RESEARCH

Elucidation of the effects of inoculum size and age on lipase production by Geotrichum candidum

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ABSTRACT

Lipases are extremely versatile enzymes with large industrial applications and they have been the subject of investigation over the last few years. A great variety of microorganisms can produce this enzyme, such as pluricellular fungi, but their growth need to be optimized due to the variations amongst experiments, which influence the results and reproducibility of the process. The aim of this work was to study the best conditions for inoculum size and age of *Geotrichum candidum* NRRLY-552, to reduce the variability of the inoculum. The optimized inoculum procedure was determined as: 1 circular area (0.79 cm^2) of the solid medium containing cells and spores added to 100 mL of culture medium (5.0 % w/v of peptone, 0.1 % w/v of $NaNO_3$, 0.1 % w/v of $MgSO_4$ and 1.0 % w/v of soybean oil) incubated for 15 h at 30 °C, 250 rpm and with an initial pH of 7.0. This procedure permitted to reduce the experimental error from 30 to 20 % and it has been applied successfully in different studies since then.

Keywords: lipase, solid inoculum, soybean oil, factorial design, *Geotrichum candidum*

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RESUMEN

Determinación del efecto del tamaño y la edad del inóculo en la producción de lipasa por *Geotrichum candidum***.** Las lipasas son enzimas extremadamente versátiles. Se utilizan en varias aplicaciones industriales, y han sido objeto de atención recientemente. Una diversidad de microrganismos puede producirlas, tales como los hongos pluricelulares. Sin embargo, para el estudio de estas enzimas se debe optimizar su crecimiento, debido a las variaciones entre los experimentos, que influyen en los resultados y en la reproducibilidad de los procesos. El objetivo de este trabajo fue investigar el tamaño y la edad idóneos del inóculo *Geotrichum candidum* NRRLY-552, con el fin de reducir su variabilidad. El procedimiento optimizado para el inóculo se determinó como una zona circular (0.79 cm^2) de medio sólido, que contenía células y esporas, añadido a 100 mL de medio de cultivo (peptona 5.0 % p/v, $NaNO_3$ 0.1 % p/v, $MgSO_4$ 0.1 % p/v y aceite de soya 1.0 % p/v) que se incubaron durante 15 h a 30 °C, 250 rpm, con un pH inicial de 7.0. Este procedimiento permitió reducir el error experimental de 30 a 20 %, y se ha aplicado con éxito en varios estudios.

Palabras clave: lipasa, inóculo sólido, aceite de soya, diseño factorial, *Geotrichum candidum*

*I*ntroduction

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are enzymes with considerable physiological significance and industrial potential [1-3]. They have several applications in organic chemistry and pharmaceutical processes, detergent formulations, biosurfactant synthesis, oleochemical industry, dairy and agrochemical industries, paper manufacture, nutrition, cosmetics, and more recently in biofuel production, among other uses [4-9]. Due to their wide-range significance, lipases remain the subject of intensive studies focused particularly on their structural characterization, elucidation of action mechanisms, kinetics, sequencing and cloning of lipase genes and general performance characterization.

Additionally, the development and management of the microorganism inoculum through various production stages have a definite effect on the subsequent performance of the process. In commercial industrial fermentation processes, it is well known that the age and density of the inoculum used directly influences on the duration of the lag phase, specific growth rate, biomass yield, sporulation and quality of the final product, and hence on production costs [10,11]. Therefore, few researchers have investigated these variables in lipases and other enzymes production [12-14].

Previously, substrate concentration, pH and inoculum size were found to be the most important factors for lipase production by P*enicillium cyclopium* by using response surface methodology [15, 16]. A strong interaction was also detected between the primary and secondary inoculums on a two-stage inoculum system [17]. Moreover, the inoculum size was an important factor in an experimental design to obtain high enzymatic activity [18]; this variable was also relevant

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in the production of an alkaline protease by *Bacillus mojavensis* [19].

Recent studies on the production, efficiency and application of lipases from *Geotrichum* sp. and *G. candidum* NRRL-Y 552 [20-24], remarked the importance of inoculum variables such as size, volume and age for lipase production by *Geotrichum* sp. through an optimized experimental design. Significantly, the best result of 35 U/mL was obtained with 0.78 cm^2 of inoculum size, 50 mL of inoculum volume medium and 12 h of inoculum age using corn steep liquor and soybean oil in fermentation medium [25].

Additionally, it is very common to obtain fungal spores for fermentation using a spore solution, but that methodology is very imprecise.

Therefore, the aim of this work was to develop an optimized inoculum procedure for lipase production by *Geotrichum candidum* NRRLY-552, including the use of a solid circular area (SCA) containing spores as an inoculum alternative methodology. This procedure allowed to reduce the inoculum variability under the tested conditions and to improve standardization, reliability and reproducibility parameters of lipase activity results. The inoculums size and age were investigated and a fractional factorial design was used to show the impact of experimental modifications on lipase production.

Materials and methods

Inoculum procedures

Geotrichum candidum NRRLY-552, an imperfect fungus, was provided by the Agricultural Research Service Collection. It was cultivated in Yeast Malt Extract Agar (YMEA) slants (0.3 % (w/v) malt extract, 0.3 % (w/v) yeast extract, 0.5 % (w/v) peptone, 1.0 % (w/v) glucose and 3.0 % (w/v) agar) for 48 h at 30 °C, and stored at 4-5 °C until use [24]. The inoculum medium was composed of (w/v): 5.0 % peptone, 0.1 % $NaNO_3$, 0.1 % $MgSO_4$ and 1.0 % soybean oil, with an initial pH = 7.0; the fungus was incubated for 24 h at 30 °C and 250 rpm.

The traditional procedure for inoculum preparation consists of transferring one loopful of cells and spores from the YMEA slants to 10 mL of inoculum medium. After incubation, the total volume was transferred to an erlenmeyer of 500 mL containing 100 mL of the same inoculum medium and incubated at the same conditions.

A different procedure was studied for the optimization of inoculum concerning the size and age. First, from the YMEA slants, spores and cells were suspended in 1 mL of sterile distilled water by scraping the surface with a loop, this volume was then poured on a Petri dish containing YMEA and spread with a Drigalski spatula; incubation proceeded for 48 h and at 30 °C. After this incubation, the surface of the agar medium on the Petri dishes were uniformly covered by the mycelium and its spores, supporting the assumption that a fixed inoculum size will always provide a very similar concentration of cells, and consequently, spores. From these YMEA Petri dishes, solid circular areas (SCA) were cut off by pressing the edge of a sterilized test tube with a specific diameter (ø) on the agar medium covered with cells and spores; the SCA were then removed carefully and transferred to an erlenmeyer of 500 mL containing 100 mL of inoculum medium volume (IMV).

Effect of the inoculum size

The effect of inoculum size was investigated in the production of lipase by *G. candidum* NRRLY-552. Fermentation medium composition and incubation conditions were the same as applied for the inoculum, except for the fermentation time which was 72 h.

Firstly, from 1 to 4, solid circular areas (SCA) of 1.54 cm^2 (ø = 1.4 cm) were used to prepare the inoculum, then, the fermentation medium was inoculated at 10 % (v/v) using the resulting inoculum. The lipase activity and pH were determined during fermentation and the best result was considered for the next test.

In sequence, two variables were studied: SCA of 0.79 and 1.54 cm^2, and IMV from 50 to 300 mL to determine the best inoculum size. The lipase activity and pH were determined during the incubation time, and the best conditions selected to make a third test to define the inoculum size.

Based on the results obtained at the first and second tests, four new fermentations were finally conducted using SCA of 0.79 and 1.54 cm^2, and IMV of 50 and 100 mL at the same fermentation conditions. The lipase activity and pH were determined during the fermentation time.

Effect of inoculum age

The effect of inoculum size was investigated in the production of lipase by *G. candidum* NRRLY-552. Fermentation medium composition and incubation conditions were the same as applied for the inoculum, except for the fermentation time which was 72 h.

The inoculum was prepared using one SCA of 0.79 cm^2 (ø = 1.0 cm) and 100 mL of inoculum medium. The incubation time of the inoculum varied from 15 to 48 h and the lipase activity and pH were determined as response during the fermentation time.

After the determination of the optimum inoculum conditions (inoculum size and age), a 2^{4-1} experimental design with 3 central points was conducted for two different inoculums procedures: traditional and optimized. For the first factorial design, the traditional inoculum was applied as described above; for the second factorial design, the optimized inoculum was obtained with 1 solid circular area (SCA) of 0.79 cm^2, 100 mL of inoculum medium incubated for 15 h. The independent variables studied were the concentrations (w/v) of: peptone (3.0-7.0 %), $MgSO_4$ (0-0.2 %), $NaNO_3$ (0-0.2 %) and soybean oil (0.5-1.5 %) in the composition of the fermentation medium; the response analyzed was the lipase activity (U/mL). Fermentation proceeded for 48 h at 30 °C and 250 rpm. The matrix, with coded and real values for the independent variables and the response values obtained, is presented in table 1; the results were analyzed using the software Statistica 7.0 Statsoft Inc.

In order to analyze the reproducibility of the process, four new trials with the optimized inoculum were carried out under the optimum conditions (central points; w/v): 5 % peptone, 0.1 % $MgSO_4$, 0.1 % $NaNO_3$, and 1.0 % soybean oil; initial pH of 7.0. Fermentation was carried out at 30 °C and 250 rpm. Lipase activity and pH were measured during fermentation time.

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Table 1. 2^{4-1} factorial designs for lipase production by *G. candidum* NRRLY-552 using different traditional (TI) and optimized (OI) inoculum procedures

Trial	Peptone (%)	$NaNO_3$ (%)	$MgSO_4$ (%)	Soybean oil (%)	Lipase activity (U/mL)	
					TI	OI
1	3.0 (–1)	0 (–1)	0 (–1)	0.5 (–1)	3.25	13.21
2	7.0 (+1)	0 (–1)	0 (–1)	1.5 (+1)	2.56	18.01
3	3.0 (–1)	0.2 (+1)	0 (–1)	1.5 (+1)	2.77	7.78
4	7.0 (+1)	0.2 (+1)	0 (–1)	0.5 (–1)	1.05	5.00
5	3.0 (–1)	0 (–1)	0.2 (+1)	1.5 (+1)	2.71	3.43
6	7.0 (+1)	0 (–1)	0.2 (+1)	0.5 (–1)	2.16	6.69
7	3.0 (–1)	0.2 (+1)	0.2 (+1)	0.5 (–1)	2.61	11.11
8	7.0 (+1)	0.2 (+1)	0.2 (+1)	1.5 (+1)	2.93	13.97
9	5.0 (0)	0.1 (0)	0.1 (0)	1.0 (0)	2.84	21.87
10	5.0 (0)	0.1 (0)	0.1 (0)	1.0 (0)	2.86	20.11
11	5.0 (0)	0.1 (0)	0.1 (0)	1.0 (0)	1.57	14.43

TI: one loop of cells and spores from an original slant were suspended in 10 mL of fresh inoculum medium; after 24 h of incubation, this volume was transferred to 90 mL of fresh inoculum medium at 10 % (v/v) and incubated for 24 h.
OI: cells and spore from an original slant were suspended in 1 mL of sterile distillated water, poured in a solid medium and incubated for 48 h; 1 solid circular area of 0.79 cm^2 was cut off and added to 100 mL of fresh medium and incubated for 15 h.

Lipase assay

Lipase activity was measured using a titrimetric assay, titrating with 0.05 M NaOH and using emulsified olive oil as substrate. The reaction mixture consisted of 19 mL of an olive oil/Arabic gum emulsion (5 % w/v olive oil and 5% w/v Arabic gum) in 100 mM potassium phosphate buffer, pH 7.0. This mixture was homogenized in a blender for 3 min and the enzyme reaction started by adding 1 mL of culture supernatant. The assay was carried out at 37 ºC and 200 rpm for 30 min. The reaction was then stopped by adding 20 mL of acetone-ethanol 1:1 (v/v), and the amount of fatty acids produced titrated with 0.05 M NaOH to pH 11.0 using an automatic titration apparatus (Mettler DL21). One unit of lipase activity (U) was defined as the amount of enzyme that releases 1 µmol of fatty acid per minute under the assay conditions [22, 24, 26].

Results and discussion

Effect of inoculum age

Initially, four experiments were carried out to evaluate the influence of the inoculum size on lipase production. In the first experiment one SCA with 1.54 cm^2 (ø = 1.4 cm) was added to the inoculums medium, while in the second, third and fourth experiments were added 2, 3 and 4 SCA with 1.54 cm^2, respectively. There was a decrease in lipase activity with an increase in inoculum size (number of SCA) during the fermentation time (Figure 1A). The highest activity (15.8 U/mL) was obtained with 1 SCA after 48 h, a value about 6 times higher than with 4 CSA, suggesting that a smaller number of spores added to the same substrate volume is better to obtain higher levels of lipase activity during fermentation.

This fact can be related to a typical phenomenon found in mycology: the self-inhibition of fungal spore germination. Many fungal spores exhibit a crowding effect [27-29], in which the spores contain

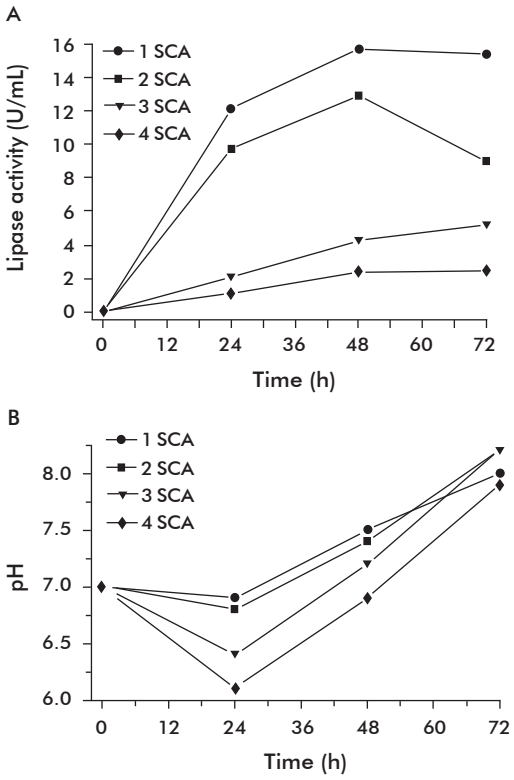

Figure 1. Effect of the inoculum size of *G. candidum* NRRLY-552 cultivation. A) Lipase activity (U /mL). B) pH. The inoculum was obtained with 1 to 4 solid circular areas (SCA) of 1.54 cm^2 and an inoculum medium volume (IMV) of 100 mL. Inoculum and fermentation medium composition (%, w/v) was: 5.0 % peptone, 0.1 % $NaNO_3$, 0.1 % $MgSO_4$ and 1.0 % soybean oil (initial pH = 7.0). Incubation proceeded at 30 °C and 250 rpm. Straight lines were used to connect the points and guide the eyes.

a prepackaged self-inhibitor that prevents germination under crowded conditions. Another effect observed was that fungi with small inoculum sizes produced a transient mycelial stage with the mycelium length inversely proportional to the inoculum size [30]. This effect was also obtained in the production of cellulase by *Trichoderma reesei* Rut C-30, in which the average dimension of the pellet seemed to be inversely proportional to the inoculum size [31].

Based on these first results, two different values of SCA were chosen: 1.54 cm^2 (ø = 1.4 cm) and 0.79 cm^2 (ø = 1.0 cm), since the first analysis suggested that the smaller the inoculum size, the more efficient the process. In addition, different IMV from 50 to 300 mL were also evaluated, their lipase activities shown in table 2; the pH data are not presented. The highest activities were obtained with a SCA of 0.79 cm^2 and an IVM of 50 mL (trial 1) and 100 mL (trial 2), were 7.53 and 7.74 U/mL obtained after 24 and 48 h, respectively. These results reinforce the hypothesis that the smaller quantity of spores in the inoculums, the greater lipase activity obtained. It was previously observed that *Geotrichum* sp. inoculum size and volume had a negative effect on lipase production [25].

Finally, based on the best results obtained so far, the effect of inoculum size was evaluated using inoculum conditions of SCA 0.79 and 1.54 cm^2 (ø = 1.0 and

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Table 2. Effect of the inoculum size on the lipase activity from *Geotrichum candidum* NRRLY-552*

Trial	SCA (cm^2)	IMV (mL)	Lipase activity (U/mL)	
			24 h	48 h
1		50	7.53	4.01
2		100	2.62	7.74
3	0.79	150	3.83	4.02
4	(ø = 1.0 cm)	200	3.12	3.37
5		250	2.93	3.33
6		300	3.60	4.83
7		50	4.73	0.21
8		100	4.49	3.05
9	1.54	150	4.23	3.34
10	(ø = 1.4 cm)	200	3.27	2.95
11		250	2.09	2.71
12		300	1.16	2.48

* The inoculum was prepared with solid circular areas (SCA) of 0.79 and 1.54 cm^2 and inoculum medium volumes (IMV) from 50 to 300 mL. Inoculum and fermentation medium composition (%, w/v) was: 5.0 % peptone, 0.1 % $NaNO_3$, 0.1 % $MgSO_4$ and 1.0 % soybean oil (initial pH = 7.0). Incubation proceeded at 30 °C and 250 rpm. ø = diameter of a circular area.

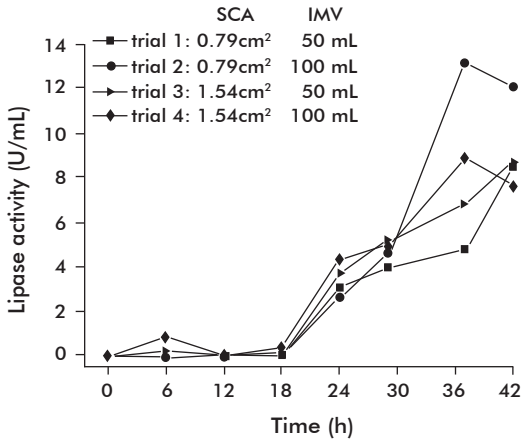

Figure 2 . Effect of the inoculum size on lipase activity from *G. candidum* NRRLY-552. The inoculum was prepared with 1 solid circular area (SCA) of 0.79 and 1.54 cm^2 and an inoculum medium volume (IMV) of 50 and 100 mL. Inoculum and fermentation medium composition (%, w/v) was: 5.0 % peptone, 0.1 % $NaNO_3$, 0.1% $MgSO_4$ and 1.0% soybean oil (initial pH = 7.0). Incubation proceeded at 30 and 250 rpm. Straight lines were used to connect the points and guide the eyes.

1.4 cm) and IMV of 50 and 100 mL. Lipase activities obtained are presented in figure 2. The highest lipase activity of 13.20 U/mL was obtained at 37 h in trial 2: SCA 0.79 cm^2 and IMV 100 mL. For comparison, the optimized inoculum defined for *Geotrichum* sp. consists of the same SCA 0.79 cm^2 but IMV 50 mL [25].

Spore cultivation on a solid medium is rarely mentioned in the literature, but in practical fermentation studies, this technique considerably reduces variations in the quantity of spores used to make the inoculums, and the results obtained were reproducible as compared with those obtained using spore measurement. There was reported the production of xylanase by *Pleurotus ostreatus* SYJ042 following the same methodology as used in the present work, *i.e.*, spore cultivation on a solid medium and the transference of SCA to the inoculum medium; the same SCA (0.79 cm^2) was determined using four 0.5 cm diameter disks (ø). The authors stated that smaller SCA and larger volumes were ideal for inoculum preparation [32]. In another study, it was shown with soil bacteria that the decreased inoculum size resulted in significant increases in the viable counts, assessed as colony forming units on solid media [12].

The pH analysis for all the tests (data not shown), revealed that pH values dropped to around 6.0-6.5 during the first 24 h of fermentation and increased almost to 8.0 at the end, when the lipase activity starts to decrease. That high pH value is not favorable to lipase production profiles, as demonstrated in Figure 1b, an effect commonly observed with *G. candidum* [22, 24].

Inoculum age

The influence of the inoculum age was analyzed using the best conditions determined for the inoculum size: 1 SCA, 0.79 cm^2, and IMV 100 mL. As shown in figure 3, a 15-h inoculum showed the best results in terms of lipase activity levels, around 16.5 U/mL from 48 to 96 h. The age of inoculum is highly variable depending on the process, cultivation conditions, medium composition and the microorganism, among other factors. The best condition for the inoculum age of *G. candidum* determined in this work was shorter than in previous studies using different microorganisms. In fact, it was optimal because of reducing the total fermentation time and increasing productivity. Inoculum age values of 18 and 96 h were previously obtained during alkaline protease production by *Bacillus mojavensis* [19] and *Bacillus* sp. [33], respectively. Nevertheless, considering lipase production from *Geotrichum* sp. [25], the inoculum age was set as 12 h, a result quite similar to that obtained in this study with *G. candidum*. The pH data are not shown but followed the same profile as described earlier (Figure 1 b).

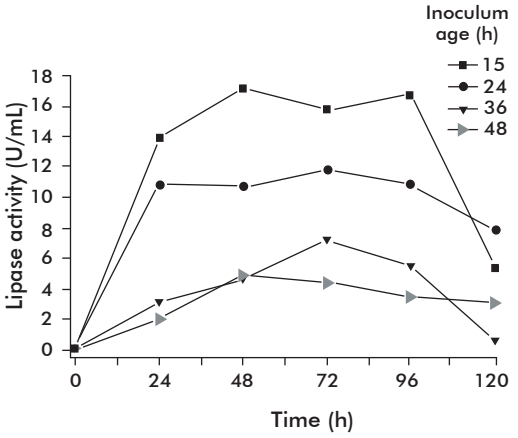

Figure 3. Effects of the inoculum age (15, 24, 36 and 48 h) on lipase activity from *G. candidum* NRRLY-552. The inoculum was prepared with 1 solid circular area (SCA) of 0.79 cm^2 in 100 mL of inoculum medium. Fermentation was conducted with a inoculation of 10 % (v/v) in 100 mL for 72 h. Inoculum and fermentation medium composition (% w/v) was: 5.0 % peptone, 0.1 % $NaNO_3$, 0.1 % $MgSO_4$ and 1.0 % soybean oil (initial pH = 7.0). Incubation conditions were: 30 °C and 250 rpm. Straight lines were used to connect the points and guide the eyes.

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Comparison of lipase production using different inoculums procedures

It is known that factorial designs can also be used to compare the results obtained with modifications not directly included in the independent variables analyzed [34]. Therefore, two 2^{4-1} factorial designs were applied to investigate the variability of lipase production with the optimized inoculums, and also with the traditional inoculum procedure (Table 1). The aim was to investigate the variability of the experiments with different inoculum procedure, not to compare both conditions in relation to their lipase activities. For that reason, the central points conditions were the same already been applied for *G. candidum* cultivation (inoculum and fermentation medium).

Considering the traditional inoculum (Table 1), the central points (trials 9, 10 and 11) resulted in an average activity of 2.42 ± 0.74 U/mL and the variables conditions (trials 1 to 8) resulted in an average activity of 2.51 ± 0.67 U/mL, respectively. The deviation observed at the central points corresponded to 30.58 % of the main value, a high deviation that must be avoided. The analysis of the effect (Table 3) showed that with the traditional inoculum, the different conditions in medium composition did not influence lipase activity and were not statistically significant, which can be seen clearly from the average activities and its deviation mentioned above and in the average activity and its deviation (2.50 ± 0.25 U/mL) considering all experiments (trials 1 to 11). This suggests that there were problems in respect to the standardization and reproducibility of the inoculum conditions, which did not respond well to the variation in medium composition because of its own variation. That effect was not observed with the optimized inoculum.

According to the results (Table 1) with the optimized inoculum, at the central points conditions (trials 9, 10 and 11) the lipase activities resulted in an average value of 18.80 ± 3.89 U/mL and at the variables conditions (trials 1 to 8) the average activity was 9.90 ± 5.01 U/mL. Considering all experiments (trial 1 to 11) the average activity was 9.90 ± 2.11 (Table 3). These values reflect the variability of results: at the same conditions, the central points presented a deviation of 20.69 % from the main value. Therefore, a well-defined inoculum avoids an extra variability to the study and allows more confidence in the responses obtained. At first, the analysis of the effects of the independent variables (Table 3) showed that with the optimized inoculum all the variables were not statistically significant in the range studied ($p < 0.10$). But after modifying the inoculum curvature it became statistically significant, indicating that the central point conditions were better for lipase production. This is in agreement with the fact that this conditions are already been used for inoculum and fermentation medium composition.

The reproducibility of the results was assessed with four new trials using the optimized inoculum at the same conditions of the central points; the results are presented in figure 4. The highest level of lipase activity was achieved between 24 and 54 h; the error in relation to the average values was around 20 %, which is acceptable for fermentation using filamentous fungi. After 48 h, lipase activity was 11.51 ± 2.52 U/mL and its deviation 21.91 % of the main value. In a previous study, using an optimized inoculum procedure and an optimized medium composition but with *Geotrichum* sp., the maximum lipase activity obtained was 35.2 ± 0.8 U/mL, its deviation corresponding to 2.27 % of the main value [25].

At this point, it is important to state that the optimized inoculum procedure here presented was lately applied for the optimization of medium composition for *G. candidum* cultivated in peptone [24], achieving a maximum lipase activity of 16.3 ± 0.8 U/mL with a deviation of 5 % of the main value. Recently, the inoculum procedure was also applied to the optimization of yeast hydrolysate and corn steep liquor instead of peptone in medium composition, and the maximum lipase activities obtained were 18.4 ± 0.8 and 21.7 ± 2.4 U/mL after 48 h, respectively [35, 36]. Deviations were equivalent to 4.3 and 9.5 % of the main value, respectively.

Table 3. Effects of the independent variables (concentration, % w/v, of peptone, $NaNO_3$, $MgSO_4$ and soybean oil) on lipase activity (U/mL) from the 2^{4-1} factorial designs for lipase production by *G. candidum* NRRLY-552 using traditional or optimized inoculum procedures

Variable	Effect (U/mL)	Standard error	t-value (5)	p-value
	Traditional inoculum			
Average	2.50	0.25	9.87	< 0.01*
Curvature	-0.16	0.97	-0.17	0.87
Peptone	-0.66	0.51	-1.30	0.25
$NaNO_3$	-0.33	0.51	-0.65	0.54
$MgSO_4$	0.20	0.51	0.38	0.72
Soybean oil	0.48	0.51	0.94	0.39
	Optimized inoculum			
Average	9.90	2.11	4.67	< 0.01*
Curvature	17.80	8.11	2.19	0.08*
Peptone	2.04	4.23	0.47	0.65
$NaNO_3$	-0.87	4.23	-0.20	0.84
$MgSO_4$	-2.20	4.23	-0.52	0.62
Soybean oil	1.80	4.23	0.42	0.69

* Statistically significant, with $p < 0.10$.

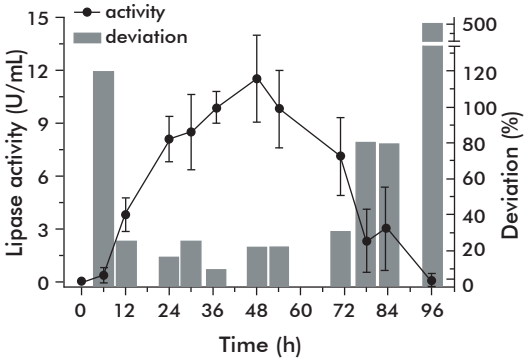

Figure 4. Validation of lipase production by *G. candidum* NRRLY-552. An optimized inoculum procedure was used: 1 solid circular area (0.79 cm^2) of Yeast Malt Extract Agar containing cells and spores in 100 mL of inoculum medium incubated for 15 h. Inoculum and fermentation medium composition (%, w/v) was: 5.0 % peptone, 0.1 % $NaNO_3$, 0.1 % $MgSO_4$ and 1.0 % soybean oil (initial pH = 7.0). Incubations were conducted at 30 °C and 250 rpm. Inoculation proceeded at 10 % (v/v) in 100 mL and the fermentations were carried in quadruplicate. Bars indicate the standard error for lipase activity; straight lines were used to connect the points and guide the eyes.

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From these results is possible to observe that the optimization of the inoculum procedure in this study was valid.

The pH data are not presented but it behaved as expected, with a slow decrease around 24 h followed by an increase until reaching 8.0 after 54 h, further increased until 8.8 after 96 h of fermentation. As previously discussed, the pH value of 8.0 is detrimental for lipase activity as can be seen in figure 4, with lipase activity showing a drop starting after 54 h to almost 1.0 U/mL at 96 h.

Conclusions

The conditions determined in this study with respect to inoculum size and age contributed to improve lipase production by *G. candidum* NRRL Y-552 and to reduce the variability on fermentation results. From this study it was possible to obtain a maximum lipase activity of 11.51 ± 2.52 U/mL after 48 h with an optimized inoculum. This result is valid since it was possible to obtain smaller deviations (~20 %) than those obtained with the traditional inoculum (~30 %), which support much more reliable studies, especially about other important factors.

The inoculum strategy presented here has been applied successfully in lipase production by *G. candidum*, resulting in 16.3 ± 0.8 U/mL (48 h) in shaken flasks [24], 21 ± 0.7 U/mL (54 h) in a bench-scale stirred reactor and 20 U/mL (30 h) in an airlift bioreactor [21]. It was also used for lipase production by *Geotrichum* sp. in shaken flasks, achieving 35.20 ± 0.80 U/mL [25], and to improve it by *Fusarium oxysporum* with a 60 % increase [2]. The errors observed by those authors were around 5 % of the main values of activities, which represent a great achievement in fungi cultivation.

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