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High-level lipase production by *Aspergillus candidus* URM 5611 under solid state fermentation (SSF) using waste from *Siagrus coronata* (Martius) Becari

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The current study evaluated lipase production by *Aspergillus candidus* URM 5611 through solid state fermentation (SSF) by using almond bran licuri as a new substrate. The microorganism produced high levels of the enzyme (395.105 U gds^{-1}), thus surpassing those previously reported in the literature. The variable moisture content, the inductor concentration, temperature and pH were analyzed. The best production conditions were initial moisture content of 75%, 0% of inductor, at 25°C, pH 5.5, in 10.0 g of substrate. The variables initial moisture and inductor contents showed significant effects on lipase production by increasing enzyme activity in about 200% when compared with the initial screening (197.44 U gds^{-1}). Optimum lipase activity was obtained at pH 2.5 at an optimum temperature of 65°C. The lipase was stable from pH 2.5 to 9.0 and at temperatures between 30 to 65°C. Results from the study are promising for the economic use and value addition from these important agro residues, which are abundantly available in Brazil. This is the first report on lipase production by *A. candidus* URM 5611 reaching a much higher yield of enzyme under SSF with almond bran licuri used as substrate.

Key words: *Aspergillus candidus* URM 5611, solid state fermentation (SSF), licuri waste, lipase characterization.

INTRODUCTION

The enzymes from microbial sources receive special attention in the global market for bioproducts, due to their current and potential application in industry, mainly in detergents, oils and fats, dairy manufactures and pharmaceutical industries (Ângelo, 2014). Lipases stand out among the enzymes produced by fungi (Contesini et al., 2010). Lipases (EC 3.1.1.3, triacylglycerol acilhydrolases) are enzymes that occupy a prominent place among biocatalysts, and they have many applications (Ali et al., 2014). For this reason, its market share in global industrial enzymes grows significantly (Reinehra et al., 2014).

In the future, this enzyme class will have industrial and commercial importance comparable to that of peptidases, which sale in industrial enzymes ranges from 25 to 40% (Barros et al., 2010; Ghasemi et al., 2011). The industrial

lipases are mainly used as additives for washing detergents, food industries, especially in the preparation of chocolate substitutes and in the production of special flavors.

Moreover, it has been widely used in the manufacture of cosmetics (tanning), in sewage treatment, and transesterification of triglycerides (Treichel et al., 2010; Kotogán et al., 2014). The widening application of microbial lipases in biotechnology has necessitated the continued research and development of novel lipases by means of extensive screening and optimisation of process parameters (Mostafa and El-Hadi, 2010). The biotechnological potential of lipases is related to the fact that these enzymes catalyze not only the hydrolysis reactions, but also the esterification and transesterification reactions, and they keep their chemical structure and activity in different organic solvents (Tan et al., 2004).

In addition, they do not often require the presence of cofactors, they catalyze various reactions at low temperature and pressure, have a broad specificity for substrates and exhibit high enantioselectivity (Reis et al., 2009; Contesini et al., 2010). They can be commonly found in nature and derive from animal, plant and microorganisms sources.

Numerous filamentous fungi species produce these enzymes, especially those from the genus *Aspergillus* (Contesini et al., 2010; Fleuri et al., 2014). The genus *Aspergillus* is currently considered as one of the largest biotechnological substance producers, with several species found in nature (Liu et al., 2006). There are about 837 (Hawksworth et al., 2011) *Aspergillus* species commonly isolated from soil and decaying plants. These fungi produce a number of extracellular enzymes (among them: lipases), many of which are applied in different industry sectors (Contesini et al., 2010; Fleuri et al., 2014).

Solid state fermentation (SSF) has become a major attraction for the production of this enzyme, like other products of high added value, due to the use of agro-industrial wastes as well as the obtained increased volumetric productivity (Ramachandran et al., 2007; Casciatori et al., 2014).

Despite functional electrical stimulation (FES) advantages, this technology is still not widely used for lipase production in large scale. This fact is due to the lack of systematic studies at bench scale, for example, the definition of microorganisms producing suitable substrates and culture conditions (Holker and Lenz, 2005; Fleuri et al., 2014).

However, there are few studies on lipase production by *Aspergillus* species through SSF. Therefore, there is the need for further studies on these microorganisms' potential for enzyme production by this method, as well as on new substrates to be used during fermentation.

This study aimed to investigate lipase production by *Aspergillus* species preserved under mineral oil in Micoteca URM Culture Collection (WDCM604) under SSF using low cost residue. It also aimed to optimize culture conditions for production and evaluate enzyme stability regarding changes in pH and temperature.

MATERIALS AND METHODS

Microorganisms and inoculum preparation

Eighteen isolates of *Aspergillus* isolated from castor bean obtained from the Micoteca URM culture collection (URM, Recife, Brazil, WDCM604) (Micoteca, 2014) were inoculated on malt extract agar (MEA (g L^{-1}): malt 20, glucose 20, peptone 1, and agar 20) and kept at 28°C. Spores of each isolate were transferred under strict aseptic conditions to 10 mL of sterile distilled water with 0.1% Tween 80. After homogenization, the spores in the suspension were counted by means of a Neubauer chamber in order to obtain the concentration of $5x10^8$ spores mL^{-1} (Sabu et al., 2005).

Solid state fermentation (SSF)

Solid substrate

Almond bran licuri (*Syagrus coronata* (Martius) Beccari) were obtained from COOPERLIC - Cooperative Lanyard sand Processing, Licuri in Caldeirão Grande, Bahia, Brazil. The waste was flushed with sterile distilled water and dried in an oven (QUIMIS, Q316M2, São Paulo, Brazil) at 55°C for 48 h.

Lipase production under SSF

As for lipase production in 250 ml Erlenmeyer flasks, 10 g bran licuri were added with 1% olive oil, in order to induce it. The mixture was moistened with phosphate buffer (0.1 mol L^{-1}). The initial moisture content was adjusted to 50% and pH 7.0. The flasks were autoclaved, cooled and inoculated with 1 mL of the spore suspension at a concentration of 5×10^8 spores mL^{-1} and incubated at 30°C (BOD, TECNAL, TE-371, São Paulo, Brazil) for 48, 72 and 96 h (Sabu et al., 2005).

Enzymes extraction

Fifty (50) mL of sterile distilled water containing 0.01% Tween 80 were added to each flask and kept on a rotary shaker (TECNAL) at 150 rpm for 10 min. The fermented substrate was subjected to filtration by using sterile lint and serial filter paper (CELAB 22.15 microns) with the aid of a vacuum pump. The resulting supernatants were assayed for lipase activity. All fermentation tests were carried out in three independent experiments (Brunk et al., 2005).

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Abbreviation: SSF, Solid state fermentation.

Table 1. Variable levels of the 2^4 experimental design for the production of lipase (U gds^{-1}) in SSF by *Aspergillus candidus* URM 5611 using bran licuri as substrate.

Variable	Levels		
	Low (-1)	Central (0)	High (+1)
Inductor (I)	5.0	10.0	15.0
Im (%)	45	60	75
T (ºC)	25	32.5	40
pH	5.5	6.5	7.5

I, inductor; Im, initial moisture content; T, temperature.

Enzyme activity

The enzymatic activity was accomplished by titrimetric method using olive oil (10% w/ v) as substrate for the enzyme dosage, which was emulsified with arabic gum (5% w v^{-1}) in distilled water. 5 mL of this emulsion was added to 1 mL of crude enzyme extract, and incubated for 1 h at 37°C, then titrated with NaOH solution (0.05 M) according to Watanabe et al. (1977). The activity dosage was performed by using phenolphthalein as indicator and the arithmetic mean of the values was used to determine the enzyme activity calculation. One lipase activity unit was defined as the amount of enzyme that releases 1 µ mole fatty acid per minute, under the conditions described above, and that can be determined through the equation by Leal (2002). The moisture of each sample was determined according to the AOAC (2003) for further conversion of the results into dry substrate (U gds^{-1}).

Experimental design and statistical analysis

The influence of temperature (T), initial moisture content (Im), inductor (I) and pH (pH) on lipase production was evaluated from the results of the experiments performed according to a 2^4 factorial design (Bruns et al., 2006), plus four central points (Table 1). All statistical analyses were performed by using Statistic 8.0 software (Statsoft, 2001).

Enzyme partial characterization

Lipase activity of the crude enzyme extract was measured at different pH and temperature values as well as the effect of pH on the enzymatic activity stability.

Effect of pH on the enzymatic activity stability

The pH effect was determined by enzymatic activity at 50°C; the crude enzyme extract was emulsified for 3 min with gum arabic (5% w/v) in olive oils at different pH, by using the following buffers: citrate - phosphate (2.5 to 3.5); acetate (4.0 to 4.5); citrate - phosphate (5.0 to 7.0) and Tris-HCl (7.4 to 9.0), all 0.05 M. In order to determine enzyme pH stability, the enzyme extract was previously subjected to the action of different pH values by using buffer solutions with pH ranging from 2.5 to 9.0 for periods of 60, 120 and 180 min and it was then subjected to the determination of lipase activity as described above. The analyses were performed in triplicate.

Effect of temperature on the enzymatic activity stability

The temperature effect was determined with pH optimal constant of 2.5 by incubating the enzyme extract with the respective substrate, with temperature variations between 30 to 80°C, with a variation range of 5°C for a period of 60, 120 and 180 min. The enzymatic activity was performed as previously described. All analyses were performed in triplicate. Aiming to determine the temperature stability of the enzyme, the extract was preincubated at temperatures ranging from 30 to 80°C for 60 min and it was then subjected to lipase activity determination. The analyses were performed in triplicate.

RESULTS AND DISCUSSION

Screening for lipase production

All the 18 tested cultures of *Aspergillus* produced high levels of lipase when bran licuri was used as substrate. Eleven of the 18 culture (61.1%) samples yielded over 100/ gds. *A. candidus* URM 5611 was the best producer with 197.44 U gds^{-1}, followed by *Aspergillus flavus* URM 5794 and *Aspergillus sclerotiorum* URM 5792 with production of 163.25 and 154.21 U gds^{-1}, respectively, (Table 2). Because of the high level of lipase (197.44 U gds^{-1}) produced by *A. candidus* URM 5611, this microorganism was selected to study the best conditions for enzyme production. Palma et al. (2000) obtained maximum lipase activity by *Penicillium restrictum* 27.8 U gds^{-1} by using babassu palm supplemented with peptone as substrate. In solid-state cultivation on a mixture of sunflower seed meal and sugarcane bagasse, Alberton et al. (2010) evaluated lipase production by *Rhizopus microsporus*. The maximum production was 26 U gds^{-1}, and it was applied to the treatment of dairy waste. When compared with the results obtained in the current study, species from genus *Aspergillus* appear to be most promising for lipase production by SSF, since they produce high levels of the enzyme. Castiglioni et al. (2009) tested lipase production by *Aspergillus fumigatus* under SSF by using shell and rice bran oil, and olive added as carbon source. The obtained value of the total activity of 117.02 U gds^{-1} was similar to that obtained in the present study by *Aspergillus caespitosus* URM 5938 with almond bran licuri and olive oil as carbon source and total activity of 117.33 U gds^{-1}. However, it was still less than the maximum value obtained with *A. candidus* URM 5611 which showed total activity of 197.44 U gds^{-1}. Fleuri

Table 2. Total lipase activity (U gds^{-1}) produced by cultures of *Aspergillus* using licuri bran as substrates and incubated for 96 h.

Access number[a] URM	Species	Lipase activity
5910	*Aspergillus awamorii* Nakaz.	99.08
5938	*Aspergillus caespitosus* Raper & Thom	117.37
5611	*Aspergillus candidus* Link	197.44
5608	*Aspergillus duricaulis* Raper & Fennell	74.33
5794	*Aspergillus flavus* Link	163.25
5698	*Aspergillus fumigatus* Fresen.	90.91
5620	*Aspergillus japonicus* Saio	106.27
5827	*Aspergillus melleus* Yukawa	112.88
5838	*Aspergillus niger* Tiegh.	84.71
5870	*Aspergillus niveus* Blochwitz	115.57
5609	*Aspergillus ochraceus* K. Wilh.	128.73
5787	*Aspergillus parasiticus* Speare	110.00
5792	*Aspergillus sclerotiorum* Huber	154.21
5615	*Aspergillus stromatoides* Raper & Fennell.	128.28
5774	*Aspergillus sydowii* (Bain. & Sart.) Thom & Church	94.90
5606	*Aspergillus terreus* Thom	81.89
5829	*Aspergillus variecolor* Thom & Raper	95.47
5701	*Aspergillus versicolor* (Vuill.)Tiraboschi	143.33

[a]Access number in the Micoteca URM culture collections (Recife, Pernambuco, Brazil).

et al. (2014), evaluated lipase production by an *Aspergillus* strain, by using wheat bran, soybean meal and soybean meal with sugarcane bagasse as substrate, through SSF. The authors observed lipase activity of 67.5, 58 and 57.3 U gds^{-1} for each substrate, respectively. Compared to the present study, *A. candidus* URM 5611 showed higher lipase activity (197.44 U gds^{-1}). Such microorganism is preserved in a Culture Collection of reference in Brazil (Micoteca URM Culture Collection (WDCM604), and it was duly identified. The fungi from genus *Aspergillus* are widely used in enzyme production. The lipase production by *Aspergillus* species has been widely reported, mainly because this enzyme is usually extracellular. Lipase (EC 3.1.1.3) (triacylglycerol lipase) also called triacylglycerol acylhydrolase, catalyzes the hydrolysis of ester bonds from oils and fats and may act in synthesis reactions (esterification, interesterification, alcoholysis, acidolysis and aminolysis) (Sharma et al., 2001). These reactions have the advantage of giving biotechnological potential to enzymes, such as stability in organic solvents, and no requirement for cofactors broad specificity (Azeredo et al., 2007). In the present study, *A. candidus* URM 5611 proved to be a major lipase producer, and it was selected for enzymatic optimization and characterization. This is the first report on lipase production by an isolate from this species.

Effects of the variables on lipase production

A. candidus URM 5611 presented efficient lipolytic activity (LA) when olive oil is used as inductor, but in the absence of this oil, there was an increase in production with higher activity. Therefore, it was selected to a study on the effect of independent variables such as temperature, pH, moisture, and induce lipase production in solid state fermentation for 48 h (Table 3). The maximum lipase activity produced by *A. candidus* URM 5611 was 397.158 U gds^{-1} in run 3 as follows: 48 h of fermentation using 0% olive oil at pH 5.5, 75% moisture at 25°C. When compared with the initial screening (197.44 U gds^{-1}), after production optimization, *A. candidus* URM 5611 had its lipase production increased by 200%. This value (397.15 U gds^{-1}) was higher than that reported in the literature so far. For example, Kempka et al. (2008) tested a strain of *Penicillium verrucosum* by using soybean meal as substrate at 27.5°C with moisture of 55% and an activity of 40 U gds^{-1} within 48 h of fermentation. Ul-Haq and Idress Rajoka (2002) found 48 U gds^{-1} activity by the fungus *Rhizopus oligosporus* by using almond cake as substrate at 30°C for 72 h. However, it is less than the lipase activity obtained by Mala et al. (2007) who evaluated the lipase production by *A. niger* at 30°C, 62% moisture using wheat bran and sesame cake as substrates, and obtained 384 U gds^{-1} at 72 h of fermentation. Thus, *A. candidus* 5611 URM showed to be most promising for lipase production under SSF at high levels. The Pareto chart shows the estimated effects of the variables (main effect or first-order) and the interactions between variables (second order effect) on the response variables (biomass and lipase activity), in order of magnitude. The length of the bars is proportional

Table 3. Experimental design results for lipase production under SSF fermentation by *Aspergillus candidus* URM 5611.

Experimental variables					Total activity (U gds^{-1})		
Run	**Temperature (°C)**	**Initial moisture (%)**	**Inductor (%)**	**pH**	**48 h**	**72 h**	**96 h**
1	25	45	0	5.5	77.66	39.55	25.55
2	40	45	0	5.5	73.90	35.44	31.83
3	25	75	0	5.5	**397.15**	153.15	62.18
4	40	75	0	5.5	112.71	38.04	43.20
5	25	45	3	5.5	45.78	20.53	20.46
6	40	45	3	5.5	**45.03**	21.56	18.94
7	25	75	3	5.5	90.73	34.33	34.61
8	40	75	3	5.5	77.04	32.16	27.15
9	25	45	0	7.5	71.33	26.63	24.32
10	40	45	0	7.5	79.38	27.61	26.93
11	25	75	0	7.5	87.92	51.14	46.52
12	40	75	0	7.5	395.10	150.10	60.18
13	25	45	3	7.5	45.75	21.17	19.59
14	40	45	3	7.5	54.21	20.03	23.13
15	25	75	3	7.5	85.36	39.14	30.78
16	40	75	3	7.5	85.95	35.08	31.69
17	32.5	60	1.5	6.5	67.17	38.75	37.10
18	32.5	60	1.5	6.5	74.92	34.99	33.53
19	32.5	60	1.5	6.5	76.61	46.85	43.68
20	32.5	60	1.5	6.5	86.81	42.67	43.31

to the standardized variable effect. The vertical line can be used to determine that the effects were statistically significant, because the bars that extend through this line correspond to statistically significant effects with confidence level of 95% (Porto et al., 2008). The effects have been demonstrated in these variables, as well as the interactions between them (Figure 1). It was observed that, at 48 h of fermentation, the humidity showed a positive effect, whereas the oil showed a negative effect, which means that higher moisture levels and lower inductor are better for lipase production. The other variables showed no effect on lipase production. Fermentation using only licuri almond meal, without the addition of olive oil and the accretion of moisture are sufficient to get a good lipase production by *A. candidus* URM 5611. This result can be explained due to high lipid content in the substrate composition (49.2 + 0.08; Source: Crepaldi et al (2001), which suggested the occurrence of inhibition by excess substrate.

Lipids generally lead to increased lipase production (Mahadik et al., 2002; Dominguez et al., 2003). However, in the current study, the increased lipid source seems to have had an inhibitory effect on enzyme production. A similar situation was observed by Vargas (2008) who evaluated lipase production by *Penicillium simplicissimum* and found that the addition of oil to the different soybean cakes used as substrate resulted in a decreased lipase production. This fact suggested the occurrence of inhibition by excess substrate. As the amount of water is always limited, controlling the level of moisture is essential to process optimization in the solid state. The water content suitable for the substrate must enable the formation of a water film on the surface in order to facilitate dissolution and transfer of nutrients and oxygen. However, the spaces among the particles must remain free to allow oxygen diffusion and heat dissipation (Gervais and Molin, 2003; Sanchez, 2009). The moisture, at very low levels, impairs the transport of nutrients and toxins through the membrane and it can cause the loss of functional properties of cellular metabolic chain enzymes (Gervais and Molin, 2003).

According to Mahanta et al. (2008), water content is a significant factor in substrate physical properties. High water content leads to decrease on substrate porosity, there by decreasing gas exchange. Moreover, low water content may result in reduction of microbial growth and in consequent decreased enzyme production. Initially, Dantas and Aquino (2010) evaluated the influence of water content between 35 and 65% on several substrates (castor cake, babassu pie, pumpkin seeds, avocado peel and coffee grounds) in lipase production by *A. niger* under SSF. Higher production of lipase (24.6 U/g) was obtained when babassu pie was used as carbon source with water content of 45%. This relationship can be better understood by observing the plot square (Figure 2). It can be seen that the highest effect values are at the base, and they correspond to moisture, but this effect is opposite in relation to inductor concentrations. The best

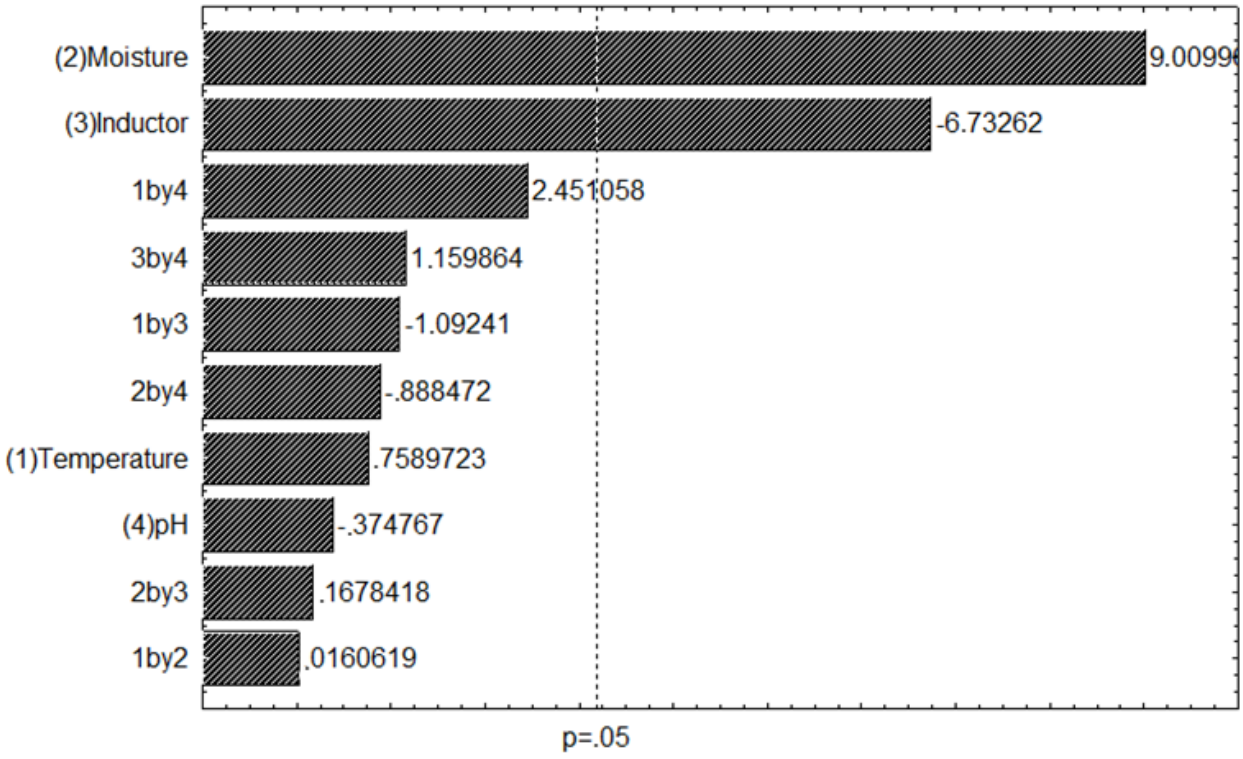

Figure 1. Pareto chart of interaction effects on the response of lipase activity produced by *Aspergillus candidus* URM 5611 after 48 h of cultivation.

conditions for enzymes production with lipolytic activity can be obtained without added inductor and increased moisture in the fermentation medium. By analyzing the fermentation costs, the current result is very interesting when a process on an industrial scale is designed. It is more advantageous to the industry to produce lipolytic enzymes using low cost substrates.

Enzyme characterization

pH effect on lipase activity and stability

As for the characterization of lipase produced by *A. candidus* URM 5611, the crude enzyme extract was chosen as the best condition for enzyme production (Table 3, Run 03). The highest lipase activity was obtained at pH 2.5. The lipase from *A. candidus* URM 5611 was stable at pH range from 2.5 to 9.0 with activity values above 65% for 180 min. The maximum stability was observed at pH 7.4 with 79% residual activity. These results show that lipase is stable over a wide pH range. Figure 3 shows the formation of two peaks, possibly due to the presence of isoenzymes. Therefore, there is a need for further studies on the purification of this enzyme. Mahadik et al. (2002) observed that lipases produced by *A. niger* NCIM 1207 had maximal activity at pH 2.5, and Falony et al. (2006) demonstrated lipase stability at a pH range of 2.0 to 10.0 for *A. niger*, thus corroborating the results from the current study. Moreover, Pera et al. (2006) reported that the optimum pH activity of *A. niger* is of approximately 6.0. Ângelo et al. (2014) characterized the lipase produced by *Fusarium oxysporum*, and found increased lipolytic activity at pH 8.5. The enzyme was stable at pH range from 5.5 to 6.5, and showed low stability at pH 8.5. It is known that lipases have isoforms, depending on the origin of the enzyme. Furthermore, assay conditions, incubation team, pH, temperature, substrate and the used methodology can directly affect enzymes kinetic properties and stability (Carvalho et al., 2005).

Effect of temperature on activity and stability

The effect of temperature on lipase activity demonstrated that the maximum relative activity was obtained at 65°C, for 180 min. The enzyme was most stable at 60 and 65°C with 120% residual activity, for 180 min. These results show that the lipase is stable over a wide temperature range. Maia et al. (2001) evaluated the optimum temperature of lipase from *Fusarium solani* and found it at 35°C. Pastore et al. (2003) studied the characteristics of the lipase produced by a strain of *Rhizopus*, and found the optimum temperature of 40°C. Saxena et al. (2003) reported that the optimum temperature of lipase from *A.*

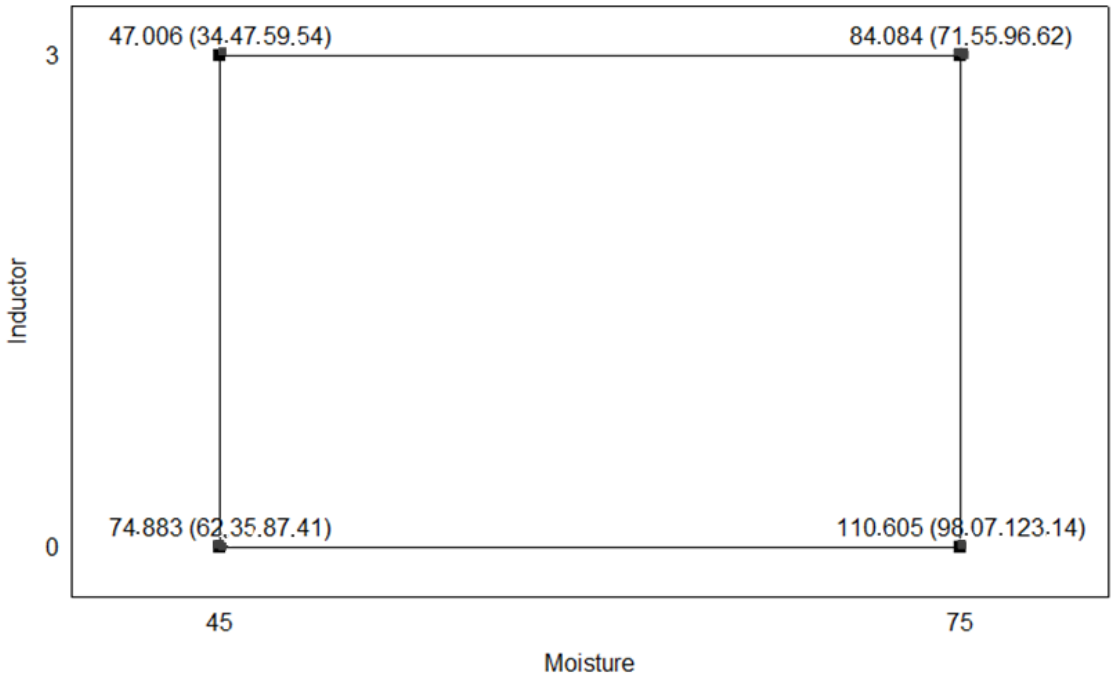

Figure 2. Square of the variables (inductor and moisture) as the response variable under lipolytic activity.

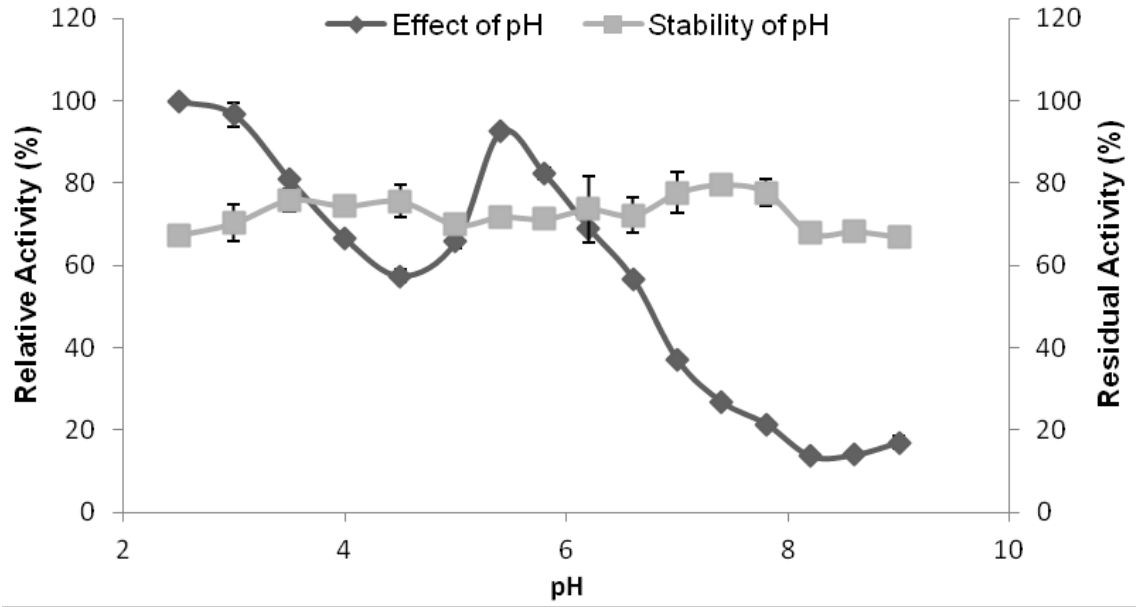

Figure 3. Effect ( ) and stability ( ) of lipase residual activity (%) on pH in SSF produced by *Aspergillus candidus* URM 5611 after 180 min of incubation with initial pH ranging from 2.5 to 9.0.

carneus was 37°C, thus differing from the results obtained in the current study, since the optimum temperature for the activity of lipase produced by *A. candidus* was of 65°C. Carvalho et al. (2005) evaluated kinetic properties of the lipase by *Geotrichum candindum* in crude extract, and found that the enzyme was stable up to 50°C. Ângelo et al. (2014) evaluated the temperature effect on the activity of lipase from *F. oxysporum*, and found the optimum temperature of 40°C. In view of the stability results obtained at different temperatures for the lipase produced by solid state fermentation of *A. candidus* URM 5611, there were thermo-

stable lipases when it is incubated for 180 min at temperatures between 30 to 80°C in constant pH of 2.5. Thermostability is important for the application of lipase in the detergent industry. Thermostable lipases have been isolated from many sources, including *Mucor pusillus*, *Rhizopus* and *Aspergillus terreus* (Singh and Mukhopadhyay, 2012). A more stable lipase alone has maximum activity at 60°C and it loses at least 90% of its activity after 15 min at 60°C (Said and Pietro, 2004).

Conflict of interests

The authors did not declare any conflict of interest.

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