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Screening of filamentous fungi from Brazilian rainforests for enzyme production

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Screening of new microbial strains for enzyme production is a key step in the improvement of industrial bioprocesses. The objective of this work was to isolate fungal strains from Brazilian rainforests and to evaluate their potential to produce industrial enzymes by solid state fermentation. First, 131 strains of filamentous fungi were isolated from Amazon rainforest and Atlantic rainforest regions. Strains selected by simple plate assay had their ability to produce lipase and tannase by fermentation of wheat bran evaluated. Maximum enzymatic activity of 1.35 and 18.7 U/mL were reached by lipase and tannase, respectively. The strain IB28a was identified by molecular and morphologic techniques as *Colletotrichum* sp. Lipase produced by this strain reached specific activity of 25.97 U/mg and showed Km and Vmax of 6.3 and 19.5, respectively. Lipase from *Colletotrichum* sp. had an optimal temperature range of 25 to 35°C and optimal pH range of 6.5 to 7.0. Moreover, the enzyme was stable after 1 h at temperatures up to 40°C and after 24 h at pH 6.5.

Key words: Bioprospection, filamentous fungi, lipase, tannase.

Introduction

The Amazon rainforest is considered the world's largest reserve of biodiversity, offering special climate features favorable to microorganism multiplication (Delabona et al., 2012). The Atlantic rainforest, located mainly along the Brazilian coast, is another important biome for microorganism bioprospection. It is considered to be one of the five principal hotspots on the planet, and today only approximately 15% of its original vegetation remains (Ribeiro et al., 2009).

Microorganisms are likely to continue to be the main source of new commercial biomolecules, because of the chemical and structural diversity of their products (Harvey et al., 2010). Enzymes obtained from filamentous fungi and yeasts are the most used. They can be produced in a short time, with high stability and great biochemical diversity and can be genetically manipulated (Anbu et al., 2013).

Current enzyme's market shows vast potential for

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profitability. The main reasons for this scenario are the high demand for novel and higher quality products, the need to reduce costs, wastes and energy consumption, and the development of the pharmaceutical and bioethanol sectors (Sarrouh et al., 2012).

Tannase (tannin acyl hydrolase, EC 3.1.1.20) is an inducible enzyme produced by a variety of microorganisms in the presence of tannic acid or some of its derivatives (Aguilar et al., 2001). It is able to hydrolyze ester and depside bonds of hydrolysable tannins (Banerjee et al., 2001). Tannase has vast potential to be applied on food and pharmaceutical industries. However, tannase application is limited by the high cost of the enzyme purification and recovery. Progress had been achieved in the last years as a result of the efforts in isolation of newly strains with higher tannase production, the optimization of fermentation systems and the development of economically viable purification methods (Chávez-González et al., 2012).

Lipase (triacylglycerol hydrolases, E.C. 3.1.1.3) catalyzes the hydrolysis of esters bonds of triglycerides into glycerol and free fatty acids in the water-oil interface (Singh and Mukhopadhyay, 2012). In a non-aqueous medium, lipase can catalyze the synthesis of acylglycerol from free fatty acids and glycerol (Macrae and Hammond, 1985). Due to their chemo-, regio- and enantio- selectivity, lipases have been used in several areas of biotechnology as: chiral drug resolution, fat modification, biofuels, cosmetics, agrochemicals, oleochemicals, flavor enhancers and detergents (Contesini et al., 2010). The search for new lipolytic strains is stimulated by the high demand for new sources of the enzyme and by the need of lipases with new catalytic characteristics and specific physical-chemical properties (Thakur, 2012).

This work is aimed at isolating new strains that are capable of producing the extracellular lipases and tannases enzyme by solid state fermentation (SSF) from two Brazilian rainforests, as well as, to identify the selected microorganisms and to biochemically characterize the produced enzymes.

MATERIALS AND METHODS

Fungal isolation

Strains were isolated from samples extracted from two Brazilian regions: the Amazon rainforest region at the State of Para (1°47'S, 48°45' W) and the Atlantic rainforest region at the State of São Paulo (23°82'S, 45°34' W). Random samples of leaves, fruits, and seeds found on the litter fall were collected, and soil samples were taken from 5 cm deep. A portion of 1 g of each sample were diluted in 10 mL of distilled water. Plates containing potato dextrose agar (PDA), supplemented with chloramphenicol 50 ppm, were inoculated by streaking a loopful of the diluted sample across the medium surface. Plates were incubated at 30°C and observed at interval of 24 h each for fungal development. Pure strains were isolated and maintained on PDA slants at 30°C until they were well sporulated. Stock cultures were kept at 4°C on PDA medium under a layer of vaseline.

Plate screening

All strains were inoculated in a selective medium which contained (g/L): Agar, 30.0; tannic acid (Tanal B- Prozyn), 10.0; $NaNO_3$, 3.0; KH_2PO_4, 1.0; $MgSO_4$. $7H_2O$, 0.5; KCl 0.5; $FeSO_4.7H_2O$, 0.01, pH 4.5 (Bradoo et al., 1996). Point inoculations were carried out and plates were incubated at 30°C for 48 h. Tannase enzymatic activity was estimated by the enzymatic index (EI), which was determined by the ratio between the halo diameter and the colony diameter (Hankin and Anagnostakis, 1975).

Plate assay to detect lipase production was conducted in a medium which contained (g/L): agar, 15.0; peptone, 10.0; NaCl, 5.0; $CaCl_2.2H_2O$, 0.1 and 10 ml of Tween 80 (adapted from Sierra, 1957). The above components were emulsified in a blender. Isolated fungi were incubated in the medium for 48 h at 30°C, next the plates were maintained at 4°C, approximately, for 48 h. The EI was determined by the same method as described above.

Solid state fermentation

The pre-inoculum was prepared by adding 1.0 ml of distilled water to remove the spores from the PDA medium.

Wheat bran enriched with 10% (w/w) tannic acid was used as substrate for tannase production. An amount of 10 g of substrate and distilled water (1:1 v/v) was taken into 250 ml flasks and sterilized at 121°C for 20 min. After sterilization, the flasks were inoculated with 1 ml of the pre-inoculum suspension (5.0×10^7 spores/ ml) and incubated at 30°C for 120 h. After fermentation, 40 ml of 0.02 M acetate buffer (pH 5.0) was added to each flask, which was shaken at 200 rpm for 1 h. Solution was filtered and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was used to determine the enzymatic activity. The enzymatic activity of a *Paecilomyces variotii* strain tannase (Battestin and Macedo, 2007) was used for comparison.

For lipase production, wheat bran and water (1:1 v/v) was used as the substrate. The flasks were inoculated as described above and incubated at 30°C for 96 h. 50 ml of distilled water was added in the flask; the solution was homogenized with a glass stick and shaken occasionally for 2 h. The enzymatic extract was obtained by filtering the cultures. *Rhizopus* sp. lipase (Macedo et al., 2003) was used as a model for comparison.

Enzymatic assay

Tannase activity was determined spectrophotometrically at 520 nm according to the method of Pinto et al. (2001), with some modifications. The amount of gallic acid released in the reaction was determined through a standard curve of gallic acid. One unit (U) of enzyme activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under the defined reaction conditions. The enzymatic activity was expressed in units per enzyme milliliters (U/mL) and the specific activity was expressed in units per enzyme milligrams (U/mg).

Lipase activity was determined using an emulsion which contained: 25 ml of olive oil and 75 ml of 7% Arabic gum solution. Lipase activity was measured in a system which contained: 5 ml of the emulsion, 2 ml of 0.1 M phosphate buffer (pH 7.0) and 1 ml of enzymatic extract. The reaction was carried out at 37°C for 30 min with orbital shaking and was stopped by adding 10 ml of an acetone- ethanol (1:1 v/v) mixture. Released fatty acids were titrated against 0.05 M NaOH using phenolphthalein indicator. The quantification was carried out with a standard oleic acid curve. One unit of lipase was determined as amount of lipase required to release one micromole of fatty acids per minute under assay conditions. Protein concentration was determined by Bradford (1976) method.

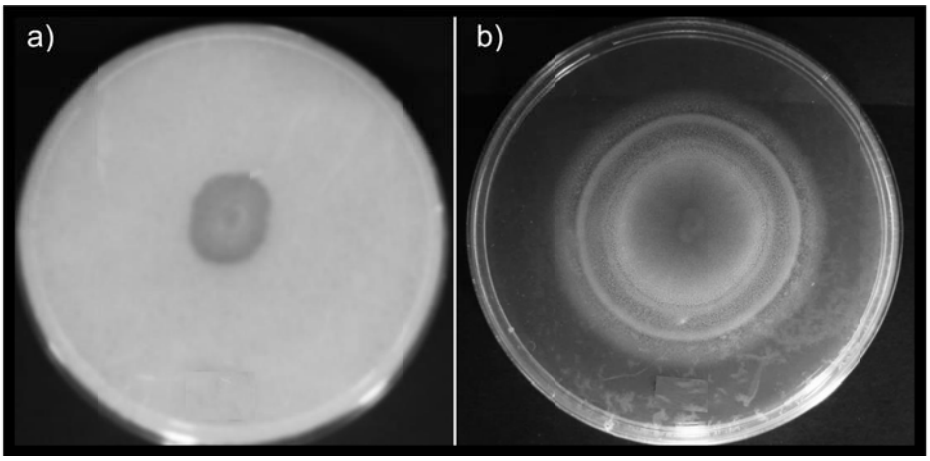

Figure 1. Halo formed by the hydrolysis of tannic acid (a) and halo formed by the deposition of calcium crystals surrounding the colony (b).

Biochemical characterization of lipase

The software Statistica® 8.0 from Statsoft, Inc. (Tulsa, Oklahoma, USA) was used for the experimental design and data analysis. A central composite rotatable design (CCRD) with three replicates at the central point and four axial points (11 runs) was used to determine the optimum temperature and pH for lipase activity. The dependent variable was lipase activity (U/mL) and the independent variables were pH and temperature.

The stability of the enzyme was examined at different pH values by incubating the enzyme in buffers at different pH values ranging from 3.5 to 9.0 for 24 h, under refrigeration (approximately 4°C). The residual activity was estimated and expressed as a percentage of the relative lipase activity. The heat stability of the enzymes was examined by incubating the samples at different temperatures ranging from 20 to 70°C for 1 h.

Kinetics parameters of Lineweaver-Burk equation (Km and Vmax) for the lipase activity were determined by measuring the enzymatic activity in a system containing diverse olive oil concentrations. The percentage of olive oil in the emulsion formed with the Arabic gum varied from 10 to 50%.

Fugal strains identification

Materials from pure cultures were stained with lactophenol cotton blue dye. Morphological observations of hyphae, conidiophore, conidia and spores were done with the help of pictures captured under light microscopy at 400 and 1000x magnification.

Strains were grown in 10 ml of Sabouraud dextrose broth (Reisner et al., 1999) at 28°C for 3 days. The mycelium suspension was washed with ultrapure water and centrifuged at 3,000 rpm for 30 s. DNA was extracted by using the phenol-chloroform method described by Raeder and Broda (1985). The pellet was dried at 37°C, resuspended with the addition of 50 µL of ultrapure sterile water and incubated at 37°C for 1 h with 1 µl of RNAse 10 mg/mL. DNA quantification assay was realized in agarose gel 1% stained with SYBR® Green using λ-DNA (50 ng/µL) as standard.

The rDNA internal transcribed spacer (ITS) region was amplified and sequenced with the primers ITS1 (5´TCC GTA GGT GAA CCT GCG G 3´) and ITS4 (5´TCC TCC GCT TAT TGA TAT GC 3´) (Zhao et al., 2001)) in GeneAmp termociclator polymerase chain reaction system 9700. All PCR reagents were obtained from Promega. PCR was performed with 1 µL of DNA and 0.05 µl of each of forward and reverse primers (5 mM). The amplification conditions were as follows: (94°C denaturation for 2 min, 94°C denaturation for 45 s, 55°C annealing for 30 s, 72°C extension for 35 s) × 30 cycles; 72°C extension for 10 min.

To confirm the amplification reaction, PCR products were electrophoresed in 1% agarose gel followed by staining with SYBR® Green. The amplification products were purified by Illustra GFX PCR DNA and Gel Band Purification kit (GE® Healthcare) following the protocol of the manufacturer. The purified products were sequenced using the dGTP BigDye Terminator Cycle sequencing ready reaction kit (Applied Biosystems) in a Genetic Analyzer 3500 XL (Applied Biosystems).

DNA sequences were assembled using the software DNA Baser Sequence Assembler and were analyzed using the BLAST algorithm. Cluster analysis was carried out using the neighbour-joining (NJ) algorithm (Saitou and Nei, 1987) and the generated tree was rooted using the outgroup option. The statistical support for the internal branches was assessed by using the Phylogeny.fr platform (Dereeper et al. 2008).

RESULTS

Isolation of microorganisms

There were 49 strains of filamentous fungi isolated from the Amazon rainforest region and 82 strains from the Atlantic rainforest region. Apart from the 131 strains isolated in this work, strains from the Unicamp's Food Science Department Collection, obtained from diverse regions of São Paulo State were also tested (n=217).

Agar plate screening

Agar plate screening assay for tannase showed that 105 strains (30.2%) exhibited a clear halo around the colony due to the degradation of tannic acid. In 26 strains (7.5%), positive results for lipase production were observed through opaque halos around the colony formed by the deposition of fat acid salts (Figure 1).

Table 1. Enzymatic activity (U.mL^{-1}) of tannase and lipase produced by filamentous by SSF.

Tannase		Lipase	
Strain	**Enzymatic activity (U.mL-1)***	**Strain**	**Enzymatic activity (U.mL-1)***
CL255	1.35 ± 0.03a	*Rhizopus* sp.	30.5 ± 0.5a
IB31c	0.93 ± 0.05b	CL316	18.7 ± 2.9b
P. variotii	0.83 ± 0.23b	IB28a	13.0 ± 3.3bc
IB34a	0.79 ± 0.24bc	IB38d	7.3 ± 4.8cd
CL43	0.79 ± 0.07bc	CL307	4.9 ± 2.4 cd
IB27a	0.72 ± 0.12bcd	CL406	3.2 ± 0.0d
IB13a	0.70 ± 0.02bcd	CL374	1.5 ± 2.3d
CL148	0.69 ± 0.21bcd	CL264	1.5 ± 0.5d
IB14a	0.67 ± 0.10bcd	CL458	0.6 ± 0.9d
IB33a	0.68 ± 0.04bcd	CL143	0.5 ± 0.0d
IB08b	0.43 ± 0.29cde		
AM1049	0.40 ± 0.05def		
IB31a	0.28 ± 0.06efg		
CL263	0.19 ± 0.04efg		
CL188	0.12 ± 0.01efg		
VL64	0.10 ± 0.05efg		
IB38d	0.04 ± 0.04fg		
IB25a	0.02 ± 0.02g		
AM1817	0.01 ± 0.02g		

*Results are presented as the mean (n= 3) ± SD, and those with different letters are significantly different, with P < 0.05 (Tukey test).

Enzyme production by SSF

Among 105 strains selected on agar plates for tannase production, all positive strains from the rainforests (n=27) and strains from the laboratory stock cultures randomly chosen, with enzymatic index (EI) greater than 3.0 (n=8), were submitted to SSF. Results of tanninolytic activity of enzymes produced by SSF are shown in Table 1. It was observed that the highest activities were reached by strain CL255 (1.35 ± 0.03 U/ml) and strain IB31c (0.93 ± 0.05 U/ml). Enzymes produced by these strains showed higher activity than the enzyme from the already known tannase producer *P. variotti* (0.83 ± 0.23). Moreover, tannase produced by strain CL255 showed specific activity approximately 34% higher (2.14 ± 0.04 U/mg) than the one produced by *P. variotti* (1.41 ± 0.32 U/mg).

All positive strains for lipase production in agar plate assay (n=26) had their capacity of producing the enzyme by SSF tested. It can be observed in Table 1 that the highest activity occurred with the *Rhizopus* sp. strain (30.5 ± 0.5 U/mL) strain, which was used as the standard, followed by strain CL 316 (18.7 ± 2.9 U/ml) and strain IB28a (13.0 ± 3.3 U/ml).

Comparison between agar plate assay and quantitative assay

Regarding the tanninolytic strains, 51.4% of the strains selected by agar plate screening gave detectable results using the enzymatic assay. Furthermore, detectable lipase production was found in 34.6% of the strains previously selected on agar plates.

The correlation between the EI measured in agar plate and the enzymatic activity, evaluated by spectrophotometry for tannase and by titration for lipase, was evaluated. No correlation was observed for the tannase assays. However, Pearson coefficient indicated a strong and positive correlation (0.96; p=0.000) between the EI evaluated in agar plates containing Tween 80 and the lipase activity measured by titration (only strains with EI higher than 1.0 U/ml were evaluated) (Figure 2).

Identification of the selected lipase producing strains

Strain IB28a developed a white cottony mass on PDA after 3 days of grown. Microscopy revealed the presence of highly branched hyphae and cylindrical conidia. Molecular identification based on ITS region revealed that strain IB28a showed high similarity with *Colletotrichum theobromicola* JX010285, *C. fragariae* JX258785 and *Colletotrichum* sp. JN390867 (Figure 3). Therefore, it was not possible to identify the strain at the species level.

Biochemical characterization of lipase from *Colletotrichum* sp. strain

The results of the assays for the activity of lipase from

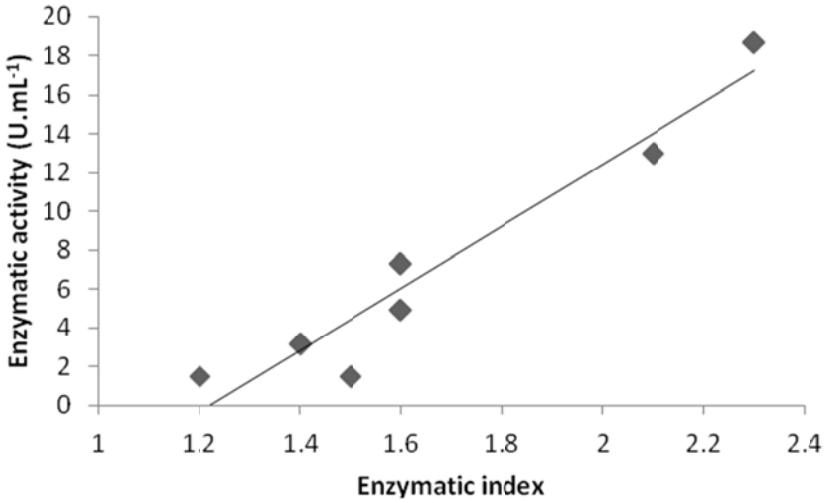

Figure 2. Correlation between data obtained by simple plate assay (EI) and by titration (enzymatic activity) from lipase producing strains with enzymatic activity higher than 1.0 $U.mL^{-1}$.

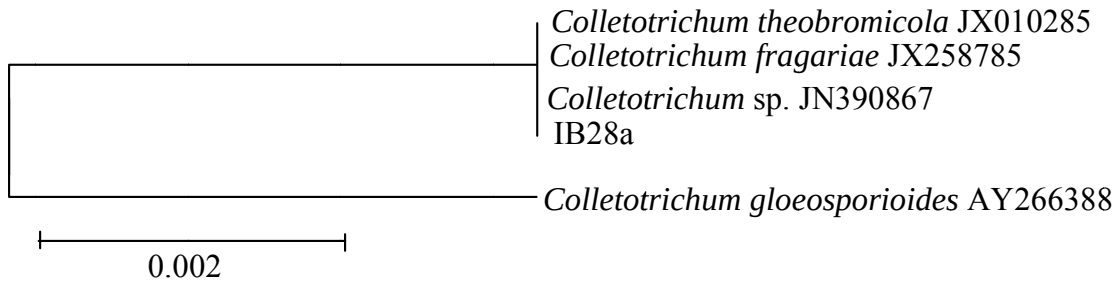

Figure 3. Neighbor-joining tree based on the study of ITS region of rDNA showing the taxonomic positions of strain CL255, adopting *C. gloeosporioide* as outgroup. The scale bar indicates the rate of nucleotide substitution.

Colletotrichum sp. strain (IB28a) are listed in Table 2 and the result of the analysis of variance (ANOVA) is shown in Table 3. The coefficient of determination value (R^2 = 0.97) indicated that the model can explain 97.0% of the experimental data's variability. The computed F-value for regression was greater than the tabulated F-value, reflecting the statistical significance of the model.

The linear and quadratic terms of pH (x_1) and temperature (x_2) showed negative and significant ($p < 0.05$) effects on the lipase activity, while the interaction term (x_1x_2) indicated a positive and significant ($p < 0.05$) effect. Equation 1 below represents the model with the significant factors for the experimental data:

$$\text{Enzymatic activity (U/ml)} = 26.54 - 2.70x_1 - 6.41x_1^2 - 3.09x_2 - 3.87x_2^2 + 5.95x_1.x_2 \quad (1)$$

Where, x_1 and x_2 are the coded values for pH and temperature, respectively.

This equation can be used for predictive purposes in determination of optimum pH and temperature for activity of *Colletotrichum* sp. lipase.

Surface response and contour plots of the models were generated as functions of the independent variables. Optimum *Colletotrichum* sp. lipase activity was observed between pH 6.5 to 7.5 and temperatures from 25 to 35°C (Figure 4). The temperature range where lipase from *Colletotrichum* sp. presents higher activity maintains more than 85% of its initial activity. Regarding the pH range, the enzyme presents 95% of the initial activity at pH 6.5, but it is not stable at higher pH values (Figure 5).

Estimated values of Km and Vmax were obtained by the Lineweaver-Burk linearization method (Figure 6). Lipase from *Colletotrichum* sp. presented Vmax = 19.5 U/mg and Km = 6.3%. The estimated kinetics parameters for lipase produced by *Rhizopus* sp. were V max = 101.0 U/mg and Km = 21.7%. Therefore, *Colletotrichum* sp. lipase showed more affinity to olive oil than lipase from

Table 2. CCRD matrix with coded and real values for the variables and responses for enzymatic activity of lipase from *Colletotrichum* sp.

Runs	x_1 pH	x_2 Temperature (°C)	Lipase activity (U.mL^{-1})
1	-1 (6.6)	-1 (30.0)	27.2a
2	-1 (6.6)	1 (60.0)	10.7d
3	+1 (9.4)	-1 (30.0)	11.6d
4	+1 (9.4)	1 (60.0)	18.9b
5	-1,41 (6.0)	0 (45.0)	18.0bc
6	+1,41 (10.0)	0 (45.0)	7.9d
7	0 (8.0)	-1,41 (23.9)	23.5 ab
8	0 (8.0)	1,41 (66.1)	12.5cd
9	0 (8.0)	0 (45.0)	27.2a
10	0 (8.0)	0 (45.0)	27.2a
11	0 (8.0)	0 (45.0)	25.3a

*Results are presented as the mean (n= 3) ± SD, and those with different letters are significantly different, with P < 0.05.

Table 3. ANOVA of the regression model for enzymatic activity (U.mL^{-1}) of lipase from *Colletotrichum* sp.

Source of variation	Sum of squares	Degrees of freedom	Mean of squares	*F*test	*P*-value
Regression	530.4	5	106.1	28.0	0.001
Residual	18.9	5	3.8		
Total	549.4	10			

$F_{0.1;5;5}$ (*F* tabulated) = 3.45, R^2 = 0.97.

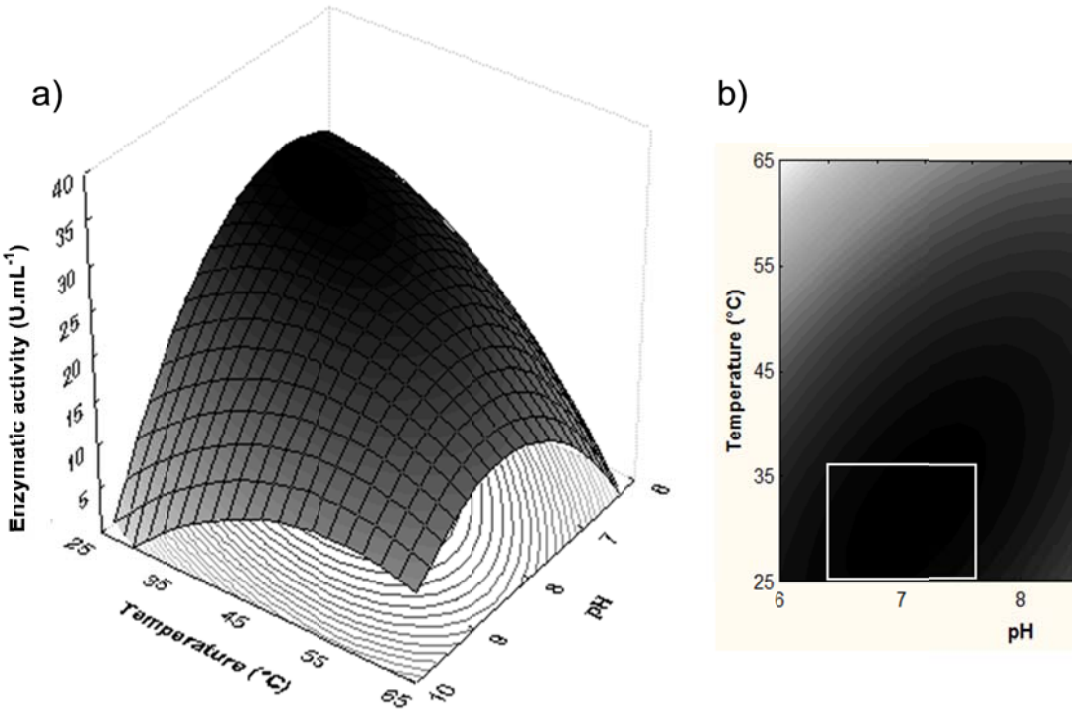

Figure 4. Response surface (a) and contour diagram (b) for enzymatic activity (U.ml^{-1}) of *Colletotrichum* sp. lipase as a function of the pH and temperature (°C).

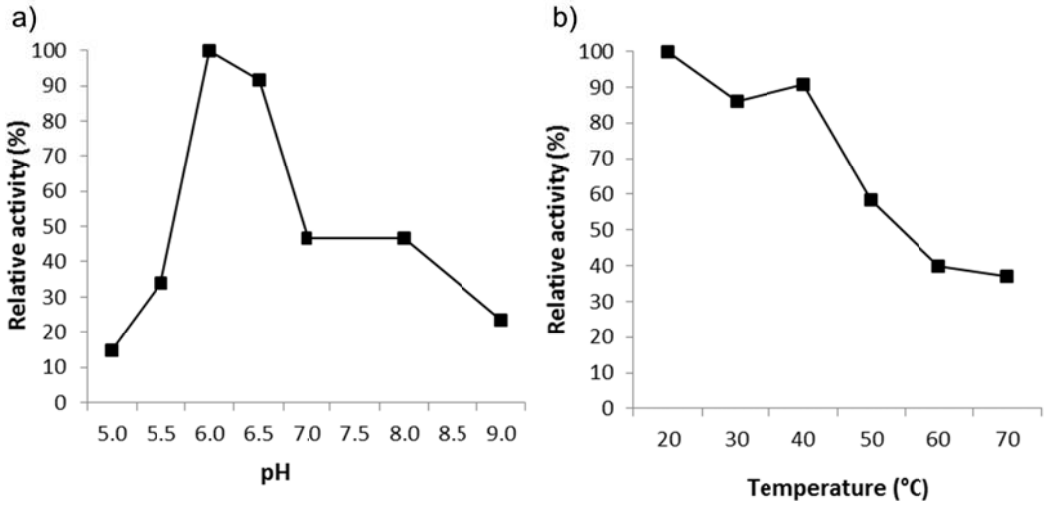

Figure 5. Effect of pH (a) and effect of temperature (b) on *Colletotrichum* sp. lipase stability.

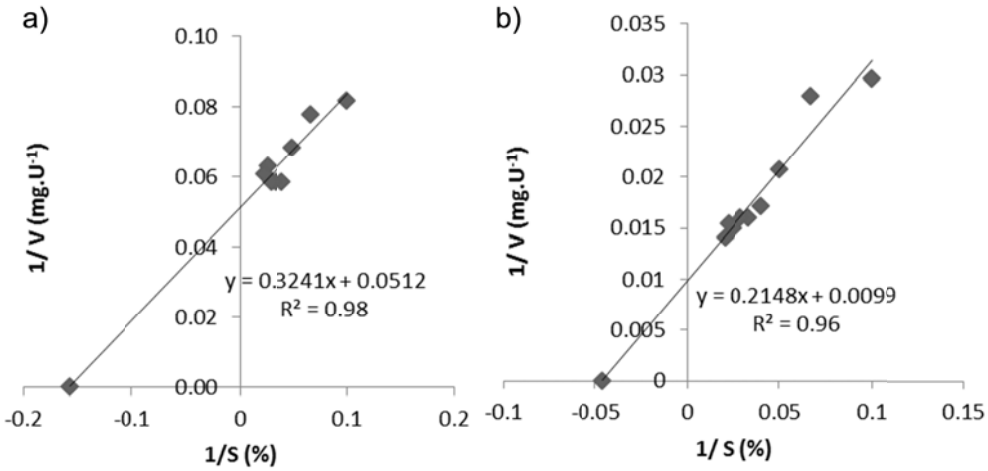

Figure 6. Lineweaver-Burk plot for *Colletotrichum* sp. lipase (a) and *Rhizopus* sp. lipase (b).

Rhizopus sp.

DISCUSSION

Fungal bioprospection is receiving increased attention in part, because of the great potential fungi have in the production of feed, food, fuel, industrial chemicals and pharmaceutical products, as well as, its application in bioremediation and biopulping (Thomas et al., 2013). Even with the advances on microbial genetics and physiology areas, screening for wild microorganisms continues to be an important field of biotechnology (Bull et al., 2000; Newman and Cragg, 2012). The Amazon rainforest and the Atlantic rainforest are suitable environments for the isolation of fungal strains capable of producing enzymes with technological application (Maugeri and Hernalsteens, 2007) (Table 4). In this work, it was possible to isolate from samples collected in tropical rainforests several strains of filamentous fungi with potential to produce extracellular lipase and tannase by SSF using wheat bran. SSF presents several advantages over submerged fermentation, such as low capital investment, reduced energy consumption and residue production, as well as, easier product recovery (Fleuri et al., 2013). Lipases produced by SSF show higher production titers and productivity, and less catabolite repression (Velasco-Lozano et al., 2012). Some advantages of tannase production by SSF are: the extracellular nature of the enzyme, higher productivity and higher stability over a wide range of pH and temperature (Lekha and Losane, 1994).

Table 4. Environments for the isolation of fungal strains capable of producing enzymes with technological application.

Region	Microorganism	Sample	Enzymes	Reference
Atlantic Rainforest	Bacteria	Soil	Lipase	Faoro et al. (2012)
	Yeast	Rotting wood	β-Glucosidase	Santos et al. (2011)
	Yeast, yeast- like strains	Water, soil, insects, plants	Esterase,lipase, protease	Buzzini and Martini (2002)
	Filamentous fungi	Soil	Cellulase	Simoes and Tauk-Tornisielo (2005)
	Basidiomycetous fungi	Basidiomes	Ligninolytic enzymes	Machado et al. (2005)
Amazon Rainforest	Bacteria	Soil, water	Cellulase, xylanase	Heck et al. (2002)
		Soils, roots	Lipase	Willerding et al. (2011)
	Yeast	Rotting wood	Xylanase	Cadete et al. (2012)
		Rotting wood	Xylan-degrading enzymes	Garcia Medeiros and Hanada (2003)
	Filamentous fungi	Soil, rotting wood	Cellulase	Delabona et al. (2012)
		-	β-Galactosidase	Tonelotto et al. (2014)
Atlantic and Amazon Rainforest	Gram-negative bacteria	Soil	Keratinolytic protease	Bach et al. (2011)
	Yeast	Flowers, fruits, soil	Lipase	Goldbeck and Maugeri Filho (2013)
		Flowers, fruits	Fructosyl transferase	Maugeri and Hernalsteens (2007)
	Yeast-like strains	Flowers, fruits, soil	Cellulase	Goldbeck et al. (2012)

The use of simple plate assays for lipase and tannase detection is highly recommended since they are rapid and low cost methods (Gopinath et al., 2013; Jana et al., 2012). Murugan et al. (2007) isolated 10 fungal strains from tannery effluent, which were subjected to screening on agar medium containing tannic acid. The formation of clear halos around the colony, demonstrating tannase production was observed in half of the assays. A lower percentage (30.2%) was observed in this study probably because tannery effluent was a favorable environment to the presence of tanninolytic microorganisms due to its high concentration of tannins. Cardenas et al. (2001) screened 960 microorganisms in agar plate containing olive oil to identify lipase producers. The authors found that 9.6% of the strains had the potential to produce the enzyme, while in the present study, a lower value was found (7.5%). Not all strains pre-selected by simple agar assay were able to produce the enzyme by SSF, most likely because the use of Tween as substrate can result in false positive, since it can also be hydrolyzed by esterases. However, Tween continues to be widely applied because of its capacity to rapidly incorporate into the medium and to promote optimal contact between cells and substrate, as well as, to provide easily visible halos of hydrolysis, avoiding the use of possibly toxic dyes (Shelley et al., 1987). The use of agar plate containing Tween was shown to be an efficient method to isolate filamentous fungi capable of producing lipase, since 34.6% of the strains selected by the simple agar assay in our work were able to produce this enzyme by SSF. Similar results were found in the study of Colen et al. (2006) where 59 fungal strains were isolated from Brazilian savanna. They tested these strains in agar medium containing tributyrin or olive oil and found that 21 were positive, and that 52.4% of the positive strains were considerate good lipase producers by SSF and SmF.

Correlation between data obtained from simple plate assay for lipase and more sensitive assays such as titration and spectrophotometry was observed in several studies. Kouker and Jaeger (1987) found that the logarithm of lipase activity from cell-free culture supernatants measured by titration was linearly correlated with the diameter of halos evaluated in a medium containing trioleoylglycerol and the fluorescent dye rhodamine B. Pereira-Meirelles et al. (1997) observed high correlation between EI of lipases, estimated in agar plate containing olive oil or babassu oil, and data obtained through spectrophotometric method using *p*-nitrophenyl laurate as substrate. Conflicting results were observed by Griebeler et al.

(2011); they compared lipases EI obtained by tributyrin agar plate assay and the enzymatic activity of lipases produced by soil bran fermentation measured by titration. The authors found that five filamentous fungi were all selected as good lipase producers by SSF and by using tributyrin as substrate, however there was one strain not screened by the simple plate assay that presented high lipase activity.

Griebeler et al. (2011) selected filamentous fungi capable of producing lipase by FES, and enzymes showed activity varying from 10.4 to 21.9 U/ml. Contesini et al. (2009) optimized the production of lipase*Aspergillus niger* by surface response analysis and were able to reach an enzymatic activity of 28.9 U/mL. Rivera-Munoz et al. (1991) studied several fungal strains regarding their lipase producing capacity such as: *A. niger* (2.9 U/ml), *Geotrichum candidum* (1.1 and 1.4 U/ml) and *Penicillium* sp. (1.7 to 36.5 U/ml).

Regarding tannase, it was observed that highest activity was reached by strain CL255 (2.14 ± 0.04 U/mg). A similar result was found in the study of El-Fouly et al. (2010) on tannase production by *A. niger* which showed enzymatic activity of 3.37 ± 0.17 U/mg.

Studies regarding lipase production by *Colletotrichum* sp. are rare. Balaji and Ebenezer (2008) tested diverse residual cheap oil substrates for lipase production by *C. gloeosporioides* and reached 2,560 U/g of dry matter using pongamia oil cake. In this study, enzyme produced by strain IB28a identified as *C. gloeosporioides* showed maximum activity of 13.0 ± 3.3 U/ml without optimization. Similar results were found in the study of Colen et al. (2006) where a *C. gloesporioides* strain was found to be the best alkaline lipase producer from 59 strains isolated from Brazilian savanna soil. They optimized the enzyme production up to 27.7 U/ml and observed its capacity to hydrolyze a wide variety of substrates such as lard, natural oils and tributyrin. In contrast, Amirita et al. (2012) did not observe lipase production by the entophytes: *C. gloeosporioides* and *Colletotrichum crassipes* in agar medium containing Tween, but the strain of *Colletotrichum falcatum* was positive for lipase activity. The authors proposed that the absence of certain active enzymes in entophytes could occur to prevent the host plant from damage.

Studies concerning the biochemical characteristics of *Colletotrichum* sp. lipase were not found. However, Maccheroni et al. (2004) evaluated the effect of the ambient pH on lipase secretion by several strains of *Colletotrichum* sp. using plate clearing assays. They concluded that, in general, lipase was secreted at neutral and alkaline pH. Fungi ensure that enzymes are secreted mostly at ambient pH values equivalent to their optimal of activity (Maccheroni and Azevedo, 1998). Therefore, the data found in the study mentioned above corroborates with our findings on *Colletotrichum* sp. lipase optimum pH values (6.5 to 7.5).

Different research groups use diverse methodologies to determine enzyme activity, therefore the comparison between distinct studies is limited. However, it is evident that the present work has a significant contribution to studies regarding production, characterization and application of lipase and tannase. It was possible to isolate from Amazon and Atlantic rainforests regions, several strains of filamentous fungus capable of producing extracellular lipase and tannase by fermentation of wheat bran. Strain IB28a, identified as *Colletotrichum* sp., isolated from the Atlantic Rainforest can be considered an interesting producer of lipase by SSF. Lipase from *Colletotrichum* sp. reached high enzymatic activity without optimization and presented higher activity at neutral pH and room temperature.

Conflict of interests

The authors did not declare any conflict of interest.

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