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STUDIES FOR LIPASE PRODUCTION ABILITY OF *Aspergillus* SP. STRAINS FROM THE MISIONES RAINFOREST OF PARANAENSE

Estudio de la capacidad de producción de lipasas de *Aspergillus* sp. provenientes de la selva misionera paranaense

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Key words: Fungi, hydrolase, enzyme activity, optimization, Plackett-Burman.

ABSTRACT

Fungi of the *Aspergillus* genus synthesize lipases, which are primarily extracellular enzymes secreted into the culture medium. These lipases have broad substrate specificity and are capable of hydrolyzing a variety of lipids, including triglycerides, phospholipids, and cholesterol esters, making them the focus of many studies on their applications. The present work carried out an exploratory analysis of the production of lipases in six fungi of the genus *Aspergillus* isolated in the Misionera Paranaense jungle (Argentina). Using simple qualitative detection techniques with tween 80% and rhodamine B as substrates, and based on the quantitative analysis carried out, it was determined that the isolate *Aspergillus* sp. LBM 054 showed the highest levels of lipase activity, reaching a total of 133 U/mL. A Plackett-Burman statistical test revealed that adding tributyrin to the culture medium significantly increased the activity of the lipase enzyme ($p < 0.05$), reaching a value of 168 U/mL. Among the concentrations evaluated, the addition of 2% tributyrin demonstrated the greatest increase in lipase activity, reaching a five-fold increase compared to the initial activity observed at the beginning of the assays. Optimum activity and stability at neutral to alkaline pH values make these enzymes suitable for various biotechnological applications. The zymogram of the selected strain showed an enzymatic profile that included a protein with a molecular mass of 38 kDa.

Palabras clave: hongos, hidrolasa, actividad enzimática, optimización, Plackett-Burman.

RESUMEN

Las lipasas sintetizadas por hongos del género *Aspergillus* son en su mayoría enzimas extracelulares que se secretan al medio de cultivo. Estas lipasas tienen una amplia especificidad de sustrato y son capaces de hidrolizar una variedad de lípidos, incluidos triglicéridos, fosfolípidos y ésteres de colesterol, lo que las convierte en el foco de muchos estudios sobre sus aplicaciones. El presente trabajo realizó un análisis exploratorio de la producción de lipasas en seis hongos del género *Aspergillus* aislados en

la selva Misionera Paranaense (Argentina). Utilizando técnicas simples de detección cualitativa con tween 80% y rodamina B como sustratos, y basándonos en el análisis cuantitativo realizado, se determinó que el aislado *Aspergillus* sp. LBM 054 mostró los niveles más altos de actividad lipasa, alcanzando un total de 133 U/mL. Una prueba estadística de Plackett-Burman reveló que la adición de tributirina al medio de cultivo incrementaba significativamente la actividad de la enzima lipasa ($p < 0.05$), alcanzando un valor de 168 U/mL. Entre las concentraciones evaluadas, la adición de 2% de tributirina demostró el mayor aumento en la actividad de la lipasa, alcanzando un aumento de cinco veces en comparación con la actividad inicial observada al inicio de los ensayos. La actividad óptima y estabilidad a valores de pH neutros a alcalinos hacen que estas enzimas sean adecuadas para diversas aplicaciones biotecnológicas. El zimograma de la cepa seleccionada mostró un perfil enzimático que incluyó una proteína con una masa molecular de 38 kDa.

INTRODUCTION

Industrial biotechnology, also known as white biotechnology, focuses on using clean and environmentally friendly technologies, such as enzymes and microorganisms, to improve industrial processes. The main objective is to replace polluting technologies with more sustainable ones that produce more biodegradable goods, generate less waste, and require less energy (El-Metwally et al. 2023). As a suitable ecological alternative to these conventional methods, using enzymes has proven to be a good option. Compared to a chemical process, the enzymes used are produced more effectively with little or no side reactions and little or no production of toxic byproducts (Hocevar et al. 2012, Chau et al. 2023). The unique catalytic properties of enzymes have driven their increasing use and importance in the industrial processes of detergents, foods, pharmaceuticals, biofuels, and various others, contributing to the growth and development of these sectors (Navvabi et al. 2018).

Lipases or triacylglycerol lipase and triacylglycerol acylhydrolase (IUBMB, EC3.1.1.3) are serine hydrolases commonly used to catalyze esterification (reverse hydrolysis), interesterification, and transesterification reactions (Okino-Delgado et al. 2017).

The mechanism of lipid hydrolysis is to collect acyl groups from glyceride molecules by lipase to create an acyl lipase complex and then transfer the acyl to the O-H group of water (Fernández-Lafuente 2010). Lipases have α-helix and β-strand structures, and their catalytic domain is formed from Ser, His, Glu, and Asp residues. This Ser is located in the conserved part, which includes Gly-X-Ser-X Gly, where X can be any amino acid residue (Vardar-Yel et al. 2024).

After proteases and carbohydrolases, lipases are the largest group of enzymes marketed and used in different industries. Lipases are produced in animals (Guerrand 2017), plants, and microorganisms (Vivek et al. 2022, Okal et al. 2023).

Among these producers, microbial lipases show a wide spectrum of industrial applications due to their higher stability, substrate specificity, and lower production costs compared to other sources. Their possible applications in biotechnology include the extraction of edible oils (Contesini et al. 2010), and the production of biodiesel (El-Metwally et al. 2023) since they can esterify both free fatty acids and triglycerides in a single step without the need for additional washing. In the pharmaceutical industry, lipases are used as biocatalysts in the synthesis of pure enantiomers of pharmaceutical compounds, which is important to avoid undesirable side effects. Lipases also have applications as modulators, activators, and inhibitors for the treatment of diseases such as obesity (Zhang et al. 2020).

Lipases are incorporated into detergent formulations to improve the removal of greasy stains, allowing washing at lower temperatures and with less water, thus reducing energy consumption and the environmental impact of washing processes (Singh et al. 2020).

In addition, lipase enzymes are used in the treatment of industrial effluents rich in fats and oils, accelerating the degradation of these contaminants (Chandra et al. 2020).

Most lipase enzymes produced by bacteria and fungi exhibit high activity between 30 and 50 ºC, withstanding temperatures of around 50 ºC (Tiquia-Arashiro and Grube 2019, Vivek et al. 2022)

On the other hand, the existence of mesophilic microorganisms capable of producing lipase enzymes

with high activity at medium or low temperatures has been reported. Demonstrating a high specific activity in the range of 15-30 ºC (DSouza et al. 2021).

Lipases with high activity at low or medium temperatures are particularly attractive for some industrial applications, such as detergent formulation, bioremediation, or food processing, for which substrate or product stability and energy savings are essential (Vardar-Yel et al. 2024)

Filamentous fungi are recognized as the best producers of lipases and are currently the preferred sources since they produce extracellular lipases, which facilitate the extraction of fermentation media (Ktata et al. 2020). The most reported fungal sources of these enzymes belong to the genera *Rhizopus* sp., *Mucor* sp., *Geotrichum* sp., *Penicillium* sp., and *Aspergillus* sp. (Kavitha 2016).

Aspergillus is a genus of fungi well known for its ability to produce lipase enzymes. These enzymes are stable under a wide range of conditions, including thermostability and pH stability. In addition, *Aspergillus* stands out for its high quality and capacity for production in large quantities, which makes them very valuable for the industry (Zhang et al. 2020, Chau et al. 2023).

Aspergillus oryzae lipase is used in detergent formulations due to its stability in alkaline solutions and the presence of anionic agents. Likewise, purified lipase from *Aspergillus fumigatus* is stable in the presence of several commercial detergents, such as "Vanish", improving the removal of oil stains by 84% under optimized conditions (Guerrand 2017, Wu et al. 2021).

On the other hand, *Aspergillus niger* lipase immobilized on titanium nanoparticles coated with gelatin has been used for the treatment of industrial effluents rich in fats and oils (Gao et al. 2022).

It is estimated that there are between 2.2 and 3.8 million fungal species worldwide, of which only approximately 120 000 have been described. This suggests great potential to discover new lipase producers with unique characteristics (Hawksworth and Lücking 2017).

Despite the important advances in studying these enzymes, there are still aspects not fully explored that could have significant applications in various industrial and environmental sectors (Vardar-Yel et al. 2024). Therefore, this justifies the search for new lipases from various sources.

In this context, this study aimed to evaluate the lipolytic capacity of fungi of the genus *Aspergillus* isolated from the Paranaense tropical forest in the Province of Misiones, Argentina.

MATERIALS AND METHODS

Fungal strains

This study used six fungal strains of the genus *Aspergillus* isolated from decomposing fruits of the rainforest of Misiones, Argentina (Zini 2013). All strains were obtained from the culture collection of the Laboratorio de Biotecnología Molecular (LBM), belonging to Instituto de Biotecnología de Misiones "Dra. Maria Ebe Reca" of the Universidad Nacional de Misiones, and were kept in solid MEA medium (12.7 g/L of malt extract, 15 g/L of agar) plates at 4 ºC and periodically sub-cultivated. They were coded with the acronym LBM for Laboratorio de Biotecnología Molecular and a corresponding order number.

Liquid culture medium for obtaining lipolytic enzymes

Czapek's medium was used to perform the different tests in a liquid medium. This medium contains sucrose 20 g/L, $NaNO_3$ 2 g/L, KH_2PO_4 1 g/L, KCl 0.5 g/L, $MgSO_4$ 0.5 g/L, $FeSO_4$ 0.01 g/L. All components were dissolved with distilled water and distributed in 100 mL Erlenmeyer, sterilized in an autoclave at 121 ºC and 1 atmosphere for 15 min. 30 mL of the medium was supplemented with olive oil 0.1 g/L sterilized by filtration. They were inoculated with a spore suspension (1×10^6 spores/mL) and incubated for six days at 28 ± 1 ºC and 140 rpm. The supernatants obtained at this stage were used for subsequent screening and quantification assays.

Qualitative screening for extracellular lipase activities in solid medium containing two substrates

A solid culture medium supplemented with Tween 80% was used for the screening qualitative of the extracellular lipolytic activity of the isolate. This medium contains Tween 80% 1 mL; $CaCl_2$ 0.01 g; Tris buffer-NaCl pH 9.2 10 mL; agar 2 g; 100 mL water.

From the center of the Petri plates that contained a solid medium for qualitative screening, 5 mm diameter cylinders were removed and cut with punch, leaving a "well" (Okino-Delgado et al. 2017, Ortellado et al. 2021). This was filled with 60 µL of the supernatant obtained from the liquid and incubated culture at 28 ± 1 ºC for four days. Hydrolytic lipolytic activity is manifested by the formation of halos as an opaque zone flanking the colonies due to the deposition of calcium salt crystals formed by the released fatty acid.

Once the lipolytic activity was determined, the presence of lipase activity was checked using olive

oil as a substrate and the fluorescent dye rhodamine B (Okino-Delgado et al. 2017, Ortellado et al. 2021). This culture medium contains 0.8% nutrient broth, 1% agar, 1% olive oil, and 0.0001% rhodamine B (w/v). Punch-cut cylinders of 5 mm diameter were removed from the Petri dish, leaving wells. These were filled with 60 µL of each supernatant obtained from the liquid culture. The lipase-producing isolates were identified four days after incubation in the plaque at 28 ± 1 ºC by the formation of fluorescent orange halos around the colonies controlled by fluorescence with UV light at 350 nm.

Spectrophotometric estimation of levels of lipase activity

The lipase activity was monitored according to (Rehman et al. 2017) using 4-nitrophenyl palmitate (4-NPP) as the test substrate. The reaction solution (1000 µL) consisting of 100 µL of 4-NPP (solution A: 0.0015 g of 4-NPP + 4 mL of isopropanol) was mixed with 800 µL of solution B (0.1 g of gum arabic + 0.4 mL of triton X 100 + 100 mL of 0.1 M phosphate buffer, pH 7) and 100 µL of the supernatant. The resulting combination was placed at 37 ºC for 10 min, and the released 4-nitrophenol (4-NP) was measured in a spectrophotometer at 405 nm. One unit of enzymatic activity was defined as the amount of enzyme that released 1 mol of 4-nitrophenol per minute (IU) under the abovementioned conditions.

Effect of different oils on lipase activity in the selected isolate

In the present study, with the isolate that shows the best activity, the effect of various oils (olive oil, tributyrin, sunflower oil, canola oil, chia oil, and corn oil) was analyzed as enhancers of lipase enzymatic activity. Using a Plackett-Burman (Rehman et al. 2017, Chaudhari and Shirkhedkar 2020) design, each variable was evaluated at a presence level and another absence (**Table I**). The Czapek medium was used as the basis for the different design combinations.

Optimization of the lipase activity produced

Based on the statistical analysis and the results obtained from the Plackett Burman design, the variable that exerted a positive effect on the activity of the lipase enzyme was selected. To determine the optimal concentration in which the highest lipase activity is obtained, a series of experiments were carried out with different concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4% of the compound that demonstrated to enhance the lipolytic activity and incubated at 28 ± 1 ºC for six days.

TABLE I. VARIABLES TESTED IN THE PLACKETT-BURMAN DESIGN WITH THEIR RESPECTIVE LEVELS.

Variable	Encoded variable (%)	
	Absence	Presence
Oliva oil	0	1
Tributyrin	0	1
Sunflower oil	0	1
Canola oil	0	1
Chia oil	0	1
Corn oil	0	1

Statistical analysis

The statistical analysis of the Plackett-Burman design was performed using Stat Graphics Centurion XVI (Inc.) in conjunction with the analysis of variance and the Bonferroni test. Graphs were made using the Windows Graphpad Prism Program 8.

Characterization of the extracellular lipolytic activity of *Aspergillus* sp. LBM 054

Effect of pH

To study the effect of pH on the lipase activity in the culture supernatant, activity measurements were carried out at a pH varying from 7 to 8.5.

The buffer solution used was sodium phosphate buffer for all pH. Lipase activity was determined with 4-nitrophenol palmitate (4-NPP) in a spectrophotometer at 405 nm.

Effect of temperature

To evaluate the effect of temperature on lipase activity in the culture supernatant, a modification was made in the protocol for determining the enzymatic activity; the reaction mixture was incubated at the optimum pH, varying the temperature (4, 20, 30, 40, 55 ºC) and keeping the other conditions constant. Lipase activity was determined with 4-nitrophenol palmitate (4-NPP) in a spectrophotometer at 405 nm.

Thermostability

To evaluate the enzymatic thermostability over time, a pre-incubation of the supernatants was carried out at 4, 20, 30, 40, and 50 ºC. The supernatants were removed at different intervals of 1, 6, 12, 48, and 72 h, and their residual lipase activity was measured. The residual activity was expressed as a percentage, taking the enzymatic activity at time zero as 100%.

pH stability

To determine the enzymatic stability as a function of pH, a pre-incubation of the supernatant was carried out at different pH 7, 7.5, 8, and 8.5 at a constant temperature of 30 ºC during different intervals of 1, 6, 12, 48, and 72 h. Then, the measurement of lipase activity was performed. The residual activity was expressed as a percentage, taking the enzymatic activity at time zero as 100%.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on resolving gels containing 12% w/v (Geoffry and Achur 2018, Ortellado et al. 2021). Electrophoresis was performed at 100 V for 2 h, by adding 1 g/L SDS to the running buffer. After protein separation, SDS was cleaned from the gels by soaking them for 30 min in 2.5% Triton X-100 at room temperature (Ortellado et al. 2021). The gels were then briefly washed (1 min) in 50 mM phosphate buffer, pH 7.0, and covered with a solution of 100 µM 4-methylumbelliferyl butyrate (MUF). After 40 min, the bands became visible only when illuminated with ultraviolet light.

RESULTS

Preliminary study and selection of lipase-producing

This study analyzed the lipolytic capacity of six *Aspergillus* fungi isolated from the tropical rainforest of Misiones (Argentina). The fungi were identified by microscopic analysis and ITS sequencing (unpublished data). The fungi were grown in a Czapek liquid medium with the addition of 0.1 g/L of olive oil at pH 7, and the supernatants obtained on the sixth day of culture were analyzed.

All *Aspergillus* isolates used in the assay showed lipolytic activity evidenced by the formation of notable halos around the wells due to the crystals of the calcium salt of the fatty acid released by lipolysis on the sixth day of culture in the assays performed with solid media with Tween 80% and nutrient medium (**Fig. 1 A**). To determine whether the isolates present specific lipase activity, an agar plate containing olive oil as substrate and fluorescent dye rhodamine B (Rehman et al. 2017, Ortellado et al. 2021), was made with seven wells where the supernatants of the six isolates obtained from day six of cultivation, and negative control were placed. All six isolates tested produced hydrolysis zones on rhodamine-olive oil agar plates characterized by a fluorescent halo visible with ultraviolet light at 350 nm (**Fig. 1 B**). Due to the potential observed in the isolates that demonstrated lipolytic activity and lipase activity, enzyme quantification by enzyme activity was investigated (Ortellado et al. 2021).

Lipase activity was quantified in all the cultures; isolate LBM 054 had the best activity of 133 U/mL. Demonstrating coincidence with the largest halos is evidenced in **figure 1 A** and **B** with the plate techniques (**Fig. 1 C**).

Considering the results obtained with the *Aspergillus* isolates, it was decided to continue the remaining assays with isolate LBM 054 because of its promising enzymatic activity in both plate assays and activity quantification.

Effect of different oils on lipase activity in the selected isolate

The Plackett-Burman statistical method was performed to detect the positive factors contributing to the potentiation of the activity. This serves as a guide to develop an effective medium composition to potentiate lipase production using oils as substrate.

The results obtained in this study showed that the highest and lowest lipase production was 402 U/mL ($p < 0.05$) in assay 17; which contained the presence of 1% sunflower oil and 1% tributyrin; no activity was obtained in several assays. To evaluate the significance of each variable on the responses sought, analysis of variance was used in combination with Fisher's test at a 95% confidence level ($p < 0.05$). Pareto plots were used to facilitate the visualization and interpretation of the statistical analyses (**Fig. 2**).

The analysis of the results showed that tributyrin was the variable with the greatest positive effect on its presence level ($p < 0.05$) on lipase activity, while the remaining components analyzed did not show statistical significance on lipase activity.

To determine the optimum concentration at which the best increase in lipase activity is obtained, different concentrations of tributyrin were evaluated. The highest increase in lipase activity was observed with the concentration of 2% tributyrin ($p < 0.05$) with 697 U/mL followed by the assay with 1% tributyrin with an activity of 379 U/mL ($p < 0.05$). The lowest lipase activity was recorded with the 4% tributyrin treatment ($p < 0.05$) with 168 U/mL (**Fig. 3**).

Characterization of the extracellular lipolytic activity of *Aspergillus* sp. LBM 054

Effect of pH

From the analysis of pH on the lipase activity of the supernatant obtained, it was observed that the

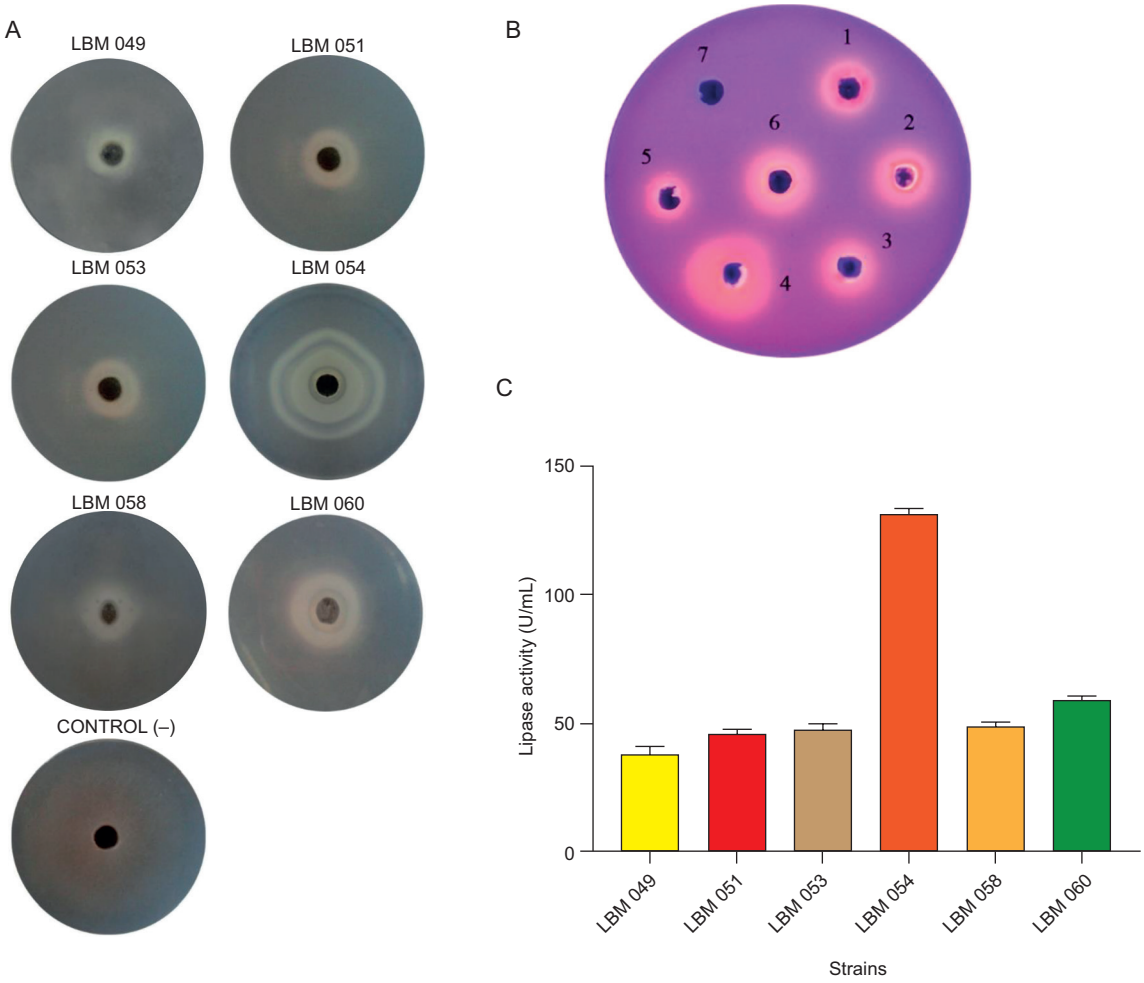

Fig. 1. Detection of lipase activity of the supernatants obtained on day six of culture in Czapek medium. **A**. qualitative screening in tween 80% plates. **B**. qualitative screening with olive oil -rhodamine B plates, in order of sowing: 1) *Aspergillus* from Laboratorio de Biotecnología Molecular (LBM) 049; 2) LBM 051; 3) LBM 053; 4) LBM 054; 5) LBM 058; 6) LBM 060; 7) A non-inoculated well was used as a negative control. **C.** Quantification of the activity by the 4-nitrophenol (4-NP) method. Tests were carried out in duplicate with the six *Aspergillus* sp. strains studied.

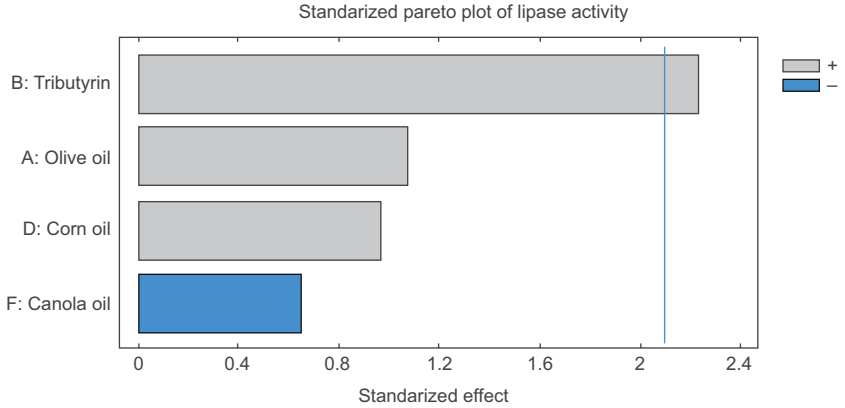

Fig. 2. Pareto plot for lipase activity in *Aspergillus* from Laboratorio de Biotecnología Molecular 054. The length of the bars in the graph is proportional to the standardized effect, the gray and light blue bars represent the factors with positive and negative effects, respectively, on lipase activity. The extension of the bars beyond the vertical line represents the variables with significant influence on the analyzed response with a confidence level of 95%.

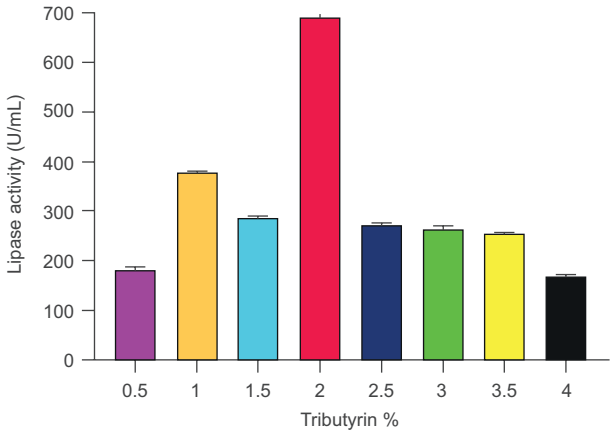

Fig. 3. Evaluation of the influence of different tributyrin concentrations on lipase activity in *Aspergillus* sp. from Laboratorio de Biotecnología Molecular 054.

maximum lipase activity ($p < 0.05$) was reported at pH 7.5 with 697 ± 2.1 U/mL, while at pH 7 the lipase activity obtained was 593 ± 1.5 U/mL. While for the case of pH 8 and 8.5, a decay in the lipase activity of the supernatant was observed (**Fig. 4**).

Effect of temperature

The effect of different temperatures was studied with the supernatant obtained by incubation at various temperatures ranging from 5 to 55 ºC.

The results showed that the optimum enzyme activity temperature for the isolation supernatant was 20 ºC ($p < 0.05$) with an activity of 694 U/mL. Followed by the activity obtained at 30 ºC with an enzyme activity of 588 U/mL; while the lowest enzyme activity was obtained at the highest temperature of 55 ºC ($p > 0.05$), being 319 U/mL (**Fig. 4**).

Thermostability

The thermostability curve obtained showed that at 20 and 30 ºC, the lipase activity of the supernatant obtained was above 50% up to 48 h. For the assay performed at 4 ºC, it was observed that the lipase enzyme activity remained above 50% up to 12 h. Moreover, for the assay at 40 ºC, it was only observed that the activity remained above 50% of the enzyme activity up to 6 h of the assay (**Fig. 5**).

pH stability

The curves obtained in the stability tests against different pH showed the lipase enzyme activity of the supernatant of *Aspergillus* sp. LBM 054 remained above 50% until 48 h in the case of pH 7, 7.5, and 8. In the case of the test carried out at pH 8.5, it was observed that the lipase enzyme activity remained above 50% until 6 h of the test (**Fig. 5**).

SDS-PAGE

To characterize the lipase enzyme profile and assess its potential variation in response to changes in the culture medium, we conducted an SDS-PAGE analysis using MUF-butyrate. The results revealed the presence of a single enzyme with an approximate molecular weight of 38 kDa (**Fig. 6**). This enzyme was detected in both the supernatant collected before the evaluation tests of various oils and the supernatant obtained after supplementation with 2% tributyrin.

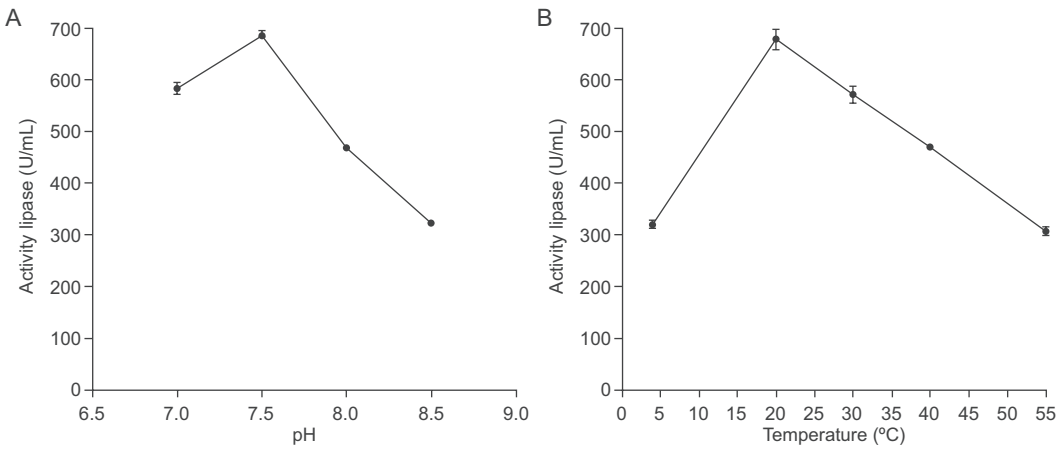

Fig. 4. Effect of temperature and pH on the lipase activity of the supernatant of *Aspergillus* sp. from Laboratorio de Biotecnología Molecular 054. **A**) pH optimum; **B**) optimal temperature.

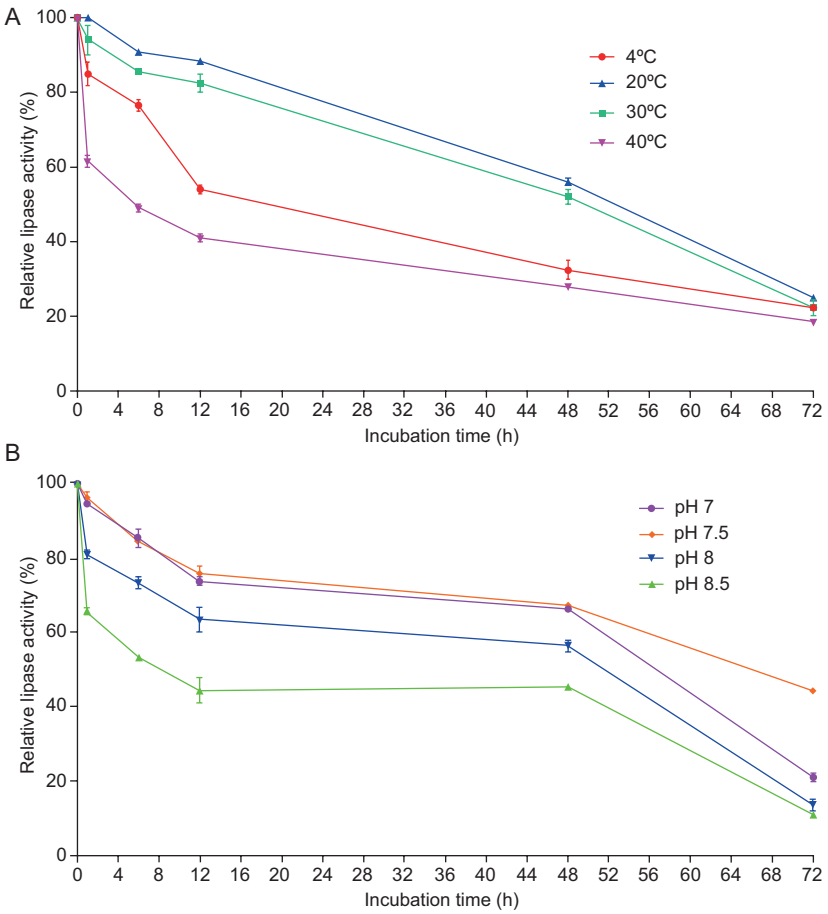

Fig. 5. Temperature and pH stability of lipase activity in the supernatant of *Aspergillus* sp. from Laboratorio de Biotecnología Molecular 054. **A**) Thermostability; **B**) pH stability. 100% corresponded to 698 ± 1.8 U/mL.

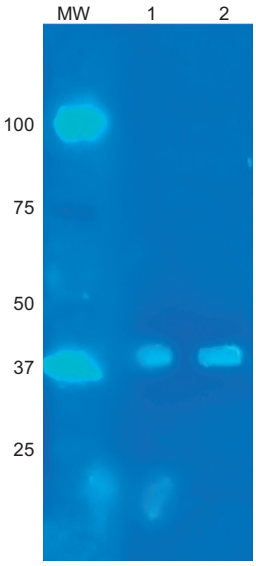

Fig. 6. Lipase detection by zymography in SDS-PAGE gels of *Aspergillus* sp. from Laboratorio de Biotecnología Molecular 054. Lipase bands were detected by fluorescence with MUF-butyrate. **MW**: molecular weight marker. **1**: Supernatant obtained from the preliminary liquid medium of Czapek medium supplemented with olive oil. **2**: Supernatant obtained from the culture medium supplemented with 2% tributyrin.

DISCUSSION

Fast-growing fungi (Eurotiales) are recognized for their ability to secrete various types of extracellular enzymes, so it is of interest to explore the potential for enzyme production that may exist in biodiversity (Zini 2013, Díaz et al. 2021, Ortellado et al. 2021). Due to its subtropical climate with high humidity, the Paraná rainforest in the province of Misiones is conducive to the development of great biodiversity. Among these stand out a great variety of fungi that produce extracellular enzymes with biotechnological potential.

The use of fungi as producers of enzymes of industrial interest has several advantages, including their ability to be used in a wide range of agricultural products, as a source of nutrients, and for the production of extracellular lipase in fermentation cultures (Toscano et al. 2011). Fungi of the *Aspergillus* genus have shown to be good producers of lipase enzymes; the existence of cold-active lipase enzymes has also been reported.

Lipases produced by fungi of the genus *Aspergillus* have important biotechnological applications. Several authors have focused on the study to identify the best conditions for producing lipases and other extracellular enzymes (Rehman et al. 2017, Wu et al. 2021).

The tween 80% plate method was used to detect lipolytic activity in the present work. It proved to be sensitive in *Aspergillus* isolates, being a simple and practical method. However, due to reports that this method usually has around 3% false positives, this results in an unacceptably high proportion of false positive results (around 84%). Therefore, the use of rhodamine B plates in the supernatants obtained from these isolates was decided.

The rhodamine B technique offers greater sensitivity and precision when detecting true lipases (Rehman et al. 2017, Ortellado et al. 2021), which is why it was used to verify the activity of the supernatants obtained. Confirmation of lipase activity can be observed when an orange halo is observed under ultraviolet light due to the interaction of rhodamine B with the fatty acids released during the enzymatic hydrolysis of triglycerides. Several authors have reported the use of this technique for the analysis of lipase production in various fungi (Ktata et al. 2020, Ariaeenejad et al. 2023). Savitha et al. (2007) used this simple technique for the selection of lipase producers starting from 32 fungal strains isolated from three different genera (*Aspergillus*, *Penicillium*, and *Mucor*).

In the present work, the combined use of these techniques was used to screen the lipase activity of six strains isolated from the Paranaense rainforest of Misiones Province, Argentina. Evidencing the presence of this enzyme in the six isolates analyzed, quantification utilizing spectrophotometry found that *Aspergillus* sp. LBM 054 presented a greater potential for the production of the lipase enzyme in its supernatant.

Evaluating different sources of carbon or nitrogen as nutrients is extremely important for evaluating the production of lipase enzymes. In the case of *Aspergillus* sp. LBM 054, it was observed that with the use of tributyrin, the best lipase activity was found in the obtained supernatant as was observed by authors such as Almeida et al. (2018), in the *Candida* fungus (Toscano et al. 2011, Almeida et al. 2019, Ariaeenejad et al. 2023).

From the analysis of optimum temperature, optimum pH, thermostability, and stability at different pHs, it was possible to observe that an increase in alkalinity plays a role in inhibiting the supernatant's lipase enzyme activity since decreases in the lipase enzyme activity were registered at pH 8 and 8.5.

Gao et al. (2022) reported *Aspergillus niger*, with an activity of 0.26 U/mL (pH 6 and 40 ºC), and *A. fumigatus*, with a lipase activity of 0.21 U/mL (pH 7 and 80 ºC) as good lipase producers. It has also been reported that *A. carneus* lipase has high pH and temperature tolerance and stability, regiospecificity 1, 3, stability in aqueous and non-aqueous media, and esterification and transesterification ability, with a lipase activity of 12.7 U/mL (Singh et al. 2020).

The lipase enzyme present in the supernatant obtained from Aspergillus sp. LBM 054 exhibited optimal activity at 20 ºC and remained stable between 20 and 30 ºC. These properties make it highly suitable for various industrial applications, including selective acylation and deacylation in the pharmaceutical industry, incorporation into cold-wash detergents, waste bioremediation, and use in the leather industry for dehairing (Ma et al. 2022; Chau et al. 2023; Abubakar et al. 2024).

Polyacrylamide gel electrophoresis with sodium dodecyl sulfate (SDS-PAGE) is the most commonly used method to determine the apparent molecular weight of enzymes (Contesini et al. 2010, Joseph et al. 2012). Lipases belonging to the genus *Aspergillus* are hydrolases whose molecular mass usually ranges between 20 and 80 kDa (Contesini et al. 2010, Vivek et al. 2022). Previous studies have reported that lipases with molecular masses between 30 and 50 kDa exhibit interesting biotechnological applications

because they usually have great versatility at different temperatures and pH (Putri et al. 2020, Ortellado et al. 2021).

In this study, the detected bands showed a molecular weight of 38 kDa, both in the supernatant of the preliminary culture medium composed of Czapek supplemented with olive oil and in the supernatant of the culture medium supplemented with 2% tributyrin, consistent with values observed in other studies for *Aspergillus* lipases. For instance, lipase from *Aspergillus niger* has been reported to have a molecular weight close to 35 kDa, while lipase from *Aspergillus oryzae* has an approximate molecular mass of 27 kDa (Mayordomo et al. 2000, Contesini et al. 2010, Vivek et al. 2022). On the other hand, other *Aspergillus* lipases, such as lipase from *Aspergillus terreus*, have reported molecular masses between 50 and 80 kDa (Toscano et al. 2011, Putri et al. 2020, Ortellado et al. 2021).

It is important to highlight that the lipases detected in the zymogram showed a higher affinity for short-chain fatty acids, as MUF-butyrate was used as the substrate. Therefore, it is plausible to assume the existence of other lipases with affinity for medium and long-chain fatty acids, which could also be of interest for biotechnological applications.

Based on these results, we have reported the presence of lipase in the supernatant of *Aspergillus* sp. LBM 054 with activity at medium temperatures. Offering an overview of the existence in nature of fungi capable of producing enzymes with potential for application in various industrial processes that require activity at low or medium temperatures that are not yet explored.

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