Catalytic and physical features of a naturally immobilized *Yarrowia lipolytica* lipase in cell debris (LipImDebri) displaying high thermostability

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Abstract

Lipase activity (337 U/g dry weight of cell debris) was detected in cell debris after ultrasound treatment of *Yarrowia lipolytica* cells cultivated in residual frying palm oil. It is a naturally immobilized lipase with protein content of 47%, herein called LipImDebri. This immobilized biocatalyst presents low hydrophobicity (8%), that can be increased adjusting pH and buffer type. Despite apparent intact cells, electron microscopy showed a shapeless and flat surface for LipImDebri and optical microscopy revealed no cell viability. Besides, an inferior mean diameter (3.4 μ m) in relation to whole cells reveals structure modification. A high negative zeta potential value (-33.86 mV) for pH 6 and 25 °C suggests that LipImDebri is a stable suspension in aqueous solution. Fourier Transform Infrared Spectra (FTIR) expose differences between LipImDebri and extracellular lipase extract signaling a physical interaction between enzyme and cell debris, which is possibly the reason for the high thermostability ($k_d = 0.246$ h⁻¹; $t_{1/2} = 2.82$ h at 50 °C, pH 7.0). A good adjustment of LipImDebri kinetic data with Hill equation ($R^2 = 0.95$) exposes an allosteric behavior related to the presence of more than one lipase isoform. These features reveal that LipImDebri can be a good catalyst for industrial applications.

Keywords Cell-bounded enzymes · Cell debris · *Yarrowia lipolytica* · Thermostability · Electron microscopy · Lipase

Introduction

Lipases from microbial source have been preferred for industrial production and by scientific community when compared to enzymes produced by plants or animals (Treichel et al. 2010). These enzymes, classified as triacylglycerol

acylhydrolases (EC 3.1.1.3), can hydrolyze and synthesize ester bounds, catalyzing esterification, interesterification, and transesterification reactions (Luković et al. 2011; Gupta et al. 2015). In this sense, lipases find new applications in several industrial areas, including the leather industry (Abol Fotouh et al. 2016; Li et al. 2020), food products segment (Hassan et al. 2019; Akil et al. 2020) and also in the energy sector (Hassan et al. 2019). Microorganisms of the genus *Aspergillus*, *Calvatia*, *Rhizopus*, *Rhodotorula* and *Yarrowia* can produce constitutive lipase (gene expression) that can be partially induced with the aid of triacylglycerols or fatty acids. The physiological role of lipases for yeast cells is, basically, to degrade oils and fatty acids and, therefore, contribute to the increase of cell mass (Baloch et al. 2019).

Yarrowia lipolytica is non-conventional yeast with numerous physiological properties and great potential for biotechnological applications. It is able to efficiently degrade a range of hydrophobic and unusual substrates as well as to synthesize enzymes with high hydrolytic power (Bankar et al. 2009). Lipases are part of this portfolio of enzymes produced by this specie which can be used in the detergent,

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food, pharmaceutical, and environmental industries. Catabolism of triacylglycerols by *Y. lipolytica* is related to its hydrolysis into free fatty acids and glycerol and, therefore, lipases can be directed to different cell compartments, such as the cytoplasm (intracellular fraction), the cell wall (cell-bound fraction) or even be secreted to extracellular medium (extracellular fraction). It has been reported that the production of these different lipase fractions is dependent on medium composition and environment condition (Pereira-Meirelles et al. 1997; Lopes et al. 2008).

Lipases bounded to the cell wall have been also reported for other microbial species. de Castro et al. (2017) used a mycelium-bound lipase of *Aspergillus westerdijkiae* as a naturally immobilized enzyme to produce biodiesel. Membrane-bound lipolytic enzymes from the microalgae *Nannochloropsis oceanica* CCMP1779 were previously identified and tested in hydrolytic reactions (Savvidou et al. 2016). Lipases bound to the cell debris of *Y. lipolytica* (LipImDebri) were tested in different conditions to promote the hydrolysis of butter fat generating an industrial food additive known as Lipolyzed Milk Fat (LMF) (Fraga et al. 2018).

Cell-bound lipases of *Y. lipolytica* were first identified by Sugiura et al. (1976) and described as lipase I with molecular weight of 39 kDa. In 2005, Fickers et al. (2005) identified LIP7 and LIP8 genes corresponding to cell-bound lipases. The utilization of naturally immobilized enzymes can generate numerous advantages in the industrial scale such as reduction of process costs (no need for support nor other process steps for immobilization), increase of enzyme stability and possible reuse of the biocatalyst (Fraga et al. 2018). However, information about the characteristics of enzymes bound to cell or cell debris are still scarce and particularly important for its application and commercialization. Therefore, the purpose of this work was to produce LipImDebri from *Y. lipolytica* and show morphological, physicochemical, and biochemical characteristics of this enzyme for possible industrial applications.

Materials and methods

Materials

Bacteriological peptone and yeast extract were acquired from Oxoid (Hampshire, UK). Glucose and agar-agar were purchased from Vetec (Rio de Janeiro, Brazil). Antifoam 204 was obtained from Sigma-Aldrich (St. Louis, MO, USA) to reduce foaming. The residual frying palm oil (RFPO) from fried potatoes was obtained from Bob's fast food restaurant (Rio de Janeiro, Brazil). This residual oil was kindly provided by Brazil Fast Food Corporation (Rio de Janeiro, Brazil) and was chemically characterized as originally a palm oil (data not shown).

4-morpholinopropanesulfonic acid was obtained from Vetec (Rio de Janeiro, Brazil). *p*-nitrophenyl laurate (*p*NP-laurate) was purchased from Sigma-Aldrich Brazil (Rio de Janeiro, Brazil) and dimethyl sulfoxide was obtained from Kasvi (Paraná, Brazil).

Lipase production in bioreactor

A *Yarrowia lipolytica* lab strain (IMUFRJ 50682/1) modified after repeated sub-culturing (Silva et al. 2019), originally isolated from Baía de Guanabara, Rio de Janeiro, Brazil (Hagler and Mendonça-Hagler 1981), was used for lipase production. Cells were stored at 4 °C after 24 h growth in test tubes with YPD-agar medium (w/v: 1% Yeast Extract; 2% Peptone; 2% Dextrose; 2% Agar agar). For inoculum, cells were cultivated at 28 °C and 160 rpm, in 500-mL flasks containing 200-mL YPD medium (w/v: yeast extract 1%; peptone, 2%; glucose, 2%). Cells from the inoculum were centrifuged (26,000 g) and used to inoculate 3.5 L of YPRFO medium (w/v: yeast extract 1%; peptone, 2%; residual frying palm oil 2.5% v/v) with sufficient amount to obtain 1-g dry weight of cells/L (Fraga et al. 2018).

The bioprocess of lipase production was performed in New Brunswick MF-114 Microferm reactor with 3.5 L of inoculated YPRFO medium mechanical agitation by 2 Rushton type stirrers (650 rpm), aeration rate of 1.5 L/min, at 28 °C for 24 h.

Ultrasound treatment to obtain lipase immobilized in cell debris (LipImDebri)

After 24 h of *Y. lipolytica* cultivation in YPRFO medium in a bioreactor, cells were harvested by centrifugation at 4 °C for 5 min (4600 g). The supernatant (extracellular lipase extract—ExLipEx) was separated in falcon tubes and stored (− 20 °C). Cells were washed with distilled water, then with 200-mM MOPS buffer pH 7.0 and centrifuged at 4 °C for 5 min (4600 g). These washed cells were suspended in MOPS buffer and submitted to ultrasound treatment: 2 cycles in 150 W and frequency of 20 kHz for 9 min in an ice bath (Nunes et al. 2014). After centrifugation, supernatant (intracellular enzymatic fraction) was separated in tubes and stored (− 20 °C) and cell debris (cells that were treated with ultrasound) was suspended in MOPS buffer, and stored (− 20 °C). Cell debris obtained after ultrasound treatment is considered the main biocatalyst in the present work, herein called LipImDebri, because of the presence of cell-bound lipase naturally immobilized in cell debris. Part of this LipImDebri was also freeze dried (LC 1500, Terroni, Sao Carlos, Brazil).

Adhesion tests

Microbial adhesion to solvents (MATS)

MATS method was described by Bellon-Fontaine et al. (1996) to determine electron donor and acceptor properties (Lewis acids/bases) of microbial cell surface through the comparison of cell affinity to a monopolar solvent and a nonpolar solvent. In this test, LipImDebri (or *Y. lipolytica* cells for comparison) was suspended in a phosphate buffer (0.1 M, pH 7.0) to an optical density of 0.7 at 570 nm (A_0). LipImDebri suspension (3.6 mL) was vortexed for 50 s with 0.6 mL of solvent (chloroform, hexadecane, ethyl acetate and decane). The optical density (570 nm) of aqueous phase was measured after 10-min rest (A) to ensure complete separation of the two phases. Cell adhesion percentage (% adhesion) to each solvent was calculated by Eq. (1):

$$\% \text{ adhesion} = \left(1 - \frac{A}{A_0}\right) \times 100. \quad (1)$$

The hydrophobicity (Hyph) in this method is defined as the cell adhesion to hexadecane. The electron donor character (EDC) is obtained by the cell adhesion to chloroform minus that to hexadecane, and the electron acceptor character (EAC) is the cell adhesion to ethyl ether minus that to hexane (Amaral et al. 2006).

Microbial adhesion to hydrocarbon (MATH)

MATH method is based on comparing microbial cellular affinity to hydrocarbons (van der Mei et al. 1995). The method consists of suspending LipImDebri (or *Y. lipolytica* cells for comparison) in various buffer solutions with pH values adjusted to 2, 3, 4, 6 and 7. Initial absorbance at 570 nm (A_0) is adjusted to, approximately, 0.7. Buffers tested were potassium phosphate—KPi (0.87 g/L K_2HPO_4 , 0.68 g/L KH_2PO_4), phosphate buffer saline—PBS (0.87 g/L K_2HPO_4 , 0.68 g/L KH_2PO_4 and 8.77 g/L NaCl) and phosphate urea magnesium sulfate—PUM (19.7 g/L K_2HPO_4 , 7.26 g/L KH_2PO_4 , 1.8 g/L H_2NCONH_2 and 0.2 g/L $MgSO_4 \cdot 7H_2O$). For this analysis, n-hexadecane (0.15 mL) was vortex mixed for 10 s with 3 mL of LipImDebri (or whole cells for comparison) suspended in different buffers. After 10 min at rest, the absorbance of the aqueous suspension (A) was measured and the aqueous phase retention ($A/A_0 \times 100$) was calculated. The first step (vortex mixing) was repeated with different vortex periods (25, 45 and 60 s) and the linear fit of the log ($A/A_0 \times 100$) versus vortex time resulted in the initial adhesion rate (R_0) as a measurement of adhesion of cells to hydrocarbon.

Physical–chemical and morphological characterization of LipImDebri

Mean diameter of the LipImDebri

A laser diffraction-type particle size distribution analyzer SALD-2000 J (Shimadzu Corp.) was used to measure particle size of LipImDebri (Kinoshita 2001).

Scanning electron microscope (SEM)

LipImDebri and *Y. lipolytica* cells were analyzed by Scanning Electron Microscope (Jeol Brand, model JSM-6510LV). The samples were fixed to 10- μ m-diameter cylindrical metal support using a double-sided carbon adhesive tape. The microscope was operated with an accelerating voltage of 15 kV.

Zeta potential

The zeta potential of LipImDebri was determined by electrophoretic light scattering by laser Doppler electrophoresis (± 150 mV) and dynamic light scattering, angle of 173° , respectively, using a Zeta sizer Nano ZS (Malvern Instruments, Worcestershire, England). The sample was first diluted in purified water (1:1 v/v), and the analyses were performed at 25 °C.

Fourier transform infrared spectroscopy (FTIR)

FTIR was performed with the purpose of identifying the presence of functional groups and vibrational modes of the molecules that would allow the differentiation of LipImDebri from the crude lipase extract (ExLipEx). The analysis was performed in an Infrared equipment (Prestige FTIR-21/ Shimadzu). All spectra were obtained between 4100 and 600 cm^{-1} and at the resolution of 4 cm^{-1} .

Biochemical characterization

Lipase hydrolytic activity

The hydrolysis of *p*-nitrophenyl laurate (*p*NP-laurate) was used to determine lipase activity. 1.9 mL of 560- μ M *p*NP-laurate was dissolved in 50-mM phosphate buffer (pH 7.0) containing 1% (v/v) dimethyl sulfoxide (DMSO) and mixed with 0.1 mL of enzyme extract at 37 °C. In the case of LipImDebri, dry weight measurement was used to determine the amount of biocatalyst in 0.1 mL. The production of *p*-nitrophenol (resultant of the enzymatic reaction) is measured by absorbance change at 410 nm in spectrophotometer

(Shimadzu Model UV-1800) for 100 s. One unit of activity (U) is defined as the amount of enzyme which releases 1 μmol of *p*-nitrophenol per minute at pH 7.0 and 37 °C.

Protein concentration

Total protein content of LipImDebri was determined by Kjeldahl method in quadruplicate (Bradstreet 1954).

Cell viability of LipImDebri

Cell counts and viability of LipImDebri were performed under standard optical microscopy based on the method developed by Lee et al. (1981) for viability analysis in yeast using a Neubauer chamber (model 1/400 mm² × 0.100 mm). Methylene blue (0.025%) staining was employed for cell viability analysis.

Enzyme stability

Thermal and pH stability of LipImDebri were determined after incubating this biocatalyst in different temperatures or pH buffers for 270 min. Determination of enzymatic activity (“Lipase hydrolytic activity”) was performed every 30 min in standard reaction conditions (37 °C, pH 7.0).

For thermal stability LipImDebri (0.5 mg) was incubated in 0.5-mL phosphate buffer (pH 7.0) at temperatures varying from 37 to 65 °C. For pH stability, same amount of LipImDebri was incubated at 37 °C with 0.5 mL of the following buffers (50 mM): sodium acetate (pH 4.0 and 5.0); potassium phosphate (pH 6.0 and 7.0) and Tris–HCl (pH 7.5 and 8.0).

Thermal and pH stability data were used to determine inactivation parameters (IP). Deactivation constant (k_d), half-life ($t_{1/2}$), and activation energy involved in thermal denaturation (E_d) were calculated. The semi-log plot of the residual lipase activity (%) versus time was used to obtain the k_d parameter. $t_{1/2}$ is calculated as $\ln(2)/k_d$ (Muley et al. 2018), and E_d determined by linearization of Arrhenius equation ($\ln(k_d)$ versus $1/T$) (Ferreira et al. 2018).

Determination of kinetic parameters

LipImDebri kinetic parameters, such as $V_{\max}$ (maximum reaction rate), $K_{0.5}$ (apparent dissociation constant), $V_{\max}/K_{0.5}$ (catalytic efficiency) and n (Hill’s coefficient) for *p*NP-laurate hydrolysis with substrate concentrations ranging from 50 to 5000 μM in 50-mM phosphate buffer at pH 7.0 were estimated by non-linear regression using the software Microsoft Office Excel®. The hydrolysis reaction was performed as described in “Lipase hydrolytic activity”.

Statistical analysis

All data were obtained in triplicates (except for protein concentration, quadruplicate analyzes were performed, “Protein concentration”). For all analyses, the means and standard deviations were calculated. All statistical analyses were performed using Excel (Microsoft® Office 2010—USA).

Results and discussion

Residual frying palm oil (RFPO) from a fast food restaurant had already been tested by our group to obtain three lipase fractions by *Y. lipolytica*: extracellular, intracellular and cell debris fractions as reported in Fraga et al. (2018). In the present study, similar values were achieved. The cell debris lipase fraction (LipImDebri) was obtained after sonicating cells (8.74 g dry weight of cells/L) harvested from a 24-h bioreactor growth in YPRFPO medium. These cells contained cell-bound lipase that were exposed by the ultrasound treatment in the conditions studied by Nunes et al. (2014) (2 cycles, 150 W, 20 kHz, 9 min) and remained attached to cell debris, generating an enzyme naturally immobilized. The hydrolysis of *p*NP-laurate resulted in a lipase activity of 337 U per gram dry weight of cell debris, confirming the production of LipImDebri.

Determination of protein concentration of LipImDebri resulted in 0.47 g of protein/ g of cell debris dry weight, i.e., around 47% of the biocatalyst. Carvalho et al. (2017) reported values of 0.315, 0.106 and 0.431 mg of protein/mL of *Y. lipolytica* lipase extract for a crude extract, an ultrafiltration purified extract and a lipase extract purified with acetone and kaolin, respectively. In a rough calculation, considering the these lipase extracts with density similar to water (around 1000 mg/mL at 25 °C), it would result in 0.000315, 0.000106 and 0.000415 mg of protein/mg of lipase extracts, respectively, i.e., 0.03, 0.01 and 0.04% of the biocatalyst, which is expected for soluble enzymes. For lipase immobilized in cellular debris of *Nannochloropsis gaditana*, the concentration of protein was approximately 6% of the cell wall mass (Savvidou et al. 2016), inferior to that of LipImDebri (47%).

Cell viability of LipImDebri

The mechanism of cell disruption by ultrasound is probably linked to cavitation phenomena as reviewed by Chisti (2003). The author also examined documents which reported that a cell can be inactivated by ultrasound at intensities less than those needed to cause disruption and that ultrasonic cell disruption generally results in cell debris. In the present study, ultrasound treatment was used to expose cell-bound lipase since whole cells did not show significant enzyme

activity (data not shown), as also depicted by Savvidou et al. (2016) for *Nannochloropsis oceanica* cells. However, to be used as a biocatalyst, this cell debris showing lipase activity (LipImDebri) was tested for cell viability, since the presence of cell metabolism in enzymatic reactions can be a problem.

Observations in optical microscopy confirm what Chisti (2003) reported. *Y. lipolytica* cells treated with ultrasound to produce LipImDebri seemed intact, not disrupted (Fig. 1a). However, methylene blue staining indicate that all cells were blue, and cell counting in Neubauer chamber showed 100% inviable cells. The cells before ultrasound treatment were also observed in optical microscopy (Fig. 1b) and no methylene blue staining was detected, indicating a 96.5% cell viability.

Aiming at releasing proteins from *Y. lipolytica* cells, Kap-trowska et al. (2012) studied the impact of ultrasound waves on the lipolytic activity of those proteins. They showed that sonication at 150 W, at an 80% duty cycle for 15 min was not enough to disrupt all the cells and that the most sensitive were hyphae forms, while oval cells were more resistant to acoustic waves. This might be the reason why Nunes et al. (2014) determined 90% of cell viability using the same ultrasound treatment conditions to treat *Y. lipolytica* cells to obtain intracellular lipases. They also showed a high percentage of hyphae-type cells in microscopy images.

LipImDebri surface characterization

Generally, supports for enzyme immobilization are chosen based on its characteristics, especially because it can interfere with enzyme catalytic site and with substrates in the reaction medium (Almeida et al. 2018). In the case of LipImDebri, the support for lipase is the cell debris. Therefore, it is important to determine its surface characteristics for a better application and to understand the modifications caused by the treatment used to obtain cell debris (ultrasound waves).

Microbial adhesion to solvents (MATS)

MATS method was designed to facilitate the characterization of cell surface and it is based on comparison between cell affinity to a monopolar solvent and a polar solvent (Bellon-Fontaine et al. 1996). This method was tested with LipImDebri, so that it was possible to identify the surface characteristics of this biocatalyst and predict the interaction between these lipases immobilized in cell debris with different substrates. Additionally, by comparing the characteristics of whole cells of *Y. lipolytica* and LipImDebri, it could be possible to identify the changes that might occur to cells when submitted to ultrasound waves.

Hydrophobicity of LipImDebri was lower than whole cells of *Y. lipolytica* (Table 1) showing that the sonication process altered the cell surface structure and properties. Ultrasound is a physical process in which vibrations generate mechanical tensions in cell suspensions promoting lysis (Gogate and Kabadi 2009). Amaral et al. (2006) identified that the high hydrophobicity of *Y. lipolytica* IMUFRJ 50682 cells cultivated in YPD medium seemed to be linked to proteins present on the cell wall because of the consistent

Table 1 MATS (Microbial Adhesion to Solvent) test for different *Y. lipolytica* strains (whole cells) and LipImDebri (cell debris obtained after ultrasound treatment with cell-bound lipase naturally immobilized)

Microbial cellular material	Hyph (%) ^a	EDC (%) ^b	EAC (%) ^c
<i>Y. lipolytica</i> whole cells	88.26 ± 12.15	− 31.72 ± 2.99	− 21.24 ± 0.59
LipImDebri	7.99 ± 0.10	− 29.87 ± 0.69	40.97 ± 0.98

^aHyph hydrophobicity—cell adhesion to hexadecane

^bEDC electron donor character—cell adhesion to chloroform minus hexadecane adhesion

^cEAC electron acceptor character—cell adhesion to ethyl ether minus hexane adhesion

Fig. 1 Optical microscopy images of LipImDebri (cell debris obtained after ultrasound treatment with cell-bound lipase naturally immobilized) (left) and *Y. lipolytica* cells (right). (Magnification of 1000 ×)

reduction of this yeast hydrophobic character after the action of pronase. Fraga et al. (2018) showed that after ultrasound treatment, the supernatant presented lipase activity, which might be from the intracellular content or released from the cell surface as also reported by Kapturowska et al. (2012). Other proteins might also be released with ultrasound treatment and this might be the reason for this hydrophobicity reduction of LipImDebri.

Table 1 depicts an extremely low electron donor character (EDC) for both cells and LipImDebri in comparison to results of Amaral et al. (2006) for *Y. lipolytica* grown in YPD medium (11.1%). Different acidic/basic character of cell surface might be related to medium composition used in cell cultivation, which influences cell surface adhesion behavior (Aguedo et al. 2005).

Microbial adhesion to hydrocarbon (MATH)

MATH test measures the interaction capacity of microbial cell surface and hydrocarbons, an indication of surface hydrophobicity. Microbial adhesion is considered, from the physical chemical point of view, a set of van der Waals forces from long range, electrostatic interactions, and short-range interactions (van der Mei et al. 1995). MATH test was performed with LipImDebri to assess the ability of this material to be removed by a hydrophobic solvent (hexadecane) in different buffers, to avoid the influence of pH in the adhesion result.

Removal of LipImDebri from various buffered aqueous phase by n-hexadecane after 10 s of vortex mixing periods is shown in Fig. 2. For every buffer tested (PBS, Kpi and PUM), retention of cell lysate by the aqueous phase was superior to 60%, which shows the high affinity to water and reduced hydrophobicity of LipImDebri, confirming the results of the MATS test. Tyfa et al. (2015) stated that a cell retention by the hydrocarbon phase of less than 20%

Table 2 Initial removal rate (R_0 , in min^{-1}) by *n*-hexadecane as a function of pH in different buffers (phosphate buffer saline—PBS, potassium phosphate—KPi and phosphate urea magnesium sulfate—PUM) obtained in the MATH (Microbial Adhesion to Hydrocarbons) test for LipImDebri (cell debris obtained after ultrasound treatment with cell-bound lipase naturally immobilized)

pH	R_0 (min^{-1})		
	PBS	KPi	PUM
2	0.015	0.018	0.026
3	0.028	0	0.012
4	0.123	0.143	0.051
6	0.010	0.082	0.399
7	0.259	0.113	0

is considered hydrophilic, which seems to be the case of LipImDebri. Depending on the ions used for buffer solution, the affinity to hydrocarbon could be increased with different pH values: pH 4 for PBS, pH 3 or 7 for KPi and pH 6 for PUM. This test was also performed varying vortex mixing period (10, 25, 45 and 60 s) and retention by aqueous phase did not change much, resulting in low initial removal rate, R_0 values (Table 2). van der Mei et al. (1995) detected a similar behavior for *S. salivarius* HBC12, which was considered a hydrophilic microorganism.

According to Table 2, the initial removal rate (R_0) of LipImDebri in hexadecane was lower compared to *Y. lipolytica* whole cells ($0.8\text{--}1.2 \text{ min}^{-1}$ [18]) and varied very little with the use of different buffers (same pH, $\Delta R_0 \leq 0.39$) or in different pH values (same buffer, $\Delta R_0 \leq 0.39$).

Physical and morphological characterization

Morphology of whole cells of *Y. lipolytica* can be compared to sonicated *Y. lipolytica* cell debris (LipImDebri) in

Fig. 2 Microbial adhesion to hydrocarbon (MATH)—Aqueous phase retention ($A/A_0 \times 100$, A_0 —initial absorbance and A final absorbance after 10-min rest, at 570 nm) in relation to buffer pH for the removal of LipImDebri of *Y. lipolytica* (cell-bound lipase naturally immobi-

lized in cell debris obtained after ultrasound treatment) by *n*-hexadecane after 10 s of vortex mixing in phosphate buffer saline—PBS (a), potassium phosphate—Kpi (b) and phosphate urea magnesium sulfate—PUM (c) buffers

Fig. 3 Photomicrographs of *Yarrowia lipolytica* whole cells (**a**, **b**) and LipImDebri—cell-bound lipase naturally immobilized in cell debris obtained after ultrasound treatment—(**c**, **d**) observed in a scan-

ning electron microscope (SEM) with magnification of 4000× for **a** and **c** and 2000× for **b** and **d**

Fig. 3. *Y. lipolytica* cells obtained during growth in YPRFPO medium are spherical and seem integral in form with smooth surfaces. It is also possible to observe cells in gemmulation phase and a small formless structure adhered to the cell surface, which is probably the oil used as carbon source in lipase production (Fig. 3a, b). This morphology is similar to that reported by Mlíčková et al. (2004) for *Y. lipolytica* cells grown in medium containing glucose.

In contrast, it is possible to observe that the original oval shape of the cells was lost after sonication, revealing a shapeless and flat surface (Fig. 3c, d). Similar to that observed by Kapturowska et al. (2012), that used same power input (150 W) but a higher sonication time (15 min), some cells are not disrupted by the treatment. However, the scanning electron microscopy indicates that cells have changed and look “empty” because its surface does not look so smooth.

Zeta potential can be defined as a measurement of the magnitude of repulsion or the electrostatic attraction of surface charges between particles. It is one of the parameters fundamentally known to affect protein stability and is directly influenced by pH and ionic strength of the aqueous medium (Chen et al. 2014).

The zeta potential value obtained for LipImDebri in pH 6 and 25 °C was -33.86 ± 5.89 mV. Strong electrostatic repulsion (high positive or negative value) can prevent aggregation by keeping colloidal particles well separated from each other and from surfaces (Al-Hezaimi et al. 2005). It is usual to use the range of -30 mV to $+30$ mV to determine that the stability of a suspension is low and probability to have aggregates increases, specially below 20 mV (Pollastri et al. 2014). Therefore, one can say that LipImDebri is a stable aqueous suspension. Values of -7 mV (Amaral et al. 2006) and -20 mV (Aguedo et al. 2005) have been reported for cells of different *Y. lipolytica* strains, which indicates that sonication has increased stability for cell lysate. A negative zeta potential value is usually observed when the suspension pH of a finely divided solid, distributed in aqueous medium, is greater than the isoelectric point of the enzyme (Fernandes et al. 2014). Thus, it is possible that the isoelectric point of LipImDebri is inferior to 6.

Regarding size (Fig. 4), a mean diameter of $3.4 \mu\text{m}$ was obtained for LipImDebri. This result reinforces the morphological analysis that cell debris have been modified, since according to Barth and Gaillardin (1997) *Y. lipolytica* cells

Fig. 4 Size dispersion curve of LipImDebri (cell debris obtained after ultrasound treatment with cell-bound lipase naturally immobilized) for pH 6 and temperature of 25 °C

are about 5–7 μm in diameter. This also indicates cell lysis that ultimately changes the shape and consequently the final size of LipImDebri.

The FTIR spectra for LipImDebri are compared to the extracellular lipase extract (ExLipEx) spectra in Fig. 4. ExLipEx is a crude enzymatic extract obtained during the growth of *Y. lipolytica* in YPRFPO after cell centrifugation (extracellular medium). Infrared spectroscopy was important to evaluate the chemical differences between the extracellular lipase extract and the enzyme bounded to the cell debris, and possibly assess the chemical interactions between the immobilized enzyme and cell debris Fig. 5.

For LipImDebri and ExLipEx, a broad and rounded band was detected in the range of 3000–3600 cm^{-1} . This band is related to the presence of hydroxyl groups because of axial deformation of the O–H band (Michell 1990). It is possible to observe a wider band in the crude enzymatic extract spectrum. Since lipase in LipImDebri is attached to the cell debris, it is possible that the vibrations of the hydroxyl groups of the enzyme are reduced and therefore, a lower band is detected.

According to Elliot and Ambrose (1950), a band around 1650 cm^{-1} is related to the “Amide I” band, which is related to the alpha helix conformation, generally observed in the region of 1650–1660 cm^{-1} . The ExLipEx band appears to be wider with overlapping peaks, most likely due to the presence of Amide I and “Amide II”, generally observed in the range of 1630–1640 cm^{-1} (β -strands conformation). For LipImDebri spectrum, this last band cannot be observed, which might be due to the interaction of these amides of lipase with cell debris. Pereira et al. (2019) observed a reduction of the band intensity in the range of 3300–3550 cm^{-1} (relative to the axial deformation of the O–H bond) for lipase immobilized in microcapsules of chitosan–alginate beads in relation to the bands of the microcapsules components, which they associated with

Fig. 5 Fourier Transform Infrared Spectra (FTIR) for crude enzymatic extract (extracellular lipase extract, ExLipEx) and LipImDebri (cell debris obtained after ultrasound treatment with cell-bound lipase naturally immobilized)

the interactions between the enzyme and the microcapsule involving, mainly, the carboxylic groups.

In 1404 cm^{-1} , it is possible to observe an axial deformation of the N–H bond related to “Amide III”. This is a combination of N–H bending, C–N stretching vibration in conjunction of a C–O in plane bending and other molecules effects (Cai and Singh 2004). A band in the position 1151 cm^{-1} is observed for LipImDebri spectrum, indicating a possible displacement of the Amide III band. This effect is usually observed when there are changes in the chemical environment, which may indicate differences between the two enzyme materials analyzed, by the presence of other chemical groups in the structure of LipImDebri or/and by the interaction (or binding) of this enzyme with cell debris.

Determination of kinetic parameters

Different substrate concentrations were related to *p*NP-laurate hydrolysis rate to obtain Michaelis–Menten kinetics

Fig. 6 Initial hydrolysis rate for *p*NP-laurate (substrate—S) by LipImDebri (cell-bound lipase naturally immobilized in cell debris obtained after ultrasound treatment) in 50-mM phosphate buffer, pH 7.0 (open diamond symbol). The solid line represents the Hill equation with the fitted parameters obtained from experimental data and the dashed line the Michaelis–Menten model

parameters, but the profile obtained for LipImDebri (Fig. 6) failed to fit this equation (Table 3). A good adjustment to the data obtained for LipImDebri was possible with Hill equation, that is indicated for allosteric enzymes, as shown in Fig. 4 and Table 3.

It has been reported that Lip7p and Lip8p are two cell-bound lipases isoforms from *Y. lipolytica* (Fickers et al. 2005) and that Lip2p is partially cell-bounded during the exponential growth phase before being released into the extracellular medium during the stationary phase (Fickers et al. 2004). Therefore, these three lipases isoforms may be present in LipImDebri, generating a homotropic effect by a cooperative action of enzymes (Cornish-Bowden 2014). An allosteric behavior was not observed for an extracellular lipase produced by *Y. lipolytica* (Pereira et al. 2019).

Kinetic parameters obtained for LipImDebri by the Hill equation (Table 3) show an inferior value of $V_{\max}$ for LipImDebri (1.56 U/mg protein, considering 47% of protein in LipImDebri) in comparison to extracellular *Y. lipolytica*

lipase (221 U/mg protein (Pereira et al. 2019); 329 U/mg protein (Carvalho et al. 2017)). Non-allosteric lipases from other microorganisms (*Fusarium verticillioides*) also show superior $V_{\max}$ values (47.71 U/mg protein Lip1 and 37.4 U/mg protein—LIP 2) (Facchini et al. 2018). Immobilized enzymes usually show lower values of $V_{\max}$, as shown by Pereira et al. (2019) and Cui et al. (2013).

Enzyme stability

Inactivation parameters (IP) (Inactivation constant, k_d and half-life, $t_{1/2}$) of LipImDebri were obtained from stability data of temperature and pH (Table 4). Higher values of the inactivation constant indicate greater susceptibility to denaturation and for $t_{1/2}$ is the opposite, higher values indicate a more stable condition (Pereira et al. 2019). Fraga et al. (2018) reported an optimum temperature and pH of 37 °C and 7.0, respectively, for LipImDebri. At those conditions, LipImDebri showed high stability, although pH 4 and temperature of 37 °C were good conditions as well (Table 3). Values obtained for LipImDebri (Table 3) were better than free and chitosan-immobilized lipase (free: $k_d = 2.16 \text{ h}^{-1}$; $t_{1/2} = 0.32 \text{ h}$; immobilized: $k_d = 2.4 \text{ h}^{-1}$; $t_{1/2} = 0.29 \text{ h}$, at 50 °C) (Pereira et al. 2019) and cutinase (free: $k_d = 1.92 \text{ h}^{-1}$; $t_{1/2} = 0.36 \text{ h}$; immobilized: $k_d = 1.74 \text{ h}^{-1}$; $t_{1/2} = 0.40 \text{ h}$, at 50 °C) (Muley et al. 2018). IP values for LipImDebri are close to that found for immobilized *C. antarctica* lipase ($k_d = 0.106 \text{ h}^{-1}$; $t_{1/2} = 6.51 \text{ h}$, at 60 °C) (Brígida et al. 2007) and immobilized *Y. lipolytica* lipase in magnetic nanoparticles ($k_d = 0.214 \text{ h}^{-1}$; $t_{1/2} = 3.24 \text{ h}$, at 60 °C) (Carvalho et al. 2020) and lower than Lipozyme TL IM ($k_d = 0.017 \text{ h}^{-1}$; $t_{1/2} = 39.6 \text{ h}$, at 50 °C), which is considered a highly thermostable lipase (Khor et al. 2010). Since in LipImDebri lipase is immobilized in cell debris by a strong bound (which was not destroyed by ultrasound treatment), this high thermostability might be related to a protection of the enzyme by the cell membrane.

The activation energy of denaturation, E_d , represents the energy barrier needed to be overcome for the irreversible denaturation process to take place (Khor et al. 2010). In this

Table 3 Kinetic parameters for *p*-nitrophenyl laurate hydrolysis by LipImDebri (cell-bound lipase naturally immobilized in cell debris obtained after ultrasound treatment)

Model	Equation	Parameter	R^2
Michaelis–Menten	$v = \frac{V_{\max} \times [S]}{K_m + [S]}$	K_m (mM)	2.52 ± 0.04
		$V_{\max}$ (U/ g LipImDebri)	1238 ± 47
Hill equation	$v = \frac{V_{\max} \times [S]^n}{K_{0.5} + [S]^n}$	$K_{0.5}$ (mM)	1.12 ± 0.17
		$V_{\max}$ (U/g LipImDebri)	731.65 ± 55.7
		n (Hill's coefficient)	4.4 ± 1.0

K_m Michaelis–Menten constant

$V_{\max}$ Maximum reaction rate

$K_{0.5}$ Apparent dissociation constant

$V_{\max}/K_{0.5}$ Catalytic efficiency

Table 4 Inactivation parameters (IP) for LipImDebri (cell-bound lipase naturally immobilized in cell debris obtained after ultrasound treatment)

E_d (kJ/mol)	IP	Temperature (°C)			
		30	37	45	50
0.7	k_d (h^{-1})	0.042	0.078	0.318	0.246
	$t_{1/2}$ (h)	16.5	14.44	2.18	2.82
	IP	pH			
		4	5	6	8
	k_d (h^{-1})	0.156	0.198	0.174	0.972
	$t_{1/2}$ (h)	4.44	3.5	3.98	0.71

For temperatures from 30 to 50 °C phosphate buffer (pH 7.0) was used. For pH stability, temperature was set at 37 °C

IP Inactivation parameters

E_d Activation energy for the thermal denaturation

K_d Inactivation constant

$t_{1/2}$ Half-life time

study, the value obtained was high, similar to immobilized lipases (Pereira et al. 2019; Ferreira et al. 2018).

Conclusions

Lipase immobilized in cell debris of *Yarrowia lipolytica* was successfully produced from residual frying palm oil after sonicating cells for 2 cycles, 150 W, 20 kHz and 9 min. The characterization of this biocatalyst showed low hydrophobicity, no cell viability and changes in surface characteristics in relation to whole cells. It was also shown that a stable suspension can be obtained with LipImDebri with reduced aggregation. An allosteric kinetic profile revealed that there is maybe more than one lipase isoform in LipImDebri and inactivation parameters indicate high thermostability for this biocatalyst. All these features indicate that LipImDebri is a good catalyst for industrial application.

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Data availability All data are available for transparency.

Code availability Not applicable.

Compliance with ethical standards

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