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Mycelium-bound lipase from *Penicillium citrinum* as biocatalyst for the hydrolysis of vegetable oils

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ABSTRACT

Mycelium-bound lipase from filamentous fungus has been extensively studied as an alternative biocatalyst used in biotransformation processes. In this work, *Penicillium citrinum* URM 4216 was assessed as a potential producer of mycelium-bound lipase and its catalytic activity was investigated to yield concentrated polyunsaturated fatty acids from vegetable oils. Under the established growth conditions and using olive oil as an inducer, *P. citrinum* was able to produce lipase having high mycelium-bound activity (271.67 ± 10.47 U g^{-1}) revealing suitable biochemical (optimum pH = 8.0 at 45 °C) and kinetic ($Km = 136.51$ μmol $L^{-1}min^{-1}$, Vmax = 267.33 μmol $g^{-1}min^{-1}$) properties and thermal stability (half-life time of 1.8 h at 60 °C) to mediate biotransformation reactions. By applying factorial design, the hydrolysis of soybean oil yielded 38% at 38 °C using oil/buffer ratio of 20% in the presence of 2.5% of an emulsifying agent for 3 h. The hydrolysis degree was increased to 96% by replacing the conventional heating system for ultrasonic irradiation and increasing the incubation time to 9 h. Similar degrees of hydrolysis (>84%) were achieved using other rich polyunsaturated vegetable oils (sunflower, olive and canola oils); confirming the specificity of this mycelium-bound lipase for polyunsaturated fatty acids, such as oleic and linoleic acids.

1. Introduction

In recent years, there has been a remarkable increase in the modification of oils and fats from both animal and plant sources (Lu et al., 2011; Bebarta et al., 2013; Zhao et al., 2014; Morales-Medina et al., 2017). Hydrolysis of oils and fats is one of the first steps for obtaining free fatty acids, which are an important component in the economic exploitation of these naturally produced renewable raw materials (Satyarthi et al., 2011; Zenevic et al., 2016). A significant number of high-value products require fatty acids in their manufactures, including coatings, adhesives, biofuels, surfactants, specially lubricating oils, shampoos, and other personal care products (Farile et al., 2017).

The reaction conditions for traditional chemical hydrolysis of oils are high-pressure and temperature (70 bar and 250 °C, for example), which can raise undesirable side reactions (oxidation or interesterification) decreasing the quality of the required product (Satyarthi et al., 2011). In this context, oil biotransformation due to its milder reaction conditions besides the high specificity and selectivity of the enzymes is considered an attractive alternative to be used to reduce limitations of the chemical processes (Goswami et al., 2013; Reis et al., 2009).

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) catalyze the hydrolysis of triacylglycerol (Goswami et al., 2013), with great potential of being used in several industrial segments such as production of biofuels, treatment of rich-lipids wastewaters, as well as on food and pharmaceutical industries (Andualema et al., 2012). The production of fatty acids from triacylglycerols catalyzed by lipases has been attempted as an energy-saving method, especially for synthesis of high value-added products from heat sensitive fatty acids (Pourzolfaghar et al., 2016). Although enzymatic processes operate in the vast majority in near room temperature conditions, processes catalyzed by lipases have certain limitations, such as slow reaction rates and significantly high cost of these enzymes (Cortez et al., 2017). To overcome such limitations, lipases bound to whole-cells are an attractive technology for enhancing reaction efficiency with lower costs. The whole-cell biocatalyst can be formed from the cell wall or the cell membrane-bound lipase (i.e., intracellular enzyme), instead of extracellular lipase (Lima et al., 2017).

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Table 1
Fatty acids composition of the tested vegetable oils [15, 25].

Oil	Free fatty acids (wt.%)				
	Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Linolenic (C18:3)
Olive	14.6	-	75.4	10	-
Sunflower	6.4	2.9	17.7	72.9	-
Canola	3.5	0.9	54.1	22.3	-
Soybean	9.8	2.9	18.3	44.7	4.72

Mycelium-bound lipases from filamentous fungus are economically attractive and can be used directly as suspended free cells without following the isolation, purification and immobilization procedures (Solarte et al., 2014). Whole-cell biocatalysts can be simply prepared by cultivation and easily recovered by filtration. Compared with conventional extracellular lipases, whole-cell catalysts have better-operating stability; due to their complete cellular structure, they act as a natural matrix that can protect the enzymes from the possible negative action of external agents, providing an effect analogous to that exerted by common matrix used for enzyme immobilization (Andrade et al., 2014; Zhou et al., 2015). To date, few strains have been studied as potential mycelium-bound lipase producers, such as *Rhizopus* sp. (Arumugan and Ponnusami, 2014; Kyeong and Yeom, 2014; Wang et al., 2015), *Aspergillus* sp. (Solarte et al., 2014; Li et al., 2015; Yan et al., 2015) and *Mucor* sp. (Andrade et al., 2014; Andrade et al., 2012; Carvalho et al., 2015). The genus, *Penicillium* is also known as a good lipase producer (Dheeman et al., 2011; Marotti et al., 2017), which is used in the dairy industries and in a number of bioconversions of industrial importance.

In this context, the objective of this work was to prepare and characterize *P. citrinum* whole-cells to be employed in the hydrolysis reaction of vegetable oils. The influence of ultrasonic radiation and emulsifier was also investigated in the hydrolysis reactions.

2. Materials and methods

2.1. Materials

Penicillium citrinum URM 4216 strain was acquired from the culture collection of the University of Recife Mycology (URM) at the Federal University of Pernambuco (Recife, PE, Brazil). Refined vegetable oils (soybean, olive, sunflower and canola) were purchased from a local market (Soya®), and their fatty acids composition is described in Table 1. Gum Arabic, KOH and salts ($NaNO_3$, $(NH_4)_2SO_4$, NH_4Cl, KH_2PO_4, $MgSO_4.7H_2O$) were purchased from Synth® (São Paulo, SP, Brazil). Soy peptone, bacterial peptone and yeast extract were purchased from Himedia® (Mumbai, India).

2.2. Medium and growth conditions

The filamentous fungal strain used in this work was cultured in submerged fermentation in a basal medium containing different vegetable oils as carbon sources at fixed concentration (30 g L^{-1}) with soybean peptone at 70 g L^{-1}. The selected carbon source was supplemented with different amounts of nitrogen source (ammonium sulfate, ammonium chloride, soy peptone, bacterial peptone and yeast extract). The pH of the culture medium was evaluated in the range of 5.0–8.0 with an incremental of 0.5 by adding concentrated HCl or NaOH solutions. The effect of the number of spores (10^5, 10^6, 10^7 and 10^8) was evaluated by performing the inoculum in 100 mL of culture medium previously sterilized (121 °C/15 min) in Erlenmeyer shake flasks (250 mL), which were incubated for 96 h at 30 °C using a reciprocal shaker (170 rpm).

2.3. Whole-cell biocatalyst preparation

After 96 h, the mycelium obtained was harvested from the culture broth by filtration using a Buchner funnel, washed with distilled water followed by acetone and dried under vacuum for 24 h to attain moisture content lower than 10%. The dry biocatalyst was then ground to powder consistency. Lipase activities in the biomass (whole-cells) and in the filtrate (culture broth) were measured by the hydrolysis method using olive oil emulsion according to the modification proposed by Andrade et al. (2014). One unit (U) of enzyme activity was defined as the amount of biomass dry weight or culture broth, that liberates 1 μmol of free fatty acid per min under the assay conditions (37 °C and pH 7.0). Moisture content (%) of whole cell-lipase was determined using Adolfo Lutz standard methods (Adolfo Lutz Institute, 2008).

2.4. Catalytic properties of mycelium-bound lipase

Biochemical properties (optima pH and temperature) and kinetic constants (K_m and V_{max}) were determined in the hydrolysis of emulsified olive oil. The effect of pH was evaluated at 37 °C in the range of 6.0–8.5 (buffer sodium phosphate 0.1 M) while the effect of temperature was investigated in the range of 30–55 °C, using 0.1 M buffer sodium phosphate at pH 7.5. The concentration of substrate (olive oil) was evaluated in the range of 5–70%, which corresponded to fatty acids concentrations of 186–2604 mmol L^{-1}. The apparent K_m and V_{max} values were calculated from Lineweaver–Burk plot, using the software Origin Pro 8.

Thermal stability was evaluated by incubation of whole-cells (0.1 M sodium phosphate buffer pH 8.0) in a thermostatic bath at 60 °C for different time intervals (30–180 min). Samples were withdrawn periodically for determining the biocatalyst residual hydrolytic activities. The denaturation rate (K_d) and half-life time ($t_{1/2}$) were calculated by Eqs. (1) and (2), respectively, where A = is the hydrolytic activity at a given time and A_0 =initial hydrolytic activity.

$$A = A_0 e^{-K_d t} \quad (1)$$

$$t_{1/2} = ln0.5/_{-k_d} \quad (2)$$

2.5. Factorial design

The enzymatic hydrolysis assays were carried out in Erlenmeyers flasks (250 mL) containing 50 g of the substrate (soybean oil emulsified in 0.1M sodium phosphate buffer at pH 8.0) with gum Arabic as emulsifier agent (2.5% wt). A full 2^2 factorial design with three assays at the center points and with addition of star points were used to optimize the hydrolysis of soybean oil. The effect of ratio oil/buffer (12.9–27.1 wt%) and temperature (26–48 °C) were investigated in eleven experiments performed at random order. To monitor the hydrolysis behavior, 0.5 g aliquot samples were taken at various time intervals and analyzed by titration. Fifty milliliters of 50:50 (v/v) mixture of acetone in ethanol were added to the sample to dissolve the oil and denature the enzyme, thus effectively freeze the reaction. The mixture was titrated with standard 0.02 mol L^{-1} potassium hydroxide solution. The hydrolysis percentage attained at 3 h reaction was taken as response variable and calculated by Eq. (3). H% is defined as the percentage weight of free fatty acids in the sample divided by its maximum theoretical amount (Freitas et al., 2007).

$$\%H = \frac{V_{KOH} \times M_{KOH} \times \overline{MW}}{W \times f} \times 100 \quad (3)$$

in which: V_{KOH} is the volume of potassium hydroxide solution (KOH) required during titration; M_{KOH} is the KOH molarity (0.02 mol L^{-1}); $\overline{MW}$ is the average molecular weight of fatty acids (g mol^{-1}); W is the weight of the sample taken and f is the fraction of the oil at the reaction starting.

The data were analyzed using *Statistica* 5® and the coefficients were

Table 2
Biomass and lipase activity obtained during cultivation of *P. citrinum* whole-cells using different nutritional sources and physical parameters.

Parameters		Dry biomass ($g\,L^{-1}$)	Lipase activity	
			Whole-cell ($U\,g^{-1}$)	Culture Broth ($U\,mL^{-1}$)
Carbon source ($30\,g\,L^{-1}$)	Olive oil	28.08 ± 0.95	188.76 ± 16.98	11.00 ± 0.28
	Canola oil	20.70 ± 1.85	83.95 ± 18.45	11.20 ± 0.57
	Sunflower oil	26.27 ± 0.69	60.76 ± 5.48	11.40 ± 0.85
Nitrogen source[a] ($g\,L^{-1}$)	BP70	24.35 ± 1.06	32.78 ± 12.01	10.60 ± 0.28
	SP35Y35	26.02 ± 0.61	131.05 ± 20.85	17.60 ± 2.83
	SP35	22.37 ± 2.12	38.03 ± 9.25	6.20 ± 0.29
	SP50	22.43 ± 2.76	80.63 ± 34.03	8.80 ± 1.13
	SP70	28.08 ± 2.45	188.76 ± 16.98	11.00 ± 0.28
pH	5.0	14.24 ± 0.42	38.42 ± 6.36	15.52 ± 0.01
	5.5	19.84 ± 1.50	33.4 ± 14.94	11.52 ± 1.58
	6.0	19.65 ± 3.65	102.51 ± 20.85	11.68 ± 0.45
	6.5	26.55 ± 0.87	169.56 ± 21.40	12.16 ± 0.23
	7.0	33.45 ± 0.91	236.96 ± 14.09	12.48 ± 1.58
	7.5	26.77 ± 2.86	159.86 ± 23.86	11.36 ± 0.81
	8.0	20.98 ± 2.89	174.54 ± 20.20	10.56 ± 0.22
Inoculum level (spores mL^{-1})	10^5	32.44 ± 2.75	211.92 ± 4.28	14.72 ± 0.01
	10^6	31.12 ± 2.95	180.36 ± 8.46	16.00 ± 0.01
	10^7	33.80 ± 2.76	271.67 ± 10.47	15.68 ± 0.01
	10^8	27.63 ± 3.88	226.91 ± 11.14	14.72 ± 0.45

[a] BP70 (bacterial peptone $70\,g\,L^{-1}$); SP35Y35 (soy peptone $35\,g\,L^{-1}$ + yeast extract 35 g L^{-1}); SP35 (soy peptone $35\,g\,L^{-1}$); SP50 (soy peptone $50\,g\,L^{-1}$); SP70 (soy peptone $70\,g\,L^{-1}$).

interpreted using Fisher's test. The accuracy and general ability of the polynomial model were evaluated by the coefficients of determination R^2. A 95% significance level ($p < 0.05$) was applied for all analyses.

2.6. Hydrolysis procedure assisted by ultrasound irradiation

The effect of ultrasound irradiation was assessed in Erlenmeyers flasks (250 mL) containing 50 g of the substrate (20% of soybean oil in 0.1M sodium phosphate buffer pH 8.0) with and without gum Arabic as emulsifier agent (2.5% wt) and 5.0 g of dry whole-cells. The reactions were carried out in an ultrasonic bath (Soniclean) at 38 °C (temperature accuracy of ±0.5 °C) with an operating frequency of 40 kHz and an electrical power rated output of 132 W. The mechanical agitation was provided using an overhead stirrer (Fisatom) at constant stirring rate of 1000 rpm for a maximum period of 9 h. The reactions were monitored by analyzing the concentration of free fatty acid by titration with KOH solution and the hydrolysis degree was calculated as in equation (3).

2.7. Downstream process and determination of fatty acids

The hydrolysis reaction was stopped by adding a mixture of chloroform: glacial acetic acid (1:0.3) to the reactor for 10 min under stirring. Then, the mixture was washed with distilled water and transferred to a separation funnel to stand for phase separation (AOAC, 2003). The chloroform phase containing free fatty acids was esterified with methanol according to ISO 12966-2 (ISO, 2011). The composition of fatty acids was determined as fatty acid methyl esters (FAME) by gas chromatography (PerkinElmer) using a split injector, flame ionization detector (FID) and Supelcowax column (L × I.D. 30 m × 0.32 mm, df 0.50 μm). The injector and detector temperatures were 250 °C, and the split injection mode was 1:10, using nitrogen at the flow rate of 5 mL min^{-1} as the carrier gas. The oven ramp temperature rate was set as follows: initial temperature from 60 °C to 210 °C at a rate of 20 °C min^{-1}; the temperature of 210 °C was maintained for 7 min, and a heating rate of 20 °C $min^{-1}$was used to reach the final temperature of 250 °C, which was maintained for 25 min. The identification of fatty acids was performed by comparing the retention times with FAME standards (MIX Supelco®, with 37 fatty acids methyl ester, Sigma-Aldrich®) and quantification was performed by normalizing areas calculated by Galaxie Chomatography Data System version 1.9 software.

3. Results and discussion

3.1. Effects of growth conditions on the mycelium-bound lipase production

The mycelium-bound lipase production by *P. citrinum* URM 4216 was investigated through a step-by-step strategy to attain whole-cells with high lipase activity. Physicochemical parameters, such as carbon and nitrogen sources, initial pH and inoculum size were studied one at a time to determine the most suitable conditions for attaining high lipase activity in the cells biomass and low activity in the culture broth.

Firstly, the influence of different oils ($30\,g\,L^{-1}$) as carbon sources was investigated by fixing the nitrogen source using soy peptone at $70\,g\,L^{-1}$. Results, summarized in Table 2, demonstrate the profiles of dry biomass, culture broth and whole-cells lipase activity according to each experiment. After 96 h of incubation, olive and sunflower oils provided the highest cellular growth (28.08 ± 0.95 and $26.27 \pm 0.69\,g\,L^{-1}$, respectively). Olive oil, in contrast to the other oils, provided the highest mycelium-bound lipase activity of $188.76 \pm 16.98\,U\,g^{-1}$, indicating that there is no direct relation between catalytic activity and growth under the studied conditions. Such fact strengthens the hypothesis that the oil composition has a direct influence on the production of the mycelium-bound lipase. Lipase activity in the culture broth was much lower (<20.0 U mL^{-1}), indicating good retention of lipase produced as mycelium-bound. These results are in line with several reports concerning the optimization of the whole-cells lipase production. This suggests that the amount of oleic acid is the major factor responsible for the production of mycelium-bound enzymes with high lipolytic activity (Zeng et al., 2006). Although the production of lipases from microorganisms is independent of the presence of lipid substrates in the culture medium, several studies indicate that lipid carbon sources are capable of increasing the level of lipolytic activity of the enzymes obtained and are considered essential for high lipase yield (Cortez et al., 2017). Yang et al. (2005) reported that among several fatty acids, oleic acid improved the production of intracellular lipase from *R. arrhizus*. Andrade et al. (2014) evaluated several vegetable oils as carbon sources in the cultivation of *Mucor circinelloides* whole-cells and observed that the highest value of lipase activity was obtained when olive oil (rich in oleic acid) was used at a concentration of $30\,g\,L^{-1}$. The authors also observed that concentrations higher than this value inhibited the cellular growth, reducing the catalytic activity.

Having defined olive oil as the most suitable carbon source among the sources studied, organic and inorganic compounds at different concentrations and combinations were evaluated as a nitrogen source. Inorganic nitrogen salts, such as ammonium sulfate ($(NH_4)_2SO_4$ $10\,g\,L^{-1}$) and ammonium chloride (NH_4Cl $10\,g\,L^{-1}$), did not support cell growth (data not shown). However, all organic nitrogen sources provided similar cell growth ranging from 22.43 ± 2.76 to $28.08 \pm 2.45\,g\,L^{-1}$ (Table 2) and the maximum lipase activity was attained with soy peptone at $70\,g\,L^{-1}$. As noted for carbon source, the lipase production was not influenced by the cellular growth. In addition, a decrease in the lipase activity was observed when the concentration of soy peptone was reduced. This suggests that both the amount and nitrogen source had influence on the lipase activity. Lipase activity in the culture broth ranged from 6.20 to 17.60 U mL^{-1}, indicating a good retention of lipase produced in the mycelium-bound. Andrade et al. [15] investigated the growth conditions for *M. circinelloides* immobilized whole-cells and reported similar results. Yang et al. (2005) reported that soy peptone improved the lipase activity of *R. arrhizus* immobilized whole-cells, due to the macromolecular protein and the composition of soy peptone. Based on these results, soy peptone at a concentration of $70\,g\,L^{-1}$ was selected as the nitrogen source for further studies.

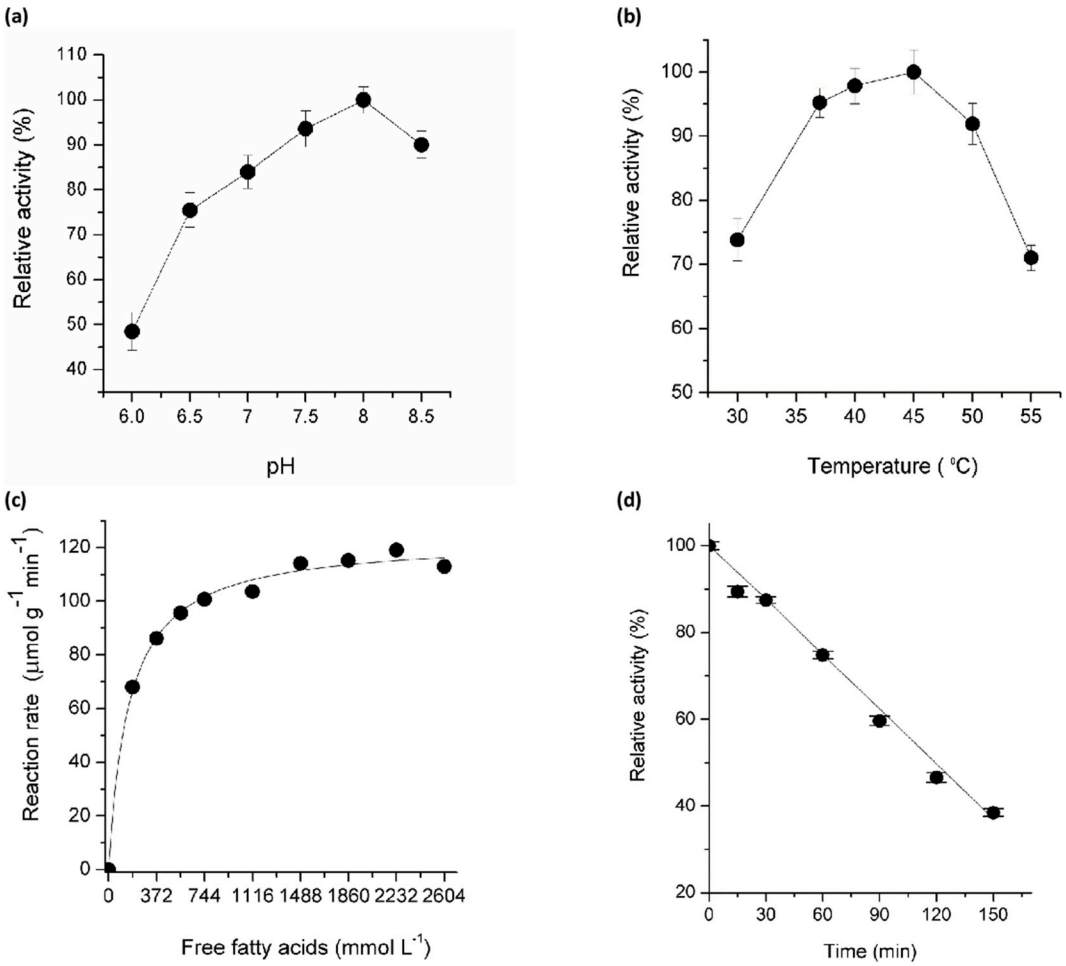

Fig. 1. Assessment the catalytic properties of mycelium-bound lipase from *P. citrinum*. (a). Determination of optimum pH (maximum activity 237.44 ± 2.97 U.g^{-1} was defined as 100% relative activity); (b). Determination of optimum temperature (maximum activity 171.86 ± 3.45 U.g^{-1} was defined as 100% relative activity); (c). Determination of kinetic parameters (Assays were performed at pH 8.0 and 37 °C); (d). Determination of thermal stability at 60 °C (Starting activity (170.04 Ug^{-1}) was taken as 100%).

Other important parameters such as pH and inoculum size that also affect the growth and lipase production of whole-cells were evaluated. It can be noted in Table 2 that higher catalytic activity (236.96 ± 14.09 U. g^{-1}) and growth (33.45 ± 0.91 g.L^{-1}) were obtained at pH 7.0. Such observation is similar to the medium without pH adjustment, i.e., at 6.8. Medium with extreme acid or alkaline pH values repressed both growth and lipolytic activity. This shows that the strain tested herein, under suitable nutritional conditions, has a balanced growth and mycelium bound lipase production at pH close to neutral. This behavior could be different for other genus and species of filamentous fungi. Essamri et al. (1998) found that pH 8.5 was the best for lipase production by *Rhizopus oryzae*, while Wang et al. (2008) described better conditions at pH 6.0 for whole-cells lipase from *Rhizopus chinensi.*

In relation to the inoculum concentration (Table 2), the highest value of enzymatic activity (271.67 ± 10.47 U.g^{-1}) occurred in medium incubated with 1×10^7 spores; although no expressive difference was found in the cellular growth. Andrade et al. (2014) reported that the concentration of inoculum can be critical to whole-cells lipase production. These authors also verified that there was no modification on growth by *Mucor circinelloides* by varying the inoculum proportion, while higher catalytic activity was obtained at low concentration of spores. Teng and Xu (2008) studied the relation between inoculum and morphology of filamentous fungi, and determined that concentrations higher than 1×10^7 spores tended to form dispersed cells, reducing the mycelium bound lipase production. In both cases, lipase activity in the culture broth was low (10–16 U mL^{-1}) indicating that the pH and inoculum did not interfere on the retention of lipase activity by the mycelium.

In summary, according to this set of experiments olive oil and soybean peptone were found to be the best carbon and nitrogen sources, respectively, to enhance the mycelium-bound lipase activity. High inoculum level and medium pH also provided suitable conditions to attain high lipase activity (271.67 ± 10.47 U g^{-1}).

3.2. Catalytic properties of the mycelium-bound lipase

The effect of pH on the whole-cells lipase activity displayed in Fig. 1a indicates that the activity increased to its maximum under a pH equals to 8.0 (237.44 ± 2.97 U.g^{-1}). Alkaline pH over 8 induced the activity to drop slightly to approximately 90% of its maximum activity. A previous study carried out by Lima et al. (2017) on the *P. citrinum* whole-cells immobilized in polyurethane foams revealed also maximum catalytic activity at pH 8.0, indicating that the immobilization did not affect the pH optimum of *P. citrinum* whole-cells. These results were slightly different from those obtained for other *Penicillium* whole-cells strains, in which the optimum pH was found to be 7.0 for mycelium-bound lipases from *P. italicum*, *P. janthinellum* and *P. purpurogenum* (Marotti et al., 2017). At pH 8.0, these authors noted a decrease in the catalytic activity by 10, 20 and 40%, respectively. Industrial demand for active lipases at alkaline pH is essential for running bioprocesses, for their use to

Table 3
Matrix of the factorial design used to evaluate the effect of mass ratio oil/buffer and temperature on the hydrolysis of soybean oil catalyzed by *P. citrinum* whole-cells. Reaction conditions: pH 8.0, 170 rpm, 3 h, 10% of biocatalyst.

Assay	Coded variable (real)		Hydrolysis (%)
	Oil/buffer mass ratio	Temperature (°C)	
1	−1 (15)	−1 (29)	1.90
2	1 (25)	−1 (29)	6.16
3	−1 (15)	1 (45)	2.55
4	1 (25)	1 (45)	4.83
5	−1.414 (12.9)	0 (37)	7.72
6	1.414 (27.1)	0 (37)	6.86
7	0 (20)	−1.414 (26)	10.36
8	0 (20)	1.414 (48)	18.37
9	0 (20)	0 (37)	30.48
10	0 (20)	0 (37)	32.20
11	0 (20)	0 (37)	36.90

concentrate target polyunsaturated fatty acids, and other biotechnological applications (Rehman et al., 2011).

The effect of temperature on the whole-cell lipase activity was evaluated in the range from 30 to 55 °C at pH 8.0 (Fig. 1b). Maximum lipase activity was attained at 45 °C (171.86 ± 3.45 U.g^{-1}), suggesting that the mycelium-bound lipase from *P. citrinum* exhibits high activity at higher temperatures, and even at 55 °C, this lipase retains about 70% of its maximum catalytic activity. Marotti et al. (2017) also found similar results with *P. italicum* whole-cells. On the other hand, in our previous study with *P. citrinum* immobilized whole-cells (Lima et al., 2017), it was observed that the immobilization process had a negative effect on the lipase activity. This is because with an increase in incubation temperature from 40 to 45 °C, the catalytic activity dropped by 25% from its maximum value.

The kinetic constant (K_m) and maximum reaction rate (V_{max}) were assessed using olive oil emulsion as substrate. The increase in the fatty acid concentration in the substrate from 186 to 1116 mmol L^{-1} resulted in significant increase in the reaction rates (Fig. 1c) and for substrate concentrations greater than 1488 mmol L^{-1}, the rate of formation of the products became essentially independent of substrate concentration. The non-linear regression showed that the experimental data fitted well the model of Michaelis-Menten ($R^2 = 0.9580$), revealing the following K_m and V_{max} values, respectively, as 136.51 mmol L^{-1} and 267.33 μmol g^{-1}min^{-1} with catalytic efficiency of 1.91. The catalytic efficiency (V_{max}/K_m) indicated the overall cumulative effect of V_{max} and K_m on the enzyme activity (Badgujar and Bhanage, 2016). In a previous study with *P. citrinum* immobilized whole-cells, Lima et al. (2017) obtained higher K_m value (158.10 mm L^{-1}) and lower catalytic efficiency of 0.78. This indicates a decrease in the substrate affinity by *P. citrinum* whole-cells caused by immobilization procedure using polyurethane foams as matrix.

Fig. 1d shows that with heat treatment the residual activity decreased over time and after 150 min of incubation, the whole-cell lipase retained 40% of its initial activity. Half-life time ($t_{1/2}$) and coefficient of thermal deactivation constant (K_d) at 60 °C were 1.8 h and $1.06.10^{-4}h^{-1}$, respectively. The literature reports that mesophilic fungi, i.e., fungi that grow and synthesize lipolytic enzymes at temperature ranges from 20 to 30 °C, tend to be less stable with heat treatment (Fernandez-Lahore et al., 1999). This may explain the low thermal stability of the mycelium-bound lipase of *P. citrinum*. However, *P. citrinum* whole-cells immobilized in polyurethane foams (Lima et al., 2017) revealed higher half-life time (2.2 h), suggesting that the immobilization process slightly increased the thermal stability of whole cells. Marotti et al. (2017) reported lower half-life times, 0.92, 1.17 and 1.21 h for mycelium bound lipases from *P. italicum, P. janthinellum* and *P. purpurogenum*, respectively.

Table 4
Effects, standard errors and *p*-values for the hydrolysis of soybean oil according to the factorial design.

Variables	Effects	Standard error	*p*-value
Mean	33.19	1.92	0.0033*
x_1	1.33	2.35	0.6281
x_1^2	−29.39	2.80	0.0089*
x_2	2.66	2.35	0.3748
x_2^2	−22.32	2.80	0.0153*
x_1x_2	−0.99	3.32	0.2979

*significant variables ($p < 0.05$).

3.3. Hydrolysis of soybean oil: effect of mass ratio oil: buffer and temperature on the hydrolysis

The optimal parameters of mass ratio (oil: buffer) (X_1) and temperature (X_2) for the hydrolysis of soybean oil were assessed using an experimental design (CCRD), maintaining the amount of the biocatalyst added at a fixed level (10 wt% of biocatalyst with average lipase activity of 156.23 ± 1.09 U.g^{-1}). As shown in Table 3, the hydrolysis percentage varied from 1.9% (run 1) to 36.9% (run 11), corresponding to the lowest (15%) and central level (20%) of mass ratio of oil to buffer, respectively.

These results were submitted to the statistical analysis, displayed in Table 4, which indicated that only the quadratic effects for mass ratio oil: buffer (X_1^2) and temperature (X_2^2) were significant at 95% of confidence level. Both effects were negative, suggesting that low amount of oil and low temperature improved the lipase performance and thus enhanced the percentage of hydrolysis. At the same confidence level, the linear terms and interactions among the studied variables were not statistically significant ($p > 0.05$).

Based on these, the experimental data were fitted to a second-order polynomial model with coded values, as shown in Eq. (5), using only the significant quadratic effects.

$$Y(\%) = 33.19 - 14.69x_1^2 - 11.15x_2^2 \quad (5)$$

where $Y(\%)$: hydrolysis percentage (%), x_1^2 mass ratio and x_2^2 temperature.

The *F*-test described in Table 5 indicated that the regression of the model was statistically significant at the 95% confidence level, since calculated *F*-value (24.65) was higher than the table *F*-value (4.35). Also, the regression coefficient ($R^2 = 0.9024$) showed that the obtained model explains 90.24% of the experimental variability.

Fig. 2 shows the response surface plotted according to the model described by equation (5). It is possible to observe that an increase of both temperature and mass ratio oil: buffer up to values around 38 °C and 20%, respectively, enhanced the hydrolysis percentage. For values higher than these, reduction of the hydrolysis percentage was noticed due to the deficient homogenization of the medium caused by the high oil concentration associated with the weekly agitation in shaker. Besides, thermal stability of the lipase limits its uses at higher temperatures. Based on this, and according to the experimental design, the highest hydrolysis degree could be attained at 38 °C and the oil: buffer mass ratio of 20%. The run conducted to study particular conditions arising from the results of the experimental design attained a hydrolysis percentage of 38.6%, showing a deviation from the predicted value in the order of 2%, which can be considered satisfactory.

3.4. Effect of ultrasound irradiation and emulsifier in the hydrolysis

The quality and stability of emulsion is crucial in the enzymatic hydrolysis reaction and is closely linked to the use of emulsifying agents and good stirring systems. In this work, stirring associated with emulsifying substrate promoted a good homogenization between biocatalyst and substrate for a maximum periodic of 3 h. For longer than this, the

Table 5
Analysis of the variance (ANOVA) for the model that represents the hydrolysis of soybean oil by *P. citrinum* whole-cells according to the face-centered composite design.

Variable	Sum of squares	Degree of freedom	Mean square	*F*-test	*p*-value	*F*-calc
x_1^2	1219.28	1	1219.28	110.40	0.01*	24.65
x_2^2	702.83	1	702.83	63.64	0.02*	
Lack of fit	141.11	6	23.51	2.13	0.35	
Pure error	22.09	2	11.04			
Total	1671.37	10				
$R^2 = 90.2\%$				$F_{3;7;0.05} = 4.35$		

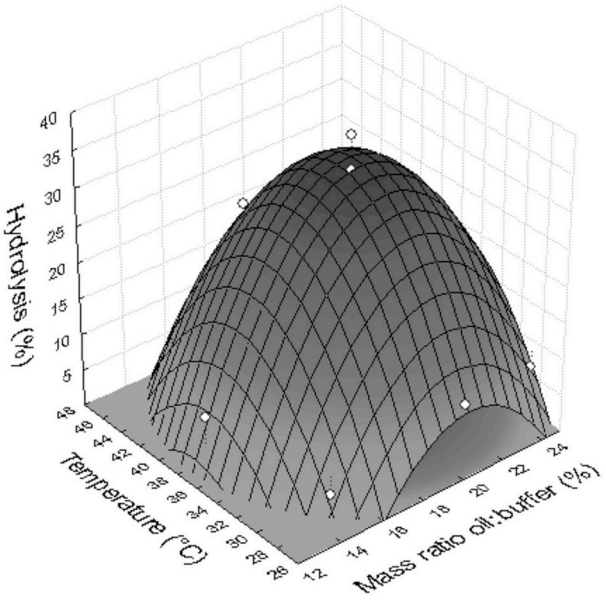

Fig. 2. Response surface of hydrolysis predicted from the quadratic model. Effect of the mass ratio oil:buffer and temperature.

emulsion destabilizes, compromising the dispersion of the biocatalyst in the reaction medium, and consequently decreasing the hydrolysis percentage. To stabilize the emulsion and enhance the heat and mass transfer in the hydrolysis reaction, the influence of ultrasound irradiation was investigated under optimal conditions previously determined (38 °C, oil: buffer mass ratio 20% and 10% wt. of biocatalyst having activity of 144.73 ± 1.99 U.g^{-1}) determined by the experimental design. In addition, reactions were performed under mechanical stirring (1000 rpm) in the presence and absence of emulsifier agent. As a control, the hydrolysis reaction was performed under shaker at the same conditions and the profile of hydrolysis percentage for both is shown in Fig. 3 (a,b).

Reactions performed under shaking exhibited similar hydrolysis profile up to 3 h incubation (Fig. 3a), then for emulsifying substrate the hydrolysis percentage maintained constant value of about 33%, while the substrate without emulsifier a decrease on the hydrolysis degree was observed, attaining values as low as 24.28%. This behavior is usually related with coalescence effect, which decreases the interface area and consequently, decreases the reaction rates. For reactions carried out using an ultrasonic system (Fig. 3b) and mechanical agitation (1000 rpm), a different behavior were verified. Both substrates (in the presence or absence of emulsifier) presented similar and low initial rates as the reaction proceed with time. However, hydrolysis percentage were progressively increased, attaining maximum values of $77.0 \pm 2.21\%$ at 9 h incubation for emulsifying substrate. Without emulsifier, the ultrasonic waves allowed to maintain suitable water/oil interface area and enhanced the formation of fatty acids attaining a maximum hydrolysis percentage of $96.06 \pm 3.07\%$. In this sense, the ultrasonic irradiations associated with mechanical stirring enhance the stability of the emulsion water/oil even without emulsifier agents. The ultrasonic irradiations promoted an effective agitation and a dispersion of phases in heterogeneous systems (Kwiatkowska et al., 2011; Chandrapala et al., 2012). According to Awadallak (2013), initial reaction rates of hydrolysis may be low due to the poor miscibility between oil and water, but as the reaction takes place, intermediate products (mono- and di-acilglycerols) are formed and act as emulsifiers in the medium. Thus, these intermediate products promote the formation of water-in-oil micelles, which can enhance the interfacial area, improving the contact

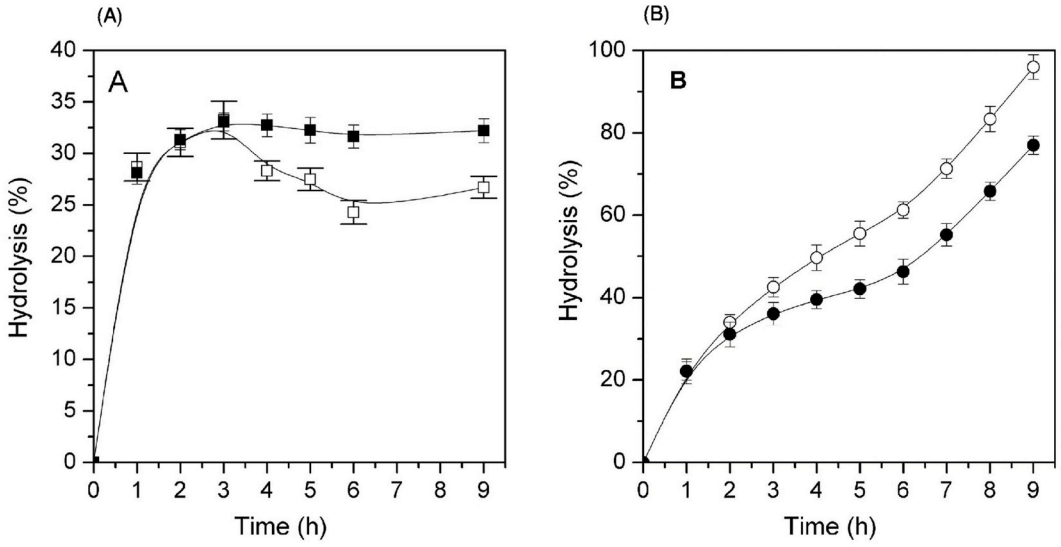

Fig. 3. Hydrolysis of soybean oil catalyzed by *P. citrinum* whole-cells. A (■) shaking bath with emulsifier and (□) shaking bath without emulsifier; B (●) ultrasound irradiation with emulsifier; (○) ultrasound irradiation without emulsifier. Reaction conditions: 38 °C, 1000 rpm, mass ratio 1:20 oil:buffer, 10% wt. whole-cells.

Table 6
Fatty acids composition of the hydrolysate of soybean oil obtained under ultrasonic irradiation in the absence and presence of emulsifier catalyzed by *P. citrinum* whole-cells. Reaction conditions: 38 °C, pH 8.0, 1000 rpm, 10% of biocatalyst.

Emulsifier	Hydrolysis (%)	Free fatty acids (%)				
		Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1)	Linoleic (C18:2)	Linolenic (C18:3)
Absence	96.06 ± 3.07	10.93	4.35	35.93	42.38	4.72
Presence	77.00 ± 2.21	9.25	3.53	24.34	31.94	3.45

Table 7
Hydrolysis of different vegetable oils under ultrasonic irradiations without emulsifier catalyzed by *P. citrinum* whole-cells. Reaction conditions: 38 °C, pH 8.0, 1000 rpm, 10% of biocatalyst.

Oil	Major fatty acid	Hydrolysis (%)	
		6 h	9 h
Soybean (control)	C18:2	73.90 ± 1.23	96.00 ± 3.07
Olive	C18:1	62.60 ± 4.70	84.33 ± 2.78
Canola	C18:1	68.53 ± 3.5	89.87 ± 2.23
Sunflower	C18:2	74.26 ± 3.76	93.51 ± 2.51

between reactants and catalyst; hence favoring the reaction. These data support Marotti et al. (2017) results that claimed an increase of at least three folds in the hydrolysis mediated by whole-cells from three species of *Penicillium* sp. assisted by ultrasonic irradiations. Ultrasound-assisted reactions show a very promising alternative for the conventional stirring methods and are technically feasible in terms of process intensification and its environment-friendly operating conditions.

Free fatty acids released in the hydrolysis carried out under ultrasonic radiations were quantified by gas chromatograph and results are shown in Table 6. For both reactions, in the presence or absence of emulsifier agent, the whole-cells lipase hydrolyzed all fatty acids from soybean oil, enriching the medium predominantly by unsaturated fatty acids, oleic (C18:1) and linoleic (C18:2) acids in accordance with soybean oil composition. For the reaction with emulsifier agent, the hydrolyzed medium was composed of approximately 42.38% linoleic and 35.93 oleic, while for the reaction not emulsified whole-cell lipase enriched the medium with 31.94% of linoleic and 24.34% of oleic acids.

3.5. Hydrolysis of vegetable oils having different compositions in fatty acids

All the experiments performed, so far, make use of soybean oil, which has rich compositions of both oleic and linoleic acids, as shown in Table 1. To confirm the selectivity of the whole-cells lipase from *P. citrinum* for these fatty acids, different vegetable oils rich in polyunsaturated fatty acids were used in the hydrolysis reaction under the optimized conditions previously established. As displayed in Table 7, the whole-cells lipase hydrolyzed the mainly fatty acids present in different vegetable oils, attaining hydrolysis percentage of 84.33, 89.87 and 93.51% for olive, canola and sunflower oils, respectively after 9 h reaction. These results are similar to those found for soybean oil (96%), confirming the specificity of whole-cell lipase from *P. citrinum* for polyunsaturated long chain fatty acids, such as oleic and linoleic acids. Different results were obtained by Marotti et al. (2017), who reported higher values of hydrolysis degree with coconut oil mediated by whole-cells from *P. purpurogenum*. Coconut oil hydrolysate was enriched with lauric acid, a saturated fatty acid; this indicates that whole-cells lipase from different species of *Penicillium* sp. can be specified for different fatty acids.

4. Conclusion

P. citrinum whole-cells were successfully applied as biocatalyst for the hydrolysis of different vegetable oils. Under maximized growth conditions, mycelium-bound lipase showed high values of hydrolytic activity and had suitable properties to be used in typical lipase reactions. The parameters, temperature and mass ratio of oil/buffer were optimized in the hydrolysis of soybean oil using a factorial design; there was maximum hydrolysis of 38% at 38 °C using 20% of oil in the presence of emulsifier agent. The percentage of hydrolysis was improved by replacing the emulsifier agent with ultrasonic irradiation, suggesting that ultrasound wave enhances the stabilization of the emulsion and improves the lipase activity. These results indicated that mycelium bound lipase from *P. citrinum* is appealing as the costs involved in the whole production process are also potentially diminished.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bcab.2019.101410.

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