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Colletotrichum gloeosporioides lipase: Characterization and use in hydrolysis and esterifications

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Colletotrichum gloeosporioides **was evaluated for its capacity to produce extracellular lipase. A crude enzyme preparation obtained after 48 h of fermentation reached 742 U/L of lipolytic activity. Estimated molecular weight of proteins responsible for this activity was about 18 kDa as determined by SDS-PAGE and zymogram analysis. Enzyme preparation showed optimum pH at 10 and stability at optimum temperature (37°C) for 5 h. It showed tolerance to a wide range of salts (NH_4^+, Mg^{+2}, Ca^{+2}, Mn^{+2} and Sn^{+2}) and to some solvents (methanol, ethanol, isopropanol, 1-butanol, acetonitrile *n*-heptane and *n*-hexane) in different concentrations. The crude enzyme preparation was applied in hydrolysis reactions on different substrates (waste cooking soybean oil, cocoa butter and palm kernel oil) reaching high yields (87.6; 80.1; 74.9%, respectively). The preparation was lyophilized and it was applied in the synthesis of pineapple flavor, by esterification with butanol and butyric acid. *C. gloeosporioides* lipolytic enzymes synthesized butyl butyrate with 70% yield, in experiments carried out for 24 h using 1:1 acid/alcohol molar ratio in *n*-heptane medium. The lyophilized preparation was also able to perform transesterification of alcohols and *p*-nitrophenyl palmitate in organic medium (*n*-hexane), showing better activity when propanol was used ($5.4.10^{-3}$ U/kg.min). This study pointed the potential of alkaline lipolytic enzyme produced by *C. gloeosporioides* in biotechnological industry.**

Key words: Lipase, *Colletotrichum gloeosporioides*, hydrolysis, esterification, transesterification.

INTRODUCTION

Lipases are hydrolases (E.C. 3.1.1.3) widely distributed in nature. These enzymes, in association with cutinases and others esterases, play an important role in the biological cycling of lipids (Villeneuve et al., 2000). They are versatile biocatalysts that catalyze the breakdown of triglycerides with release of diacylglycerols, mono-acylglycerols and glycerol. They can catalyze, in non-aqueous media, esterification, interesterification and

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transesterification reactions, and acting on a broad range of substrates as natural oils, synthetic triglycerides and esters of fatty acids (Houde et al., 2004). They usually show good chemoselectivity, regioselectivity and enantio-selectivity, acting over a wide range of temperatures (Joseph et al., 2008).

Lipases have attracted great interest as a group of catalysts with proven potential to be used in biotechnological processes, still under explored, of lipid technology bio-industry (Treichel et al., 2010). Due to its versatility, its catalytic employment covers different areas such as obtaining food and esters used as flavors, production of detergents, application in optically active drugs resolution, production of fine chemicals, agrochemicals, biopolymers, as well as in use as biosensors, in bioremediation, and, even, for cosmetics and perfumes production (Hasan et al., 2006; Joseph et al., 2008).

The demand for new biocatalysts have boosted commercial production of enzymes and this expansion has led to projections of a global market for enzymes of 8 billion dollars in 2015 (Li et al., 2012). However, the industrial use of lipase is still limited by its commercial cost, particularly for applications that require large contingents of biocatalysts and generate low-value product (Ribeiro et al., 2011). Thus, more research is needed to achieve cost reduction and increased catalytic efficiency such as improvement of enzymes (by physical, chemical or genetic properties). Prospecting for microbial groups that form enzymes with new properties regarding specificity and stability to pH and temperature is also a modern trend on this area (Li et al., 2012).

Phytopathogenic fungi produce numerous extracellular enzymes to degrade cell wall polymers from plants in order to obtain nutrients and ensure infection during invasion process of plant tissues (Annis and Goodwin, 1997). The filamentous fungus *Colletotrichum gloeosporioides* is recognized as causing anthracnose (Huang et al., 2014). Especially, in tropical and subtropical fruits, affecting high value crops as mango, strawberry, avocado, citrus, papaya, cashew and passion fruit (Serra et al., 2011; Cannon et al., 2012). *Colletotrichum* is considered the eighth largest phytopathogenic genus in economic impact and scientific interest (Dean et al., 2012). *Colletotrichum* species are reported as producing cutinase, which allows the phytopathogen to penetrate through the cuticular barrier of the hosts (Dickman et al., 1982; Ettinger et al., 1987; Bonnen and Hammerschmidt, 1989). Other enzymes secreted by *Colletotrichum* described in literature are pectate lyase (Drori et al., 2003; Miyara et al., 2008), proteinases (Dunaevsky et al., 2007), lipases (Balaji and Ebenezer, 2008), celulases, amylases and esterases (Venkatesagowda et al., 2012).

In spite of the studies on enzymes produced by this organism, and their pathogenicity importance, there are only a few studies in the literature on biotechnological potential of *C. gloeosporioides* lipolytic enzymes. Thus, the objective of this work was to evaluate *C. gloeosporioides* lipase production by submerged fermentation, characterize the crude enzyme preparation obtained and test enzymatic activities in synthesis (in organic medium) and hydrolysis reactions to produce biotechnologically important products.

MATERIALS AND METHODS

All solvents used were of analytical grade and were obtained from Vetec Química fina LTDA (Rio de Janeiro, Brazil). Cocoa butter used was kindly donated by Cargill Agrícola S.A. Palm oil was obtained in trade from Salvador City, Bahia (Brazil) and the waste cooking soybean oil was donated by the academic community. Aloe and olive oil were obtained in trade from Belo Horizonte City, Minas Gerais (Brazil).

Fungus

A strain of *C. gloeosporioides* with known lipolytic activity from Food Microbiology Laboratory of Universidade Federal de Minas Gerais was used. It was maintained in potato dextrose agar (PDA) plates at 25°C for 5 days and after growth, agar plugs were taken and placed in distilled water at 25°C to long term preservation (Castellani, 1967).

Culture conditions and enzyme production

The isolates were grown on PDA plates for 5 days at 25°C. Spores suspension with concentration of 10^7 spores per milliliter was prepared. For production of lipase, 125 mL Erlenmeyer flasks containing 25 mL of fermentation medium (Colen et al., 2006), $g.L^{-1}$: peptone (10), $MgSO_4$. $7H_2O$ (0.6), KH_2PO_4 (1.0), NH_4NO_3 (1.0) and olive oil (4 mL) at pH 6.0 were inoculated with 1 mL of spore suspension. Cultivation was carried out under agitation (150 rpm) at 30°C for to 96 h. Each 24 h, three flasks were removed and their contents were filtered on Whatman (number 1) membrane, for biomass separation to obtain the crude enzyme preparation. Mycelia retained in the paper filter were used to determine biomass dry weight. In the crude enzyme preparation, were determined pH, total protein content and lipase and protease enzyme activities. Lipase specific activity was calculated (lipase activity/total protein content), each 24 h. Monitoring of enzyme production was carried out to determine higher production time, under conditions employed.

Biomass dry weight

Material retained on the filtration membrane was washed twice with ethanol and dried at 80°C until constant weight (Stone et al., 1992).

Total protein content

It was determined as described by Bradford (1976). Bradford reagent was added in microplates with the crude enzyme preparation (directly or after dilution if necessary). The mixture was gently stirred, left at rest for 15 min before reading absorbance at

595 nm. A bovine serum albumin (Merck) standard curve was built for comparison.

Lipase activity

Methodology was adapted from Winkler and Stuckmann (1979), using the *p*-nitrophenyl palmitate (*p*NPP) as substrate. A 3 mg/mL *p*NPP solution in isopropanol was mixed (1:9) with TRIS-HCl at pH 8.0 (0.09 M), arabic gum (2 $mg.mL^{-1}$) and triton X-100 (14 $mg.mL^{-1}$). The crude enzyme preparation was added to this mixture (directly or after dilution if necessary) and absorbance was determined immediately (at 410 nm) against a blank (without enzyme). Reaction took place for 10 min at 37°C. A standard curve using a *p*NP solution in isopropanol was used to calculate lipase activity. A unit of lipase activity was defined as the amount of enzyme required to release 1 µmol of *p*-nitrophenol (*p*NP) per L of crude enzyme preparation added to the test, by minute.

Protease activity

Protease activity was determined according to Charney and Tomarelli (1947). Substrate (1 mL) containing casein 2.5 % (w/v) in bicarbonate buffer (50 $mmol.l^{-1}$), at pH 8.3 was incubated at 37°C. The reaction was initiated with addition of crude enzyme preparation (1 mL) and was paralyzed after 30 min, with 8 mL of trichloroacetic acid 8% (w/v). After filtration, NaOH 0.5 $mol.l^{-1}$ (5 mL) was added to the filtrate (5 mL) and absorbance was read at 445 nm. For each sample, a blank with crude enzyme preparation denatured (boiled for 10 min) was prepared. A unit of protease activity was defined as the amount required to release 1 µmol of azocasein peptides per mL of crude enzyme preparation added to the test, by minute.

Molecular weight estimation

Molecular weight determination was accomplished by submitting the crude enzyme preparation to a SDS-polyacrylamide gel electrophoresis as reported by Laemmli (1970). It was carried out on a 12.5% gel at room temperature. Then, this gel was treated with triton X-100 (2.5%) to remove the SDS obtaining the proteins in native form. A zymogram was carried out by overlaying this gel with a layer of chromogenic substrate solution (Phenol red 0.01%; olive oil 1%; 10 $mmol.L^{-1}$ $CaCl_2$ and 2% agar at pH 7.4). Once synthetic molecular weight markers were used in the same gel used for running the zymogram, degradation of the substrate changed gel color where the catalytic protein was. In this way, molecular weight of lipase was estimated comparing location of the band holding catalytic activity with molecular weight marker bands.

Enzyme storage

Lipolytic activity was monitored by two months at room temperature, the 4 (refrigerator), -18 (freezer) and -80°C (ultrafreezer). Crude enzyme preparation was maintained at -80°C for following tests.

Enzyme stability

Stability tests were conducted according to Castro-Ochoa and collaborators (2005) methodology, modified, using *p*NPP as substrate. All analyses were made in triplicate and lipase activities of the sample without any organic solvent, ion or detergent were taken as the control (100% of activity). Residual activity was measured by *p*-NPP spectrophotometric assay at 37°C, at pH 8.0.

Thermal stability was determined by incubation of crude enzyme preparation (0.5 mL) at different temperatures (30, 40, 50, 60 and 70°C) by 1 h, followed by enzymatic activity quantification. The temperature at which the enzyme was more stable by 1 h was also evaluated after 24 h. For stability in solvents, crude enzyme preparation (0.5 mL) was incubated with three concentrations (6, 30 and 50% v/v) of water miscible solvents (methanol, ethanol, isopropanol, 1-butanol, acetonitrile) and three concentrations (30, 50 and 75% v/v) of water immiscible solvents (toluene, *n*-hexane, *n*-heptane and dichloromethane) at 37°C for 1 h and then enzymatic activity was dosed. For pH stability, crude enzyme preparation was incubated at 37°C for 1 h with different buffers (0.05 mol/L) (citrate-phosphate to pH 4-6; TRIS-HCl to pH 7-9 and glycine-NaOH to pH 10-11) and enzymatic activity was dosed. To study the behavior of the activity and stability of lipase towards different ions, crude enzyme preparation (0.5 mL) was incubated at 37°C for 1 h with aqueous solutions (0.5 mL) containing 1 and 10 $mmol.L^{-1}$ of NH_4^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Sn^{2+} and EDTA and then, enzymatic activity was dosed.

Results were submitted to analysis of variance (ANOVA, single-factor), followed by Tukey test with 95% confidence to evaluate possible statistically significant differences.

Optimum pH and temperature of reaction

Based on lipase thermal stability, optimum temperature was determined by lipolytic assay as described previously, in a 30 - 45°C range of temperature. Optimum pH was determined by lipolytic assay, in a 4-11 range of pH, buffered (50 mM) with same pH solutions.

Applications of lipase

Hydrolytic activity

To evaluate the hydrolytic capacity of lipase in crude enzyme preparation, titrations tests were carried out (Colen et al., 2006), using different oils (olive, palm kernel and aloe oils, waste cooking soybean oil and cocoa butter) as substrates. The enzymatic reactions, prepared in Erlenmeyer flasks were incubated on a reciprocal shaker at 45 cycles/min, at 30°C for 10 min. Each reaction contained 5.0 mL of oil emulsion (25 % v/v) in polyvinyl alcohol (2 % v/v) as substrate, 5 mL of buffer TRIS-HCl 0.1 $mol.L^{-1}$, pH 8.0 and 1 mL of crude enzyme preparation natural or after denaturation process by boiling at 100°C for 10 min (blank reaction). Reaction was stopped by adding 10 mL of acetone/ethanol (1:1). After the reaction, the released fatty acids were determined by titration with NaOH 0.05 N using thymolphthalein (0.2 %) as a pH indicator. Hydrolytic activity was expressed in percentage of hydrolysis (fatty acids released) by min, per mL, under test conditions, and olive oil hydrolysis value was taken as 100%.

Transesterification activity

To assess lipase activity in medium under low water content, a transesterification test was carried out as described by Teng and Xu (2007) and modified. This method is based on transesterification catalyzed by lipase between esters of fatty acids and alcohol, in the absence of water. *p*-nitrophenyl palmitate (*p*NPP) and different alcohols (methanol, ethanol, isopropanol, 1-butanol and dodecanol)

were used as substrate to transesterification. Crude enzyme preparation was lyophilized and weight equivalent to 20 mg of protein was suspended in 0.5 mL of substrate solution (*p*NPP 10 mmol.L^{-1} in *n*-heptane). Reaction was initiated with the addition of alcohol (30 µL) to the mixture, and the reaction was incubated at 37°C for 30 min under agitation (200 rpm). After the mixture settled for 30 s, the clear supernatant (100 µl) was mixed with NaOH 0.1 mol.L^{-1} (1 mL). The *p*NP released, extracted from the alkaline aqueous phase, was dosed at 410 nm against a blank (without enzyme) in a UV-visible spectrophotometer (Varioskan Flash Multimode reader, Thermo Scientific). A standard curve of *p*NP in *n*-heptane was built in order to obtain quantitative results.

Esterification activity

Following Silva and collaborators (2014), modified, a mixture of butanol (250 mmol.L^{-1}) and an acid (butyric, acetic or oleic, at 250 mmol.L^{-1}), diluted with *n*-heptane was used as substrate. This reaction medium (5 mL) was incubated with 1 g of lyophilized crude enzyme preparation containing *C. gloeosporioides* lipase, at 37°C, 200 rpm, during 24 h. The esters yields were quantified by measurements of residual acid concentration in the reaction mixture. An aliquot of 500 µL was taken, diluted with ethanol/acetone 50:50 (v/v), homogenized and titrated with NaOH solution (20 mmol.L^{-1}) using phenolphthalein as indicator. Experiments were carried out in triplicates.

RESULTS

Obtaining crude enzyme preparation

Monitoring the fungus growth, under incubation in liquid substrate fermentation, showed a maximum production of biomass after 48 h, reaching 5 g.L^{-1} of growth medium. Maximum production of lipase, 742 U/L (enzyme units per liter of filtered fermented broth), also occurred in 48 h, with a specific activity of 4.68 (units of lipase per mg of protein per mL of filtrate). Maximum protease activity (2.7 U/L), was detected after 24 h of fermentation, but its activity did not affect lipase activity, that remained virtually unchanged until 96 h of incubation. Then, a period of 48 h of incubation was chosen for crude enzyme production.

Some industrial scale applications of enzymes demand a partial characterization of enzymatic crude extract, in order to suit process conditions to the peculiarities of the enzymes. In this context, an estimation of enzyme molecular weight was conducted, as well as investigation of best storage conditions and evaluation of *C. gloeosporioides* lipolytic enzymes stability towards solvents, ions, pH and temperature. To estimate molecular weight, a SDS-PAGE gel of crude enzyme preparation was compared with a zymogram containing a lipid emulsion at a concentration of 1% and using a pre-stained molecular weight marker. The fraction with enzymatic activity presented approximately 18 kDa (Figure 1), resembling that found by Chen and collaborators (2007) from another lipolytic enzyme of *Colletotrichum.*

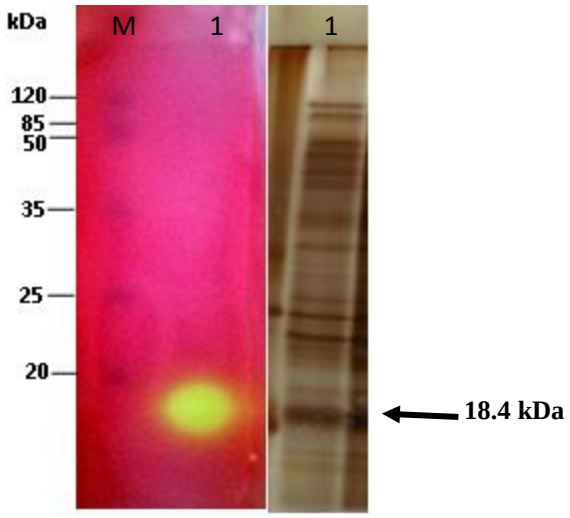

Figure 1. Molecular weight estimation of *C. gloeosporioides* crude enzyme preparation with lipolytic activity by comparison between zymogram (left) and SDS-PAGE analysis (right).

Enzyme storage

Lipolytic activity present in crude enzyme preparation was stored over 60 days at different temperatures. The activity was measured and demonstrated to be very unstable when stored at room temperature (25°C): significant decrease occurred over time (α=0.5), remaining around 50% of initial activity after 60 days. At lower temperatures (4 and -20°C), at the same incubation period, 86.8 and 90% of initial activity were maintained, respectively. Greater stability has been detected when the enzyme preparation was stored on ultrafreezer (-80°C). In this case, enzyme preparation showed greater activity than before being stored, suggesting stability of enzyme activity under freezing conditions. These data corroborate results obtained with carboxilesterases stored at low temperature (Corrêa et al., 2013).

Effect of temperature on enzyme activity and stability

In order to characterize the properties of the crude enzyme preparation some of its characteristics were assayed. Crude enzyme preparation retained 100% of lipolytic activity after 1 h of incubation at 30 and 40°C (Figure 2). Studies with *Fusarium graminearum* lipases, also pointed stability and activity at that temperature range (Nguyen et al., 2010). From 50°C, thermal stability of *C. gloeosporioides* enzyme was drastically reduced,

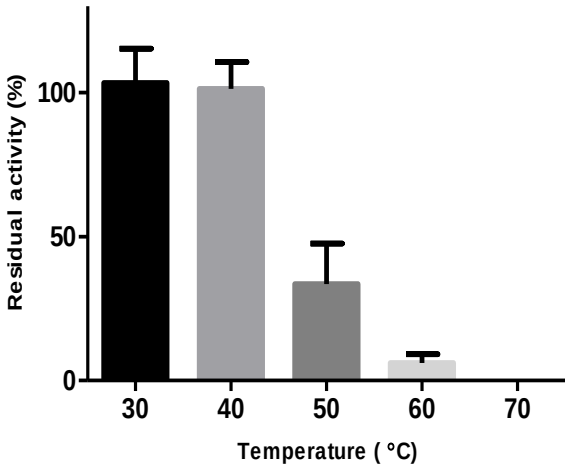

Figure 2. Temperature effect on lipase from *C. gloeosporioides* stability. The lipase was incubated for 60 min at 30, 40, 50, 60 and 70 °C, and then residual activity was measured using pNPP at pH 8.0, 37 °C. Different letters represent statistically significant difference for 95% confidence.

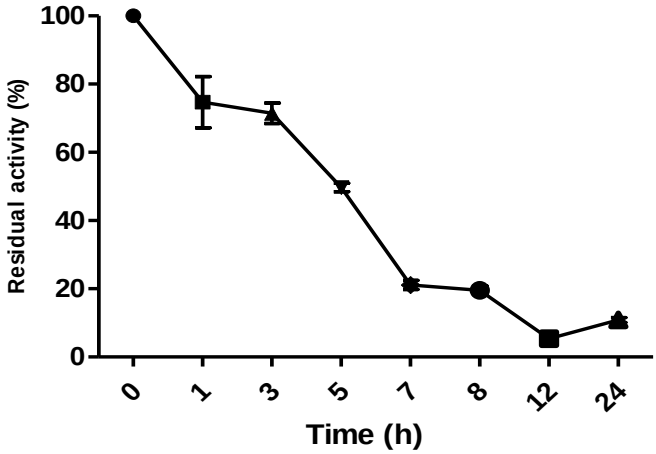

Figure 3. Temperature effect on lipase from *C. gloeosporioides* stability for 24 h. The lipase was incubated for 24 h at 37°C, and then residual activity was measured using *p*NPP at pH 8.0, 37°C.

retaining one-third of its activity, while, at 60°C only 6% of activity was preserved. Total activity loss was verified at 70°C. The lack of activity at higher temperatures suggests modifications in three-dimensional structure and, consequently, loss of function.

Catalytic activity of *C. gloeosporioides* lipase was detected at 37°C. Optimal temperatures close to 37°C have been reported for *Mucor hiemalis f. corticola* lipases (Ulker and Karaoglu, 2012). Then, in the present study, enzyme thermal stability was evaluated at this temperature for 24 h (Figure 3). During the first three hours, lipase activity was maintained, holding 80% of initial activity. Half-life of the enzyme occurred after 5 h of incubation, with sharp decline after this period. These results show a special behavior of *C. gloeosporioides* lipase. Li and collaborators (2013) for instance, evaluating *Stenotrophomonas maltophilia* lipase, observed enzyme half-life as 40 min at 40°C. Therefore, the results are very interesting since long term stability is a required characteristic for enzyme industrial applications.

pH effect on enzyme activity and stability

C. gloeosporioides lipase has remained relatively stable in the pH range 5 to 8 (60% recovery activity or more), with greater stability at pH 6.0, after 1 h at 37°C (Figure 4). It was more unstable at more alkaline pH, keeping about 30% of original activity at pH 9 and 10; retained about 20% of the activity at pH 11. Despite having been shown little resistance to alkaline pH (less than 1 h), the crude enzyme preparation presented optimum pH (higher activity) at 10.

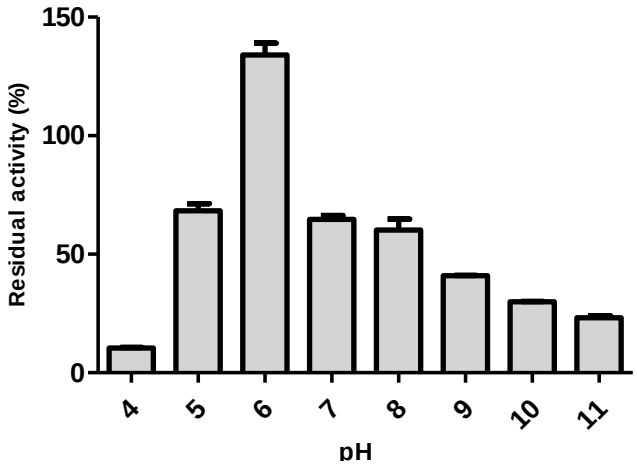

Figure 4. Effects on stability of lipase from *C. gloeosporioides*. The lipase was incubated for 60 min at 37°C in the presence of different buffer (50 mM): citrate-phosphate to pH 4-6; TRIS-HCl to pH 7-9 and glycine-NaOH to pH 10-11, and the residual activity was measured using *p*NPP at pH 8.0, 37°C. Different letters represent statistically significant difference for 95% confidence.

Solvent stability

Stability of lipase in organic medium is important for esterification reactions (Ulker and Karaoglu, 2012).

Table 1. Effects of water miscible and immiscible solvents on stability of *C. gloeosporioides* lipase. The lipase was incubated for 60 min at 37°C in the presence of different solvents in different concentrations (% v/v). Then, the residual activity was measured using *p*NPP at pH 8.0, 37°C.

Concentration (% v/v)	Residual activity (%)			
	6	30	50	75
Methanol[a]	132.5	30.9	13.1	[c]
Ethanol [a]	143.1	28.1	5.6	[c]
Isopropanol [a]	124.9	23.7	3.8	[c]
Butanol [a]	150.6	24.1	18.9	[c]
Acetonitrile[a]	100.8	3.1	0.0	[c]
Toluene [b]	[c]	223.5	5.4	0.0
Heptane [b]	[c]	84.3	118.7	223.3
Dichlorometane [b]	[c]	85.5	80.6	57.1
Hexane [b]	[c]	89.5	122.0	127.8

[a] Miscible solvents; [b]immiscible solvents; [c]not determined.

Enzyme stability was tested in five different water miscible solvents (methanol, ethanol, isopropanol, 1-butanol and acetonitrile) in three concentrations (6, 30 and 50%, v/v). *C. gloeosporioides* lipase exhibited tolerance to low concentrations (6% v/v) of all solvents (Table 1). At higher concentrations, the enzyme was most active in methanol (30% v/v) and butanol (50% v/v). Total loss of activity was observed when acetonitrile concentration was higher than 30% v/v.

This lipase also proved to be tolerant to water immiscible solvents: it was stable in *n*-heptane and *n*-hexane in all concentrations tested, with highest activity with 75% v/v of *n*-heptane (Table 1). In toluene and dichloromethane, the enzyme was more stable at low concentration (30% v/v), with total loss of activity in toluene 50% v/v. Stability of a lipase from *Mucor hiemalis f. hiemalis* in *n*-hexane and *n*-heptane has been previously reported (Hiol et al., 1999).

Effect of ions and EDTA on enzyme stability

Ionic components may interfere with the activity and stability of lipase present on a crude enzyme preparation. *C. gloeosporioides* enzyme was stable against all the tested solutions at 1 mmol.L^{-1} (Figure 5). The ions NH_4^+ and Sn^{2+} stimulated lipolytic activity in both concentrations assayed (1 and 10 mmol.L^{-1}). Yu and collaborators (2013) showed that optimal levels of NH_4^+ lead to increased lipolytic activity in *Rhizopus oryzae*. Stimulation of lipolytic activity in the presence of NH_4^+ has also been demonstrated by lipases from *Nomuraea rileyi MJ* (Supakdamrongkul et al., 2010) and *Aspergillus carneus* (Saxena et al., 2003).

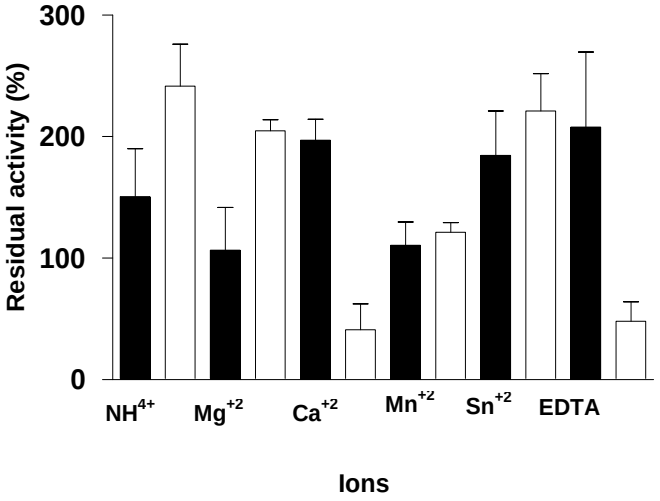

Figure 5. Ions effects on stability of lipase from *C. gloeosporioides*. The lipase was incubated for 60 min at 37°C in the presence of different ions: $NH4^+$, Mg^{2+}, Ca^{2+}, Mn^{2+}, Sn^{2+} and EDTA in two different concentrations: 1 mmol.L^{-1} (columns in black) and 10 mmol.L^{-1} (columns in white) and then the residual activity was measured using *p*NPP at pH 8.0, 37°C.

As well as calcium, chelating agent EDTA affected lipase in different ways, enhancing lipolytic activity at a concentration of 1 mmol.L^{-1} but, at 10 mmol.L^{-1} loss of lipolytic activity by 50% was observed. This effect observed for EDTA suggests that *C. gloeosporioides* lipase may be dependent on divalent ions.

Ions may interfere with enzyme activity, acting directly in enzyme catalytic site (Lee and Rhee, 1993; Supakdamrongkul et al., 2010). They can also act in the substrate, due to formation of complexes between metal ions and ionized fatty acids, increasing their solubility and modifying their behavior on an oil/water interface.

Enzyme applications

C. gloeosporioides lipase showed capacity to catalyze esterification, hydrolysis and transesterification reactions. The hydrolytic activity on different substrates is shown in Figure 6. This enzyme was able to hydrolyze all substrates tested. Considering olive oil hydrolysis as the reference, the highest hydrolysis degree occurred using waste cooking soybean oil (87.6%) and the lowest hydrolysis degree was detected for aloe oil (32.7%). The results showed that *C. gloeosporioides* lipase have broad hydrolytic activity, corroborating with literature for lipases from other sources (Chen et al., 2007; Balaji and Ebenezer, 2008).

In this context, it was observed that *C. gloeosporioides* lipase was able to perform transesterification in organic medium (with *n*-hexane), showing greater activity on short chain alcohols. The major activity of transesterification

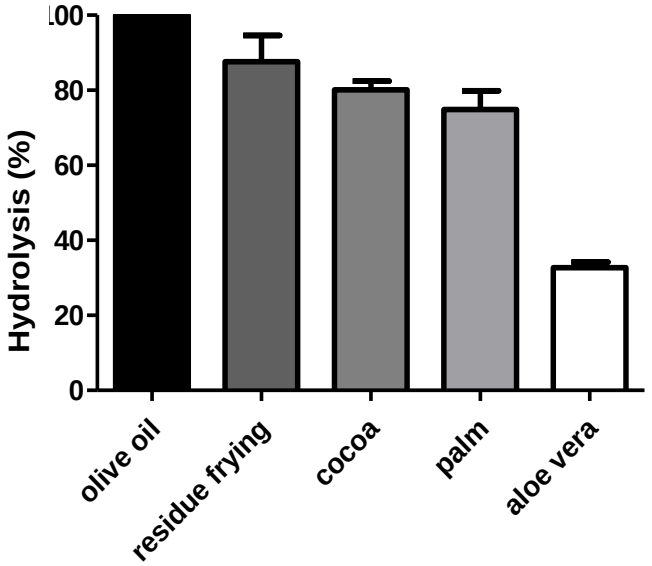

Figure 6. Percentage of hydrolysis of different oils (25 % v/v) using lipase from *C. gloeosporioides* incubated under agitation (45 cycles/min) at 30°C for 10 min. Each reaction contained 5.0 mL of oil emulsion in polyvinyl alcohol (2% v/v), 5 mL of buffer TRIS-HCl 0.1 mol.L^{-1}, pH 8.0 and 1 mL of crude enzyme preparation natural or after denaturation process by boiled to 100°C for 10 min (blank reaction). Olive oil hydrolysis value was taken as 100%.

occurred using propanol ($5.4.10^{-3}$ U/kg.min) and the smallest activities were observed for butanol and dodecanol. According to these results, there is evidence of lipase increased efficiency in transesterification reactions using short chain alcohols (e.g. methanol, ethanol and propanol instead of butanol or dodecanol). This is an interesting feature since other lipases, such as that obtained from *Aspergillus niger* showed better activity using pentanol instead of ethanol and methanol in esterification with lauric acid (Mustranta et al., 1993).

The capacity of *C. gloeosporioides* in esterification was also evaluated in the synthesis of butyl butyrate, butyl acetate (short chain esters used in food industry to produce, for instance, pineapple flavor) and butyl oleate (biodiesel additives). Esterification experiments were carried out for 24 h under 1:1 acid/alcohol molar ratio in *n*-heptane medium and absence of water. The amounts of synthesized butyl butyrate, butyl acetate and butyl oleate were 70, 14 and 40%, respectively. Bayramoglu and collaborators (2011) showed a yield of 42.6% of butyl butyrate synthesis using *Mucor miehei* lipase, in similar conditions.

DISCUSSION

Although lipolytic enzymes from phytopathogenic fungus have been widely studied in order to apply them to infections control, little have been undertaken on the physico-chemical characteristics of *C. gloeosporioides* lipase, to evaluate its potential industrial applications. This study showed individual characteristics of *C. gloeosporioides lipase* that suggests some potential uses.

C. gloeosporioides has recognized lipolytic activity (Colen et al., 2006; Chen et al., 2007; Balaji and Ebenezer, 2008). In this work, this fungus produced a crude enzyme preparation containing lipolytic activity obtained by fermentation in liquid substrate. Despite having detecting proteolytic activity in crude enzyme preparation, activity remained low and virtually unchanged throughout the remaining fermentation time, without generating negative interference on lipolytic activity present in the fermented broth. Composition of the culture medium used, with low protein content, may have influenced the formation of enzyme with negligible proteolytic activity.

C. gloeosporioides lipase presented long thermal stability at its optimum temperature. This long stability is interesting to lipase application once long industrial processing demand stable enzymes in these conditions. This lipase presented better stability at pH around 6, similarly to what was observed for *Mucor griseocyanus* lipase (Coca and Dustet, 2006). However, results found for *C. gloeosporioides* lipase demonstrate a wide range of pH stability and predilection by mild alkaline condition, in agreement with other study, which described other alkaline enzymes produced by this species (Dunaevsky et al., 2007).

The optimal pH alkaline, around 9-10, has been previously described for lipolytic enzymes of *C. gloeosporioides* (Dickman et al., 1982), *Colletotrichum lagenarium* (Bonnen and hammerschmidt, 1989) and *Fusarium solani f. pisi.* (Purdy and Kolattukudy, 1975). Lipases stable below 60°C, which tolerate alkaline pH, are interesting for application in detergent industry (Sangeetha et al., 2010) because enzymes used in this industry need to be active and stable under laundry conditions.

Lipases present in detergent formulations should be compatible with oxidizing and surfactants agents and present wide substrates specificity (Jellouli et al., 2011; Chauhan et al., 2013). Broad substrate specificity of detergent formulations is interesting because it increases their effectiveness on different types of dirt from different sources. On this aspect, the lipase from *C. gloeosporioides* also seems interesting since it was able to act on different fatty materials (olive oil, palm kernel oil, aloe oil, waste cooking soybean oil and cocoa butter).

Other interesting characteristic showed by crude enzyme preparation was a good stability in waste cooking oil, catalyzing oil hydrolysis with great yields. Waste cooking oil is receiving much attention recently because it can be applied as low cost substrate for lipase-mediated biodiesel production (Lam et al., 2010). This application, however, demands lipase stability in the oil, once they have high levels of free fat acids. According to the results

of this study, *C. gloeosporioides* behavior in waste cooking oil associated with its transesterification capacity indicates its applicability in transesterification reactions to produce biodiesel on these low-cost substrates (waste cooking oil).

Other good indicator is the pronounced stability of *C. gloeosporioides* lipase towards immiscible solvents, as well as its tolerance to low concentrations of miscible solvents. Lipases can perform esterification reactions in low water content conditions (Singh and Mukhopadhyay, 2012). Immiscible organic solvents are the preferred reaction medium because they allow greater solubility of substrates. Unlike miscible solvents, they do not contribute to disappearance of the aqueous layer, which is fundamental to folding and stability of the enzyme (Tamalampudi et al., 2007). However, miscible solvents such as short-chain alcohols are common substrates in lipase-mediated esterification reactions (Mustranta et al., 1993; Tan et al., 1996). Thus, stability of these enzymes in front of miscible solvents is also required for an efficient performance of these catalysts.

The stability showed by crude enzyme preparation in water immiscible solvents, associated with its ability in catalyze the synthesis of short chain esters, with particular efficiency in formation of butyl butyrate, when still in free form the enzyme reached high conversion rates, indicating its use in industry to synthesis esters used as food flavoring agents.

Conflict of interest

The authors declare that they have no conflict of interest.

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