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Applied Biocatalysis with an Organic Resistant Partially Purified Lipase from *P. aeruginosa* During FAME Production

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Abstract: A partially purified lipase from the *Pseudomonas aeruginosa* strain (PSA-01) isolated from the palm oil fruit *Elaeis guineensis* was used as biocatalyst to produce fatty acid methyl esters (FAME). Lyophilized lipase supernatant (LLS) was used during the first step to screen the main variables (pH, temperature, stoichiometric oil:methanol ratio, water content and type of oil). Other variables which were identified during the screening assays (scale of reaction recipient, LLS amount and use of hexane to solubilize methanol forming a methanol-oil microemulsion) were tested during a second step. The response variable was % molar yield of FAME. It was quantified by GC. Additionally, the LLS work parameters were optimized and compared to a partially purified lipase (PPL) during a final assay. The first-order interactions between the analyzed factors were significant ($p<0.05$). The highest yield was 4.16% w/w (respect to oil) using a partially purified lipase (PPL) with pH 8, refined, bleached, and deodorized oil (RBD), 5% water (by volume) in oil and 10% hexane (by volume), and a stoichiometric ratio of 1:170 oil:methanol. The final assay was carried out at 54°C and 200 rpm for 48 hours. It resulted in a 34.68% conversion using PPL. It also showed a 13-fold improvement versus the initial yield with LLS, suggesting the need for a better purification process. During this research, the lipase was partially purified and used at an alkaline pH. It showed resistance to organic compounds such as methanol and hexane. This implies great potential to act as an effective biocatalyst in the implementation of biodiesel production processes.

Keywords: Biocatalysis, lipase, *P. aeruginosa*, FAME.

INTRODUCTION

Lipases are used as biocatalyst in transesterification reaction of fatty acids to produce FAME. They are classified as triacylglycerol hydrolases (EC. 3.1.1.3) [1]; and they are associated with lipid bioconversion in living organisms to obtain a large amount of energy. Lipases are grouped in extracellular and intracellular [2]. Many microbial strains have been used to obtain lipases, but the most frequently reported enzyme sources are *Candida sp., Pseudomonas sp.* and *Rhizopus sp.* Lipase producer microorganisms have been studied from different sources, mainly from soil, marine water, waste water and industrial waste [3]. One of the most widely used feedstocks to produce FAME is the Palm oil from *Elaeis guineensis*.

In transesterification reaction, a catalyst such as an acid, a base or an enzyme, is needed to make the process faster and more profitable. Otherwise, this process would be slow and uninteresting. This process is also reversible, requiring a driving force toward the right to produce methyl esters (FAME). The most widely proposed way to overcome this imbalance is through the addition of alcohol in excess to the reaction mixture according to the principle of *Le Chatelier* [4]. Obtaining biodiesel from oils involves conversion of the triacyl glycerides into three molecules of fatty acid alkyl esters through a reaction with a short chain alcohol; a free glycerol molecule is formed as a byproduct in a stepwise reaction [5].

Promising catalytic processes based on lipases according to the different reactions they can catalyze (hydrolysis, interesterification, transesterification) have been reported [6]. Enzymes have advantages over acidic and basic catalysts: less energy consumption (room temperature reaction conditions), easier product separation, reduce treatment costs related to chemical catalyst recovery, regeneration and reuse when it is immobilized, a final product with neither alkalinity nor acidity, less water consumption and minor waste water treatment. Enzymes also guarantee that both free fatty acids and triglycerides are converted to fatty acid alkyl esters [3, 7, 8]. However, enzymatic biodiesel production has drawbacks related to actual industrial enzymes which are characterized by high production costs, inhibition by secondary products, low yields, low reaction rates, and low enzyme stability in the presence of methanol in excess [9].

Due to the long reaction time and high cost of commercial enzymes, enzymatic biodiesel production is more expensive than alkali-catalysis processes [10]. Each purification step in a enzyme production process results in a increases cost significance of catalyst. Feedstock's cost is the major economic factor for industrial biodiesel production, as approximately seventy to ninety-five percent of the total production biodiesel cost are related to raw materials (triglycerides source, alcohol, catalyst, co-solvent, etc) [11]. The purpose of this work is to evaluate a partially purified lipase from *Pseudomonas aeruginosa* to obtain alkyl esters, as an alternative to reduce the enzymatic production process

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cost, identifying the best transesterification process conditions to modify palm oil with methanol to obtain the higher possible yield of FAME.

MATERIALS AND METHODS

Reagents and Biocatalyst

Methanol was chosen as acyl acceptor. Short chain alcohols like methanol and ethanol are suitable for transesterification with *Pseudomonas* lipases. However, better yields are reported with methanol because of its small molecular weight and higher polarity, which allows a major diffusion in the reaction media [12].

P. aeruginosa was obtained from the palm fruits of Manuelita S.A crops in a previous work by Uscategui *et al.* [13]. Lipase was named as LPSA-01 according to the name of the strain. The crude palm oil (CPO) and the refined, bleached, and deodorized oil (RBD) were supplied by Del Llano S.A. The reagents were RA grade and were obtained from different suppliers.

LLS was obtained by 24-hour liquid/submerged fermentation (LF/SmF) in an enriched broth using CPO as inducer. Supernatant was filtered, concentrated and dialyzed using tangential filtration with a MILLIPORE Pellicon XL Biomax 5 cassette (MILLIPORE Labscale TFF System, USA) to remove proteins smaller than 5 kDa. Afterwards, the dialysate was lyophilized at 0.04 mbar, -40°C (Labconco Freezone 1, USA). LLS was used directly in the reaction. PPL was obtained using molecular exclusion chromatography with BIO-RAD Bio-gel P-60 (BIO-RAD BioLogic LP system, USA) following the manufacturer protocol to obtain a fraction containing a partially purified lipase (PPL). Protein quantification by modified Bradford assay [14], catalytic activity by p-nitrophenol liberation [15] and electrophoresis were carried out during each purification step (data not shown).

First Assay-LLS

Standard reaction conditions were established by a factorial experiment design using Analysis of Variance (ANOVA and Response Surface Methodologies (RSM) in JMP 10.0.0 software (SAS Institute Inc). Transesterification experiment was carried out in 100 ml erlenmeyers in an incubator-shaker (JEIO IS-971, Korea) during 48 hours at 200 rpm. CPO or RBD oil was loaded first on each flask. Then methanol and lipase at defined pH (buffer) were loaded according to the factorial design. A final sample of each treatment was treated with hexane and FAME concentration was obtained by Gas Chromatography (GC, Agilent Technologies 7890A GC System, CA USA. HP88 column, FID, flow:1mL/min, and argon as carry gas). The response variable in all experiments was % FAME yield, calculated using Eq. 1.

$$FAME\ yield\ (\%) = DF\ x \frac{M_{FAME}}{M_{Used\ oil}} x\ 100\%$$

Equation 1. Porcentual conversion of oil to methyl esters.

DF is dilution factor, M_{FAME} is mols of FAME, and $M_{used\ oil}$ is initial mols of CPO or RBD oil in the reaction mixture.

First assay was a 2^5 full factorial design with 32 experimental units. Coded variables are provided in Table **1**.

Table 1. Coded variables for first assay.

Variable	-1	+1
Temperature (°C)	42	58
pH	6	8
Oil	Crude (CPO)	Refined (RBD)
Oil:methanol (volume) **(Stoichiometric relation)**	1:4 (1:100)	1:6 (1:149)
Water amount (%V/V water-oil)	10	30

Collateral Assays

These assays were carried out to analyze secondary variables such as scale of reaction, lipase concentration and addition of co-solvent, which were identified during first assay. These variables were combined with the first assay variables at different levels using full factorial designs. Reaction was carried out at 42°C for 48 hours at 200 rpm. Experimental design variables and levels are summarized in Table **2**.

Table 2. Variables and levels for collateral assays (first and third assays were carried out with 2 replicates and the second assay was carried with 1 replicate).

Assay	Variables	Levels	Variable Code
1	Vessel scale (ml)	10	-1
		100	1
	Stoichiometric relation oil-methanol	1:6	-1
		1:140	1
2	Catalyst amount (mg/ml)	20 (1.35% w)	-1
		80 (5.39% w)	0
		140 (9.44% w)	1
	Stoichiometric relation oil-methanol	1:56	-1
		1:106	0
		1:156	1
3	Sotoichiometric relation oil-methanol	1:6	-1
		1:156	1
	Cosolvent	No cosolvent	-1
		Hexane-oil (v/v) 1:1	1
	Water	2%	-1
		10%	1

Final Assay

A 2_V^{5-1} fractional factorial with 5 central points and 21 experimental units was carried out, as described by Gutiérrez [16]. Response surface was used to identify the most significant variables for improving FAME production. The model equation used to perform this analysis is shown in Eq. 2

$$Y = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} X_i + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{kij} X_i X_j$$

Equation 2. Model used for response surface process improvement.

In this model, Y is the response variable (%Yield), B_{k0}, B_{ki}, B_{kij}, are constants (estimated parameters), X_i and X_j are independent decoded variables. The central points included a 7.5% water content, a 20% co-solvent content, a 1:156 oil: methanol stoichiometry, pH 10, and without lipase. The idea was to evaluate any individual first order pH effect due to a high buffer pH. Fixed variables were RBD oil, 4.16% catalyst (due to the availability of the PPL), and a temperature of 54°C (determined on a parallel work assay, data not shown). All assays proceeded for 48 hours at 200 rpm.

RESULTS

First Assay

Data analysis (JMP 10.0 software) of the first LLS assay using SLS (R^2 = 0.8213; α=0.05) showed significant individual effect (P<0.0001) for pH, oil, water and stoichiometric ratio. Temperature was not significant (P=0.3500), but it presents a first order interaction with oil (Fig. **1**). Moreover, pH showed a first order interaction with oil, obtaining better results with RBD oil at pH 8. The highest response was obtained with pH 8, RBD oil, 42°C, stoichiometric relation oil:methanol 1:149 and 10% v/v water: oil. The mean response in this assay was 2.41% FAME conversion yield.

According to reviewed literature [17, 18], a minimum amount of water is required to guarantee lipase activity and this quantity is unique for each lipase. This point will be discussed later in this work.

Collateral Assays

The best results were reached when reaction was carried out in small scale vessels (10-mL vials instead of 100-mL Erlenmeyer flasks), due to the fact that small reaction volumes propitiate more collisions between reactive. In addition, increasing the amount of lipase up to 9.44% lipase-oil in the reaction resulted in a significant yield improvement (highest concentration tested due to the availability of the lipase, Fig. **2**).

The lipase was effective up to oil-methanol stoichiometric relations approximately 1:156 despite widely recommended stoichiometry 1:6 mols of methanol per oil mol. Higher stoichiometric relations showed a decreased FAME production (Fig. **2**). The water content was reduced from 10% to 2% v/v of oil (3.11% and 2.71 mean yield response, respectively), increasing the amount of FAME obtained in the first assay. Additionally, co-solvents helped to homogenize the mixture by forming a microemulsion with reverse micelles, facilitating interactions between the reagents. When a co-solvent was added (relation 1:1 volume hexane-oil) to the reaction with the lowest water content (2%), a positive interaction occurred between these two variables (2.81% and 2.23%, to 2% and 10% of water amount respectively).

Under these conditions, the assay molar mean yield was improved from 2.41% to 3.98% FAME molar mean yield. The levels of each variable that showed the best response were chosen for the final assay (a 10 ml vessel scale, stoichiometric relation 1:156, use of solvent and less amount of water), except catalyst concentration, due the availability of PPL.

Final Assay

RSM using 2_V^{5-1} fractional factorial with 5 central points design was used to determine the optimal levels of the five significant factors identified in the previous assays. The coded levels for each factor are shown in Table **2**. The effects of the analyzed factors and the first order interactions were predicted by the second-order polynomial function obtained on the regression analysis.

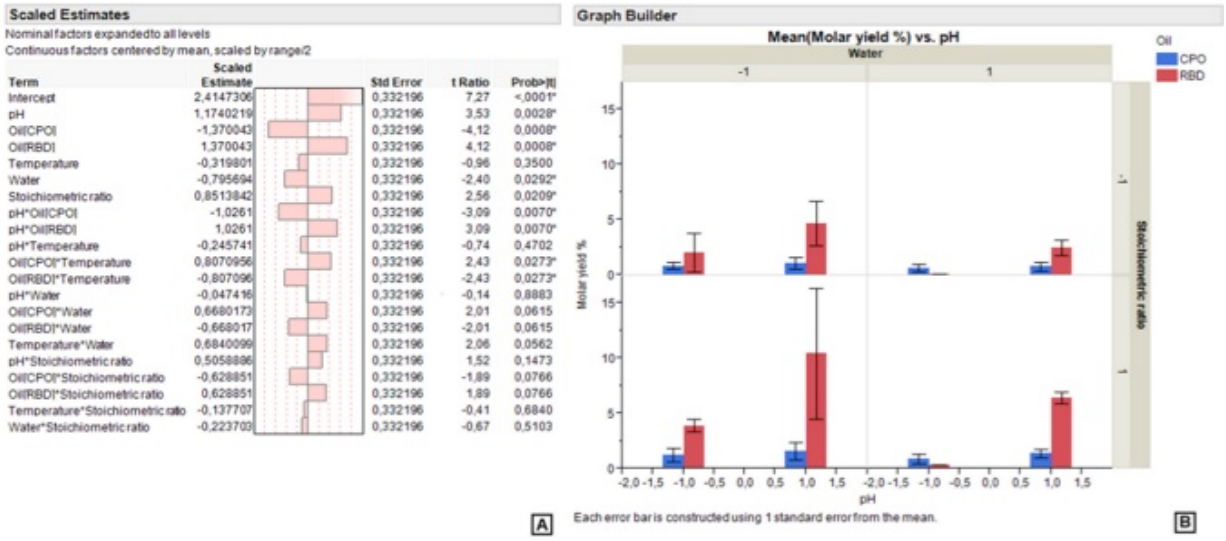


Fig. (1). A) Effects test report for the first assay analyzed factors and first order interactions. **B**) Interaction plot of significant individual effect variables.

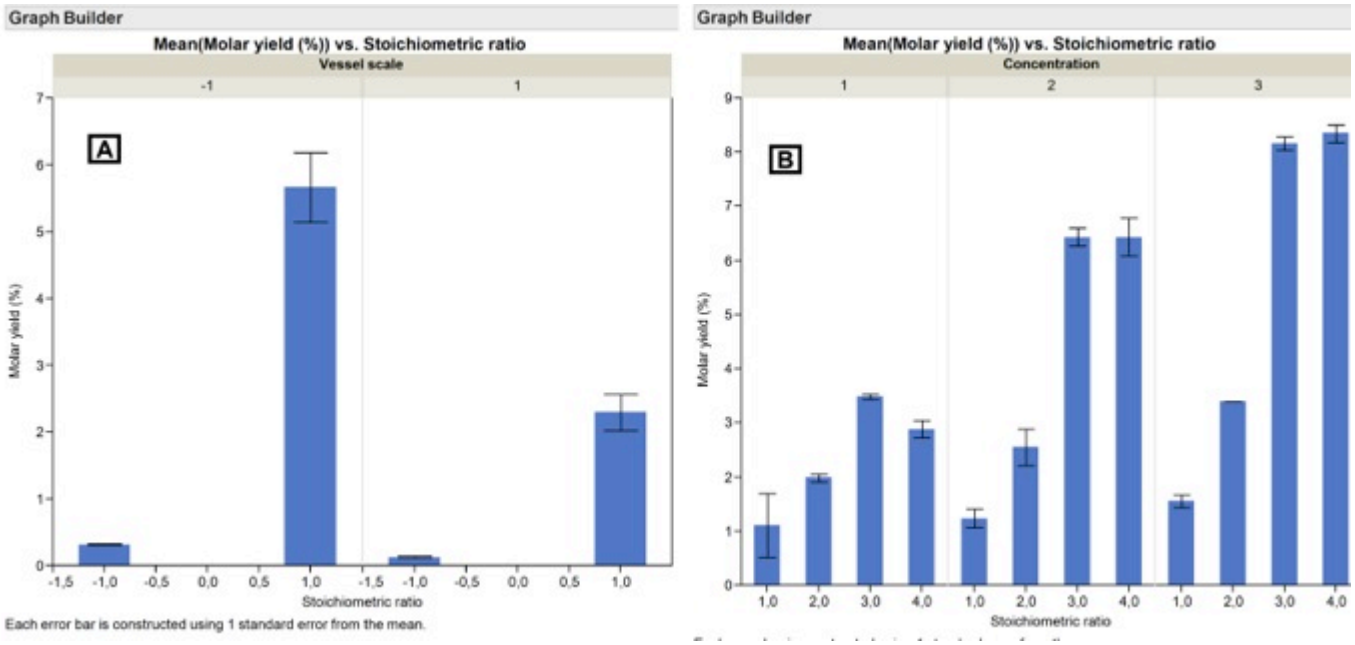


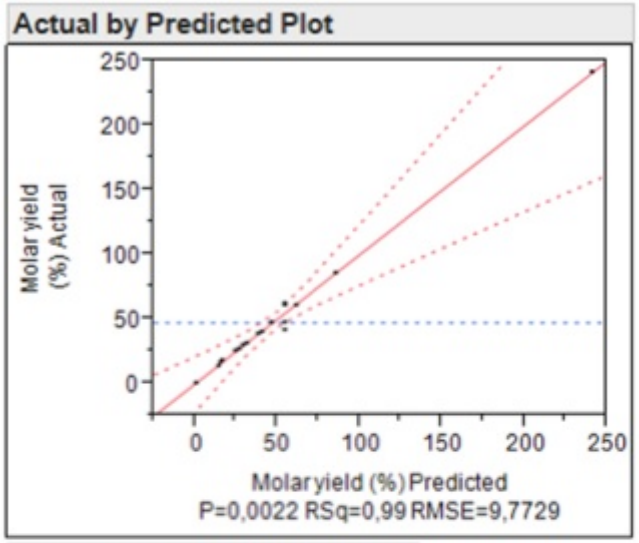
Fig. (2). A) Molar yield with two reaction scales (10 and 100 ml, -1 and 1 respectively) and 1:6 and 1:156 oil/methanol molar ratios. **B**) Reaction yields at different biocatalyst concentrations (20, 80 and 140 mg of LLS/ml, coded variables 1, 2 and 3) and stoichiometric ratios (1:56, 1:106, 1:156 and 1:206 stoichiometric ratios, coded 1, 2, 3 and 4).

$$\text{Molar Yield (\%)} = 35.1145 + (-8.6901 * \text{A}) + (-22.31 * \text{B}) + (-15.177 * \text{C}) + (-7.5756 * \text{D}) + 19.4842 * \text{E} + \text{A} * (\text{A} * 9.6362) + \text{A} * (\text{B} * 11.9214) + A * (C * 9.326) + B * (C * 10.9045) + A * (D * 7.6225 + B * (D * 10.4091) + C * (D * 19.5154) + A * (E * (-20.2376)) + B * (E * (-16.2933)) + C * (E * (-7.2036)) + D * (E * (-9.5453))$$

In the function, factor A is catalyst (coded variable, Table **2**), B is the % water added, C is % of co-solvent, D is alcohol mols of the oil-methanol molar ratio, and E is pH of the buffer added to carry out the biocatalyst. The statistical significance of the response surface regression model was checked by *F* test and ANOVA (Fig. **3**). The analysis showed that the second order polynomial model with the significant variables ($P < 0.05$) is adjusted to the experimental data (Adjusted $R^2 = 0.9602$). It explains 96% of the variability in the response of the assay (Molar yield (%)).

Once the work parameters were determined, they were tested and adjusted to compare the LLS against PPL (coded variables -1 and 1, respectively). In this assay, all the first order interactions between the variables were significant. The most important individual effect was water content, while the most significant interaction of first order was catalyst/pH. The response surface of this interaction showed the best response with LLS at pH 10 (Fig. **4A**).

The interaction between water content and catalyst shows that reducing the water content increases the yield of FAMEs for both LLS and PPL (Fig. **4B**).



Summary of Fit

RSquare	0,992043
RSquare Adj	0,960217
Root Mean Square Error	9,772897
Mean of Response	47,09551
Observations (or Sum Wgts)	21

Analysis of Variance

Source	DF	Sum of Squares	Mean Square	F Ratio
Model	16	47632,857	2977,05	31,1702
Error	4	382,038	95,51	Prob > F
C. Total	20	48014,895		0,0022*

Fig. (3). Outcoming ANOVA report (JMP 10.0) for the second order polynomial model for FAME molar yield (%).

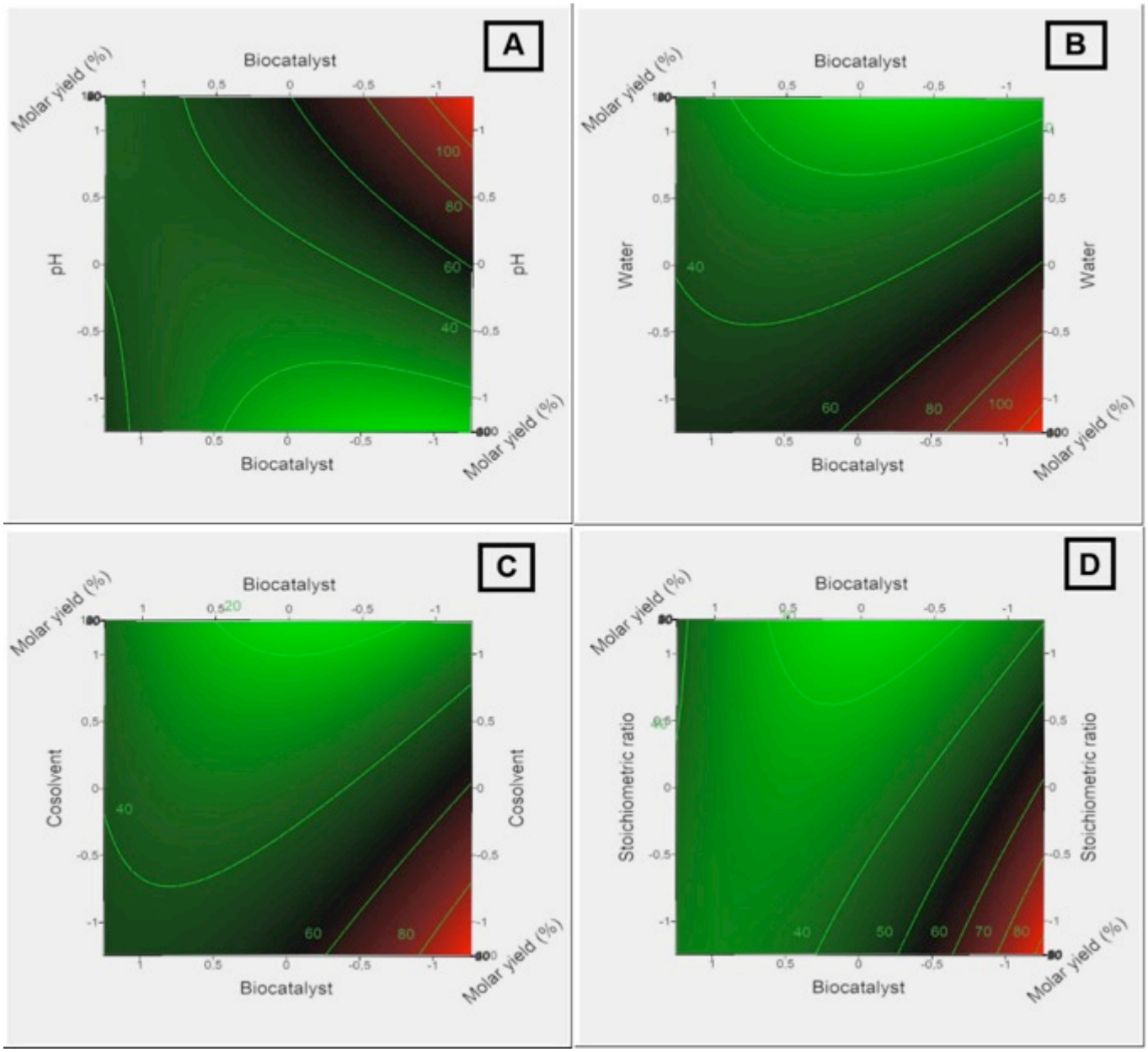


Fig. (4). Response surface for FAME molar yield (%) by LLS and PPL (coded variables -1 and 1 respectively) and interactions between each catalyst with: **A)** pH, **B**) water, **C**) co-solvent and **D**) stoichiometric ratio.

The response surface between the co-solvent/catalyst showed that reducing the co-solvent content caused an increased yield, showing the highest response with LLS (Fig. **4C**).

The interaction between the stoichiometric ratio and the catalyst implies an increased response for the LLS when decreasing the stoichiometric ratio. PPL remained unaffected (Fig. **4D**).

Nevertheless, to avoid any possible influence by atypical data points as described by Gutiérrez [16], we analyzed the catalyst interactions using the median instead of the mean. The best response was found with PPL at pH 8 (35.5%, Fig. **5A**). Additionally, we found that pH 10 has a negative individual effect with a higher impact on LLS (Fig. **5A**). Gao *et al.* [19] explains that this effect is undesirable because the typical alkali transesterification is promoted by high pH, avoiding the biocatalysis.

The median analysis showed that increasing the stoichiometric ratio from 1:140 to 1:170 with LLS increased FAME yield (15.77% and 25.29% respectively). PPL presented the highest molar yield and remained unaffected by the increase of stoichiometric ratio (31.83% and 33.34% respectively, Fig. **5B**). This behavior was also observed for PPL during the interaction between the co-solvent and biocatalyst (Fig. **5C**), indicating that PPL could be resistant to organic solvents, such as methanol and hexane.

The interaction between the stoichiometric ratio and co-solvent shows that reducing the hexane content to 10% versus oil with a stoichiometric ratio of 1:140 promotes higher FAME production (Fig. **6A**).

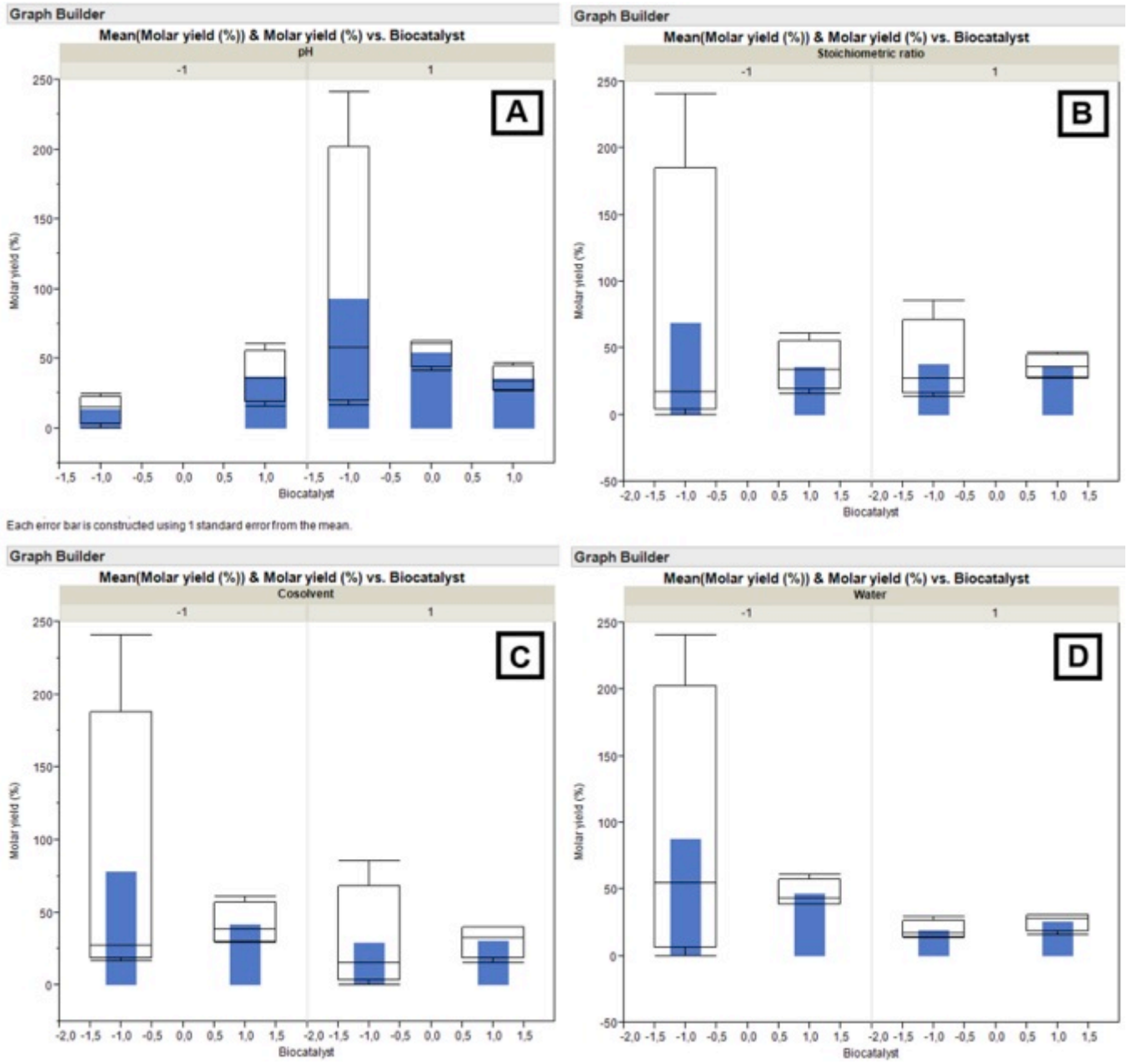


Fig. (5). Bars and boxplots for the catalyst interactions with: **A**) pH, **B**) stoichiometric ratio, **C**) co-solvent, and **D**) amount of water.

The interaction between co-solvent and water amount showed the best response when the amount of water and co-solvent was decreased (5% and 10%, respectively, Fig. **6B**). Stoichiometric ratio and water content interaction showed an increase of FAME production after reducing the value of both variables. The best results were achieved with a 1:140 stoichiometric ratio and 5% water content (Fig. **6C**). Otherwise, the interaction between stoichiometric relation and pH showed an increased response with pH 10. However, the molar yield increased with lower stoichiometric ratio (1:140, Fig. **6D**).

Finally, the best response was achieved with PPL and 5% of water, 10% of hexane, stoichiometric relation oil-methanol 1:140 and pH 10.

The median analysis showed a deviation caused by atypical data. As a result, PPL showed a higher response than LLS (Fig. **5**). PPL data were more homogeneous, and the partial purification process improved FAMEs yield during lipase biocatalysis. LLS average performance was improved by approximately 8-fold, from 2.41% to 20.61% FAMEs molar yield. On the other hand, PPL exhibited the highest response with 34.68% FAMEs molar yield. Therefore, a minimum purification step is desirable for a more efficient process and for future industrial scaling.

DISCUSSION

Surface response methodology has proved suitable for optimizing processes that involve numerous variables. This methodology analyzes first order interactions and enables the combination of the best levels of each variable [16]. Previous research on transesterification conducted by Gao *et al.* [19],

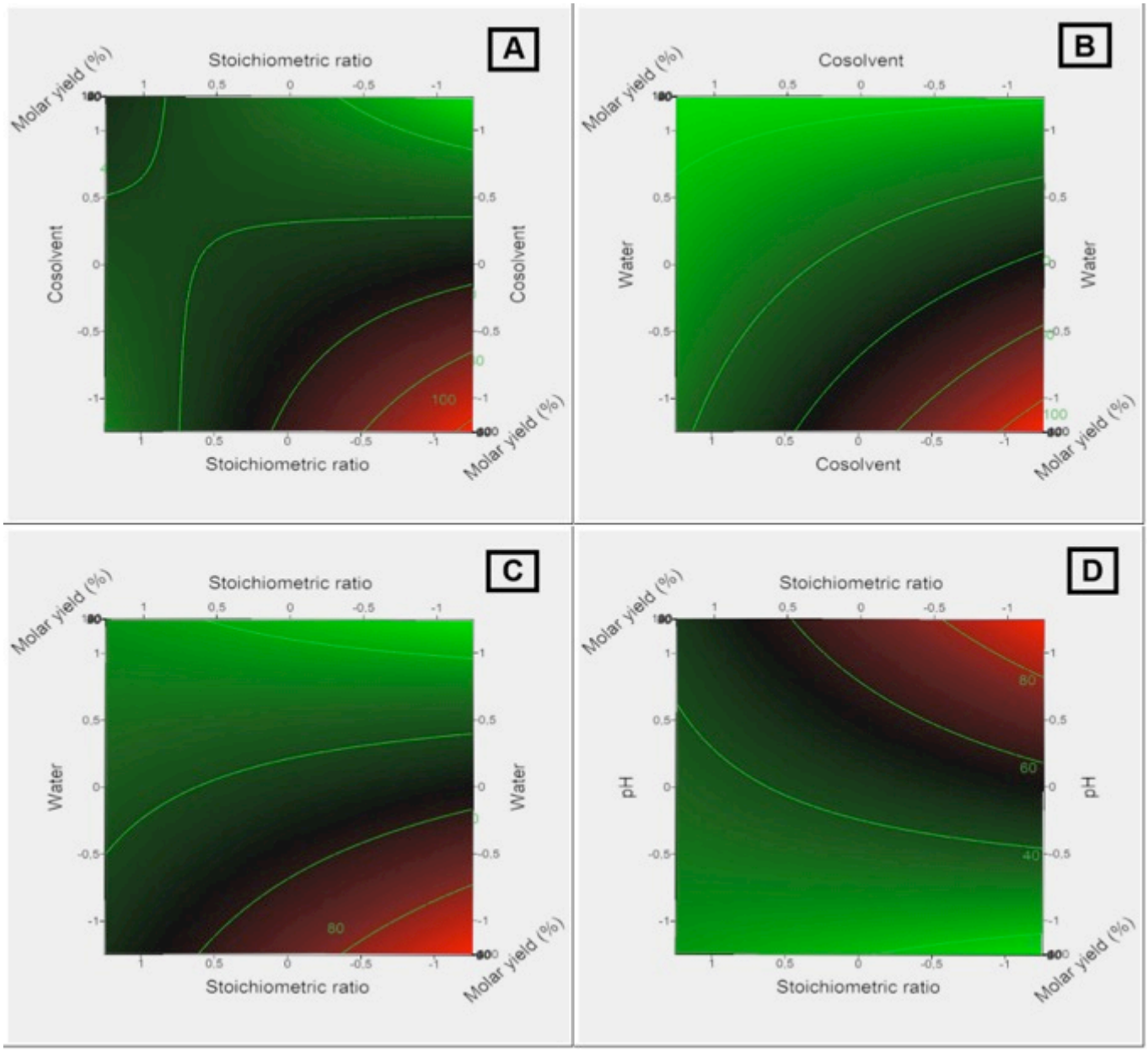


Fig. (6). Response surface plots. **A**) Stoichiometric ratio and co-solvent. **B**) Water amount and co-solvent. **C**) Water amount and stoichiometric ratio. **D**) pH and stoichiometric ratio.

Rodrigues [20], Shie *et al.* [21] and Sim *et al*. [22], with different kinds of catalysts showed excellent results using this methodology. However, possible mean deviations due to atypical data points have not been analyzed yet. When data are uniformly distributed, mean and median may be near each other [14]. When atypical points appear, median analysis avoid deviations and is more reliable than mean, evidencing a real data tendency. In this work, an atypical observation was identified giving a mean slant in the response surface analysis for biocatalyst interactions plots, due to the fact that biocatalyst was a nominal variable. However, this slant does not affect the numerical variables.

According to the reaction media, lipases can catalyze reactions such as hydrolysis, inter-esterification and alcoholysis. The enzyme activity changes depending on the amount of water or solvent in the surroundings of the protein. In non-aqueous media, the water amount has strong influence on the stability and catalytic activity of any lipase. Consequently, some amount of water is required to retain its activity in organic solvents, but an excess of water might influence the equilibrium in a transesterification reaction towards hydrolysis. Lipases act at the organic/aqueous interface, and water helps to increase the available interfacial area. In addition, water helps to keep hydrogen bonds and sulfide interactions, aiding in the folding of the protein. As a result, water enhances lipase activity. Therefore, to maximize the enzyme activity, the optimum water content required must be determined. However, the water amount for a certain reaction is dependent on the nature of feedstock, the lipase, the immobilized support and the organic solvent used [23].

In addition, each lipase attains the highest catalytic activity only at its specific temperature and pH due to structural properties from different sources lipases. This might be the reason for showing different activity on different oil substrates. Thus, there is a need for optimizing the process [24].

Previously reported conditions related to the same type of *P. aeruginosa* lipases were similar to results shown in this research. Best responses were achieved at alkaline pH and temperatures within 20°C-70°C, which are proper for mesophilic lipases catalysis [25]. However, the highest reaction temperature in the first assay (58°C) was near the boiling point of methanol (64.7°C), increasing the possibility of biocatalyst denaturation after long periods, due to the high energy of the reaction system [26].

Temperature does not exert an individual effect and has similarity to the behavior trend of different mesophilic lipases reported [25]. This fact confirms that used PPL from *P. aeruginosa* is mesophilic and then, the equilibrium conversion for enzymatic biodiesel production will not be significativelly influenced within the available temperature range. Additionally, an increase of temperature enhances the reaction rate, but too high temperature will decrease the enzyme stability. Besides, low temperature and long time reaction yields are comparable to high temperature and fast reactions [5]. Consequently, interaction between the operational stability of the lipase and the reaction rate of transesterification is a key factor to determine the optimum temperature for enzymatic transesterification [12].

In the final assay, the highest yield was achieved at 54°C after 48 hours, remaining within the working range reported for this type of lipase on Brenda database [27]. In addition, the most efficient lipases produce conversions higher than 90% at temperatures between 30°C and 50°C. Reported reaction times varied from 8 hours for immobilized enzymes to 90 hours for the same free enzymes, depending on the feedstock and alcohol [25]. Moreover, temperature of 55°C as the working temperature for lipases from *P. aeruginosa* has been reported [28, 29]. This temperature showed, for all the cases, the best balance between stability and reaction rate. As result, the highest quantity of FAME was obtained in a constant time of reaction using the whole potential of the enzyme as biocatalyst.

The interaction between the oil**:** methanol ratio/type of oil in the first assay showed the best behavior with the highest methanol content and RBD oil. The purity of the triacyl glycerides influenced the reaction because the phospholipids in the crude oils add to the lipase surface, blocking the active site and causing inhibition. Thus, it was necessary to eliminate phospholipids by additional process to avoid mass transfer problems and improve the reaction yield [22, 30, 31].

This research shows that the lipase is effective under high methanol concentrations. The best conversion during first assay was achieved with a stoichiometric ratio of 1:149 oil: methanol, revealing an unusual behavior. In addition, oil-methanol molar ratio in the final assay was 1:170 for LLS and PPL (median analysis). These ratios are desirable for industrial lipases because they enable the reuse and cost reduction, as proposed by some authors [6, 17, 32, 33]. However, it has drawbacks related to the high costs of the required alcohol. This issue must be carefully analyzed depending on the final use of the enzyme.

The stoichiometric ratio is a crucial variable in transesterification reactions. A lack or excess of alcohol may cause incomplete reactions or inhibit the enzyme. The average recommended stoichiometric ratio is 1:6, including a 3 mol excess of methanol to ensure complete reaction without lipase inhibition due to the fact that catalytic sites are occupied by the insolubilized short chain alcohol molecules [5, 32]. This ratio varies based on the resistance of the lipase (4 to 18 alcohol/oil mol), where the high values represent high resistant lipases as reported by Ana *et al*. [34] and Stamenkovic *et al.* [35]. Another way to enhance the reaction towards FAME production is through a continuous products removal [3]. However, it needs a different experimental assembly (a continuous reaction equipment instead batch reactors).

An enzyme low molecular weight may be attributed to the increase in compactness of the enzyme molecule which led to increase the intermolecular bonding (hydrogen bonding, van der waals and ionic forces). Furthermore, Saranya *et al*. [36] and Lee *et al*. [37] proposed that the increased intermolecular bonding and low molecular weight of the lipase can increase thermal and solvent resistance. Probably, the amino acid composition of PPL used in this research has hydrophobic characteristics. Thereby, this fact might give the tolerance feature exhibited to the lipase. Yet, there is a need for further molecular studies and additional tests.

The lowest amount of water (2%) and the presence of an organic co-solvent in1:1 oil/co-solvent ratio, increased FAME yield as compared to the treatment without co-solvent. In the parallel and final assays, the aqueous phase shows interactions with all the analyzed factors. We found the highest yields with 10% in parallel assay and 5% in the final assay. This finding has been confirmed by other researchers. For example, Kaieda found in a methanolysis reaction catalyzed by a *Pseudomonas cepacia* lipase that reducing the water content facilitates FAME production, while, water in excess shifts the reaction equilibrium towards hydrolysis [17]. Moreover, when the lipase is non-resistant toward the solvent, it needs a minimum amount of water [18, 23, 25]. On the other hand, the low amount of water on the final product makes the process profitable as compared to alkali transesterification. Low water lipase catalysis eliminates the need for glycerol separation; avoid alkali traces, and additional process to eliminate water excess. High water amount accelerates the natural oxidation process and reduces the quality of the final product. Additionally, natural antioxidants of biodiesel such as tocopherols, sterols, and tocotrienols, are destroyed during distillation and purification steps in alkali catalyst reactions [38].

Co-solvent enhances the reaction kinetics due to solubilization of triacylglycerols and methanol in the reaction media forming reverse micelles. Therefore, inhibition by insolubilized methanol is avoided [39, 40]. PPL resistance to high methanol concentration and the activity in presence of hexane, indicate that the lipase retains its activity in organic solvents. According to Gaur *et al*. [15], this property is rare because organic solvents displace the water

molecules needed for protein folding and occupy active sites. Thus, the lipase may lose its catalytic activity. It is also important to consider the free lipases aggregates formation in the presence of co-solvent because the mass transfer process is hindered. This condition could prevent the lipase activity [41].

In the final assay we identified a first order interaction between methanol-oil ratio/pH exhibiting a better FAME conversion at high stoichiometric ratios and pH 8. Murray *et al.* [42] revealed that pH is critical for protein folding and enzyme catalytic activity. In addition, pH 8 was the best for the *P. aeruginosa* lipase, confirming previous research [15, 29, 43]. It is generally accepted that lipases need an specific pH range to conserve their appropriate folding, which will depend on its isoelectric point [42].

In the parallel assay it was observed that the reactor capacity affects the reaction yield. Smaller capacities facilitate interactions between the substrate, lipase and alcohol. The reaction yield has been tested in packed bed reactors [44-46], showing a higher efficiency when the reaction media has low flows through the stationary phase, increasing the contact between catalyst and reactants. Likewise, the enzyme amount influences the yield and the rate of the reaction, due to increased number of enzyme molecules able to react with the substrate [18,22], as explained by Murray *et al.* [42].

Lipase purification positively affects the reaction yield. We carried out a partial purification process that must be improved further according the results. Other researches had reached lipase purification factors of approximately 300, improving their activity approximately 1000-fold compared to the enzyme supernatant [15,28,47]. Also, protein engineering is widely used to improve the lipase production yields and enzymatic activity [48,49].

CONCLUSION

The partially purified lipase obtained from *P. aeruginosa* was isolated directly from palm oil fruit and it transesterified the palm oil triglycerides to FAME, making it suitable for biodiesel production. Each lipase requires individualized process conditions with appropriate control over them to improve the reaction yield. In this case, a partially purified lipase exhibited an improvement of the reaction yield compared to the lyophilized supernatant, producing more than 68% of FAME (34.68% and 20.61% respectively). However, the lipase production and purification process yield must be improved due the protein losses on each step. Protein engineering is needed to improve the enzyme properties, making a profitable biocatalysis process for recommend it as a biocatalyst for oleochemical process and other industrial purposes.

ABBREVIATIONS

CPO = Crude palm oil

GC = Gas chromatography

LLS = Lyophilized lipase supernatant

PPL = Partially purified lipase

RBD = Refined, bleached and deodorized

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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