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Partial purification of crude lipase extract from *Yarrowia lipolytica*: Precipitation, aqueous two-phase systems (ATPS), and immobilization methods

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ABSTRACT

Efforts have been concentrated on developing alternative methods of enzyme purification that are less costly and highly efficient. In this work, we evaluated three different methods for lipase purification from Yarrowia lipolytica, such as precipitation using ammonium sulfate, ethanol, or acetone; aqueous two-phase systems (ATPS) based on polyethylene glycol (PEG) and potassium phosphate; and direct immobilization. It was impossible to obtain stable precipitates of the enzyme due to the low concentration of total protein and the presence of biosurfactant produced by the microorganism. Different mixture compositions were selected for the partitioning study. Three ATPS showed selective partitioning of the target enzymes, i.e., lipase and protease migrated to opposite phases. In the ATPS composed of 13 wt% PEG-4000 and 10 wt% salts, it was possible to achieve a purification factor for lipase of 4.2. Purification by immobilization performed by lipase-lipase interactions showed three lipases of distinct sizes in the crude extract. In the immobilization method by hydrophobic supports, phenyl-agarose and butyl-agarose were more selective in immobilizing than octyl-agarose. In the ion exchange immobilization method, only the lipases identified at 66 kDa and 41 kDa have an attraction for DEAE-agarose (anionic) and sulfopropyl-agarose (cationic) matrices.

1. Introduction

The yeast *Yarrowia lipolytica* is classified as "unconventional" and has been one of the most studied species in recent years regarding its physiology, genetics, molecular biology, and biotechnological applications. Such interest in this species is related to the substances naturally secreted by its metabolism and its high potential for enzyme production (lipases, proteases, phosphatases, and esterase) (Barth and Gaillardin, 1997; Botelho et al., 2020; Santos et al., 2018). Lipases are among the most used enzymes at both academic and industrial levels because of their high stability and activity under a great diversity of reaction conditions and their capability of using many different substrates (Arana-Peña et al., 2021; Rodrigues et al., 2019). Due to the high lipase activity produced by *Y. lipolytica*, this enzyme is often used for biotransformation. It is considered nonpathogenic, and several processes based on this organism were classified as generally recognized as safe (GRAS) by the Food and Drug Administration (FDA, USA) (Marcelo and Priscilla, 2016; A.I.S. Brígida et al., 2014).

Submerged fermentation is the main technique for extracellular enzyme production and has been widely used on an industrial scale. Nevertheless, lipase (due to its protein structure) can be used as substrate by the protease and lose its catalytic activity. This lipolytic degradation can occur in the fermentation process or enzyme extract storage, impeding its application as a final product. Thus, efficient downstream processing techniques to separate lipase from protease are of crucial importance for its commercial success (Ventura et al., 2011; de Souza et al., 2019). For the successful commercial production of enzymes and proteins, efficient downstream processing techniques are essential. Moreover, when these processes are applied to biological materials, rigorous purification steps, delicate enough to preserve the biological activity, are required. It is important to emphasize that the required level of purification is associated with the product's application. The impact of the total cost

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in the production process depends significantly on the type and concentration of the product obtained at the end of the cycle. Thus, studies aimed at developing and improving techniques for the extraction and purification of biocompounds have been intensifying with the increasing demand from the industrial sector (Molinho et al., 2013; Soares et al., 2015). The enzyme purification process should be chosen considering the need for a good recovery of enzyme activity combined with a high purity degree, characteristics of the lipase, and the extract obtained (Barbosa et al., 2015).

Precipitation is one of the most common purification techniques, which can remove impurities or isolate a target protein in a mixture. Among the precipitation techniques, ammonium sulfate is the most widely used. This behavior is because most proteins are precipitated at high molarity and have a density that does not interfere with the sedimentation of a wide range of these biocomposites. Thus, this technique promotes a protein concentration effect with simultaneous purification. However, precipitation is considered to have low separation resolution due to low specificity (Chaurasiya and Hebbar, 2013; Liu et al., 2021; Mariam et al., 2015).

Another effective and economically viable method of enzyme purification is aqueous two-phase systems (ATPS). Phase separation occurs by a mixture of two mutually incompatible solutes in water by which two aqueous phases are formed with different compositions (Ventura et al., 2011; Buarque et al., 2020). This is a widely known technique, biocompatible environment, high selectivity, and adaptability to continuous sample processing and retention of biological activity. The purification is based on partitioning solutes between the two phases depending on their relative affinity for each phase (Capela et al., 2020; Santos et al., 2022; Buarque et al., 2022). Polymer-based ATPS (polymer-polymer or polymer-salt) are suitable for microbial cells because of the high-water content, low interfacial tension, and a relatively biocompatible environment that can stabilize the cell (Buarque et al., 2021). The phenomenon used to separate enzymes without compromising their activity is the mixture of a polymer and a high ionic strength salt (such as polyethylene glycol (PEG)-potassium phosphate) since the salt retains a considerable amount of water (Marcelo and Priscilla, 2016). (Santos et al., 2022) studied the partitioning of lipase by extractive fermentation from *Y. lipolytica*. Lipase migrated to PEG-2000-rich phase (partition coefficient of 2.22). The microorganism's tolerance to the phase-forming components was also evaluated, where PEG (2000 and 4000) and potassium phosphate showed tolerance values of 20 and 23 wt%, respectively. It was found in the work of (De Carvalho et al., 2018) that an optimized system formed by PEG 1500 + potassium phosphate at pH 6 and 4 °C in a 4 L bioreactor was superior to traditional methods (ultrafiltration and precipitation with acetone and kaolin) in purifying crude lipase extract produced by *Yarrowia lipolytica* IMUFRJ 50,682. The lipase in this study was partitioned to the bottom phase (salt-rich phase) and reached a partition coefficient value of 19.79.

A technique that has also been widely applied is the immobilization of lipases on hydrophobic supports of low ionic strength. This method allows these enzymes to undergo interfacial activation with different hydrophobic surfaces, either by a hydrophobic protein or by another lipase. Thus, this strategy provides lipase immobilization/purification in one step (Arana-Peña et al., 2021; Rodrigues et al., 2019; de Almeida et al., 2018). Immobilization of biocatalysts is economically beneficial for the operation of continuous bioprocesses. Immobilization facilitates product separation, improves thermostability, and provides more flexibility with enzyme/substrate contact (Braga and Belo, 2013). Many interesting applications have been found for lipases; however, these applications are limited for economic reasons. Thus, immobilization is described as the most efficient and economical method to improve lipases' stability and biological activity (Alloue et al., 2008). (Cunha et al., 2008) studied the immobilization of lipase from *Y. lipolytica* by diethylaminoethyl cellulose, phosphate cellulose, an octyl-agarose adsorption method, octadecyl-agarose, and MANAE-agarose. The authors also report higher stability by octadecyl and a 97% yield. In addition, CNBr-agarose was used as a support for covalent bond immobilization, which immobilization by covalent bond led to the complete inactivation of the enzyme.

Within this context, the present work reports the purification of the crude lipase extract from *Yarrowia lipolytica* obtained by submerged fermentation. The conventional precipitation method was studied with ammonium sulfate, ethanol, acetone, and dialysis against air. In addition, purification by aqueous two-phase systems (ATPS) based on PEG + phosphate salt was also carried out. A study of the effect of PEG and salt concentration; and variation of pH, tie-line, and polymer molecular weight was carried out. Finally, the purification of lipase by direct immobilization was also studied. For this purpose, immobilization by hydrophobic interactions (octyl-agarose, butyl-agarose, and phenyl-agarose), lipase-lipase interactions, and ion exchange was performed.

2. Experimental procedures

2.1. Materials

The polyethylene glycols (4000 and 8000 g mol^{-1}), phosphate salts, and Triton X-100 were purchased from Vetec (>98% pure). The reagent p-nitrophenyl laurate, sucrose laurate, azocasein, bovine serum albumin, CNBr-agarose, and α-naphthyl acetate (>99% pure) were acquired from Sigma-Aldrich.

2.2. Microorganism

A wild strain of *Y. lipolytica* (IMUFRJ 50,682), isolated from an estuary of Guanabara Bay in Rio de Janeiro and was used in this study (Santos et al., 2018). Cells were stocked at 4 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose, and 3% agar).

2.3. Analytical methods

Lipase activity was estimated by absorbance (410 nm) in a microplate reader (Molecular Devices, SpectraMax M2e) due to the oxidation of p-nitrophenyl laurate (p-NPL) at a concentration of 0.162 mg mL^{-1} in potassium phosphate buffer (0.05 M) at pH 7.0 (A.I.S. Brígida et al., 2014).

Protease activity was determined by hydrolysis of azocasein. The 0.5% (w/v) azocasein solution was prepared in 50 mM acetate buffer, pH 5.0. 1 mL of the enzyme extract was added to 1 mL of the azocasein solution, and the mixture was incubated for 40 min at 32 °C. Then, 1 mL of trichloroacetic acid (15% w/v) was added to precipitate the protein molecules not hydrolyzed by the proteases. Afterward, the mixture was centrifuged for 15 min at 3000 rpm, and then 2 mL of the mixture was added to 2 mL of 5 M KOH (Botelho et al., 2020). The absorbance reading of the sample was performed at $\lambda = 428$ nm, and the proteolytic activity was calculated according to Eq. (1):

$$A = \frac{ABS_{sample} - ABS_{blank}}{(0.01)*(\Delta t)*V_S} \quad (1)$$

where: A = enzyme activity (U L^{-1}); Abs = absorbance; Δt = analysis time (in minutes); Vs = volume of the enzymatic solution.

The total protein content in the crude extract was determined according to (Bradford, 1976), using bovine serum albumin (Sigma-Aldrich) as standard.

2.4. Purification

2.4.1. Precipitation

Ammonium sulfate solution (25 to 90%) or ethanol or acetone was added to 15 mL of the crude extract, in an ice bath at 5 °C. This saturated solution was kept at 10 °C for 24 h. Dialysis was carried out to remove the traces of ammonium sulfate. Afterward, a cellulose membrane of 18 kDa diameter was used. Then, 20 mL of crude extract was held against air until 6 mL of concentrated extract was obtained.

2.4.2. Aqueous two-phase system (ATPS)

The mixtures compositions used in the enzyme partitioning were chosen based on the phase diagrams determined by (Glyk et al., 2014). The systems were formed by 12 to 18 wt% of PEG and 12 to 18 wt% of phosphate salt. All systems were prepared in graduated tubes with a total weight of 10 g, containing 2 mL crude lipase extract. Then, the system constituents were vigorously stirred for 5 min on a vortex mixer and centrifuged at 26,000 g for 15 min. Finally, both phases were carefully separated. Three independent replicates of each experiment were carried out.

The partition coefficient of lipase, protease, and protein was calculated as the ratio of lipase activity, protease, or protein concentration in the top phase (C_T) and bottom phase (C_B) (Eq. (2)). The specific lipase activity (U mg^{-1}) was calculated according to Eq. (3). The purification factor (PF - fold) was calculated as the ratio between the specific activity of lipase after (SA) and before (SA_i) the partitioning procedure according to Eq. (4).

$$K = \frac{C_T}{C_B} \tag{2}$$

$$SA = \frac{EA}{C_P} \tag{3}$$

$$PF = \frac{\mathrm{SA}}{\mathrm{SA_i}} \tag{4}$$

where: EA is the enzyme activity (lipolytic or proteolytic activity in U mg^{-1}) and C_P is the protein concentration in mg mL^{-1}.

2.4.3. Immobilization by hydrophobic interactions

Immobilization was performed with the butyl-, phenyl- and octyl-agarose hydrophobic supports, where the crude lipase extract was diluted in 5 mM sodium phosphate buffer pH 7. For each 1 g of support, 10 mL of lipase solution was added. The system was kept under stirring at room temperature. Thus, the immobilization was followed until the yield achieved between 90 and 100%. Finally, the activities of derivatives were measured, and an electrophoresis gel to identify the immobilized protein bands was prepared. Desorption analyses with Triton X-100 and sucrose laurate were also performed for fractional separation.

2.4.4. Immobilization by lipase-lipase interaction

Pseudomonas fluorescens lipase immobilized on glyoxyl-agarose by multipoint bonding as support was used for lipase-lipase interaction immobilization. For each 1 g of support, 10 mL of lipase solution was added. The system was kept under stirring at room temperature. Immobilization was followed until the immobilization yield was between 90 and 100%. Subsequently, the activities of the derivatives were measured, and an electrophoresis gel to identify the immobilized protein bands was prepared.

2.4.5. Immobilization by ion exchange

Immobilization by ion exchange was performed using DEAE-agarose and SP-agarose as supports. For each 1 g of support, 10 mL of crude extract was added. The system was kept under stirring and at room temperature. The immobilization was followed up to a yield of 90 to 100%. At the end of the immobilization process, the activities of the derivatives were measured, and an electrophoresis gel was used to identify the immobilized protein bands. Desorption analyses with NaCl at different concentrations at room temperature were also performed for fractional separation.

2.4.6. Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples in known concentrations were added to the buffer (glycerol 10%, β-mercaptoethanol 5%, SDS 2.3%, Tris–HCl pH 6.8 0.0625 M) in a 1:1 ratio. Posteriorly, the mixture was boiled for three minutes, centrifuged rapidly, and applied to the gel. The 12% SDS-polyacrylamide gels 1 mm thick were subjected to a current of 30 mA at a constant voltage. After the run, the gels were stained with silver nitrate according to protocols described in the literature (Oakley et al., 1980). Low molecular weight markers were used as standards.

Table 1
Specific activity of lipase and protease on ammonium sulfate precipitation.

Sample	Lipase (U mg^{-1})	Protease (U mg^{1})
Crude extract	890	0.11
50% saturation	560	0.07
75% saturation	640	0.06
White layer 50%	950	0.07
White layer 75%	1020	0.06

Table 2
Specific activity of lipase, protease, and protein content on dialysis against air.

Extract	Lipase (U mg^{-1})	Protease (U mg^{1})	Protein (mg mL^{-1})
Crude	3.78×10^5	0.10	5.12
Dialyzed	2.78×10^5	0.17	13.15

2.4.7. Zymogram

To perform the zymogram (native electrophoresis), a gel prepared as described in 2.4.6 was used. However, at 10% polyacrylamide and the absence of SDS and β-mercaptoethanol. Electrophoresis in native conditions was prepared in 1% electrolyte and a 2.5% triton x-100 sample. The determination of the activity in the sample retained in the gel occurs by the presence of a different coloration in the part of the gel where the enzyme is located when it is placed in contact with some substrate. For this purpose, three reactions were used.

The chromogenic substrate was prepared by dissolving 2 mL of p-NPL solution (0.018 g mL^{-1} dimethylsulfoxide (DMSO)) in 100 mL of pH 7 buffer containing agar (1%, w/v). The solution was homogenized and cooled to solidify. The agar containing the substrate was overlaid with the native gel and then incubated at 37 °C for 1 h. The presence of lipase was evidenced by the formation of a yellow trace in the translucent medium from the presence of p-nitrophenol released in the hydrolysis of p-NPL.

3. Results and discussion

3.1. Purification by precipitation

The purification of lipase from *Y. Lipolytica* by the precipitation method was performed using ammonium sulfate, ethanol, or acetone as precipitating agents. It was not possible to observe any precipitates at the used concentrations of ammonium sulfate; however, a superficial white layer was visualized at concentrations of 50 and 75% ammonium sulfate (Table 1). Similarly, the analyses with ethanol or acetone did not show any precipitate or superficial white layer.

Table 1 shows the specific activities in the crude extract and the surface layer formed. Thus, the specific lipolytic activity increased in the white layer while the specific activity of the protease decreased. These results were expected in extracts with low protein concentration since their high dispersion in the medium makes the molecules' aggregation process difficult (A.I.S. Brígida et al., 2014). Although the extract concentration is within the range usually used in purification processes, dialysis studies against air were performed to concentrate the proteins in the extract.

The protein concentration was increased by 2.6-fold, while a loss in specific lipase activity can be seen in the results obtained from dialysis against air (Table 2). Precipitation analysis was performed with ammonium sulfate at 50% saturation with the dialyzed extract. From Table 3, unstable precipitate formation with high specific lipase activity can be

Figures

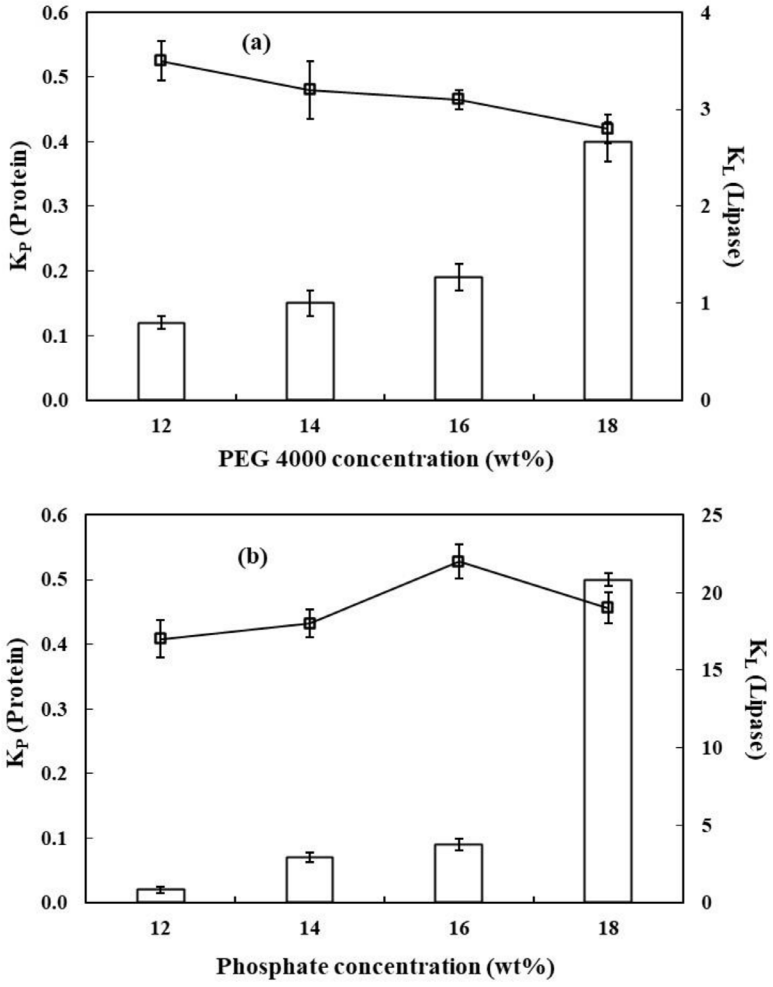

Fig. 1. Partitioning of lipase from *Y. lipolytica* (K_L, bar) and total protein (K_P, symbol - square) in ATPS based on PEG 4000 + phosphate: (a) - effect of polymer concentration and phosphate at 14 wt%; (b) - effect of salt concentration and PEG at 12 wt%.

Table 3
Specific activity of lipase, protease, and protein content on ammonium sulfate precipitation after dialysis against air.

Sample	Lipase (U mg^{-1})	Protease (U mg^{-1})	Protein (mg mL^{-1})
Dialyzed	2.78×10^5	0.175	13.15
Supernatant	1.43×10^5	0.07	8.96
Precipitate	2.00×10^6	0.08	2.32

observed. (Amaral et al., 2006) reported that this yeast strain *Y. lipolytica* IMUFRJ 50,682, could produce biosurfactants under specific conditions. Thus, for a better understanding, the emulsification index was determined, which presented a value of 65.5%, confirming the presence of biosurfactants in the extract. Verifying this in the medium can influence the aggregation of proteins, preventing them from forming stable aggregates. Thus, the use of precipitation as a purification step becomes unfeasible.

3.2. Purification using ATPS PEG-potassium phosphate

Fig. 1a shows the influence of PEG concentration on the partition of lipase from *Y. lipolytica* in a PEG-potassium phosphate system. The behavior of the lipase showed little influence by varying the concentration of PEG 4000 (12 to 16 wt%). A more expressive increase was only observed when 18 wt% of the polymer was used. It is known that the affinity of enzymes with polymers, such as PEG 4000, is directly associated with their hydrophobic character (Show et al., 2014). According to (Bassani et al., 2010), this observation may be attributed to the fact that when the concentration of this polymer increases, the PEG forms a compact and hydrophobic structure with a great capacity to interact with the active site of lipase and a high affinity for hydrophobic ligands. (Barbosa et al., 2011) observed a similar behavior of lipase from *Bacillus* sp. ITP-001. The authors reported that increasing the PEG concentration favored greater partitioning of the lipase.

In contrast, studies of the influence of potassium phosphate concentration on the partitioning phenomenon (Fig 1b) showed that the increase of the salt concentration favors the migration of lipase to the PEG-rich phase, reaching a partition coefficient of 23 (18 wt% of salt). According to (Babu et al., 2008), the solubility of biomolecules in the bottom phase decreases with an increase in salt concentration, resulting in increased lipase partitioning to the top phase. This process is known as the salting out effect. (Barbosa et al., 2011) also observed that increasing the concentration of phosphate salt promotes a higher migration of lipase from *Bacillus* sp. ITP-001. Similar behavior was also obtained by (Gulati et al., 2000) for lipase from *Aspergillus terreus* in ATPS composed of PEG 6000 and potassium phosphate. Regarding the partitioning of total proteins, it was observed that they are not influenced by the variation of polymer and salt concentration in the range studied in this work (Fig 1a and b). Thus, it is possible to verify that the higher

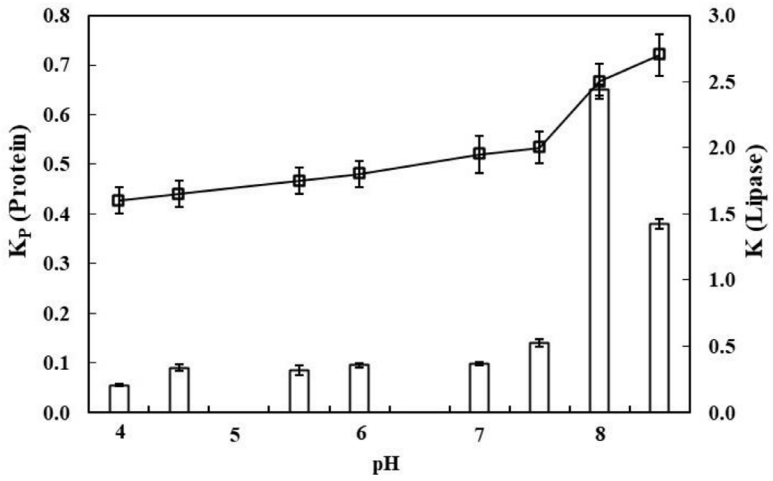

Fig. 2. Effect of pH on lipase (K_L, bar) and total protein (K_P, symbol - square) partitioning in ATPS based on 18 wt% of PEG 4000 + 20 wt% of phosphate.

the partitioning, the higher the specific activity of lipase in the top phase.

Based on these results, the PEG 4000 (18 wt%) + potassium phosphate (20 wt%) system was selected for pH variation studies. The crude extract from *Y. lipolytica* obtained in multiphase reactor produces high protease activity in the fermented broth. This protease can become a concern to the lipase production process since the lipase (due to its protein structure) can be used as substrate by the protease and lose its catalytic activity. The protease activity was carried out in each phase to verify its behavior. In the salt-rich phase, no activity of this enzyme was found. However, it is possible to observe its presence in the top phase (polymer-rich phase), as seen in Fig. S1. The system formed by 18 wt% PEG and 20 wt% potassium phosphate also showed the highest protease activities, indicating a similar trend to lipase behavior.

Concerning pH, the proteins show partition behavior broadly related to their positive or negative charges considering their isoelectric points. Generally, positively charged proteins partition to the bottom phase, while negatively charged proteins are directed to the top phase. Only the electrostatic effects are considered in the pH value close to the isoelectric point (Marcelo and Priscilla, 2016; Barbosa et al., 2011; Padilha and Osório, 2019). Fig. 2 shows the dependence of the partition coefficient of lipase and total protein on the pH of the system solution. At low pH values (4–6), lipase was preferentially partitioned in the phosphate-rich phase (bottom phase). It is known that lipase enzymes have isoelectric pH in the range of 6–7, which induces a positive charge lipase surface in pH = 6 (Marcelo and Priscilla, 2016). Such phenomenon was also observed in purification processes in systems formed by PEG + potassium phosphate of lipase *Bacillus* (Barbosa et al., 2011), lipase from *Burkholderia cepacia* (Padilha and Osório, 2019), and lipase from *Yarrowia lipolytica* IMUFRJ 50,682 (Marcelo and Priscilla, 2016).

Although a maximum value of K was observed at pH 8, at pH 8.5, a decrease in the value of the partition coefficient of this enzyme was observed. This behavior at pH values higher than the isoelectric point is usually characteristic of PEG of higher molecular weight, where the steric barriers are higher (Bassani et al., 2010). The protease activity showed higher specific activities between pH 6 and 7.5 (Fig. S2), indicating that the isoelectric point of the proteases produced during the culture of *Y. lipolytica* is lower than 6. At pH 8, lower specific protease activity was also observed in the top phase, although no activity was observed in the bottom phase.

To obtain a system in which lipase and protease are partitioned mainly to opposite phases, different mixture points in PEG-potassium phosphate ATPS were evaluated based on different tie-lines. For this purpose, PEG-based systems of molecular weight 4000 and 8000 were used at pH 7. The choice of maintaining pH 7 in the studies instead of pH 7.5 was due to the stability of the enzyme at this pH being higher than the others, thus avoiding false results by denaturation. (A.I.S. Brígida et al., 2014) reported the effect of pH on stability in lipase from *Yarrowia lipolytica*, which found that the lipase remained active at pH between 7 and 8 with an optimum point at pH 7. This result also corroborates with the study of (Destain et al., 1997), where purified lipase from *Y. lipolytica* showed higher stability at pH 7 in an active pH range between 6 and 10.

Fig. 3a shows the effect of different tie lines on the partitioning of lipase and protease to the PEG 4000-based ATPS. The partitioning profile was independent of the tie-line. No similarity could be observed in the system behaviors with increasing volume in the top phase. System A1 (see Tables S1 and S2) showed very distinct partition values of these enzymes, in which a partition to opposite phases (lipase to top phase and protease to bottom phase) can be verified. Furthermore, under these conditions, a low purification factor of lipase in the top phase (PF = 1.5) was also observed. The D3 system also showed opposite partitioning for the enzymes. However, the value of the purification factor was lower; for the other systems, lipase and protease migrated preferentially to the top phase (PEG-rich phase). C1 showed a better partition coefficient and purification factor (PF = 7) value among the systems in that the enzymes were partitioned to the same phase. The influence of different tie lines on the partitioning of lipase and protease for the PEG-8000-based system was also studied and is shown in Fig 3b. The partitioning profile showed the same behavior as PEG 4000, exhibiting no similarity between the mixture points with increasing volume in the top phase. In the A3 system (see Tables S1 and S2), it is possible to note the partitioning of the enzymes to opposite phases with a purification factor of 4.2. In addition, in the B2 system, despite the positive partition coefficient in the top phase for protease (Log $K = 0.75$), it was lower than that obtained for PEG-4000-system C1 (Log $K = 3$). Comparing the ATPS based on PEG 4000 and 8000, three systems showed partitioning of lipase and protease to opposite phases:

- 14.46 wt% of phosphate and 4.35 wt% of PEG 4000
- 14.23 wt% of phosphate and 1.07 wt% of PEG 8000
- 4.23 wt% of phosphate and 27.14 wt% of PEG 8000

Thus, the choice of which system to use will depend on the application for which it is intended. For example, the A1 (Tables S1 and S2) PEG 4000 system can be used in direct applications where the presence of PEG does not interfere, and the presence of protease must be minimal or non-existent. In situations where salt or PEG is an interfering factor, the 4.23 wt% phosphate and 27.14 wt% PEG 8000 system are best due to the low PEG concentration in the bottom phase and the easy salt removal using dialysis. Aiming for a higher degree of purity in the enzyme fraction, C1 of PEG4000/phosphate is the most suitable as it has a partition coefficient and higher purification factor.

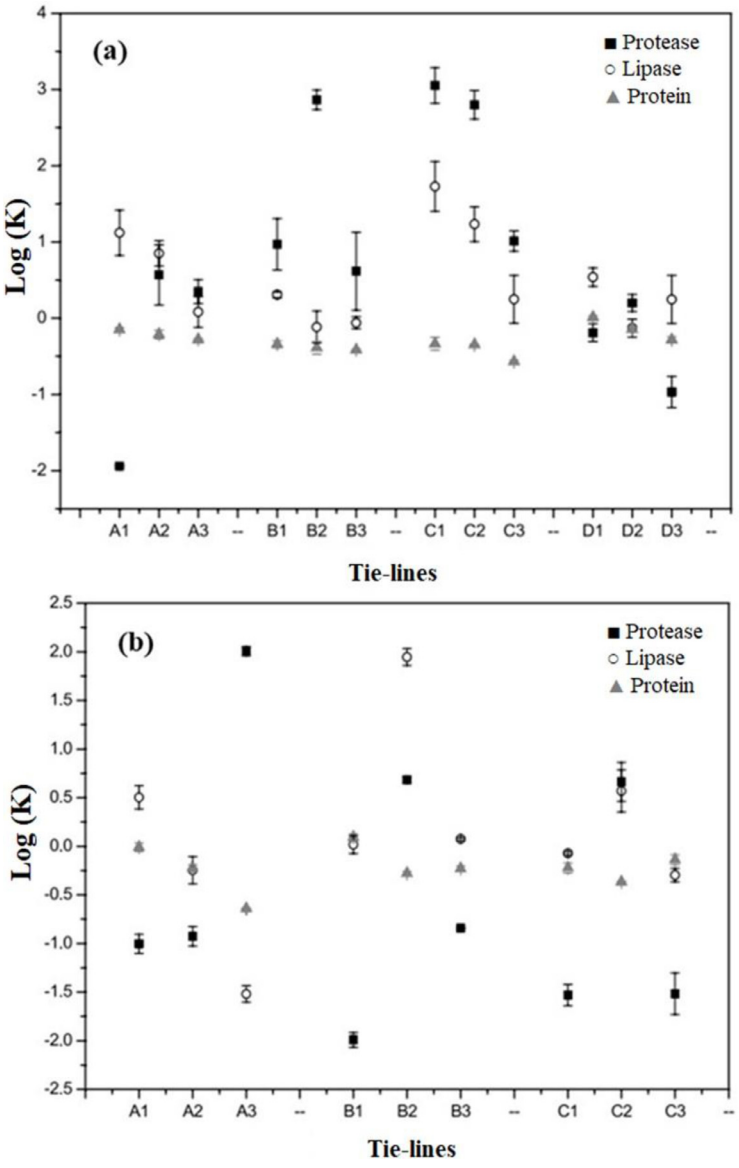

Fig. 3. Partitioning of lipase, protease, and protein in different tie-lines of ATPS formed by PEG + phosphate salt at pH 7: (a) PEG 4000 and (b) PEG 8000.

The purification factor obtained for C1 (Tables S1 and S2) of PEG4000/phosphate was slightly higher than other studies using the same ATPS constituents for lipase recovery (Bassani et al., 2010). (Marcelo and Priscilla, 2016) studied purification using ATPS-based PEG 1500 + potassium phosphate buffer (pH 6, 7, and 8) at room temperature of *Yarrowia lipolytica* lipase produced in a 4 L bioreactor (3 L culture medium). The authors report that in systems at pH 7, the proteins precipitated at the system interface. However, for pH 6, the lipase was partitioned to the salt-rich phase (PF = 1.38), whereas the protein migrated preferentially to the top phase. Nevertheless, an opposite behavior is observed at pH 8 for lipase (PF = 0.54). (Ooi et al., 2009) reported a purification factor of 12.42 lipase from *Burkholderia pseudomallei* in systems consisting of PEG 6000 + potassium phosphate buffer (pH 7) + NaCl (used as adjuvant).

3.3. Purification by immobilization

3.3.1. Immobilization by hydrophobic interaction

Lipases have a high affinity for hydrophobic supports, so immobilization studies of lipases present in the crude extract of *Y. lipolytica* on supports with different hydrophobicity strengths were performed.

A diluted crude extract solution (1.170 U L^{-1}) at pH 7 was used for immobilization by adsorption on octyl-agarose. In 1 h of adsorption, 100% immobilization yield was observed. However, the derivative showed only 476 U kg^{-1} and a recovered activity of 28.5%. This low activity is probably due to a strong interaction of the enzyme to the support (causing deformation in the active site) or immobilization promoting steric hindrance. The yield in this work was higher than that reported by (Cunha et al., 2008) during the immobilization of Lip2 lipase from *Y. lipolytica* on octyl-agarose (70%). Column 2 of Fig. 4 shows the profile of the proteins immobilized on the octyl-agarose derivative. Thus, it was expected to find only bands immobilized near 40 kDa (molecular mass value of the lipases reported for *Y. lipolytica*). However, other bands were also observed, such as protease, which could be other proteins excreted by the yeast. The immobilization of other bands, besides the band of interest, in immobilizations from crude lipase extract in octyl-agarose was also recently reported (Volpato et al., 2010).

Desorption analyses with different concentrations of Triton X-100 and sucrose laurate were performed to obtain a fractional separation of the bands. Most bands were observed when 0.2% Triton X-100 or sucrose laurate was used, with the observation of only 4 bands (Fig. 4, column 3). In addition, this compound at 0.2% and 0.5%, and 1.5%

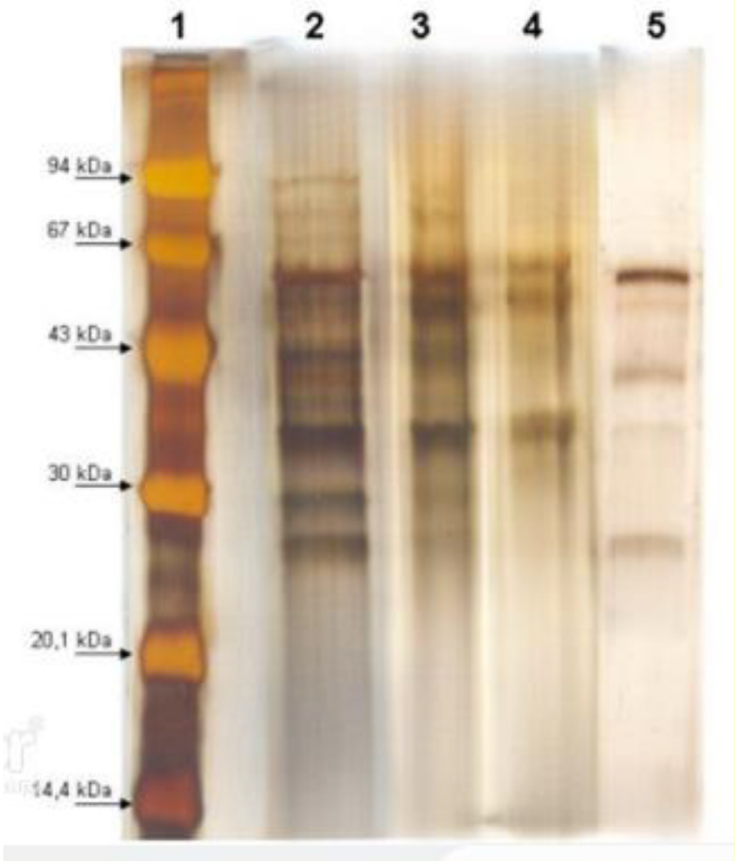

Fig. 4. Electrophoresis of the derivatives obtained after immobilization and desorption of lipase in octyl-agarose. (1) Protein marker; (2) Derivative obtained from immobilization of *Y. lipolytica* extract in octyl-agarose; (3) Derivative (2) after sequential desorption with 0.2 and 0.5% (v/v) triton x - 100; (4) Derivative (2) after sequential desorption with 0.2 and 0.5% (v/v) triton x – 100, and 1.5% (w/v) sucrose laurate; (5) Derivative obtained from immobilization of *Y. lipolytica* extract in octyl-agarose in the presence of 0.1% triton x – 100.

sucrose laurate were used sequentially to eliminate the 43 kDa band. The activity of the desorbed lipases could not be quantified since the detergent concentration was at values that promote the inhibition of lipases from *Y. lipolytica*.

Triton X-100 was used in the immobilization process as an additive. This detergent was studied in an aqueous solution of crude extract of *Y. lipolytica* with lipolytic activity values of 2060 U L^{-1}. The yield obtained in the immobilization process was 98.7% after 1 h. However, the immobilized exhibited 224 U kg^{-1} of recovered activity. Thus, it can be stated that this process favored the immobilization of the band near 60 kDa (LYL2) (Fig. 4, column 4). These results showed the need to quantify the number of lipases in the extract. In the analyses of the purification studies, a zymogram of this extract using tributyrin as substrate was performed, and only one positive band was observed (Fig. S3a). However, another zymogram was performed using α-naphthyl acetate as substrate with the purified extract (Fig. S3b). Thus, it was possible to observe the presence of 3 active bands. Since it cannot be stated that the bands visualized refer to the last 3 bands observed (Fig. 4), studies aimed at a fractional separation of these bands should be performed to identify and characterize them. From octyl-agarose, it was only possible to obtain two fractions: one rich in the band between 30 and 40 kDa (LYL1) and another rich in the two bands near 60 kDa (LYL2).

The lipases were immobilized in CNBr-agarose by one-pot ligation and characterized their activity profile against substrates of different sizes to obtain their characteristics. The derivatives obtained were compared with other commercial enzyme derivatives (Fig. S4). The fraction LYL1 (bands near 40 kDa) showed a minor difference between the activity values obtained for p-nitrophenyl laurate (p-NPL) and p-nitrophenyl palmitate (p-NPP). The two products obtained from purified fractions showed different ratios than that obtained for the crude extract. This result may be related to the substrate partition with the support and/or the proportion of active lipases. Since it was impossible to completely desorb the lipases present in the crude extract of *Y. lipolytica*, studies with immobilization on butyl-agarose were performed. As shown in Fig. 5, the only bands in the derivative are those near 60 kDa. However, desorption experiments and immobilization of the supernatant obtained from the desorption showed that the other bands are in smaller proportions. Furthermore, visualization of the adsorbed enzymes is obtained by desorption in electrophoresis breakthrough buffer, so it is possible that they are firmly bound and do not desorb even under denaturing sample preparation conditions for SDS-PAGE. Despite the questions about the bands present, the derivative obtained showed, for 2090 U L^{-1} offered, 3100 U kg^{-1} of activity in an immobilization process with 91.9% yield and 242% of recovered activity.

The immobilization behavior on phenyl-agarose of lipases from *Y. lipolytica* obtained from the crude extract was also evaluated. On this support, a profile like that found in the octyl derivative after desorption with 0.2% Triton X-100 was obtained (Fig. 6). Desorption with 0.5% Triton X-100 showed a derivative with 2 bands close to 60 kDa, making it an excellent alternative to isolating these proteins. As for the immobilization process, from 2090 U L^{-1} offered, a derivative of 793 U kg^{-1} was obtained with a 99.2% yield and 57.3% recovered activity. Although with lower values than those obtained with butyl, phenyl-agarose proved more efficient than octyl-agarose in getting a mixed derivative for direct application and as a matrix for separation.

Some recent studies support our choice of using the surfactant Triton X-100 for the immobilization of lipases, (Ramakrishna et al., 2021) evaluated the effect of the anionic surfactant Triton X-100 on the immobilization of lipase from *Pseudomonas fluorescens*. The Triton X-100 was able to significantly improve the substrate activity and selectivity of immobilized lipases by interacting with the free lipase, altering the secondary structure of the lipase. (Choudhary et al., 2023) studied the using Triton X-100–0.81% (v/v) in lipase production of *Pseudomonas plecoglossicida S7*, which a 2.81-fold increase in lipase values was ob-

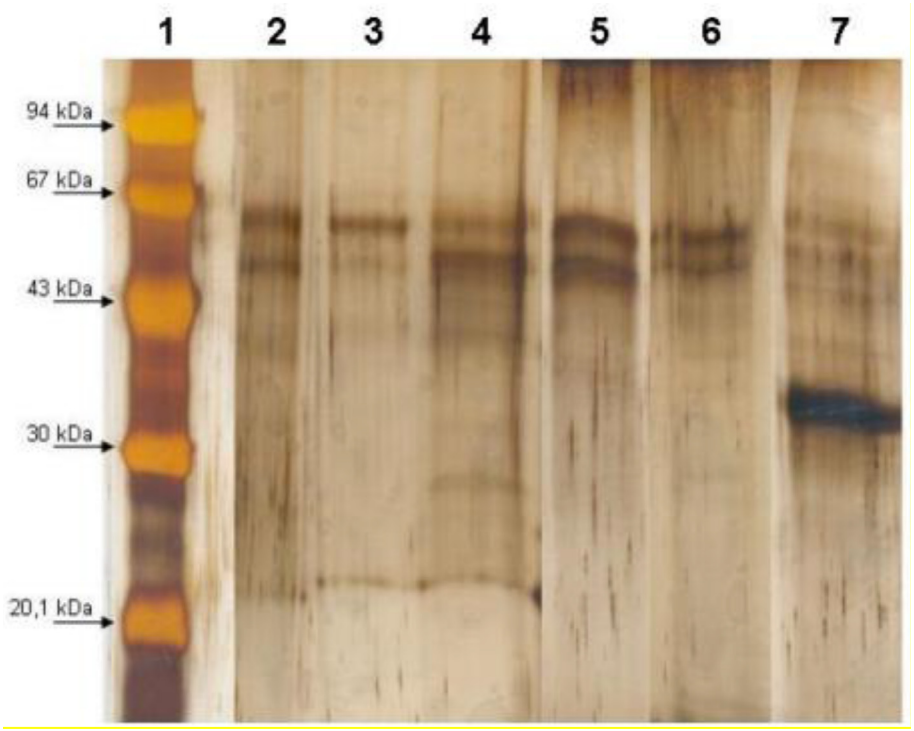

Fig. 5. Electrophoresis of the derivatives obtained after immobilization and desorption of lipase in butyl-agarose. (1) Protein marker; (2) Derivative obtained from immobilization of *Y. lipolytica* extract in butyl-agarose; (3) Supernatant of the desorption of derivative (2) with 1% (v/v) sucrose laurate; (4) Derivative (2) after desorption with 1% (v/v) sucrose laurate; (5) Derivative (2) after desorption with 2% (v/v) sucrose laurate; (6) Derivative (2) after desorption with 2% (v/v) triton x - 100; (7) Derivative obtained from immobilization of the supernatant (3) in butyl-agarose.

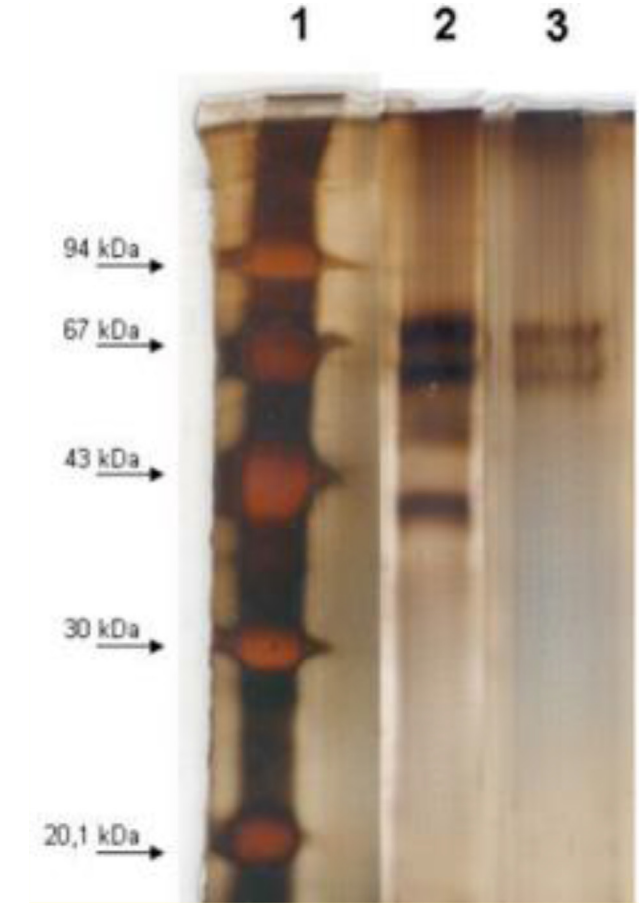

Fig. 6. Electrophoresis of the derivatives obtained after immobilization and desorption of lipase in phenyl-agarose. (1) Protein marker; (2) Derivative obtained from immobilization of *Y. lipolytica* extract in phenyl-agarose; (3) Derivative (2) after desorption with 0.5% (v/v) triton x – 100.

served with addition of the surfactant. (Ferreira Gonçalves et al., 2021) observed an increase in lipolytic activity in immobilized porcine pancreatic lipase extract by hydrophobic adsorption using the surfactant Triton X-100. (Sánchez-Otero et al., 2022) noted that immobilization of *Geobacillus thermoleovorans* lipase in the presence of Triton X-100 led to improved lipolytic activity but decreased thermal stability, probably due to an enzyme conformational change.

3.3.2. Immobilization by lipase-lipase interaction

Although the zymogram showed the presence of 3 lipases in the crude extract of *Y. lipolytica*, the immobilization results of octyl, butyl, and phenyl make doubt that these are representative bands. Thus, an immobilization by lipase-lipase interaction was performed. Lipases are known to form agglomerates through adsorption between hydrophobic sites on their surface (Palomo et al., 2006). Therefore, the immobilization of lipases from a crude extract with another lipase immobilized by multipoint linkages to a matrix ensures that the obtained derivative will have only lipases.

The immobilization time of 0.5 h was sufficient to obtain a derivative with 1180 U kg^{-1} of activity in a yield of 96% and 145.7% of recovered activity. The electrophoresis of the derivative (Fig. 7) showed the 3 bands referring to lipases present in the extract of *Y. lipolytica*, which presented molecular masses of approximately 38 kDa, 41 kDa, and 66 kDa. An extracellular lipase of 38 kDa was reported by (Yu et al., 2007), which was purified by ion-exchange chromatography on FF Sepharose Q, followed by hydrophobic interaction chromatography on an FF Sepharose butyl matrix. Its affinity for the butyl group was also confirmed in immobilization studies on butyl-agarose (Fig. 5, column 7). (Song et al., 2006) also reported the purification of two *Y. lipolytica* extracellular lipases of 41 kDa, with distinct characteristics of optimum temperature and specificity. As for the 66 kDa lipase, to the present time, no work has been found reporting it, and the molecular mass of *Y. lipolytica* lipases is reported to be between 38 and 41 kDa.

Despite immobilizing the three lipases identified by zymogram with immobilization on LPF-glioxyl-agarose, (Volpato et al., 2010) reported different immobilization selectivity, which depended on the lipase used on the support. From a crude extract of Staphylococcus warneri, vari-

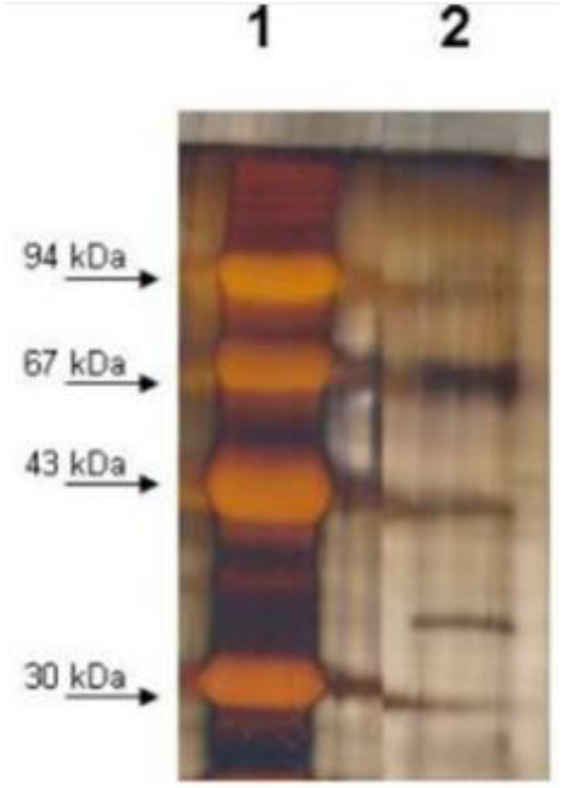

Fig. 7. Electrophoresis of the derivatives obtained after immobilization of lipase from *Y. lipolytica* in lipase of *Pseudomonas fluorescens*-glyoxyl-agarose. (1) Protein marker; (2) Derivative obtained from immobilization of *Y. lipolytica* extract in phenyl-agarose.

ous derivatives were obtained. For example, the authors reported that BTL2 immobilized on glyoxyl-DTT showed selective immobilization for only one lipase of 30 kDa. In parallel, 2 lipases of 28 and 40 kDa were immobilized on PFL-glyoxyl. Despite the high cost of lipase-based support, this process is quite promising in cases where immobilization is selective for a single enzyme.

3.3.3. Immobilization by ion exchange

In searching for selective support for the separation of the lipases in the *Y. lipolytica* extract, immobilization analysis by ion exchange was also performed on ionic character matrices, DEAE-agarose (anionic) and sulfopropyl-agarose (cationic). Fig. 8 shows the profile of the proteins immobilized on DEAE and SP-agarose after 1.5 h of contact. In the DEAE matrix, a more intense coloration of the bands is observed with increasing contact time. Despite 4 well-defined bands, traces of 2 other bands were observed. In the SP-agarose matrix, 5 well-defined bands were observed. Among the 3 lipases in the extract, only the lipases identified at 66 kDa and 41 kDa were attracted to the matrices. Furthermore, desorption analysis efficiently removed most of the proteins, except for the two bands near 66 kDa.

Immobilization in ion exchange matrices may promote easier desorption. However, it was observed that the amount of enzyme immobilized in ion exchange matrices (based on supernatant activity values) is lower compared to hydrophobic matrices (Fig. S5). In addition, variations in the derivative activity values were high. However, the profile of the immobilized proteins does not change (Fig. 8). The variations in the activities of derivatives observed with time are typical of systems that are not in equilibrium. Immobilization studies are needed at longer contact times. These data diverge from those followed by (Cunha et al., 2008) in Lip2 extract from *Y. lipolytica* when immobilized in MANAE-agarose. In this study, the time required to immobilize 97% of the offered enzymes was 0.5 h. At longer times, the system was observed to be in equilibrium, and no variations in the activity of the supernatant or the derivative were found.

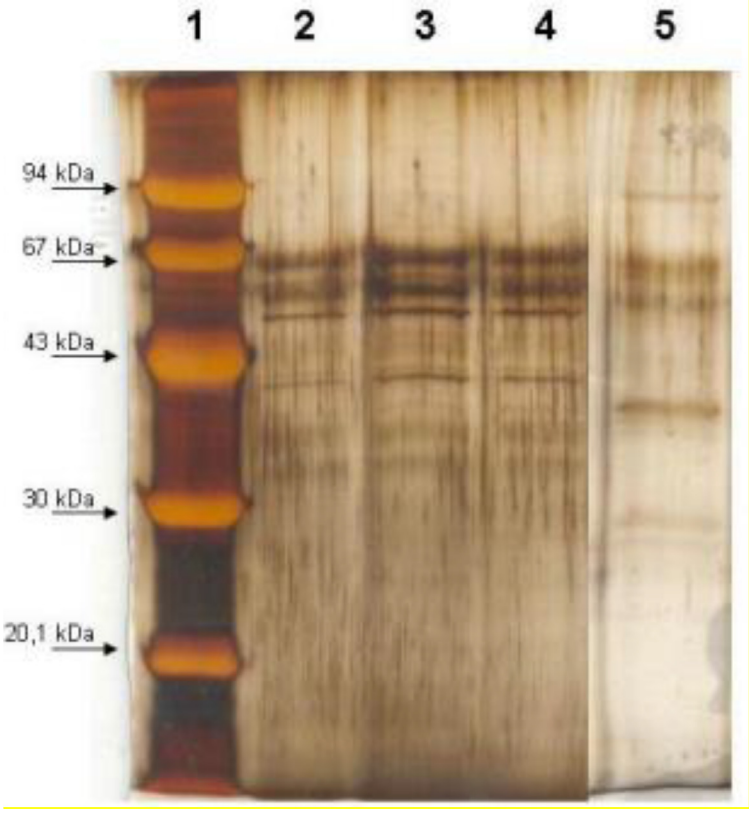

Fig. 8. Electrophoresis of the derivatives obtained after immobilization and desorption of lipase in DEAE-agarose. (1) Protein marker; (2) Derivative obtained from immobilization of *Y. lipolytica* extract in DEAE-agarose, 0.5 h of immobilization; (3) Derivative obtained from immobilization of *Y. lipolytica* extract in DEAE-agarose, 1.0 h of immobilization; (4) Derivative obtained from immobilization of *Y. lipolytica* extract in DEAE-agarose, 1.5 h of immobilization; (5) Derivative obtained from immobilization of *Y. lipolytica* extract in sulfopropyl-agarose, 1.5 h of immobilization.

4. Conclusion

The partial or total separation of lipases from *Y. lipolytica* was evaluated by different methods, precipitation with ammonium sulfate and dialysis against air, ATPS-based PEG-potassium-phosphate, and direct immobilization. The low total protein concentration in the obtained extract and the presence of biosurfactant (produced by the microorganism under lipase production conditions) did not form stable precipitates, so the method is not recommended for this extract as a purification step. The ATPS composed of PEG 4000 + potassium phosphate showed optimal partitioning values and purification factors. However, these values were obtained in a pH range greater than or equal to 7, in which lipase offers low stability, limiting the use of ATPS in this pH range. It was observed that in 3 systems, the lipases and proteases were partitioned into opposite phases. Each scenario presents a different behavior. The choice of which system to use will depend on the final application for which it is intended. In the immobilization purification studies, it was possible to identify 3 lipases of distinct sizes in the crude extract through zymogram analysis and lipase-lipase interaction. Thus, from a comparison between the 3 hydrophobic supports used, phenyl-agarose and butyl-agarose were the most selective in immobilizing the lipases compared to octyl-agarose. Furthermore, it was impossible to find detergent concentrations where we had desorption of only 1 lipase since the 38 and 41 kDa lipases always desorbed at close concentrations. The lipase of approximately 67 kDa remained immobilized even after 2% detergent in the three supports, which shows a strong interaction. Regarding the 3 lipases in the extract, only the lipases with molecular weights of 66 kDa and 41 kDa are attractive for DEAE-agarose (anionic) and sulfopropyl-agarose (cationic) matrices.

Ethical approval

This article does not contain any studies on human or animals.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Filipe Smith Buarque reports financial support was provided by Carlos Chagas Filho Foundation for Research Support of Rio de Janeiro State. Maria Alice Zarur Coelho reports financial support was provided by National Council for Scientific and Technological Development. Maria Alice Zarur Coelho reports was provided by Coordination of Higher Education Personnel Improvement.

CRediT authorship contribution statement

Ana I.S. Brígida: Conceptualization. **Filipe S. Buarque:** Writing – original draft, Writing – review & editing. **Vanessa L.R. Nogueira:** Methodology. **Vânia M.M. Melo:** Methodology. **Jose M. Guisán:** Formal analysis, Investigation. **Bernardo D. Ribeiro:** Formal analysis, Investigation. **Luciana R.B. Gonçalves:** Supervision. **Maria A.Z. Coelho:** Supervision.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.clce.2023.100105.

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