High Lipase Production from *Geotrichum candidum* in Reduced Time using Cottonseed Oil: Optimization, Easy Purification and Specificity Characterization

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Abstract: Despite the wide variety of potential applications of lipases within industrial processes, the high cost of production and purification is still their main limiting factor. The aim of this work is to optimize the production of *Geotrichum candidum* lipase (GCL) using submerged fermentation with a combination of statistical experimental design and surface methodology analysis, in order to give a higher production within a shorter time at the lowest possible cost and easy purification. Cottonseed oil, a low-cost by-product of cotton processing, was used as both an inducer and a carbon source. A maximum lipase activity of 27.17 IU mL^{-1} was achieved after 30h fermentation in a 5L stirred tank bioreactor under optimal conditions: 2.3% (m/v) of casein peptone, 0.8% (v/v) of cottonseed oil and 0.05% (m/v) of $MgSO_4$ and $NaNO_3$. The lipase purification in a single step by immobilization on PHB particles was verified. The combination of these two steps allowed a significant decrease in this lipase cost of production. Moreover, the produced lipase showed high specificity to hydrolyze long-chain fatty acids with cis-9 double bonds, such as oleic and linoleic acids, having an excellent potential for modifying oils in order to produce different bio-products in industrial applications.

Keywords: Fermentation, optimization, lipases, bioproducts, bioprocessing.

1. INTRODUCTION

The application of enzymes in industrial processes has advanced significantly in the recent years. Characteristics, such as high catalytic efficiency, specificity and the ability to accelerate specific chemical reactions without the formation of undesirable by-products, are aspects that have contributed to accelerating the use of these biocatalysts in different industrial sectors [1]. The promising application of lipases in industrial processes is due to their versatility in catalyzing reactions in aqueous (hydrolysis) and organic (esterification, transesterification and interesterification) media. Moreover, many lipases have shown high specificity, providing products that could have not been obtained by conventional chemical processes. This enzyme has been widely applied in the modification of oils and fats, the synthesis of organic compounds, detergents, polymers, biofuels and supplements, as well as in analytical procedures [2].

However, the high cost of lipases is still the main obstacle for exploiting its potential in the industrial sector. The cost value of any enzyme is determined by the prices of the raw materials used in its production, the produced amount and the purification process [3, 4]. In order to decrease these enzymes' costs of production, many studies reported in literature have suggested the replacement of synthetic materials by complex media, including by-products and wastes from the agro-industrial sector [4, 5]. Although these materials are cheaper than the synthetic media, they could hinder the purification process of the enzyme, requiring the addition of several steps for purification. Usually, the purification step contributes substantially to the overall cost of obtaining an enzyme.

Therefore, the replacement of the components in the production medium, by low-cost materials, should provide a saving in the lipase production, without hampering or endearing the cost of the subsequent purification process. Thus, a combined strategy of using a cheaper inducer and carbon source (e.g. cottonseed oil), with a simple purification process, could significantly reduce the cost of lipase production [4, 6].

Geotrichum candidum (G. *candidum*) has been described as a microorganism able to produce different

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lipases in the presence of an inducer in culture medium, such as triacylglycerols, especially olive oil [7]. These lipases exhibit high specificity towards different fatty acids. High specificity is very interesting for industrial applications such as the specific modification of engineering-triacylglycerols, the production of useful fatty acids or the resolution of racemic mixtures [8, 9].

In a previous study, performed in our laboratory, we reported the production of a lipase from *G. candidum* (GCL) in stirred flasks. This enzyme was also purified by immobilization on PHB particles and it was used to catalyze ethyl linoleate synthesis in a heptane medium [10]. In the present study, we focused on the optimization of the lipase production, using experimental designs, aiming to obtain a high enzyme production with the lowest possible cost, in a 5L stirred tank bioreactor, but allowing easy purification of the culture medium through the immobilization method. This study also included the substrate specificity's characterization of the produced lipase, through its application in the hydrolysis of vegetable oils with different fatty acid compositions (olive and babassu oils).

2. MATERIALS AND METHODS

2.1. Microorganism

G. candidum NRRL Y-552 was obtained from André Tosello Foundation, Campinas, Brazil. A colony with a diameter of 5mm was removed from Sabouraud dextrose agar plate and inoculated in a 1000mL shaker flask containing 100mL of inoculum medium at 30°C and at 250rpm for 24h. The inoculum medium was composed of 2% (m/v) casein peptone, 0.1% (m/v) yeast extract, 0.05% (m/v) of $MgSO_4$ and $NaNO_3$ and 1% (v/v) cottonseed oil for all the performed experiments [10].

2.2. Culture Medium Component Evaluation

2.2.1. Plackett-Burman Design (PB Design)

The culture medium containing cottonseed oil, as both inducer and carbon source, was evaluated by the PB design with twelve assays and three central points. The fermentation assays were performed for 60h and the samples were collected in 12h intervals. The activity of the produced lipase was used as a response of the PB design. All experiments were performed in triplicate.

2.2.2. Factorial Design

The culture medium previously evaluated by PB design was optimized by the central composite rotatable design (CCRD). A 2^2 full factorial design, with tree assays in the central point and four-star points totalizing eleven assays, was employed. The levels of each independent variable were chosen based on the results obtained from the PB design experiments. The parameters and their levels were peptone (1-3% m/v) and cottonseed oil (0.5-2.5% v/v). The concentrations of $MgSO_4$ and $NaNO_3$ were fixed at 0.05% (m/v) and the fermentations were carried out for 48h.

Lipase activity was used as a response of the factorial design. The results were analysed using Statistica version 5.0 software to fit the following second-order polynomial, as shown:

$$Y = b_0 + \sum b_i x_i + \sum b_{ii} x_i^2 + \sum \sum b_{ij} x_i x_j + \varepsilon$$

where: Y is the response variable, b_0, b_i, b_{ii} and b_{ij} are the constant coefficients, x_i and x_j are the coded independent variables and ε is the random error.

2.3. Lipase Production in a Bioreactor

In order to validate the optimization made by CCRD, a fermentation assay was carried out for 60h in a 5L stirred tank fermenter (BIOFLO 310-Newbrunswick) equipped with two Rushton impellers and containing 4L of the optimized medium: 2.3% (m/v) of peptone, 0.8% (v/v) of cottonseed oil, 0.05% (m/v) of $MgSO_4$ and $NaNO_3$ at initial pH of 7.0 and 30°C. The agitation speed and specific gas flow rate were 300rpm and 1vvm respectively [11]. The samples were collected in intervals of 6h, in order to determine lipase activity, biomass concentration and depletion of carbon source.

2.4. Purification of the *G. candidum* Lipase by Immobilization on PHB

The purification capacity of the produced lipases from fermented broth, in just one step, was carried out immobilizing the lipase on hydrophobic support, polyhydroxybutyrate (PHB) particles, *via* physical adsorption [6, 10]. Five mL of crude extract (fermented broth without cells) was added to 1g of PHB, previously incubated with 5mL ethanol (95% m/m) [10]. The suspensions were kept under continuous agitation in an orbital shaker (200rpm), at room temperature for 12h. The support with the adsorbed lipase was filtered (What man filter paper 41) under vacuum and washed with distilled water (volume ratio 1:5). The prepared

biocatalyst was subsequently incubated in 1% (m/v) Triton X-100 for 2h. The supernatant of the immobilization and the surfactant solution were analyzed by electrophoresis.

2.5. Analytical Methods

2.5.1. Hydrolytic Activity

The fermentative broth was filtered (Whatman filter paper) under vacuum and the resulting supernatant was used as crude lipase extract (GCL). The lipase activity in this crude extract was assayed in the hydrolysis of olive oil emulsion [12]. The substrate was prepared by mixing 50mL of olive oil with 50mL of Arabic gum solution (7% m/v). The reaction mixture containing 5mL of the emulsion, 4mL of 100mM sodium phosphate buffer at pH 7.0 and 1mL of GLC was incubated for 5min at 37°C under continuous agitation in an orbital shaker (200rpm). The reaction was stopped by adding 15mL of acetone and ethanol mixture (1:1 v/v). The liberated fatty acids were titrated with standard 20mM sodium hydroxide solution in the presence of phenolphthalein as indicator. The reaction blanks were made by adding 1mL of culture medium. One international unit (IU) of activity was defined as the amount of enzyme required to liberate 1µmol of FFA per minute under the experimental conditions described above.

2.5.2. Substrate Specificity

The free fatty acids (FFA) liberated from babassu and olive oils through the hydrolysis reactions catalyzed by produced GCL, were separated and analyzed by methodology described by Borgstrom, Belfrage and Vaughan [13, 14]. After the hydrolysis of the oil emulsion (without a titration), as described above, a volume of 15mL of hexane was added and shaken in order to extract the oleaginous compounds. The hexane, containing FFA liberated, was separated by centrifugation, and the remaining aqueous solution was re-extracted with 15mL of hexane. The organic fractions were combined and dried in a rotary evaporator. Then, 2g from this material were dissolved in a mixture of three organic solvents: methanol (10mL); chloroform (8.9mL) and heptane (7.1mL). Phenolphthalein was added as indicator and the mixture was titrated with standard sodium hydroxide solution (0.1M), saponifying only the FFA liberated by the hydrolysis. The volume of the added sodium hydroxide solution was measured and the mixture was completed up to 10mL with distilled water. The system was vigorously stirred using a vortex for 30 s and then the phases were separated by centrifugation. The upper phase, containing the saponified FFA was collected, and a second extraction procedure was performed with the bottom phase. The upper phases were combined and a solution of hydrochloric acid aqueous solution (1:1) was added, dropwise, until the pink color disappeared, when the FFA were back to its free form. The FFA were extracted twice with petroleum ether (20mL each extraction), then the organic phases were combined and evaporated. Derivatization of the FFA was carried out according to the methodology proposed by Hartman and Lago [15].

2.5.3. SDS–PAGE Analysis

SDS-PAGE analysis was performed in the Mini-Protean II Dual-Slab Cell (BioRad, USA), according to Laemmli [16].

2.5.4. GLC Analysis

The fatty acid composition was determined using a gas chromatographic system, with FID detector; capillary column Thermo Sientific – TR-BD, length 30 m, internal diameter 0.25mm and film thickness 0.25µm. The operational conditions were: 1.5mL.min^{-1} flow rate; 260°C injector temperature; 260°C.min^{-1} detector temperature; oven temperature of 45°C for 2min, 250°C at 7°C.min^{-1}, 250°C for 7min; nitrogen as carrier gas; injected volume of 1.0 µL.min^{-1} and Split of 1:50.

2.5.5. Biomass Concentration

To determine the biomass concentration, the suspension was filtered (Whatman paper n° 44), followed by washing with distilled water. The washed cells were dried at 45°C until constant mass.

2.5.6. Carbon Source

The depletion of the carbon source concentration in the supernatant was determined by an enzymatic method using a triglycerides GPO-PAP test kit (Laborlab, Brazil).

3. RESULTS

3.1. Plackett-Burman Design

Five variables were selected, corresponding to culture medium components (casein peptone, cottonseed oil, $MgSO_4$, yeast extract and $NaNO_3$) (Table **1**). Their levels were chosen based on the previous experiments carried out in our laboratory and on the literature concerning the produced lipase from

Table 1: Levels of the Five Variables Utilized in Plackett-Burman Design for GCL Production by Submerged Fermentation

Variables		Levels		
		-1	0	+1
x_1	Casein peptone (% m/v)	3	5	7
x_2	Cottonseed oil (% v/v)	0.5	1.0	1.5
x_3	$MgSO_4$ (% m/v)	0	0.05	0.1
x_4	Yeast extract (% m/v)	0	0.05	0.1
x_5	$NaNO_3$ (% m/v)	0	0.05	0.1

Table 2: Coded Levels for Plackett-Burman Design, and the Lipase Activity obtained throughout the Submerged Fermentation Process

Assays	Casein peptone (%)	Cotton Seed Oil (%)	$MgSO_4$ (%)	Yeast Extract (%)	$NaNO_3$ (%)	Lipase Activity (IU/mL)				
						12h	24h	36h	48h	60h
1	1	-1	1	-1	-1	2.92±0.60	12.89±0.70	9.48±0.47	2.94±0.02	17.30±1.44
2	1	1	-1	1	-1	5.20±0.52	3.09±0.83	9.05±0.15	3.92±0.29	7.45±0.05
3	-1	1	1	-1	1	10.47±0.59	14.73±1.18	19.00±0.02	22.31±0.76	18.06±0.93
4	1	-1	1	1	-1	1.48±0.20	9.44±0.55	2.03±0.47	4.56±0.27	11.73±0.60
5	1	1	-1	1	1	6.88±1.05	8.15±2.22	9.18±0.30	7.76±0.92	6.24±0.28
6	1	1	1	-1	1	2.03±0.59	6.24±1.23	7.38±1.56	10.61±0.59	13.29±1.21
7	-1	1	1	1	-1	4.46±2.13	5.97±0.47	9.02±0.28	14.53±0.07	13.93±0.36
8	-1	-1	1	1	1	4.83±1.66	7.19±1.59	7.68±1.00	4.19±0.09	14.23±1.02
9	-1	-1	-1	1	1	9.22±0.88	5.56±0.29	1.69±0.52	1.87±0.12	3.20±0.05
10	1	-1	-1	-1	1	1.80±0.36	9.29±0.31	15.14±0.33	16.89±0.28	11.12±0.29
11	-1	1	-1	-1	-1	3.44±0.67	10.96±0.24	17.33±0.77	4.42±1.24	6.84±0.62
12	-1	-1	-1	-1	-1	10.48±0.05	15.68±0.28	14.89±0.23	3.88±2.05	9.64±0.28
13	0	0	0	0	0	7.84±0.47	9.48±0.25	12.03±0.62	14.64±1.67	13.01±0.10
14	0	0	0	0	0	6.19±2.77	11.58±0.88	12.88±1.34	14.60±0.02	13.38±1.67
15	0	0	0	0	0	6.50±2.50	10.40±0.50	13.55±0.29	14.59±0.10	12.76±0.64

Geotrichum strains [6, 11, 17-19]. The lipase activity varied from 1.48 ± 0.20 $IU.mL^{-1}$ for 12h of fermentation to 22.31 ± 0.76 $IU.mL^{-1}$ for 48h of fermentation (Table **2**).

3.2. Factorial Design

According to the results obtained in the PB design, two variables were selected for the culture medium optimization by CCRD: peptone and cottonseed oil concentrations (Table **3**).

The statistical analysis showed significant linear (x_1) and quadratic (x_1^2) coefficient for casein peptone concentration and linear coefficient for cottonseed oil concentration (x_2) at 95% of confidence level.

According to the statistical method, the experimental data were fitted to a response surface model, to effectively evaluate the relation between the response (lipase activity) and the variables. A second-order polynomial model was obtained using coded values from data estimation, as shown:

$$Y\,(IU/mL) = 18.96 + 2.32x_1 - 3.89x_1^2 - 3.24x_2 - 1.70x_2^2 - 1.83x_1x_2$$

Table 3: Matrix of Factorial Design (CCRD) Used to Investigate the Activity of GCL after 48h of Submerged Fermentation

Assays	Coded (Actual) Variables		Lipase Activity ($IU.mL^{-1}$)	
	Casein Peptone (% m/v)	Cottonseed Oil (% v/v)	Experimental	Predicted
1	-1 (1.29)	-1 (0.79)	13.90 ± 0.14	12.28
2	1 (2.71)	-1 (0.79)	22.80 ± 0.13	20.60
3	-1 (1.29)	1 (2.21)	8.90 ± 0.39	9.47
4	1 (2.71)	1 (2.21)	10.47 ± 0.57	10.46
5	-1.41 (1)	0 (1.5)	7.50 ± 0.42	7.95
6	1.41 (3)	0 (1.5)	13.24 ± 0.24	14.51
7	0 (2)	-1.41 (0.5)	17.44 ± 0.17	19.81
8	0 (2)	1.41 (2.5)	11.38 ± 0.33	10.68
9	0 (2)	0 (1.5)	18.33 ± 0.03	18.96
10	0 (2)	0 (1.5)	20.80 ± 0.06	18.96
11	0 (2)	0 (1.5)	17.76 ± 0.04	18.96

where Y is the lipase activity after 48h fermentation, and x_1 and x_2 represent the coded values for both casein peptone and cottonseed oil concentrations, respectively.

The analysis of variance (ANOVA) (Table **4**) allowed to generate the response surface for the analysis of variable effects on the lipase activity after 48h of fermentation. The response surface obtained can be observed in Figure **1**.

In order to validate the optimization of the lipase production by CCRD, a fermentation assay was performed for 60h in a 5L stirred tank fermenter: with 2.3% m/v casein peptone, 0.8% v/v of cottonseed oil, 0.05% m/v of $MgSO_4$ and $NaNO_3$, controlled agitation speed of 300rpm and specific gas flow rate at 1vvm [11] (Figure **2**).

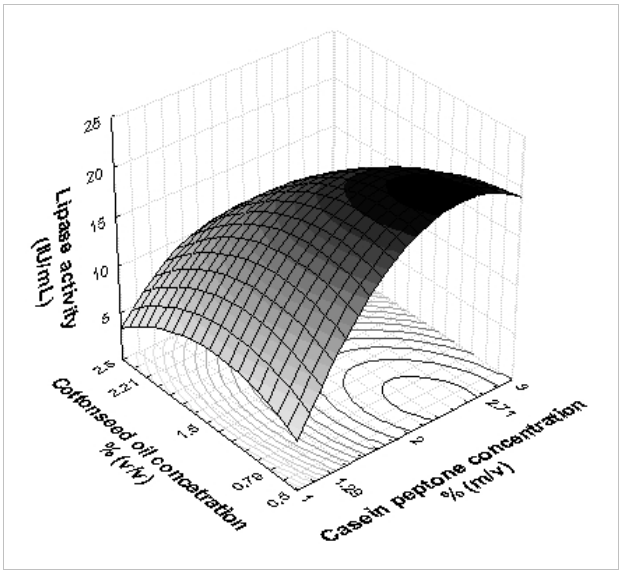

Figure 1: Response surface predicted for GCL activity after 48 of submerged fermentation as a function of the casein peptone and cottonseed oil concentrations in CCDR.

Table 4: Analysis of Variance (ANOVA) for the Model that Represents the Lipase Activity after 48h of Submerged Fermentation

Source of Variation	Coefficient	Degrees of Freedom	Mean Quadratic	F Test	p-Value
Regression	229.17	5	45.8342	11.02	0.009902
Residual	20.80	5	4.1606		
Lack of fit	15.58				
Pure error	5.22				
Total	249.98	10			
Coefficient of determination: R^2 = 91.7%					
$F_{5;5;0.05}$= 5.05					

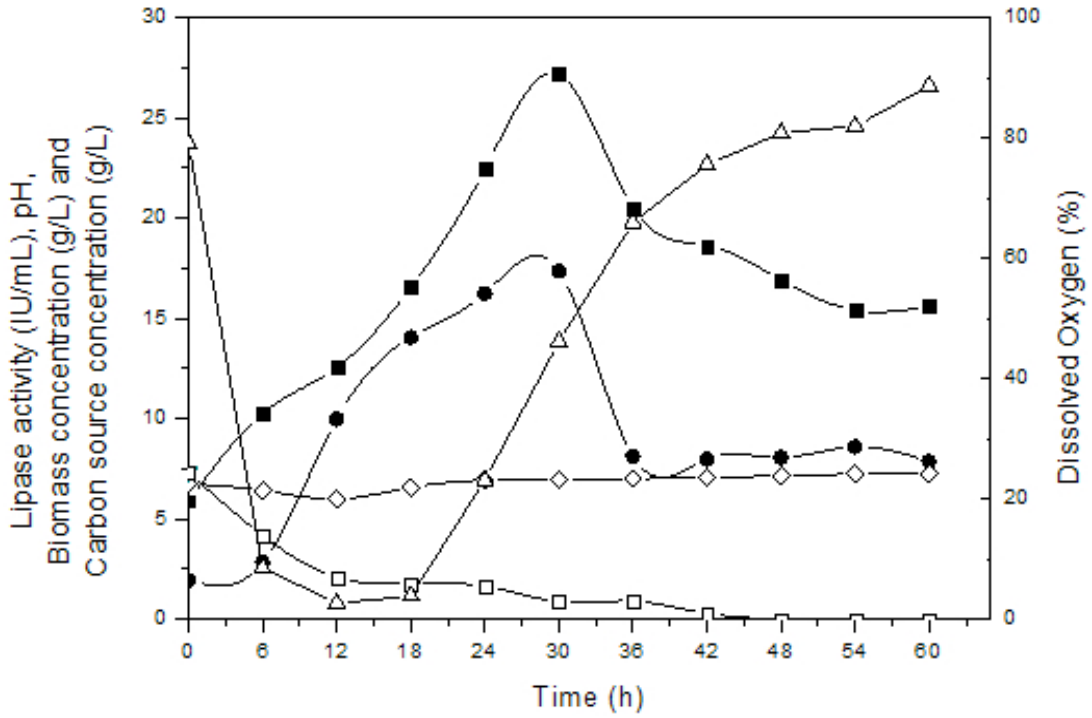

Figure 2: Time courses of biomass concentration (●), pH (◊), lipase activity (■), carbon depletion (□) and dissolved oxygen (Δ) obtained for lipase production from *G. candidum* during 60h of submerged fermentation in bioreactor, using agitation speed of 300rpm and 1vvm.

3.3. Lipase Purification by Immobilization Procedure

The electrophoresis analysis (Figure **3**) showed a single band of protein after 30h fermentation, with an apparent molecular mass of 65 kDa, that corresponded to the lipase produced by *G. candidum* [10]. The fermented broth contains multiple impurities present in the culture medium but despite this it is possible to affirm that most of the proteins present in it were produced lipases by microorganism [19].

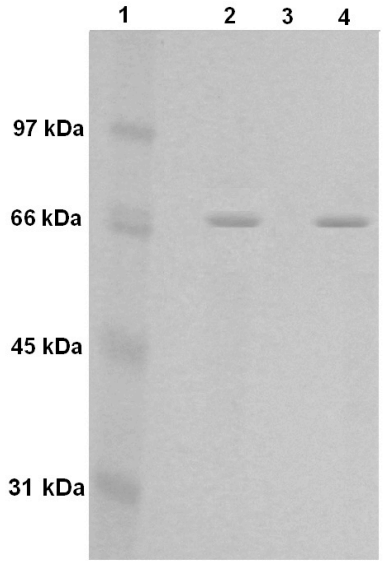

Figure 3: SDS–PAGE analysis for GCL after 30h of submerged fermentation in bioreactor. Line (1) Molecular mass markers; (2) Crude lipase extract after 30h; (3) Supernatant of immobilization; (4) Lipase desorption with 1% Triton X-100.

The behavior for the selected support (PHB) for purification process by lipase immobilization was verified. GCL was immobilized by offering a volume of 5mL of crude enzymatic extract, corresponding to 0.5mg of protein, per gram of PHB particles. The prepared biocatalyst was subsequently incubated in 1% (m/v) Triton X-100 for 2h and it was analyzed by electrophoresis (Figure **3**).

3.4. Substrate Specificity of Produced Lipases in Hydrolysis Reactions

The fatty acid specificity of the produced GCL was evaluated in hydrolysis reactions of olive and babassu oils, performed at 37°C for 5minutes under agitation in an orbital shaker (200rpm). These oils were chosen due to their distinct fatty acid composition (see Table **5**). The free fatty acids (FFA) liberated from babassu and olive oils, through the hydrolysis reactions catalyzed by produced GCL, were separated from glycerol, tri, di and monoglycerides by the methodology described by Borgstrom, Belfrage and Vaughan (see

Table 5: Initial Composition of FFA in Vegetable Oils, Final Composition of FFA Obtained in the Mixture after the Hydrolysis of olive and Babassu Oils by GCL, and the Ratio between the Initial and Final FFA Composition Percentages

Initial Composition of FFA in Vegetable Oils (% m/m)			Final Composition of FFA in the Mixture (% m/m)		Ratio between Initial and Final FFA Composition (% m/m)	
	Olive	Babassu	Olive	Babassu	Olive / Olive	Babassu / Babassu
Caprylic ($C_{8:0}$)	-	3.68	-	1.06	-	0.29
Capric ($C_{10:0}$)	-	4.73	-	1.07	-	0.23
Lauric ($C_{12:0}$)	-	47.00	-	20.29	-	0.43
Myristic ($C_{14:0}$)	-	18.40	-	4.03	-	0.22
Palmitic ($C_{16:0}$)	11.54	10.20	8.72	10.68	0.76	1.05
Stearic ($C_{18:0}$)	2.63	-	1.88	-	0.71	-
Oleic ($C_{18:1}$)	81.58	15.98	81.70	62.87	1.00	3.93
Linoleic ($C_{18:2}$)	4.25	-	7.70	-	1.81	-

analytical methods) [13, 14]. After this extraction, the FFA were derived and analyzed by a gas chromatographic system (GLC).

The final composition of FFA obtained in the mixture after the hydrolysis of the olive and babassu oils by GCL, was taken as 100%. Therefore, it was possible to observe bigger proportions of certain fatty acids in this final mixture than they are used to be in the vegetable oils' composition (initial) (Table **5**). This is the major evidence that GCL acts differently in the hydrolysis of different fatty acids, because if it acts equally the ratio between initial and final FFA composition will always be 1.

4. DISCUSSION

4.1. Plackett-Burman Design

The analysis of PB design, at 90% of confidence level, showed that the yeast extract was a significant variable for 24, 36 and 60h of fermentation and the values of the effects were all negative (-5.06, -7.43 and -3.25) indicating that an increase in yeast extract in the fermentation medium resulted in a decrease in activity of the produced lipase. Casein peptone was a significant variable for 12h of fermentation, and the effect has also been negative. Both, peptone and yeast extract are usually used in fermentation media as organic nitrogen sources, however the yeast extract is a more complex nitrogen source than peptone, because other components, like vitamins, are also present [20]. The microorganism preferred to consume peptone as nitrogen source, since the PB design showed that the negative effects observed during the fermentation of peptone were lower than the negatives effects observed for yeast extract after 24 and 36h of fermentation. Similar results were reported for production of extracellular lipases from *Candida albicans* and *Aspergillus flavus* that have preferred peptone as nitrogen source instead of yeast extract [21].

Furthermore, the extracellular lipase activity from microorganisms was described as more dependent on carbon than on nitrogen source concentration [18]. For these reasons, in the next step corresponding to the optimization of the culture medium by CCRD, the yeast extract was withdrawn and the level of the peptone was reduced.

$MgSO_4$ was statistically significant after 60h of fermentation, but a higher value for hydrolysis activity was observed after 48h of fermentation (22.31 ± 0.76 $IU.mL^{-1}$) and, in this set of experiments, $MgSO_4$ was not statistically significant. $NaNO_3$ was also not statistically significant in all the analyzed range. However, traces of salt in the culture medium were described as required for lipase production by *G. candidum*, therefore, in the CCRD step, the values of the salts were fixed at 0.05% (m/v) corresponding to a minimal amount used in the PB design [19].

The variation of cottonseed oil concentration was not statistically significant in the analyzed range, however the highest hydrolytic activity was found for the +1 level, corresponding to a maximal cottonseed oil concentration used in the PB design. Once that

cottonseed oil acts as both lipase inducer and carbon source, we chose to amplify the analyzed oil concentration range, in the DCCR, in order to verify if an increase in this concentration would have any effects on the lipase production.

4.2. Factorial Design

A maximum activity was observed for assay 2. This value was very close to the maximum activity obtained by the PB design when 3% (m/v) peptone and 1.5% (v/v) cottonseed oil were employed. However, less amount of peptone and cottonseed oil were used here to obtain a similar lipase activity (2.71% m/v and 0.79% v/v respectively) demonstrating that the optimization was very useful in reducing the total production costs of this lipase.

The analysis of variance (ANOVA) showed that the pure error was very low (5.22), indicating good reproducibility of the obtained experimental data. The F-test value (11.02) was higher than the table F (F5; 5: 0.05 = 5.05) showing the predictive capacity of the model (Eq. 1). The determination coefficient (91.7%) was satisfactory, allowing a good correspondence between experimental and predicted values for lipase activity. Thus, the model given by Eq. (2) can be used to generate the response surface for the analysis of variable effects on the lipase activity after 48h of fermentation.

The response surface (Figure **1**) showed that the lipase activity was strongly improved with the decrease in cottonseed oil concentration for the value levels used, indicating that a high concentration of cottonseed oil could start repressing the enzyme production [22].

In order to validate the optimization of the lipase production by CCRD, a fermentation assay was performed under the best conditions for lipase activity and containing 4 L of the optimized medium (Figure **2**).

Hlavsová *et al*. reported that the lipase secretion by *G. candidum* only started when about 50% of the carbon source was consumed, indicating that in the beginning the lipase was intracellular, being released later into the fermentation broth and reaching maximum levels at the end of the cell growth [23]. These results are in accordance with the results obtained in this work. In Figure **2**, it was possible to observe that the initial lipase activity detected was around 50% of carbon source depletion.

The highest hydrolytic activity of 27.17 $IU.mL^{-1}$ was found after 30h fermentation (Figure **2**). Burkert *et al*. reported that the maximum lipase activity of 22.0 $IU.mL^{-1}$ was obtained under optimum conditions, using a culture medium with soil oil in a stirred tank bioreactor after 54h fermentation [18]. Thus, the cottonseed oil was allowed to reach a high lipase production in a shorter time, almost half of the time spent when soil oil was used as inducer. This means a great saving in energy that when added to the lower cost of cottonseed oil could contribute to a considerable reduction in the cost of production of this lipase *via* submerged fermentation.

After 30h the lipase activity decreased, probably due to the high depletion of the carbon source (cottonseed oil).

4.3. Lipase Purification by Immobilization Procedure

In order to decrease the costs of the enzyme production, many authors have described the replacement of a synthetic media by a complex media, including by-products and wastes from the agro-industrial sector. This practice should be carried out more carefully, because it could hinder the enzyme's purification process, requiring the addition of several purification steps, losing the savings generated by replacing the synthetic medium.

Jacobsen *et al*. proved that *G. candidum* produced a very few contaminant proteins together with the lipases when a synthetic medium was used for fermentation, justifying our option to utilize it instead of a complex medium in the present work [19]. The use of a cheaper inducer and carbon source (cottonseed oil), with a simple purification process, could be a good strategy to reduce significantly the cost of lipase production.

G. candidum lipase exhibited high affinity for PHB particles. The immobilization yield was 98% and the recovered activity percentage 20%. The lipase was fully desorbed by incubation in Triton X-100. SDA-PAGE (Figure **3**) showed that only the lipase was adsorbed in PHB particles and that it was totally desorbed after incubation of 1% of Triton X-100, as previously described in our prior work [10]. Therefore, the enzyme produced with few contaminating proteins allowed the lipase purification in just one step, reducing the downstream process cost.

4.4. Substrate Specificity of Produced Lipases in Hydrolysis Reactions

According to Table **5** the proportion of free oleic acid obtained after hydrolysis of babassu oil by GCL, was 3.5 to 4 times higher in relation to the other fatty acids present in this oil. The proportions of free caprylic, capric, lauric and myristic acids were between a 25 and 50% lower when compared to the other fatty acids in the original oil sample. This behavior demonstrates that the lipase was more specific in the hydrolysis of ester bonds formed by the oleic acid than by saturated fatty acids. Even with a higher amount of lauric acid in the babassu oil (about three times higher than oleic acid) the enzymatic action was preferentially on the oleic acid esters.

The GCL was also very efficient in the hydrolysis of the oleic acid present in olive oil, but it was even more suitable to hydrolyze the linoleic acid groups. Since the oleic acid corresponded to 80% of the fatty acids in olive oil, high proportions of it were expected after hydrolysis. Moreover, despite the reduced presence of the linoleic acid in olive oil, its proportion, relative to other fatty acids, increased from 50 to 100%, indicating a significant specificity towards it by the produced lipase during the hydrolysis process.

Therefore, the GCL was more effective on both oleic and linoleic acids' esters from olive and babassu oils, indicating a specificity of this produced enzyme towards these two fatty acids.

Reports indicating that the composition of a culture medium, in particular the type of the lipidic substance used as a carbon source or inducer can result in a production with different substrate specificities (typo selectivity) [24, 25]. Thus, once GCL was produced in a culture media with an oil rich in oleic and linoleic acids (cottonseed oil), it was expected that GCL showed a high specificity for these fatty acids.

As far as we know, this is the first report on statistical optimization of medium components using cottonseed oil as both inducer and carbon source that obtained a high production of a lipase with high substrate specificity, in the shortest time ever reported, with a very low-cost for a synthetic medium ($ 3.2 L^{-1}). The optimization medium in this study can be used for the production of GCL that can be purified from fermentative broth using a single-step (by immobilization on hydrophobic supports), contributing further to the reduction of its cost of production, thus encouraging its application in industrial processes.

CONCLUSIONS

The optimization of the culture medium, using cottonseed oil as both inducer and carbon source, allowed a high lipase production from *G. candidum* (27.17 IU/mL after 30h fermentation). This is the highest activity value already described for this lipase in literature and it was obtained in a shorter time period than what had been already described. This optimization has also had other advantages, as the use of very small amounts of synthetic culture medium components and the removal of expensive components as yeast extract. The lipase purification in a single step by immobilization on PHB particles was verified. GCL, with an apparent molecular mass of 65 kDa, showed high specificity in hydrolyzing ester bonds of oleic and linoleic acids, which are components of various vegetable oil triacylglycerols.

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