Production And Partial Characterization Of a Thermostable, Alkaline And Organic Solvent Tolerant Lipase From Trichoderma atroviride 676

Thiago Andrade Marques, Cristiani Baldo, Dionísio Borsato, Joao Batista Buzato, Maria Antonia Pedrine Colabone Celligoi

Abstract: The several industrial applications of lipases have stimulated interest in isolation of new enzymes from novel sources. In this study, the lipase production from *Trichoderma atroviride* 676 was optimized by statistical design methods. The response surface methodology based on 3(3-1) fractional factorial, showed that the yeast extract and $MgSO_4$ played a significant role on lipase production, yielded an enzymatic activity of 101.75 U/ml. Using Box-Benhken design, the optimization of the temperature levels, pH and agitation rate resulted in the maximum enzyme production of 175.20 U/ml, obtained at 28 ºC, pH 6.0 and 105 rpm. The enzyme was optimally active at pH 8.0 and 35ºC, and was stable at pH 3.0-8.0 and temperature of 25ºC-75ºC. In addition, the lipase was highly stable on non-polar hydrophobic solvents as kerosene, n-dodecane and hexane. The new lipase from *T. atroviride* 676 could be considered a potential candidate for industrial and biotechnological applications.

Index Terms: fermentation, lipase, optimization, organic solvents, thermo-alkaline, *Trichoderma atroviride* 676.

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1 INTRODUCTION

Lipases (EC 3.1.1.3) are ubiquitous hydrolytic enzymes which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids over an oil–water interface. Indeed, lipases also catalyze the hydrolysis, transesterification and synthesis of other esters and exhibit enantioselective properties. Due to the capacity of lipases to catalyze precise chemical transformation, they have progressively widespread in the detergent, cosmetic, food, organic synthesis, and pharmaceutical industries [1], [2], [3].The numerous industrial applications of lipases have stimulated interest in isolation of new lipases from novel sources and strong efforts have been concentrated on the engineering of enzymes with specific properties or better performance for industrial applications. In this sense, lipases from microorganisms have gain great attention of researchers and industries, due to the great variety of catalytic activity, higher yields, ease of genetic manipulation and no seasonal fluctuation [4]. The filamentous fungi are considered the best producers of extracellular lipases for large scale production [5].

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Trichoderma sp. has been reported as a producer of multiple enzymes, including cellulose, xylanase and β- glucosidase [6], [7], [8], [9]. However, the lipase production by Trichoderma sp. is little explored [10]. In this paper, we report the production and the partial biochemical characterization of a thermostable, alkaline and organic solvent tolerant lipase from a newly isolated *T. atroviride* 676 strain. The optimization of the enzyme production was carried out by statistical approach using the response surface methodology (RSM). This tool has been used by various researchers for optimization of culture conditions and present great advantages compared to conventional methods that fail to consider the interaction of different factors involved on the enzyme production [11], [12]. To our knowledge, this is the first report on the production of an organic solvent tolerant, thermostable and alkaline lipase from *T. atroviride* 676.

2 MATERIALS AND METHODS

2.1 Microorganism and inoculum preparation

T. atroviride 676 (IOC 4503) was originally isolated from Amazon forest soil and are available at Coleção de Culturas de Fungos Filamentosos, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil. The strain was grown in a potato dextrose agar medium (PDA), in Erlenmeyer flasks (125 ml), at 28 ºC for 4 days, using an inoculum of 10^6 spores/ml. The strain was subculture every 15 days, and preserved at 4 ºC.

2.2 Fermentation medium

The lipase production by *T. atroviride 676* was investigated employing the Vogel minimal salts medium supplemented with Tween 80 (0,5% v/v), containing olive oil at a concentration of 1% (v/v) and different concentrations of glycerol, olive oil, tween 80 and Vogel's medium, according to statistical design of experiments. The pH was adjusted to 6.0 and then sterilized at 121°C, for 15 minutes. The cultures were incubated at 120 h, 28 ºC, under orbital shakers at 120 rpm. Parameters such as pH, temperature and agitation rate also varied according to experimental designs. One percent (1 % v/v) of the prepared inoculum was added to each 25 ml of medium in Erlenmeyer flasks (125 ml). The cell-free supernatant was used as a source of extracellular lipase and the biomass concentration (dry cell weight) was quantified gravimetrically.

2.3 Optimization of lipase production by statistical approach

The influence of medium composition and the physical parameters of fermentation process on lipase production was evaluated by two experimental designs: $3^{(3-1)}$ fractional factorial and Box-Benhken. In both cases, the concentration of olive oil was fixed at 1% (v/v). In the first one, 3 variables at 3 levels in 12 experiments, including 4 center points were studied. The independent variables were: yeast extract (0, 15, 30 g/l), $(NH_4)_2SO_4$ (0.5, 2.5, 4.5 g/l) and $MgSO_4.7H_2O$ (0.5, 2.0, 3.5 g/l). In the second experimental design, 15 experiments, including 3 center points, with 3 variables at 3 levels were used. The independent variables were: temperature (21; 28 and 35 °C), pH (4.0; 6.0 and 8.0) and agitation rate (90; 120 and 150 rpm). The enzymatic activity was taken as the response of the design (U/ml). Both factorial designs were based on the quadratic model (Eq.1):

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x^2{}_{ii} + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the response variable (lipase productivity), β_0 is the intercept, β_i is the coefficient for the linear effect, β_{ii} is the coefficient for the quadratic effect, β_{ij} is the coefficient for the interaction effect and *x* is the coded level of the variable. For model validation the lipase activity were estimated and compared with the predicted values. Results were assessed by an analysis of variance (ANOVA) and multiple regression analysis at the 5 % significance level using Statistica 9.0 software.

2.4 Determination of lipase activity

The lipase activity was assayed using *p*-nitrophenyl-palmitate (*p*NPP) as substrate, according to Winkler e Stuckmann [13]. The cell-free supernatant was incubated with *p*NPP (8.0 mM dissolved in isopropanol), 50mM Tris-HCl buffer (pH 8.5) containing Triton X-100 (0.4 % w/v), at 37 °C, for 10 minutes. The release of *p*NPP was measured at 410nm. One unit of enzyme activity (U/ml) was defined as the amount of enzyme required to release 1μmol/min de *p*-NPP.

2.5 Effect of pH and temperature on crude lipase activity

The optimum temperature for the lipase was determined by incubating the enzyme with the substrate (*p*-NPP) at temperatures ranging from 25 to 75°C, in 50 mM Tris buffer, pH 8,5 for 10 minutes. The thermal stability was studied by incubating the enzyme at each desired temperature for 2 hours. The optimum pH for the crude lipase was determinate by incubating the enzyme with the substrate (*p*-NPP) at various pH ranging from 6.0 to 9.0 at optimized temperature value in the following buffers: 50mM citrate phosphate buffer (pH 6.0-6.5), 50mM Tris buffer (pH 7.0-9.0), for 10 minutes. The pH stability was studied by incubating the lipase at each desired pH, and then measuring the lipase activity after 2 hours. The lipase activity was measuring according to Winkler e Stuckmann [13].

2.6 Effect of detergents and organic solvents on crude lipase activity

The stimulatory or inhibitory effect of organic solvents and detergents on the enzyme activity was carried out by the addition of 10% (v/v) of isopropanol, ethanol, acetonitrile, acetone, hexane, n-dodecane, kerosene, Tween 80 and Triton X-100 on the enzyme solution, for 2 hours, at 30°C. After incubation, the residual activity of the enzyme was measuring according to Winkler e Stuckmann [13].

3. RESULTS AND DISCUSSION

3.1 Optimization of culture conditions

The RSM, an effective and quick experimental design tool, was apply to define the optimal conditions for lipase production by *T. atroviride* 676. In the first experimental design, the effect of yeast extract, $(NH_4)_2SO_4$ and $MgSO_4$ on lipase production were investigated. The lipase production (i.e. the response) for each individual run is shown in Table 1. The lipase activity ranged from 1.92 to 108.70 U/ml, and the highest enzymatic activity (101.75 U/ml, average of center points) occurred at 15 g/l of yeast extract; 2.5 g/l of $(NH_4)_2SO_4$ and 2.0 g/l of $MgSO_4$. The lowest production was observed in run 3 (with no yeast extract; 4.5 g/l of $(NH_4)_2SO_4$ and 2.0 g/l of $MgSO_4$). The regression analysis was carried out to fit the response function (lipase production) with the experimental data. Yeast extract and $MgSO_4$ stimulated the lipase production, while $(NH_4)_2SO_4$ showed a negative effect. Only the variable X_1 (p= 0.0014) and the interaction between X_1 and X_2 (p= 0.0394) significantly influenced the enzyme synthesis (Table 2). In agreement with these results, Sifour and co-workers [14] also related that $MgSO_4$ and $(NH_4)_2SO_4$ had no significant effect on lipase production by *G. stearothermophilus*. On the other hand, Hasan-Beikdashti and co-workers [12] described the positive effect of yeast extract, which constitute a nitrogen source and a supplier of vitamins, on lipase production from *Stenotrophomonas maltophilia*. The positive effect of yeast extract and the inhibiting effect of $(NH_4)_2SO_4$ on lipase synthesis by *Pseudomonas gessardii* also was described [15]. Based on the experimental results, the following second order polynomial equation was obtained (Eq. 2):

$$Y_1 = 101.75 + 23.11\,x_1 - 1.40\,x_2 + 2.67\,x_3 + 11.01\,x_1x_2 - 55.55\,x_1^2 - 6.89\,x_2^2 - 23.38\,x_3^2 \quad (2)$$

where Y_1 is the lipase activity response, x_1, x_2 and x_3 are the codified variables, yeast extract, $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$, respectively. The lack of fit (p= 0.42) was not significant, and the high coefficient of determination obtained (R^2 = 0.99) point to the accuracy of the model.

Table 1. Fractional factorial design to investigate the effect of yeast extract, $(NH4)_2SO_4$ and $MgSO_4$ on lipase production by *T. atroviride* 676, showing the real and coded values along with the experimental responses.

Runs	X_1	X_2	X_3	Y_1	Y_2
	Yeast extract (g/l)	$(NH_4)_2SO_4$ (g/l)	$MgSO_4$ (g/l)	Lipase activity (U/ml)	Biomass (g/l)
	0 (-1)	0.5 (-1)	0.5 (-1)	2.56	12.00
2	0 (-1)	2.5 (0)	3.5 (+1)	4.24	24.40
3	0 (-1)	4.5 (+1)	2.0 (0)	1.92	19.20
4	15 (0)	0.5 (-1)	3.5 (+1)	73.70	10.40
5	15 (0)	4.5 (+1)	0.5 (-1)	69.26	32.40
6	30 (+1)	0.5 (-1)	2.0 (0)	54.67	18.80
7	30 (+1)	2.5 (0)	0.5 (-1)	41.41	11.20
8	30 (+1)	4.5 (+1)	3.5 (+1)	51.33	32.40
9	15 (0)	2.5 (0)	2.0 (0)	97.48	27.60
10	15 (0)	2.5 (0)	2.0 (0)	99.70	26.00
11	15 (0)	2.5 (0)	2.0 (0)	108.70	16.40
12	15 (0)	2.5 (0)	2.0 (0)	101.11	17.60

The factors and levels of the variables were chosen based on literature review and on previous experiments (data not published), aiming to select important parameters for the lipase production.

Table 2. Regression analysis obtained from the $3^{(3-1)}$ fractional factorial showing the effect of yeast extract, $(NH_4)_2SO_4$ and $MgSO_4$ on lipase production by *T. atroviride* 676.

Factor	Coefficient	Error	t-value	p-value
Intercept	101.75	2.40	42.47	0.000002*
Yeast extract (X_1) (L)	23.11	1.96	11.82	0.0014*
Yeast extract (X_1) (Q)	-55.55	3.09	-17.96	0.0004*
$(NH_4)_2SO_4(X_2)$ (L)	-1.40	1.96	-0.72	0.5311
$(NH_4)_2SO_4(X_2)$ (Q)	-6.89	3.09	-2.23	0.1160
$MgSO_4 .7H_2O$ (X_3) (L)	2.67	1.96	1.37	0.2714
$MgSO_4 .7H_2O$ (X_3) (Q)	-23.38	3.91	-5.97	0.0098*
X_1X_2	11.01	3.09	3.56	0.0394*

*Lack of fit: p=0.42; R^2=0.99; *Significance at p<0.05.*

The three dimensional response surface was plotted to study the interaction among the selected variables and to determine the optimum levels of the variables to attaining maximum lipase production. The Fig. 1 shows that the maximum lipase production is situated in the center point of yeast extract and $MgSO_4$. The highest experimental production was 101.75 U/ml, occurring at 15 g/l of yeast extract; 2.5 g/l of $(NH_4)_2SO_4$ and 2.0 g/l of $MgSO_4$. The predictive model indicated 101.75 U/ml as the maximum theoretical production, at the conditions of the center points.

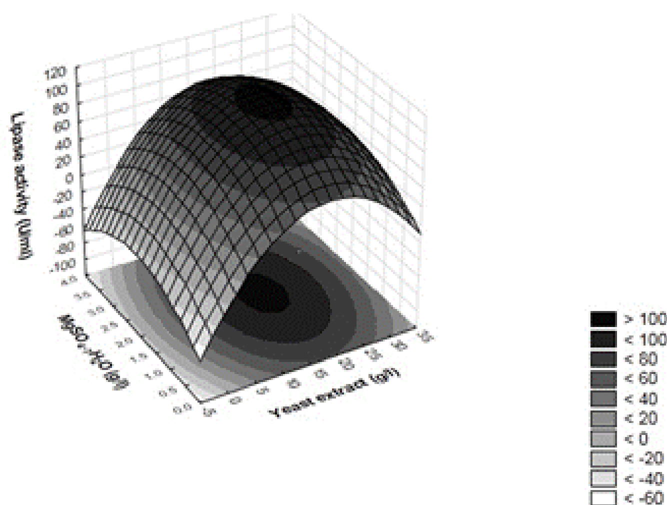

Fig. 1. Response surface plots showing the effect of yeast extract and $MgSO_4$ (g/L) on the lipase production by *T. atroviride* 676. The $(NH_4)_2SO_4$ concentration was fixed at 2.5 g/l

Searching for increase the lipase productivity by *T. atroviride 676*, the influence of temperature, pH and agitation on the enzyme production was evaluated by Box-Benhken design (Table 3). The highest enzymatic activity (163.01 U/ml, average of center points) occurred at 28 °C, pH 6.0 and 120 rpm. Statistical analysis showed that the temperature, pH and agitation had a significant effect on lipase production, as well as the interaction between X_1 (temperature) and X_3 (agitation). Among the variables studied, the temperature profoundly influenced the fermentative process (p= 0.0098) and presented a negative effect on the enzyme production (Table 4). Comparing runs 1 and 2 (Table 3), which had the same pH and agitation, is possible to observe that an increase of temperature from 21 ºC to 35 ºC resulted in the absence of enzymatic activity. According to the literature, the temperature is one of most critical parameters for lipase production and diverges according to the microorganism studied [14]. Experimental data from lipase production were analyzed in order to construct the second order polynomial equation (Eq.3):

$$Y_1 = 163.01 - 24.04\,x_1 - 22.31\,x_2 - 23.94\,x_3 + 6.67\,x_1x_2 + 18.20\,x_1x_3 + 3.17\,x_2x_3 - 79.20\,x_1^2 - 65.30\,x_2^2 - 32.84\,x_3^2 \quad (3)$$

where Y_1 is the lipase activity response, x_1, x_2 and x_3 are the codified variables, temperature, pH and agitation, respectively. The lack of fit (p= 0.059) was not significant, and the coefficient of determination (R^2) was 0.96, indicating that the proposed model can be used for predictive purposes. The three dimensional response surface showed sub-regions more suitable to high lipase production values with the variable x_1 = 0 and the variable x_3 = 0 in codified values (Fig. 2.). The greatest experimental production was 163.01 U/ml (28 ºC, pH 6.0 and 120 rpm) and the value predicted was 166.77 U/ml (28 ºC, pH 6.0 and 105 rpm). To confirm the validity of the statistical model, five repetitions of the predicted conditions were performed and the average production was 175.20 U/ml. This result did not show a significant difference from the predicted optimum value.

Table 3. Box Behnken design to investigate the effect of temperature (°C), pH and agitation (rpm) on the lipase production by *T. atroviride* 676, showing the real and coded values along with the experimental responses.

	X_1	X_2	X_3	Y_1	Y_2
Runs	Temperature (°C)	pH	Agitation (rpm)	Lipase activity (U/ml)	Biomass (g/l)
1	21 (-1)	4 (-1)	120 (0)	50.37	7.20
2	35 (+1)	4 (-1)	120 (0)	0	0
3	21 (-1)	8 (+1)	120 (0)	23.70	5.60
4	35 (+1)	8 (+1)	120 (0)	0	0
5	21 (-1)	6 (0)	90 (-1)	121.42	9,60
6	35 (+1)	6 (0)	90 (-1)	25.89	10.00
7	21 (-1)	6 (0)	150 (+1)	39.64	8.80
8	35 (+1)	6 (0)	150 (+1)	16.93	7.20
9	28 (0)	4 (-1)	90 (-1)	131.19	13.60
10	28 (0)	8 (+1)	90 (-1)	48.96	8.00
11	28 (0)	4 (-1)	150 (+1)	74.44	7.20
12	28 (0)	8 (+1)	150 (+1)	4.90	3.20
13	28 (0)	6 (0)	120 (0)	156.22	18.40
14	28 (0)	6 (0)	120 (0)	163.04	19.20
15	28 (0)	6 (0)	120 (0)	169.78	18.80

Table 4. Regression analysis obtained from the Box Behnken design to investigate the effect of temperature (°C), pH and agitation (rpm) on the lipase production by *T. atroviride* 676.

Factor	Coefficient	Error	t-value	p-value
Intercept	163.01	12.40	13.15	0.00004*
Temperature (X_1) (L)	-24.04	7.59	-3.17	0.0098*
Temperature (X_1) (Q)	-79.20	11.17	-7.09	0.0020*
pH (X_2) (L)	-22.30	7.59	-2.94	0.0114*
pH (X_2) (Q)	-65.30	11.17	-5.84	0.0029*
Agitation (X_3) (L)	-23.94	7.59	-3.15	0.0099*
Agitation (X_3) (Q)	-32.84	11.17	-2.93	0.0113*
X_1X_2	6.67	10.73	0.62	0.1881
X_1X_3	18.20	10.73	1.70	0.0330*
X_2X_3	3.17	10.73	0.30	0.4482

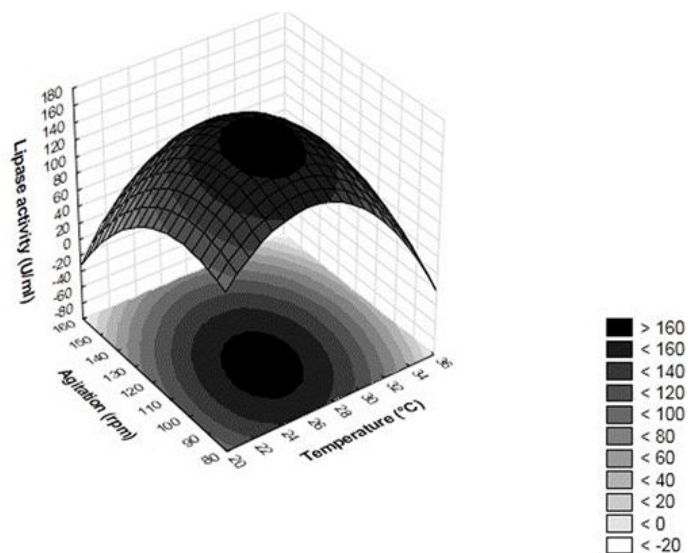

Fig. 2. Response surface plots showing the effect of temperature (°C) and agitation (rpm) on the lipase production by *T. atroviride* 676. The pH was fixed at 6.0.

3.2 Determination of optimum reaction conditions

3.2.1 Effect of pH and temperature on lipase activity and stability

The crude lipase was active in the studied range of temperature from 25 to 75ºC with higher activity at 35ºC. However, a decline in relative lipase activity was observed at temperatures of 55ºC, 65ºC and 75ºC with the relative activity of 21,95%, 15,55% and 9,25%, respectively (Fig. 3.). Similar results were observed for lipases obtained from others microorganisms showing the optimal temperature varying from 30ºC to 40°C [16], [17], [18], [19].The thermo stability assay showed that the lipase from *T. atroviride* 676 retained 95,5% and 78,9% of its relative enzymatic activity at 25ºC and 65ºC, respectively (Fig. 3.). The maximum relative activity of lipase was observed at pH 8.0, and an evident decrease of activity was detected at pH of 6.0, 7.0 and 9.0. In the pH stability studies, the lipase retained approximately 98% of its relative activity at abroad range pH values between 3.0-8.0 (Fig. 4.). A mild decrease of enzymatic activity was detected at pH 9.0. The maximum lipase activity obtained at pH 8.0 and the stability results are in agreement with the results obtained from lipases produced by *Aureobasidium pullulans* [18], Ralstonia sp [20], Geotrichum sp. [21], Pseudomonas sp. [22] and *Thermomyces lanuginosus* [23]. Lipases active and stable in alkaline media are very attracting and present great potential for application in the detergent industry. In this way, the lipase produced by *Acinetobacter radioresistens*, for example, has an optimum pH of 10 and it was stable over a pH range of 6–10 [24]. Horchani and co-workers [25], also reported the biochemical characterization of a thermoactive, alkaline and detergent-stable lipase from *Staphylococcus aureus*. In addition, alkaline and thermostable lipases could be used for synthesis of biodiesel and biopolymers and production of pharmaceuticals, cosmetics, flavors and agrochemicals [26]. In this sense, the lipase from *T. atroviride* 676 was stable in the broad range of temperature and pH and could be applied in wide industrial applications.

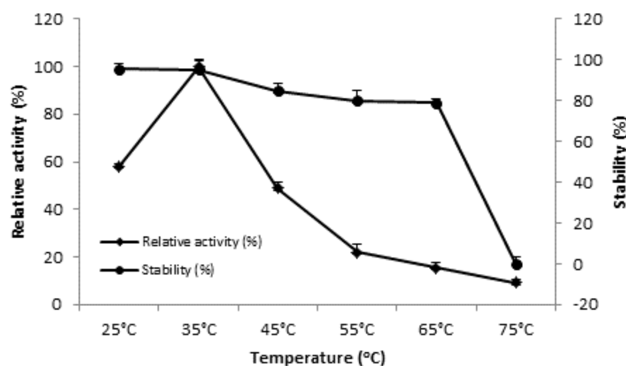

Fig. 3. Relative lipase activity and stability of lipase from *T. atroviride* 676 at different temperature. All measurements are the mean of three experimental data.

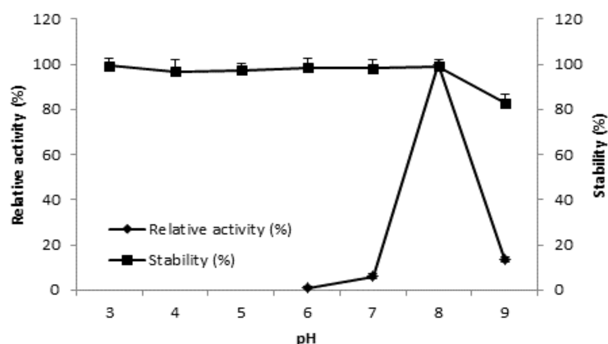

Fig. 4. Relative lipase activity and stability of lipase from *T. atroviride* 676 at different pH. All measurements are the mean of three experimental data.

3.2.2 Effect of detergents and organic solvents on crude lipase activity

The effect of different organic solvents on the stability of lipase from *T. atroviride* 676 is showed on Fig. 5. Interestingly, the non-polar hydrophobic solvents showed a strong stimulatory effect with relative activity of 194,24 % (kerosene), 190,29 % (n-dodecane) and 139,17 % (hexane). In contrast, the exposure to the hydrophilic solvents such as isopropanol, ethanol, acetone and acetonitrile showed a drastic reduction on the lipase residual activity. These results are in agreement with the general behavior of lipases in non-polar hydrophobic solvents [27], [28], [29], [30]. The increased of enzyme activity in presence of non-polar hydrophobic solvents may be due to the surface–solvent interaction leading to interfacial activation [31]. The high stability of the lipase from *T. atroviride* 676 in organic solvents makes it a potential candidate for organic syntheses applications. In this study, the addition of Tween 80 induced a mild decrease of the relative activity (96,32 %) and Triton X-100 did not modify the relative enzymatic activity (Fig. 5.). This agrees with findings of Castro-Ochoa et al. [32] that observed a decrease on lipase activity from *B. thermoleovorans* when incubated with Tween 80. In contrast, lipase from Streptomyces sp. showed an increase of enzymatic activity when incubated with Triton X-100, Tween 20 e Tween-80 [29]. The stability of lipase from *T. atroviride* 676 on surfactants is a desirable characteristic for detergents formulations purposes.

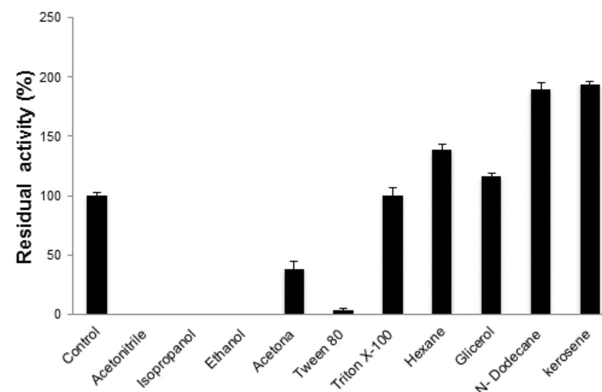

Fig. 5. Effect of organic solvents and detergents on lipase activity. Activity without organic solvents or detergents was set as 100% (control). All measurements are the mean of three experimental data.

4. CONCLUSION

In summary, the optimum conditions for lipase production by *T. atroviride* 676 were found to be temperature (28 ºC), pH 6.0 and agitation rate (105 rpm) reached a production of 175.20 U/ml. The productivity exhibited by *T. atroviride* 676 was greater than lipase activity described for other microorganisms such as *A. carneus* (12.7 U/ml) [20], *C. cylindracea* (20.26 U/ml) [11] and *R. arrhizus* (3.98 U/ml) [21]. The enzyme showed the maximum lipase activity at pH 8 and 35ºC, and was stable at pH 3.0-8.0 and temperature 25ºC-75ºC, and was highly stable on non-polar solvents as kerosene, n-dodecane and hexane. Considering the overall properties of the studied lipase, it may be used on different biotechnological applications such as organic solvent synthesis and detergents formulations. Consequently, new studies searching for the purification of lipase are essential for further applications of this promising enzyme.

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