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Production and Partial Biochemical Characterization of Multiple Forms of Lipases

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HIGHLIGHTS

- Multiple forms of lipases can exhibit different properties and applications.
- Mycelium-bound lipases can be helpful for the oleochemical industry.
- All of the multiple forms of lipase were studied and presented hydrolysis activity.
- Partial biochemical characterization of multiple forms of lipases was realized.
- *Trichoderma koningii* was suggested as a good producer of mycelium-bound lipases.

Abstract: The total cost of processes that use lipases can be reduced by using the mycelium or not purified crude extract with lipolytic activity. These catalysts can be used without complex purification and can show desirable properties to emerging applications like hydrolysis and biotransformation of vegetable oils. To explore the potential of multiple forms of lipases (produced by the same filamentous fungus species), this work investigated intracellular and extracellular lipases produced by *Trichoderma koningii* (intracellular lipases were never studied), *Penicillium spp.* F02 (isolated from industrial grease trap) and *Aspergillus niger*. For this, batch fermentations were conducted for 96h using olive oil as a carbon source. The submerged culture was interrupted with vacuum filtration to isolate the mycelium and crude extract. The lipolytic activities were determined by the hydrolysis method of olive oil. The best results were obtained for intracellular activity with *T. koningii* (81.5 U/g of dry biomass) and for extracellular activity with *A. niger* (10.6 U/mL of crude extract). Both were attained in 96h of fermentation. *T. koningii* showed an accumulation of intracellular lipases and a depreciated extracellular activity in the initial 24 hours, which can be reasonable indications that there is strong retention of lipases in the mycelium. Statistical analysis within the range of 37°C to 44°C associated with pH from 7.8 to 8.0 favored the activity of lipases from *T. koningii*. All these findings are promising for

future studies in the enzymatic synthesis of biodiesel with lipases (intracellular and extracellular) from *T. koningii*.

Keywords: Hydrolysis; FFA; Mycelium-bound lipases; Crude extract lipases; Response surface methodology.

INTRODUCTION

In the oleochemical industry, one of the main requirements for adequate Sustainable Development is the replacement of conventional thermochemical processes by enzymatic routes. Well-recognized benefits of the enzymatic process include the generation of biodegradable products, mild reaction conditions, and wide adaptability for feedstocks. The high cost of enzymes is the major impediment to widely commercializing the enzymatic process compared to chemical catalysts [1]. The enzymes used in the oleochemical industry are known as lipases.

Lipases (triacylglycerol hydrolases E.C. 3.1.1.3) catalyze hydrolysis and synthesis of esters formed from glycerol and long-chain fatty acids [2]. Besides the before-mentioned industrial use, lipases are commonly utilized in biodiesel production, dairy industry and can improve the bioremediation of greasy liquid effluents [3]. These enzymes can be obtained from several sources: animal, vegetable, and microbiological [4]. Among these well-known sources, lipases from filamentous fungi and yeasts are aimed for their high stability [5].

Isolation of novel biocatalysts for industrial processes is still being considered the deciding factor for cost-effective industrial production using enzymes, such as lipases [6]. The species *Penicillium*, *Fusarium*, and *Aspergillus* are screened on a large scale for lipase production [7]. In industrial applications, these lipases are used in multiple configurations, e.g., soluble, free, immobilized, lyophilized, or mycelium bound.

The use of mycelium-bound lipases (MBL) has been studied since before the 1970s and may represent an advantage over extracellular lipases, although the use of these lipases in crude extracts can be helpful for several industries cause is often more stable than purified preparations of lipases [8;9]. MBL is economically attractive because it does not require purification or immobilization [3]. This preparation of lipase can be used as suspended free cells or as a whole-cell biocatalyst [9], and it shows excellent prospects for numerous applications [10].

Among the diverse applications involving lipases can highlight the hydrolysis and subsequent enrichment of vegetable oils. The free fatty acids (FFA) and partial glycerides obtained can be employed as intermediates to produce esters with higher added value than the triglycerides present in the starting material [11]. Examples of applications of such a class of compounds are the synthesis of food additives [12;13], flavors and fragrance esters [14;15], and biosensors [16;17]. Considering that MBL can be used with almost no downstream operations, employing them in the hydrolysis stage of vegetable oils is suggested to reduce the total cost of catalyzed reactions involving lipases obtained from filamentous fungi [8].

Some recent studies (Marotti and coauthors [18] and Lima and coauthors [19]) have already demonstrated the potential of MBL from filamentous fungi in the biotransformation of vegetable oils. All these mentioned works studied the production and partial biochemical characterization, which are mostly determinations of kinetic parameters (Michaelis constant [K_m] and maximum reaction rate [$V_{max.}$]) and physic-chemicals parameters (ideal pH and Temperature, deactivation constant [K_d] and half-life time [$t_{1/2}$] on multiple temperatures) lipases produced from the genus *Penicillium*. The biochemical characterization may be a form to identify different lipases produced by the same microorganism and select one type and each most adequate use.

Lima and coauthors [19] presented *P. citrinum* URM 4216 isolate as a great producer of MBL, with intracellular activity (272 U/g of dry cells using olive oil as an inducer) and high thermal stability (half-life time of 1.8 h at 60°C). *Penicillium* is recognized for its ability to secrete various enzymes, and it is therefore of interest to explore the potential of each isolate [20], which can be extended to other genera like *Aspergillus* and *Trichoderma*.

Aspergillus niger produces a variety of extracellular lipases with good biological properties and industrial applications. The U.S. Food and Drug Administration generally recognizes these lipases as safe [21] due to the practical use and industrial production of foods and beverages such as cheese, miso, and sake [22]. A few recent studies have explored extracellular lipases [23] (production and determination of the effect of pH and Temperature on enzyme activity) and the production of MBL for enzymatic synthesis of biodiesel [24; 25] by *A. niger*. All those studies previously cited [23-25] produced lipases via submerged fermentation.

The species *Trichoderma koningii* can be a filamentous fungus able to produce MBL at a low cost and with interesting properties. In the literature, there is a need for more publications about the production and determinations of biochemical parameters of lipases obtained from *T. koningii*. The production and

characterization of ideal pH and Temperature of extracellular lipases by *T. koningii* via submerged culture have already been investigated in a master's thesis by Marques [26]. Dairy effluents as a culture medium have increased lipolytic activity (11 U/mL to 1327 U/mL) when supplemented in the culture medium with olive oil, Tween 80, and basic Vogel medium.

Furthermore, this present work aimed to evaluate the production of intracellular lipases (MBL) and extracellular lipases (crude extract) produced by three different microorganisms (*A. niger*, *T. koningii*, and *Penicillium spp.* F02) and determine the effect of Temperature, pH, and substrate concentration on lipase activity. The total absence of studies on MBL produced by the species *T. koningii* and the discovery of new biocatalysts produced by microorganisms isolated from oily residues (case of *Penicillium*) are plausible justifications for the realization of this study. Still, there are a few studies on the biochemical characterization for multiple forms of lipases produced by the same microorganism.

MATERIAL AND METHODS

Microorganisms

Penicillium spp. F02 was isolated from the scum of the grease trap and was provided by the Sanitation Laboratory of the Department of Environmental Engineering of the Federal University of Espírito Santo (LABSAN/DEA/Ufes). *A. niger* [National Institute of Quality Control (INCQS) 40018] and *T. koningii* (INCQS 40331) were obtained from the Oswaldo Cruz Foundation. All microorganisms were stored in Petri dishes with Potato Dextrose Agar (PDA) at 4°C and frozen in glycerol solution at 20 % (-80°C).

Preparation of the inoculum

The microorganisms were cultivated in PDA (4.2 g/L) at 28°C. After 5 days, the spores were scraped into 10 mL of Tween 80 solution (0.1% v/v) and were counted in the Neubauer chamber [27].

Culture medium

The medium for lipase production was composed of bacteriological peptone (3 g/L), yeast extract (0.5 g/L), NaCl (0.5 g/L), commercial olive oil with low acidity content (30 g/L), KH_2PO_4 (0.1 g/L) and $MgSO_4.7H_2O$ (0.05 g/L) [18; 28].

Materials

Bacteriological peptone and yeast extract were purchased from Himedia® (India). NaCl, $MgSO_4.7H_2O$, and KH_2PO_4 were analytical grades.

Submerged culture

Batch fermentations were conducted in Erlenmeyer flasks (250 mL) containing 100 mL of the culture medium. The flasks were autoclaved (121°C / 20 minutes) and manipulated in laminar flow for inoculum addition to obtain a final concentration of spores equal to 10^6 spores/mL [28]. The trials were performed on an orbital shaker at 200 rpm and maintained at 28°C. After 96 h of incubation, the media were vacuum filtered in a Buchner filter using a paper filter of 80 g/m² (Whatman, UK). The mycelium was washed with pure acetone to remove residual olive oil.

Analysis

Lipase's activity

The activities of intracellular lipase (mycelium) and extracellular lipase (filtrate/crude extract) were performed by the hydrolysis method of olive oil emulsion [29;18].

The substrate was prepared by an emulsion of 10 mL of olive oil and 90 mL of an aqueous solution of Arabic gum (30 g/L), vigorously stirred in a mixer for 3 minutes. In Erlenmeyer, flasks of 125 mL were added: 5 mL of the substrate, 4 mL of sodium phosphate buffer solution 10mM (pH 7.0), and 0.3 g of mycelium (wet base) or 0.5 mL of the crude extract. In the blank trials, the enzyme was replaced by 0.5 mL of sodium phosphate buffer. The reactional flasks were incubated at 37°C for 15 minutes on an orbital shaker (100 rpm).

After the incubation period, the reaction was stopped by the addition of 10 mL of a 1:1 solution of acetone and absolute ethanol. Free fatty acids were titrated with a standard solution of KOH 20mM, using

phenolphthalein as an indicator. One unit of lipase (U) was defined as the amount of enzyme [dry mycelium or crude extract] that releases 1 µmol of free fatty acids per minute of reaction under the assay conditions.

Dry biomass

The dry biomass concentration (DBC) of microorganisms was determined by dry weight every 24h at 80°C.

Effect of pH and Temperature on enzyme activity

The effect of Temperature and pH on lipolytic activity (response variable) was investigated by a complete face-centered experimental design 2^2. Tree repetitions were performed at the central point (CP). Table 1 presents the levels of the variables studied. The experimental design results were analyzed using Statistica trial version 14.0 (StatSoft Inc., USA), and the values were considered significant when p-value<0,10.

Table 1. Variables and levels used in the experiments

Variables/Levels	-1	0*	+1
Temperature (°C)	30	37	44
pH	6.0	7.0	8.0

*Central point

Effect of substrate concentration

To determine the K_m and the $V_{max.}$ emulsified media (Arabic gum 30 g/L and commercial olive oil) were prepared, with mass proportions of olive oil ranging from 2.5 to 30%, equivalent to a concentration of 93 to 1114mM of fatty acids [20].

RESULTS

Table 2 presents the DBC and lipolytic activities attained for each filamentous fungi cultivated via submerged fermentation. Notably, the results of extracellular activity (EA) and intracellular activity (IA) are different since the extracellular activity was measured by mL of broth and intracellular activity per gram of microorganisms.

The highest DBC (Table 2) for all strains was achieved after 96h of incubation at 28°C and using 30 g/L of olive oil as a carbon source. *T. koningii* showed the highest DBC, equal to 19.3 g/L, while *A. niger* demonstrated the lowest, only 8.6 g/L. About the EA kinetics of lipases (Table 2), it is noted that *A. niger* presented the maximum lipase activity among all experiments after 96h of incubation (10.6 U/mL). Although, *T. koningii* had a slightly lower enzyme production (9.1 U/mL) in the same period.

Table 2. Dry biomass concentration and lipolytic activities were attained for each microorganism studied under submerged fermentation up to 96 hours of incubation

	Aspergillus niger			*Trichoderma koningii*			*Penicillium spp.* F02		
Fermentation time (hours)	EA	IA	DBC	EA	IA	DBC	EA	IA	DBC
0	NP	NP	0.05	NP	NP	0.11	NP	NP	0.04
24	3.8	9.4	2.1	1.1	42.9	7.1	5.6	7.8	2.3
48	4.4	23.7	6.9	4.3	44.8	12.1	6.4	19.3	10.6
72	5.1	31.4	7.7	8.1	69.8	13.5	7.6	26.2	13.7
96	10.6	71.6	8.6	9.1	81.5	19.3	6.7	40.9	15.9

NP = not performed, EA = Extracellular activity of lipase (U/mL of crude extract), IA = Intracellular activity of lipase (U/g of dry mycelium), DBC = Dry biomass concentration (g/L)

Figures 1 and 2 represent the effect of substrate (olive oil emulsion) on reaction rates presented by intracellular and extracellular lipases produced in this study under submerged fermentation, respectively. Table 3 demonstrates the biochemical parameters obtained in this study for all lipases produced.

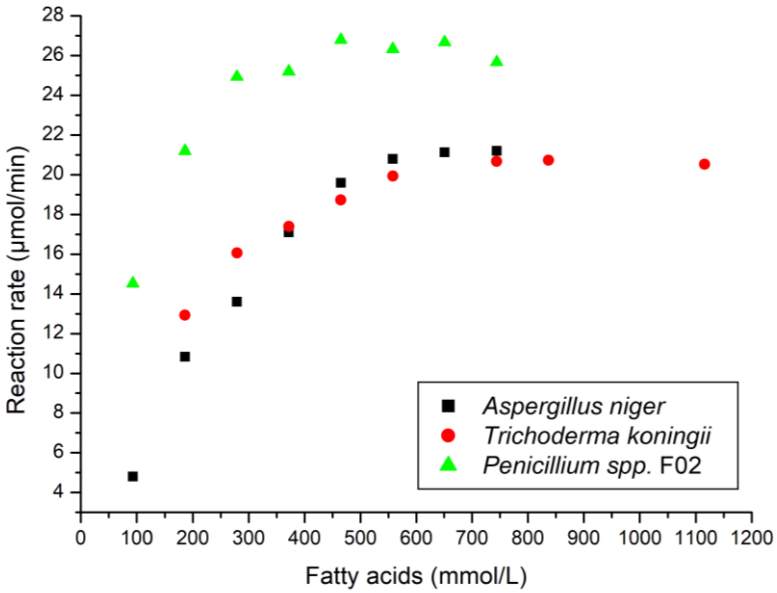


Figure 1. Profile of reaction rates of intracellular lipases produced under submerged culture

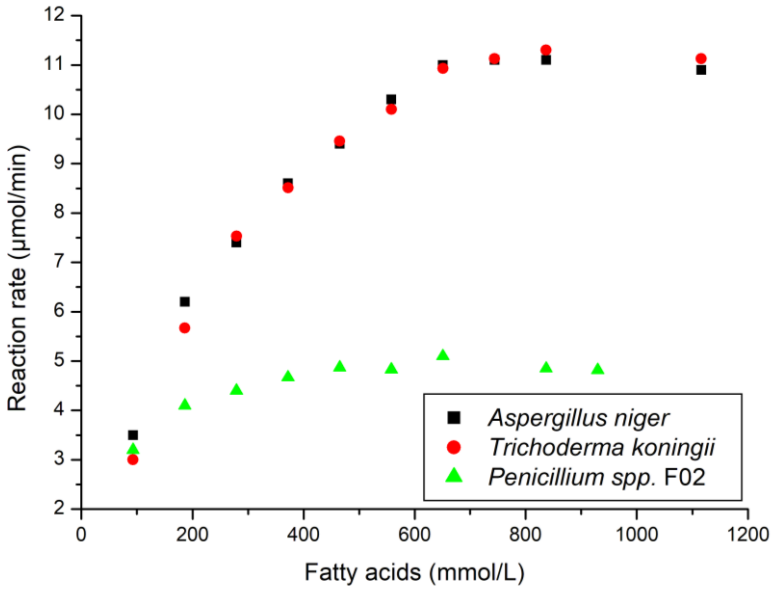


Figure 2. Profile of reaction rates of extracellular lipases produced under submerged culture.

Table 3. Biochemical parameters (K_m and $V_{max.}$) for intracellular and extracellular lipases produced in this study.

	K_m	$V_{max.}$	R^2
IL *A. niger*	380.2	33.7	0.9689
EL *A. niger*	307.1	15.7	0.8827
IL *T. koningii*	153.5	24.5	0.8465
EL *T. koningii*	421.1	17.9	0.9379
IL *Penicillium spp.*	103.6	32.2	0.9182
EL *Penicillium spp.*	67.5	5.6	0.9676

IL = Intracellular lipase, EL = Extracellular lipase

The K_m and $V_{max.}$ were assessed by the Eadie-Hofstee plot method [31]. The linear adjustment (Table 3) showed that the experimental fits well in the model of Michaelis-Menten [19]. The intensification in the concentrations of the fatty acids in the substrate from 93 to 465mM resulted in a considerable increase in the reaction rates (Figure 1 and Figure 2), and for substrate concentrations superior to 558mM, it is noted that there is a plateau on the profile of reaction rate, which can indicate a probable saturation of the enzyme by high concentrations of olive oil emulsions (relative to interval studied).

Table 4 shows the results of each run proposed on experimental design to determine the effect of studied factors on lipase activity. The highest values of IA were observed in run 9 (82.6 U/g) and run 8 (81.4 U/g).

For EA, the best result was found in run 8 (13.2 U/mL). Table 5 shows the residual errors calculated for intracellular and extracellular lipase activity. For the statistical analyses of EA, run 6 was disregarded since the residual error calculated for this run was higher than the other runs.

Table 4. Results of the study of pH and Temperature on activity of lipases produced by *T. koningii* (IA and EA)

Run	Temperature	pH	IA	EA
1	-1 (30)	-1 (6.0)	22.8	2.3
2	0 (37)	-1 (6.0)	18.7	8.0
3	+1 (44)	-1 (6.0)	8.4	3.5
4	-1 (30)	0 (7.0)	68.8	8.4
5**	0 (37)	0 (7.0)	69.8	8.4
6	+1 (44)	0 (7.0)	62.1	12.7*
7	-1 (30)	+1 (8.0)	75.9	12.8
8	0 (37)	+1 (8.0)	81.4	13.2
9	+1 (44)	+1 (8.0)	82.6	10.5
10**	0 (37)	0 (7.0)	71.4	9.9
11**	0 (37)	0 (7.0)	70.7	9.2

*Disregarded statistical analyses, **Central points

Table 5. Observed, Predicted and Residual Values for statistical analyses on activity of lipases produced by *T. koningii*

	IA			EA		
Run	O	P	RV*	O	P	RV*
1	22.8	23.029	-0.229	2.30	2.827	-0.527
2	18.7	19.191	-0.491	8.00	5.33	2.67
3	8.4	7.68	0.721	3.50	5.643	-2.143
4	68.8	68.658	0.142	8.40	8.53	-0.13
5	69.8	70.095	-0.295	8.40	10.158	-1.758
6	**62.1**	**63.858**	**-1.758**	**12.7**	**9.597**	**3.104**
7	75.9	75.813	0.087	12.8	12.143	0.657
8	81.4	82.525	-1.125	13.2	12.897	0.304
9	82.6	81.563	1.034	10.5	11.46	-0.961
10	71.4	70.095	1.305	9.90	10.158	-0.258
11	70.7	70.095	0.605	9.20	10.158	-0.958

O = Observed, P = Predicted, RV = Residual values, *RV = O - P

A variance analysis (Table 6) was conducted to fit a mathematical model to the experimental data to identify an optimal region for the studied response. The following second-order polynomial equation can describe the predicted model.

Table 6. Analysis of variance (ANOVA) for response surface model obtained from experimental design for lipases from *Trichoderma koningii*.

	ANOVA EA				ANOVA IA			
	SS	df	F	p	SS	df	F	p
(1) T (L)	1.21*	1	2.147*	0.28*	34.56	1	53.72	0.018
T(Q)	13.192	1	23.417	0.04	37.294	1	57.97	0.017
(2) P (L)	85.882	1	152.453	0.007	6016.667	1	9352.332	0.001
P (Q)	0.713*	1	1.265*	0.378*	937.475	1	1457.216	0.001
1L x 2L	3.063*	1	5.436*	0.15*	111.302	1	173.009	0.006
Lack of Fit	6.435*	2	5.712*	0.149*	7.142*	3	3.701*	0.220*
Pure Error	1.127*	2	-	-	1.287*	2	-	-
Total SS	110.596*	9	-	-	7327.709*	10	-	-

T= temperature, P = pH, L = linear, Q= quadratic, IA = Intracellular activity, EA = Extracellular activity, SS = Sum of Squares, df = degree of freedom, * = not significant statistically

The ANOVA for the multiple forms of lipases from *T. koningii* was obtained from the experimental design given in Table 6. ANOVA confirmed the acceptability of the quadratic model and explained whether this model satisfactorily fitted the variation observed in lipase production with the designed level of variables. As the F-test for the model was significant at the 10% level ($p < 0.10$), this model was considered fit and could effectively explain the variation observed.

The regression equations which generated each response surface are presented below (Eq. 1 and Eq. 2). Also, the equations presented the coefficients of determination (R^2), which present the number of experimental observations is satisfactorily explained by the statistical model. It is worth mentioning that the validity of the models is restricted to the conditions of the experimental design ($30 < T < 44^oC$ and $6.0 < P < 8.0$).

$$IA = -992.501 - 0.078 \times T^2 + 273.144 \times P - 19.251 \times P^2 + 0.758 \times T \times P \quad (1)$$

$$R^2 = 0.99$$

$$EA = -88.504 + 4{,}705 \times T - 0.053 \times T^2 \quad (2)$$

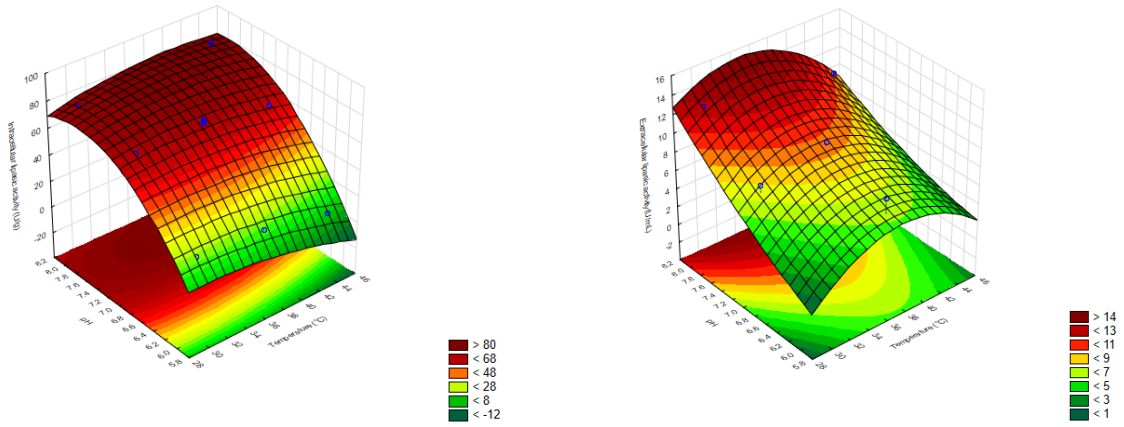
$$R^2 = 0.84$$

Were,

IA = Intracellular lipolytic activity in U/g

EA = Extracellular lipolytic activity in U/mL

The surface response obtained is presented in Figure 3. For extracellular lipases, it was observed that the response surface (Figure 3b) presented a saddle shape. On the only identified publication about the biochemical characterization of *T. koningii* [26] was determined that the optimal temperature and pH conditions for the enzymatic extract of *T. koningii* were 37°C and pH 8.0, which is like behavior presented by the response surface obtained in this study.



(a) (b)

Figure 3. Response surfaces were obtained from the face-centered experimental design 2^2. a) Surface response to intracellular lipolytic activity and b) Response surface to extracellular lipolytic activity

Table 7 shows statistically significant effects of studied variables for lipases produced by *T. koningii* under submerged culture. For IA, all factors (linear, quadratic, and interaction) were considered statistically significant. In contrast, only the factors quadratic temperature and linear pH presented a p-value lower than 0.10 for extracellular activity. This difference demonstrates the particularity of each of the forms of lipase studied.

Table 7. Statistically significant effects for intracellular and extracellular activities of *Trichoderma koningii*

	IA			EA		
Factor	**Effect**	**SD**	**p-value**	**Effect**	**SD**	**p-value**
Mean	54.704	0.255	0.000	8.171	0.249	0.001
(1) Temperature (°C) (L)*	-4.823	0.661	0.018	-	-	0.276
Temperature (°C) (Q)**	3.816	0.508	0.017	2.590	0.524	0.039
(2) pH (L)	63.347	0.661	0.000	7.600	0.600	0.006
pH (Q)	19.251	0.508	0.001	-	-	0.369
1L x 2L	10.611	0.809	0.006	-	-	0.142

L = linear; Q = quadratic, SD = standard deviation, - = not significant statistically

DISCUSSION

The *Penicillium spp.* presented a slight reduction in the EA of lipase after 72h of fermentation (Table 2). This can be enlightening, considering that it may have occurred in the production of proteases. The proteases may have been produced when the nutrients are near to end. In this case, these enzymes' actuation can be deactivated as part of the extracellular lipases. In this case, it is suggested that an assessment in future works with this species short time fermentation to prevent the formation of secondary metabolites and save energy, considering expected large-scale production [23].

For IA kinetics of lipases (Table 2), the highest value was observed for *T. koningii* at 96 h (81.5 U/g). A similar IA of 81.6 U/g of dry cells was obtained by Marotti and coauthors [18] using the microorganism *P. citrinum* CCT3281 under submerged culture (30°C, 200 rpm, and 72h) and olive oil (30 g/L) as a carbon source. In a submerged culture of *Mucor circinelloides* and using olive oil (30 g/L) as a carbon source, Andrade and coauthors [29] obtained a maximum IA of 66.8 U/g of cells. All previously mentioned studies measured the lipase activity by the hydrolysis method using olive oil emulsion as substrate, and the mentioned essays are conducted on identical conditions of Temperature and pH in this present work (37°C and pH 7.0).

Trichoderma presented differentiated dynamics of production of MBL (Table 2) because in their initial 24 h, the IA is about 35% of the maximum, only reached after 96h of fermentation. This fact indicates a tendency of this microorganism to retain lipases in the mycelium and not to secrete this enzyme into the environment, which can be proven by a low relative EA presented by *T. koningii* (1.1 versus 3.8 and 5.6 U/mL). Additionally, the highest production of MBL (42.9 U/g) and a significant increase in DBC on the first day of fermentation (0.11 to 7.1 g/L) can be evidence that *T. koningii* can be a good producer of MBL.

Lima and coauthors [19], with *P. citrinum* URM 4216 and using olive oil (30 g/L) as a carbon source, 10^7 spores/mL, and with a time of 96h, found an EA of lipase in the culture broth equal to 11 U/mL for olive oil [19]. Lima and coauthors [23] conducted a study with a wild strain of *A. niger C*, which showed an EA of lipase equal to 13.12 U/mL for olive oil. The submerged fermentation was conducted with the following conditions: 15.0 g/L of sucrose; 4.0 g/L of $(NH_4)_2SO_4$; 4.0 g/L de soybean oil; 1.0 g/L of yeast extract and pH 7.0. Remarkably, the different conditions of the assays of determination lipase activity cannot allow direct comparations.

Considering the promising results obtained in the stage of production of lipases by *T. koningii*, along with the total absence of publications on intracellular lipases or about the biochemical characterization of multiple forms of lipases of the mentioned species, the study of the effect of the Temperature and pH on lipase activity was only performed in the present study for lipases from *T. koningii.* Besides that, in previous work [34] (a review on biochemical characterization of lipases from filamentous fungi) was presented that the genera *Aspergillus* and *Penicillium* are more characterized in literature than genus *Trichoderma* and optimal temperatures are typically between 35 °C and 60 °C and for optimal pH is generally range to 6.5 and 7.5. These are reasonable justifications for excluding the essays of lipase optima's pH and Temperature for genera *Aspergillus* and *Penicillium* in the present study.

Santos and coauthors [2014] evaluated a $V_{max.}$ equal to 27.6 µmol/min for EL produced by *A. niger*, with olive oil as substrate [32]. K_m values equal to 117 and 77mM for lipases produced by *A. niger*, with the emulsion of commercial olive oil as substrate, were reported by [32] and [33], respectively. Regarding *T. koningii*, no studies were found in the literature on determining kinetic parameters.

Only the parameter K_m is independent of the amount of enzyme present in the reaction. The smaller K_m was calculated to EL of *Penicillium spp.* (67.5mM). A small K_m indicates that (for the same concentrations of the substrate) the enzyme can catalyze the transformation of this substrate at a higher rate than when the K_m is large.

The effect of the linear pH factor on intracellular activity (Table 7) is about 13 times higher when compared to the effect of the linear temperature factor and indicates a better influence of the pH factor on intracellular lipolytic activity. The response surfaces were presented in Figure 3 and presented a similar behavior regarding the location of zones of maximum lipolytic activity (intense red color). It is noted that for intracellular activity (Figure 3a), the increase in pH and the maintenance of reactional temperatures around the central point led to an increase in the response, noticed by the intense red color zone. The set of conditions formed by temperatures slightly higher than 37°C (39°C) and pH slightly below 8.0 (pH 7.86) maximized intracellular lipolytic activity (maximum predicted is 83.4 U/g).

MBL properties desirable for biocatalysts are mainly great hydrolysis activity and good esterification and transesterification capacity in organic media [16]. Only the hydrolytic activity of triacylglycerol was identified in this study. It is a clear the need for new efforts to prove the ability of the MBL produced by *T. koningii* for industrial applications, but the results obtained are hopeful for future studies. In this, new strategies can be

used. One of them is to evaluate the delipidated biomass, which in the study conducted by Romano and coauthors [30] presented *A. flavor-furcations* as the most promising microorganism in screening from seven isolates from Amazonian Forest. This mentioned species showed high synthesis activity of ethyl palmitate by transesterification in an organic medium (668.5 mU/g), with a great prospect in the pharmaceutical and biodiesel industries [30].

It was presented in a highly cited study (19 times cited on Google Scholar) [35] that an isolated identified as *Aspergillus westerdijkiae* has shown no lipolytic activity (titrimetric method) on the culture filtrate. Only the mycelium showed lipolytic activity, and this form of lipase is classified as an inexpensive alternative for biodiesel production. This work observed that all fungi presented hydrolysis activity on mycelium and in the filtrate. This fact can reinforce the importance of promoting new studies about lipases produced by species that were not being studied, like the *T. koningii* and the new isolate *Penicillium spp.* F02.

To explore the full potential of multiple forms of lipase produced by *T. koningii* and the other microorganisms evaluated in this study, it is suggested to investigate the potential recyclability of MBL, inhibitors, and activators (molecules that interfere in the catalysis) of lipolytic activity, operational stability in organic solvents [35] and substrate specificity to diverse vegetable oils [18] to determine the application of these low-cost enzymes in the hydrolysis and biotransformation of vegetable oils.

CONCLUSION

All the filamentous fungi studied (*A. niger*, *Penicillium spp.*, and *T. koningii*) presented both intracellular and extracellular lipolytic activities of hydrolysis. The best results are obtained for *T. koningii* (81.5 U/g) and *A. niger* (10.6 U/mL), both were attained at 96h. This study was the first at describes the MBL of the species *T. koningii.* Their profile of intracellular activity suggests a strong and relatively fast accumulation of lipases in the mycelium, which can indicate that this microorganism is an excellent candidate to be a producer of MBL. K_m values range from 67.46mM to 421.07mM and for $V_{max.}$ A variation of 5.56 to 33.68 U was observed. Statistical analysis indicated that the reactional temperatures in the range of 37 to 44°C and the pH from 7.8 to 8.0 favored the enzymatic activity of lipases produced by *T. koningii*. More studies are needed, but the results are hopeful to use the MBL and crude extract of *T. koningii* in the enzymatic hydrolysis of vegetable oils to produce FFA or partial glycerides at low cost considering the high relevance of these in multiple industrial applications.

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