PRODUCTION AND CHARACTERIZATION OF LIPASES FROM *Aspergillus flavus* ISOLATED FROM A SOIL SAMPLE OF THE AMAZON RAINFOREST

Abstract

Lipases (E.C.3.1.1.3) hydrolyze triglycerides producing fatty acids and glycerol and can be produced by animals, plants and microorganisms. These enzymes are used in the food industry, biofuels, waste treatment, cosmetics and fine chemicals. Due to the great diversity of microorganisms in the Amazon rainforest and the fact that little is known about them, the present study aimed to produce and characterize lipases from fungi isolated from soil samples from the Amazon rainforest. Therefore, initially the six isolates belonging to the Collection of Microorganisms of Medical Interest at INPA were identified at the genus level by micromorphological analysis. Submerged bioprocesses were performed to determine the best lipase producer. Sequencing of the ITS region of the rDNA was performed to identify the isolate highlighted in the production of lipases. Univariate experiments were carried out studying the influence of carbon sources, nitrogen, initial pH, agitation, and inoculum size on the production of lipases by the isolated isolate. Finally, the enzymes produced were submitted to semi-purification and determination of their optimal temperature, optimal pH and thermal stability. As a result, the six isolates were identified at the genus level, 5 of which belonged to the *Aspergillus* genus and 1 to the *Penicillium* genus. The isolate highlighted in the lipase production screening was *Aspergillus flavus* INPA 83. The most suitable culture conditions to produce lipases by *Aspergillus flavus* INPA 83 were: soybean oil as carbon source, yeast extract as nitrogen source, pH initial temperature between 6 and 8, orbital agitation of 100 rpm and inoculum size between 10^3 and 10^6 cells/mL. The semi-purified enzymes showed an optimal temperature between 40°C and 50°C, an optimal pH between 6 and 7 and a thermal stability of 72% after 24 hours of incubation at 30°C. In view of the above results, it was concluded that *Aspergillus flavus* INPA 83 is a potential Amazonian source of lipases, the latter being a potential bioproduct for the bioeconomy development strategies in the Amazon.

Keywords: Enzymes, fungi, Amazon rainforest.

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INTRODUCTION

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) are an enzymatic group whose biological function is to catalyze the hydrolysis of triacylglycerols, forming diacylglycerols, monoacylglycerols, glycerol and free fatty acid. Its natural substrates are long-chain triacylglycerols with low water solubility, which gives lipases the characteristic of catalysis at an oil/water interface (Kavitha et al. 2021).

These enzymes are obtained from animals, plants and microorganisms, and microbial lipases stand out in the industrial sector due to their wide application in several sectors. This is possible due to the various reactions catalyzed by them, since lipases, in addition to hydrolyzing long-chain triacylglycerols, are also capable of catalyzing the reverse reaction, that is, they can carry out the esterification reaction in fatty acids and glycerol, thus forming triacylglycerols. Furthermore, it can catalyze transesterification reactions in media with low water concentration (Jaeger and Eggert 2002, Kavitha et al. 2021).

Lipases of microbial origin can be used in detergent production processes, in the food industry to enhance the flavor of dairy products, in the processing of meats, vegetables, fruits and beers, in the leather industry to remove lipids, in the pharmaceutical and cosmetic industry as dyes, perfumes, sunscreens, makeup additives, among others (Jaeger and Eggert 2002, Kavitha et al. 2021).

This diversity of application of lipases promotes interest in the search for new sources that produce this enzyme and that have different and attractive characteristics for the industrial sector, such as lipases that are stable under different physical and chemical conditions (Griebeler et al. 2011, Howard et al. 2012). Therefore, it is necessary to isolate and select new producing microorganisms, as well as to study the optimization of their production, purification, characterization and application.

The Amazon rainforest is known for its biodiversity of fauna, flora and microorganisms, which is an environment conducive to being increasingly explored and with great potential for discovering new sources of enzymes. There are still not many published works on lipase-producing fungi in our region, therefore, the study of this region is of total regional and economic importance to search for new sources that produce lipase. Therefore, the present study aimed to produce and characterize fungal lipases isolated from Amazonian soil samples.

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MATERIALS AND METHODS

Microorganisms

The six isolates used in the present study (INPA 28, INPA 52, INPA 53, INPA 59, INPA 83 and INPA 131) are part of the Microbiological Collection of the Instituto Nacional de Pesquisas da Amazônia. These were isolated from the soil of the Adolpho Ducke Forest Reserve (Lat.: 02º95'43"S; Long.: 59º93'39"W) and from the Instituto Nacional de Pesquisa do Amazonas (INPA) (Lat.: 3º09'41 "S, Long.: - 59º98'77"W). The isolates were preserved in PDA (potato dextrose agar) medium under mineral oil. For reactivation, a fungal loop was seeded in Petri dishes (9 cm) containing PDA with chloramphenicol (250 mg/L) and incubated at room temperature until growth.

Substrates and chemicals

The carbon sources investigated were soybean oil (Liza-Cargill Agrícola SA, São Paulo), Olive oil (Galo Extra Virgin- Sendas Distribuidora S/A, produced: Vitor Guedes, IND.COM. SA- Portugal), coconut (Ducoco-Bebedouro Linhares-ES) and animal lard (Aurora, São Miguel do Oeste-SC) and the nitrogen sources investigated were yeast extract (Acumedia - Neogen Culture Media- England), bacteriological peptone (Acumedia - Neogen Culture Media- England), ammonium sulfate (Dinâmica Química Contemporânea LTDA. – SP -Brazil), meat extract (KASVI – PR -Brazil) and sodium nitrate (Dinâmica Química Contemporânea LTDA. – SP -Brazil). Other media components used in the experiment were obtained from Hi-media (Mumbai, India). All the chemicals were of analytical reagent grade.

Fungi identification

The fungi were identified based on their morphological characteristics, evaluating the characteristics of the colony, shape, color of the aerial hyphae, growth rate, margin characteristics, surface texture, spores or reproductive structures. The spores and mycelia were picked from the purified colonies, placed onto glass slides and stained using lacto phenol cotton blue (1% v/v) before examining under a light microscope (IC et al. 2020). The taxonomic status was classified using a handbook on fungus identification (LACAZ et al. 2002).

The isolate highlighted in the production of lipases was identified to specie by analyzing the nucleotide sequence from the ITS region from the rDNA. Fungal DNA was extracted from the mycelium by the Phenol:Chloroform:Isoamyl Alcohol method,

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following the protocol previous described (Ferrer et al. 2001). The fungal were identified according to their internal transcribed spacer ribosomal DNA (ITS-rDNA) sequences. Polymerase chain reaction (PCR) was then conducted to amplify a pair of universal primers ITS1 (sequence: 5′-TCC GTA GGT GAA CCT GCG G-3′) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) of the extracted DNA under the following conditions: initial denaturation at 95°C for 10 min, 34 cycles of 95°C for 1 min, 55°C for 1 min, 72°C for 1.30 min and final extension at 72°C for 10 min. The PCR products were analyzed in 1.5% agarose gel in Tris-Borate-EDTA buffer, stained with ethidium bromide and visualized under UV light. The PCR products were sent to be purified and sequenced at MacrogenTM (Seoul, Korea). The sequences were compared with the sequences available in GenBank via BLAST searches.

Screening of lipases producers

To identify which fungi isolated from the soil had the best potential for lipases production, submerged fermentation was performed. Initially, the fungal isolates were transferred to tubes containing PDA and incubated at room temperature (25 ± 2 °C) for 72 h. After 3 days, the fungus spores of each isolate were suspended and counted using a Neubauer chamber (1×10^4 spores/mL). This spore suspension was used to inoculate Erlenmeyer flasks (250 mL) with 50 mL of soybean oil (15 g/L), tween 20 (10 g/L), yeast extract (1 g/L), Na_2HPO_4 (0.9 g/L), KH_2PO_4 (2 g/L), $MgSO_4$.7H2O (0.1 g/L) and $(NH_4)_2SO_4$ (1 g/L) and concentration of 1×10^4 spores/mL medium. The flasks were incubated at room temperature (25 ± 2 °C), orbital agitation of 100 rpm for 5 days.

For cell removal, manual separation was performed and then the culture medium was centrifuged at 5000 g for 10 minutes. The supernatant was used to measure the enzyme activity.

Determination of lipase activity

Lipase activity was determined by hydrolysis with p-NPP (p-nitrophenyl palmitate) as described by 28 with the following modifications: 0.8 mL of Tris-HCl buffer (40 mM, pH 8.0), 0 .1 ml p-NPP (20 mM p-nitrophenyl palmitate) as substrate and 0.1 mL culture broth. The reading was performed in a spectrophotometer at 410 nm. Under the experimental conditions, the assumed extinction coefficient for pNPP (p-nitrophenyl palmitate) was 1500 mol.L^{-1}.cm^{-1} (Maia et al. 2001). After 30 minutes of incubation at

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37 °C, the absorptivity of the solution was again quantified in a spectrophotometer at 410 nm. One unit of lipase (IU) was defined as the amount of enzyme that produced 1 μmol of p-nitrophenol per minute (equation 1).

Equation 1

$$UI/L = \frac{(Abs_f - Abs_i).(1,0)}{T.(\varepsilon).(0,1)}$$

Onde:

Abs_f = Final Absorbance

Abs_i = Initial absorbance

1,0 = Volume assay

T= Time

ε = Extintion coeficient

0,1= Enzyme extract

Effect of carbon and nitrogen sources on pigment production

To investigate the influence of the culture medium on lipase production, the culture medium was prepared with modified carbon (30 g/L) and nitrogen (3 g/L) sources. The carbon sources evaluated were soy oil, olive oil, coconut oil and animal lard. The nitrogen sources analyzed were yeast extract, peptone, ammonium sulfate, meat extract and sodium nitrate. The experiments were performed in triplicate, where the bioprocess was conducted as previous described.

Effect of pH, agitation and inoculum size on lipases production

We investigated influence of bioprocess factors, the influence of pH, agitation and inoculum size on lipase production. The investigated pH were 2, 4, 6, 8, and 10; orbital agitation were: 0, 50, 100, and 150 rpm and inoculum concentrations were: $1x10^2$, $1x10^3$, $1x10^4$, $1x10^5$ and $1x10^6$ spores/mL [41.9]. The experimental conditions were the same as previous described.

Lipases characterization

Initially, a submerged bioprocess was carried out with the optimized conditions and subjected to precipitation. The proportions of ethanol in relation to the enzyme filtrate (sample: ethanol) were: 1:1, 1:2, 1:3 and 1:4. Once the proportions were defined,

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precipitation was carried out for 24 hours at -20 °C. The precipitated material was centrifuged for 20 minutes at 5000 g at 4 °C to sediment. The enzymatic sample was stored at -20°C. This sedimentation was solubilized in distilled water at 4 °C and, later, the partial biochemical characterization of the enzymatic sample was performed (Kareem et al. 2017).

The semi-purified enzyme was characterized to determine its temperature and optimal pH and thermal and pH stability.

Effect of Temperature and Temperature stability:

To identify the effect of temperature lipase and its stability, the experiment was carried out in the range of 30-70°C at standard pH 8.0 in improved medium. A relative activity was calculated as the ratio of the activity of free enzyme measured at different temperatures to the activity of enzyme at the standard conditions (pH 8.0, 37°C). Free Enzyme thermo-stability was also determined in the improved medium by exposing free lipase to temperature (30, 50 e 70°C) for 1 hr. The enzyme was then cooled to room temperature and activity was measured under standard conditions. Residual activity was calculated as the ratio of the activity of lipase enzyme measured after incubation to the activity of the enzyme under standard condition.

Effect of pH

The influence of the pH was investigated in the range of 4.0-9.0 at 55°C in the improved medium. A relative activity was calculated as the ratio of the activity of free enzyme measured at different pH to the activity of enzyme at the standard conditions (pH 8.0, 37°C).

Statistical analysis

All tests were carried out in triplicate, and the findings were reported as mean values. We used parametrical T-test as the statistical test to compare the variance between two samples.

RESULTS

Fungal screening

The fungal isolates selected for the present study were micromorphologically identified at the genus level. They belonged to the *Aspergillus* and *Penicillium* genera and were named as: *Aspergillus* INPA 28, *Aspergillus* INPA 52, *Aspergillus* INPA 53, *Aspergillus* INPA 59, *Aspergillus* INPA 83 and *Penicillium* INPA 131.

Bioprocesses were performed to evaluate the production of lipases by the isolates. The maximum activity (IU/L) and productivity in 72hs (IU/L.h-1) were determined (Table 1). The isolate *Aspergillus* INPA 83 (0.45 IU/L.h-1) had the highest productivity.

Table 1 – Maximum activity (IU/L) and productivity (IU/L.h-1) of lipases produced by fungi isolated from soil samples from the Amazon region

Strain	Lipase ativity (UI/L)*	Productivity 72 hs (UI/L.h^{-1})
Aspergillus INPA 83	32.8 ± 0.3 ^{a}	0.45
Aspergillus INPA 53	21.2 ± 0.4^{b}	0.29
Aspergillus INPA 52	15.7 ± 0.2^{b,c}	0.22
Aspergillus INPA 59	9.11 ± 0.2^{c}	0.12
Aspergillus INPA 28	1.38 ± 0.2^{d}	0.02
Penicillium INPA 131	0.03 ±0.01^{e}	0.0004

* Different letters indicate significant differences in access treatment means from repeated-measures ANOVA ($P < 0.05$)

Aspergillus INPA 83 was submitted to identification by sequencing (ITS region of the rDNA) due to its prominence in the production of lipases. This assay demonstrated 95% sequence similarity (BLAST: Basic Local Alignment Search Tool) with the NCBI (National Center for Biotechnology Information) accession ID number GCUD71P7015 (83 R) and GCUWYPH2015 (83 F) related to the species *Aspergillus flavus*. This isolate was then called *Aspergillus flavus* INPA 83 and was selected for all other stages of the work.

Optimizing the lipase production from *Aspergillus flavus* INPA 83

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Aspergillus flavus INPA 83 was subjected to tests based on univariate experiments in order to investigate the influence of bioprocess factors. The influences of nutritional factors (carbon source, nitrogen source) and also of physicochemical factors (pH, orbital agitation velocity and inoculum size) on lipase production were investigated.

Coconut oil, olive oil, animal fat and soybean oil substrates were investigated as carbon sources (Figure 1). Soybean oil was the substrate that resulted in the highest enzymatic activity (32 IU/L). In addition, the influence of nitrogen sources peptone, meat extract, sodium nitrate, ammonium sulfate and yeast extract was investigated (Figure 1). Yeast extract was the nitrogen source that resulted in the highest enzyme production (32.8 IU/L).

The influence of factors pH, orbital agitation and inoculum size on lipase production by *Aspergillus flavus* INPA 83 was investigated (Figure 2). Under experimental conditions, pH 6 and 8, orbital agitation of 100 rpm and inoculum sizes between $1x10^{-3}$ to $1x10^{-6}$ resulted in the highest lipase activities by *Aspergillus flavus* INPA 83.

Characterizing the lipase production from *Aspergillus flavus* INPA 83

In order to characterize the lipases, the culture media was submitted to a semi-purification with ethanol. Then, the purification product was rehydrated, and the characterization of the pH and optimum temperature of the lipases was performed (Figure 2).

The lipase obtained showed optimal activity at pH 6 and 7. Significant reductions in activity (60%) were observed at pH 4 and 9.

The optimal temperature of the enzymes produced by *Aspergillus flavus* INPA 83 is shown in Figure 4. The lipases obtained showed optimal activity in the range of 40 ^{o}C and 50 ^{o}C.

The stability of lipases produced by *Aspergillus flavus* INPA 83 was studied under different temperatures. Lipases lost 95% of their activity after 4 hours of incubation at 70°C (Figure 2). In the other hand, these enzymes maintained 78% of their activity even after 24 h of incubation at 30°C (Figure 2).

5. Discussion

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The findings from this study suggest that *Aspergillus flavus* INPA 83 (a fungal strain isolated from Amazon soil) is a good lipase producer. We isolated this microorganism from Amazon soil, submitted it into a lipase screening and we characterized the lipases produced. Our results highlight the biotechnological potential of amazon strains as a potential source of lipases for industrial use.

The genera *Aspergillus* and *Penicillium* stood out in producing lipases. This result is in agreement with the literature that highlights these genera as good producers of lipases (Rigo et al. 2010, Toscano et al. 2014, Roden and Schuetz 2017, Kavitha et al. 2021). The isolate *A. flavus* INPA 83 was the major producer of lipases under the experimental conditions. The species *A. flavus* is highlighted in the production of lipases and previous works demonstrate the potential of this species to produce lipases of industrial interest (Toscano et al. 2014, Colla et al. 2016, Kavitha et al. 2021, Abdulmumini et al. 2022).

Lipases from *A. flavus* INPA 83 showed optimal temperature and pH of 6-7 and 40-5 ^{o}C, respectively. These optimal enzyme operating conditions were expected. Previous works demonstrate that the lipases of these species present these optimal parameters. This result is positive since a significant part of the lipase applications are in these operating ranges(Aguieiras et al. 2015, Kavitha et al. 2021, Abdulmumini et al. 2022).

The present work opens perspectives for carrying out work on the biochemical and molecular characterization of these enzymes. In addition, it opens perspectives for production optimization experiments using amazon residues as substrates. In conclusion, it was possible to find a new source of lipases to be potentially explored by the growing bioeconomy of the Amazon region. Undoubtedly, lipases from the Amazon region produced with regional residues are a viable option for cosmetics, medicines and fine chemicals.

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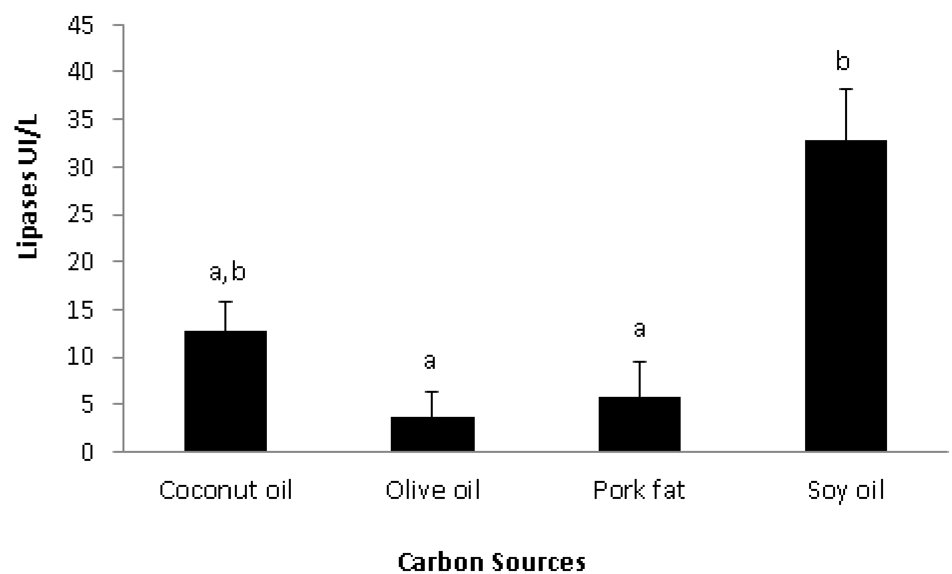
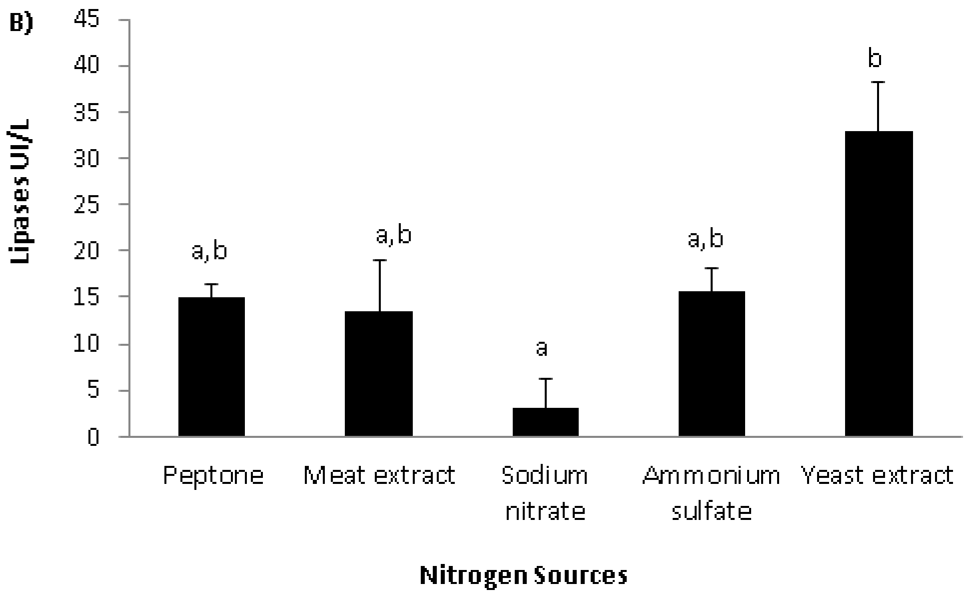
Figure 1- Influence of different carbon and nitrogen sources on lipase production by the isolate *Aspergillus flavus* INPA 83. The means presented with the same letters do not show statistical difference by the "t" parametric method (confidence level of 95).

Figure 2- Influence of pH, orbital agitation and inoculum size (A, B and C) on lipase production by the isolate *Aspergillus flavus* INPA 83. The means presented with similar letters do not present statistical difference by the method.

Figure 3- Optimal pH, optimal temperature and stability of the lipase produced by *Aspergillus flavus* INPA 83. The means presented with similar letters do not show statistical difference by the "t" parametric method (95% confidence level).





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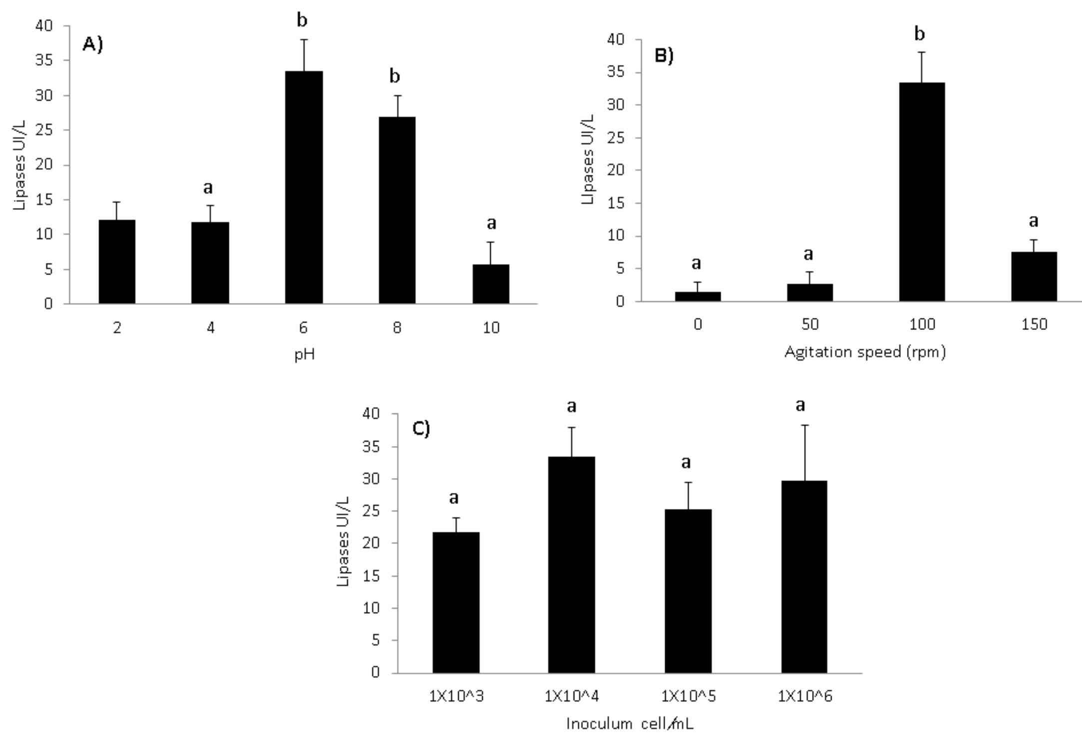

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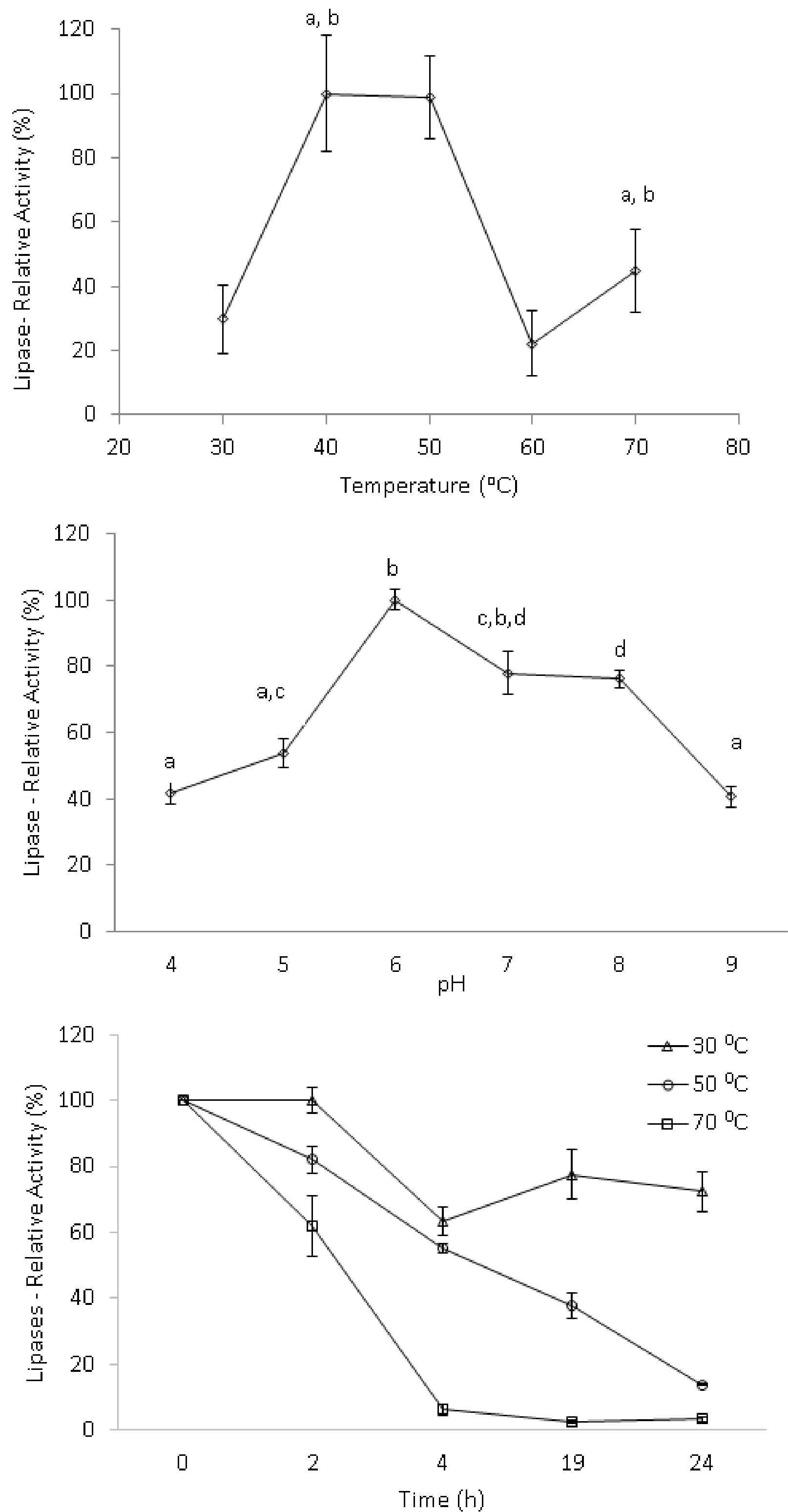

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