Immobilized Lipase-Catalyzed Ethanolysis of Sunflower Oil in a Solvent-Free Medium

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ABSTRACT: The ethanolysis of sunflower oil (SFO) with Lipozyme (immobilized 1,3-specific *Mucor miehei* lipase) in a medium solely composed of substrates was investigated. The effects of oil/ethanol molar ratio, temperature, added water content, and amount of enzyme were analyzed. The optimal values were, respectively, 1:3, 50°C, 0% (vol/vol), and 0.4 g of Lipozyme per 5.7 mmol of SFO. The use of immobilized lipase made the reuse of enzyme feasible, and the enzyme could be recovered easily from the reaction mixture and recycled to reduce the cost of catalyst. In the last three consecutive runs of enzyme reuse, the final conversion yield of SFO from ethanolysis with added silica gel support was higher than that obtained from ethanolysis under standard conditions. The lipase-catalyzed alcoholytic reaction is potentially useful in the production of alkyl esters of specific interest.
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KEY WORDS: Alcoholysis, alkyl esters, biocatalysis, immobilized lipase, reuse of immobilized lipase, sunflower oil.

Alcoholysis of vegetable oils is an important reaction that produces fatty acid alkyl esters, which are valuable intermediates in oleochemistry, and methyl esters and ethyl esters, which are excellent substitutes for diesel fuel (1). "Biodiesel" is the term applied to ester-based fuel oxygenates that are derived from biological sources and intended for use in compression–ignition engines (2). Among the attractive features of the use of biodiesel as a fuel are the facts that (i) it is plant-, not petroleum-derived, and as such, its combustion does not increase current net 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) relative to conventional diesel fuel, its combustion products have reduced levels of particulates, carbon monoxide, and, under some conditions, nitrogen oxides (2). As a consequence, there is considerable interest in exploring and developing the use of biodiesel as a fuel (2,3). For most technical applications, methyl esters are produced because methanol is available as an absolute alcohol. But, especially for diesel fuel substitutes, it is preferable to prepare ethyl esters because ethanol can be produced from biomass and is less toxic than methanol. Industrially, alcoholysis is usually carried out by heating vegetable oils in alcohols at 100 to 200°C. Because of the high energy cost of the conventional chemical process and the lower prices of enzymes, industrial application of lipase in the oleochemical industry has become more attractive (4,5).

In recent years, the use of lipases as catalysts for transformations of fatty acids has been widely investigated (6). The main technical application of lipases is to modify the fatty acid compositions of triacylglycerols by interesterification (7). For catalyzed hydrolysis of triacylglycerols, as well as the direct synthesis of esters (8), a few reports deal with the use of lipases for the alcoholysis of vegetable oils in a solvent-free medium.

In spite of the great technical importance of fatty acid esters (9,10), lipase-catalyzed transesterifications that involve high-molecular-weight fatty acids have only recently been investigated (1,4). Lipase-catalyzed alcoholysis in a solvent-free medium is important in industrial applications. Such a system presents an enormous advantage by avoiding the problems of separation, toxicity, and flammability of organic solvents, thus lowering the cost of the final product and permitting recovery of product without a further organic solvent evaporation 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 lipase in ethanol can convert SFO to the respective fatty acid esters in high yield. Several reaction parameters influence both reaction rate and composition at equilibrium. Some of these parameters have been studied here, aiming to obtain optimal conditions for the ethanolysis of SFO in a medium solely composed of substrates.

MATERIALS AND METHODS

Materials. All reactions were catalyzed with Lipozyme, a 1,3-specific lipase from *Mucor miehei,* immobilized on a macroporous anion exchange resin, and generously provided by Novo Nordisk Industry A/S (Bagsvaerd, Denmark). A refined edible grade of SFO was used, and its fatty acid composition was palmitic (7%), stearic (4%), oleic (25%), and linoleic (64%). From that composition, an average molecu-

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lar mass of 876.4 was determined. Absolute ethanol was purchased from Carlo Erba (Milan, Italy). Butyl laurate was purchased from Sigma (St. Louis, MO). Chloroform (99%) was obtained from Janssen Chimica (Geel, Belgium). Fatty acid alkyl esters and mono- , di-, and triacylglycerols of the fatty acids mentioned previously were obtained from Sigma. Silica gel 100 support was purchased from Fluka Chemica (Buchs, Switzerland).

Gas chromatograph (GC) analysis. The fatty acid alkyl esters and mono-, di-, and triacylglycerols were analyzed with a Varian (Fernando, CA) 3400 GC, equipped with a flame-ionization detector, a compact low-thermal mass on-column capillary injector, and a Varian 4290 data integrator. The GC was fitted with a 12 m × 0.32 mm × 0.25 μm SGE BPX5 column (Ringwood, Victoria, Australia). 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 was then programmed first at 50°C/min to 280°C and then at 10°C/min to 369°C, which was maintained constant for 10 min. Twenty microliters of the reaction medium (enzyme was first allowed to settle) was dissolved in 1 mL chloroform that contained lauric acid butyl ester as internal standard (1.72 g/L). Two microliters were directly injected into the GC. For the determination of calibration curves, solutions of pure fatty acid alkyl esters and mono-, di-, and triacylglycerols were used. The amounts of each component were calculated as percentages of the initial amount of fatty acid (11), and the sum of molar fractions of ethyl esters, mono-, di-, and triacylglycerols was taken as 1.

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 50°C and 250 rpm on a rotary shaker, and placed in an incubation chamber, before adding 0.4 g of the immobilized *Mucor miehei* lipase (Lipozyme). All reactions took place in a covered, capped glass vial. The progress of the reaction was followed by withdrawal of 20 μL of the reaction medium at various time intervals, which was analyzed by the GC method described above. Further alcoholysis reactions were carried out with varying substrate molar ratios, enzyme quantity, added water, and different temperatures. As described by Berger *et al.* (12), a corresponding quantity (0.527 g) of silica gel was mixed to the standard reaction medium to investigate its effect on the equilibrium yield during reuse of the same quantity of enzyme preparation. The immobilized enzyme was recovered from the reaction medium by filtration.

RESULTS AND DISCUSSION

Time course of SFO alcoholysis. Alcoholysis is a transesterification reaction, in which the ester bond of triglycerides is cleaved by lipase to produce fatty acids that consequently 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 as a function of time has been analyzed. As shown in Figure 1, the triglyceride concentrations in the bulk rapidly decrease during the first hour. Of the initial free fatty acids, 66% is converted to alkyl esters after 1 h. Mono- and diglyceride concentrations increase to a maximum of 19.3 and 8.3% within 1 h, respectively. Alkyl esters appear in the bulk and reach a maximum value of 83% within 5 h. This suggests that the water present in the enzyme preparation is a better nucleophile than ethanol, and therefore, fatty acid is produced first and is then esterified by the enzyme with ethanol acting as the nucleophile.

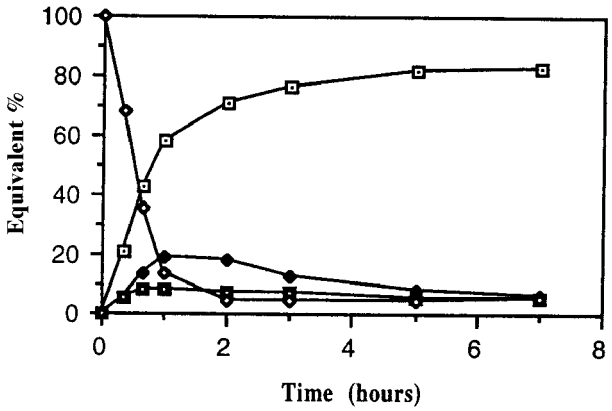

FIG. 1. Progress curve of intermediates (ethyl esters, □; monoacylglycerols, ◆; diacylglycerols, ■; triacylglycerols, ◇) of sunflower oil (SFO) ethanolysis (substrates' molar ratio 1:3, 50°C, and 0.4 g of Lipozyme).

Substrate molar ratio. The aim was to obtain a maximum SFO conversion with no or little residual ethanol. In this medium, solely composed of substrates, the optimal yield (83%) was obtained with stoichiometric amounts (oil/ethanol molar ratio is 1:3) (Fig. 2). When an ethanol excess was used, SFO conversion was always low, and the product mixture contained large quantities of residual ethanol, and sometimes mono- and diglycerides and oil. The relative ethyl ester yield decreased with an increase in the ethanol molar excess within 7 h. For a 1:2 molar ratio, the ethyl ester yield at 7 h was 60%,

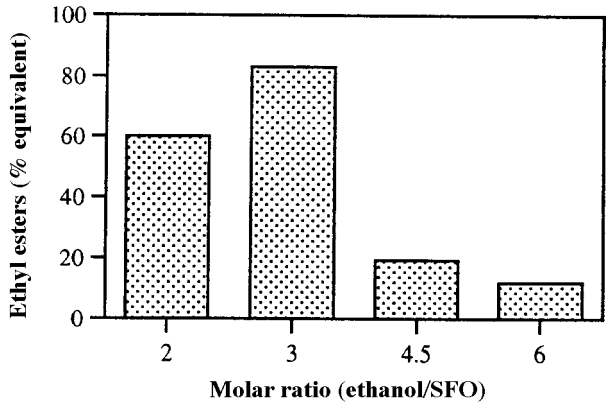

FIG. 2. Effect of substrate molar ratio on SFO ethanolysis at equilibrium after covered batch (5.432 mL of SFO, 50°C, and 0.4 g Lipozyme). For abbreviation see Figure 1.

whereas for 1:4.5 and 1:6 molar ratios, the yields at 7 h were 19.5 and 12%, respectively. The decreased activity and equilibrium yield at higher ethanol concentrations may reflect the ability of the ethanol excess to distort the essential water layer that stabilizes the immobilized enzyme (13,14). Consequently, the molar ratio of 1:3 was used in most of the subsequent trials.

Lipase quantity. As could be expected, an increase in immobilized lipase quantity increased the initial rate and total alkyl ester formation during the first few hours. As an example, Figure 3 illustrates alkyl ester formation as a function of time with 0.2, 0.3, 0.4, and 0.5 g of immobilized lipase, substrate molar ratio of 1:3 in solvent-free medium. Under these conditions, the yield of alkyl esters is 63% within 40 min with the highest lipase quantity (0.5 g), whereas with 0.4, 0.3, and 0.2 g, the yields are 43, 28, and 9%, respectively. Nevertheless, even for the lowest quantity of lipase a nearly theoretical yield of alkyl ester formation was obtained within 7 h.

Added water. The importance of the control of water content in lipase-catalyzed esterifications has been frequently emphasized. To get a better view of the effect of added water on SFO ethanolysis at 50°C, when the substrate molar ratio was 1:3 at a lipase level of 0.4 g, the composition of the reaction medium as a function of time was analyzed (Fig. 4). With 0.5% (vol/vol) of added water, about 12% of alkyl esters was reached in 1 h, but an increase in time did not result in a further increase in alkyl ester formation. With 0.25% (vol/vol) of added water, about 41% of alkyl esters was reached in 1 h, and only about 72% was reached in 7 h. Interestingly, the reaction proceeded nearly identically with 0% added water and with 0.125%. The water (*ca.* 10% w/w of lipase) present in the lipase preparation was essentially sufficient to give a nearly complete conversion of SFO in 7 h. Additional increases of water did not result in further improvements in SFO conversion. Thus, in comparison to the result obtained when working with an organic solvent (1), an opposite effect of added water on SFO ethanolysis has been obtained when working in a solvent-free medium. Therefore, direct lipase-catalyzed ester synthesis may be directly compared with transesterification in a medium solely composed of substrates, a fact we have not yet been able to explain.

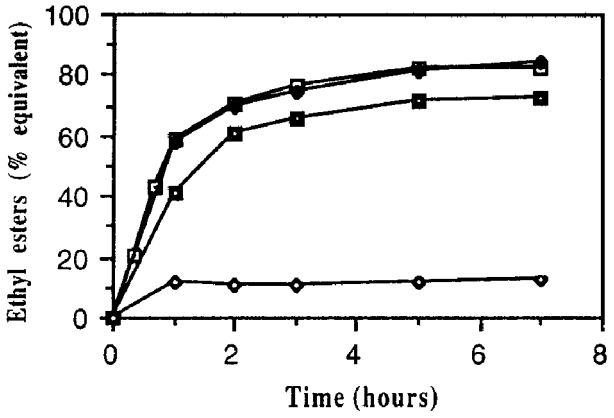

FIG. 4. Effect of added water on SFO ethanolysis (substrate molar ratio 1:3, 50°C, and 0.4 g Lipozyme). Water: 0%, □; 0.125%, ◆; 0.15%, ■; 0.5%, ◇. For abbreviation see Figure 1.

Temperature. Figure 5 illustrates the effect of temperature on the time course of SFO ethanolysis. In contrast to 35°C, at 50 and 60°C, little difference was observed, and the reaction proceeded nearly identically, with about 71 and 74% of ethyl esters reached within 2 h, respectively, and the same conversion yield at 4 h. These results agreed with Mittelbach (1), according to whom the optimal temperature for *Mucor miehei* lipase-catalyzed SFO alcoholysis in organic solvent is 40–70°C. Consequently, the temperature of 50°C was used in most of the subsequent trials.

Lipase reuse. Alcoholysis parameters previously presented concern only for batch conditions, but such data may also be useful for the development of continuous production of alkyl esters. To complete this study, the effect of reaction run number on SFO ethanolysis with the same quantity of immobilized lipase was studied (Fig. 6). For the four consecutive runs

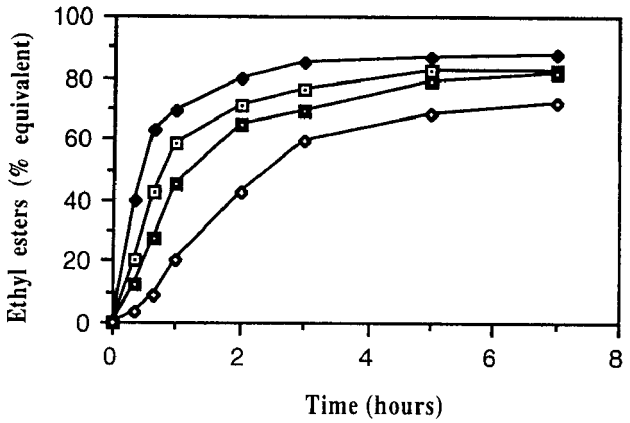

FIG. 3. Effect of lipase preparation quantity on SFO ethanolysis (substrate molar ratio 1:3, 50°C, and 0% added water): 200 mg, ◇; 300 mg, ■; 400 mg, □; 500 mg, ◆. For abbreviation see Figure 1.

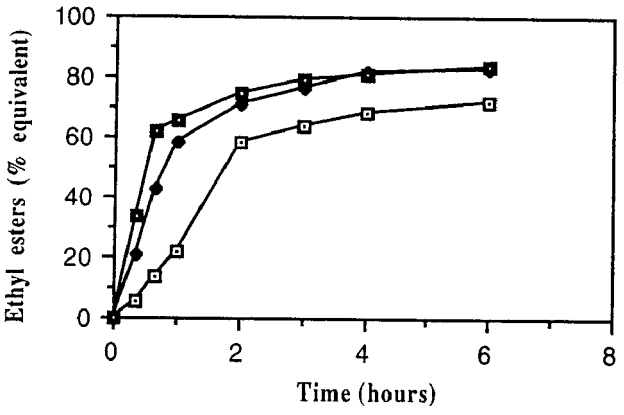

FIG. 5. Effect of temperature on SFO ethanolysis (substrate molar ratio 1:3, 0% added water, and 0.4 g Lipozyme). 35°C, □; 50°C, ◆; 60°C, ■. For abbreviation see Figure 1.

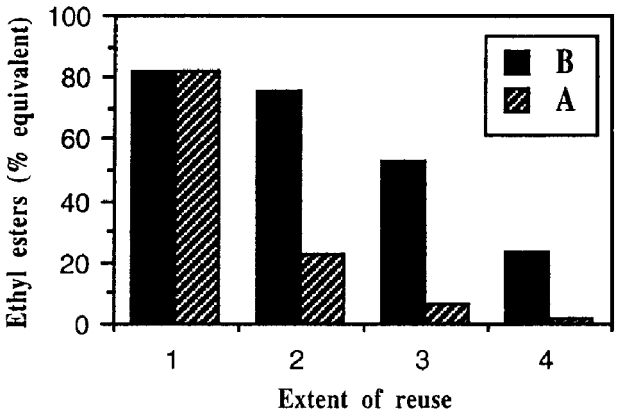

FIG. 6. Effect of lipase reuse on SFO ethanolysis without (A) or with (B) added silica gel. After each run the reaction medium was filtered. In runs 2, 3, and 4 the reaction medium was operated under the same condition as in Figure 1. For abbreviation see Figure 1.

of ethanolysis, a comparison was made between the equilibrium yield of ethyl ester synthesis under standard conditions, without adding silica gel (solid bars, Fig. 6), and that with added silica gel (0.527 g) (hatched bars, Fig. 6) as support to the polar product (glycerol) produced with the progress of alcoholysis during 24 h of incubation. Under standard conditions, equilibrium is reached at 7 h as shown in Figure 1, and the final ethyl ester yield in the first run (Fig. 6) is 83% of fatty acids. After that, there were no substantial changes in the concentration of the components involved in the ethanolysis.

As compared to the first run, in the last three runs a decrease of ethyl ester synthesis is the most nonfavorable effect that could be detected. This decrease could be explained by losses in activity of the immobilized lipase.

To clarify the reasons for decreases of ethyl ester equilibrium yield and immobilized lipase activity during the last three consecutive 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 and under standard conditions (Fig. 6). As opposed to standard conditions, in the last three runs, the added silica gel induced an increase in the equilibrium yield of ethyl esters. This increase of the equilibrium yield is the most favorable effect that could be detected. The study of ethyl ester synthesis from SFO and ethanol with added silica gel in a medium, solely composed of substrates, shows that the presence of silica gel improves significantly the yield of ethyl esters at equilibrium for the different runs (exept for run 1). The following explanation for the critical role of silica gel in this type of reaction is proposed: the silica gel has a strong affinity for the glycerol (15), behaves as a glycerol "collector," and plays a protective role for the immobilized enzyme by avoiding produced polar substrate (glycerol) adsorption on the Lipozyme support. Similar results have been obtained in the synthesis of triacylglycerol without (16) or with (17) organic solvents. Moreover, the adsorption of glycerol by the added silica gel support facilitates the contact between Lipozyme, SFO, and ethanol.

In summary, good yields of ethyl esters from SFO ethanolysis in a medium solely composed of substrates can be achieved with a 1,3-specific immobilized lipase (Lipozyme). Stoichiometric amounts of substrates are necessary and sufficient to achieve the best SFO ethanolysis in a solvent-free system. Furthermore, the water content of the enzyme preparation is sufficient to yield good SFO conversion. Moreover, the crucial role of added silica gel, which behaves as a glycerol "collector" in these reactions, was demonstrated. The added silica gel support improves significantly the yield of ethyl esters at equilibrium for the different reaction runs of SFO ethanolysis with the same enzyme quantity (except run 1). Unlike the explanation given by Berger *et al.* (12), the formation of a liquid–liquid interface with the help of silica gel was not the only crucial role. Silica gel plays a protective role against the blockage of the immobilized enzyme by avoiding adsorption of the produced polar substrate (glycerol) on the Lipozyme support.

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