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Production and Characterization of Whole-Cell *Rhizopus oryzae* CCT3759 to be Applied as Biocatalyst in Vegetable Oils Hydrolysis

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Abstract

The present study aims to produce a mycelium-bound lipase of the fungus *Rhizopus oryzae* CCT3759 by submerged fermentation in order to be applied as biocatalyst in the hydrolysis of different vegetable oils. Optimal cultivation conditions have been achieved in a medium containing olive oil as inducer for 72 h of fermentation, thus obtaining 30.5 g/L of dry biomass concentration and hydrolytic activity of 389.1 U/g, which corresponds to a total lipase activity around of 12,000 U/L. Maximum hydrolytic activity was observed at pH 6.0 and 40 °C. Kinetic parameters concerning apparent Michaelis–Menten constant ($K_m = 50.5$ mM) and maximum reaction rate ($V_{max} = 815.4$ μmol/g min) have been determined in olive oil emulsion hydrolysis. Thermal stability tests revealed that the enzyme retained 75% of its initial activity after 4 h at 50 °C, whose thermal inactivation constant (K_d) and half-life ($t_{1/2}$) was 0.073 h^{-1} and 9.4 h, respectively. The effect of biocatalyst concentration, expressed as activity units—U (200 and 400 U), on the hydrolysis of vegetable oils was investigated under fixed conditions: oil/buffer mass ratio of 25% (m/m), 100 mM buffer sodium phosphate pH 6.0, 40 °C and the mechanical stirring frequency of 600 rpm. As expected, increasing the initial activity from 200 to 400 U leads to higher values of initial reaction rates and hydrolysis percentage. However, initial reaction rate values were similar for six different vegetable oils due to the high accessibility of the lipase to the substrate under such experimental conditions. A complete hydrolysis of olive, cottonseed, sunflower and canola oils has been achieved after 26–30 h of reaction using 400 U of activity. These results suggest a promising application of the produced biocatalyst in the production of free fatty acids, an important class of compounds for oleochemical industries.

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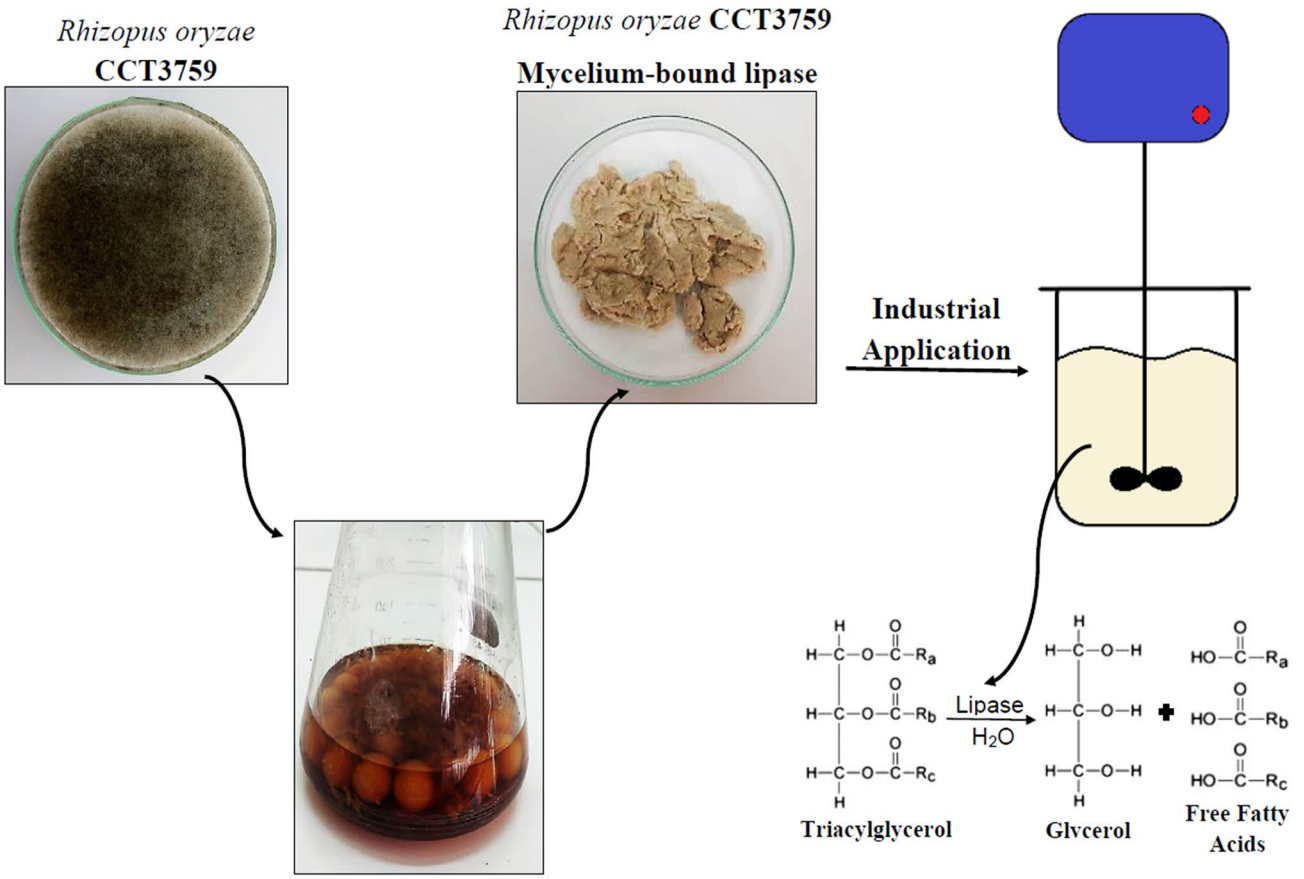
Graphic Abstract



Keywords Mycelium-bound lipase · *Rhizopus oryzae* · Characterization · Oil hydrolysis

1 Introduction

The worldwide consumption of vegetable oils has been increasing yearly and their production has reached 200 million metric tons in 2020/2021 globally, and Brazil is an important oilseed producer in the world. These data refer to the production of main vegetable oils, such as coconut oil, cottonseed, palm, palm kernel, peanut, rapeseed, soybean and sunflower [1].

Despite the fact that the food industry is the main sector of consumption of vegetable oils, they have also been used as feedstocks for obtaining FFA and glycerol, which are important precursors for the pharmaceutical, cosmetic and oleochemical industries to produce biodiesel [2–5], biosurfactants [6–8], flavors esters [9, 10], biolubricants [11–15], and structured lipids [16–18].

A well-known commercial process for obtaining FFA is the Colgate-Emery Process, which is carried out at high temperatures (250 °C) and pressures (50 bar). In these abrupt operating conditions, undesirable reactions of oxidation, dehydration and interesterification of oils and fats require steps of separation and purification of the final products [19–21].

In order to overcome problems involved in the thermochemical process, an enzymatic hydrolysis of vegetable oils has been yielding satisfactory results. The use of lipases as biocatalysts has several advantages over thermochemical methods, as they can act under moderate conditions of temperatures and pressure, thus reducing energy costs and facilitating recovery and purification of the final product [19, 22, 23]. These enzymes have been widely used for producing FFA from refined vegetable oils/animal fats or waste oils with high FFA content in their compositions as feedstocks [3, 19, 22, 23].

Lipases (triacylglycerol acylhydrolases EC 3.1.1.3) are enzymes that act in the hydrolysis of ester-carboxylic bonds present in oils and fats, which result in releasing FFA and glycerol. However, they can act in esterification, interesterification and transesterification reactions in non-aqueous media. In addition, they have important features

such as chemo-, regio-, enantioselectivity and high versatility towards a variety of substrates—natural and non-natural esters [24–27]. Such very attractive properties indicate that lipases can be applied in different industrial segments, such as detergents, textiles, cosmetics, pharmaceuticals [28, 29].

Lipases can be obtained from animal, vegetable or microbial origins, however those obtained from microbial origins are the most commonly used by industries due to their favorable characteristics to the industrial sector [30, 31]. Among their attractive characteristics to the industrial sector, the following can be highlighted: high activity and stability over a wide range of temperature and pH values, and in organic solvents. In addition, they have higher production yield, possibility of genetic manipulation and rapid cell growth in low-cost media [32, 33].

Lipases produced by microorganisms can be either extracellular or intracellular [30, 33, 34]. Among intracellular lipases, there are those bound to the mycelium, which are defined as lipases that are associated with fungal biomass, thus being naturally immobilized. Although being mycelium-bound, lipases are still active, therefore they can be used as biocatalysts so as to partially eliminate costly steps, such as purification, recovery and immobilization by different protocols [35–37].

The fungus *Rhizopus oryzae* has already been reported as good lipase producer. It is a filamentous fungus of the genus *Rhizopus* which is capable of producing different metabolites such as enzymes (cellulases, lipases, proteases, tannases), organic acids (lactic and fumaric acid), aromatic compounds and dyes, in addition to having the advantage of being categorized as a GRAS fungus (Generally Recognized as Safe), thus it is safe for applications in the food industry [38, 39].

Rhizopus oryzae strains contain two types of lipase, a 34 kDa lipase bound to the cell wall and a 31 kDa lipase bound to the membrane and cell wall [40]. Most of its lipases require a pH value ranging between 6.0 and 8.5 and their ideal temperature ranges between 30 and 45 °C to express the high hydrolytic activity. These enzymes are most active for esters containing fatty acids with 8 to 18 carbon atoms [41].

There are still few studies in literature on the application of mycelium-bound lipase of the fungus *Rhizopus oryzae* in the hydrolysis of vegetable oils for producing FFA of great relevance to the oleochemical industry, which makes it a promising niche for the development of new research [42]. Therefore, the present work aims to investigate the potential of a strain of *Rhizopus oryzae* CCT3759 as a producer of mycelium-bound lipase, its biochemical and kinetic characterization and application in the hydrolysis of different commercial oils in order to obtain FFA.

2 Materials and Methods

2.1 Microorganism

The used strain was that of the fungus *Rhizopus oryzae* CCT3759 obtained from the André Tosello Tropical Research and Technology Foundation (Campinas, SP, Brazil). In order to obtain and maintain culture spores, fungal cells had been previously inoculated on Sabouraud agar medium under aseptic conditions. The culture was incubated at 30 °C and 72 h, or until they reached the highest sporulation status. Cells were washed with 10 mL sterile distilled water to obtain spore suspension under aseptic conditions.

2.2 Materials

Olive oil (Carbonell™); cottonseed oil and canola oil (Vitalliv™); corn oil (Sinhá™); sunflower oil and soybean oil (Liza™) were purchased at a local market (Alfenas, MG, Brazil). Sabouraud agar medium and soybean peptone were acquired from HiMedia Laboratories (Mumbai, MH, India). Gum Arabic, monobasic potassium phosphate, monobasic sodium phosphate, bibasic sodium phosphate were acquired from Dinâmica Química (Indaiatuba, SP, Brazil); and magnesium sulfate heptahydrate, sodium hydroxide, sodium nitrate, ethanol solution (70% v/v) from Vetec Química (São Paulo, SP, Brazil). All other reagents and organic solvents of analytical grade were purchased from Vetec Química.

2.3 Culture Medium and Experimental Conditions

The culture medium consisted of 30 g/L of vegetable oil (cottonseed, olive oil, canola, sunflower, corn or soybean oils), 70 g/L of soybean peptone, 1 g/L of $NaNO_3$, 1 g/L of KH_2PO_4 and 0.5 g/L of $MgSO_4.7H_2O$, and all of which have been previously autoclaved (121 °C and 15 min). Cultivations were performed in 250 mL-Erlenmeyer flasks containing 100 mL of autoclaved medium and inoculated with a suspension of 1×10^6 spores at 30 °C and orbital shaking at 180 rpm. Spore concentration was determined by counting cells in a Neubauer chamber using an Olympus® binocular microscope (Olympus Corporation, Tokyo, Japan). At the end of the culture process, the produced biomass was separated from the medium by vacuum filtration, washed with water and acetone and quantified for hydrolytic activity and humidity by drying the wet biomass (0.25 g) in a microwave oven (180 W per 5 min) [43]. Subsequently, the fungal biomass were stored at 4 °C prior to use.

2.4 Determination of Submerged Culture Conditions Of *Rhizopus oryzae* CCT3759 for Mycelium-Bound Lipase Production

In each culture cycle, the mycelium-bound lipase production was evaluated in terms of dry biomass concentration (g/L) and hydrolytic activity (U/g) by the method of olive oil emulsion hydrolysis described by Marotti et al. [37], and total lipase activity (U/L) that was defined as the units of hydrolytic activity produced per liter of cultivation [44]. To determine better experimental conditions for producing whole-cells with high catalytic activity, six vegetable oils with different fatty acid compositions such as olive, cottonseed, canola, sunflower, corn or soybean oils (Table 1) have been evaluated. In this study, the influence of the submerged cultivation time was also evaluated for each studied carbon source at every 24, 48, 72 and 96 h of submerged cultivation. Enzymatic activity (U) is defined as the amount of dry biomass or culture broth required for the release of 1 µmol of FFA per minute under experimental conditions (0.1 g of biomass or 1 mL of culture broth at 37 °C and 100 mM buffer sodium phosphate pH 7.0).

2.5 Characterization of Biochemical and Kinetic Properties of Mycelium-Bound Lipase

Biochemical and kinetic properties of lipase bound to the mycelium were characterized by olive oil emulsion hydrolysis. The effect of temperature was evaluated in the range of 25–60 °C using a 100 mM buffer sodium phosphate at pH 6.5, while the pH effect was investigated in the range of 4–5.5 (100 mM buffer sodium citrate) and from 6.0 to 8.0 (100 mM buffer sodium phosphate) at 40 °C. The influence of substrate concentration (olive oil) was investigated in the range of 5 and 40% m/m (corresponding to 186 to 1488 mM of FFA) under optimal conditions (100 mM buffer sodium phosphate pH 6.0 and 40 °C). Apparent Michaelis–Menten kinetic constants (K_m) and maximum reaction rate (V_{max}) were determined according to a non-linear model using the software Origin Pro version 5.0. Thermal stability tests were performed by incubating the biomass in a 100 mM buffer sodium phosphate pH 6.0 and a thermostatic bath at 50 °C by 4 h. Samples were removed periodically to determine residual hydrolytic activity. The thermal denaturation constant (K_d) and half-life time ($t_{1/2}$) were respectively determined as follows (Eqs. 1 and 2):

$$\ln A = \ln A_o - K_d.t \tag{1}$$

$$t_2^1 = \frac{0,693}{K_d} \tag{2}$$

where ln A is the residual activity after the heat treatment during a incubation period and ln A_0 is the initial enzyme activity.

2.6 Hydrolysis Reactions of Vegetable Oils in Stirred-Tank Reactors

In 250 mL glass jacketed reactors, 100 mL of the substrate composed by the emulsion of 25 g of vegetable oil in 100 mM buffer sodium phosphate pH 6.0, using Gum Arabic as an emulsifier (3% m/v) were prepared. The tests were carried out at 40 °C and the ratio of enzyme units was set at 200 and 400 U (which corresponds to an average mass of approximately 0.9 and 1.8 g of dry biomass, respectively) and 600 rpm of mechanical stirring, which was performed by using an overhead motor stirrer with a steel helical impeller. A 50:50 (v/v) mixture of acetone and ethanol was added to the aliquots (0.5 g) that have been removed periodically, and FFA concentration was quantified by titration with a 20 mM sodium hydroxide solution (NaOH) using phenolphthalein as indicator. Hydrolysis percentage (%) was calculated by Eq. (3) [46].

Table 1 Fatty acids composition and average molecular mass of FFA and vegetable oils used in this study [45]

Fatty acid	Composition (% m/m)					
	Cottonseed	Olive	Canola	Sunflower	Corn	Soybean
C16:0—Palmitic	25.2	11.3	4.5	6.3	11.9	11.5
C18:0—Stearic	1.8	2.8	2.0	3.9	2.1	4.1
C18:1—Oleic	16.5	74.5	60.4	20.9	27.2	23.5
C18:2—Linoleic	54.8	9.8	21.2	67.6	57.7	53.3
C18:3—Linolenic	0.2	0.5	9.4	0.2	0.6	6.8
Average molecular mass of FFA (g/mol)	274.7	279.3	280.5	279.5	278.2	276.0
Average molecular mass of vegetable oils (g/mol)	865.1	878.9	882.5	879.5	875.6	869.1

$$\text{Hydrolysis}(\%) = \frac{(V_a - V_b).C_{NaOH}.10^{-3}.M}{m.f} \quad (3)$$

where: V_a is the volume of NaOH (mL) solution required in the sample; V_b is the volume of NaOH solution required in the control (mL); C_{NaOH} is the molar concentration of NaOH (20 mM); M is the average molecular mass of FFA in the vegetable oil (Table 1); m is the sample mass (0.5 g); f is the oil fraction (0.25).

Initial reaction rates have been analyzed by the formation of FFA (mM) in the first 12 h of reaction. The results were plotted using the software Origin Pro version 5.0 to obtain a linear equation for initial hydrolysis reaction rates of each vegetable oil. The calculation of FFA concentration is performed as described in Eq. (4).

$$FFA(\text{mmol/L}) = \frac{V_a - V_b.C_{\text{NaOH}}.10^3}{m} \quad (4)$$

where: V_a is the volume of NaOH in the sample (mL); V_b is the volume of NaOH in the control (mL); C_{NaOH} is the molar concentration of NaOH (20 mM); m is the sample mass (0.5 g).

3 Results and Discussion

3.1 Selection of Culture Conditions for the Mycelium-Bound Lipase Produced from *Rhizopus oryzae* CCT3759

The growth and regulation of metabolic activities of microorganisms are directly influenced by physical–chemical conditions of the culture medium and the characteristics of each microorganism, for example, different oils can be applied as inducers in lipase production [47]. The first stage of this study consisted in investigating the best culture conditions for obtaining a mycelium-bound lipase with high catalytic activity. For such purpose, six vegetable oils with different fatty acid compositions (Table 1) have been studied as inducers for producing a mycelium-bound lipase from *Rhizopus oryzae* CCT3759. These vegetable oils were selected due to their different fatty acid compositions that can be easily obtained in our country (Brazil).

Table 2 shows the average values of dry biomass concentration and the hydrolytic activity of the biomass produced. According to the results, all oils showed greater lipase retention onto the mycelium of fungal biomass, since the lipase produced was mostly retained onto the fungus mycelium due to lower values of hydrolytic activity in the fermentation broth (< 30.0 U/mL, see Table 3).

The results obtained in cultures with olive oil and canola oil showed the best results regarding biomass concentration (based on dry biomass), reaching 35.0 ± 2.0 and 17.2 ± 0.6 g/L, respectively. In fact, the results obtained by using olive oil as inducer showed higher values than other vegetable oils in all studied cultivation times (24, 48, 72 and 96 h), with its lowest biomass concentration being 24.5 ± 4.0 g/L, that is higher than maximum values obtained for the other evaluated vegetable oils (see Table 2). Olive and canola oils also reached the highest values of hydrolytic activity, 389.1 ± 16.2 and 364.5 ± 13.2 U/g, respectively,

Table 3 Influence of cultivation time of *Rhizopus oryzae* CCT3759 on hydrolytic activity in the fermentation broth (U/mL)

Vegetable oil	Hydrolytic activity (U/mL)			
	24 h	48 h	72 h	96 h
Cottonseed	25.0 ± 0.5	20.1 ± 1.0	21.0 ± 0.9	19.8 ± 0.7
Olive	23.9 ± 0.4	27.4 ± 0.6	23.3 ± 0.4	25.4 ± 0.3
Canola	24.5 ± 0.8	28.0 ± 3.1	28.7 ± 1.3	24.9 ± 0.3
Sunflower	26.2 ± 1.2	25.7 ± 1.1	26.2 ± 1.8	19.3 ± 3.7
Corn	23.2 ± 1.6	25.8 ± 1.4	23.1 ± 1.7	20.6 ± 1.4
Soybean	22.8 ± 4.1	28.2 ± 1.2	23.3 ± 0.6	19.6 ± 0.9

Table 2 Influence of cultivation time of *Rhizopus oryzae* CCT3759 on biomass concentration (g/L) and hydrolytic activity of the mycelium-bound lipase (U/g of micellium)

Vegetable oil	24 h		48 h		72 h		96 h	
	Hydrolytic activity (U/g)	Biomass concentration (g/L)	Hydrolytic activity (U/g)	Biomass concentration (g/L)	Hydrolytic activity (U/g)	Biomass concentration (g/L)	Hydrolytic activity (U/g)	Biomass concentration (g/L)
Cottonseed	450.0 ± 20.6	3.2 ± 1.0	764.3 ± 36.0	5.6 ± 0.3	715.0 ± 57.8	7.9 ± 1.2	209.8 ± 11.0	13.1 ± 4.5
Olive	291.9 ± 9.5	24.5 ± 4.0	275.2 ± 30.6	30.9 ± 1.9	389.1 ± 16.2	30.5 ± 2.1	322.9 ± 18.0	35.0 ± 2.0
Canola	267.8 ± 4.9	1.6 ± 0.1	336.8 ± 13.4	5.8 ± 1.0	364.5 ± 13.2	17.2 ± 0.6	245.9 ± 16.7	13.2 ± 3.0
Sunflower	252.1 ± 15.1	3.0 ± 0.6	388.2 ± 30.7	6.7 ± 0.6	263.7 ± 12.1	10.1 ± 0.9	181.4 ± 20.1	10.3 ± 0.4
Corn	335.0 ± 13.0	3.1 ± 0.9	285.7 ± 56.0	4.3 ± 0.6	233.2 ± 20.2	5.7 ± 0.6	189.5 ± 20.6	7.8 ± 0.2
Soybean	385.4 ± 35.0	4.5 ± 3.8	251.4 ± 36.1	3.1 ± 0.2	287.8 ± 37.1	6.1 ± 0.1	195.8 ± 7.4	7.0 ± 0.7

but lower than the results provided by cottonseed oil (764.3 ± 36.0 U/g) after 72 h of cultivation.

Both vegetable oils (olive and canola oils) showed results that suggest greater activity retention onto the mycelium after reaching maximum activity after 72 h of cultivation. After this period, an activity reduction of 17.0% was observed for olive oil (from 389.1 to 322.9 U/g) and 32.5% for canola oil (from 364.5 to 245.9 U/g) for 96 h of cultivation. A drastic activity reduction of 72.5% was also obtained for the lipase produced using cottonseed oil (764.3 U/g in 72 h to 209.8 U/g at the end of 96 h of cultivation). These results may be due to the need for a new carbon source after substrate consumption (oils/fats and/or by-products), in which there was a consumption of primary metabolite (enzyme) to maintain cell growth.

In this study, total enzyme activity for the selection of vegetable oil as a carbon source was also evaluated, as shown in Fig. 1. According to these results, a maximum total enzyme activity around of 12,000 U/L was achieved after 72 h of fermentation using olive oil as inducer.

According to Table 2 and Fig. 1, the difference in enzymatic activities and total lipase activity presented by lipase produced over cultivation time can be explained by the different composition of fatty acids present in all oils. Studies suggest that *Rhizopus oryzae* cells using vegetable oils with higher concentration of oleic and linoleic acids in their composition efficiently obtained a fungal biomass with high catalytic activity [48, 49]. The same behavior was observed in this study, once olive oil has the highest percentage of oleic acid (74.5% m/m), which provides whole-cell lipases with greater activities [37, 47]. In this sense, while olive oil and canola oil are rich in oleic acid, over 60%, the other studied oils present percentages below 28% (Table 1), thus explaining the low yields of hydrolytic activity obtained using the other vegetable oils.

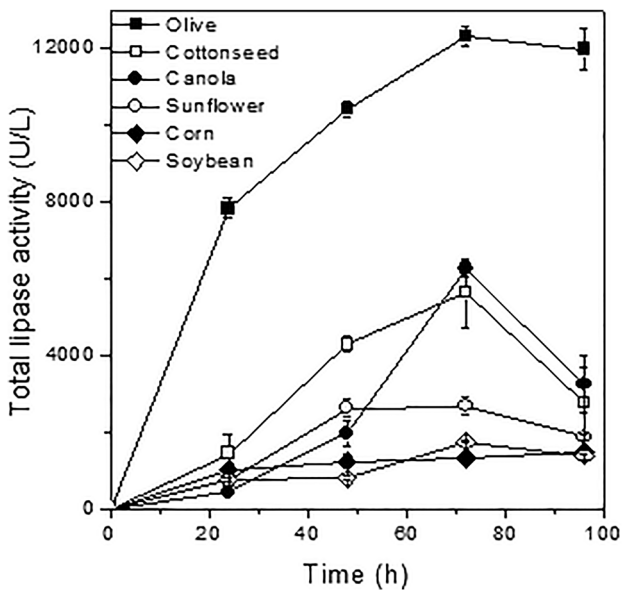


Fig. 1 Effect of vegetable oil and cultivation time of *Rhizopus oryzae* CCT3759 on the total lipase activity (U/L)

Similar results have been achieved in other studies involving the production of whole-cells from *Rhizopus oryzae* with high hydrolytic activity [44, 50–52]. Hama et al. [48] demonstrated that, with the use of oleic acid or olive oil as inducer to produce *Rhizopus oryzae* lipases, it was found a strong inhibition of lipase secretion and a high amount of lipase located in the cell wall and membrane. Andrade et al. [53] and Lima et al. [54] evaluated the effect of different vegetable oils on cell growth and the catalytic activity of *Mucor circinelloides*, moreover, it was observed that the highest values of hydrolytic activity and cell growth have been achieved in both studies by using olive oil as carbon source due to a high concentration of oleic acid in its composition. Marotti et al. [37], under the same cultivation conditions selected in this study, carried out a selection of species of fungi belonging to the genus *Penicillium* that produce a mycelium-bound lipase and the highest values of hydrolytic activity and biomass concentration have also been obtained from different strains using olive oil as carbon source.

Based on these results, further tests were conducted using 30 g/L of olive oil and a submerged cultivation time of 72 h.

3.2 Biochemical Characterization of Mycelium-Bound Lipase

Biochemical and kinetic characteristics of the produced mycelium-bound lipase are presented in Fig. 2. The effect of temperature on the mycelium-bound lipase activity was in the range of 25 °C to 60 °C and pH 6.5 (Fig. 2a) which showed that, in these experimental conditions, the produced lipase has greater catalytic activity at 40 °C with hydrolytic activity of 738.1 ± 11.8 U/g (relative activity of 100%). Lipase has been proved capable of acting in a wide temperature range (25–60 °C) with relative activity above 80%. Temperatures above 40 °C resulted in a progressive reduction of hydrolytic activity due to the enzyme thermal inactivation. Similar results have been observed in previous studies [55, 56].

Sebsequently, the effect of pH on the hydrolytic activity of the mycelium-bound lipase was also evaluated at 40 °C (Fig. 2b). Under experimental conditions, it is observed that the produced lipase showed higher values of catalytic activity at pH 6.0 (maximum activity of 869.5 U/g ± 8.8 U/g) and a slight increase in pH promoted a slight decay of enzyme, maintaining 72% of its maximum activity at pH 6.5 (631.4 ± 8.2 U/g). Similar results have been reported for whole-cell *Rhizopus oryzae* S3 lipase [56].

The apparent Michaelis constant (K_m) and maximum reaction rate (V_{max}) of the reaction were determined through

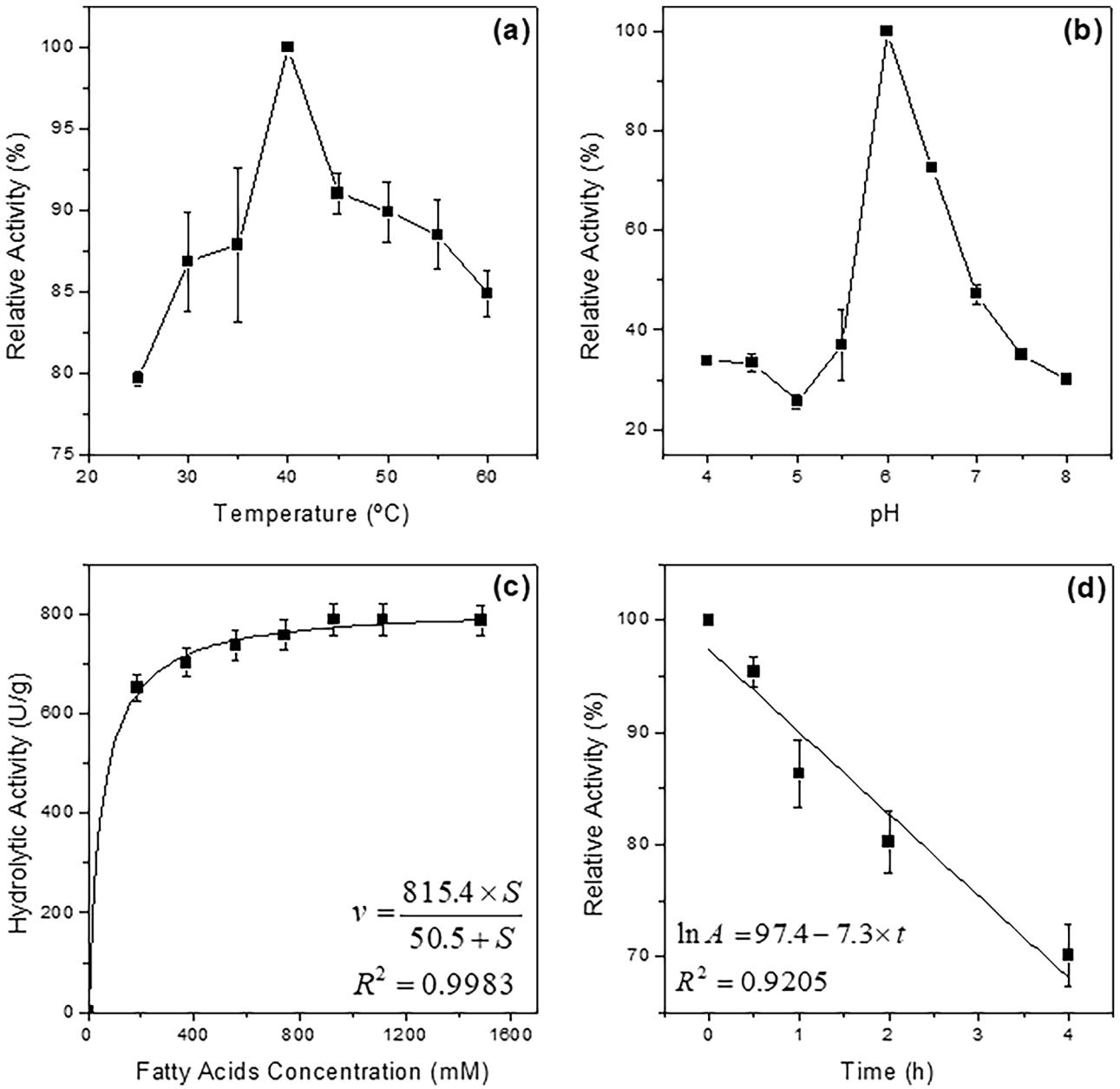


Fig. 2 Biochemical and kinetic characterization of the lipase bound to the mycelium of Rhizopus oryzae CCT3759 in the hydrolysis of olive oil emulsion. **a** Effect of reaction temperature (maximum activity of 738.1 ± 11.8 U/g, defined as 100% relative activity); **b** Effect of pH (maximum activity of 869.5 ± 8.8 U/g, defined as 100% relative activity); **c** Effect of olive oil concentration on the hydrolytic activity and estimation of apparent kinetic parameters; **d** Thermal stability tests at 50 °C and estimation of inactivation parameters

olive oil hydrolysis emulsified with Gum Arabic in the range from 5 to 50% m/m, which is equivalent to FFA concentration from 186 to 1488 mM (Fig. 2C). The reactions have been carried out under the optimum conditions determined above (100 mM buffer sodium phosphate pH 6.0 and 40 °C). These parameters were determined by a non-linear adjustment of the Michaelis–Menten model, thus obtaining a high correlation coefficient (R^2) of 0.9983. The apparent values of K_m and V_{max} were 50.5 mM and 815.4 µmol/g.min, respectively. The produced lipase showed higher V_{max} and greater affinity (K_m) to the substrate (olive oil) than those produced using different fungus species, such as *Penicillium italicum* (539.1 µmol/min, 151.3 mM); *Penicillium janthinellum* (387.6 µmol/min and 123.6 mM); *Penicillium purpurogenum* (493.8 µmol/min and 141.4 mM) [37]; *Penicillium citrinum* (123.2 U/g and 158.1 mM) [36]; *Penicillium citrinum* (136.5 mM and 267.3 µmol/g min) [54]; *Mucor circinelloides* (186.9 µmol/g min and 115.7 mM) [57].

The enzyme inactivation profile is shown in Fig. 2d, in which the lipase was maintained at pH 6.0 (100 mM buffer sodium phosphate) and 50 °C. After 4 h, the lipase showed retention of approximately 75% of its initial activity (866.87 ± 5.4 U/g). The linear decay model fitted well to the experimental data ($R^2 = 0.9202$), in which it was possible to determine thermal inactivation constant (K_d) and half-life ($t_{1/2}$)—0.0733 h^{-1} and 9.4 h, respectively. This result is of great industrial interest, because the longer the enzyme remains active and stable, the lower the number of required replacements of the biocatalyst and therefore have to reduce the costs involved. The produced lipase showed greater thermal stability than other studies found in literature. Essamri and Deyris and Comeau [55] reported that lipase was inactivated at 40 °C for 30 min of incubation time. Razak et al. [56] reported that there was retention of 70% of initial activity after incubation at 50 °C for 3 h.

3.3 Vegetable Oils Hydrolysis in a Tank-Stirred Reactor

After determining the best cultivation experimental conditions and evaluating the parameters that maximize the hydrolysis reaction such as pH, reaction temperature and substrate concentration, as described above, it was evaluated the performance of produced whole-cells from *Rhizopus oryzae* CCT3759 as biocatalyst in the hydrolysis of six different vegetable oils using two different concentrations (200 U and 400 U) in order to maximize the FFA production. Enzymatic hydrolysis reactions of vegetable oils were carried out under the best reaction conditions obtained for olive oil emulsion described above (100 mM buffer sodium phosphate pH 6.0 and 40 °C). These tests were carried out using an oil/buffer mass ratio of 25% (m/m), since a good dispersion of whole-cells in the reaction medium was observed in this condition, which resulted in maximum enzyme activity (see Fig. 2C). In preliminary tests performed at a concentration of vegetable oils at 50% m/m that is maximum concentration assessed in tests for determining apparent kinetic parameters (see Fig. 2d), it was observed a strong aggregation of oil droplets to the mycelium under such experimental conditions. The enzymatic hydrolysis profiles of vegetable oils and the determination of initial reaction rate values obtained using 200 U and 400 U of enzymatic activity are shown in Fig. 3.

According to Figs. 3a, b and Table 4, the increase in activity from 200 to 400 U resulted in higher initial reaction rates (see "v values" in Table 4) in the first 12 h of reaction using canola oil (36.8–46.9 mM/h), sunflower oil (33.1–51.1 mM/h) and soybean oil (26.2–40.7 mM/h), as expected. On the other hand, similar values of initial reaction rates for cottonseed oil (42.2–42.5 mM/h), olive oil (43.6–47.7 mM/h) and corn oil (42.5–44.9 mM/h) have been obtained in the same conditions. These results could be due to high selectivity of this lipase to hydrolyze preferentially vegetable oils containing high concentration of oleic and linoleic acids in their compositions, as aforementioned (see Sect. 3.1).

According to Fig. 3a, oils hydrolysis tests conducted with 200 U has achieved hydrolysis percentages ranging from 66.7 ± 1.3 to 86.4 ± 0.4% after 48 h of reaction. As expected, higher amounts of enzyme in the reaction (400 U) also increased the percentage of hydrolysis and reduced reaction time (Fig. 3b). In fact, a complete hydrolysis of olive, cottonseed, canola and sunflower oils was achieved after 26–30 h reaction. Under these same conditions, hydrolysis percentage of sunflower and soybean oil of 96 and 90%, respectively, was achieved after 30 h of reaction (Table 4).

Based on these results, olive oil was the one that achieved the highest hydrolysis percentage and, thus, FFA concentration (Fig. 3b). This indicates that the obtained lipase has high selectivity for vegetable oils containing high concentration of oleic acid ($C_{18:1}$) in their composition as olive oil, about 74.5% m/m. In fact, high hydrolysis percentage was also achieved for cottonseed and sunflower oils due to their high concentration of oleic acid and linoleic acid ($C_{18:2}$), since both present a similar concentration of this unsaturated fatty acid, as aforementioned. The results obtained in the hydrolysis of soybean oil suggest that *Rhizopus oryzae* CCT3759 lipase has less activity for oils composed of higher proportions of linolenic acid ($C_{18:3}$). These results corroborate the previous results of lipase production, since the microorganism produces lipase to enable the assimilation of vegetable oil as a carbon source for energy production and, consequently, cell growth, production of enzymes and other compounds.

4 Conclusion

An application of whole-cells in biocatalysis reactions consists in using microbial biomass with high catalytic activity as biocatalyst, which is a technology that offers advantages such as low production costs, ease of operation, reduced recovery costs, purification or immobilization of lipases. The best culture conditions for obtaining catalytic cells were evaluated in 72 h of submerged culture using olive oil as a carbon source. Under fixed reaction conditions, hydrolysis percentages greater than 90% after 26–30 h of reaction in a stirred tank reactor for all evaluated oils have

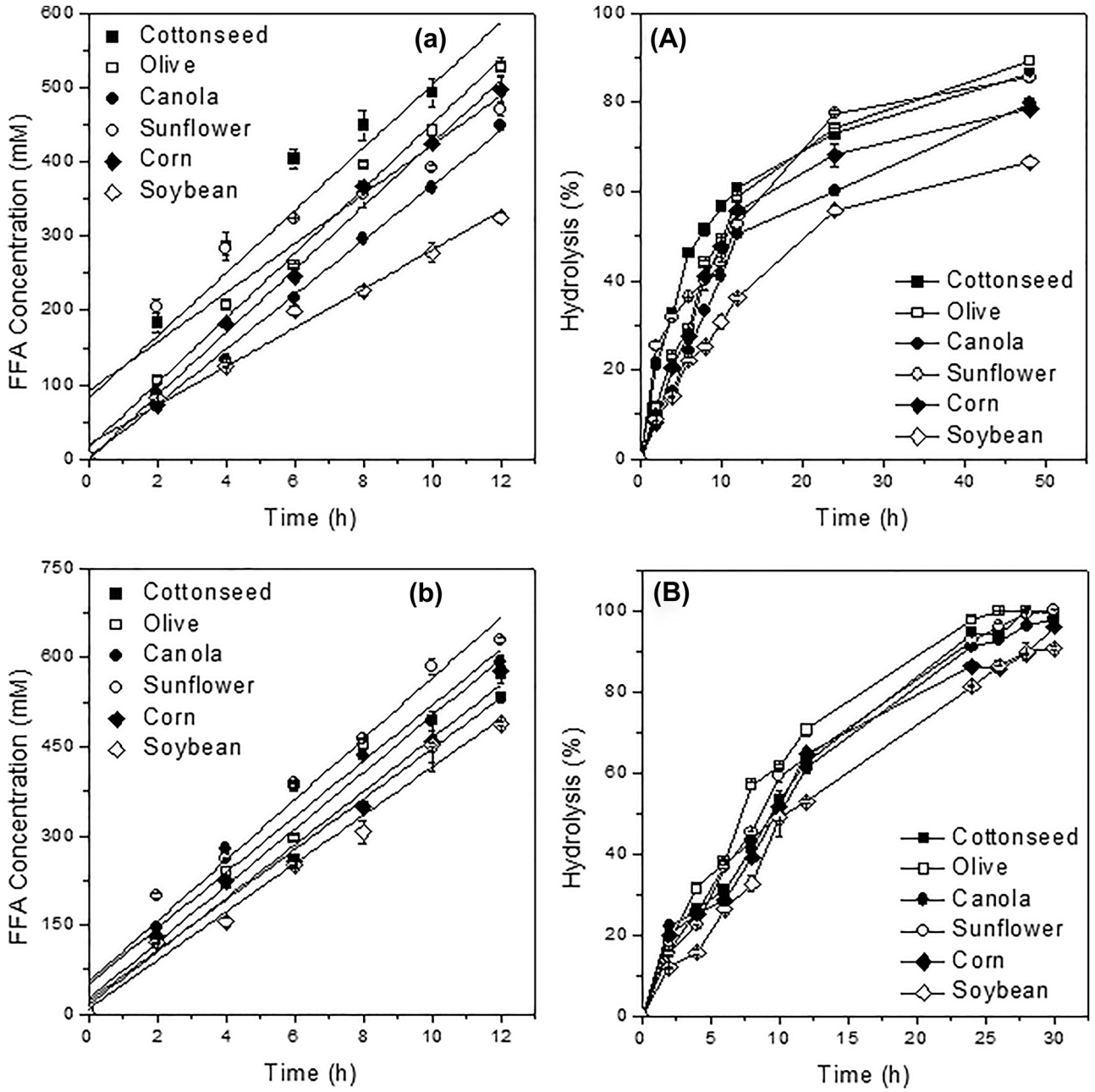


Fig. 3 Effect of mycelium concentration on the initial reaction rate (**a** 200 U and **b** 400 U) and hydrolysis percentage (**A** 200 U and **B** 400 U) of vegetable oils. The reactions were carried out using oil/buffer mass ratio of 25% containing 3% m/v of Gum Arabic, 40 °C, pH 6.0 (100 mM buffer sodium phosphate) and mechanical stirring frequency of 600 rpm

been obtained. Therefore, this study has demonstrated that whole-cell *Rhizopus oryzae* CCT3759 is an interesting biocatalyst to produce FFA due to its high catalytic activity in mild reaction conditions and selectivity to catalyze ester bonds hydrolysis containing high monounsaturated fatty acids in their composition as oleic acid. Moreover, a more in-depth study is currently being performed by the present research group using residual oils (frying oil) as inductor to produce whole-cell *Rhizopus oryzae* to be subsequently used as biocatalysts in non-aqueous media, in addition to industrial esters production (emollient esters and biolubricants) by esterification reactions.

Table 4 Effect of initial activity (U) on reaction rate (v) and hydrolysis percentage of vegetable oils conducted in stirred-tank reactors catalyzed by whole-cell *Rhizopus oryzae* CCT3759

Vegetable oil	Initial activity (U)	Hydrolysis (%)	Time (h)	Linearized equation	R^2	v (mmol/h)
Cottonseed	200	86.4 ± 0.4	48	$y = 81.23 + 42.23x$	0.9027	42.2
	400	99.7 ± 0.3	28	$y = 21.70 + 42.55x$	0,9876	42.5
Olive	200	89.2 ± 1.4	48	$y = 14.87 + 43.65x$	0.9886	43.6
	400	~ 100	26	$y = 25.57 + 47.67x$	0.9791	47.7
Canola	200	79.5 ± 0.8	48	$y = 0.368 + 36.79x$	0.9960	36.8
	400	98.0 ± 2.0	30	$y = 50.84 + 46.92x$	0.9616	46.9
Sunflower	200	85.5 ± 0.9	48	$y = 91.10 + 33.13x$	0.8576	33.1
	400	99.0 ± 1.0	28	$y = 54.57 + 51.14x$	0.9703	51.1
Corn	200	78.5 ± 0.6	48	$y = 0.351 + 42.50x$	0.9924	42.5
	400	96.1 ± 0.7	30	$y = 15.36 + 44.93x$	0.9805	44.9
Soybean	200	66.7 ± 1.3	48	$y = 19.48 + 26.18x$	0.9827	26.2
	400	90.1 ± 2.0	28	$y = 8.81 + 40.73x$	0.9783	40.7

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Declarations

Conflict of interest The authors declare no conflict of interest.

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