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Application of lipase from the physic nut (*Jatropha curcas* L.) to a new hybrid (enzyme/chemical) hydroesterification process for biodiesel production

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ABSTRACT

The aim of this study was to characterize a new plant enzyme (with high lipase activity) extracted from germinated physic nut seeds, and to apply this lipase to the production of biodiesel using a new process of enzyme/chemical hydroesterification. The lipase activity was detected only in the vegetable enzyme extract from germinated seeds (VEEG). Similar activities were obtained for substrates with different lengths of fatty-acid chains (111 ± 19 U/g for tributyrin (C4:0), 106 ± 49 U/g for tricaprylin (C8:0), and 96 ± 4 U/g for olive oil (C18:1)). The VEEG, obtained by a controlled processes of seed germination, was able to hydrolyze a wide range of biodiesel raw materials (vegetable oils, tallow, and biodiesel waste); of these, soy and physic nut oil showed especially high hydrolysis conversion (97% FFA). The biodiesel (fatty acid methyl esters) was produced by the hydrolysis of the physic nut oil using the VEEG, and subsequent esterification of the generated fatty acids with methanol by heterogeneous acid catalysis (niobic acid in pellets). The resulting biodiesel was of excellent quality, with the following properties: viscosity (5.5 mm^2/s), ester content (97.1%), total glycerol (0.09 % w/w), max. methanol (0.05 % w/w), and CFPP (0 °C).

1. Introduction

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) are a group of enzymes that catalyze hydrolysis, esterification, and transesterification reactions. They act on several ester compounds [1,2], and their natural substrates are acylglycerols.

The process of lipid modification using lipases is currently attracting great interest. The reasons for these enzymes great biotechnological potential, besides the different types of reaction that they can catalyze, include their high stability in the presence of organic solvents, the lack of need for cofactors, and their ability to catalyze reactions with chemo-, regio-, and enantioselectivity [3,4].

Plant lipases are especially interesting because of their low cost, their ease of purification, and their wide availability from natural sources, with no need for molecular genetic technology to produce them [3]. Plant lipases also show good substrate selectivity and specificity, which makes them valuable to the oil and chemical industry [5,6].

The physic nut lipase has been little studied. Abigor et al. [7] found lipase activity in germinated and dormant seeds of this plant, but, Staubmann et al. [8] achieved lipase activity only in the germinated seed. The optimum pH and temperature described in these studies for the germinated enzyme extract were 7.5 and 37 °C [7] and 8.0 and 45 °C [8].

With the heightened environmental concerns about the emission of atmospheric pollutants by fossil fuels, greater attention is being given to alternative and renewable energy sources such as biodiesel [9]. Nowadays, the most widely used process for making biodiesel is the alkaline transesterification of triacylglycerols with methanol in the presence of an alkali catalyst [10]. The reaction conditions usually employed are: molar ratio of alcohol/oil 6:1, 1% (w/w) of sodium hydroxide related to the amount of oil, temperature of 60 °C, and reaction time of 90 min [11–13]. Although this process reaction time and yield (98%) are good, it does have some drawbacks: the difficulty of separating the catalyst from the glycerol, the production of highly alkaline waste, and the necessity for high-quality raw materials (acidity less than 0.5%) [12].

The production of biodiesel by the route of hydroesterification circumvents these drawbacks, since this process is conducted in two stages. The first stage is the hydrolysis of tri-, di- and monoa-

Abbreviations: CFPP, cold filter plugging point; VEEG, vegetable enzyme extract germinated; VEED, vegetable enzyme extract dormant; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEMED, N,N,N′,N′-tetramethylethylenediamine; MUF, methylumbelliferyl; FFA, free fatty acid.

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cylglycerols, producing FFA and glycerol. After this step, the organic phase that contains the FFA is separated from the aqueous phase which contains the glycerol and the VEEG. In the second step, the fatty acids are esterified with an alcohol to obtain biodiesel. The hydroesterification process also allows the use of any fatty raw material [14,15] (animal fat, vegetable oil, used cooking oil, acid waste from vegetable oils production, among others) independently of its acidity and water content [16]. This gives it an advantage over the conventional transesterification process, which inevitably generates soaps in the presence of fatty acids [10,17], inactivating the catalyst, and making it difficult to separate the biodiesel from the glycerol, which affects the yield of these plants [4,13,14,18,19]. Some studies have examined the whole process of hydroesterification employing heterogeneous acid catalysts (which can be reused) or reactions without a catalyst, with conversion rates above 90% [20].

In the present study, we evaluated a new hybrid (enzyme/chemical) hydroesterification process for biodiesel production. The hydrolytic stage of the hydroesterification process using a new plant enzyme catalyst has advantages over the more widely used process for hydrolyzing oil and grease (Colgate–Emery process), which uses around 4.83 MPa and about 250 °C to obtain 96–99% conversion in 2 h reaction time [21]. Enzyme hydrolysis uses a lower temperature (30–60 °C) and atmospheric pressure, which in turn leads to the formation of fewer undesirable byproducts and reduced purification costs [13,22,23], and, particularly in this case, with no need to use any type of emulsifier. During the esterification stage, the use of niobic acid (Nb_2O_5) in pellets as a chemical catalyst is a potentially attractive alternative, as many studies have shown that lipases are easily deactivated by the alcohols that are normally used in the synthesis of biodiesel [24,25].

Therefore, the aim of this study was to characterize and apply a new plant enzyme (with high lipase activity) extracted from germinated physic nut seeds, and to use it in the production of biodiesel by a new process of enzyme/chemical hydroesterification.

2. Materials and methods

2.1. Source of lipase (plant enzyme extract)

The enzyme extract was produced by triturating and washing the germinated and dormant physic nut seeds in acetone, according to the methodology described by Cavalcanti et al. [26]. The acetone powders obtained were termed the vegetable enzyme extract from germinated (VEEG) and dormant (VEED) physic nut seeds.

2.2. Lipase characterization

2.2.1. SDS-PAGE zymogram

This technique was used to identify the protein band corresponding to the lipase(s) found in the enzyme extracted from the seed (VEED and VEEG).

SDS-PAGE gel was made at a 10% concentration with a separation solution consisting of 4.0 mL water; 3.3 mL acrylamide mix (30%); 2.5 mL 1.5 M Tris–HCl buffer, pH 8.8; 0.1 mL SDS (10%); 0.1 mL ammonium persulfate (10%); and 4 μL Temed. The concentration solution was prepared with: 3.4 mL water; 830 μL acrylamide mix (30%); 630 μL 0.5 M Tris–HCL buffer, pH 6.8; 50 μL SDS (10%); 50 μL ammonium persulfate (10%); and 5 μL Temed. The analyzed extracts were pre-treated with solutions of Triton X-100 (2%), Tween 80 (2%) and 0.1 M Tris–HCl buffer, pH 8.0. After electrophoresis, the gel was washed in 2.5% Triton X-100 (30 min) and then quickly with 0.05 M phosphate buffer, pH 7.0, for protein renaturation. Next, 100 μL of 4-methylumbelliferyl heptanoate (prepared with 7.2 mg MUF-heptanoate in 1.0 mL ethylene glycol monomethyl ether) was added to the gel. The MUF-heptanoate was used as a substrate for detecting lipase activity. The presence of fluorescent bands (which were revealed by shining ultraviolet light on the gel) indicated that the substrate had been hydrolyzed by the lipase. Next, the gel was covered in a Coomassie Blue solution to stain all the bands in the gel [27].

2.2.2. Preference for substrates with fatty acids of different chain lengths

Hydrolytic activity was established using tributyrin (C4:0), tricaprylin (C8:0) and olive oil (C18:1) as a substrate, all at 5% (w/v) and emulsified with Triton X-100 and 0.1 M Tris–HCl buffer, pH 8.0. The VEEG (90 mg) was added to 10 mL emulsion and incubated at 40 °C while stirring at 200 rpm. The reaction was stopped by the addition of 20 mL ethanol and 10 min incubation at 40 °C while stirring (200 rpm) to extract the FFA. The reaction blanks were made by adding VEEG after the ethanol. The FFA were titrated with 0.04 N NaOH solution in an automatic titrator. One unit of lipase activity (U) was defined as the amount of enzyme (g) that catalyzes the production of 1 μmol fatty acid per minute under assay conditions [28].

2.3. Hydrolysis reaction of different raw materials available for biodiesel production

The hydrolysis reactions were carried out in thermostated reactors, where the substrate (10%, w/v), 0.1 M Tris–HCl buffer, pH 8.0, and the VEEG (10%, w/w) were incubated under magnetic stirring. The substrates used were refined and unrefined palm oil, olive oil, physic nut oil, acid waste from palm oil processing, castor oil, biodiesel waste, and animal tallow. The hydrolysis reaction conversion rates were evaluated by the production of FFA. The pH was chosen according to the literature, where the optimal pH for the activity of these enzymes is reported as between 7.5 [7] and 8.5 [8].

2.4. Extraction of free fatty acids

The reaction was stopped for extracting FFA from the reaction medium. Anhydrous sodium sulfate was added to remove any traces of water from the product, which was then filtered and concentrated in a rotary evaporator. In order to evaporate any traces of solvent in the product, it was incubated overnight at 70 °C.

2.5. Evaluation of hydrolysis reaction

The FFA content was determined by titration. The samples (0.5 g) were solubilized in 10 mL of a solution composed of Triton X-100 (25%), 0.05 M acetate buffer (50%) in distilled water and 10 mL ethyl alcohol, and were titrated with 0.04 N NaOH solution until pH 11.0 in an automatic titrator. The blanks were made by titrating the solution with no sample. The acidity, which indicates the percentage of FFA (w/w) in the oil, was established according to equation:

$$\text{acidity}\,(\%\text{FFA}) = \frac{N \cdot (V_s - V_b) \cdot \text{MM}}{10 \cdot m}$$

where N is the NaOH normality, V_s the volume of base used in sample titration (mL), V_b the volume of base used in blank titration (mL), MM the molecular mass of the predominant fatty acid (g), and m is the sample mass (g).

2.6. Biodiesel production and analysis

2.6.1. Hydrolysis of physic nut oil

The hydrolysis reactions were carried out at 450 rpm in a batch stirred-tank reactor (1.5 L volume). The hydrolysis reactions were

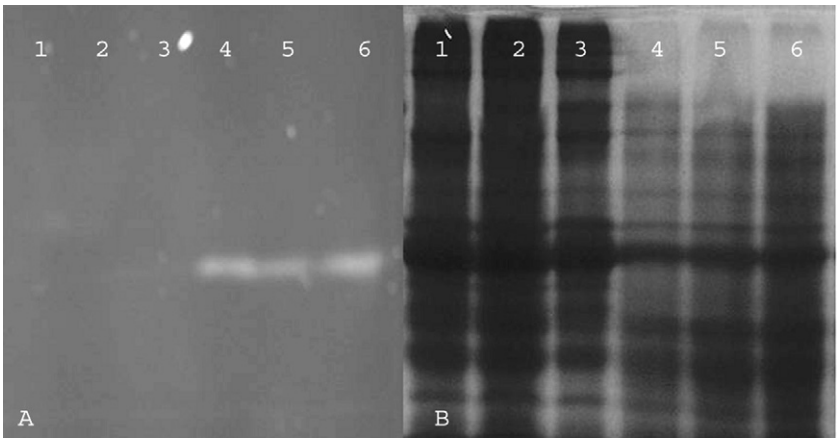

Fig. 1. (A) Hydrolysis of MUF-heptanoate in buffer, Triton X-100 and Tween 80, respectively; catalyzed by VEED (lanes 1, 2 and 3) and catalyzed by VEEG (lines 4, 5 and 6). (B) Gel stained with Coomassie Blue in buffer, Triton X-100 and Tween 80, respectively; lanes 1, 2 and 3 catalyzed by VEED; lines 4, 5 and 6 catalyzed by VEEG.

conducted with 50% substrate (w/v), 0.1 M Tris–HCl buffer, pH 8.0, and the VEEG (10%, w/w), for 2 h.

2.6.2. Esterification reaction

The esterification reaction took place in an autoclave-type reactor (Parr Instruments Inc., Model 4842) at 500 psi and 200 °C for 2 h with constant stirring (500 rpm). The reaction was carried out using methanol and the FFA derived from the hydrolysis of the physic nut oil using VEEG. A substrate molar ratio of 1:3 (fatty acid/alcohol) was used, with 20% (w/w of FFA) catalyst (niobic acid in pellets).

The acid catalyst had been previously calcinated at 200 °C for 1 h. It was removed after the reaction by filtration and dried in a muffle furnace for subsequent reuse.

2.6.3. Biodiesel analysis

The biodiesel analyses were carried out according to the standard methodologies published by ASTM (American Society for Testing and Materials) and ABNT (*Associação Brasileira de Normas Técnicas* – Brazilian Technical Standards Association). The analyses done were appearance, specific mass at 20 °C, kinematic viscosity at 40 °C, water and sediment, flashpoint, ester content, carbon residues, sulfate ash, total sulfur, sodium, and potassium, free glycerol, total glycerol, monoglycerides, diglycerides, triglycerides, methanol or ethanol, stability to oxidation at 110 °C, CFPP, distillation, and 90 vol.% recovered.

The results were compared with the values established by Resolution No. 7 of 19/03/2008, of the ANP (National Agency of Petroleum, Natural Gas and Biofuels), which establishes the specifications necessary for the sale of biodiesel in Brazil. The Brazilian standards are similar to their European and US counterparts, though they have some added flexibility to take account of the characteristics of Brazilian raw materials.

3. Results and discussion

In order to check for the presence of lipase activity in the VEED and VEEG, a hydrolytic assay was undertaken on these extracts (5%) using soybean oil (10%), buffered to 0.1 M Tris–HCl pH 8.0, at 40 °C, for 2 h. The VEEG showed excellent hydrolytic capacity, converting up to ~99% of the triacylglycerols into FFA, whereas the VEED hydrolytic capacity was practically non-existent (~1%), in agreement with the findings of Staubmann et al. [8]. These results indicate that the controlled germination of physic nut seeds is fundamental for the production of this plant lipase. This differs from seeds of castor bean (*Ricinus communis*), another member of Euphorbiaceae, which show lipase activity both when the seeds are dormant and when they are germinated [29].

SDS/PAGE zymography was used to identify the band that corresponded to the lipase in the enzyme extracts (both germinated and dormant). In Fig. 1 we can see the band relating to the lipase found only in the VEEG. Triton X-100 and Tween 80 were added to both extracts to solubilize the lipase(s) present in the extracts, but no major difference was found between the results for the different buffer solutions.

In order to investigate whether the VEEG showed a preference for substrates of a particular carbon-chain length, assays were undertaken using olive oil (C18:1), tricaprylin (C8:0), and tributyrin (C4:0) as substrates, in equimolar quantities. The lipase activities found in this assay were: 111 ± 19 U/g for tributyrin, 106 ± 49 U/g for tricaprylin, and 96 ± 4 U/g for olive oil. It was possible to show that the VEEG not only had high hydrolytic activity, but also showed no selectivity for the chain length of the substrates tested. This is an important characteristic for the production of biodiesel by hydrolysis, followed by esterification using raw materials with different fatty acid contents.

When the VEEG was used to hydrolyze biodiesel raw materials (Fig. 2), it was able to satisfactorily convert almost all the tested substrates in only 2 h of reaction time. Soybean and physic nut oil showed the best conversion, higher than 98%. This was expected, because physic nut oil is the natural substrate of the VEEG lipase, and soybean oil has a very similar chemical composition (mostly oleic and linoleic acids). Hydrolysis of soybean oil catalyzed by different types of lipase was also studied by Park et al. [30], who

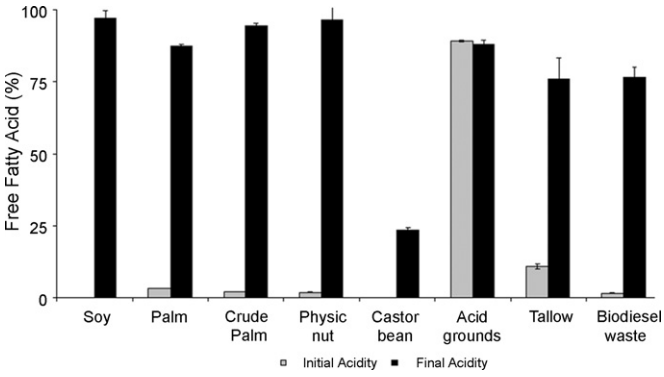

Fig. 2. Hydrolysis of different biodiesel raw materials by VEEG the hydrolysis reactions were carried out with 10% w/v of substrate, 0.1 M Tris–HCl buffer, pH 8.0, 10% w/w of VEEG, for 2 h.

Table 1
Analysis of the biodiesel obtained by hydroesterification.

Properties	Result	Unity	Method	Resolution 7 – ANP	
				Min.	Max.
Aspect	L II	–	–	–	–
Density 20 °C	879	kg/m^3	ASTM D 1298	850	900
Viscosity kinematic 40 °C	5.50	mm^2/s	ASTM D 445	3.0	6.0
Water, max.	0.04	vol.%	ASTM D 2709	–	0.05
Flashpoint, min.	128	°C	ASTM D 93	100	–
Ester	97.1	%	EN 14103	96.5	–
Carbon residue	0.01	% w/w	ASTM D 4530	–	0.05
Ashes, max.	0.01	% w/w	ASTM D 874	–	0.02
Sulfur, max.	5	mg/kg	ASTM D 5453	Note	50
N + K, max.	3	mg/kg	EN 14108/14109	–	5
Free glycerol, max.	0.01	% w/w	ASTM D 6584	–	0.02
Total glycerol, max.	0.09	% w/w	ASTM D 6584	–	0.25
Monoglycerides	0.24	% w/w	ASTM D 6584	Note	Note
Diglycerides	0.14	% w/w	ASTM D 6584	Note	Note
Triglycerides	0.095	% w/w	ASTM D 6584	Note	Note
Methanol or ethanol, max.	0.05	% w/w	EN 14110	–	0.2
Oxidation stability 110 °C	>6	h	EN 14112	6	–
CFPP	0	°C	ASTM D 6371	Note	19
Distillation; 90 vol.%	335	°C	ASTM D 1160	–	360

compared the activities of these enzymes in the isolated and combined forms. After 10 h of reaction, it was found that the combined systems of microbial lipases, such as *Penicillium* sp. (lipase G), and *Rhizopus niveus* (lipase N) or *Penicillium* sp. (lipase G), and *Rhizopus delemar* (lipase D), provided higher yields (98% and 99.5% hydrolysis) than those obtained in systems containing only one lipase (7.2% – G lipase, 42% – N lipase, and 44.4% – lipase D). These results, when compared to those found in this study for the vegetable lipase from physic nut, highlight its immense potential, since the hydrolysis obtained in our study was 98%, with only 2 h of reaction, and without combination with other imported and costly sources of lipase.

Palm oil (refined) and crude palm oil (unrefined), which contains a higher proportion of palmitic acid, were also well hydrolyzed, with 87 and 95% conversion, respectively. Noor et al. [31], working with crude palm oil and lipase SP 398 (Novo Nordisk A/S), obtained 100% hydrolysis in 90 min of reaction. However, these authors employed a lower substrate concentration (2.5%). The palm oil waste product is already very acidic (about 90%), which is probably what inhibited the enzyme activity, as no hydrolysis activity was identified for this waste material.

Castor oil, which contains about 90% ricinoleic acid, was not hydrolyzed satisfactorily (24% conversion), even though this oil is formed of long-chain fatty acids (C18:1-OH). Most probably this was because of the presence of the –OH group in its chain. Puthli et al. [32] obtained 43% conversion in 6 h of reaction using the commercial lipase from *Humicola lanuginose*, termed Lipolase.

With clarified tallow (animal fat that remains in slaughterhouses) and biodiesel lipid waste (lipids that were not esterified in biodiesel production), the hydrolysis level reached 75% of FFA.

The results shown in Fig. 2 indicate that the VEEG could be used in hydroesterification processes for biodiesel production involving a wide range of raw materials [9], including waste, with excellent conversion.

At the hydrolysis stage, a better quality, potentially food-grade glycerol was produced, as it did not come into contact with methanol or chemical catalysts. Highly pure glycerol (>99%) is widely used in the food, cosmetics, and pharmaceuticals industries, and it is technically difficult and costly to obtain pure glycerol from the alkaline route of biodiesel production [33], since this glycerol is contaminated with methanol and the alkaline catalyst. Glycerol is now the main byproduct of the oil and chemical industry, and accounts for 10% of all biodiesel production [23,24].

While the Colgate–Emery hydrolysis procedure does give similar conversion rates and yields to the process described in this study, it uses far more energy and also causes the thermal degradation of the products.

Because of the excellent hydrolytic capacity of VEEG when used with physic nut oil, this substrate was selected for the hydrolytic stage of the hydroesterification process for biodiesel production. The other factors that support this choice are that physic nut oil would not compete with the food industry, it has better oxidation stability than soybean oil, lower viscosity than castor oil, and a lower pour point (the temperature where it starts to solidify) than palm oil. In addition, the biodiesel is stable in storage [34]. The profile of the fatty-acid composition corresponding to these features was C14:0 (1.4%), C16:0 (15.6%), C18:0 (9.7%), C18:1 (40.8%), C18:2 (32.1%), and C20:0 (0.4%) [35].

In the esterification step, we opted to use a chemical catalyst (niobic acid), since it has been shown in several studies that lipases are easily deactivated by the alcohol used in the synthesis of biodiesel [24]. The degree of deactivation is inversely proportional to the number of carbon atoms of the alcohol [24]. The application of this chemical acid catalyst in the form of pellets also has the advantages of ease of separation and reuse.

Table 1 shows the properties of the biodiesel produced, and the respective specifications published by ANP. It can be seen that the enzyme/chemical hydroesterification process produced a top-quality fuel that meets all the ANP specifications and has similar qualities to the biodiesel obtained by the traditional alkaline transesterification.

4. Conclusions

This new process for obtaining biodiesel from mixed enzyme/chemical hydroesterification has several advantages over the conventional process: similar yield and conversion rate to the chemical-hydrolysis process, but with much lower energy expenditure; fewer undesirable byproducts (contaminated glycerol and degraded fatty acids); generation of potentially food-grade glycerol; and the production of biodiesel from physic nut oil (no competition with the food industry). This process also makes it possible to produce and use an efficient, low-cost plant enzyme catalyst in the production of a top-quality biodiesel (within the specifications required by the ANP), with possible applications in other biotechnology processes.

The development of new biocatalysts and low-cost processes is of the utmost importance for obtaining a commodity such as biodiesel.

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