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Optimization of ethyl ester production from olive and palm oils using mixtures of immobilized lipases

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ABSTRACT

Although reactions of transesterification are generally catalyzed by one specific lipase preparation, the concept of "*combi*-lipase" could be better explored for the production of biodiesel, since oils are heterogeneous substrates. In this research, we tested this concept by evaluating the enzymatic transesterification of olive and palm oils, two diverse fatty acid compositions, using standalone or mixtures of three immobilized lipases as biocatalysts: Novozym 435 (CALB), Lipozyme TL-IM (TLL), and Lipozyme RM-IM (RML). For olive oil, the combination of 29.0% of TLL, 12.5% of RML, and 58.5% of CALB was the best, allowing for 95% conversion efficiency in 18 h of reaction, up from 50% for the best individual lipase (CALB). For palm oil, the best enzyme combination was 52.5% of TLL and 47.5% of RML, resulting in 80% of conversion of ethyl esters in 18 h, compared to only 44% when standalone TLL was used. Repeated batches of reaction were carried out in order to test the operational stability of the *combi*-lipase systems, with results showing that they could be used for at least seven cycles keeping higher than 80% of their initial activities.

1. Introduction

The transesterification of vegetable oils and animal fats using short-chain alcohols (methanol and ethanol) to produce alkyl esters to be used, as biodiesel fuel has been extensively researched [1–3]. In industrial scale, biodiesel is mainly produced following the conventional chemical process where basic catalysts are used, which is efficient in terms of reaction time and yields. However, the chemical synthesis presents some drawbacks in terms of glycerol recovery, removal of salt residues, large amounts of wastewater, and high-energy costs [4]. In addition, the basic catalysis cannot be carried out using acid oils. Acid catalysts, which could be used with these acid substrates, are generally less efficient [5]. In this context, the research on enzyme catalysis for biodiesel synthesis, in special using lipases, is in continuous development to overcome setbacks of the chemical catalysis [6].

Lipases (EC 3.1.1.3) are largely used enzymes to catalyze alcoholysis, hydrolysis, esterification, and transesterification of fat acids. Lipases have excellent catalytic activities and stabilities in non-aqueous media and their regiospecificity, regioselectivity, and enantioselectivity can be successfully used for many applications in organic synthesis, including the production of biodiesel [7]. As for regiospecificity (positional specificity), the lipases are classified into three groups: sn-1,3-specific, which hydrolyzes ester bonds at sn-1 and sn-3 positions; non-regiospecific (or random), which acts on all three positions; and fatty acid-specific lipases, which hydrolyze esters that are formed from long-chain fatty acids containing double bonds between C-9 and C-10 [8,9].

The main advantages of using lipases, as compared to the conventional chemical reaction, are their substrate specificity and selectivity, besides their ability to react at low temperatures, avoiding high costs of energy input [5]. The reaction is controlled by the molecular properties of the enzyme in use, the structure of the substrate, and factors affecting binding of the enzyme to the substrate [10]. These factors avoid the production of undesired by-products, allowing the production of purer biodiesel and glycerol [11].

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Table 1
Fatty acid composition of palm and olive oils used in this research.

Fatty acid	Structure	Average composition (%)	
		Palm	Olive
Palmitic	16:0	52.71	10.2
Palmitoleic	16:1	–	–
Stearic	18:0	3.80	2.50
Oleic	18:1	36.71	78.10
Linoleic	18:2	6.70	7.10
Linolenic	18:3	–	0.76

On the other hand, enzyme regiospecificity to one type of fatty acid is a reason for decreased conversions, which also depends on the type of oil or fat in use as substrate [12]. This fact reflects the extensive research for finding best sources of lipases for optimal reaction rates and conversions for one specific substrate [8,13,14]. Vegetable oils, which are the main raw materials for biodiesel synthesis, are heterogeneous substrates, containing triglycerides formed by very different fatty acids, glycerides with lower substitutions, and even free fatty acids [15]. Therefore, it is difficult to find a lipase that optimally works for all available substrates [16].

In a previous work, we proposed the concept of *combi*-lipase biocatalyst for heterogeneous substrates. The concept is based on the fact that a composed biocatalyst of a mixture of different lipases should be more effective on heterogeneous substrates than one specific lipase [17]. It was applied for the hydrolysis of soybean oil, with results showing that the mixture of lipases from *Rhizomucor miehie* (RML) and *Candida antarctica* (CALB) was more efficient than the lipase from *Thermomyces lanuginosus* (TLL), which was the most active enzyme, when used individually [16].

In this context, the objective of this work was to evaluate the concept of *combi*-lipase biocatalyst for the transesterification reaction in the synthesis of biodiesel. Three vegetable oils, with very different compositions of fatty acids, olive oil and palm oil were used. Ethanol was used as acyl acceptor because this alcohol poses lower environmental impacts than methanol. The commercial immobilized lipases from *C. antarctica* (CALB, Novozym 435), *T. lanuginosus* (TLL, Lipozyme TL-IM), and *Rhizomucor miehei* (RML, Lipozyme RM-IM) were used to compose the *combi*-lipase biocatalyst. The enzymes were also tested individually in order to verify their independent activities. The transesterification reaction parameters were optimized for the best *combi*-lipase composition. In addition, the recycling of the *combi*-lipase biocatalyst was tested by multiple batches of reuse in order to test its stability and activity.

2. Experimental

2.1. Materials

TLL immobilized on acrylic resin (Lipozyme TL-IM), RML immobilized on anion-exchange resin (Lipozyme RM-IM), and CALB immobilized on macroporous resin (Novozym 435) were kindly donated by Novozymes (Novozymes, Spain). Olive, palm, and sunflower oils were purchased at a local market and were used without any treatment. The composition of fatty acids of these oils is presented in Table 1 [18,19]. All other chemicals were of analytical or chromatographic grade.

2.2. Reaction of transesterification and its analysis

The transesterification reactions were carried out in 50 mL Erlenmeyer flasks containing 1 g of oil and appropriated amounts of ethanol, temperature, and enzyme content according to the experimental design. The amount of ethanol (molar ratio used in the reaction) was determined based upon the calculated average molecular weight of palm and olive oils knowing their fatty acid compositions [18–20]. The reactions were carried out in an orbital shaker at 180 rpm.

Table 2
Experiments performed in the mixture design.

Experiment	TLL	RML	CALB	Conversion (%)	
				Olive	Palm
1	1.00	0.00	0.00	48.57	23.29
2	0.00	1.00	0.00	32.19	20.12
3	0.00	0.00	1.00	56.04	16.48
4	0.50	0.50	0.00	49.77	38.18
5	0.50	0.00	0.50	57.19	26.00
6	0.00	0.50	0.50	44.17	20.98
7	0.33	0.33	0.33	54.91	30.46
8	0.33	0.33	0.33	55.34	33.11
9	0.33	0.33	0.33	54.30	35.86

After reaction completion, 5 mL of distilled water was added, followed by centrifugation (2500 × *g*, 7 min, 4 °C). The upper phase, containing esters, was analyzed by gas chromatography (Shimadzu, model GC-17A) equipped with a flame ionization detector (FID) and DB5 capillary column (30 m × 0.25 mm id × 0.25 mM; J&W Scientific). The injector temperature was 300 °C, split ratio = 1:30, and the FID detector temperature was 310 °C. The carrier gas used was nitrogen at a flow of 1.0 mL min^{-1}. The chromatographic conditions were: initial column temperature of 50 °C, heating rate of 10 °C min^{-1} reaching a final temperature of 310 °C. The amount of sample injected was 1 μL, and total time of the analysis was 30 min.

Methyl heptadecanoate, which was used as an internal standard, was mixed with heptane to prepare a stock solution. After a sample was accurately weighted, an internal standard stock solution was added to the sample. A standard FAEE (Fatty Acid Ethyl Esters) mix (C4-C24) from Supelco was used to identify the peaks at different retention times and to correct the peak area using the response factors of the compound. The FAEE content was calculated using the compensated normalization method with internal standardization, based on the European standard DIN EN 14103 [21].

2.3. Reactions using the combination of lipase mixtures

A 3-factor mixture design and triangular surface analyses were performed to evaluate the best combination of lipases. The simplex-centroid design with interior points composed of seven experiments with two replications at the center point is shown in Table 2.

The reactions were carried out in 50 mL Erlenmeyer flasks at 40 °C in an orbital shaker (180 rpm) for 18 h. The conditions were: substrate molar ratio, 6:1 (ethanol:oil); temperature, 40 °C; biocatalyst content 10% as oil mass. All reaction conditions, including time, were defined based on previous studies [22–24]. The biocatalyst content corresponds to individual or mixtures of lipases according to Table 2.

2.4. Central composite design

A central composite design (CCD) of three variables was carried out in order to obtain the optimal conditions for the transesterification reaction (the optimum mixture of enzymes was selected as in 2.3 and used as the biocatalyst). The variables and their coded and uncoded values are presented in Table 3, whereas in Table 4 are shown the 17 treatments obtained for the three variables, each at five levels. The design was constructed of eight factorial points, six axial points (two axial points on the axis of design variable), and