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Thermotolerant lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583: Effect of carbon sources on enzyme production, biochemical properties of crude and purified enzyme and substrate specificity

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ABSTRACT

In this study, *Penicillium* sp. section *Gracilenta* CBMAI 1583 was used to produce lipase under submerged conditions. The enzyme was purified and the biochemical properties of both the crude and purified enzymes were evaluated. Maximum lipase production (1.62 U mL^{-1}) was obtained using olive oil 0.5% (w v^{-1}) after 72 h of cultivation, representing a 90% increase in the lipase initially produced. The enzyme was purified using hydrophobic interaction chromatography (phenyl Sepharose) under conditions which allowed its interfacial activation. The partially purified sample showed an enzyme with esterase activity (65.4 kDa) on α- and β-naphthyl acetate and other with lipase activity (52.9 kDa) on octyl oleate. Optimum activity of crude and purified lipase was observed at pH 4.0 and 70 °C. The purified lipase was activated by NaCl, $BaCl_2$, NH_4Cl, $MnSO_4$ and $MgSO_4$; it also presented high stability in organic solvents such as hexane, 2.2.4-trimethylpentane, acetone, DMSO and toluene. Maximum enzyme activity was observed with *p*-nitrophenyl decanoate as substrate; and the enzyme kinetics showed to be directly affected by Triton X-100. The enzyme shows potential application in processes that operate in acid pH, such as treatment of dairy and industry effluents, resolution of esters in the pharmaceutical industry or in the food industry, as well in synthesis reaction under non-aqueous conditions.

1. Introduction

Lipases (triacylglycerol acyl hydrolases, E.C. 3.1.1.3) constitute an important class of enzymes that catalyze the hydrolysis of triacylglycerol ester bonds at oil/water interface (Manoel et al., 2015). Microbial lipases stand out in the current industrial scenario due to its broad application spectrum, which may be related to their diverse functions. In addition to hydrolysis of long-chain triglycerides, these enzymes can catalyze the reverse reaction in organic media, performing esterification of fatty acids and glycerol into triglycerides, as well as acting on transesterification, and alcoholysis reactions, among others (Tan et al., 2015; Kumar et al., 2016).

Lipases are considered an environmentally friend alternative for many industrial processes, contributing to reduce the amount of largely employed chemicals. In detergents, they can be used as biodegradable and non-toxics additives, resulting in harmless residues (Sharma et al., 2017). In the production and refinement of oleochemicals, lipases are efficiently applied to release fatty acids and glycerol from lipids (Ibrahim et al., 2008). In the food industry, lipases are applied to enhance dairy flavor and in the processing of meat, vegetables, fruits and beer; in addition, they can also be used to reduce calories of lipids by fatty acids transesterification (Houde et al., 2004; Aravindan et al., 2007). In pharmaceutical and cosmetic industries, lipases are used to obtain mono-, di- and triglycerides that may act as colorants, fragrances, and sunscreen or makeup additives. Lipases may be also applied on leather manufacture for lipids removal (Gandhi, 1997).

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Filamentous fungi are excellent enzymes producers under submerged and solid-state cultivation conditions. Lipases from *Penicillium* species show high potential in fermentation platforms: lipase from *Penicillium simplicissimum* was produced with triolein as carbon source, and the enzyme purified showed molecular weight of 56 kDa and stability in the pH from 5 to 7 and at 50 °C (Sztajer et al., 1992). In other studies, lipase from *P. simplicissimum* was obtained after solid state cultivation using mixed substrate and low-cost nitrogen sources (Gutarra et al., 2007; Vargas et al., 2008) and the enzyme was purified and immobilized using hydrophobic supports under interfacial activation conditions (Cunha et al., 2009). *Penicillium wortmanii* was screened as the best lipase producer using 5% (w v^{-1}) olive oil as the carbon source; and the crude lipase presented optimum activity at pH 7.0 and 45 °C; besides the enzyme was highly stable at 40 and 45 °C and retained 55% of activity at 50 °C (Costa and Peralta, 1999). Lipase from *Penicillium restrictum* was obtained in solid waste from the babassu oil industry supplemented with peptone, olive oil or starch. Lipase activity was very sensitive to the type and level of supplementation, and it was decreased by increasing protease level and pH of the media (Gombert et al., 1999). An economic analysis of lipase production by *P. restrictum* in both submerged and solid state fermentations performed at 100 m^3 lipase/year production scale, showed that the submerged process is economically unfeasible, with the unitary product cost 68% higher than the product selling price, however the solid-state fermentation is very attractive from an economic point of view with the product cost 47% lower than the selling price, presenting 1.5 years payback time, 68% investment return of and 62% internal return rate in a 5-year-project life (Castilho et al., 2000).

The *Penicillium* strain used in this work was isolated from Atlantic forest soil and previously screened as lipase producer (Tauk-Tornisielo et al., 2005). The immobilization was previously evaluated using hydrophobic supports, *i.e.* agarose based butyl-(But), phenyl-(Phe) and octyl-Sepharose (Oct), acrylic Toyopearl (Toyo), and macroporous Lewatit VP OC 1600 (Lew) and octadecyl Sepabeads (Sep) — to obtain highly active and stable biocatalysts. The properties of the immobilized enzyme were compared to the cyanogen bromide derivative which emulates the properties of the soluble enzyme, but without problems caused by molecular interaction. The derivatives were characterized and applied in reactions to obtain Omega-3 fatty acids and ethyl esters from fish oil, a very appreciated product in food and pharmaceutical industries (Turati et al., 2017). The aims of this work were to evaluate the culture conditions using pure and complex carbon sources for lipase production under submerged cultivations. The crude lipase was purified by using the interfacial activation strategy in hydrophobic interaction chromatography and the purified enzyme was biochemically characterized.

2. Materials and methods

2.1. Microorganism

The *Penicillium* strain was isolated from soil of Atlantic Rainforest, at Juréia-Itatins Ecological Station, São Paulo, Brazil (Ruegger and Tauk-Tornisielo, 2004), and preliminarily identified as a lipase producer (Almeida 2013). Morphological and molecular taxonomy revealed that the *Penicillium* strain belongs to the section *Gracilenta*; however, it was not possible to classify at the specie level. The strain is available at the Brazilian Collection of Environmental and Industrial Microorganisms – CBMAI/CPQBA – UNICAMP, Paulínia, São Paulo, Brazil, under registration number CBMAI 1583.

2.2. Strain maintenance and inoculum preparation

Penicillium strain was periodically cultivated on 3% (w v^{-1}) oat-agar slants for five days at 28 °C and stored at 4 °C. Inoculum was prepared by suspending conidia from five days-old cultures in sterile distilled water. One milliliter of the conidia suspension adjusted to 10^7 conidia mL^{-1} was used to inoculated the culture media.

2.3. Enzyme production

Cultures were carried out in Erlenmeyer flasks (125 mL) containing 25 mL of culture medium (g L^{-1}): KH_2PO_4 2.0, bacto-peptone 5.0, yeast extract 1.0, $NaNO_3$ 0.5, KCl 0.5, $MgSO_4{\cdot}7H_2O$ 0.5, olive oil 10.0, pH adjusted to 5.5 (Dheeman et al., 2011). Cultivation was performed at 160 rpm, during 120 h and 28 °C. The crude extract was obtained separating the mycelium by filtration. The filtrate was used as enzyme source and biomass was dried at 105 °C until constant weight.

2.4. Effect of carbon sources

Pure carbon sources (glucose, lactose, glycerol, citric acid, tributyrin, triolein, trioctanoate, and lauric, palmitic, myristic, stearic, oleic and linoleic acids) and natural triacylglycerol (canola, castor, corn, linseed, olive, palm, soybean and sunflower oils, chicken fat, lard and beef tallow) were individually evaluated at 1.0% (w v^{-1}). Fatty acids, lard and beef tallow were previously emulsified with 0.1% (w v^{-1}) Tween 80 under vigorous agitation for 5 min. Controls were prepared without carbon source. Olive oil was further evaluated at 0.25%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5% and 3.0% (w v^{-1}). Cultivation was carried out in pH 5.5, 160 rpm for 72 h at 28 °C.

2.5. Influence of temperature

Cultivation was carried out at 24, 26, 28, 30 and 32 °C; at pH 5.5, 160 rpm for 72 h.

2.6. Lipase activity and protein assays

Lipase activity was assayed according to Almeida et al. (2012), using *p*-nitrophenyl-palmitate (Sigma-Aldrich). The reaction was carried out in McIlvaine buffer pH 7.0 at 37 °C for 5 min and interrupted by heat shock (90 °C, 1 min) followed by the addition of 1 mL saturated sodium tetraborate solution. The released *p*-nitrophenolate (*p*NP) was measured at 405 nm (ε = 18,033 M^{-1} cm^{-1}). Controls were prepared without enzyme. One unit of enzyme activity was defined as the amount of enzyme capable of releasing 1 μmol of product per minute.

Protein was determined with the modified Bradford method (Sedmak and Grossberg, 1977), using bovine serum albumin as standard. All assays were performed in duplicates.

2.7. Enzyme purification by hydrophobic interaction chromatography

The culture filtrate was dialyzed against 0.05 M ammonium acetate buffer pH 6.9 (6 h, 3 changes, 4 °C). After dialysis, the sample was applied to HiprepTM 16/10 Octyl Sepharose FF column (GE Healthcare) previously equilibrated in the same buffer. The flow rate was set to 2.5 mL min^{-1} and 3.0 mL fractions were collected. After column-wash with the same buffer, adsorbed proteins were eluted with 100 mL of a 0.0–1.0% (w/v) Triton X-100 linear gradient prepared in the same buffer. Fractions with high lipase activity were pooled.

2.8. Electrophoresis

2.8.1. Sample preparation

Triton X-100 was removed from the sample by precipitation with chloroform 1:1 (v v^{-1}). After vortexing, samples were centrifuged at 3000 rpm for 30 min at 4 °C. The upper phase containing the enzyme solution with less than 0.03% (w v^{-1}) Triton X-100 was recovered (Horikawa and Ogawara, 1979). Residual Triton X-100 was further removed with HiPPR™ Detergent Removal Spin Column Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

3. SDS-PAGE

Denaturing electrophoresis was carried out according to Hames (1987) with a 3.75% polyacrylamide stacking gel and 8–18% polyacrylamide gradient resolution gel. Gels were stained with 0.1% (w v^{-1}) Coomassie Brilliant Blue R-250 (Hames, 1990).

3.1. Zymogram

Non-denaturing electrophoresis was carried out following previous SDS-PAGE protocol, however, the loading buffer was prepared with 0.05% (w v^{-1}) SDS and without β-mercaptoethanol. Stacking and resolution gels, as well as the running buffer were prepared without SDS. After the run, the gel was washed with 1% (w v^{-1}) Triton X-100 to remove SDS followed by abundant wash with distilled water. Zymograms were based on hydrolysis and esterification reactions. For hydrolysis, 1- and 2-naphthyl acetate (Sigma-Aldrich) were used as substrates according to Mueller et al. (1975) with modifications. The reaction medium was composed by 25 mg of each substrate solubilized in 1 mL 1-propanol and then diluted to 50 mL with McIlvaine buffer pH 7.0 containing 25 mg of Fast Blue RR dye (Sigma-Aldrich). The gel was incubated at 37 °C until visualization of the bands and the reaction was stopped by soaking in 7.5% (v v^{-1}) acetic acid. For esterification, a modification of the method proposed by Kwon et al. (2011) was utilized. The reaction medium contained 1.5% (w v^{-1}) oleic acid (18:1 Δ^9) and 1.5% (v v^{-1}) octanol in McIlvaine buffer pH 6.0. For substrate emulsification, the medium was subjected to ultrasound for 2 h at room temperature. Bands were visualized after incubation of the gel in this solution for 13 h at 37 °C.

3.2. Enzyme characterization

3.2.1. Effect of pH and temperature on lipase activity and stability

Optimal pH was determined by performing activity assays in the pH range from 2.0 to 8.5, with 0.5 unit intervals, at 37 °C. The reaction time was 5 min. The following buffer systems were utilized: 0.05 M glycine–HCl pH 2.0–3.0; McIlvaine pH 3.0–7.5; 0.05 M Tris–HCl pH 7.5–8.5. The effect of pH on enzyme stability was analyzed by measuring residual activity after incubating enzyme samples, previously 1:2 (v v^{-1}) diluted in the above-mentioned buffer systems, for 24 h at 4 °C.

Optimal temperature was determined by carrying out enzyme assays in temperatures from 40 to 80°C, with 5 °C intervals. The reaction time was 5 min. Thermal stability was evaluated by incubating the culture filtrate at 40, 50, 60 and 70 °C, without substrates. Aliquots were periodically withdrawn, immediately transferred to an ice bath and the residual activity was determined. All assays were performed in duplicates. Thermal deactivation constant (K_d) and half-life time ($T_{1/2}$) were calculated at each temperature according to Eqs. (1) and (2):

$$\ln A = \ln A_0 - K_d t \quad (1)$$

where A is lipase activity at time t (min) and A_0 is lipase activity at time zero;

$$T1/2 = \frac{\ln 2}{Kd} \quad (2)$$

3.3. Effect of chemical compounds and ions

The lipase activity was assayed in the presence of NH_4Cl, $HgCl_2$, $CaCl_2$, $BaCl_2$, $CuCl_2$, $MgSO_4$, $MnSO_4$, $ZnSO_4$ and $Pb(CH_3COOH)$, sodium dodecyl sulfate (SDS), tetra sodium ethylenediaminetetracetate (EDTA), phenylmethanesulfonylfluoride (PMSF), β-mercaptoethanol and 1,4-dithiothreitol (DTT) at 2 and 10 mM a final concentration. The activity was expressed in relation to the control (without any substance).

3.4. Effect of surfactants and emulsifiers on lipase stability

The purified lipase was incubated with 0.5%, 1.0% and 5.0% (w v^{-1}) sodium dodecyl sulfate (SDS), sodium deoxycholate, gum Arabic, Triton X-100, Tween 20 and Tween 80, at 25 °C without substrate. After 1 h, the residual activity was determined and expressed in relation to the control (without any substance). All assays were performed in duplicate.

3.5. Enzyme kinetics

The activity of lipase was assayed with *p*-nitrophenyl palmitate at concentrations from 0.0 to 1.0 mM. The Michaelis-Menten constant (K_m) and maximum reaction velocity (V_{max}) were estimated from the Lineweaver-Burk plot (Lineweaver and Burk, 1934). The experiments were performed with samples containing 0.5% (w v^{-1}) Triton X-100 and in the absence of this surfactant.

4. Results and discussion

4.1. Lipase production

Initially, the time-course of *Penicillium* sp. section *Gracilenta* CBMAI 1583 growth and lipase production was evaluated for seven days, 160 rpm and 28 °C (Fig. 1). Enzyme production was directly related to the microbial growth, increasing up to the third day, when it reached the maximum production (0.849 ± 0.15 U mL^{-1} and 0.223 ± 0.01 g). High lipase production by other *Penicillium* spp is usually obtained after five days of cultivation (Costa and Peralta, 1999; Vargas et al., 2008; Dheeman et al., 2011). After the third day, fungal growth declines what may probably be associated to the lack of nutrients and/or accumulation of toxic metabolites, and the decrease in enzyme activity may be related to an increase in protease production what is commonly associated to the stationary growth phase.

4.2. Effect of pure carbon sources

Addition of pure carbon sources was evaluated to verify if the lipase production by *Penicillium* sp. sect *Gracilenta* CBMAI 1583 is under the control of inductive or constitutive promotors. Lipase production was verified in carbon sources such as carbohydrates, alcohols, organic acids, fatty acids and pure triacylglycerols (Table 1). Higher lipase production was induced by stearic acid (0.585 U mL^{-1}), followed by palmitic, oleic and myristic acids (0.294, 0.133 and 0.115 U mL^{-1},

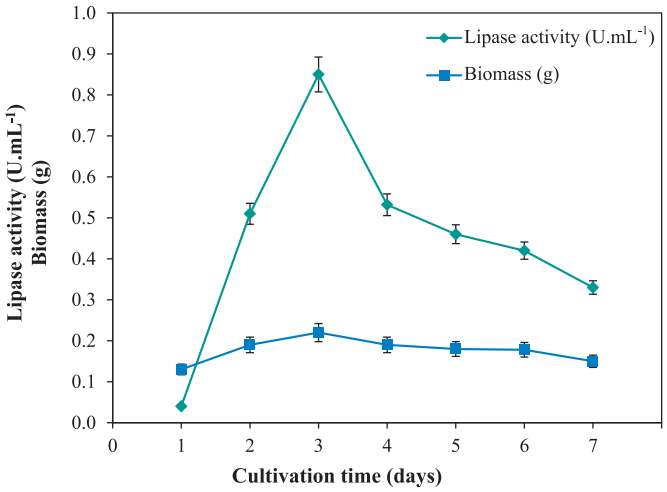

Fig. 1. Time-course of fungal growth and lipase production by *Penicillium* sp. section *Gracilenta* CBMAI 1583. Cultures were carried out in pH 5.5, 160 rpm, 3 days and 28 °C.

Table 1
Lipase production, mycelial growth and fermentation parameters from *Penicillium* sp. section *Gracilenta* CBMAI 1583 using pure carbon sources.

Carbon source (1% w v^{-1})	Dry biomass (g)	Lipase activity (U mL^{-1})	Specific activity (U μg prot^{-1})	$Y_{P/X}$ (U g^{-1})	P_L (U h^{-1})
Glucose	0.045 ± 0.00	0.018 ± 0.01	0.013 ± 0.13	10.00	0.14
Lactose	0.021 ± 0.00	0.019 ± 0.01	0.014 ± 0.00	22.61	0.31
Glycerol	0.096 ± 0.01	0.001 ± 0.00	0.001 ± 0.04	0.26	0.00
Citric acid	0.021 ± 0.00	ND	ND	ND	ND
Tributyrin	0.056 ± 0.00	0.030 ± 0.01	0.026 ± 0.05	13.40	0.18
Trioctanoate	0.123 ± 0.03	0.053 ± 0.00	0.002 ± 0.03	10.78	0.15
Triolein	0.218 ± 0.01	0.174 ± 0.03	0.054 ± 0.06	19.95	0.28
Lauric acid	0.079 ± 0.04	0.032 ± 0.03	0.013 ± 0.13	10.13	0.14
Myristic acid	0.105 ± 0.03	0.115 ± 0.03	0.058 ± 0.18	27.38	0.38
Palmitic acid	0.134 ± 0.00	0.294 ± 0.19	0.150 ± 0.16	54.85	0.76
Estearic acid	0.184 ± 0.01	0.585 ± 0.04	0.265 ± 0.04	79.50	1.10
Oleic acid	0.203 ± 0.00	0.133 ± 0.02	0.076 ± 0.03	16.38	0.23
Linoleic acid	0.104 ± 0.02	0.107 ± 0.01	0.078 ± 0.09	25.72	0.36

$Y_{P/X}$ – yield product on biomass (U g^{-1}); P_L – lipase productivity; ND not detected. Cultures were carried out in pH 5.5, 160 rpm, 3 days and 28 °C.

respectively). Notably, lipase production with stearic acid was 32.5-fold greater than that observed in medium supplemented with glucose, indicating that lipase production is inducible by lipidic carbon sources. Some non-lipidic carbon sources, in turn, acted as weak inducers of lipase production (0.018 U mL^{-1}–0.056 U mL^{-1}). These results suggest that the lipase production by this fungal strain is induced by long-chain fatty acids. Fojan et al. (2000) related that esterases preferentially break ester bonds from short-chain fatty acids, while lipases display much broader substrate range than esterases. The physical state of the substrate is a probable factor contributing to the specificity for substrate. Long-chain fatty acids are typically insoluble or at least poorly soluble. Thus, the lipase must can identify insoluble or strongly aggregated substrates. Since lipase is active towards aggregated substrates, lipase activity is directly related to the total substrate area and not to the substrate concentration (Verger, 1997).

A possible relation among lipase production and fatty acids chain-length and unsaturation level was also evaluated (Fig. 2). Lipase production was induced by long-chain saturated fatty acids, *e.g.*, stearic and palmitic acids (C18:0 and C16:0, respectively), while 12- and 14-carbon saturated fatty acids, as well as unsaturated linoleic (C18:2) and oleic acids (C18:1) resulted in low lipase production. An analysis of the fermentation parameters indicated the efficiency of saturated fatty acids on the lipase production; since stearic acid showed $Y_{P/X}$ 76.50 U.g of biomass^{-1} and P_L 1.10 U.g of biomass^{-1} h^{-1}, followed by palmitic acid ($Y_{P/X}$ = 27.38 U.g of biomass^{-1}; P_L = 0.76 U.g of biomass^{-1} h^{-1}). Specific activities, measured for further enzyme purification procedures, was higher in cultivation with stearic acid (0.265 U.μg of protein^{-1}), followed by palmitic acid (0.150 U μg of protein^{-1}).

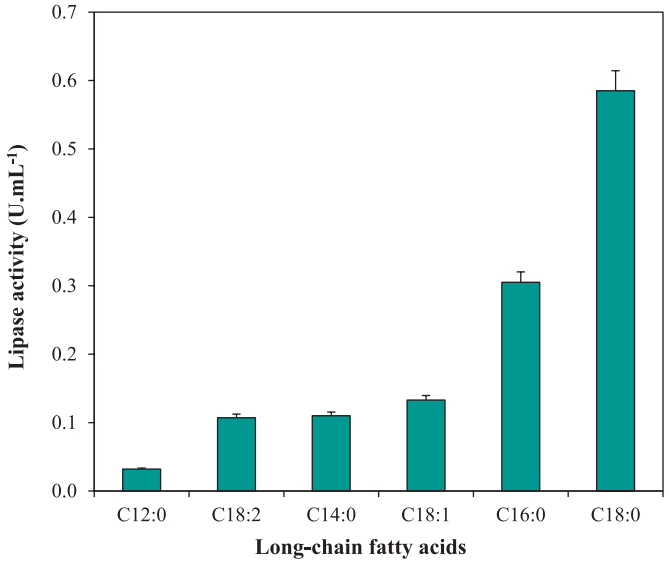

Fig. 2. Lipase production by *Penicillium* sp. section *Gracilenta* CBMAI 1583 on long-chain fatty acids with different chain-length and unsaturation degree. CX:Y - X correspond to the number of carbons and Y to the number of unsaturation. Cultures were carried out in pH 5.5, 160 rpm, 3 days and 28 °C.

4.3. Effect of complex carbon sources

When complex triacylglycerols were evaluated for lipase production (Fig. 3), olive oil was the best natural inducer for lipase production (1.139 U mL^{-1}), followed by linseed oil (0.865 U mL^{-1}). Under these conditions, the highest values using olive oil were also observed for specific activity (1.62 U μg of protein^{-1}), lipase yield on biomass (115.96 U g of biomass^{-1}) and productivity (0.324 U g of biomass^{-1} h^{-1}) (Table 2). Linseed oil presented values of specific activity (1.03 U.μg of protein^{-1}), lipase yield on biomass (86.859 U g of biomass^{-1}) and productivity (0.264 U g of biomass^{-1} h^{-1}) (Table 2). Mycelial growth did not differ among cultivation with different natural triacylglycerols (not shown), indicating that this strain was able to use these complex materials as energy and carbon sources. Olive oil is widely recognized as a good inducer for lipase production due the high concentration of oleic acid (~80%) and other lipidic compounds, *e.g.,* vitamins and tocopherols, which usually stimulate microbial growth and lipase production by filamentous fungi and yeast (Almeida et al., 2012). Linseed oil presents approximately 20% unsaturated fatty acids (18:1, oleic acid 18:2, linoleic acid) and 53% polyunsaturated fatty acid (18:3, linolenic acid). These fatty acids (18:1 and 18:2) showed

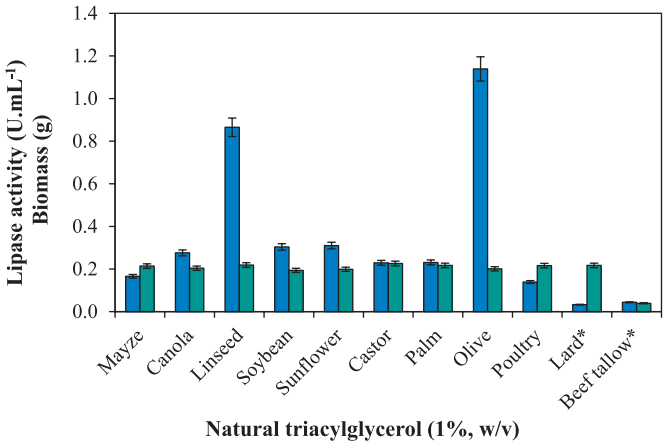

Fig. 3. Growth and lipase production by *Penicillium* sp. section *Gracilenta* CBMAI 1583 on natural triacylglycerols. Cultures were carried out in pH 5.5, 160 rpm, 3 days and 28 °C. *addition of 0.1% (w/v) Tween 80. (■) Dry biomass (g); (■) Lipase activity (U/mL).

Table 2
Lipase activity and fermentation parameter from *Penicillium* sp. section *Gracilenta* CBMAI 1583 using complex carbon sources.

Natural triacylglycerol (1% w v^{-1})	Predominant fatty acid (%)	Specific activity (U μg prot^{-1})	$Y_{P/X}$ (U g^{-1})	P_L (U h^{-1})
Mayze	18:2 (57%); 18:1 (28%)	0.283 ± 0.05	19.284	0.057
Canola	18:1 (61%); 18:2 (21%)	0.457 ± 0.00	30.121	0.085
Linseed	18:3 (53%); 18:1and 18:2 (~20%)	1.035 ± 0.03	86.859	0.264
Soybean	18:2 (54%); 18:1 (25%)	0.594 ± 0.06	32.564	0.087
Sunflower	18:2 (66%); 18:1 (22%)	0.540 ± 0.02	33.547	0.092
Castor	18:1 ω-OH (90%)	0.354 ± 0.03	20.537	0.064
Palm	16:0 (48%); 18:1 (36%)	0.452 ± 0.07	22.859	0.068
Olive	18:1 (76–79%)	1.624 ± 0.08	115.96	0.324
Poultry	18:1 (55%); 16:0 and 18:2 (18%)	0.225 ± 0.03	13.811	0.041
Lard*	18:1 (44%); 16:0 (29%)	0.082 ± 0.01	3.596	0.010
Beef tallow*	18:1 (35%); 18:0 (26%); 16:0 (30%)	0.013 ± 0.02	26.872	0.014

$Y_{P/X}$ – yield product on biomass (U g^{-1}); P_L – Lipase productivity; ND not detected. Cultures were carried out in pH 5.5, 160 rpm, 3 days and 28 °C. *addition of 0.1% (w v^{-1}) Tween 80.

moderate decrease in the lipase production when evaluated pure fatty acids for lipase production. Consequently, other triacylglycerols sources such as oils from mayze, canola, soybean, sunflower, castor, palm which also present high levels of linoleic (18:2) and oleic acid (18:1) in their composition, may have contributed to decrease the lipase production by *Penicillium* sp. sect *Gracilenta* CBMAI 1583. Almeida et al. (2012) also verified a decrease in lipase production by *Candida viswanathii* using linseed oil under submerged conditions. In that work, the *C. viswanathii* strain did not produced lipase using linoleic acid. Castor oil is considered an inhibitor of lipase production due the anti-nutritional effect of the ricinoleic acid (90% of total composition) and unsaturated omega-9 fatty acid that holds in its structure an unusual hydroxyl linked to the 12-carbon. Considering beef tallow and lard are solid at room temperature, mechanical emulsion could not promote an adequate dispersion of these substrates into the liquid medium, resulting in low lipase production.

Lipase production was strongly affected by lipid concentration in the medium (Fig. 4). Low lipid concentration in the medium cannot support cell growth and enzyme production due the fast consumption of carbon source by the fungus; on the other hand, high lipid concentration may cause repression of lipase production because of the excess of free fatty acids in the culture or due to weaker oxygen transfer into the medium, which can alter fungal metabolism and consequently the lipase production (Elibol and Ozer, 2000). The lipase production by *Penicillium* sp. section *Gracilenta* CBMAI 1583 using media with different lipid concentration showed that the highest values were found with 0.5% olive oil (1.62 U mL^{-1}). This result shows a 1.82-fold increase on

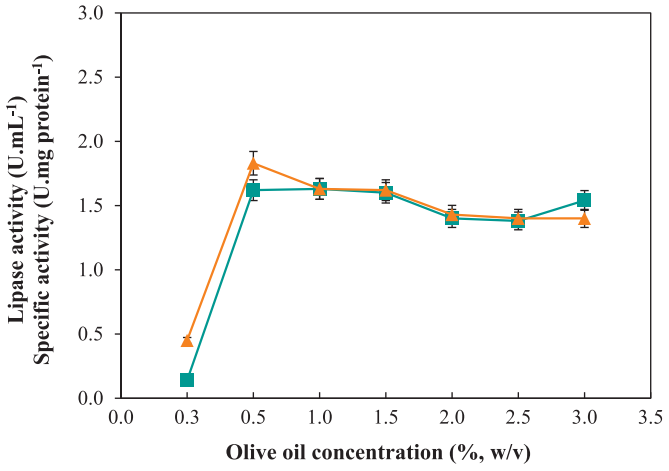

Fig. 4. Effect of olive oil concentration on lipase production by *Penicillium* sp. section Gracilenta CBMAI 1583. Culture conditions: pH 5.5, 160 rpm, 3 days and 28 °C. (■) Lipase activity (U mL^{-1}). (▲) Specific activity (U.mg of protein^{-1}).

lipase production. Under these conditions, fermentation parameters were also higher compared to the other concentrations of lipids ($Y_{P/X}$ = 354.65 U g^{-1}; P_L = 4.92 U g of biomass^{-1} h^{-1} and specific activity of 1.83 U μg of protein^{-1}). Thus, a high concentration of olive oil in the culture medium increase biomass but decrease lipase production.

4.4. Lipase purification

The dialyzed extracellular filtrate from *Penicillium* sp. section *Gracilenta* CBMAI 1583 produced under optimized conditions was subjected to octyl Sepharose chromatography using ammonium acetate buffer. Most of non-target proteins were eluted in the initial fractions, and elution of bound proteins was further performed with a Triton X-100 gradient. The fractions presenting high lipase activity were pooled and submitted to non-denaturant electrophoresis and SDS-PAGE. By following this chromatographic procedure, partial purification of the lipase was obtained, and two protein bands (65.4 and 52.9 kDa) could be observed in the gel (Fig. 5). A zymogram performed with this sample showed that both enzymes hydrolyzed α- and β-naphthyl acetate, while only the 52.9 kDa protein presented esterification activity on octyl oleate. In this sense, it can be concluded that the fungus produces a 65.4 kDa esterase and a 52.9 kDa lipase with reduced specificity, since this enzyme could hydrolyze short-chain esters and esterify long-chain fatty acid and octanol (Table 3).

Both enzymes presented similar properties in terms of hydrophobicity and MW, then, another chromatographic step was carried out to purify this lipase. The dialyzed extract was subjected to phenyl Sepharose chromatography using ammonium acetate buffer (0.05 M, pH 6.9). Elution of bound proteins was performed with a 0.75% Triton

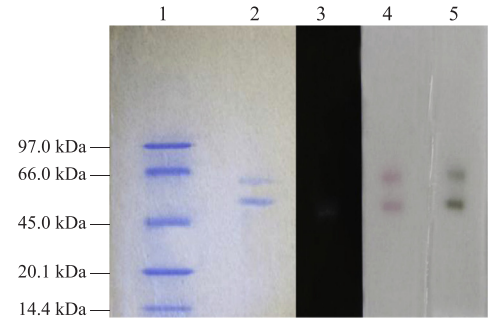

Fig. 5. SDS-PAGE (lane 1 and 2) and zymogram (lanes 3–5) of partially purified lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583. Lane 1: MW markers phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa); lane 2: sample eluted from size exclusion chromatography in a Sephadex G-100 column; lane 3: synthesis of oleic acid and octanol; lane 4: hydrolysis of 2-naphthyl acetate; lane 5: hydrolysis of 1-naphthyl acetate.

Table 3
Growth and fermentation parameters from *Penicillium* sp. section *Gracilenta* CBMAI 1583 using different olive oil concentration.

Concentration (% w v^{-1})	Dry biomass (g)	$Y_{P/S0}$ (U g^{-1})	$Y_{P/X}$ (U g^{-1})
0.25	0.069 ± 0.00	20.656	1.493
0.50	0.129 ± 0.00	249.876	12.108
1.00	0.149 ± 0.02	125.725	10.818
1.50	0.217 ± 0.06	79.022	7.184
2.00	0.262 ± 0.04	53.058	5.191
2.50	0.298 ± 0.02	41.569	4.412
3.00	0.289 ± 0.04	39.040	5.194

$Y_{P/S0}$ - $Y_{P/X}$ – yield product on biomass (U g^{-1}); P_L – Lipase productivity; ND not detected. Cultures were carried out in pH 5.5, 160 rpm, 3 days and 28 °C.

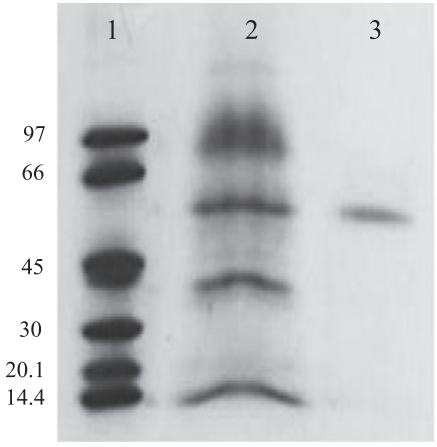

Fig. 6. SDS-PAGE of the purified lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583. Lane 1: MW markers phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and lactalbumin (14.4 kDa); Lane 2: crude dialyzed filtrate; lane 3: pooled fractions eluted from phenyl Sepharose column.

X-100 gradient. Fractions with high lipase activity were pooled and submitted to SDS-PAGE (Fig. 6). The enzyme was purified with 80.8% yield and 516-fold enrichment (Table 4). Almeida et al. (2016) used a similar purification step to isolate a lipase produced by *Candida viswanathii*. After selecting octyl Sepharose resin and 0.02 M ammonium acetate buffer pH 6.9 as initial conditions, two proteins peaks corresponding to non-bound proteins eluted in the initial fractions and the second peak eluted with 1.0% (w w^{-1}) Triton X-100 with high lipolytic activity. In that process, the enzyme purification presented 84.5% yield and 47.0-fold enrichment. The use of hydrophobic resins as an affinity-like process to purify lipases is possible due to the interfacial activation phenomenon which occurs in presence of natural substrates drops, allowing adsorption of these enzymes to the hydrophobic interface via a very hydrophobic area formed by the internal face of the lid and/or surroundings of the active site (Bastida et al., 1998; Manoel et al., 2015).

4.5. Enzyme characterization

4.5.1. pH activity and stability

The effects of pH and temperature on the crude and purified lipase

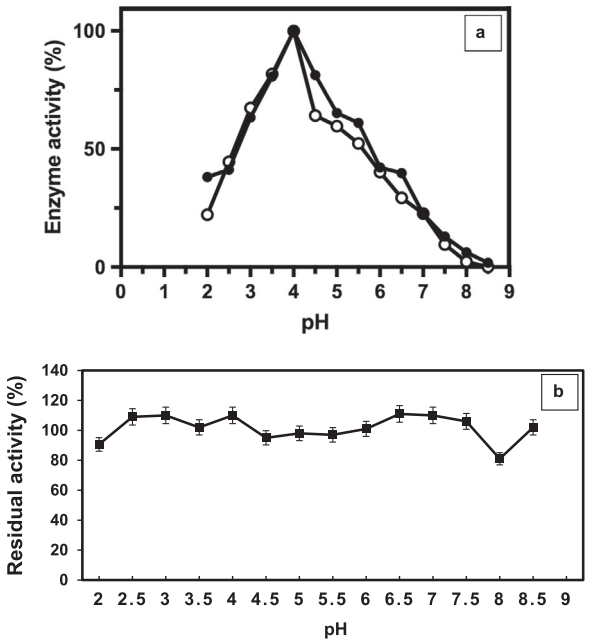

Fig. 7. Effect of pH on activity (a) of the crude and purified lipase and stability (b) of the purified lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583. Lipase activity was assayed in the following 50 mM buffer systems: glycine-HCl pH 2.0, 2.5 and 3.0; McIlvaine pH 3.5–7.0; and Tris-HCl pH 7.5, 8.0 and 8.5. (a) Lipase activity of the (○) crude and (•) purified enzyme. (b) (■) Lipase activity of the purified enzyme.

are presented in Fig. 7. Optimal activity was observed in pH 4.0, and increasing pH to alkaline conditions gradually decreased enzyme activity up to pH 8.5 (Fig. 7a). Optimum activity in pH range 4.0 – 6.0 were also found for the lipase from *Penicillium simplicissimum*, *Penicillium chrysogenum* and *Penicillium roqueforti* (Mase et al., 1995; Bancerz et al., 2005; Gutarra et al., 2009). However, *Penicillium* species frequently produces alkaline lipases with optimum activity in the pH range from 7.0 to 9.0 (Druet et al., 1992; Pimentel et al., 1994; Lima et al., 2003; Tan et al., 2004; Menoncin et al., 2010).

The stability of the crude and purified lipase was evaluated by incubating enzyme samples at different pH in the absence of substrate for 24 h at 4 °C. The crude enzyme fully retained its activity in the pH range from 5.0 to 7.0, while in the pH ranges 2.0–4.5 and 7.5–8.5 the activity remained between 80% and 90%. The purified enzyme was fully stable in a broader pH range (from pH 2.5–7.5) and activities no lower than 80% were verified in pH 2.0, 8.0 and 8.5 (Fig. 7b). Stability in acid pH is very important to industrial application in oleochemicals sector in which the hydrolysis of triacylglycerol occur in alkaline condition and requires acidification of the formed soaps to obtain fatty acids (Lee and Foglia, 2000). Besides, the hydrolytic activity and stability in acid conditions can be considered as an alternative route without the acidification of the formed soap to obtain fatty acids.

4.6. Temperature activity and stability

Lipase activity was evaluated from 40 to 80 °C (Fig. 8). Crude lipase presented maximum activity at 70 °C; while purified lipase presented

Table 4
Purification of the lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583.

Step	Total activity (U)	Total protein (mg)	Specific activity (U mg prot^{-1})	Yield (%)	Purification (fold)
Dialyzed crude filtrate	115.0	124.5	0.9	100.0	1.0
Phenyl Sepharose	2.9	0.2	464.5	80.8	516.1

Lipase activity assay was carried out in McIlvaine buffer pH 4.0 at 70 °C.

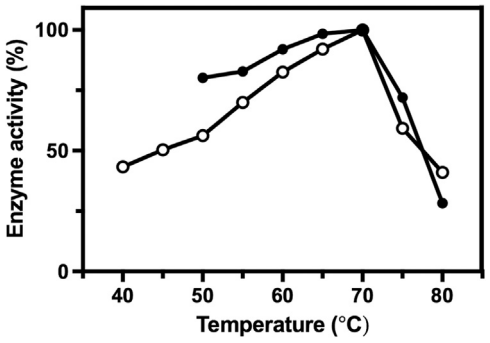

Fig. 8. Effect of temperature on activity of crude and purified lipase produced by *Penicillium* sp. section *Gracilenta* CBMAI 1583. Lipase activity was assayed in McIlvaine buffer pH 4.0. Lipase activity of the (○) crude and (•) purified enzyme.

maximum activity at 65–70 °C. At 50 °C, the enzyme presented 80% of its activity, while above 70 °C the activity decreased sharply. This optimum temperature is higher than that from other *Penicillia* lipases such as that from *Penicillium simplicissimum* produced under solid state cultivation, which presents maximum activity at 45–50 °C (Gutarra et al., 2009) or that from *Penicillium aurantiogriseum* with optimum activity at 60 °C. According to Sharma et al. (2001), most of the industrial processes in which lipases can be employed operates above 45 °C, then, an enzyme intended to be applied should exhibit optimum activity around 50 °C.

Thermal inactivation of the crude and purified lipase was carried out by incubating enzyme samples without substrate at 40, 50, 60 and 70 °C. Both enzyme forms were fully stable up to 40 °C. Above this temperature, a biphasic inactivation pattern was observed, as described by a first order kinetic model for enzyme inactivation, indicating the presence of thermolabile and thermostable regions in the enzyme structure (not shown). The parameters K_d and $T_{1/2}$ of the enzyme showed that the crude enzyme was 22-, 11- and 12-fold more stable than the purified enzyme at 50, 60 and 70 °C, respectively (Table 5).

4.7. Effect of chemical compounds and metallic ions on enzyme activity

The effect of ions and other chemical compounds on the activity of purified lipase is shown in Table 6. In general, most of the chemical compounds at 2 mM did not exhibit any influence on enzyme activity. Exceptionally, non-ionic detergents such as Tween 20 and Tween 80 activated the enzyme by 15%, while PMSF inhibited enzyme activity by 57%. At 10 mM, EDTA DTT also showed a slight negative effect on enzyme activity. PMSF was a strong inhibitor and SDS fully denatured the enzyme.

At 2 mM, Ba^{2+} was a strong activator, while Ca^{2+} was an intermediate inhibitor. Mn^{2+}, Mg^{2+}, NH^{4+}, Na^{+} and Co^{2+} activated by 10 – 20% the lipase from Penicillium sp. section Gracilenta CBMAI 1583.At 10 mM, Ca^{2+} inhibiting effect was much more pronounced, and the activity was almost fully depleted; furthermore, showed an opposite effect Co^{2+} partially inhibiting the enzyme while both Hg^{2+} and Zn^{2+} were strong inhibitors. Among monovalent ions, only NH_4Cl and NaCl at both concentrations presented a slight stimulatory effect.

Table 5
Thermal parameters of the crude and purified lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583.

Temperature (°C)	K_d (min^{-1})		$T_{1/2}$ (min)	
	Crude enzyme	Purified enzyme	Crude enzyme	Purified enzyme
40	0.002	–	360	–
50	0.008	0.167	90	4.1
60	0.002	1.107	6.3	0.6
70	0.008	3.759	2.3	0.2

Lipase activity assay was carried out in McIlvaine buffer pH 4.0 at 70 °C.

Table 6
Effect of ions and chemical compounds on the activity of purified lipase from *Penicillium* sp section *Gracilenta* CBMAI 1583.

Substance	Relative activity (%)	
	2 mM	10 mM
Control	100	100
EDTA	95.4	88
PMSF	43.0	13.9
β-mercaptoethanol	93.3	93.7
DTT	91.9	86
Urea	104.7	107.8
SDS	90.5	ND
Tween 20	115.6	98.7
Tween 80	115.6	82.0
$HgCl_2$	105.9	13.0
$CuCl_2$	103.6	ND
$CoCl_2$	110.1	61.9
NaCl	112.0	120.1
$CaCl_2$	48.2	11.8
$BaCl_2$	183.9	NE
NH_4Cl	110.3	114.6
$ZnSO_4$	95.5	28.5
$MnSO_4$	114.2	128.4
$MgSO_4$	118.1	109.4
$Pb(CH_3COO)_2$	110.7	108.4

Lipase activity assay was carried out in McIlvaine buffer pH 4.0 at 70 °C for 5 min. The control corresponded to 26.9 U mL^{-1}. Standard deviation was lower than 0.3%. ND: not detected. NE: not evaluated.

The effect of different ions and chemical compounds varies considerably between different lipases and any generalization can be quite difficult. In general, Ca^{2+} stimulates activity of lipases as verified for those from *Penicillium candidum* (Bancerz et al., 2005), *Penicillium chrysogenum* (Li and Zong, 2010). However, like our work, the *P. aurantiogriseum* lipase was inhibited by this ion, as well as by other divalent cations such as Cu^{2+} and Ba^{2+} (Lima et al., 2003). Hg^{2+} inhibits numerous enzymes, including many lipases. This effect is associated to its interaction with thiol groups of cysteine, which can be important for catalysis (Sugihara et al., 1996).

4.8. Effect of organic solvents on enzyme activity

Organic solvents are widely used in reactions with lipases because they facilitate the manipulation of hydrophobic substrates, and they can also modulate and/or enhance the activity and selectivity of lipases. The effect of organic solvents on the stability of the purified lipase is shown in Table 7. The solvents were ranked according to the increase of log *P*, which is the partition coefficient of the solvent between n-octanol and water, a useful quantitative parameter to represent the hydrophobic or hydrophilic nature of a substance (Sangster, 1989). Thus, the more negative the log *P* value the more hydrophilic is the substance, and vice-versa.

The enzyme was stable in most solvents retaining more than 70% of activity after incubation for 1 h at 25 °C. With n-butanol and xylol the enzyme showed 62.1% and 63.7% activity loss, respectively. In general, polar solvents are more destabilizing to protein structure due to water removal from the hydration layer, which helps protecting the enzyme native structure (Castro-Ochoa et al., 2005). Notably, the *Penicillium* sp. CBMAI 1583 lipase was more stable than the *P. aurantiogriseum* lipase which is poorly stable when incubated in methanol, ethanol, 1-propanol, acetone and n-butanol for 1 h at 28 °C (Lima et al., 2003).

More hydrophobic solvents such as n-hexane (log $P = 3.50$) and isooctane (log $P = 4.51$) slightly stimulated lipase activity. This increase may be associated to an interfacial activation of the lipase in the

Table 7
Stability on organic solvents of the purified lipase from *Penicillium* sp section *Gracilenta* CBMAI 1583.

Solvent	Log *P*	Relative activity (%)
Control	–	100.0
Glycerol	−1.67	72.8
DMSO	−1.38	98.4
Propylene glycol	−0.92	75.9
Methanol	−0.76	71.3
Acetonitrile	−0.4	94.7
Ethanol	−0.24	72.3
Acetone	−0.23	99.9
2-Propanol	0.07	72.7
1-Propanol	0.25	72.8
n-Butanol	0.8	37.9
Toluene	2.5	95.9
Xylol	3.15	36.3
n-Hexane	3.5	111.3
2.2.4-trimethylpentane	4.51	109.2

Incubation was carried out with 10% ($v.v^{-1}$) solvent at 25 °C for 1 h in sealed flasks. Lipase activity was assayed in McIlvaine buffer pH 4.0 at 70 °C. Control corresponded to 26.9 $U.mL^{-1}$. Standard deviation was lower than 0.3%. Log *P*: logarithm of partition coefficient for each solvent between n-octanol and water (Sangster, 1989).

presence of hydrophobic surfaces, which alters the equilibrium between the open and closed forms of the enzyme, rendering stabilization to the open form changes in substrate and product solubility in the reaction medium (Guncheva and Zhiryakova, 2011). Lipase activation in the presence of hydrophobic solvents was also verified for the lipases from *P. aurantiogriseum* (Lima et al., 2004) and *P. chrysogenum* (Bancerz et al., 2005). It was possible to observe a relationship between the log *P* values and the residual lipase activity, but the stability in various organic solvents suggests the use of this enzyme in reactions in non-aqueous media such as for organic synthesis.

4.9. Specificity for substrates

The specificity for substrates of the purified lipase was studied using various chain lengths *p*-nitrophenyl alkyl esters substrates (Fig. 9). The highest activity was verified with *p*-nitrophenyl decanoate. The activity on laurate, palmitate and stearate *p*-nitrophenyl esters was around

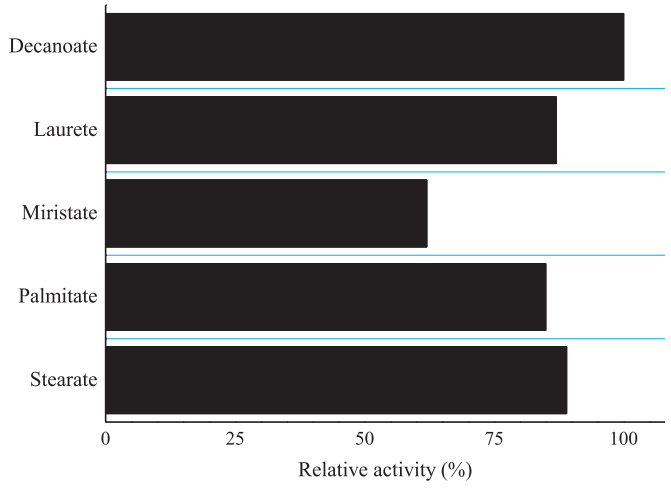

Fig. 9. Activity on synthetic *p*-nitrophenyl alkyl esters of the purified lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583. The activity was assayed with 0.5 mM of substrates in McIlvaine buffer pH 4.0 at 70 °C for 5 min. The control lipase activity corresponded to 39.5 U mL^{-1}. Standard deviation was lower than 0.3%. Number of carbons in each substrate is indicated after each bar.

Table 8
Kinetic parameters of purified lipase from *Penicillium* sp. section *Gracilenta* CBMAI 1583 with *p*NPP as substrate.

Sample	Parameter			
	K_m (mM)	V_{max} (μmol min^{-1} mg $prot^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
With Triton X-100	0.018	1050.4	886.3	4.9×10^7
Without Triton X-100	0.024	1281.0	1129.4	4.7×10^7

Lipase activity was assayed in McIlvaine buffer pH 4.0 at 70 °C for 5 min.

80–90%, while 66% of activity was verified against *p*-nitrophenyl myristate. The activity against short-chain esters could not be evaluated due to spontaneous substrates hydrolysis in the assay conditions. The specificity for substrates of different lipases is variable, *i.e.*, whereas *P. candidum* lipase has preference for long chain fatty acids *p*-nitrophenyl esters (C16) (Ruiz et al., 2001), *P. roqueforti* lipase has preference for short chain (C4 and C6) *p*-nitrophenyl esters (Mase et al., 1995).

4.10. Kinetics of purified lipase

Substrate hydrolysis reactions were performed for the purified lipase with *p*-NPP (0.0–1.0 mM) to determine K_m and V_{max}. From these values, the turnover number (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) in presence and absence of Triton X-100 (0.5% $w.v^{-1}$) were calculated (Table 8). The *p*-NPP hydrolysis in presence and absence of Triton X-100 presented a hyperbolic curve following the classical Michaelis-Menten model. Some lipases follow the Michaelian model as observed for those from *Rhizopus oryzae*, *Mucor miehei*, *Candida rugosa*, *Candida antarctica* A, while others such as those from *Thermomyces lanuginosus* and *Penicillium camemberti* present sigmoidal non-hyperbolic curves (Nini et al., 2001).

The enzyme, in absence of Triton X-100, showed K_m 0.024 mM, V_{max} 1281 μmol.min^{-1}.mg of protein^{-1}, k_{cat} 1129.4 s^{-1} and k_{cat}/K_m 4.7×10^7 M^{-1} s^{-1}, while in presence of Triton X-100 it was observed K_m 0.018 mM, V_{max} 1050.4 μmol min^{-1}.mg of protein^{-1}, k_{cat} 886.3 s^{-1} and k_{cat}/K_m 4.9×10^7 M^{-1} s^{-1}. Schaffner et al. (1978) related that kinetic experiments in different concentrations of Triton X-100 indicate that an apparent optimum detergent: substrate ratio is required to obtain maximal hydrolysis rates. An excess of detergent results in competitive inhibition of the enzyme, whereas suboptimal amounts of detergent also reduce the velocity of hydrolysis. Excessive surfactant may act as a competitive inhibitor for two reasons: (1) a given amount of substrate distributed over the surfaces of an increased number of micelles decreases its effective concentration and thus the rate of enzyme substrate interaction at the molecular level; (2) reversible binding of the enzyme's active site to the detergent may occur. Redondo et al. (1995) studied the effect of Triton X-100 on the lipases A and B from *Candida rugosa*. Kinetic parameters for the experiment performed in the molar fraction of substrate fix and the bulk concentration of substrate and Triton X-100 was varied, a similar Michaelis-Menten behavior was observed with both lipases; the curve fitting gave k_{cat}/K_m values of 3.0×10^5 and 5.6×10^5 s^{-1} M^{-1} for lipases A and B, respectively.

5. Conclusions

In this work, the lipase production by *Penicillium* sp. section *Gracilenta* CBMAI 1583 was almost 2-fold increased by studying substrate and cultivation conditions. Initial purification studies revealed the fungus produces a 65.4 kDa enzyme with esterase activity and a 52.9 kDa enzyme with lipolytic activity. Lipase purification was successful using phenyl Sepharose chromatography under interfacial condition. The purified enzyme is optimally active in acid pH (4.0) and

high temperatures (70 °C). The enzyme presents low specificity, hydrolyzing *p*-nitrophenyl esters with chain-length from 10 to 18 carbons. Maximal activity was observed with p-nitrophenyl decanoate, suggesting a possible preference for intermediate-chain *p*-nitrophenyl esters. The observed characteristics indicate potential industrial application in processes that operate in acid pH, such as treatment of dairy and industry effluents, resolution of esters in the pharmaceutical industry or in the food industry. The stability of lipase in organic solvents also suggests that this enzyme is a candidate to act in organic synthesis reactions in non-aqueous media.

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Conflicts of interest

The authors confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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