J. Chem. Chem. Eng. 10 (2016) 68-76
doi: 10.17265/1934-7375/2016.02.003

Selection of Strains Producing Lipase with Transesterification Activity and Its Characterization

Gabriel Luis Castiglioni[1*], Jorge Alberto Vieira Costa[2], Giovana da Silva Padilha[3] and Ranulfo Monte-Alegre[3]

1. Department of Food Engineering, School of Agronomy, Federal University of Goiás, Rodovia Goiânia/Nova Veneza, Km 0, Goiania, GO 74690-900, Brazil

2. Department of Chemical Engineering, Federal University of Rio Grande, Rua Engenheiro Alfredo Huch, Rio Grande 96201-900, Brazil

3. Department of Food Engineering, University of the State of Campinas, Rua Monteiro Lobato 80, Campinas 13083-862, Brazil

Abstract: The interest for lipase production is due to the ability of this enzyme to catalyze some reactions, such as the transesterification. Although industrial biodiesel is produced chemically, there are several problems associated with this technology that can be prevented through the use of lipases. The present work aimed to select microorganisms with potential for production of lipase with transesterification activity. The lipase from *Burkholderia cepacia* was the one with the most promising results for this type of reaction, showing results of hydrolytic activity at 37 °C and pH 8.0. The pH and volume of crude enzyme extract that showed favorable for synthesis of biodiesel is at about pH 6.0 and 3.75 mL, respectively, which represents approximately 42% of water in the system, ensuring the conversion of nearly 60% to biodiesel.

Key words: Biodiesel, *Burkholderia cepacia*, characterization, lipase, transesterification.

1. Introduction

Lipases (triacylglycerol acyl hydrolase EC 3.1.1.3) represent 10% of the enzyme market and are widely used in different processes of industrial importance. In favorable thermodynamic conditions, they are also capable of catalyze reactions of synthesis in various substrates with high selectivity. The versatility of these enzymes make them promising in many biotechnological applications [1].

Among the various reactions that lipases can catalyze, the most noteworthy are hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. One of the industrial applications of lipases is the biofuel production [2]. Lipases have gained prominence due to the high stability in organic solvents, no requirement for cofactors, high specificity for different substrates and high enantioselectivity [3]. The characteristics related to the versatility, specificity and ease of production make lipases a group of enzymes with a broad of biotechnological applications, such as biodiesel production [4].

Biodiesel has received attention in recent years as an alternative to petroleum-derived diesel, being suitable for use in existing engines. The researchers show promising results of use of lipases for biodiesel synthesis because their high level of activity, stability at alkaline pH and resistance to organic solvents [5].

The main reaction for producing biodiesel is the transesterification, which can be catalyzed either chemically or enzymatically. The transesterification of triglycerides provides an environmentally more attractive option compared to the conventional process. The advantages related to this technique are less energy-intensive, ease of recovery of glycerol and production of high concentrations of free fatty acids [6, 7].

Considering these observations, the present study aimed to select microorganisms producing lipases

[*]**Corresponding author:** Gabriel Luis Castiglioni, Ph.D, research field: food engineering.

with transesterification activity and its characterization.

2. Materials and Methods

2.1 Microorganisms and Maintenance

It's showed in Table 1 that the microorganisms are used to verify the production of lipase. Except for *Bacillus licheniformis*, all strains were maintained at 4 °C in test tubes with nutrient agar pH 7.0. The maintenance medium for *Bacillus licheniformis* contained 10 g tryptone, 5 g yeast extract, 10 g NaCl, 5 g glucose, 15 g agar, 1 L distilled water and pH adjusted to 7.0. For solid phase fermentation, microorganisms were maintained at 4 °C in PDA (potato dextrose agar).

2.2 Fermentation

From test tubes containing the microorganisms, scraping was done with 5 mL of sterile distilled water (non-sporulated microorganisms) and 5 mL of 0.2% Tween 80 solution (sporulated microorganisms), which were used as inoculum for fermentation.

Semi-solid fermentations were carried out using 40 g of culture medium with 55% moisture content (adjusted with sterile water) in 250 mL Erlenmeyer flasks at 30 °C for 144 h. The brans were sieved to ensure particle size near Tyler 40.

The preincubation of submerged fermentations was performed using the same fermentation conditions, but with lower concentrations of soybean oil (0.5% *v/v*) and 48 h incubation time. Later, these fermentations were performed in 125 mL Erlenmeyer flasks with 15% inoculum at 30 °C under shaking at 150 rpm. The working volume of the fermentation broth was 60 mL.

2.3 Extraction and Lipase Activity

The separation of bacterial cells through submerged fermentation was done by centrifugation at 1,372 g for 15 min to obtain the supernatant containing extracellular lipase. During the solid fermentation, extraction was

Table 1 Microorganisms, culture media and type of fermentation used to produce lipase.

Microorganism	Strain	Culture media	Fermentation
Burkholderia cepacia (¥)	ATCC 25416	1	SF
Burkholderia cepacia (¥)	CCT 1145	1	SF
Burkholderia cepacia (¥)	ATCC 10856	1	SF
Zymomonas mobilis (£)	Not identified	1	SF
Bacillus subtilis (£)	NRRL 744	1	SF
Bacillus subtilis (£)	NRRL 744*	1	SF
Bacillus subtilis (£)	NRRL 41044**	1	SF
Bacillus licheniformis (£)	Not identified	2	SF
Saccharomyces cerevisiae (£)	ATCC 7754	3	SF
Wild yeast (£)	Not identified	3	SF
Aspergillus fumigatus (€)	NRRL 164	4^{P1}	SSF
Aspergillus fumigatus (€)	NRRL 166	4^{P2}	SSF
Aspergillus (£)	Not identified	4^{P3}	SSF

(¥) strains acquired in André Tosello Tropical Foundation for Research and Technology; (£) strains acquired in the Microbiology Laboratory of the Faculty of Food Engineering from Unicamp; (€) strains acquired in the United States Department of Agriculture Research, Education and Economics Agricultural Research Service. * Mutant obtained by ultraviolet radiation for 1 min; ** Mutant obtained by selection with antibiotic (streptomycin 500 $\mu L \cdot mL^{-1}$). Culture media: 1: 5 $g \cdot L^{-1}$ peptone, 5 $g \cdot L^{-1}$ yeast extract and 3% (*v/v*) soybean oil; 2: 2 $g \cdot L^{-1}$ yeast extract, 5 $g \cdot L^{-1}$ peptone, 5 $g \cdot L^{-1}$ NaCl, 0.05% $CaCl_2$ and 1% (*v/v*) soybean oil; 3: 0.5 $g \cdot L^{-1}$ peptone, 0.3 $g \cdot L^{-1}$ yeast extract and 1% (*v/v*) soybean oil; 4: Medium containing 0.04 mL spore suspension 0.35 $mL \cdot g^{-1}$ medium nutrient solution composed of 0.5 $g \cdot L^{-1}$ $MgSO_4.7H_2O$, 3.0 $g \cdot L^{-1}$ $NaNO_3$, 1.0 $g \cdot L^{-1}$ KH_2PO_4, 1.0 $g \cdot L^{-1}$ yeast extract, 0.3 $g \cdot L^{-1}$ peptone and 3% (*v/v*) soybean oil. P1: Proportion in weight of rice husk and wheat bran (22:110); P2: Proportion in weight of rice husk and soybean bran (22:80); P3: Proportion in weight of rice husk and rice bran (15:85); SF: submerged fermentation; SSF: semi-solid fermentation.

performed with 50 mM phosphate buffer pH 7.0 at a ratio of 10 mL for 1 g of fermented medium. After adding the buffer, the samples were homogenized by shaking at 150 rpm and 37 °C for 30 min and filtered under vacuum in Buchner funnel, obtaining the crude extract for the lipolytic activity.

The supernatant from the submerged fermentation was used as enzyme source for the analysis of lipase activity. The method described by Macêdo, et al. [8] was used with some modifications, using olive oil emulsion and 7% gum arabic solution in the proportion of 25:75 respectively. The reaction mixture was composed of 5 mL of emulsion, 2 mL of 10 mM phosphate buffer pH 8.0 and 1 mL enzyme extract, which was homogenized in a shaker at 150 rpm and 37 °C. The volume of 1 mL of the culture medium was transferred to a flask containing 5 mL of deionized water at 0 (white) and 30 min of reaction. The fatty acids released during the reaction were titrated with 0.01 N NaOH. One unit of lipase activity was defined as the amount of enzyme capable of release 1 μmol of fatty acid per minute.

2.4 Effect of Temperature on Lipase Activity and Stability

To determine the optimum temperature for lipase activity, assays were performed between 25 °C and 50 °C. For the thermal stability tests, samples of the enzyme extract were incubated in a water bath at 40 °C, 50 °C and 60 °C, by taking aliquots in 0, 15 min, 30 min, 60 min, 120 min for determination of enzymatic activity (described in the previous item). From these results, the thermal inactivation constant (K_d) was calculated for each condition used, represented by the slope from the first-order kinetic of denaturation. To determine the half-life of lipase, the enzyme concentration (A) after the time of exposure was considered half of the initial concentration (A_0).

2.5 Effect of pH on Activity and Stability of Lipase

The determination of the optimum pH for lipase activity was studied in the range between 3 and 11, using 10 mM phosphate buffer. Stability tests were performed with buffers at pH 5, 8 and 11 (0.1 N) for 240 min of exposure. After collecting, lipase activity was determined according to the methodology previously described, however using 0.11 N phosphate buffer pH 8.0.

2.6 Sds-Page Electrophoresis and Isoelectric Focusing

The samples were analyzed in Mini Protean III (BioRad, USA) using polyacrylamide gel according to Laemmli [9]. The concentration of 7.5% was used for the samples treated with buffers containing SDS (sodium dodecyl sulfate) in denaturing and reducing conditions. Aliquots of 15 μL of sample and 5 μL of marker present in the gels were subjected to 180 *V*. In isoelectric focusing electrophoresis, Phast System (Pharmacia, Sweden) was used at 410 V with different gradients of acrylamide gels at pH between 3 and 9, as described by the manufacturer. In both techniques, gel staining was performed with silver nitrate according to Morrissey [10].

2.7 Transesterification

For determination of the transesterification activity of lipases produced by different microorganisms, two experiments were conducted. In the first, pH gradients from 6.0 to 8.0 were tested maintaining constant the volume of enzyme extract. The experiments were conducted in 25 mL test tubes for the reaction of 5.0 mL of soybean oil, 1.5 mL of ethanol, 0.7 mL of 0.2 N phosphate buffer and 1.5 mL of crude enzyme extract (1.4 $U \cdot mL^{-1}$). The reaction conditions were maintained at 40 °C under agitation 20 rpm in homogenizer AP 22, Phoenix Luferco. The samples were qualitatively analyzed by thin layer chromatography.

In the second experiment, a 2^2 rotational central composite design was proposed with triplicate at the central point considering the variables volume of enzyme extract (0.732-6.767 mL of crude enzyme

extract with 1.12 $U \cdot mL^{-1}$ of activity), and pH (5.292-7.707) of the reaction medium. In this experiment, the samples were analyzed by gas chromatography.

2.8 Chromatographic Analysis of the Transesterification Reaction Products

The thin layer chromatography analyses were made in Merck Cromatosheet plates-aluminum CCF-C/25. Samples were prepared by diluting 30 μL in 300 μL of hexane, using as eluting and revealing solutions petroleum ether:acetone (97:3) and methanol:sulfuric acid (1:1), respectively. Five microliters of each sample was applied in the plate which was placed vertically in contact with the eluent solution. After approximately 25 min, the plate was removed and allowed to stand for 5 min, and sprayed the revealing solution followed by drying for 5 min at 120 °C.

For quantification of the reaction products, a Cromacon gas chromatograph, model Simple Crom, with flame ionization detector was used. The injector and detector temperatures were respectively 245 °C and 250 °C. 1 μL of the prepared sample was injected. In a first step the column was maintained at 120 °C for 30 s and then elevated to 300 °C at a rate of 10 °C min^{-1}. The compounds were identified by comparison of retention times with standards and quantified by area normalization.

3. Results and Discussions

Among the microorganisms selected for lipase production, the most productive strains were *Burkholderia cepacia* (isolated), wild yeast, *Aspergillus fumigatus* NRRL 164 (using medium containing soy and rice brans), *Aspergillus fumigatus* NRRL 166 (in medium containing wheat bran) and *Aspergillus* (not identified). The other strains did not show satisfactory lipase activity. The results of experiments of lipase production and their transesterification activities are presented in Table 2.

In addition to the type of microorganism, the composition of the medium and fermentation conditions are extremely important variables for optimization of lipase production.

Moreover, the high production cost restricts the use of lipases in industrial scale, creating a challenge by

Table 2 Lipase production by various microorganisms and production of biodiesel by transesterification.

Microorganism	Lipase activity ($U \cdot mL^{-1}$)	Biodiesel (%)
Zymomonas mobilis	0.00	0.00
Burkholderia cepacia isolated	0.55	19.50
Burkholderia cepacia ATCC 25416	0.14	4.10
Burkholderia cepacia CCT 1145	0.10	2.05
Burkholderia cepacia ATCC 10856	0.00	0.00
Bacillus subtilis NRRL 744	0.00	0.00
Bacillus subtilis NRRL 744 1' UV	0.00	0.00
Bacillus subtilis NRRL 41044 with streptomycin	0.00	0.00
Bacillus licheniformis	0.14	2.20
Wild yeast (Not identified)	0.83	6.90
Saccharomyces cerevisiae ATCC 7754	0.00	0.00
Aspergillus fumigatus NRRL 164 (Rice bran)	0.14	1.10
Aspergillus fumigatus NRRL 164 (Wheat bran)	0.00	0.00
Aspergillus fumigatus NRRL 164 (Soybean bran)	0.69	1.55
Aspergillus fumigatus NRRL 166 (Rice bran)	0.55	1.50
Aspergillus fumigatus NRRL 166 (Wheat bran)	1.93	2.90
Aspergillus fumigatus NRRL 166 (Soybean bran)	0.00	0.00
Aspergillus (Not identified)	1.10	2.10

*Percentage of oil converted to biodiesel.

the exploitation of different microorganisms, substrates and culture conditions to obtain the best options for industrial production and viability [11].

Thus, it can be noted that the experiments conducted with different compositions of culture medium were determinant for the production of lipase from *Aspergillus fumigatus*. The addition of wheat bran in the fermentation medium with the strain NRRL 166 as well as the addition of soybean bran for the strain NRRL 164 significantly increased the enzyme production.

Besides the evaluation of lipase activity, another determinant parameter for the selection of the enzyme-producing microorganism was the transesterification activity.

Although lipolytic activity values of the Aspergillus strains were those expected (except for the strains of *Aspergillus fumigatus* NRRL 164 and *Aspergillus fumigatus* NRRL 166, using respectively wheat and soybean bran in the fermentation medium) the yields related to transesterification remained below 7%. This shows that lipases from these strains can efficiently hydrolyze triglycerides, however with limitations in the transesterification reaction to which they were submitted.

The literature reports that the specificity of lipases is controlled by molecular properties of the enzyme, substrate structure and factors affecting the enzyme-substrate binding [12]. The various types of specificity of lipases can help to explain the results in Table 2, since the reactions to which they were subjected have specific characteristics. Among the specificities of these enzymes it is important to emphasize those related to the substrate, as they have different rates of hydrolysis for triacylglycerols, diacylglycerols or monoacylglycerols. The position of enzyme attack also affects the reaction rate, whereas the hydrolysis occurs differently with primary, secondary or tertiary esters, or can be nonspecific, releasing fatty acids from the three positions. A third specificity refers to fatty acids that compose the substrate. There is preference to specific fatty acids, mainly in terms of the chain length and number of unsaturations. The stereospecificity should also be considered, since the discrimination between the enantiomers in racemic substrates is observed.

The results showed that the transesterification of *Burkholderia cepacia* lipase reached about 20% of conversion into biodiesel with 1 mL of crude enzyme extract. This specificity in the reaction makes the enzyme promising for biodiesel synthesis, even with low hydrolytic activity.

Once lipase from *Burkholderia cepacia* was chosen, the characterization of this enzyme was initiated. Evaluating the temperature range between 25 °C and 50 °C, the highest activity has been found at 37 °C (1.20 $U.mL^{-1}$). The largest difference for this activity was 20.83% at 20 °C, showing that its activity did not suffer very rapid changes in the temperature range used. These results are shown in Fig. 1.

Yuan et al. [13] studied the effect of temperature on the activity and stability of lipase produced by *Burkholderia* sp. and observed the best results at 30 °C with less variation in enzyme stability when compared to the present work. During the evaluation of lipase stability, it was observed that exposure for 2 h at 40 °C decreased 15% of the activity. However, when subjected to 50 °C to 60 °C, there was no significant difference in enzyme inactivation, reaching 45% of reduction in enzyme activity at 50 °C for 120 min.

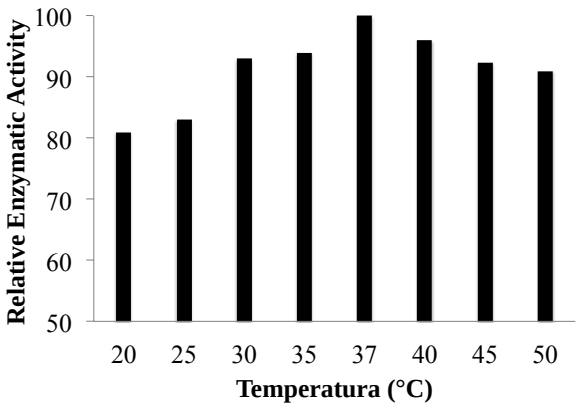


Fig. 1 Enzymatic activity of lipase produced by *Burkholderia cepacia* in different temperature conditions.

From the construction of Fig. 2, it was determined the coefficients of thermal inactivation (Kd) for each condition used.

The results found in the first hour of exposure were similar to the first order model. The Kd values were 0.0015 min^{-1}, 0.0051 min^{-1} and 0.0052 min^{-1} and half-lives of 462.10 min^{-1}, 135.91 min^{-1} and 133.30 min for temperatures of 40 °C, 50 °C and 60 °C, respectively. Results were reported by Kojima et al. [14], for lipase from *Pseudomonas fluorescens* AK102 in temperatures between 40 °C and 50 °C.

Evaluating the effect of pH on the activity and stability of lipase, it was observed that, especially in acidic pH, its activity had a less drastic decrease compared to those more alkaline. The highest lipase activity was observed at pH 8.0 (1.28 $U \cdot mL^{-1}$) as shown in Fig. 3.

Yuan et al. [13] observed that the best conditions for lipase activity of a new species of *Burkholderia* was in 30 °C and pH around 10. Similar results were also found by Jin, et al. [15], however with lipase from *Burkholderia anthina* NT15, characterizing these lipases as alkaline.

Sakiyama, et al. [16] evaluated the effect of pH on the activity of lipase produced by *Pseudomonas* sp. and found results in the pH range between 8 and 9, similar to those results found in this study.

Evaluating the stability of lipase from *Burkholderia cepacia* in relation to the pH of preincubation, the treatment with pH 8.0 and 37 °C showed an increase in lipolytic activity (approximately 27%) after 240 min. The results are shown in Fig. 4.

An atypical result in lipase activity was observed at pH 5.0. Initially there was an increase, remaining practically constant between 90 min and 180 min, followed by decline. Furthermore, in pH 11.0 there was a decrease of approximately 55.6% during 250 min of enzyme exposure when compared to treatment at pH 8.0.

Under normal conditions, the enzyme exhibits a native and catalytically active structure which is

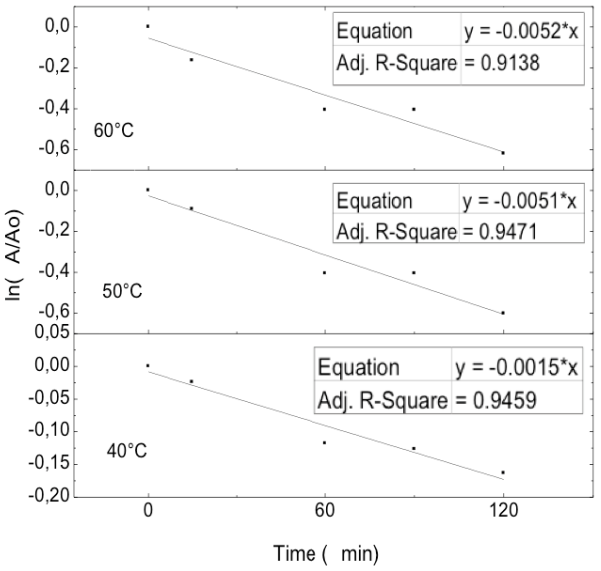


Fig. 2 Thermal inactivation kinetics of lipase from *Burkholderia cepacia*.

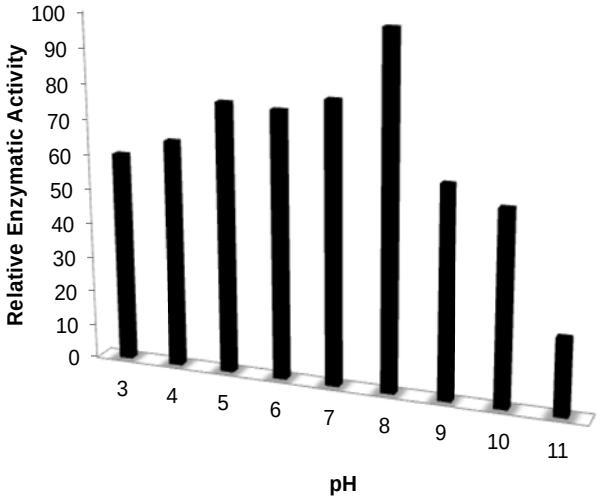


Fig. 3 Activity of lipase produced by *Burkholderia cepacia* in the pH range between 3 and 11 at 37 °C.

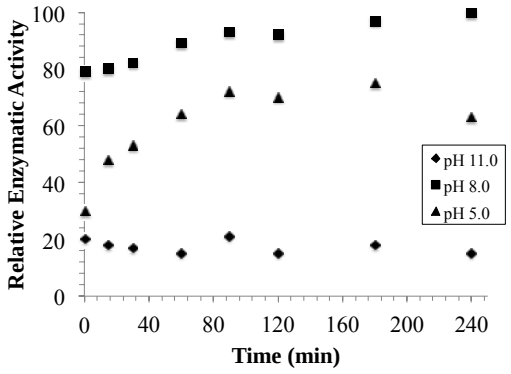


Fig. 4 Relative lipolytic activity versus time of preincubation of lipase from *Burkholderia cepacia* at different pH values.

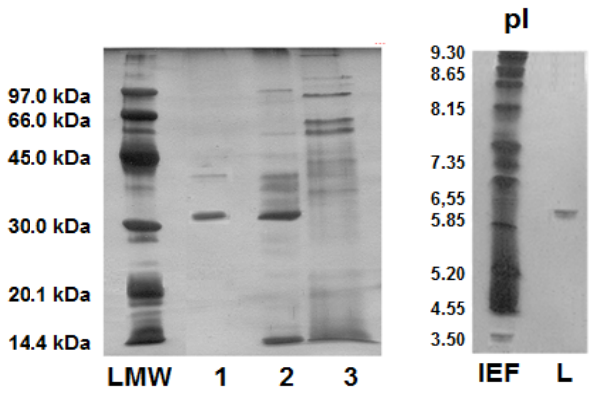
maintained by a balance of non-covalent forces. Under pH and extreme temperature conditions, all these forces decrease, except the hydrophobic interactions, and the enzyme loses activity by acquiring a more disordered conformation.

The obtained molar mass of lipase from *Burkholderia cepacia* by SDS-PAGE (Fig. 5(a)) was approximately 31 kDa. This result indicates a monomeric constitution for this enzyme. In Fig. 5(b) is also shown the electrophoresis gel with the result of the isoelectric point of lipase from *Burkholderia cepacia*. Jin, et al. [15] found a value of 44.5 for the molecular weight of *Burkholderia anthina* NT15. Yang, et al. [4] evaluated the molecular mass of lipase from *Burkholderia cepacia* strain G63 and found similar results to the present study (33 kDa).

The observed isoelectric point for lipase was approximately 6.0. This result can be easily achieved in a process of separation, which is interesting and crucial for their subsequent use at industrial level.

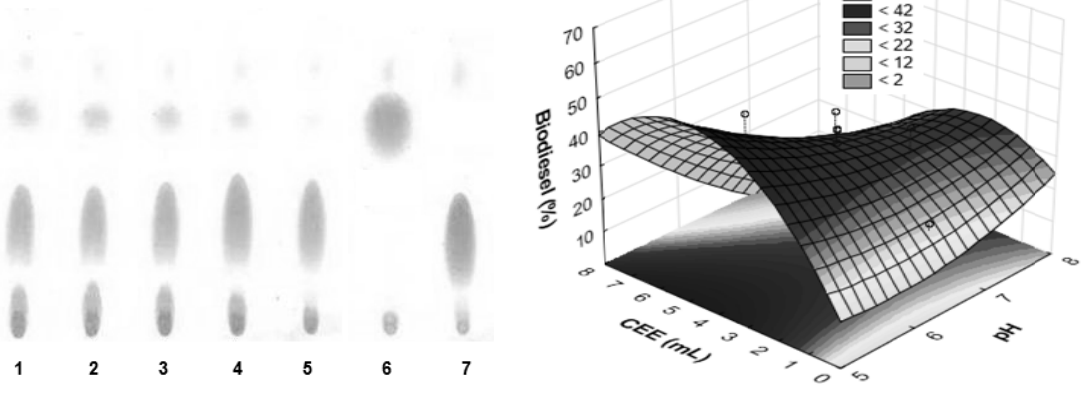
In Fig. 6(a) and (b), the experimental results of thin layer chromatography and gas chromatography are showed, respectively, for the evaluation of the effect of pH in the transesterification reaction.

The results show that the lower the pH, the greater is the conversion to biodiesel during the transesterification reaction. Changes in the pH of the system reflect the interaction between the reactants and residues of the catalytic site of the enzyme, regulating its activity. This statement suggests that one possible explanation for the results is the presence of ionizable groups in the active site, which can affect the affinity of the enzyme for the substrate and the rate of synthesis.



(a) (b)

Fig. 5 (a) SDS-PAGE in 12.5% gel, sample under denaturing and reducing conditions. Where: LMW (low molecular weight) marker; Sample of *Burkholderia cepacia* lipase purified in aqueous two-phase system PEG/phosphate salt at pH 6.0 (1); Commercial lipase from *Burkholderia cepacia* (2); Crude enzyme extract (3). (b) Isoelectric focusing electrophoresis pH 3 to 9. Where: IEF marker (isoelectric point); Sample with lipase from *Burkholderia cepacia* (L).



(a) (b)

Fig. 6 Results of the transesterification reaction of soybean oil at different experimental conditions using enzyme extract from *Burkholderia cepacia*. (a) Thin layer chromatography experiments using enzymatic extract with phosphate buffer pH 6.0 (1); 6.5 (2); 7.0 (3); 7.5 (4); 8.0 (5); Ethyl ester (6); and soybean oil (7). (b) Response Surface of Rotational Central Composite Design 2^2 for biodiesel synthesis.

Relating the effect of pH with the amount of extract containing lipase in a RCCD (rotational central composite design), the same behavior is also observed, that is, more acidic conditions favoring synthesis of biodiesel, confirming the results previously found. The negative effects found in the RCCD for pH and volume of enzyme were 12.68 (quadratic effect) and 19.39 (linear effect) with 0.0485 and 0.0047 of significance, respectively.

Regarding the amount of enzyme extract (aqueous medium), the best results were found near the central point. To obtain better yields in the reaction is also necessary to control the volume of water in the system, which was approximately 42%. Most important than increase the amount of enzyme using different volumes of extract is the relation between the concentration and the amount of water present in the system. This procedure enables to avoid the dilution effect caused by the addition of crude extract, providing the best reaction conditions in mixed systems.

In several reactions that require the presence of water molecules, their concentration in the reaction medium is an extremely important factor. The reaction of triglyceride hydrolysis involves the presence of such molecules to produce glycerol and fatty acids, however, in the reaction of transesterification, the water concentration should be low to minimize the formation of such products and allow the synthesis of fatty acid esters. Shifting the reaction equilibrium towards the products or in reverse direction is controlled by the amount of water present in the reaction mixture [17].

Efficient conversion results have been reported in the literature, such as those found by Lee and Kim [18], where values of conversion into biodiesel were 86.4% in 24 h of reaction using lipase from *Staphylococcus haemolyticus* L62. Baron, et al. [19] has achieved 90% of conversion to fatty acid ethyl esters in 6 h, with lipase from *Burkholderia cepacia* LTEB11 immobilized on a hydrophobic support. These results show the potential of the results of this work once values exceeding 50% of conversion were found in mild conditions using crude extract of lipase.

4. Conclusions

The strain of *Burkholderia cepacia* was the microorganism among those tested with the highest potential for lipase production for transesterification reaction. The characterization has shown this lipase to be a small molecule capable of a more ready catalysis of the hydrolysis at 37 °C and pH 8.0. However, in the transesterification reactions, the pH exerts a positive significant effect in low acidity conditions in the presence of approximately 42% water in the system.

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