic solvent was 40–65°C. Consequently, 45°C was used in most subsequent trials.

Lipase reuse. Although the alcoholysis parameters presented previously apply to only batch conditions, such data also may be useful for the development of a continuous production process for alkyl esters. To complete this study, the effect of the reaction run number on SFO ethanolysis with the same quantity of immobilized lipase was studied (Fig. 6). For four consecutive runs of ethanolysis, a comparison was made between the equilibrium yield of ethyl esters synthesized under standard conditions, either without added silica gel or with silica gel (0.527 g) added as support for the polar product (glycerol) obtained by alcoholysis during a 24-h incuba-

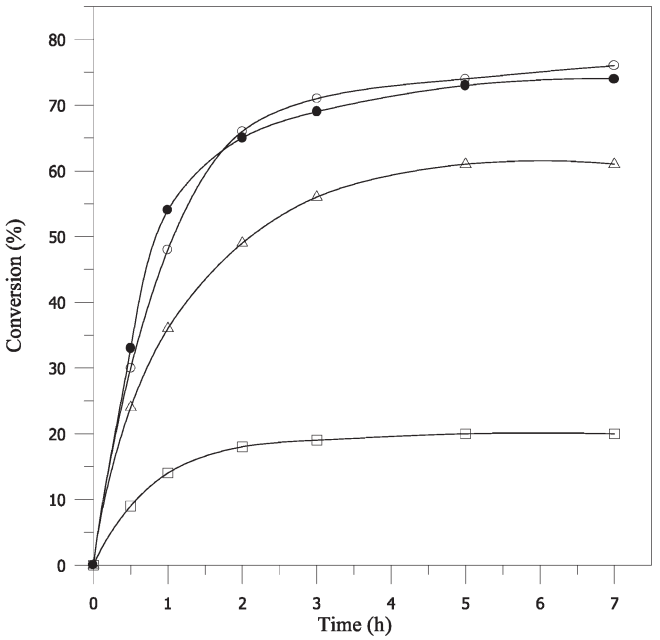

FIG. 4. Effect of added water on SFO ethanolysis (substrate molar ratio 1:3, 45°C, and 500 mg lipase): (○), 0.125 (●), 0.15 (△), or 0.5% (□). For abbreviations, see Figure 1.

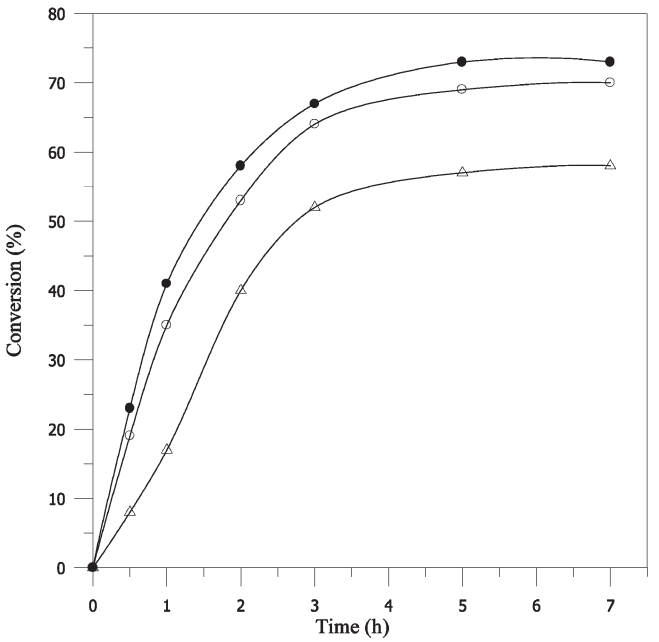

FIG. 5. Effect of temperature on SFO ethanolysis (substrate molar ratio 1:3, 0% added water, and 500 mg lipase): 35 (△), 45 (●), or 55°C (○). For abbreviations, see Figure 1.

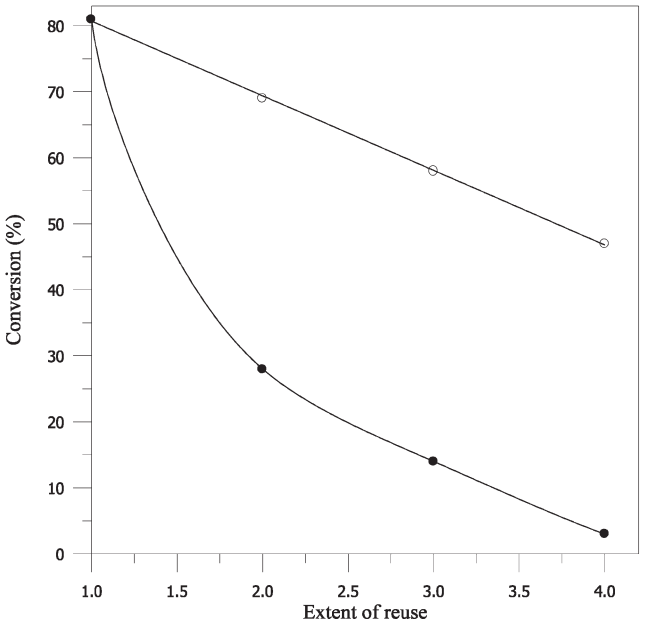

FIG. 6. Effect of lipase reuse on SFO ethanolysis without (●) or with (○) added silica gel. After each run the reaction medium was filtered. In runs 2–4 the reaction was performed under the same conditions as in Figure 1. For abbreviations, see Figure 1.

tion. Under standard conditions, equilibrium was reached in 7 h (Fig. 1), with an ethyl ester yield of 81% (Fig. 6).

A decrease of ethyl ester synthesis took place on reuse of the lipase. This decrease could be explained by loss of activity of the immobilized lipase due to glycerol inhibition.

To clarify the reasons for the decreased yield of ethyl esters at equilibrium and immobilized lipase activity during the last three runs, a given quantity of silica gel was added to the reaction medium before adding the immobilized lipase. Interestingly, in run 1, the equilibrium yield of ethyl esters was the same with added silica gel under standard conditions (Fig. 6). As compared with standard conditions, in the last three runs the added silica gel reduced the loss in yield of ethyl esters. Similar results were reported in the synthesis of TAG without (16) or with (17) organic solvents. The silica gel in these types of reactions has a strong affinity for the glycerol (18) and probably behaved as a glycerol "collector," playing a protective role for the immobilized enzyme by binding the polar substrate (glycerol) from it.

In summary, good yields of ethyl esters from SFO ethanolysis in a medium composed solely of substrates were achieved with a 1,3-specific immobilized lipase. Stoichiometric amounts of substrates were necessary and sufficient to achieve the best SFO ethanolysis. Furthermore, the water content of the enzyme preparation was sufficient to yield good SFO conversion. The added silica gel improved the yield of ethyl esters in different SFO ethanolysis reactions with the same quantity of enzyme. However, the immobilized lipase could not be reused, since enzyme activity decreased with each cycle even in the presence of silica gel.

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