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VEGETABLE OILS TRANSESTERIFICATION CATALYZED BY IMMOBILIZED NON-COMMERCIAL LIPASE

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ABSTRACT

Industrial biodiesel synthesis has several issues, such as requirement of high-cost purified oils and large generation of basic effluents. The use of lipases may be an alternative in place of conventional alkaline transesterification and allows usage of diverse low-cost feedstocks with high free fatty acids content. The utilization of non-commercial lipases contributes to further reduce inherent process costs and its immobilization allows for reuse of the catalysts. In this study, fatty acid ethyl esters (FAEEs) were produced by transesterification of different vegetable oils with anhydrous ethanol using non-commercial lipase immobilized on hydrophobic supports. Lipase from *Rhizomucor miehei* produced by solid state fermentation was extracted and immobilized. The maximum yield of FAEEs was approximately 50% in the transesterification of babassu oil catalyzed by lipase immobilized in Purolite® C18, after 72 hours of reaction.

Keywords: Biodiesel. Lipase. Transesterification. Vegetable Oils. Immobilization.

1 INTRODUCTION

The production of alternative fuels, such as biodiesel, has increased mainly due to environmental problems associated with the use of fossil fuels: pollution and global warming, becoming a priority in national and global energy policy strategies[1]. The use of biocatalysts represents an alternative route to eliminate problems associated with the application of classical homogeneous catalysts. However, for enzymatic technology to compete industrially with the conventional process, the cost of these biocatalysts must be reduced, as well as their performance improved to obtain greater conversions in shorter reaction times[2].

The use of lipases obtained from microorganisms produced by solid state fermentation (SSF) or submerged fermentation using waste as a source of nutrients represents a promising technology for reducing the costs of these biocatalysts, which is important to make their application in biodiesel synthesis viable. Furthermore, as lipases catalyze esterification and transesterification reactions, a wide variety of raw materials, including lower-cost acidic oils, can be used to produce biodiesel via the enzymatic route[3].

In order to solve some problems associated to enzymatic route, enzyme immobilization can become a strategy to improve their performance, enzymatic stability, activity, specificity or selectivity[4]. Immobilization can also increase the range of conditions under which the enzyme can be used, reduce inhibitions or inactivation caused by the substrate[5]. Furthermore, the recovery and reuse of the biocatalyst is important for the commercial viability of enzymatic biodiesel.[6].

Therefore, the aim of this work was to analyze biodiesel production from different vegetable oils using lipase produced by solid-state fermentation and immobilized on hydrophobic supports, Purolite® C18 or silica magnetic microparticles functionalized with C18 groups (SMM C18).

2 MATERIAL & METHODS

Materials: Anhydrous ethyl alcohol P.A 99,8% and acetone was purchased from Êxodo Científica (Sumaré, SP, Brazil). Babassu oil was from COPPALJ (Lago do Junco, Maranhão, Brazil). Castor oil was from Farmax (Divinópolis, MG, Brazil). Coconut oil was from Copra Indústria Alimentícia Ltda (Maceió, AL, Brazil). Linseed oil was from Cisbra Agroindustrial Ltda (Ijuí, RS, Brazil). Soybean oil was from Cargill Agrícola S.A. (Rio Verde, GO, Brazil). Triton X-100 was from Neon Comercial Regentes Analíticos Ltda (Suzano, SP, Brazil). Purolite Lifetech® ECR8806F (Purolite® C18) was kindly donated by Purolite® Lta. (Wales, UK). SMM C18 was purchased from Kopp Technologies (São Carlos, SP, Brazil). Other reagents were used in analytical grade.

Lipase production: The fermented solid was obtained by SSF of babassu cake, using a strain of *Rhizomucor miehei* (IDAC accession number 071113-01) and produced by the Microbial Biotechnology Laboratory - IQ/UFRJ. Fermentations were carried out according to Aguieiras *et al.*[3] for 72 hours. The fermented solids were dried in a lyophilizer until achieving a moisture content of less than 3 wt%, stored at 4 °C until use and named enzymatic solid preparation (ESP).

Lipase extraction: Protocol 1 - Enzymatic extraction was carried out by adding 5 mL of 0.1 M sodium phosphate buffer pH 7.0 to 1g of ESP, which was kept under stirring at 200 rpm, at 35 °C for 20 minutes. Subsequently, the ESP was pressed manually, and

the supernatant was centrifuged at 4 ºC, 10000 rpm for 15 minutes (common procedure for other protocols). Protocol 2 - Enzymatic extraction was carried out by adding 10 mL of 0.1 M sodium phosphate buffer pH 7.0 to 1g of ESP, which was kept under stirring at 200 rpm, at 35 °C for 1 hour. Protocol 3 - Enzymatic extraction was carried out by adding 10 mL 0.1 M sodium phosphate buffer pH 7 to 1g of defatted ESP, which was kept under stirring at 200 rpm, at 4 °C for 15 hours. Protocol 4 - Enzymatic extraction was carried out by adding 10 mL 0.1 M sodium phosphate buffer pH 7 containing 1% Triton X-100 to 1g of defatted ESP, which was kept under stirring at 200 rpm, at 4 °C for 15 hours.

Immobilization: The lipase was immobilized by adsorption on support (Purolite® C18 or SMM C18) in 5 mM sodium phosphate buffer solution pH 7 (1:10 support/enzyme solution ratio, g/mL) and remained under gentle stirring at room temperature until complete immobilization. The biocatalysts were recovered by filtration or applying a magnetic field and washed with distilled water. The parameters of immobilization yield (IY) and recovered activity (RA) were calculated[7]. A control solution containing buffer and enzyme was kept under the same immobilization conditions. Protein concentration was measured spectrophotometrically in an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden) using the Bradford method[8] and activity was measured by titrating the tributyrin hydrolysis with a 20mM KOH solution using an automatic titrator, according to Beisson's method[9] (Titrando 907 titrator, Metrohm (Herisau, Switzerland).

Transesterification: The reaction medium was composed of different vegetable oils (soybean, castor, coconut, babassu or linseed oil), a molar ratio of 1:6 oil/anhydrous ethanol and lipase as ESP or immobilized on a solid support. Reactions were carried out at 40°C in closed bottles with stirring at 250 rpm. Samples were centrifuged at 6000 rpm for 10 minutes. The light phase was removed, washed with hot distilled water, centrifuged again (washing 3 times) and dried overnight at 60°C.The samples were analyzed in an Agilent Technologies 7890A gas chromatograph (Santa Clara, CA, USA) for the content of fatty acid ethyl esters (FAEEs) in accordance with European standard EN 14103.

3 RESULTS & DISCUSSION

The extraction of lipase from ESP to the soluble phase carried out according to protocol 1 produced an enzymatic solution with activity of 17.5 U_{TBU}/mL and a total protein concentration of 1.03 mg/mL. Due to the low enzymatic activity, the extraction procedure was modified to protocol 2, which produced an enzymatic solution with activity of 11.7 U_{TBU}/mL and a total protein concentration of 0.65 mg/mL. Despite the longer incubation time, this new protocol did not significantly increase the total amount of enzyme extracted (Table 1).

Table 1 Total enzymatic activity of the lipase extraction process according to protocols 1 and 2.

Extraction	ESP before extraction (U_{TBU})	Enzyme solution after extraction (U_{TBU})
Protocol 1	83.48	87.5
Protocol 2	83.48	117.0

The total activity data in Table 1 shows the extracted enzyme activity was greater than that initially offered for extraction. This indicates that the enzymatic activity in ESP was probably underestimated. Therefore, a step of removing oil from ESP was added before extraction (addition of acetone to ESP in the proportion 1:10 m/v, incubation under rotary agitation at 4 ºC, 200 rpm for 4 hours and subsequent filtration), since that the oil from babassu may be interfering in the measurement of enzymatic activity. After washing with acetone, the enzymatic activity measured in the defatted ESP was 523.4 U_{TBU}/g, indicating that the previously measured activity (83.48 U_{TBU}/g) was underestimated. Furthermore, the extraction protocols 1 and 2 were not sufficient to extract all the lipase from ESP. Therefore, two new strategies for extracting lipase from defatted ESP were evaluated using protocols 3 and 4, which changed the temperature and incubation time. In general, the activity of the soluble enzyme was greater than the activity of the enzyme naturally immobilized in ESP, probably due to diffusive problems (Table 2). Furthermore, the presence of Triton X-100 (protocol 4) significantly increased the enzymatic activity of the soluble enzyme, since it is well documented that this surfactant promotes interfacial activation of lipases (Table 2). Although protocol 4 was apparently more efficient than protocol 3 for lipase extraction, protocol 3 was selected for the remaining experiments, since a test carried out confirmed that the surfactant influenced the measurement of enzymatic activity.

Table 2 Total enzymatic activity of the lipase extraction process according to protocols 3 and 4.

Extraction	Defatted ESP before extraction (U_{TBU})	Enzyme solution after extraction (U_{TBU})	Defatted ESP after extraction (U_{TBU})
Protocol 3	2497.5	2400.0	1629.5
Protocol 4	2497.5	4309.0	671.0

Figure 1 shows the immobilization profile of *R. miehei* lipase on Purolite® C18 (A) and SMM C18 (B) supports. The IY for Purolite® C18 was approximately 72% after 4 hours of immobilization and for SMM C18 it was approximately 73% after 24 hours. Although the IYs were similar for both supports, the RA of lipase in Purolite® C18 (100%) was higher than the RA of lipase in SMM C18 (15%). As already observed in the group's work, the SMM support can make it difficult to measure hydrolytic activity due to partition effects due to its high hydrophobicity[10]. Therefore, the hydrolytic activity of the SMM C18 derivative may be underestimated.

The synthesis of enzymatic biodiesel produced was evaluated through the transesterification of different vegetable oils (soybean, linseed, castor and coconut oil), anhydrous ethanol (1:6 oil/ethanol molar ratio) and ESP at a concentration of 10% wt. The production of enzymatic biodiesel was evaluated through the yield of FAEEs (on a mass basis), the main component of biodiesel, obtained in transesterification reactions. The mass yields of FAEEs achieved in the transesterification of vegetable oils were 5.92 ± 0.28% for soybean oil, 1.32 ± 0.17% for linseed oil, 14.16 ± 1.67% for castor oil and 8.77 ± 0.37% for coconut oil (Figure 2). Lipases present specificity in relation to the type of fatty acid in the substrate, the length of the fatty acid chain, the presence of unsaturations and regiospecificity (specificity regarding the position of the fatty acid in the triglyceride). Therefore, it is important to evaluate different vegetable oils as substrates in transesterification to obtain higher yields of FAEEs. The low yields obtained

in this case may have occurred due to a low enzymatic load offered in the reaction, since, if we increased the mass of ESP offered, homogenization would be impaired.
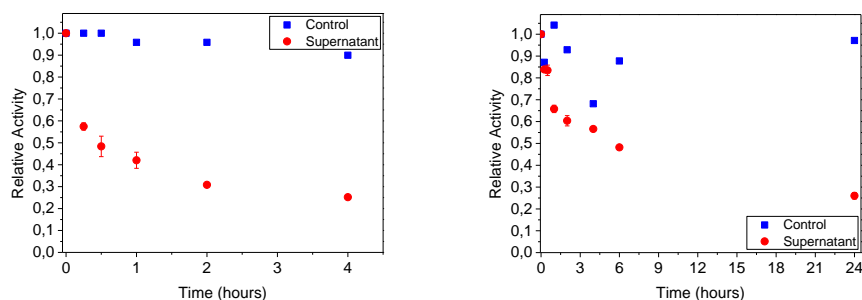

Figure 1 Immobilization profile of *R. miehei* lipase on (A) Purolite® C18 (protein loading 2.0 mg/g of support) and (B) SMM C18 (protein loading 2.3 mg/g of support). (■) Relative activity of the free enzyme control solution and (●) Relative activity of the supernatant.
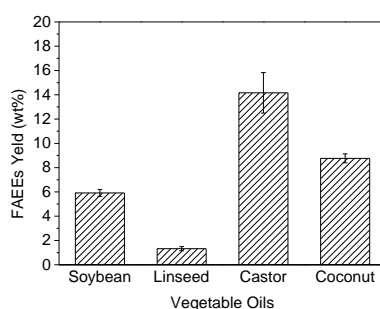

Figure 2 Yield of FAEEs from transesterification (72 h) of different vegetable oils with anhydrous ethanol catalyzed by (A) ESP 10 wt% (11.93 U_{TBU}/g oil) and (B) Purolite® C18, 10 wt% (604, 7 U_{TBU}/g oil).

Immobilization of lipase in Purolite® C18 produced a biocatalyst with high enzymatic load, so transesterification experiments were carried out using this derivative and castor oil, since the highest yield of FAEEs was obtained using this same oil. In this experiment, babassu oil was also tested, which is a low-cost extractive oil (vegetable) from the Amazon. The mass yields of FAEEs were approximately 50% with babassu oil and 39% with castor oil, both using an enzymatic load of 604 U_{TBU}/g oil for 72 hours of reaction. According to international standards, biodiesel (B100) must have (on a mass basis) a minimum of 96.5% fatty acid esters[11]. The use of lipase immobilized in Purolite® C18 made it possible to conduct the transesterification reaction with a high concentration of enzyme in the reaction medium, leading to better yields of FAEEs compared to the use of ESP in the first transesterification.

4 CONCLUSION

This study evaluated the extraction of lipase from *R. miehei* obtained from solid-state fermentation in babassu cake to the soluble phase, as well as its immobilization on hydrophobic solid supports and its performance in the transesterification of vegetable oils. The Purolite® C18 support achieved better results about immobilization parameters compared to the SMM C18 magnetic support, producing derivatives with high enzymatic load. Among the vegetable oils tested in the transesterification reaction, babassu oil stood out, with a mass yield of FAEEs of approximately 50%, and castor oil with a mass yield of approximately 39%. Although yields in FAEEs of at least 96.5% (international standard for biodiesel) were not achieved, this work showed the viability of a non-commercial lipase immobilized on a hydrophobic support to be very promising.

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