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ORIGINAL ARTICLE

Comparing submerged and solid-state fermentation of agro-industrial residues for the production and characterization of lipase by *Trichoderma harzianum*

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Abstract Lipase production by *Trichoderma harzianum* was evaluated in submerged fermentation (SF) and solid-state fermentation (SSF) using a variety of agro-industrial residues. Cultures in SF showed the highest activity (1.4 U/mL) in medium containing 0.5 % (w/v) yeast extract, 1 % (v/v) olive oil and 2.5 C:N ratio. This paper is the first to report lipase production by *T. harzianum* in SSF. A 1:2 mixture of castor oil cake and sugarcane bagasse supplemented with 1 % (v/w) olive oil showed the best results among the cultures in SSF (4 U/g ds). Lipolytic activity was stable in a slightly acidic to neutral pH, maintaining 50 % activity after 30 min at 50 °C. Eighty percent of the activity remained after 1 h in 25 % (v/v) methanol, ethanol, isopropanol or acetone. Activity was observed with vegetable oils (olive, soybean, corn and sunflower) and long-chain triacylglycerols (triolein), confirming the presence of a true lipase. The results of this study are promising because they demonstrate an enzyme with interesting properties for application in catalysis produced by fermentation at low cost.

Keywords Lipase · *Trichoderma harzianum* · Solid-state and submerged fermentation · Castor bean · Sugarcane bagasse · Cassava cake · Corn husk

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Introduction

Lipases are enzymes belonging to the group of serine hydrolases (EC 3.1.1.3), and their natural substrates are long chain triacylglycerols. In addition to their hydrolytic action, lipases catalyze the reverse reactions such as esterification, transesterification and lactonization, where the water activity of the reaction medium is one of the determining factors for each class of reaction (Jaeger et al. 1999). Because of their ability to catalyze different reactions, lipases are among the most used enzymes in biocatalysis, with applications in the detergent and food industries, as well as in the synthesis of biosurfactants and pharmaceuticals (Rodrigues and Fernandez-Lafuente 2010). Lipases are distributed widely in nature, and fungal enzymes have been chosen for industrial applications due to their generally recognized as safe (GRAS) status and the ease of cultivation of these microorganisms (Jaeger et al. 1999).

Lipases have been produced traditionally by submerged fermentation (SF) because the recovery of extracellular enzymes and determination of biomass are facilitated by being performed by simple filtration or centrifugation. Solid-state fermentation (SSF) has also been used for the production of lipases, and has some advantages over SF, such as enabling the use of agricultural waste, demanding less water and energy, and easy aeration of the medium. However, there are few examples of lipase production by filamentous fungi in SSF. Other groups have produced lipases with *Penicillium restrictum* using babassu cake (Palma et al. 2000) and *Pencillium simplicissimum* using soybean cake (Di Luccio et al. 2004). Fernandes et al. (2007) not only reported lipase production in SSF using the bacteria *Burkholderia cepacia*, but also developed a methodology for using the fermented substrate

containing cells and extracellular enzymes directly in the reaction medium for biocatalysis, avoiding the steps of concentration, purification and immobilization of enzyme.

The objective of this work was to study conditions for the production of lipase from *T. harzianum* in SF and SSF and to perform biochemical characterization of the enzyme produced.

Materials and methods

Microorganism

The *Trichoderma* sp. FCLA 501 strain of filamentous fungi was selected from among 30 strains, and identified as producing lipase in preliminary tests with Rodamine B. This strain was maintained in test tubes containing potato dextrose agar (PDA) under refrigeration (5 °C).

For identification, genomic DNA was extracted using a procedure described by Liu et al. (2008). PCR reactions for amplification of ITS regions were performed using primer pairs and protocols proposed by Liu et al. (2008) and Meincke et al. (2010). PCR products were sequenced using the procedure proposed by Sanger et al. (1977). Obtained sequences were aligned using BioEdit (Hall 1999) and consensus sequences were identified by BLASTn [Basic Local Alignment and Search Tool (Altschul et al. 1990)], TrichoKEY 2.0 and TrichoBLAST 1.0 (Druzhinina et al. 2005).

Submerged fermentation

The inoculum was obtained by adding 1 mL spore suspension (10^8 spores/mL) to a 125 mL Erlenmeyer flask with 25 mL culture medium as described in Lima et al. (2003). The flasks were incubated in a rotary shaker at 180 rpm for 48 h at 28 °C. The total volume of inoculum was transferred aseptically to a 500 mL Erlenmeyer flask containing 125 mL of the same culture medium and maintained under the same conditions as above. The cultures were monitored for up to 168 h. At the end of fermentation, the cell mass was separated by filtration and the filtrate was used for analytical determinations.

To study different nitrogen sources, yeast extract was replaced by 5 g/L peptone, 2 g/L KNO_3 or 10 g/L NH_4NO_3, with 1 % (v/v) olive oil remaining as a source of carbon. The effects of different carbon sources were studied by substituting olive oil for 1 % (v/v) corn, soybean, or sunflower oil or 1 % (w/v) glucose, with 5 g/L yeast extract remaining as a nitrogen source. The carbon:nitrogen (C:N) ratio was studied by varying the concentration of yeast extract to obtain C:N ratios of 1; 2.5; 5 and 10, keeping the concentration of olive oil at 1 % (v/v), as described by Lima et al. (2003).

Solid-state fermentation

For production of lipase by SSF, the strain was grown with different agroindustrial residues as substrate, such as castor bean cake, sugarcane bagasse, cassava cake and corn husk. Castor bean is a residue from the extraction of castor oil; sugarcane bagasse comes from sugar/alcohol industry; cassava cake is a residue from the extraction of flour and corn husk originates from the cultivation of maize. Lipase production on solid medium was tested using castor bean cake, sugarcane bagasse and combinations of castor bean cake with cassava cake (castor/cassava medium), corn husk (castor/corn medium) or sugarcane bagasse (castor/cane medium) in a 1:1 ratio. The substrates were analyzed for their biochemical composition using Weende and Kjeldahl methods according to AOAC (1993), sieved and the particle size between 1.0 and 2.8 mm was used. Fermentation was conducted in a 250 mL Erlenmeyer flask containing 10 g substrate moistened with 0.1 mol/L phosphate buffer pH 7.0, to obtain 55 % moisture and 1 % (v/w) olive oil. The flasks were inoculated with 1 mL spore suspension (10^8 spores/mL) and incubated at 28 °C for 96 h.

To monitor enzyme production, the SSF was subjected to an extraction procedure by adding 40 mL 1 % (w/v) NaCl solution to the flasks and keeping them under agitation at 180 rpm for 30 min at 28 °C. The contents of the flasks were then filtered through gauze and centrifuged (6,000 *g*, 15 min, 5 °C). The enzymatic activity of the supernatant, called crude enzyme extract, was determined by analytical methods. Statistical analyses were performed by ANOVA.

Analytical methods

The lipolytic activity of the enzymatic extract was tested against several synthetic esters of *p*-nitrophenyl (pNP: acetate, caproate, caprate and palmitate), different triacylglycerols (tricaprylin, triolein and tributyrin) and vegetable oils (olive, soy, corn and sunflower oils). Activity using synthetic esters was measured spectrophotometrically (Winkler and Stuckmann 1979) at 410 nm. A unit of enzyme activity was defined as the release of 1 μmol pNP per minute reaction and expressed as unit per mL (U/mL) on SF, or in unit per gram dry substrate (U/g ds) on SSF. Activity using triacylglycerols and vegetable oil was measured using titrimetric method (Stuer et al. 1986) preparing a emulsion with Triton X-100 and 0.05 mol/L phosphate buffer, pH 7.0. The fatty acids were quantified with NaOH (0.05 mol/L). A unit of enzyme activity was defined as the release of one fatty acid per minute reaction and expressed as unit per milliliter (U/mL).

Characterization of crude enzymatic extract

After cultivation in SF, ammonium sulfate was added to the culture supernatant to 80 % saturation. This solution was

held for 12 h at 4 °C, then centrifuged at 11,000 *g* for 20 min at 4 °C. The precipitate was resuspended in 0.05 mol/L phosphate buffer, pH 7.0, and dialyzed for 12 h at 4 °C. The dialyzed precipitate was called enzymatic extract and was used in biochemical and kinetic characterizations.

The enzymatic extract was analyzed by SDS-PAGE according to Laemmli (1970). The gel was stained with silver nitrate according to the method described by Blum et al. (1987).

The kinetic parameters of enzyme activity were determined in the standard test conditions indicated above: the reaction, containing varying amounts of substrate pNPP (0.1–10 mmol/L), was monitored for 1 min at 50 °C. The Michaelis constant (K_m) and maximum velocity (V_{max}) values were calculated by the Hanes-Woolf plot (Segel 1982).

To analyze the effect of pH on enzyme activity and stability, the pH of the reaction medium was varied between 2.6 and 10.0 (adapted from the method described by Lima et al. 2004).

To determine the effect of temperature on lipase activity, the hydrolysis of pNPP was followed at temperatures between 30 and 70 °C in 0.05 mol/L phosphate buffer, pH 8.0. The effect of temperature on enzyme stability was determined by pre-incubation of the enzymatic extract at temperatures between 0 and 70 °C for 60 min in 0.05 mol/L phosphate buffer, pH 6.0. The activity was then measured at 37 °C using pNPP phosphate buffer, pH 8.0.

The enzymatic extract was incubated in acetone, ethanol or isopropanol for 60 min at 30 °C in different concentrations of solvents (25 % to 100 %, v/v). For nonpolar solvents, the enzymatic extract was adsorbed on 1 cm^2 Whatman No. 4 filter paper and incubated in the presence of butanol, toluene, hexane, isooctane or *n*-heptane for 60 min at 30 °C. The filter paper was then transferred to tubes with 1 mL 0.02 mol/L phosphate buffer pH 7.0, for desorption of the enzyme and measurement of the residual activity against pNPP, as described above. Relative activities were calculated for the enzyme adsorbed on filter paper without the addition of organic solvent.

Results and discussion

Microorganism identification

PCR products were obtained with primer pairs ITS1F/ITS4TrR, ITSTrF/ITSTrR and T230F-T397R. After sequencing, comparison of consensus sequences using BLASTn, TrichoKey and TrichoBLAST revealed that the sequence analyzed showed high similarity (up 99 %) with that of *Hypocrea lixii* / *Trichoderma harzianum*. Meincke et al. (2010) developed the primer system used here to describe the composition of *Trichoderma* communities in the potato rhizosphere in Germany. Our data demonstrated that this approach was also very useful to identify *Trichoderma* sp. FCLA 501 strain as belonging to *Hypocrea lixii/Trichoderma harzianum* complex with high confidence and accuracy.

Production of lipases in SF

Lipase production by *T. harzianum* occurred only in cultures using organic sources of nitrogen (Table 1). The same maximum enzyme activity was observed in cultures with yeast extract as compared to those with peptone (1.4 U/mL). However, in cultures with yeast extract, maximum activity was obtained after a shorter time of cultivation (48 h for yeast extract and 96 h for peptone).

The microorganism was cultivated with olive, soy, corn or sunflower oils as a carbon source, and lipase activity was determined. Glucose was used as a comparison. Table 1 presents the best results obtained. The highest enzyme activity was obtained with olive oil (1.4 U/mL), followed by soybean and corn oils (mean 0.7 and 0.8 U/mL, respectively) after 48 h of culture. Sunflower oil yielded lower lipase activity (0.34 U/mL), and required a longer time of cultivation (144 h) than the other oils.

Table 1 Effect of different nitrogen and carbon sources and C:N ratio on lipase production by *Trichoderma harzianum* in submerged fermentation (SF)

Culture medium	Activity (U/mL)
N source (w/v) + olive oil 1 % (v/v)	
Yeast extract (5 g/L)[a]	1.44±0.43
Peptone (5 g/L)[b]	1.35±0.40
KNO_3 (2 g/L)	0.0
NH_4NO_3 (10 g/L)	0.0
C source 1 % (v/v) or (w/v) + yeast extract 0.5 % (w/v)	
Olive oil[a]	1.44±0.43
Soybean oil[a]	0.72±0.23
Corn oil[a]	0.80±0.25
Sunflower oil[c]	0.34±0.06
Glucose[c]	0.77±0.70
C:N ratio[d]	
1	1.17±0.75
2.5	1.40±0.40
5	0.52±0.15
10	0.07±0.03

[a] 48 h of culture

[b] 96 h of culture

[c] 144 h of culture

[d] Experiments were carried out after 48 h of culture, varying the concentration of yeast extract and remaining fixed concentration of olive oil, 1 % (v/v)

After culturing the microorganism in increasing concentrations of yeast extract in order to achieve a C:N ratio between 1 and 10 while keeping the concentration of the carbon source constant (1 % (v/v) olive oil), lipase production by *T. harzianum* was higher in experiments with a lower C:N ratio (Table 1).

Although the use of organic sources of nitrogen is commonly cited for the production of lipases, inorganic sources have been studied because of their low cost and to facilitate the subsequent stages of purification of the enzyme. In the present work, enzyme activity was not observed in cultures with inorganic sources. However, in one of the few reports on lipase production by *Trichoderma* (Hypocreacea family), Kashmiri et al. (2006) obtained 7.3 U/mL in cultures of *Trichoderma viride* with NH_4Cl at 4 g/L. Ulker et al. (2011), reported peptone as the best nitrogen source for production of *T. harzianum* lipase, as also observed here. Studies with *Fusarium* (Nectriaceae family) reported lipase production with either peptone (Maia et al. 1999) or $NaNO_3$ (Maia et al 2001). Using *Penicillium aurantiogriseum* cultures (Trichomaceae family), Lima et al. (2003) obtained a 30 % higher activity with 1 % (w/v) $(NH_4)_2SO_4$ than with peptone and yeast extract. Independent of nitrogen source, high concentrations of nitrogen, and therefore lower C:N ratios, generally favor lipase production by filamentous fungi in SF. This was observed in this work and previously in cultures of *Fusarium solani* (Maia et al. 1999), *P. aurantiogriseum* (Lima et al. 2003) and *Aspergillus niger* (Adham and Ahmed 2009).

As observed in this work, lipase production is often induced by the presence of vegetable oils and repressed by the presence of carbohydrates (Falony et al. 2006; Lima et al. 2003; Maia et al. 1999). Among the oils most cited are olive, soybean, corn, babassu, sunflower, canola and sesame oils; the best results are generally obtained with olive oil (Falony et al. 2006; Lima et al. 2003; Wang et al. 2008). Kashmiri et al. (2006) described the production of lipase by *T. viride* using olive oil (20 g/L). Similar to our results with cultures of *T. harzianum*, Wang et al. (2008) observed an inhibitory effect on lipase production in *Rhizopus chinensis* with the addition of sunflower oil to the culture medium. However, the present results contradict those reported by Ulker et al. (2011), which indicated glucose as a better carbon source for *T. harzianum* lipase production.

Production of lipases in SSF

This paper is the first to report lipase production by *T. harzianum* in SSF, for which different agro-industrial residues were used singly or in combination (Table 2). The highest enzyme activity, 4.0 U/g ds at 96 h of culture, was observed in castor/cane medium. There was a significant difference between the activities produced with castor/cane and other culture substrates (ANOVA, $P=0.0029$).

Table 2 Production of lipase by *T. harzianum* in solid state fermentation (SSF) using different agro-industrial wastes. Culture conditions: 28 °C, 96 h of incubation. Tests carried out in triplicate. ANOVA statistical test. C:N ratio = (nitrogen-free extract + ether extract + 1 % olive oil)/crude protein

Culture medium	Activity (U/gds)	C:N
Castor bean cake	2.16±0.44	1.16
Sugarcane bagasse	0.20±0.32	8.28
Castor bean and cassava	2.70±0.44	3.7
Castor bean and corn husk	2.70±0.38	3.1
Castor bean and sugarcane bagasse	4.04±0.32	2.23

Supplementation with olive oil was necessary because, in the absence of oil, enzymatic activity was not detected. The same phenomenon was observed in cultures of *A. niger* by Falony et al. (2006). Although all tests were performed with the same supplementation, the composition and therefore the fat content of the substrate varied between tests. The chemical analyses (Table 3) of castor bean and sugarcane indicated lipid contents of 1.2 % and 3.5 %, respectively, a value 10–30 times higher than that of corn husk or cassava cake. However, tests with isolated substrates (castor bean or sugarcane) resulted in lower enzyme activities despite the same cultivation time (2.0 and 0.2 U/g ds for castor bean and sugarcane, respectively), indicating that although the fat content is important, it is not the only factor responsible for the possible synergism between these substrates.

Another factor to consider is the percentage of protein available in solid substrates. Castor bean and sugarcane have crude protein contents of 28 % and 7 %, respectively, whereas the other substrates have less than 2 %. Thus, the higher concentrations of nitrogen in these substrates may have contributed to the greater lipolytic activity seen with castor/cane medium, because high concentrations of nitrogen have been shown to be favorable to the production of fungal lipases (Adham and Ahmed 2009; Lima et al. 2003; Maia et al.

Table 3 Bromatological analysis of agro-industrial residues used for lipase production in SSF

Analysis	Sugarcane bagasse	Castor bean cake	Cassava cake	Corn husk
Non-lignocellulosic carbohydrates	46.98 %	24.38 %	82.75 %	63.60 %
Ether extract	3.52 %	1.15 %	0.13 %	0.11 %
Crude fibre	20.02 %	37.37 %	14.02 %	32.89 %
Dry material	92.15 %	89.41 %	89.11 %	89.01 %
Total digestive nutrient	48.02 %	56.47 %	73.82 %	52.55 %
Crude protein	6.95 %	27.91 %	1.78 %	1.41 %
Ash	22.53 %	9.19 %	1.32 %	2.49 %
Moisture	7.85 %	10.59 %	10.89 %	10.99 %

1999). The results obtained in SF support this hypothesis because the highest activities were observed in tests with higher concentrations of yeast extract and lower C:N ratios. Furthermore, by calculating the C:N ratios of the different media used as substrates for SSF, considering the composition and presence of olive oil, it was found that castor bean medium, despite having the lowest C:N ratio (1.16), provided lipolytic activity similar to sugarcane medium, whose C:N ratio was the largest among the media tested (8.28). A mixture of substrates, however, seems to promote a favorable balance, and with a C:N ratio of 2.23, castor/cane medium yielded the highest lipase activity. The castor/cassava and castor/corn media have C:N ratios of 3.7 and 3.1, respectively.

Chemical analyses of corn husk and cassava cake (Table 3) showed the highest values of non-lignocellulosic carbohydrates (63.6 % and 82.7 %, respectively). Both carbohydrates and lipids can be used as sources of carbon by fungi, but lipase production is induced by oils and catabolically repressed by glucose. The production of glycosidases by *T. harzianum* has been reported (Delabona et al. 2012; Azevedo et al. 2000), with the possible inhibition of lipase production by the presence of amylases and cellulases hydrolysis products. The higher content of carbohydrates in these substrates, therefore, may explain the lower levels of enzyme activity and greater time required to reach a peak of activity (120 h). This result is consistent with that seen in SF, in which higher enzyme activity was obtained in a shorter time with vegetable oils than with glucose (Table 1). The same effect was observed by Falony et al. (2006)

Direct comparison between the volumetric activities obtained in SSF and SF is difficult because the activity measured in SSF experiments is dependent on the extraction protocol used (such as volume of extraction solution, contact time and agitation). Still, considering the same volume (40 mL), total enzyme activity was 30 % higher in SF (0.5 % yeast extract and 1 % olive oil) than in SSF (castor/cane medium) (Table 4). Moreover, considering the cost of refined olive oil and the volumes added in SF and SSF to obtain such total activity, with SSF, the cost of this carbon source is approximately ten times lower, which compensates greatly for the lower yield in activity. In SF with soybean oil, the total activity is half that obtained with olive oil. However, taking into consideration the cost and large-scale production of these oils in Brazil, the production of lipase with soybean oil represents only 50 % of the cost with olive oil. Despite the lower cost of soybean oil compared to olive oil, the relations activity/g oil and cost/activity with SSF using castor/cane medium are better than SF using soybean oil.

Table 4 Economic evaluation of the culture medium in SSF and SF for lipase production by *T. harzianum*

Process/culture medium	Total activity (U)[b]	Activity (U/g oil)	Refined oil price (US$/kg)[c]	Cost US$/U(million)
SSF/castor bean-sugarcane[a]	40	434	4.65	0.98
SF/olive oil	57	154	4.65	11.11
SF/soybean oil	28	76	1.11	5.37

[a] Calculation considering the amount of olive oil added to the solid substrate (0.092 g/10 g solid substrate)

[b] (U total): enzymatic activity unit in 40 mL crude extract in SF or 10 g solid substrate in SSF after extraction with NaCl 1 % (w/v) solution

[c] Source: Ministry of Agriculture, Livestock and Supply, Brazil, February and March 2010

Kinetic and biochemical characterization

Due to the greater volumetric activity obtained with SF, kinetic characterization was performed on enzymes produced by this method. Cultures were stopped when lipase activity was maximal (1.44 U/mL, 48 h). After precipitation of lipase by ammonium sulfate precipitation, the enzymatic extract had activities of 5 U/mL and 2 U/mg.

SDS-PAGE of the enzymatic extract revealed the presence of a single intense band corresponding to 48 kDa, i.e., close to the 40–50 kDa range of most lipases (Lima et al. 2004). Electrophoretic analysis also demonstrated the presence of two other weakly visible bands with molecular masses above 66 kDa (Fig. 1).

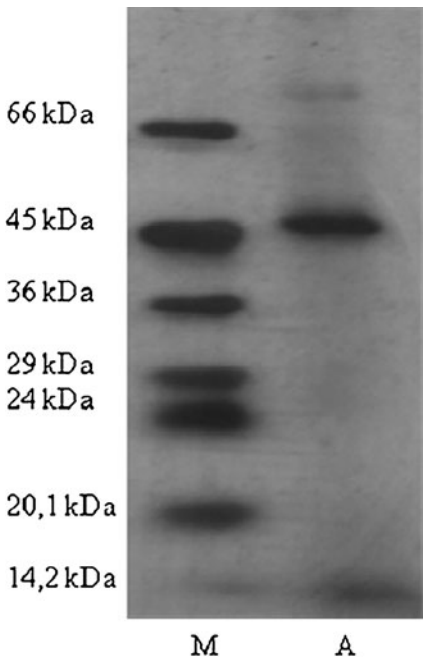

Fig. 1 SDS-PAGE of the enzymatic extract from *Trichoderma harzianum* after precipitation by ammonium sulfate to 80 % saturation. Lanes: *M* Standard marker, *A* enzymatic extract

Effects of pH and temperature on activity and stability

Lipolytic activity of the enzymatic extract was highest (5.33 U/mL) in citrate-phosphate buffer, pH 6.0, but activity was also significant at pH 4.0 and 5.0 (2.4 and 3.6 U/mL, respectively). Unlike the activities of most fungal lipases, activity decreased rapidly at pH values above 7.0. At pH 8.0, for example, lipolytic activity in phosphate buffer was only 0.9 U/mL, and 0.8 U/mL in Tris-HCl. In glycine-NaOH, pH 9.0 and 10.0, the activities were 0.87 and 0.28 U/mL, respectively. Maximum activity was observed when incubation occurred at pH 6.0 and at 50 °C (tested within a range of temperatures between 20 and 70 °C) in the presence of pNPP substrate for 5 min (data not shown). This data differ from those reported by Ulker et al. (2011) for *T. harzinum* lipase. The latter authors described the highest activity at pH 8.5 and 40 °C.

Enzyme activity was stable between pH 5.0 and 8.0 (60 min, 28 °C) and between 20 and 40 °C (60 min, pH 6.0) (data not shown), while retaining 40 % initial activity after incubation for 30 min at 50 °C. Lipases are generally stable in neutral pH and very stable in alkaline pH (Lima et al. 2004). Normally, fungal lipases are not stable at temperatures above 40 °C. Enzyme activity remained stable even after 30 days of storage at −22 °C and 4 °C.

Effects of different substrates on enzyme activity

Among the vegetable oils tested as substrates, the highest lipolytic activities were obtained with corn oil (0.50 U/mL) and olive oil (0.46 U/mL, Table 5). Olive oil is distinguished by its high content of monounsaturated fats, especially oleic acid (18:1 Δ^9), and very low content of polyunsaturated fats. Moreover, the oils of corn, soybean and sunflower are notable for their high (between 40 % and 70 %) content of linoleic acid (C18: 2 $\Delta^{9,12}$). Despite having the same size chain (18 carbons), variations in degree of saturation in the fatty acids present in oils can influence lipase-mediated catalysis. This was observed in the work of Ogino et al. (2000), in which the lipase from *Pseudomonas aeruginosa* showed maximum activity in the presence of trilinolein (C18: 2 $\Delta^{9,12}$); activity was 50 % lower with triolein (18:1 Δ^9) and almost non-existent with tristearin (18:0). The lipase from *Pseudomonas* sp. (Zaliha et al. 2005), in the presence of soybean oil, displayed only 56 % of the activity measured with olive oil. The lipase from *Pseudomonas aeruginosa* showed a two-fold greater activity with castor oil than with olive oil (Ogino et al. 2000).

Lipase-mediated hydrolysis was observed in all the triacylglycerols tested (Table 5). Triolein and tricaprylin showed the same activity despite the different sizes of their carbon chains. When different esters of *p*-NP were tested, the highest lipase activity was obtained with pNPP (16:0). Trioleins, with their long acyl chains, are ideal substrates to measure lipase and are not hydrolyzed by other esterases (Jaeger et al. 1999). Therefore, the ability to hydrolyze this triacylglycerol and *p*-NP of long-chain acyl esters indicates the presence of true lipases in the enzymatic extract of *T. harzianum*.

Table 5 Lipolytic activity of the crude extract with different substrate

Substrate	Activity (U/mL)	Method
Olive oil	0.46±0.004	Titrimetric
Soy oil	0.31±0.009	Titrimetric
Corn oil	0.5±0.002	Titrimetric
Sunflower oil	0.36±0	Titrimetric
Triolein [18:1 (Δ^9)]	0.47±0	Titrimetric
Tricaprylin (6:0)	0.46±0	Titrimetric
Tributyrin (4:0)	0.28±0.004	Titrimetric
p-Nitrophenyl acetate (pNPA, 2:0)	2.3±1.3	Spectrophotometric
p-Nitrophenyl caproate (pNPC, 6:0)	2.2±1.26	Spectrophotometric
p-Nitrophenyl caprate (pNPCA, 10:0)	4.35±3.05	Spectrophotometric
p-Nitrophenyl palmitate (pNPP, 16:0)	5.0±0.57	Spectrophotometric

The effect of substrate concentration on lipase activity of *T. harzianum* was evaluated by varying the concentration of pNPP between 0.1 and 10 mmol/L at 50 °C. A hyperbolic line was obtained as a result (plot V_o vs substrate concentration), linearized by the Hanes-Woolf plot, indicating Michaelian behavior. The kinetic constants were calculated as V_{max} 1.892 U/mL and K_m 0.6535 mmol.

Stability in organic solvents

The polarity of the solvent affects enzymatic stability in biocatalysis, and log P—the octanol-water partition coefficient—is

Table 6 Residual activity of the lipolytic crude extract after preincubation in various solvents

Organic solvent	Log P	Residual activity (%)			
		Solvent concentration % (v/v)			
		25	50	80	100
Methanol	−0.76	95.4±0.04	42.6±0.01	0	0
Isopropanol	−0.28	97.2±0.02	21.3±0.02	0	0
Ethanol	−0.24	79.6±0.02	26.8±0.02	0	0
Acetone	−0.23	85.2±0.07	32.4±0.04	0	0
Butanol	0.80				0
Hexano	3.50				20.0±0.04
Toluene	2.50				15.5±0.01
n-Heptane	4.00				31.6±0.12
Iso-octane	4.51				17.4±0.02

used to measure the hydrophobicity of the solvent. Hydrophilic solvents (−2.5<log P <0) are generally incompatible with enzyme activity because they remove the water layer surrounding the protein, causing its denaturation (Azevedo et al. 2001; Lima et al. 2004), whereas solvents immiscible in water (2<log P <4), such as alkanes and haloalkanes, maintain catalytic activity (Zacks and Klibanov 1988; Lima et al. 2004). However, the lipase from *T. harzianum* showed the opposite profile. After 1 h in the presence of 25 % isopropanol, methanol, acetone or ethanol, there was residual activity of 97 %, 95 %, 86 % or 85 %, respectively (Table 6). This stability allows, for example, the use of enzyme in catalysis because methanol and ethanol can be used in the reaction medium, as in the transesterification of vegetable oil for biodiesel production. When using hydrophobic solvents, the best result was obtained with *n*-heptane, which recovered 30 % of enzyme activity.

Shimada et al. (1993) found that the activity of the lipase of *Fusarium heterosporum* remained at 73 % and 54 % when incubated for 20 h in the presence of 50 % methanol and ethanol, respectively, but was only 6 % after incubation in isopropanol. Gaur et al. (2008) found similar results with the lipase from *Pseudomonas aeruginosa*, with a residual activity of 97.4 %, 100 % and 102 % with 25 % methanol, isopropanol and ethanol, respectively. High activity in hydrophilic solvents was also reported by Zhang et al. (2009) for the lipase from *Pseudomonas fluorescens* JCM5963. However, these three authors also observed high activity in hydrophobic solvents.

Conclusions

Under the conditions studied, the highest lipase activity (U/mL) was obtained in SF, while the highest activity/g oil was obtained with SSF. Olive oil was superior in lipase production compared to soybean, corn and sunflower oils. Under the conditions compared, the use of olive oil in castor/cane medium by SSF was the most economic bioprocess to produce lipase. Characterization of the crude enzyme verified the presence of a genuine lipase, with potential use in catalysis due to its stability in organic solvents such as methanol, which is used widely for transesterification reactions to produce biodiesel.

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