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Deep eutectic solvents as modulators of *Yarrowia lipolytica* lipase activity

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ABSTRACT

The use of deep eutectic solvents (DES) as co-solvents offers a promising strategy for modulating the biocatalytic activity of enzymes. This study explored the DES effects on the activity of *Yarrowia lipolytica* lipase (YlLip2). Both experimental analysis and molecular docking simulations were employed. The experimental results indicated that the lipolytic activity is significantly influenced by the chain length of the ammonium-based hydrogen bond acceptor (HBA) and the fatty acid chain length of the hydrogen bond donor (HBD). The exception was tetraoctylammonium chloride ($[N_{8881}]Cl$) which improved the activity values compared to the control, and its combination with hexanoic acid showed similar values. According to molecular docking studies, $[N_{8881}]^+$ exhibited the highest of affinity energy with the YlLip2 structure, suggesting a strong interaction potential. None of the HBA/HBD combinations directly interacted with the amino acids forming the catalytic triad of YlLip2. However, significant interactions were found with the allosteric sites. DES components with higher affinity energies also showed more interactions with the allosteric site. Particularly, $[N_{8881}]Cl$ was unique in exhibiting hydrophobic interactions with residues His49 and Phe50, which are part of the allosteric site. These specific interactions might be responsible for the observed activating effect on YlLip2's catalytic activity in the DES presence.

1. Introduction

Lipases (EC 3.1.1.3) are enzymes found across a vast array of living organisms and are members of the α/β-hydrolase structural family [1,2]. These enzymes exhibit tremendous biotechnological potential owing to their remarkable catalytic versatility. Lipases are capable of catalyzing a wide range of chemical reactions, including the complete or partial hydrolysis of triacylglycerols and various lipid modifications through esterification, interesterification, and transesterification [3–5]. The growing interest in enhancing lipase production aligns with its expansive application across numerous industrial sectors, such as food processing, pharmaceuticals, detergents, wastewater treatment, and bioenergy. Moreover, lipases exhibit significant regioselectivity and enantioselectivity, overcoming traditional chemical catalysts [6,7]. In industrial applications, lipases are extensively utilized, and their market value reflects their broad utility. As of 2023, the enzyme market, including lipases, was valued at approximately USD 613 million. Projections indicate a significant growth trajectory, with the market expected to nearly triple to USD 1.63 billion by 2033, growing at a compound annual growth rate (CAGR) of 10.3% [8]. This surge underscores the increasing demand and expanding applications of lipases in various commercial processes.

Microbial lipases hold greater industrial value compared to their plant or animal counterparts due to their variety of catalytic activities available, high yield production, simplicity of genetic manipulation, absence of seasonal fluctuations, regular supply, and more stability. Furthermore, microbial lipases are typically safer and more convenient to handle, and microorganisms grow rapidly in economically viable media [9]. Given this context, the yeast *Yarrowia lipolytica* emerges as a highly promising biotechnological candidate due to its exceptional ability to secrete enzymes with potent lipase activity. Among these, Lip2 stands out as the most extensively studied enzyme of *Y. lipolytica*. It is renowned for its high extracellular production and exhibits relevant catalytic properties, including high substrate selectivity and remarkable

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stability across a broad range of pH and temperature [10–13]. Lipase applications from *Y. lipolytica* as biocatalysts have been reported in the area of food, such as flavor synthesis [14], in the lipolyzed milk fat production [15], in the cheese and sausage maturation process, and even in the treatment of fat-rich effluents from the food industry [16].

The extensive application of lipases in industrial processes has spurred a surge in studies focused on their activity and stability in the presence of different solvents. Recent research has highlighted the benefits of using deep eutectic solvents (DES) as solvents or additives to enhance lipase-catalyzed reactions. These studies suggest that DES can significantly improve the efficiency and outcomes of lipase-mediated processes [17,18].

DESs are a mixture of hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) containing anionic and/or cationic species (e.g., ammonium or phosphonium salts) mixed with non-ionic species. The great interest in DES results from their unique features, such as low volatility, low vapor pressure, high thermal stability, and non-flammability. Some physicochemical properties can be easily adapted by a suitable HBD/HBA design and different proportions [19–22].

DESs can change the substrate's solubility, increasing enzyme activity, selectivity, and stability. However, due to the high number and variability of these solvents, their effects on enzyme-based processes are unpredictable [23,24]. Some studies have highlighted the potential of choline chloride (ChCl)-based DESs in lipase activation and stabilization [25–28]. In contrast, other research has indicated that certain DESs can adversely impact enzymatic performance [18,29,30]. Notably, despite extensive research, there is a gap in the literature concerning the influence of DESs on the production and activity of lipase from *Yarrowia lipolytica*. Furthermore, since lipase stabilities and biological activities depend on the corresponding source, specific studies are always necessary for each microbial source [31].

In the context of bioprocess applications, DES/water systems are relevant for lipase-catalyzed reactions in which enzyme hydration, substrate solubility, and reaction equilibrium must be simultaneously controlled. In these media, water helps preserve the active enzyme conformation, while DES components can modulate the local solvent environment and influence catalytic performance. Such systems may be useful in hydrolysis, esterification, and transesterification processes, as well as in integrated extractive schemes involving hydrophobic substrates. Therefore, understanding how DES constituents affect YlLip2 activity is an important step toward the rational design of greener and more efficient reaction media for future bioprocess applications [25–28].

Given the limited studies on and the significant biotechnological relevance of lipases from *Yarrowia lipolytica*, this study aims to evaluate the impact of DES based on ammonium salts combined with fatty acids as co-solvents on lipase production and activity. Specifically, the ammonium-based hydrogen bond acceptors (HBAs) used in this study include tetraoctylammonium chloride ([N_{8888}]Cl), methyltrioctylammonium chloride ([N_{8881}]Cl), methyltrioctylammonium bromide ([N_{8881}]Br), tetrabutylammonium chloride ([N_{4444}]Cl), tetrabutylammonium bromide ([N_{4444}]Br), and choline chloride (ChCl). The chosen hydrogen bond donors (HBDs) are hexanoic acid, octanoic acid, and decanoic acid.

In order to provide insights into the molecular mechanisms involved in the effects of these DES on lipase activity, molecular docking studies were employed. Molecular docking is a computational method used to predict the binding affinity and mode of interaction between a receptor-ligand complex, providing valuable information about the molecular interactions that influence biocatalytic processes. Through this approach, the preferential binding and selectivity of the lipase towards different DES combinations can be predicted. These insights are essential as it allows for the rapid and efficient development of optimized biocatalytic reactions, enhancing our understanding of how DESs can modulate enzyme activity at a molecular level [21,32].

2. Materials and methods

2.1. Materials

A wild strain of *Y. lipolytica* (IMUFRJ 50682), isolated from an estuary of Guanabara Bay in Rio de Janeiro, was used in this study [33]. Cells were stocked at 4 °C in YPD medium (1% yeast extract, 2% peptone, 2% glucose, and 3% agar). The DES constituents, [N_{8888}]Br, [N_{8881}]Cl, [N_{8881}]Br, [N_{4444}]Cl, [N_{4444}]Br, and [Ch]Cl, as well as the hexanoic, octanoic, and decanoic acids, were supplied by Sigma-Aldrich with a purity > 98 wt%. The culture medium components used were bacterial peptone, purchased from Oxoid; yeast extract, purchased from Sigma-Aldrich; and glucose (purity >98%), supplied by Vetec.

2.2. Preparation of DES

All DES studied here were formed using the heating method, where specific hydrogen bond acceptors (HBAs) ([N_{8888}]Br, [N_{8881}]Cl, [N_{8881}]Br, [N_{4444}]Cl, [N_{4444}]Br, ChCl) were combined with hydrogen bond donors (HBDs) (hexanoic acid, octanoic acid, and decanoic acid) in a 1:1 molar ratio. The components were mixed in sealed glass vials and subjected to continuous heating at 80 °C while being stirred for one hour [19,34]. Then, each DES was diluted to a concentration of 20 wt% in aqueous solutions. The 20 wt% DES concentration was selected as a fixed comparative condition based on the biological tolerance of *Y. lipolytica* to DES-containing media. Previous toxicity assays performed in our group (unpublished data) indicated that higher DES concentrations caused marked inhibition of biomass growth, thereby compromising lipase production. Therefore, this composition was adopted as a compromise between enabling the assessment of DES

Table 1
Abbreviations and composition of prepared DESs.

DES	HBA	MM (g mol^{-1})	HBD	MM (g mol^{-1})	Molar ratio
[N_{8888}]Br:HexA	[N_{8888}]Br	546.79	Hexanoic acid	116.16	1:1
[N_{8888}]Br:OctA	[N_{8888}]Br	546.79	Octanoic acid	144.21	1:1
[N_{8888}]Br:DecA	[N_{8888}]Br	546.79	Decanoic acid	172.26	1:1
[N_{8881}]Cl:HexA	[N_{8881}]Cl	404.16	Hexanoic acid	116.16	1:1
[N_{8881}]Cl:OctA	[N_{8881}]Cl	404.16	Octanoic acid	144.21	1:1
[N_{8881}]Cl:DecA	[$_{N8881}$]Cl	404.16	Decanoic acid	172.26	1:1
[N_{8881}]Br:HexA	[N_{8881}]Br	448.61	Hexanoic acid	116.16	1:1
[N_{8881}]Br:OctA	[N_{8881}]Br	448.61	Octanoic acid	144.21	1:1
[N_{8881}]Br:DecA	[N_{8881}]Br	448.61	Decanoic acid	172.26	1:1
[N_{4444}]Cl:HexA	[N_{4444}]Cl	277.92	Hexanoic acid	116.16	1:1
[N_{4444}]Cl:OctA	[N_{4444}]Cl	277.92	Octanoic acid	144.21	1:1
[N_{4444}]Cl:DecA	[N_{4444}]Cl	277.92	Decanoic acid	172.26	1:1
[N_{4444}]Br:HexA	[N_{4444}]Br	322.37	Hexanoic acid	116.16	1:1
[N_{4444}]Br:OctA	[N_{4444}]Br	322.37	Octanoic acid	144.21	1:1
[N_{4444}]Br:DecA	[N_{4444}]Br	322.37	Decanoic acid	172.26	1:1
ChCl:HexA	ChCl	139.62	Hexanoic acid	116.16	1:1
ChCl:OctA	ChCl	139.62	Octanoic acid	144.21	1:1
ChCl:DecA	ChCl	139.62	Decanoic acid	172.26	1:1

effects and preserving microorganism viability. The components and their respective molar ratios used to prepare the DESs are shown in Table 1.

2.3. Lipase production

To cultivate an inoculum, yeast cells were initially grown in 500 mL Erlenmeyer flasks containing 200 mL of YPD medium (1% yeast extract, 2% peptone, and 2% glucose). The flasks were incubated at 28 °C on a rotary shaker set to 160 rpm for approximately 72 h. Post-cultivation, the concentration of yeast cells was measured by optical density at 570 nm [12]. Subsequently, 1 g p.s. L^{-1} of yeast biomass was resuspended and transferred into fresh YPD medium (20 mL) enriched with 20 wt% of DES. The lipase production was maintained in 50 mL Erlenmeyer flasks and incubated for cell growth at 28 °C with agitation at 250 rpm for 72 h. To ensure sterility, all experimental materials and culture media were autoclaved at 1 atm for 25 min.

2.4. Lipolytic activity

Lipase activity was quantified by spectrophotometric detection (410 nm) of the hydrolysis of p-nitrophenyl laurate (p-NFL) at a concentration of 0.162 mg mL^{-1} in potassium phosphate buffer (0.05 M) at pH 7.0. The assay was monitored at 37 °C in a microtiter plate reader for 10 min. Samples (20 μL) were incubated in the microtiter plate for five minutes, and the reaction was started by adding 180 μL of p-NFL solution [12]. The activity was determined according to Eq. (1):

$$A = \frac{(\Delta Abs) * D * f * V_r}{(\Delta t) * V_S} \quad (1)$$

where A = enzyme activity (U L^{-1}): is defined as the amount of enzyme able to produce 1μmol of p-nitrophenol per minute at pH 7 and 37 °C; ΔAbs = absorbance variation in the time interval Δt (in minutes); D = dilution of the enzymatic solution; f = conversion factor is the angular coefficient obtained from the p-nitrophenyl standard curve (p-NF); Vr = reaction volume; Vs = volume of the enzymatic solution.

2.5. Structural analysis of lipase from Yarrowia lipolytica (YlLip2)

The amino acids Ser162, Asp230, and His289 form the catalytic triad of YlLip2, responsible for its lipolytic activity. This identification was founded on the detailed study by [35], which elucidated the structure of the *Yarrowia lipolytica* lipase and highlighted the importance of these three residues in enzymatic catalysis.

The Protein Allosteric Sites Server (PASSer), a molecular dynamics model that meticulously analyzes the dynamic behavior and structural characteristics of the target protein, was selected to identify potential allosteric regions. PASSer integrates three sophisticated machine learning approaches: an ensemble model that combines Extreme Gradient Boosting (XGBoost) with a Graph Convolutional Neural Network (GCNN); an automated machine learning framework powered by AutoGluon from Amazon Web Services (AWS); and a classification model (LTR) for ranking allosteric sites. This ranking is facilitated by a pre-trained LightGBM algorithm, which classifies potential pockets based on their likelihood of being the primary allosteric sites [36–38].

2.6. Molecular docking

The structure of the lipase enzyme (PDB ID: 3o0d) was obtained from the Protein Data Bank (PDB). To accurately prepare the enzyme for computational studies, the protonation states of titratable residues were calculated using the Protein Prepare tool available on the PlayMolecule web server (playmolecule.org) [39]. This step was performed at pH 7.4, with all water molecules and ligands from the original PDB file being removed to ensure a clean structure for further analysis. For ligand preparation, HBA and HBD, rigid roots and 3D atomic coordinates were generated using Discovery Studio v21 (Accelrys, San Diego, CA, USA) and applied to the Chem3D-MM2 protocol for energy minimization to create the ligand files [40]. Then, AutoDockTools (ADT) [41,42] was used to convert all ligand into input files (.pdbqt), setting all possible rotatable bonds defined as active by torsions, and convert the YlLip2 PDB file into.pdbqt input files. The coordinates at the center of the grid box (x-, y-, and z-axes) were 3.271 × -0.796 × 2.263. The grid dimension was 120 Å × 110 Å × 110 Å to cover the whole YlLip2 structure. The model with the lowest docking score was obtained using the following configuration: exhaustiveness = 100, num_modes = 10, and seed = 100.

3. Results and discussion

The studies by van Osch et al. [43], Crespo et al. [44] and Florindo et al. [45,46] described the characterization and fundamentals of a set of hydrophobic DES. These studies demonstrated, through melting point, viscosity, density, FTIR NMR, and water stability measurements, that such mixtures exhibit eutectic behavior, with significant melting point depression and the formation of hydrogen bond networks between the carboxyl group of the fatty acid and the ammonium cation.

From a physicochemical perspective, these systems have densities between 889 and 942 $kg{\cdot}m^{-3}$ and moderate viscosities (173–783 mPa·s), values lower than those of hydrophilic DES. Furthermore, it has been demonstrated that the hydrophobic nature of these systems is directly related to the size of the alkyl chain of the ammonium cation. Salts such as [Ch] show a higher tendency to leach into the aqueous phase, while more hydrophobic salts, like $[N_{8888}]^+$, maintain high stability and minimal transfer of components to the aqueous phase. This difference also explains the increased efficiency of $[N_{8888}]^+$-based systems in the extraction of nonpolar compounds. Thermodynamic modeling based on solid-liquid equilibrium diagrams and described by equations such as NRTL and PC-SAFT reinforces the low non-ideality of these liquids and the combined contribution of hydrophobic interactions and hydrogen bonds to their formation. These physicochemical characteristics, associated with low miscibility in water and structural versatility, justify the choice of these DES as hydrophobic green solvents suitable for evaluating modulating effects on biocatalysts [44].

Regarding choline chloride-based DES, the reported results [47] demonstrate that the formation of eutectic liquid is driven by strong interactions between the chloride anion and the carboxyl group of fatty acids, with stability increasing progressively from acetic acid to long-chain acids such as decanoic, dodecanoic, and tetradecanoic acids. However, the addition of water progressively alters the intermolecular organization and physicochemical properties of these media. Recent studies on choline chloride-carboxylic acid systems further showed that water content strongly affects viscosity, density, conductivity, and hydrogen-bond organization, indicating that the significance of water addition in these systems differs significantly from that observed for hydrophobic ammonium-based media [48]. Therefore, under the present assay condition (20 wt% DES in water), ChCl-based mixtures are more appropriately interpreted as aqueous ChCl-based HBA/HBD systems rather than as DESs preserved in the strict structural sense.

For some DES systems, specifically those based on $[N_{8888}]$Br, $[N_{8881}]$Cl, $[N_{8881}]$Br, $[N_{4444}]$Cl, and $[N_{4444}]$Br combined with fatty acids, biphasic DES-water systems are formed. However, previous results from our group [10–13] demonstrated preferential partitioning of extracellular *Y. lipolytica* lipase to the more hydrophobic quaternary ammonium-rich phase, indicating that exclusive localization of the enzyme in the aqueous phase is not supported for hydrophobic ammonium-based media. Accordingly, the hydrophobic ammonium-based systems and the ChCl-based mixtures should not be discussed as equivalent media under the present aqueous condition. For the former, a DES-rich hydrophobic phase is maintained; for the latter, the observed effects should be interpreted as arising from diluted

aqueous HBA/HBD environments acting on the enzyme microenvironment.

It is known that in aqueous solutions containing 20 wt% hydrophilic DES, part of the hydrogen-bond network between HBA and HBD is disrupted. In contrast, among the DES studied here, those formed by $[N_{4444}]^+$, $[N_{8881}]^+$, and $[N_{8888}]^+$ with fatty acids are hydrophobic, being immiscible in water and leading to stable biphasic systems. In these cases, as reported by van Osch et al. [43] and Florindo et al. [45,46], the DES-rich phase retains its hydrogen bond even after contact with water, acting as a functional microenvironment capable of extracting nonpolar compounds, including lipase.

On the other hand, ChCl-based DES are hydrophilic and readily dissolve in aqueous medium, which plausibly results in partial disruption of HBA-HBD interactions in the presence of 80% water. This reorganization of intermolecular interactions upon dilution has already been described by Durand et al. [49] and Shehata et al. [26]. Nevertheless, this aspect does not invalidate the observed effects, since even in diluted aqueous media, the ChCl-based DES constituents specifically influence the enzyme microenvironment, altering selectivity, stability, and catalytic activity [17,25]. Molecular dynamics simulations further support that water addition promotes new hydrogen bonds between the HBD and water molecules at the expense of those between DES constituents [50]. Therefore, two distinct behaviors can be identified: (i) hydrophobic DES preserve their eutectic properties and form biphasic systems in which lipase migrates to the DES-rich phase, maintaining its activity; (ii) hydrophilic DES partially dissociate in aqueous medium but still negatively modulate lipase activity through specific interactions. This difference does not compromise the validity of the results but rather reveals the distinct nature of the action of each DES type on the biocatalyst.

3.1. YlLip2 activity

In a work reported by our group [11], the lipase production by *Yarrowia lipolytica* was demonstrated, showing a production peak at 32 h in YPD culture media, coinciding with the longest cell growth time. Based on these findings, this study evaluated the impact of different DES on YlLip2 activity at the same time, ensuring that this approach allows for investigating the role of different DES in modulating the enzyme's behavior during its most active phase.

This work evaluated YlLip2 activity in the presence of 18 ammonium-based HBA/HBD systems prepared from $[N_{8888}]$Br, $[N_{8881}]$Cl, $[N_{8881}]$Br, $[N_{4444}]$Cl, $[N_{4444}]$Br, and ChCl combined with hexanoic, octanoic, and decanoic acids under a fixed aqueous condition (20 wt% DES in water). In addition to these combinations, the study also evaluated the impact of individual HBA on enzyme activity. The enzyme activity was compared to the control (culture medium without the DES components). The results are depicted in Fig. 1, which illustrates how the alkyl chain length of both the HBA and HBD influences the enzymatic activity of YlLip2. For a better overview and understanding analysis, the DES were divided into two sets based on the type of halide ion in the HBA: chloride-based (Fig. 1a) and bromide-based (Fig. 1b). Table S1 in Supporting Information offers a detailed breakdown of the enzyme activity values for each DES component alongside their corresponding pH levels.

Fig. 1a displays the impact of chloride-anion-based DES on the enzymatic activity. The data indicate a significant increase in YlLip2 activity with increasing HBA alkyl chain length and decreasing HBD chain length. The values ranged from 1.87 ± 0.33–73.79 ± 4.45 L^{-1}. It is important to emphasize that HBA-$[N_{8881}]$Cl (individually) was higher than the control, which had an activity of 45.00 ± 4.57 L^{-1}. This suggests that $[N_{8881}]$Cl acts as a potent activator of YlLip2. Additionally, the DES $[N_{8881}]$Cl: hexanoic acid exhibited activity values comparable to the control, indicating activity preservation in the presence of a DES. Overall, the trend observed is that the presence of DES generally results in lower enzymatic activity compared to their respective single HBA

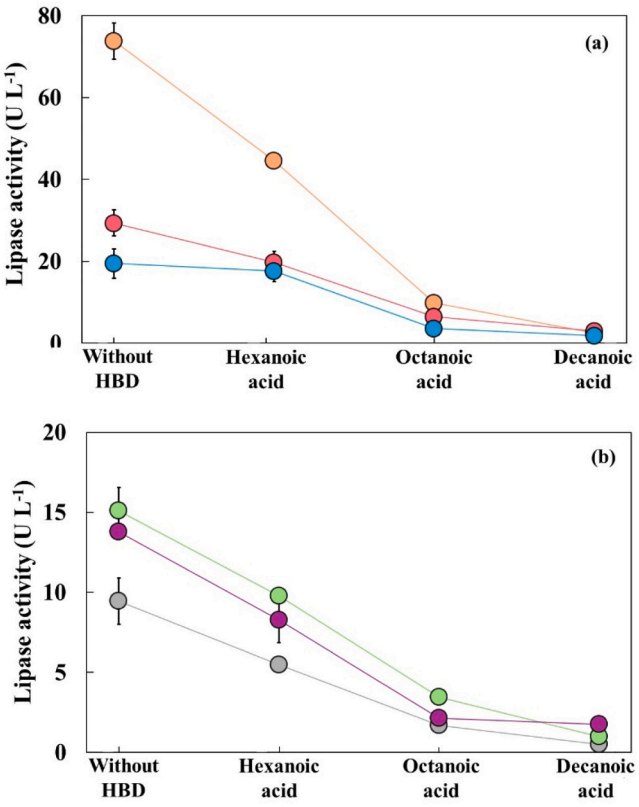

Fig. 1. Lipase activity from *Yarrowia lipolytica* in aqueous media containing 20 wt% DES and the corresponding isolated HBA components. (a) chloride-based systems, including hydrophobic DESs formed by tetraalkylammonium salts and fatty acids and hydrophilic ChCl-based systems: $[N_{8881}]$Cl (●), $[N_{4444}]$Cl (●), ChCl (●). (b) bromide-based hydrophobic DESs: $[N_{8881}]$Br (●), $[N_{4444}]$Br (●), and $[N_{8888}]$Br (●). The control exhibited an enzymatic activity of 45.00 ± 4.57 L^{-1}.

counterparts. This suggests that the inclusion of the HBD component in DES formulations may have an inhibitory effect on lipolytic activity.

The $[N_{8881}]$Cl reached 73.79 ± 4.45 L^{-1}, whereas the conventional control reached 45.00 ± 4.57 L^{-1}, corresponding to a 1.64-fold increase (+64%). Over 32 h, this corresponds to volumetric activities of 2.31 and 1.41 U L^{-1} h^{-1} , respectively. Literature reports based on p-nitrophenyl laurate (pNPL) assays under screening-type or non-intensified cultivation conditions often describe extracellular lipase activities in the range of 10–10^2 U L^{-1}, although the absolute values are strongly affected by strain, medium composition, inducer type, and cultivation regime. For example, Fabiszewska et al. [51] evaluated extracellular lipase activity in several *Yarrowia lipolytica* strains, including KKP 379 and W29 (ATCC 20460), cultivated in shake flasks using rich YP or minimal YNB media supplemented with 20 g L^{-1} vegetable oils, and reported average activities around 70 L^{-1}. Likewise, Diniz et al. [52] observed that cultivation of *Y. lipolytica* IMUFRJ 50682in yeast extract without the pomegranate-seed residue inducer resulted in relatively low lipolytic activity, with a maximum of 90 L^{-1} after 20 h, indicating that removal of an inducing matrix can maintain activity within the same general range observed here. Sipiczki et al. [53] screened several *Yarrowia* strains in YPD medium supplemented with olive oil and/or Tween 80 and found that *Y. lipolytica* 854/4 reached only 25 U L^{-1} at 48 h, whereas other isolates showed maxima in the range of 15–41 U L^{-1}, highlighting the strong dependence of extracellular lipase production on strain background and medium formulation. In addition, Stolarzewicz et al. [54] reported supernatant activities of 80 L^{-1} at 40 h and 13 L^{-1} at 70 h for *Y. lipolytica* KKP 379 cultivated in batch bioreactor mode (0.2 vvm; initial pH 5.0), further illustrating how cultivation time and process

conditions can shift activity within a comparable order of magnitude.

Among the various DES studied, those based on ChCl with fatty acids consistently showed a deactivating effect on YlLip2. These ChCl-fatty acid DES resulted in decreased enzymatic activity, indicating that, under these conditions, the presence of ChCl contributes to inhibitory effects. Nevertheless, it is important to note that the influence of ChCl depends on the combination with HBD, and other studies have reported neutral or even beneficial effects of ChCl-based DES when combined with sugars or polyols [55]. Xue et al. [56] evaluated the impact of choline-based solvents on the lipase activity of *Candida rugosa* and demonstrated that the oxygen atoms of the choline cation disrupt the hydrogen bonding network with water molecules surrounding the lipase. This disruption reduces the nucleophilicity of water, which in turn diminishes the enzyme's catalytic efficiency. Our previous work, Buarque et al. [57], evaluated the inhibitory role of DES on the *Yarrowia lipolytica* metabolism, especially on hexokinase (YlHxk), an enzyme vital for growth, since it catalyzes glucose metabolism through phosphorylation in the glycolytic pathway. It was found that DES based on ammonium salts with medium- and long-chain fatty acids had inhibitory effects on the growth of *Yarrowia lipolytica*. However, an exception was observed for the $[N_{8881}]$Cl ammonium salt and hexanoic acid combination, which showed higher tolerance by the yeast. Computational analyses of the YlHxk tunnels revealed that ligands transported through the same tunnel as the natural substrate (glucose), $[N_{8881}]^+$ and hexanoic acid, exhibited binding trends similar to the control (medium without DES), which explains their lower interference. In contrast, ligands that accessed alternative tunnels displayed a stronger propensity to cause strong inhibition. The importance of recognition at the enzymatic level was also emphasized, demonstrating that the DES effects cannot be predicted only by the macroscopic properties of the solvent.

Similarly, Deive et al. [58] reported that a ChCl aqueous solution decreased the lipolytic activity of lipase from *Thermomyces lanuginosus* by 50%. In contrast, studies by Lai et al. [59] and Zhao [60] highlighted a different behavior for ammonium-based cations. These cations, characterized by their ability to form hydrogen bonds with water due to their hydroxylated nature, may assist in maintaining or restoring the enzyme's active conformation. This property can promote better stabilization and activation, counteracting the deactivating effects observed with choline-based DES. These insights align with our observations on YlLip2 activity.

The effects of bromide-based DES on YlLip2 activity are shown in Fig. 1b, where a significant reduction in enzyme activity was observed in the presence of HBA ($[N_{4444}]$Br, $[N_{8881}]$Br, and $[N_{8888}]$Br) + fatty acids (HBD) compared to the control (no HBA and/or HBD). the enzyme activity values ranged from $0.49 \pm 0.12\ L^{-1}$ to $25.10 \pm 0.44\ L^{-1}$, significantly lower than the control activity level of $45.00 \pm 4.57\ L^{-1}$. This reduction in activity is primarily attributed to the properties of the anion. Bromide ions have a higher propensity to form hydration complexes compared to chloride ions. This characteristic weakens the strength of the cation-anion network within the HBA-DES, thereby diminishing the beneficial interactions between the lipase and the solvent. When these interactions are reduced, it adversely affects the enzyme's mobility and conformation, impeding the formation of the enzyme-substrate complex [49,58,61]. Regarding the influence of the HBA and HBD components, an increase in the alkyl chain length of either the cation (HBA) or the fatty acid (HBD) enhances their hydrophobicity. This increased hydrophobicity reduces the accessibility of the substrate to the enzyme's active site, corroborating our results. Deive et al. [58] and Xue et al. [56] have also reported that longer alkyl chains in DES can hinder substrate access to the catalytic site, thereby diminishing enzyme activity. This effect is particularly evident in our study, where longer alkyl chains had lower lipase activity values.

Yang et al. [62] suggested that interactions between DES and cells can impact cell membrane integrity, thereby influencing membrane permeability. Disrupted membranes enable substrates to access enzymes more easily and facilitate the diffusion of products out of the cells. In a related study, Mbous et al. [63] examined the permeability of cell membranes in media containing DES, observing intracellular fluorescence, which implied increased porosity. In contrast, control cells exhibited only extracellular fluorescence. These findings demonstrate the capacity of DES to permeate cell membranes.

It is important to distinguish the behavior of lipases and esterases toward fatty acids. While esterases generally show preference for short-chain substrates and may be inhibited by longer chains, lipases such as YlLip2 have a high affinity for medium- and long-chain substrates, a feature related to their hydrophobic lid domain. Previous studies have confirmed the catalytic efficiency of YlLip2 toward fatty acid esters [64,65], reinforcing that the presence of these compounds as HBDs in DES formulations does not compromise the enzyme's stability or catalytic activity. These results emphasize the complex interaction between DES components and lipase activity, highlighting the importance of appropriate selection of HBA/HBD for biotechnological applications to avoid inhibitory impacts on enzymatic processes. Given this context, molecular docking was applied to better elucidate the responsible interactions between the HBA ions and the HBD fatty acids in the protein structure, elucidating the role of these compounds in modulating the lipase.

3.2. Computational analysis

The lipase from *Yarrowia lipolytica* is a glycosylated polypeptide of 301 amino acids with a dynamic lid domain that covers the active site [66]. This lid undergoes conformational changes to control access to the active site, opening in the presence of a hydrophobic interface and closing in aqueous environments, thus regulating enzyme activity [67–69]. The lid region is composed of an α helix (α2) and is formed by the amino acids Thr88 - Leu105. Notably, this lid region protects the catalytic triad responsible for the lipolytic activity formed by the amino acids Ser162, Asp230, and His289 (Fig. 2 and Table S2). Furthermore, the oxyanion hole is formed by two amino acid residues (Thr88 and Leu163), hydrogen bond donors that stabilize the tetrahedral intermediate [35]. Allosteric sites, unlike active sites, can often be more accessible. These sites play a role as active modulators, potentially altering the enzyme's behavior and promoting different enantioselectivity patterns [70,71]. Amino acids constituting the three high-probability allosteric sites identified in YlLip2 as predicted by PASSer, are shown in Fig. 2 and Table S2 in Supporting Information.

The molecular docking provides a comprehensive understanding of the docking sites of DES components within the protein structure, detailing their binding affinity and the specific amino acids involved in these interactions. This computational analysis is crucial for advancing biocatalysis and optimization of enzymatic processes [72]. In general,

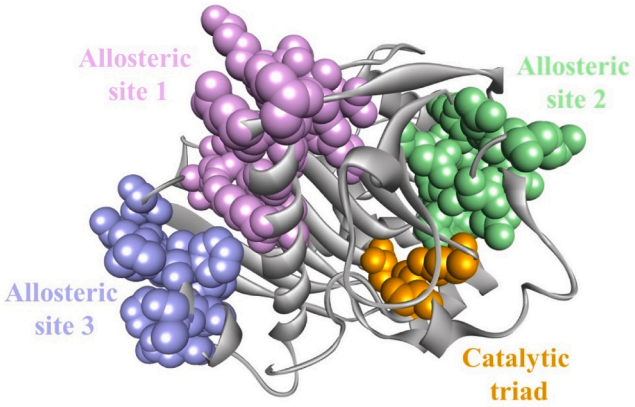

Fig. 2. Three-dimensional structure of *Yarrowia lipolytica* lipase (YlLip2) (PDB ID: 3o0d) with catalytic and allosteric sites identified: catalytic site (dark yellow), allosteric site 1 (light purple), allosteric site 2 (light green) and allosteric site 3 (light blue).

ligand and amino acid interactions can involve hydrogen bonding, hydrophobic, electrostatic, and van der Waals. The absolute affinity value (kcal mol^{-1}) of each ligand for YlLip2, the best binding pose and docking affinities, and the type of interaction and geometric distance (Å) are reported in Tables S3 and S4. Figures S1 and S2 display the docking pose with the highest absolute affinity value between the enzyme structure and HBA/HBD. The higher these absolute energy values, the stronger the energy interaction between the ligand and the receptor [32].

The ammonium-based HBAs were categorized into chloride-based and bromide-based for a more comprehensive analysis and are depicted in Figs. 3 and 4. The docking affinity energy values for chloride-based HBAs showed a distinct trend where the affinity decreased as the lipolytic activity of YlLip2 increased: $[N]^{+}_{8881}$ (-5.5 kcal mol^{-1}) > $[N_{4444}]^{+}$ (-4.7 kcal mol^{-1}) > $[Ch]^{+}$ (-3.2 kcal mol^{-1}). Similarly, for bromide-based HBAs, we observed a consistent pattern that aligned with our experimental data: $[N_{8881}]^{+}$ (-5.5 kcal mol^{-1}) > $[N_{8888}]^{+}$ (-5.3 kcal mol^{-1}) > $[N_{4444}]^{+}$ (-4.7 kcal mol^{-1}) (Fig. 3 and Table S3, in Supporting Information). These results reflect the complex interaction dynamics between the enzyme and DES components. In contrast, the behavior of fatty acid (HBDs) presented an inverse relationship: as the affinity energy values reduced, the lipolytic activity of YlLip2 also decreased [73]. The affinity energy values follow the order: hexanoic acid (-4.2 kcal mol^{-1}) > octanoic acid (-4.4 kcal mol^{-1}) > decanoic acid (-4.6 kcal mol^{-1}) (Fig. 4 and Table S4, in Supporting Information). Thus, longer chain fatty acids with higher affinity energies could inhibit lipase function more effectively.

These affinity energy values align well with our experimental findings and are consistent with trends reported in the literature. For instance, Barbosa et al., (2019) demonstrated through molecular docking that a larger cation size of the phosphonium-based ionic liquids, lower affinity energy values with lipase from *Burkholderia cepacia*, correlating with reduced enzyme activity. Similarly, Brandão et al. [74] observed a comparable relationship between fatty acids in their docking studies.

Notably, none of the studied HBA or HBD components directly interacted with the amino acids constituting the YlLip2 catalytic site, indicating that the observed effects on lipase activity are likely mediated through indirect or allosteric interactions rather than direct active site inhibition. To further assess the influence of these ligands on YlLip2, the enzyme's allosteric sites were explored. Interestingly, despite the biotechnological importance of *Y. lipolytica* lipase, no allosteric effects have been documented for this enzyme to date. Therefore, the PASSer software was used to predict the allosteric sites on YlLip2. PASSer provides a detailed analysis of protein structures, classifying regions based on their probability of being active allosteric sites [75,76]. Some studies have been successful in identifying allosteric regions using this software. For instance, Faisal et al. [77] employed PASSer to successfully pinpoint the allosteric site of the SARS-CoV-2 NSP10/NSP16 methyltransferase, underscoring the tool's effectiveness in identifying critical regulatory regions in proteins. Menden et al. [70] identified the allosteric sites of *Candida rugosa* lipase using a computational approach and determined inhibitors and activators with possible medical and biotechnological applications.

Table S2 presents the amino acids forming the three predicted allosteric sites of YlLip2 and their associated probability values as determined by the PASSer software. These sites are potential regions where allosteric modulators can interact to influence the enzyme's function. For the cation-based HBA, the interactions with the amino acids at these allosteric sites follow a trend based on the length of the cations' carbon chains. The cation $[N_{8881}]^{+}$ demonstrated the highest number of interactions, forming 15 bonds with the allosteric sites. This is followed closely by $[N_{8888}]^{+}$ with 14 interactions, $[N_{4444}]^{+}$ with 13 interactions,

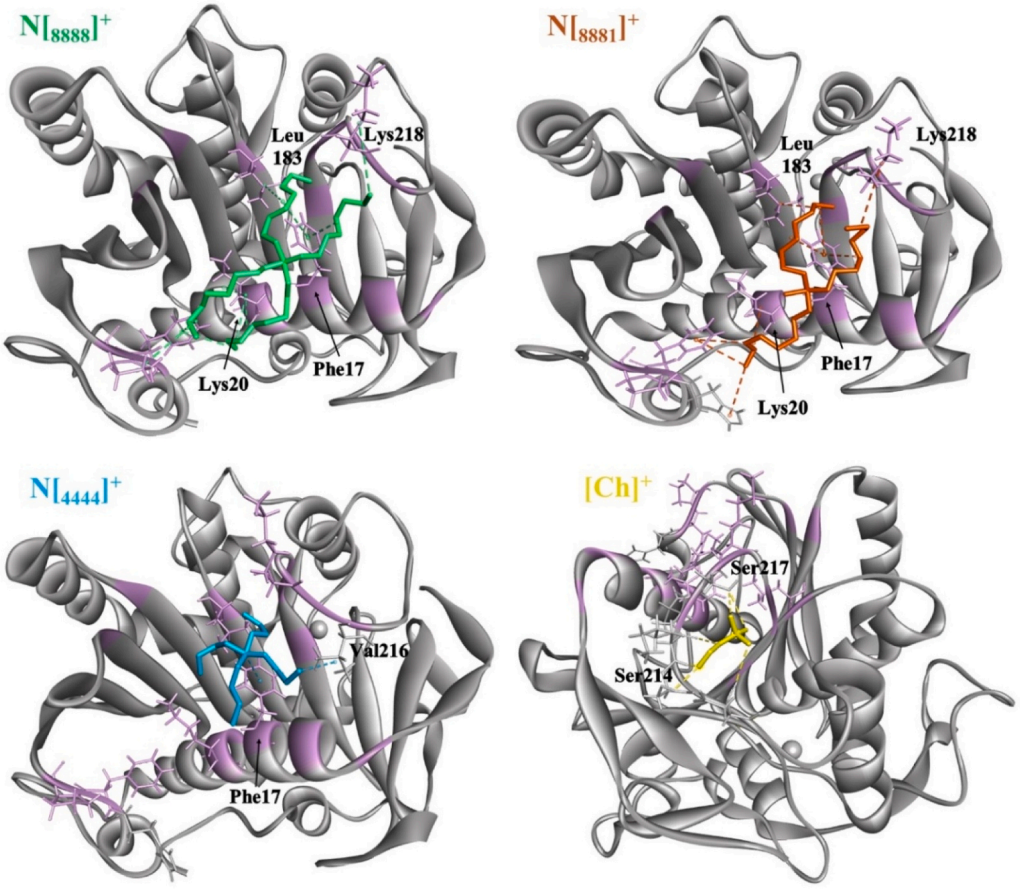

Fig. 3. Molecular docking analysis between the HBA-forming cation and amino acids constituents of the allosteric sites of YlLip2.

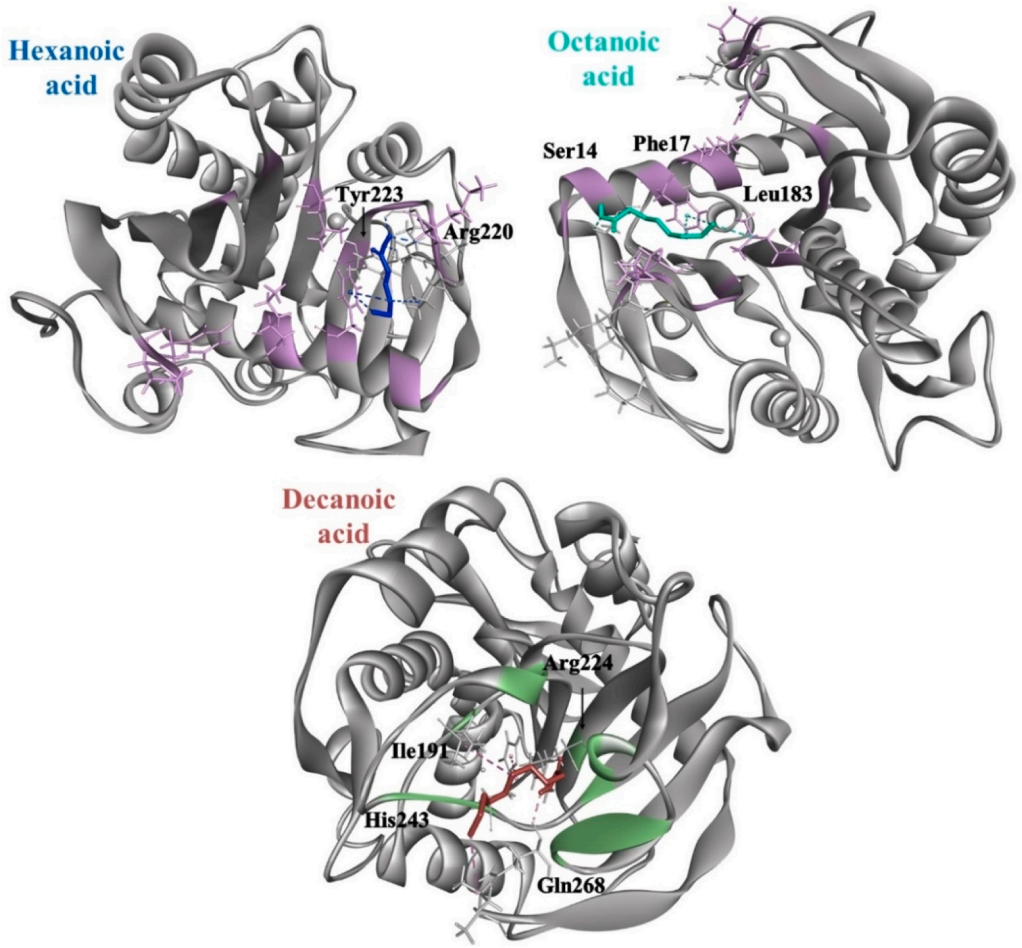

Fig. 4. Molecular docking analysis between the fatty acids (HBD) and amino acids constituents of the allosteric sites of YlLip2.

and lastly choline, which only formed 2 interactions. This pattern suggests that ammonium compounds with longer carbon chains enhance their ability to engage with the allosteric regions of YlLip2. A similar trend was observed with the fatty acids. Shorter fatty acid chains resulted in fewer interactions with the allosteric sites: hexanoic acid (10 bonds), octanoic acid (11 bonds), and decanoic acid (12 bonds). This indicates that as the length of the fatty acid chain increases, the number of interactions with the enzyme's allosteric sites also increases. These data are in agreement with lipase activity experimental data, where increasing the length of the cation or fatty acid chain in most of the HBAs and HBDs studied decreased the enzymatic activity.

The results indicate that HBAs and HBDs binding to the amino acids at YlLip2's allosteric sites may function as a "lid blocker". By occupying these sites, these compounds potentially obstruct the substrate's access to the enzyme's active site; this obstruction becomes more pronounced with the increasing chain length of the ammonium-based HBAs and fatty acids [73]. This is consistent with the observed trend: longer chains result in more interactions with the allosteric sites and higher absolute affinity energy values.

Remarkably, the $[N_{8881}]$Cl-HBA, individually, exhibited a higher YlLip2 activity compared to the control, suggesting it does not block the active site. Additionally, the DES formed by $[N_{8881}]$Cl and hexanoic acid demonstrated enzyme activity levels comparable to the control, indicating a balanced interaction where neither the DES components significantly hinder substrate binding. The $[N_{8881}]$Cl cation has the highest absolute affinity energy value among the HBAs studied, indicating a strong interaction with the YlLip2 structure. Notably, $[N_{8881}]$Cl was the only one that interacted with the amino acids of allosteric site three, specifically forming hydrophobic interactions with residues His49 and Phe50.

Allosteric site three, which has the highest predicted probability (29.22%) of being a key regulatory site, may be playing a significant role in modulating enzyme activity. The stronger interaction at this site likely induces conformational changes that enhance YlLip2's catalytic behavior. In contrast, other HBAs that interact exclusively with this allosteric site tend to exhibit reduced enzymatic activity relative to the control, implying a potential inhibitory effect when their interactions are limited to this region. Moreover, decanoic acid was the only HBD found to interact with amino acids at allosteric site two. This interaction may contribute to the observed inhibition of lipolytic activity, indicating that allosteric site two also has a role in regulating enzyme function.

Interestingly, despite $[N_{8881}]^+$ interacting with allosteric site one, its interactions with allosteric site three appear to be more influential. These interactions seem to promote beneficial conformational changes, leading to an overall increase in enzyme activity compared to the control. Therefore, the specific interactions between $[N_{8881}]^+$ and the residues His49 and Phe50 at allosteric site three are likely the primary drivers behind the enhanced catalytic performance of YlLip2 in the presence of the DES containing $[N_{8881}]$Cl. These insights underscore the complexity of enzyme regulation by allosteric sites and highlight how targeted interactions at specific sites can lead to either activation or inhibition of enzyme function. Understanding these dynamics is decisive for optimizing biocatalytic processes using DES.

4. Conclusion

In this study, the enzymatic activities of YlLip2 were assessed in the presence of 18 DES composed of ammonium-based HBA and fatty acid-

based HBD. The results revealed that enzyme activity tended to increase with longer alkyl chains of the cation-HBA and shorter fatty acid-HBD chains. Nevertheless, substituting a chloride anion with a bromide anion resulted in greater inhibition of lipase activity. Most DES displayed lower YlLip2 activity values than the control. Notably, HBA-$[N_{8881}]Cl$ (individually) and DES $[N_{8881}]Cl$: hexanoic acid would exhibit higher and similar values to the control, respectively. Molecular docking analyses supported these findings by showing that higher absolute affinity energy values correspond to stronger interactions between the DES components and the lipase structure. This interaction strength was positively correlated with the length of the alkyl chains in both the cation-HBA and HBD. Importantly, none of the DES components interacted directly with the catalytic triad amino acids of YlLip2, indicating that the catalytic site was unaffected. Instead, the binding occurred at allosteric sites, where the interactions followed the same trend as the decreasing order of affinity energy values. An important observation was that $[N_{8881}]Cl$ was the only component to interact with allosteric site three, specifically forming hydrophobic interactions with residues His49 and Phe50. These interactions at site three likely facilitate an enhancement in the catalytic activity of YlLip2 in the presence of DES. This suggests a potential activation mechanism through the allosteric modulation of YlLip2's structure and function. In conclusion, both molecular docking simulations and experimental data underscored the diverse interactions of different HBAs and HBDs with YlLip2. It was evident that $[N_{8881}]Cl$ can serve as an effective activator of YlLip2, opening avenues for reactions that are typically challenging in conventional solvents, such as those required in fermentative extraction processes.

CRediT authorship contribution statement

Matheus M. Pereira: Writing – review & editing, Validation, Supervision, Investigation, Formal analysis, Conceptualization. **Buarque Filipe Smith:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Bernardo D. Ribeiro:** Validation, Investigation, Formal analysis. **Mara G. Freire:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Coelho Maria A. Z.:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.procbio.2026.04.007.

Data availability

Data will be made available on request.

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