A USEFUL METHODOLOGY TO SELECT LIPASE-CATALYZED TRANSESTERIFICATION AIMING BIODIESEL APPLICATION

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ABSTRACT

The application of lipases in various fields has been notably increased in the last few decades and qualitative/quantitative improvements need to be done. However, many methodologies of screening are described in order to find a good lipase producer and statistical optimization is a necessary tool to improve lipase production. In this work, an isolation of filamentous fungi lipase producers and a transesterification capacity screening was evaluated. Four fungi were chosen to the transesterification reaction assays and the best fungus selected was submitted to a submerged fermentation. Parameters of the culture medium were optimized using response surface methodology. Selected liquid medium was SR at 30 °C, 72 h, 100 rpm. Corn oil was the best carbon source and together with Tween 80 increased two-fold the lipase activity. After the experimental design, the new medium optimized were 3.5-fold higher than the original liquid medium and was composed by 0.5% corn oil, 0.012% $MgSO_4.7H_2O$, 0.015% KH_2PO_4, 0.05% $NH_4H_2PO_4$. Hence, the lipase produced proved its transesterification capacity and can be used for biodiesel production.

Keywords: Lipase; Transesterification; Biodiesel

UMA METODOLOGIA ÚTIL PARA SELECIONAR LIPASES COM ATIVIDADE DE TRANSESTERIFICAÇÃO VISANDO APLICAÇÃO NA PRODUÇÃO DE BIODIESEL

RESUMO

A aplicação de lipases em vários campos tem aumentado notavelmente nas últimas décadas e melhorias qualitativas/quantitativas precisam ser feitas. Entretanto, a fim de encontrar um bom produtor de lipase muitas metodologias de seleção são descritas, além disso, a otimização estatística é uma ferramenta necessária para melhorar a produção de lipases. Este trabalho apresenta o isolamento de fungos filamentosos produtores de lipase e a avaliação da capacidade de transesterificação dessas enzimas. Quatro fungos foram selecionados para os ensaios de transesterificação e o melhor fungo isolado foi submetido a fermentação submersa. Parâmetros do meio de cultivo foram otimizados usando metodologia de superfície de resposta. O meio de cultivo líquido selecionado foi o SR, a 30 °C, 72 h e 100 rpm. Óleo de milho foi a melhor fonte de carbono e, quando adicionado de Tween 80 aumentou duas vezes a atividade de lipase. Após o planejamento experimental, o novo meio de cultivo otimizado, composto por

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óleo de milho 0,5%, extrato de levedura 0,45%, peptona 0,2%, $MgSO_4.7H_2O$ 0,012%, KH_2PO_4 0,015% e $NH_4H_2PO_4$ 0,05%, foi 3,5 vezes melhor que o original. Dessa forma, a lipase produzida demonstrou capacidade de transesterificação e pode ser utilizada para a produção de biodiesel.

Palavras-chave: Lipase; Transesterificação; Biodiesel

INTRODUCTION

Lipases (E.C. 3.1.1.3) are hydrolases which catalyze the hydrolysis and synthesis of long-chain acylglycerols. Furthermore, they can be used in fat and oil modification by esterification and interesterification and transesterification reactions; and other characteristic properties like substrate specificity, stereospecificity and regiospecificity (JAEGER & EGGERT 2002). These characteristics make then important catalysts in the production of specific fatty acids or glycerides from vegetable oils in biodiesel production via transesterification of oil and fat with alcohols (methanol and ethanol) (FACCHINI et al., 2013). These enzymes can be secreted by several microorganisms and can be easily found in several habitats, mainly when contaminated with oil. Thus, they stimulate the biodiversity exploitation for searching new microorganisms able to produce these enzymes (FACCHINI et al., 2013).

Fungal lipase productions have been described by many genera with desirable properties and potential applications in a number of different areas. *Fusarium oxyporum* is well-known specie as good lipase producer (PRAZERES et al., 2006) and improvements on its production could be an important tool to optimize its application (KAUSHIK et al., 2010).

Screening is a key step in directed evolution methods and an important issue for developing better biocatalysts. Due to the wide range of applications, researchers have made attempts to isolate lipase producing fungi. The study of isolation and screening enlarges the number of species that potentially produce lipases, contributing to the development of innovative commercial strategies, once these new species can present different temperatures and pH ranges, stabilities, specificity to certain fatty acids, and enantioselectivity (GUPTA et al., 2003).

Interesting on biodiesel production has incentivized researchers to develop methodologies in order to determine both esterification and transesterification activities of lipolytic microorganisms. Methods based on gas chromatography, high-performance liquid chromatography, or titrimetric determination has been proposed. However, when these methods are used to screen a large numbers of lipases with transesterification capacity, generally is necessary to determine the best reaction conditions and it easily can lead to several hundred individual experiments becoming extremely time-consuming and expensive. Thus, a simple and practical tool to determine the transesterification activity has been proposed and could be used to evaluate the lipase synthetic activity in organic solvent (TENG & XU 2007; GOUJARD et al., 2009). In this regard, the purpose of this work was the isolation and screening of lipase producer fungi and, consequently, a screening in three steps of these selected lipolytic fungi to decide on the best lipase catalyzed-transesterification capacity. Furthermore, this work was directed to produce lipase from *Fusarium oxysporum*, aiming to find different catalytic properties of commercial interest as biodiesel production.

MATERIAL AND METHODS

Filamentous fungi isolates

The fungi were isolated from several sources as soil, grease traps, recycling lubricating oil industry and buriti (*Mauritia flexuosa*) seeds decomposing. The isolation and cultivation was carried out on Petri dishes using the oatmeal agar medium, plus 10 mg/mL of ampicillin, at 30 °C. The cultures were maintained at 4 °C. The chosen strain was identified as *Fusarium oxysporum* Shiltdl by Universidade Federal de Pernambuco (UFP), Pernambuco, Brazil.

Screening of lipolytic microorganisms

Firstly, microorganisms that produce lipases were pre-selected, using solid medium constituted by 0.001% Rhodamine B, 2% agar, 0.0001% Tween 80 and 1% olive oil, pH 5.5 (Mc Ilvaine buffer) and pH 8.0 (Tris-HCl buffer) (SHELLEY et al., 1987). Culture plates were incubated at 30 °C, for 144-168 h. The fluorescent halos were evidenced when irradiated at 350 nm, and the diameter of the colonies (d) and total clear hydrolytic halos (D), including the colonies, were determined. The strains that yielded higher halos (D-d) were selected as potential microorganisms for lipase production. Designated filamentous fungi were submitted to a submerged fermentation in SR (RIZZATTI et al., 2001) liquid medium supplied with 0.05% glucose and 1% olive oil, at 30° C, 100 rpm. For each fungus, aliquots were taken in different times (72h, 96h and 120 h) to determine the best time of production of lipase.

Hydrolytic lipase activity and protein determination

The extracellular lipase activity was assayed using *p*-nitrophenyl palmitate (pNPP) as substrate (PENCREACH, 1996). The enzyme was incubated in Mc Ilvaine buffer (pH 5.0) or Tris-HCl 0.5M, pH 8.0 and Triton X-100 (0.4%, w/v) at 40 °C, using as substrate pNPP (7.9 mM, dissolved in isopropanol). The absorbance was measured at 410 nm. All experiments were done in triplicates. One unit of lipase activity is defined as the release of 1 µmol of *p*-nitrophenol (pNP) per min, in the assay conditions. Protein content was estimated by the Bradford method (BRADFORD, 1976) using bovine serum albumin as standard.

Screening of lipases-catalyzed transesterification

Transesterification reaction: The enzymatic transesterification was carried out in a non-aqueous medium, with a molar ratio of 1:9 (oil:ethanol/methanol), in a final volume of 20 mL, including 6% n-hexane and 4% lyophilized lipase from crude extracts. Ethanol was added in two steps to prevent any interference in the enzyme activity. All experiments were performed in triplicates and at 40 °C, 150 rpm for 24 h.

Thin layer chromatography analysis (TLC): The transesterification results were obtained by analyzing the ester released by TLC. The reaction carried out on silica gel G-60 plates (Merk). The solvent consisted in a mixture of n-hexane: ethyl acetate: acetic acid (90:10:1). Chromatograms were revealed in an iodine vapor atmosphere (SHAH et al. 2004).

Transesterification assay by gas chromatography (CG-MS): The products of enzymatic transesterification samples were diluted in 1% methanol and analyzed using an HP gas chromatograph, model GC-2012 5890, equipped with a Shimadzu column (model BP-1), 30 m length and 0.25 mm internal diameter. The mobile phase consisted of helium gas flow (1.47 mL/minute). An injector temperature of 200 °C was employed, and analysis was accomplished by using a temperature ramp from 80 to 200 °C. The mass spectra of the main chromatographic peaks was monitored in a mass spectrometer model GCMS-QP 2010 Plus, which was coupled to the chromatograph. Standard curves were performed using ethyl oleate with 23

min of retention time. All the esters produced between 23 and 26 min were considered as lipase product.

Colorimetric transesterification-based assay: The spectrophotometric method was performed according to TENG & XU (2007) with modifications. 0.1 g of each lyophilized crude extract (approximately 60 U/g of hydrolytic activity) was mixed with 10 mL of pNPP stock solution (10 mM in n-hexane). The mixture was incubated at 40 °C, with 60 μL of anhydrous methanol to start the enzyme reaction. After 1h, the reaction was stopped with 1 mL of 0.1 M NaOH. The released pNP was detected at 410 nm. One unit of enzyme activity was defined as the release of 1μmol of pNP, per minute, per gram of enzyme (U/g). All reactions were performed in triplicate.

Optimization of fermentation parameters on *Fusarium oxysporum* lipase production

Cultures were carried out in Erlenmeyer flasks (150 mL) containing 1% soy oil and 25 mL of liquid media: SR (RIZZATTI et al., 2001); Khanna (KHANNA et al., 1995); Adams (ADAMS, 1990) or Czapek (WISEMAN, 1975), at 30°C, 100 rpm. Aliquots were taken each 24h and the lipase activity analyzed. The effect of 1% vegetable oils as olive, canola, soy, corn, palm, pequi, castor, nim, sunflower, macauba pulp and sesame oils; 0.25% surfactants as Tween 80, Tween 20, PEG 4000, Triton X-100, SDS, PEG 8000 and glycerin were also studied.

Experimental design and optimization by response surface methodology (RSM)

In order to determine the optimal process conditions for the enzyme production, a two-level full factorial design with four center points were employed. The independent variables investigated (peptone, corn oil and yeast extract concentrations), in the two-level full factorial, are present in Table 1.

Table 1. Coded and actual values of the factors for two-level factorial design

Experimental factor (%)	Coded levels		
	-1	0	1
Peptone	0	0.2	0.4
Yeast extract	0	0.45	0.9
Corn oil	0.5	1.0	1.5

A second-order polynomial equation (Eq. 1) was generated to determine the predicted response: (Eq. 1) $Activity = \beta_0 + \beta_1 P + \beta_2 Y + \beta_3 C + \beta_{12} PY + \beta_{13} PC + \beta_{23} YC + \beta_{123} PYC$

Where: the activity was the predicted response; B0 was the intercept; B1, B2, B3 were the linear coefficients; B11, B12, B23 were the squared coefficients; B123, interaction coefficients with P, Y, and C corresponding to the main factors: peptone, yeast extract and corn oil, respectively.

Analysis of data

The analysis of variance (ANOVA) and surface response was obtained by the Statistica 7.0 software (Stat Soft Inc., OK, USA). The model was evaluated through the analysis of *F*-test and R^2 with significance at 5% ($p<0.05$). The equation obtained by the model was used to determine the optimum conditions of activity.

RESULTS AND DISCUSSION

Screening of lipolytic producers

A total of 33 fungi were isolated from different habitats. Each one received a code according to the local where they were collected. The screening on agar plate with Rhodamine B (Figure 1) presented 12 fungi with halos in both acid (pH 5.5) and alkaline medium (pH 8.0), 10 fungi in acidic medium and 4 fungi in alkaline medium (Figure 2). The detection of halos generated by the enzymatic hydrolysis is an easy and valuable method to make lipase screening and can be done using Rhodamine or other dye as Victoria Blue (HASAN et al., 2009; AKBARI et al., 2011). The fungi grew up better in alkaline medium than in acidic medium, consequently the halos measured needed to have different scales. Therefore, were selected for the next step only fungi that presented halos D-d $\geq$ 0.5 cm (acid medium) and $\geq$ 1.0 cm (alkaline medium), in order to select the best producers.

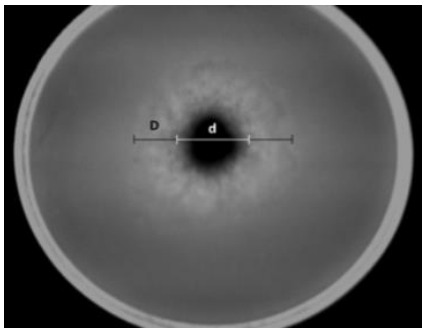

Figure 1. Fungi were grown on Rhodamine plates. D = halo diameter; d = mycelium diameter

A total of 21 fungi – including other fungi from our collection, well known as lipase producers (PEREIRA et al., 2014) – were submitted to cultivation in liquid medium order to quantify the lipase activity (Table 2). Consequently, according to the higher activities obtained 6 fungi were selected in order to verify the transesterification capacity.

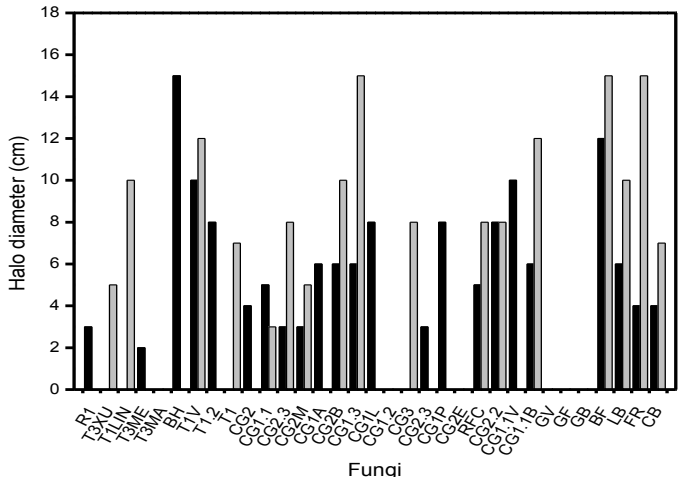

Figure 2. Halo diameters (D-d) produced by the isolated fungi in Rhodamine plates. Symbols: Black bars, acid lipase; gray bars, alkaline lipase.

Transesterification screening

A prior test on TLC, was done to check if any of the fungi lipases were able to promote the transesterification (Figure 3). Although TLC is a qualitative analysis, all fungi have shown transesterification activities and better results were obtained when ethanol was used, instead of methanol, in the reaction.

Table 2. Lipase activity determined from cultures incubated by different times

		Lipase activity (U/mL)	
Fungi	**Time (h)**	**Acid**	**Alkaline**
T1LIN	96	Nd	0.103
BH	72	0.065	Nd
T1V	72	0.015	0.180
T1.2	96	0.084	Nd
CG2B	72	Nd	0.101
CG1.3	72	Nd	0.394
CG1L	72	0.400	Nd
CG1P	120	Nd	Nd
CG 2.2	72	0.249	Nd
CG1.1B	72	Nd	0.311
BF	120	0.060	0.045
FR	72	Nd	0.724
LB	72	0.233	0.234
CG1.1V	71	0.020	Nd
Aspergillus phoenicis	96	0.410	Nd
Fusarium oxysporum	72	0.450	Nd
Aspergillus terréus	72	0.102	Nd
Aspergillus. nidulans var *cristatus*	96	0.257	Nd
Aspergillus japonicus	96	0.174	Nd
Aspergillus niger	96	0.163	Nd
Fusarium verticillioides	96	1.800	0.779

*Nd = not detected.

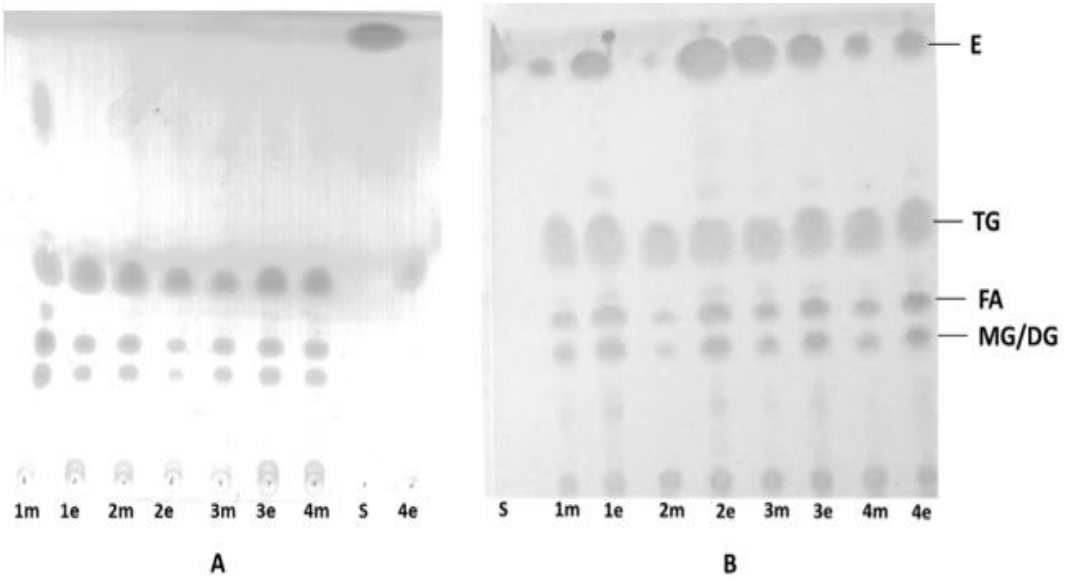

Figure 3. TLC of the selected fungi; (A) control samples, (B) samples treated with lipases from different fungi. S = ethyl oleate standard; 1= *F. oxysporum*; 2= *F. verticillioides*, 3= *A. phoenicis*; 4= FR (unidentified strain), E = methyl or ethyl esters, TG= triacylglycerol, FA= fatty acids, DG= diacylglycerol, MG= monoacylglycerol. The lower case letters: "m"- methanol; "e"- ethanol.

The second step was the analysis by GC-MS of the final product formed, the presence of ester on the reaction. The CG-MS was performed in order to qualitatively corroborate the transesterification capacity and select some fungi, because the lipase extracts were not pure and many other compounds could be detected. Therefore, the aim of this analysis was to identify any vestige of ester in the product. Esters were detected in all four selected fungi in the end product.

After the confirmation of the transesterification capacity, it was interesting to test this transesterification capacity quantitatively. For that, a spectrophotometric method was performed. *F. verticillioides* and *F. oxysporum* had the best activity, followed by *A. phoenicis* and FR (Table 3). However, due to the low results presented by *F. verticillioides*, when methanol was used as alcohol in the transesterification reaction, *F. oxysporum* was selected as the best fungi and some fermentation parameters were improved for its better application in biodiesel production. GOUJARD et al. (2009) showed that this method was effective and is in accordance with the different levels of transesterification activity measured among commercial lipases, though the immobilized lipase showed better results than the crude extract. According to these authors, the lipase from *F. oxysporum* showed higher transesterification activity (0.577 U/g) than the crude enzymatic extract from *Carica papaya* (0.1 U/g) and *Ananas comosus* (0.001 U/g).

Table 3. Transesterification activity of selected fungi by the colorimetric method

Fungi	Activity (U/g)
Aspergillus phoenicis	0.376
FR	0.341
Fusarium oxysporum	0.577
Fusarium verticillioides	0.598

Lipase production by *F. oxysporum*

Different liquid media were initially tested varying from 48 to 144 h of cultivation (Figure 4). SR and Khanna presented higher activities in 72 h and 96 h, respectively, possibly due to their rich composition including higher concentration of organic nitrogen source (SR medium) and elevated concentration of many salts presented in both media. However, due to its poor composition, the lipase production in Czapek medium was weak. These results agree with CAMARGO-DE-MORAIS et al. (2003) obtaining 72h as the best production time, for the same fungi specie, *F. oxysporum*. On other hand, some authors obtained lipase activity only after 5 days of cultivation (ABBAS et al., 2002; ACIKEL et al., 2011; RAPP, 1995). Investigations of the carbon source effects have been done in order to find good lipase inducers and enhance lipase production (PRAZERES et al., 2006; ANDRADE et al., 2013). RAPP et al. (1995) mentioned the use of olive oil added to glucose to produce lipases by *F. oxysporum*. Lipase production using vegetable oils by same strain was published after, but low activity was found (CAMARGO-DE-MORAIS et al., 2003) or the oil presence did not affect the lipase production (ABBAS et al., 2002). In contrast, the presence of oil was sufficient to promote the lipase production (LIMA et al., 2003).

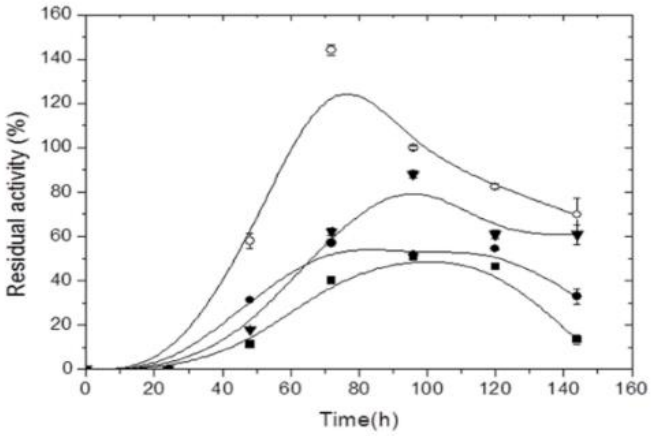

Figure 4. Effect of the composition of different culture media on lipase production. Symbols: (▼) Khanna; (●) Adams; (■) Czapek and (○) SR media.

Many types of oils and surfactants were tested to determine their effect on the growth of *F. oxysporum* and lipase production (Figure 5A). As result, the lipase levels from corn oil medium was three fold higher than olive, palm and canola oils, and soy oil has the second best activity. *Rhizopus delemar* lipase had a maximum activity when sunflower oil was included in the fermentation medium (ACIKEL et al. 2011). WANG et al. (2008) and LIMA et al. (2003) observed that the effect of olive oil was the best on the growth and lipase activity. The presence of surface-active materials can stimulates the release of the enzyme. They could affect the permeability of the cell, increasing the secretion of lipase, or have a surfactant effect on cell-bound lipase (ACIKEL et al., 2011). In this context, surfactants were added to the medium as inducers. Figure 5B shows that the presence of Tween 80, Tween 20 and glycerin enhanced the lipase production. Tween 80 was an excellent lipase inducer and had a triple effect on lipase activity when compared to the control. Tween 20 enhanced 2.5-fold the lipase production. It is miscible with water and can be used as a carbon source, did not inhibiting the fungal growth. PEG 8000 and 4000, SDS and Triton X-100 discourage the lipase

production. ACIKEL et al. (2011) described that variation of Tween 80 concentration enhanced the lipase activity of *R. delemar* about two-fold. Span-80 (Sorbitan oleate) had the best results enhancing the lipase activity from *R. chinensis* (WANG et al., 2008).

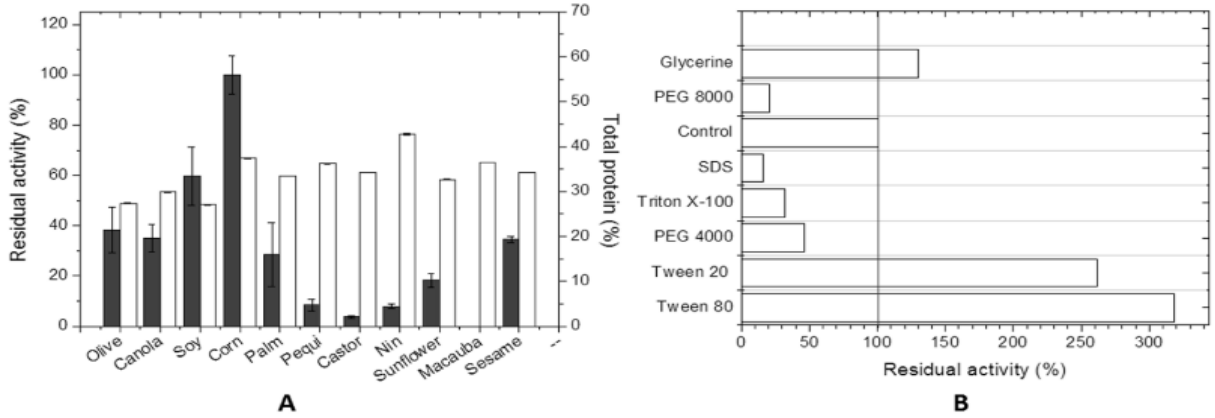

Figure 5. Effects of (A) carbon source and (B) surfactant in the growth and lipase production of *F. oxysporum*. Symbols (A): black columns = lipase activity; gray columns = total protein.

Optimization of medium composition using RSM

The optimum concentrations and the interaction of yeast extract, peptone and corn oil were studied by experimental design and RSM. Table 4 shows the lipase activity of the experiment for each individual test, along with the predicted responses of two-level factorial design. The lipase production varied from 0.3 to 17.1 U, depending on the medium composition after 72h fermentation. The maximum activity was achieved with 0.5% corn oil and absence of yeast extract and peptone, at 30°C, for 96h and 100 rpm.

Table 4. Experimental design and results

	Coded variables			Activity
Test	**Y**	**P**	**C**	**(U)**
1	-1 (0)	-1 (0)	-1 (0.5)	17.1
2	-1 (0)	-1 (0)	+1 (1.5)	5.5
3	-1 (0)	+1 (0.4)	-1 (0.5)	1.1
4	-1 (0)	+1 (0.4)	+1 (1.5)	5.2
5	+1 (0.9)	-1 (0)	-1 (0.5)	2.5
6	+1 (0.9)	-1 (0)	+1 (1.5)	0.3
7	+1 (0.9)	+1 (0.4)	-1 (0.5)	16.5
8	+1 (0.9)	+1 (0.4)	+1 (1.5)	4.1
9	0 (0.45)	0 (0.2)	0 (1.0)	5.2
10	0 (0.45)	0 (0.2)	0 (1.0)	5.1
11	0 (0.45)	0 (0.2)	0 (1.0)	5.0
12	0 (0.45)	0 (0.2)	0 (1.0)	4.5

Then, a regression analysis (Table 5) was carried out to fit the mathematical model to the experimental data. The predicted model, the significant factors and the interaction effects could be described by second-order polynomial equation (Eq. 2):

(Eq. 2) Lipase activity (U) = 6.52 -2.77(C) +4.26(YP) -3.24(CYP)

The results indicated that corn oil was significant ($p>0.05$) and in low concentration enhanced the lipase activity; while the interaction between yeast extract and peptone had a positive effect in lipase activity. This linear model indicated that $F_{calc}>F_{list}$, with a regression coefficient (R^2 = 0.9528), suggesting a good predictive capacity and was statistically valid.

Table 5. Analysis of variance for 2^2 factorial design for the lipase production

Source of variation	Sum square	Degrees of freedom	Mean square	F_{calc}	F_{list}
Model	289.51	7	43.51	11.5	6.09
Residual	15.07	4	3.77		
Total	304.58	11			

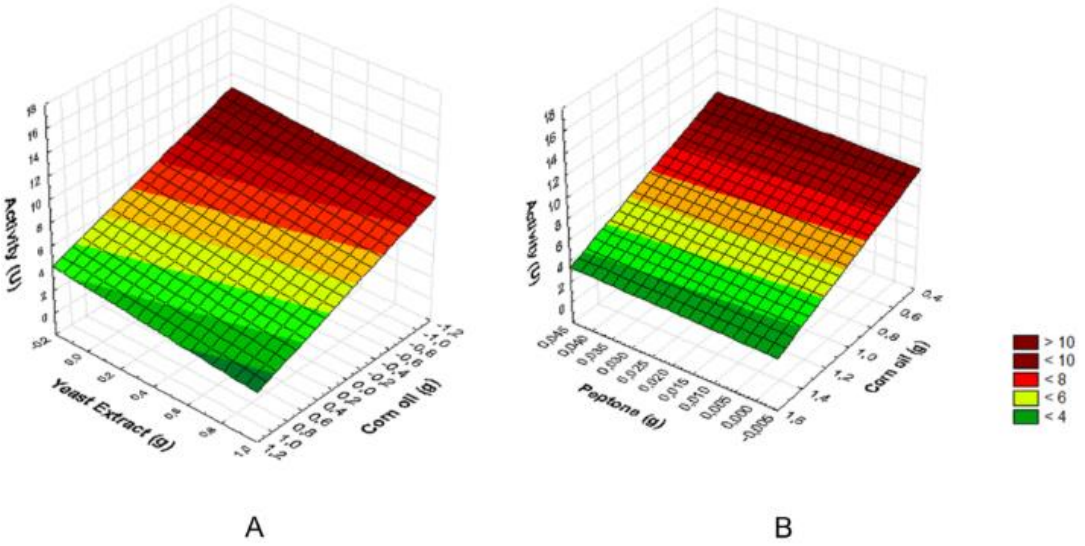

Figure 6. Response surface of lipase production by *F. oxysporum*. Interaction between (A) yeast extract and corn oil; (B) peptone and corn oil

The correspondent response surface plots clearly confirmed the influence of corn oil on lipase production (Figure 6). However, the interaction between yeast extract and peptone also had influence on lipase production. It could be verified in experiment 7, Table 4. For that reason is acceptable include this two component on lipase production in its lower concentration, as in central points. Therefore, a new SR standardized medium was purposed to lipase production by *F. oxysporum* using 0.5% corn oil, 0.45% yeast extract, 0.2% peptone and the salt mixture present in SR medium (0.012 % $MgSO_4.7H_2O$, 0.015 % KH_2PO_4, 0.05 % $NH_4H_2PO_4$). This new medium was approximately 3.5-fold as against the yield obtained by the original medium and decreases the total cost of enzyme production due to the yeast extract and peptone are not present. KAUSHIK et al. (2010) obtained almost 2.8-fold increase in lipase activity with the optimized medium (1.5% corn oil; 0.3% sodium nitrate; 0.05% casein, at 200 rpm, for 96h). Optimal enzyme production was also obtained by experimental design and RSM when soybean meal was used as substrate (LIU & ZHANG, 2011).

CONCLUSIONS

The three steps were essential to find a good producer of lipase-catalyzed transesterification and was a simple and complete methodology of screening but colorimetric method was a key to define the best producer. The initial lipase screening was also interesting in order to find acid or alkaline lipases once depending on the application, alkaline lipases are required. Corn oil was the most significant carbon source and the presence of Tween 80 notably enhanced the lipase production. The two-level full factorial model was a useful tool employed to predict the combined effects of cultivation conditions on the lipase production followed by RSM. The maximum activity was obtained with the optimized medium (0.5% corn oil, 0.45% yeast extract, 0.2% peptone and SR salt mixture). The model was valid and proved to be suitable in predicting the optimum fermentation conditions. This lipase can successfully be used for biodiesel production.

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