Enzymatic Transesterification of Sunflower Oil in an Aqueous-Oil Biphasic System

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ABSTRACT: A biphasic oil-aqueous system for FAME production by enzymatic catalysis was studied. The transesterification of sunflower oil with methanol was catalyzed by free or immobilized lipases from *Rhizomucor miehei* (Palatase 20 000 L) and *Humicola insolens* (Lipozyme TL 100 L). The effects of protein amount, temperature, pH, and molar ratio of methanol to sunflower oil on the enzymatic reaction using free lipase were evaluated; the best results were obtained with *H. insolens*, at pH 5, 40°C, and 36.8 mg of protein. By using this lipase immobilized in Hypol® 2002 (64.8 mg of protein) at a 6:1 methanol/oil molar ratio and a 2:1 volumetric oil/water phase ratio, an ester content of 96.1% and a conversion of 91.2% were achieved. The immobilized lipase could be reused, although a 30% reduction in conversion efficiency was observed after four uses.

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KEY WORDS: Aqueous-oil system, fatty acid methyl esters, lipases, sunflower oil, transesterification.

FAME, the compounds obtained by transesterification of the glycerides present in vegetable oils with methanol, can be used as a substitute for conventional automotive diesel or in admixture with this fuel. Biodiesel use has some environmental benefits since it leads to a decrease in the emission of CO, hydrocarbons, and particulate matter and to the elimination of SO_x emissions; the global balance of CO_2 emission is null, and consequently a decrease in the greenhouse effect is achieved.

Although biodiesel is usually produced by chemical processes using basic or acid catalysts, it can also be obtained using an enzyme as catalyst. The enzymatic processes may be advantageous because they do not promote secondary reactions, thereby reducing the number of purification steps, and the presence of the enzyme in the glycerol phase can even increase its value as an animal feed. Furthermore, the enzyme can be immobilized on solid supports and thus be used in continuous mode or reused in batch processes, which is economically advantageous for industrial purposes. However, the high cost of enzymes and some problems concerning methanol and/or glycerol inhibition still cause enzymatic processes to be unattractive for the large-scale production of biodiesel.

The enzymatic transesterification of several oils and alcohols has been performed in solvent (1,2) and in solvent-free media (1,3–5) by immobilized lipases. For example, the transesterification of high-oleic sunflower oil with butanol by immobilized Lipozyme led to a 95% conversion, when the reaction was carried out in *n*-hexane, and to only 60% for a solvent-free system (1). The stepwise addition of methanol allowed a degummed soybean oil transesterification of 93.8% in a solvent-free system, and the reuse for 25 cycles of immobilized *Candida antarctica* lipase, without any loss of activity (6). To avoid the stepwise addition of methanol and/or the presence of an organic solvent in the biodiesel production process, methyl esters may be synthesized in water-oil biphasic systems (7–10). A lipase from *Rhizopus oryzae* that efficiently catalyzed the methanolysis of soybean oil in a water-containing system, without an organic solvent, was described. A methyl ester content of 80–90% was reached by stepwise additions of methanol (7). The methanolysis of rice bran oil by *Cryptococcus* spp. S-2, carried out in a one-step methanol addition system at a 4:1 methanol/oil molar ratio and a water content of 80 wt% (by weight of substrate), provided a methyl ester content of 80.2% (9).

In this work, the enzymatic transesterification of sunflower oil with methanol in an aqueous–oil biphasic system was studied, in order to obtain high degrees of transesterification and high contents of methyl esters. To establish the best operational conditions, a comparative study of several parameters was performed using *Rhizomucor miehei* and *Humicola insolens* lipases. The reusability of the immobilized lipase was also investigated.

EXPERIMENTAL PROCEDURES

Materials. Commercial food-grade sunflower oil (AAA girasol) from Sovena (Portugal) and methanol (99.8%) from Merck (Darmstadt, Germany) were used as transesterification reaction substrates. The enzymatic reactions were catalyzed by liquid preparations of lipases from *R. miehei* (18.4 mg protein/mL enzyme solution) and *H. insolens* (27.5 mg protein/mL enzyme solution), commercially available as Palatase 20 000 L and Lipozyme TL 100 L, respectively, and kindly supplied by Novozymes A/S (Bagsværd, Denmark). Hydrophilic polyurethane foams, namely, Hypol® 2002 [toluene-diisocyanate 1–7%; 4,4-methylenedi(cyclohexyl isocyanate) < 1.3%], Hypol® 3000 (toluene-diisocyanate 7–10%), and Hypol® 5000 (diphenylmethane-4,4′-diisocyanate > 25%), were used as lipase immobilization supports. These supports were kindly provided by Hampshire Chemical Corporation (Owensboro,

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KY). The chemicals used for the analysis, namely, heptane and petroleum ether, were supplied by Merck, and the GC standards for FAME were obtained from Sigma-Aldrich (Diesenhofen, Germany).

Transesterification experiments. The enzymatic transesterification of sunflower oil with methanol was performed in 250-mL Erlenmeyer flasks, with 200 rpm orbital shaking. The biphasic system used consisted of 50 mL organic phase—the sunflower oil— and an aqueous phase composed of a buffer solution (phthalate-NaOH, sodium phosphate, or glycine-NaOH buffer, 100 mM) to which methanol and the lipase were added. To establish the best operational conditions, the effects of the following parameters on the transesterification reaction were evaluated: lipase amount, temperature (30–60°C), pH (5–9), methanol–to-oil molar ratio (3:1, 4:1, 5:1, and 6:1), and volumetric organic to aqueous phase ratio (1:1, 2:1). Samples were taken after 48 h of reaction and centrifuged. The ester phase (20–25 mg) was analyzed directly by GC after the addition of 100 μL internal standard (methyl heptadecanoate, 5.3 mg/mL) and 900 μL *n*-heptane. Transesterification reactions were carried out in triplicate, and the results are reported as mean ± SD.

Enzyme immobilization. Lipozyme TL 100 L was immobilized by entrapment in Hypol 2002, Hypol 3000, and Hypol 5000. For all experiments, 2.5 mL of Lipozyme TL 100 L lipase solution was added to 2 g of immobilization support and vigorously mixed. The enzyme–support contact time was 2 h at room temperature, at which time there was no visible liquid phase in the preparation. At this time, the foam was cut into small pieces (<1 mm) by using a household coffee mill, washed with phthalate-NaOH buffer (100 mM, pH 5), and recovered by vacuum filtration. The amount of protein present in the washing solution was determined spectrophotometrically by the method of Lowry *et al.* (11), and the amount of immobilized protein was calculated by mass balance.

Use of recycled immobilized lipase. After each transesterification batch reaction, the immobilized lipase (Lipozyme TL 100 L) was recovered by vacuum filtration and washed with 50 mL phthalate-NaOH buffer (100 mM, pH 5). The immobilized lipase was then used in the next batch reaction consisting of fresh aqueous and oil phases. The percent conversion determined after 48 h of reaction was expressed as a relative conversion, considering that the conversion achieved in the first batch corresponds to 100%. The amount of protein present in each aqueous (after 48 h of reaction) and washing phase was determined spectrophotometrically by the method of Lowry *et al.* (11).

Analytical methods. The sunflower oil was characterized in terms of the acid, iodine, and peroxide values according to Portuguese Standard NP-903 (12), the Kaufmann method (13), and method 965.33 described in the *Official Methods of Analysis of the AOAC* (14), respectively. To determine the FA composition of the sunflower oil, oil samples (150 mg) were chemically transesterified in petroleum ether (boiling range: 60–80°C) in the presence of a solution of potassium hydroxide (2 N) in methanol (15). After organic phase evaporation, the sample was dissolved in *n*-heptane and analyzed by GC using methyl heptadecanoate (5.3 mg/mL) as internal standard. Standards of FAME were used to identify the FAME in the sample. FA composition was calculated as percentage of the total FA present in the sample determined from the peak areas. The values obtained allowed calculation of the M.W. of the sunflower oil.

Transesterification products were quantified by GC using a Varian 3300 chromatograph (Walnut Creek, CA,) equipped with an FID and a Supelcowax 10 column (30 m × 0.32 mm i.d.; film thickness 0.25 μm; Bellefonte, PA). Helium was used as carrier gas, and the injector and detector temperatures were kept at 200 and 250°C, respectively. The oven temperature was maintained at 200°C for 11.5 min and then increased to 225°C at 10°C/min.

The percent conversions and the methyl ester contents were defined as [(mmol methyl esters/mmol sunflower oil FA) × 100] and [g methyl esters/g biofuel) × 100], respectively.

RESULTS AND DISCUSSION

Sunflower oil characteristics. The sunflower oil used in this work was analyzed according to some parameters that are relevant when the final purpose is to obtain biodiesel (Table 1). Exception for the PV, all the values are consistent with the ones available in the literature (16,17). The results obtained showed a low amount of FFA, indicating a low level of oil decomposition; the iodine value reflects the unsaturation of the FA in the oil, and the PV is a measure of oil autoxidation, which is an important factor to consider when storage is concerned. The low PV is consistent with the intended use of the oil for food purposes; the oil is therefore bottled under nitrogen to reduce oxidation.

The sunflower oil was composed mainly of oleic (23.9%) and linoleic (66.1%) acids (Table 1). Some slight variations between these values and the ones presented in the literature can be due to different seed sources. Oil analysis by GC also allowed the determination of the M.W. of the oil (880 g/mol).

Establishing operational conditions for the transesterification reaction using two different lipases. The effect of lipase amount (Palatase 20 000 L and Lipozyme TL 100 L, 18.4 and 27.5 mg protein/mL of enzyme solution, respectively) on the transesterification reaction of 0.306 mol of methanol with 0.051 mol of sunflower oil (6:1 methanol-to-oil molar ratio),

TABLE 1
Properties and Composition of "AAA" Commercial Sunflower Oil[a]

Characteristics	
Iodine value	132.4 ± 4.2 g I_2/100 g
Acid value	0.089 ± 0.004 mg KOH/g
PV	2.57 ± 0.02 mequiv peroxide/kg
FA composition (wt%)	
Palmitic	6.1 ± 0.5
Stearic	4.2 ± 0.9
Oleic	23.9 ± 0.4
Linoleic	66.1 ± 0.5
Arachidic	0.6 ± 0.08

[a]Values are reported as the mean of three determinations ± SD.

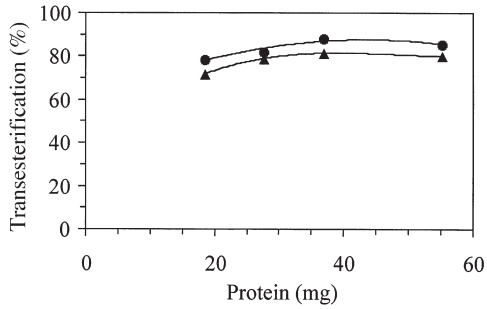

FIG. 1. Effect of protein amount (free ▲ Palatase 20 000 L and ● Lipozyme TL 100 L) on the transesterification of sunflower oil FA (6:1 methanol-to-oil molar ratio, 1:1 volumetric phase ratio, T = 40°C and pH = 5). Data correspond to the mean of three independent experiments, with SD always lower than 3%.

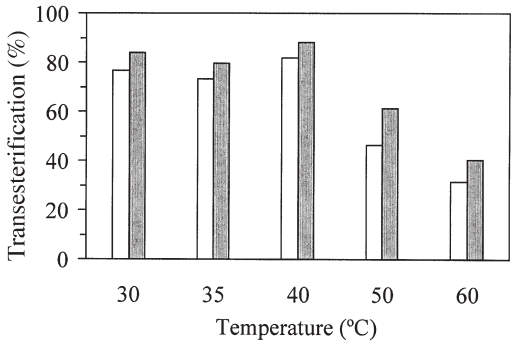

FIG. 2. Effect of temperature on sunflower oil transesterification using free Palatase (□) and Lipozyme (■), at a 6:1 methanol/oil molar ratio, a 1:1 volumetric phase ratio, 36.8 mg protein, and pH 5. Data correspond to the mean of three independent experiments, with SD always lower than 3%.

at a 1:1 volumetric phase ratio, pH 5 and 40°C was evaluated. From the data obtained after 48 h of reaction (Fig. 1), it was evident that transesterification increased with increasing protein amount up to 36.8 mg. From this point onward a constant conversion was obtained, with values of 81.5 and 88.1% using the liquid preparations of Palatase and Lipozyme, respectively.

The effect of temperature on the formation of FAME by the transesterification of sunflower oil with methanol (6:1 methanol-to-oil molar ratio, 1:1 volumetric phase ratio) at pH 5, catalyzed by free Palatase 20 000 L and Lipozyme TL 100 L (36.8 mg protein), is shown in Figure 2. The best temperature was 40°C for both enzymatic systems, giving conversions of 81.5 or 88.1% when Palatase or Lipozyme was used, respectively. Above this temperature, the conversion values did not exceed 61.2%, possibly owing to some enzymatic deactivation and/or methanol evaporation.

The effect of aqueous media pH values on the percent conversion of sunflower oil catalyzed by free Palatase 20 000 L and Lipozyme TL 100 L (36.8 mg protein), at a 1:1 volumetric phase ratio, a 6:1 methanol-to-oil molar ratio, and 40°C was evaluated. Similar degrees of transesterification were observed over the pH range studied (pH 5–9). The conversion values obtained ranged from 78.4 to 81.5% when using Palatase and from 86.7 to 88.1% with Lipozyme (SD always lower than 3%). Thus, a broad pH optimum was observed with both enzymes.

The comparative study performed with *R. miehei* (Palatase 20 000 L) and *H. insolens* (Lipozyme TL 100 L) lipases in their free forms, at a 1:1 volumetric phase ratio and a 6:1 methanol-to-oil molar ratio, showed that similar activities were obtained despite the different lipase sources. In both cases a lipase amount of 36.8 mg, a temperature of 40°C, and a pH of 5 may be selected as the most adequate operational conditions for the transesterification reaction. However, a higher sunflower oil percent conversion (88.1 ± 0.9%) and a higher content of methyl esters (92.3 ± 0.9 wt%) were reached with Lipozyme TL 100 L. Under the same conditions, a conversion of 81.5 ± 1.0% and a methyl ester content of 84.6 ± 1.0% were obtained when Palatase 20 000 L was used. This fact led to the choice of Lipozyme TL 100 L for subsequent studies. (Note: The values 88.1 vs. 92.3, and 81.5 vs. 84.6 are considered to be comparable given the methods used to determine them.)

Effect of methanol-to-sunflower oil molar ratio on the transesterification reaction using free and immobilized Lipozyme TL 100 L. The influence of the methanol-to-sunflower oil molar ratio on the transesterification reaction was investigated to determine whether excess methanol was required to obtain the best conversions, as happens in chemical transesterification (16). In addition, the effect of the water content (different volumetric oil-to-aqueous phase ratios), which is an important parameter for reactions in biphasic systems, was also considered. To obtain high sunflower oil conversions, the aqueous phase should be reduced to avoid methyl ester hydrolysis. However, the aqueous phase must be large enough to prevent enzyme inactivation by the alcohol. Therefore, in these biphasic systems, the amount of aqueous phase will be a compromise between those two factors. To study the effect of the parameters described above on sunflower oil percent conversion by free lipase, the reaction was carried out at a 2:1 volumetric oil-to-aqueous phase ratio and at various methanol-to-oil molar ratios, pH 5, 40°C, and 36.8 mg protein. As shown in Table 2, the sunflower oil conversion and the methyl ester content increased with an increasing concentration of methanol in the aqueous phase, corresponding to an increase in the methanol/oil molar ratio. When free lipase was used, the highest conversion (90.8%) and methyl ester content (95.5%) were obtained with a 6:1 methanol/oil molar ratio as described for chemical transesterification (16). In the methanolysis of rice bran oil with *Cryptococcus* spp. S-2 lipase (9) and extracted palm oil with *R. oryzae* lipase (8), a similar behavior was observed up to a molar ratio of 4:1. On the contrary, at a 6:1 methanol/oil molar ratio, a decrease in the methyl ester content was observed and was attributed to enzyme denaturation by methanol.

The presence of different water contents in the reaction mixture using free lipase, at a 6:1 methanol/oil molar ratio, showed no significant effect on sunflower oil transesterification. In fact, conversions of 90.8 and 88.1% were obtained for water con-

TABLE 2
Effect of Methanol-to-Oil Molar Ratio on Sunflower Oil Percent Conversion and Methyl Ester Content[a]

	Methanol/oil (mol/mol)			
	3:1	4:1	5:1	6:1
$Methanol_{aq}$ (%)	24.3	32.4	41.6	50.0
Free lipase				
Conversion (%)	75.6 ± 0.6	83.7 ± 1.3	85.2 ± 1.4	90.8 ± 2.1
Methyl esters (% w/w)	79.0 ± 0.6	87.9 ± 1.3	89.7 ± 1.4	95.5 ± 2.1
Immobilized lipase				
Conversion (%)	—	80.1 ± 1.7	83.6 ± 1.3	91.2 ± 1.9
Methyl esters (% w/w)	—	84.3 ± 1.7	87.9 ± 1.3	96.1 ± 1.9

[a]Experiments performed at a 2:1 volumetric oil-to-aqueous phase ratio, pH 5, and 40ºC, using Lipozyme TL 100 L in its free form (36.8 mg protein) or immobilized in Hypol® 2002 (2 g of immobilized lipase preparation containing 32.4 mg immobilized protein/g support) as catalyst. Values are reported as the mean of three independent experiments ± SD.

tents of 28 and 83% by weight of vegetable oil, respectively (volumetric oil/aqueous phase ratio of 2:1 and 1:1, corresponding to methanol concentrations in the aqueous phase of 50 and 25%, respectively) (Table 2, Fig. 1). A different behavior was described for the methanolysis of extracted palm and rapeseed oils by *R. oryzae* lipase, in which the conversion was dependent on the amount of water present in the reaction mixture (8). In these systems, at a 6:1 methanol/oil molar ratio, a conversion decrease of about 20% was achieved when the water content was lowered from 75 to 33% by weight of vegetable oil.

In taking into account the results obtained in the experiments with free lipase, and in an effort to improve the application of enzymatic catalysis to the large-scale production of biodiesel, the lipase was immobilized in hydrophilic polyurethane foams. The lipase immobilized in Hypol 2002 (2 g of immobilized lipase preparation containing 32.4 mg immobilized protein/g support) was used in the biphasic system at a 2:1 volumetric oil/aqueous phase ratio with various methanol-to-oil molar ratios at pH 5 and 40°C. As observed in the free lipase system, the increase in the methanol-to-oil molar ratio led to an increase in sunflower oil conversion (Table 2). At the 6:1 methanol/oil molar ratio, a product containing 96.1% of methyl esters, a value that is close to the one required for a biodiesel to be used as fuel in diesel engines (96.5%) (EN 14214) (18), was obtained with a 91.2% conversion. Since the free and the immobilized lipases led to similar conversion values, the use of these kinds of supports for lipase immobilization in the transesterification processes is very promising.

Some tests were also performed using Lipozyme TL 100 L immobilized in polyurethane foams of higher porosity (Hypol 3000 and Hypol 5000) at a 2:1 volumetric oil/aqueous phase ratio and a 5:1 methanol/oil molar ratio. The conversion obtained with the lipase immobilized in Hypol 3000 (85.8%) was slightly higher than the one achieved using Hypol 2002 under the same reaction conditions (83.6%). On the contrary, a lower conversion was observed with Hypol 5000 immobilized lipase (77.8%). Although the washing step of the immobilization process led to a release of protein in all cases, only with Hypol 5000 was the remaining protein amount (29.2 mg) lower than 36.8 mg—the protein threshold amount for best conversion (Fig. 1). This fact could explain the decrease observed in the degree of sunflower oil transesterification.

Recyclability of the immobilized lipase. The Hypol 2002 immobilized Lipozyme (32.4 mg immobilized protein/g support) was used in successive sunflower oil/methanol transesterification reactions, at a 5:1 methanol–to-oil molar ratio and a 2:1 volumetric organic-to-aqueous phase ratio. A conversion decrease with lipase reuse was observed (Fig. 3), the relative conversion after the fourth reaction being 30% lower than the initial one. At this point, a decrease of about 1.9-fold in the amount of immobilized protein was detected (Fig. 3). This protein loss resulted from the release of immobilized protein during the transesterification processes and the washing steps. However, this fact was not responsible for the behavior observed since a protein amount of 36.7 mg (detected after 4 reuses) should be high enough to carry out the transesterification reaction efficiently (Fig. 1). The reduction in lipase efficiency was probably due to enzyme denaturation by the addition of high concentrations of methanol (41.6%) in the beginning of each reuse experiment. This negative effect of the alcohol was also observed when using lipase PS from *Pseudomonas cepacia*, immobilized by entrapment within a sol–gel structure, in the transesterification of soybean oil with

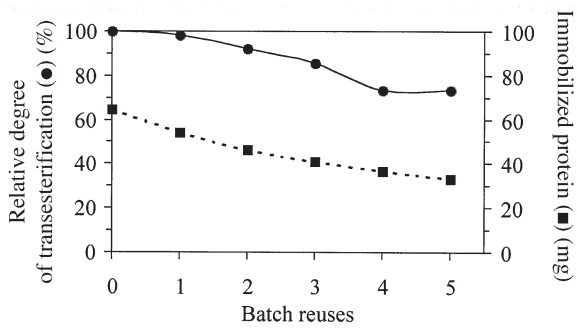

FIG. 3. Reusability of immobilized Lipozyme TL 100 L in the transesterification reaction, at a 5:1 methanol/oil molar ratio, a 2:1 volumetric oil/aqueous phase ratio, pH 5, and 40°C. (●) Relative transesterification and (■) initial immobilized protein in each batch reuse.

methanol (19). In this case, an activity loss of about 25% was achieved after repeated use of the immobilized lipase.

Since the discontinuous reuse of the immobilized lipase led to a loss of efficiency, with subsequent decrease in biofuel quality, the implementation of a continuous process should be considered.

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