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Production, Extraction and Characterization of Lipases from the Antarctic Yeast *Guehomyces pullulans*

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Abstract: The production of extracellular lipases from the Antarctic yeast *Guehomyces pullulans* is induced using an olive oil medium as an inductor substrate and a first characterization of its enzyme, using the protein extract obtained from the medium, is described. For this, the effect of pH and temperature on the lipase activity are evaluated and the enzyme kinetic for the lipase is determined. Lipase production was 0.27 U/mL, a high value compared to lipolytic activities in non-optimized media. However, this value can be increased by optimizing the culture medium. The lipase of *G. pullulans* has maximum activity at pH 8.0 and 40°C (thermal stability 40-50°C). Regarding the kinetic parameters, a $K_M = 3.7\times10^{-4}$ M was obtained, a value located in the range of industrial lipases. In addition, its kinetics presented the phenomenon of interfacial activation. The results presented in this work show the biotechnological potential of the lipase due its biochemical properties and are useful for later work directed to study other factors that affect the enzyme activity and potential biotechnological applications of the *Guehomyces pullulans* lipase.

Keywords: *Guehomyces pullulans*, Antarctic Yeast, Lipase, Extracellular Production, Induction, Characterization

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are hydrolases which constitute the third most important category of enzymes, after carbohydrases and proteases (Kavitha, 2016). They are ubiquitous enzymes, produced by plants, animals and microorganisms. Lipases act on insoluble substrates emulsified in water and catalyze the hydrolysis (cleaving ester bonds) of triacylglycerides composed of long-chain fatty acid (more than ten carbon atoms). Lipases differ from esterases because the latter act on water-soluble substrates, such as simple esters with short-chain fatty acids (less than six carbon atoms) (Bussamara *et al.*, 2010; Lux *et al.*, 2007; Sharma *et al.*, 2001). In addition to the hydrolytic activity on triacylglycerides, lipases catalyze two groups of chemical reactions in non-aqueous media: Esterification and transesterification (Damaso *et al.*, 2013; Hasan *et al.*, 2009).

Currently, lipases are mainly obtained from a wide range of bacteria, fungi and yeasts (Thakur, 2012; Treichel *et al.*, 2010). Yeasts are the main source of biotechnological products in the world, exceeding in production other industrial microorganisms. Enzymes such as lipases are part of the multiple products with biotechnological and industrial potential (Kurtzman *et al.*, 2011). Lipases have gained attention due to the amount of chemo-, regio- and stereoselective transformations they catalyze, thanks to their characteristics such as substrate specificity, regional specificity and chiral selectivity (stereoselective). For this reason, new biotechnological applications have been established using lipases in the synthesis of biopolymers and biodiesel, enantiopure pharmaceuticals, agrochemicals, biosensors, additives for detergents and food processing (Sharma *et al.*, 2001; Shimada *et al.*, 2002; Tan *et al.*, 2010). Among the commercially important lipase-producing yeasts or those with potential biotechnological applications are: *Candida* sp., which includes *Candida rugosa* and *Candida antarctica*, also *Yarrowia lipolytica* and others species of *Pichia* sp., *Rhodotorula* sp. and *Trichosporon* sp. Due to their high performance and enzymatic properties, the genes encoding lipases from *Candida* sp., *Trichosporon* sp. and *Y. lipolytica* have been cloned and overexpressed (Sharma *et al.*, 2011; Thakur, 2012; Treichel *et al.*, 2010; Vakhlu and Kour, 2006).

Although most studies on yeast lipases refer to the species mentioned above, there are some lipase-producing yeasts that have potential for biotechnological innovation (Bussamara *et al.*, 2010; Thakur, 2012) In addition, about 2% of the microorganisms have been proven as enzyme sources (Hasan *et al.*, 2006). This figure allows us to understand the extensive research that can be carried out to discover and characterize new enzymes. This is the case of the basidiomycete yeast *Guehomyces pullulans* used in the study, which has been specially studied for the production of the enzyme β-galactosidase, an enzyme with hydrolytic activity on lactose (Kurtzman *et al.*, 2011). The production of lipases by *G. pullulans* was reported in a bioprospecting carried out by Martorell *et al*. (2017). Consequently, in this research is performed a lipase production from *G. pullulans* and is carried out an enzyme characterization where the effect of temperature, pH and enzymatic kinetics are analyzed. This provide a useful approach aimed at obtaining a new biocatalyst with the desired enzymatic capacity.

Materials and Methods

Induction to the Secretion of the Lipase

Microorganism

The yeast *Guehomyces pullulans* (strain CLQCA-ANT-073) belongs to the Quito-Catolica Yeast Collection of the Pontificia Universidad Catolica del Ecuador (CLQCA). It was selected on the basis of a lipase/esterase screening of 49 independent yeast isolates, including 7 Antarctic species, from the CLQCA.

Culture Conditions

Guehomyces pullulans (CLQCA-ANT-073) was incubated on YM agar medium for 5 days at 25°C. For lipase production, yeast biomass was inoculated in a liquid culture medium composed of: 0.2% glucose, 0.5% peptone, 0.01% $MgSO_4$, 0.1% K_2HPO_4, 5% gum Arabic and 2% olive oil as inducer (Bussamara *et al.*, 2010; Dhiman and Chapadgaonkar, 2013). The pH was adjusted to 5.0 and the culture medium was sterilized at 121°C for 15 min. The yeast culture (50 mL) was incubated by shaking at 200 RPM for 40 days at 20°C.

Protein Extraction and Purification

The inducer medium was centrifuged at 14,000 RPM for 10 min at 4°C. Proteins in the supernatant were precipitated by salting-out with 75% ammonium sulfate for 5 h at 4°C. A protein pellet was obtained by centrifugation and resuspended in 50 mM phosphate buffer, pH 7.0. Aliquots were stored at -30°C for later use (Bae *et al.*, 2014; Bussamara *et al.*, 2010; Joseph *et al.*, 2012; Kumar *et al.*, 2005). Protein concentration was determined by spectrophotometry using the Bradford method, with BSA as a standard.

Detection of Lipase Activity

The chromogenic medium used for lipase detection was: 1% olive oil, 10 mM $CaCl_2$, 2% agar and 0.01% phenol red as an indicator of the pH shift. Medium pH was adjusted with 0.1 M NaOH (to fuchsia-red coloration), sterilized at 121°C for 15 min and dispensed in Petri dishes (Nath and Hindumathy, 2012; Singh *et al.*, 2006).

Six wells were made in the middle of the medium. In each well, 100 µL of protein extract was added at different concentrations: 0.4, 0.04 and 0.004 mg/mL diluted in phosphate buffer pH 7 and 0.04 mg/mL diluted in water. In addition, water and phosphate buffer were used as controls. The plate was incubated at 30°C for 2.5 h.

Characterization of the Lipase

Quantitative Determination of Lipase Activity

Lipase enzymatic activity was determined by spectrophotometry using *p*-Nitrophenyl Palmitate (*p*NPP) as a substrate. The activity was quantified by measuring (at 410 nm) the increase of *p*-nitrophenol (*p*NP) released by the protein extract incubated for 30 min at 37°C and pH 7.0, against a blank without the enzyme source. To initiate the reaction, 0.1 mL of the lipase solution (protein extract) was added into 0.9 mL of substrate/buffer solution containing 3 mg of *p*NPP dissolved in 1 mL of isopropanol and 9 mL of the following solution: 4.4 mg/mL Triton X-100 and 1.1 mg/mL gum arabic in 50 mM phosphate buffer pH 7.0. A progress curve was constructed and enzymatic activity (U/mL) was calculated. A Lipase Unit (U) was defined as the amount of enzyme that release 1 µmol of *p*NP per minute under the experimental conditions. A calibration curve was prepared using *p*NP as standard (Bussamara *et al.*, 2010; Damaso *et al.*, 2013; Garcia Roman, 2005; Mercado-Malebran, 2014; Wrolstad *et al.*, 2005).

Enzymatic Kinetic Study

Kinetic parameters were determined by studying the effect of *p*NPP concentration (7.9, 79, 400, 790 and 1600 µM) on the reaction rate. Enzymatic activity for each concentration was measured during 30 min at 37°C and pH 7.0. The experiment was carried out in triplicate. V_{max} and K_M were calculated by fitting the curve to the Michaelis-Menten model by the Lineweaver-Burk method.

Thermal Stability and pH Effect

Lipase thermal stability was estimated by incubating 0.1 mL of the protein extract for 1 hour at different temperatures (30, 40, 50 and 60°C) in the absence of substrate. Then, the residual activity was measured at 50°C and pH 7.0 for 30 min using *p*NPP as substrate (Bussamara *et al.*, 2010; Juntachai *et al.*, 2011). Four repetitions were performed for each trial. Subsequently,

the enzymatic activity average was calculated. For the statistical analyze, a completely randomized design ANOVA was performed for unequal samples with a Tukey post hoc test.

Optimum pH was determined by quantifying the enzymatic activity at different pH (6.0, 7.0, 8.0, 9.0 and 10.0) (Bussamara *et al.*, 2010; Juntachai *et al.*, 2011). Four repetitions were performed at 37°C for 30 min and the relative enzymatic activity was calculated. For the statistical analyze, a completely randomized design ANOVA was performed for unequal samples with a Tukey post hoc test.

Results and Discussion

Detection of Lipase Activity

The presence of lipases in the protein extract was determined by a chromogenic method. At the end of the incubation period, a color change from fuchsia to yellow was observed in the well of the most concentrated sample (0.4 mg/mL), forming a 1 cm in diameter ring (Fig. 1A). The samples diluted in phosphate buffer (0.04 and 0.004 mg/mL) displayed a blurred and almost imperceptible halo (Fig. 1B and 1C). The dilution of the sample in water did not show ring formation (Fig. 1F). In the control samples (Fig. 1D and 1E) there was no change in coloration, which rules out any interference in the reaction.

The chromogenic medium evidenced the presence of lipase activity. The fatty acids released by the enzymatic action lead to the medium acidification, which takes on a yellow coloration when the pH is acidic (Nath and Hindumathy, 2012). This result shows the presence of lipases in the protein extract.

The similarity of B and C rings even though there is a 10-fold reduction in concentration, could be explained by the sensitivity of the method. While the method has been reported by Singh *et al*. (2006) to be highly sensitive (can detect as low as 0.5 enzyme units within 15 min), the concentrations of samples B and C were below this minimum range. Consequently, a relative lack of sensitivity probably occurred leading to no difference between B and C rings. Also, at concentrations below this minimum range, the amount of fatty acids released is not enough to produce the color-change into a brilliant yellow, even when a drop in pH have occurred and a change to red color is observed, as in B and C samples (Singh *et al.*, 2006). This also contributed to the absent of the ring in the sample diluted in water (F). However, the difference between F and B, that have the same concentrations, suggests that the phosphate buffer pH 7.0 in dilutions B and C favored the enzymatic activity, in contrast to the water-diluted sample (F), since most lipases work in an optimal pH range between 7.0 and 8.0 (Sharma *et al.*, 2001).

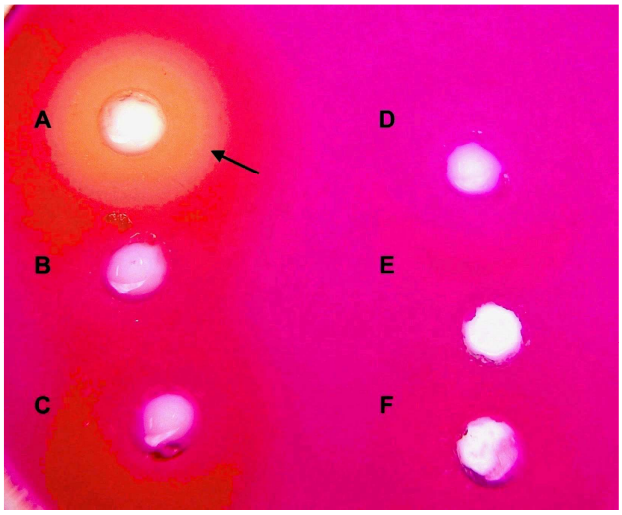

Fig. 1: Detection of lipase activity in the chromogenic medium. The medium contained phenol red as a colorimetric indicator. (A). Sample 0.4 mg/mL. (B). Sample 0.04 mg/mL. (C). Sample 0.004 mg/mL. (D). Buffer sodium phosphate pH 7.0. (E). Distilled water. (F). Sample diluted in water 0.04 mg/mL. The formation of a yellow ring around A, indicates the presence of lipases in the extract

Lipase Enzymatic Activity

The lipase produced with olive oil as inducer showed an activity of 0.27 U/mL. Enzyme production is a function of microbial growth, the inductor substrate used and the different ways of optimizing the medium (media composition, pH, presence of both activators and inhibitors that affect cell growth and lipases production, agents that affect the interface, processes fermentation, etc.) (Dhiman and Chapadgaonkar, 2013; Hadeball, 1991; Jonsson and Snygg, 1974; Sharma *et al.*, 2001).

Lipase production studies (Bussamara *et al.*, 2010; Cardenas *et al.*, 2001; Rapp and Backhaus, 1992; Singh and Mukhopadhyay, 2012; Thakur, 2012) distinguish between enzymatic activities in optimized and in non-optimized culture media. The lipase activity for *G. pullulans* reported here (0.27 U/mL) was from a nonoptimized medium and it is considered high when compared with enzymatic activities between 0.002-0.3 U/mL reported by Bussamara *et al*. (2010) for 29 yeast isolates in non-optimized induction media. In addition, it was also higher to those reported by Rapp and Backhaus (1992) for 15 yeast isolates in non-optimized media (0.002-0.15 U/mL). However, in optimized culture media it is possible to significantly increase the enzymatic activity. For example, *Candida rugosa*, cataloged as the best lipase producer, can increase its activity from 20-40 U/mL to 117 U/mL by several optimization processes (Tan *et al.*, 2003), even when starting from activities between 0.1-0.7 U/mL, as reported by early studies (Dalmau *et al.*, 2000).

Incubation time and temperature are also factors that influence lipase production (Park *et al.*, 2013). In this study, *Guehomyces pullulans* (CLQCA-ANT-073) was incubated for 960 h at 20°C. Incubation times reported in the literature are from 24 to 180 h, depending on the cultivation method, microorganism and the different ways of optimizing (Singh and Mukhopadhyay, 2012). However, in the case of cold adapted yeasts, the incubation times are longer: 240, 348 and 624 h with temperatures of 15-30°C, showing high activities at 15°C and 20°C (Bae *et al.*, 2014; Joseph *et al.*, 2012; Park *et al.*, 2013).

Guehomyces pullulans was recently reported as a lipase-producer in a bioprospection of 102 isolates from Antarctic yeasts (Martorell *et al.*, 2017). Therefore, there is no additional information regarding the factors and conditions that control its biosynthesis and secretion. For this reason, the results reported here should be considered as complementary contribution for the optimization process in order to improve the production of lipases from *Guehomyces pullulans.*

Kinetic Parameters

The kinetic curve for *G. pullulans* lipase showed the phenomenon of interfacial activation (Fig. 2) as it occurs in the majority of lipases (Reis *et al.*, 2009; Sanchez Ferrer, 1998; Sharma *et al.*, 2001; Verger, 1997). In this interfacial kinetics, the reaction rate increases after a substrate concentration close to 400 μM, being this value defined as an approximation to the critical micellar concentration.

Kinetic parameters were calculated by the Lineweaver-Burk linearization. V_{max} and K_M were 6.7 μmol/min/mL and 3.68×10^{-4} M, respectively. This K_M was lower than the one reported for *Pichia lynferdii* Y-7723 lipase (1.68×10^{-3} M) (Bae *et al.*, 2014), suggesting that the affinity of the *G. pullulans* enzyme is relatively high. On the other hand, K_M for *Candida rugosa* lipase B (4.2×10^{-4} M) (Pereira *et al.*, 2001) was similar to the one in *G. pullulans*.

K_M for *G. pullulans* lipase was higher than those reported for *Yarrowia lipolytica* NCIM 3639 lipase (2.2×10^{-5} M) (Sathish Yadav *et al.*, 2011) and for *C. rugosa* lipase A (3.92×10^{-5} M) (Gonzalez *et al.*, 2010), suggesting that the substrate affinity of the *G. pullulans* enzyme is relatively lower than those. It is worth noting that substrate affinity is within the range (10^{-1}–10^{-5}) of most industrial lipases (Bae *et al.*, 2014). Consequently, this result suggests that the lipase from *G. pullulans* (CLQCA-ANT-073) would have a potential industrial use.

Thermal Stability and pH Effect

The residual activity of the *G. pullulans* lipase was evaluated after incubation for 1 hour over a wide range of temperatures (30-60°C; Fig. 3). The maximum activity was found at 40°C. The enzyme retained 80% of its maximum activity at 50°C. In contrast, the enzyme only retained 45% activity at 30 and 60°C. The optimal stability temperature was defined between 40 and 50°C by the Tukey test. This rules out *G. pullulans* lipase as a cold-active enzyme, but it shows its greater thermostability compared to the cold-active lipase of *Pichia lynferdii* Y-7723 (5-30°C) (Bae *et al.*, 2014) and with *Candida rugosa* lipase (35-40°C) (Vakhlu and Kour, 2006).

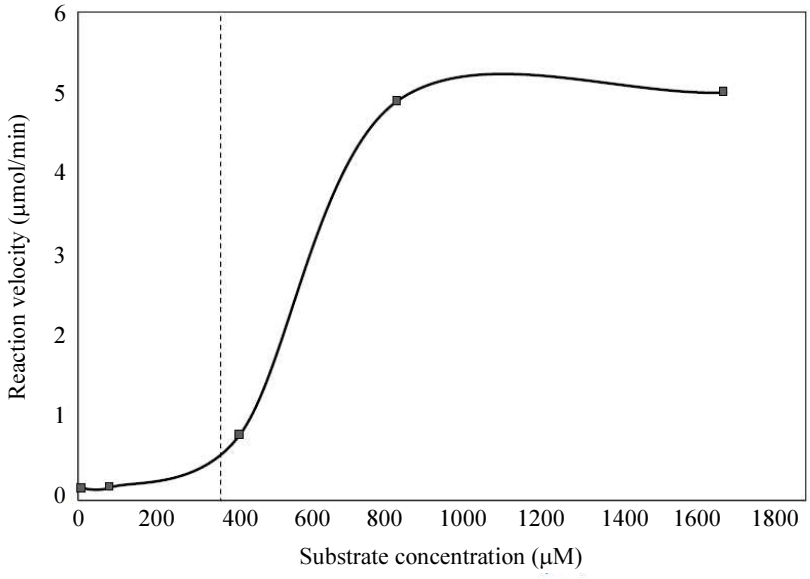

Fig. 2: Interfacial enzymatic kinetics of the lipase from *Guehomyces pullulans*. Five concentrations of substrate were used for each activity assay. The dotted line represents the moment of interfacial activation at a concentration close to 400 μM. This point indicates the critical micelle concentration

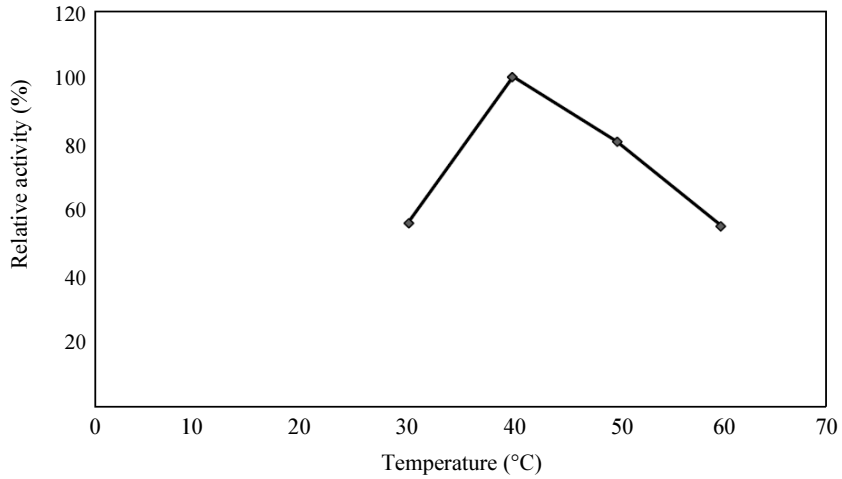

Fig. 3: Effect of temperature on the thermal stability of the lipase from *Guehomyces pullulans*. Enzyme activity assays were performed after incubating the lipase at the indicated temperatures for 1 hour. The optimum temperature is in the range of 40-50°C

Commonly, cold-active lipases exhibit high activity at low temperatures (0-20°C), but reduced thermostability (Bae *et al.*, 2014; Kavitha, 2016). On the other hand, highly thermostable lipases show optimal temperature values of 50-75°C (Gonzalez *et al.*, 2010; Sharma *et al.*, 2011). Consequently, the optimum temperature of the lipase of *G. pullulans* (40-50°C) is highly favorable, since being thermostable up to 50°C requires relatively less activation energy than the highly thermostable ones and, in turn, it is sufficiently high to promote lower risks of microbial contamination in industrial processes (Hasan *et al.*, 2006; Pereira *et al.*, 2001).

The development of this thermostable lipase (40-50°C) in *G. pullulans*, even though it is a cold-adapted yeast, could be explained by the kind of cold-adapted isolate we used in our work. Martorell *et al*. (2017) have determined that *G. pullulans* could be psychrophilic or psychrotolerant. These two forms of microorganisms colonize cold habitats such as Antarctica permanently or temporarily. However, only psychrophilic microorganisms, which are widely prevalent in permanently cold habitats, are still capable of growing at 0°C (or below) due to their unique physiological adaptations, such as the synthesis of cold active enzymes (flexible but not thermostable enzymes). Also, we could consider them "obligate psychrophiles" as they failing to grow above 20°C. In contrast, the psychrotolerant grow at approximately 0°C, but have an optimal growth above 20°C and are predominant in environments with periodic low temperatures. That means that psychrotolerant organisms evolved their physiology to tolerate cold, but they are not as specialized as psychrophiles, so the production of cold-active enzymes does not occur. Therefore, their enzymes can have more thermostability but less efficiency in low temperatures than the cold-active enzymes (Baeza *et al.*, 2017; Kavitha, 2016; Martorell *et al.*, 2017; Shivaji and Prasad, 2009).

The production of the *G. pullulans* thermostable lipase suggests that this enzyme comes from a psychrotolerant isolate, capable to synthesize a thermostable lipase. Another example is the lipase CAL-A of the Antarctic yeast *Candida antarctica*, that is considered the most thermostable lipase known (Kirk and Christensen, 2002; Shivaji and Prasad, 2009). It also can be possible since, although the Antarctic climate is mainly cold, the presence of several geothermal sites, including thermal springs, fumaroles, hot soils and hydrothermal vents, provides ideal environments for the development of thermophilic and hyperthermophilic microorganisms (Flores *et al.*, 2018).

To determine the optimum pH, the activity of the lipase from *G. pullulans* was assayed in a pH range (6.0-10.0; Fig. 4). The results showed a significant difference in the enzymatic activity, which indicates the dependence on pH. The maximum activity was recorded at pH 8.0. At neutral pH the enzyme still retained 83% of its maximum activity. On the other hand, at pH 6.0 and 9.0 the enzyme retained 36 and 42%, respectively. The lower activity was recorded at pH 10.0, where the enzyme only retained 10% of its activity. The optimum pH was defined at 8.0 using the Tukey test, which indicates that lipase activity is best at a slightly alkaline pH.

Most lipases reported in the literature have optimal activity at neutral or slightly basic pH values, as is the case of *C. rugosa* lipases, whose optimal values of 7.8 resembles the recorded in this study for *G. pullulans* lipase (Bussamara *et al.*, 2010; Sharma *et al.*, 2001; Vakhlu and Kour, 2006). It is known that a change in pH can lead to ionizable amino acids to undergo a change in their charges, followed by possible conformational changes in the enzyme active site (Bae *et al.*, 2014; Berg *et al.*, 2014).

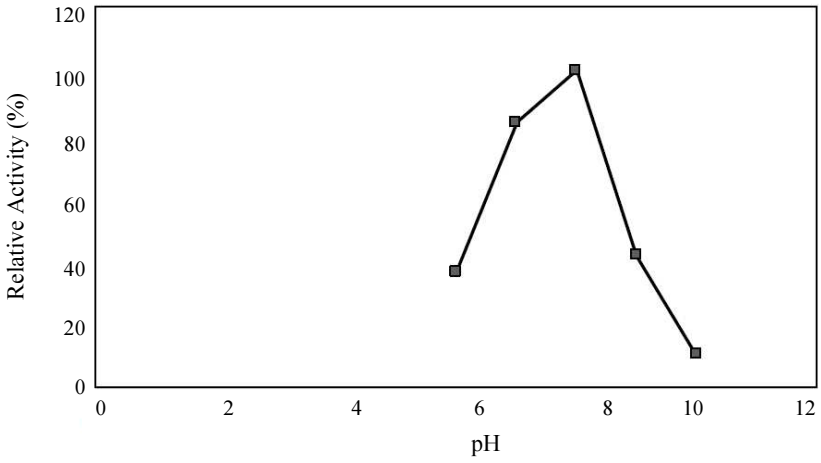

Fig. 4: Effect of pH on the enzymatic activity of the lipase of *Guehomyces pullulans*. The activity tests were carried out at the indicated pH and a temperature of 37°C. The optimum pH value was 8.0

It has been proposed that, in lipases, this tendency to alkaline pH could be due to an electrostatic repulsion between the negatively charged active site and the ionized fatty acids at alkaline pH, which leads to a rapid release of the reaction products of the interface (Reis *et al.*, 2009).

Conclusion

In this study, the lipase production from *Guehomyces pullulans* (CLQCA-ANT-073) in an olive oil inducer medium was high for a non-optimized medium. This shows that olive oil is a good inducer, which, however, can be replaced by another lipid substrate for lipases that could increase enzyme production in a media optimization process in future studies in order to have more enzyme for biotechnological proposes.

The lipase showed a significant dependence on pH and temperature to achieve maximum enzymatic activity, acting better in slightly alkaline conditions and at moderate temperatures. Regarding the kinetic parameters of the lipase, these are comparable to those of the most important reported lipases from other yeasts and show the enzyme potential for possible industrial uses.

The results present in this study constitute a first characterization of the lipase of *Guehomyces pullulans,* recently cataloged as a lipase-producer and could be useful for later work directed to study other factors that affect the enzyme activity, including inhibitors and activators and potential biotechnological applications.

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Author's Contributions

Luis Lascano Demera: Conducted literature research, carried out the whole laboratory work, designed and performed the experiments, wrote the manuscript and performed the data analysis.

Patricia Portero Barahona: Provided criteria for the design of microbiology experiments, kinetics, and revised the manuscript.

Enrique Javier Carvajal Barriga: Was the scientific director of the project. He established the research objectives; supported the design of experiments, data analysis, manuscript writing and collected the yeast strains at the Ecuadorian Antarctic Scientific Station by February 2012.

Ethics

The authors declare no ethical issues for the publication of this research work.

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