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Thermal stabilization of lipase by choline chloride- and betaine-based deep eutectic solvents: A molecular and functional study

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ABSTRACT

Deep eutectic solvents (DES), a new generation of (green) solvents, are increasingly used in enzymatic reactions as stabilization media. However, the roles of individual components and water content remain poorly understood. We investigated the activating and thermostabilizing effects of various DES compositions and their water dilutions on immobilized lipase B from Candida antarctica. Our results show that enzyme thermostabilization strongly depends on both factors. Certain hydrogen-bond donor (HBD) and acceptor (HBA) combinations act synergistically even at high dilutions, while others exhibit inhibitory effects dominated by a single component. The highest relative activity of 13.1 was observed in choline chloride (ChCl):Glycerol (1:1 ratio; 10 wt% water), whereas a 75 wt% aqueous solution of ChCl exhibited relative activity of 10.7. Molecular docking simulations with individual DES components showed binding of choline near the enzyme's catalytic triad, suggesting stabilization without competing with substrate binding. Our findings highlight the importance of HBD/HBA selection, water content, and component-specific enzyme interactions, and offer experimental and atomistic insights for efficient and targeted design of DES formulations for biotechnological applications.

1. Introduction

Deep eutectic solvents (DES) are a new generation of (green) solvents generally based on readily available and inexpensive compounds [1–6]. They are mixtures of two or more components, typically Lewis or Brønsted acids and bases, which act as hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs). When HBDs and HBAs interact, they primarily form hydrogen bonds, producing a mixture whose eutectic point is far below the temperature of an ideal liquid mixture [7,8]. Their properties can be finely tuned by selecting different HBDs and/or HBAs, adjusting the HBD:HBA ratio, and varying the water content, making them highly versatile solvents with rapidly expanding applications. DES are mostly non-toxic, with low volatility and flammability, and high solvency [9]. They also have high compatibility with enzymes [10] and have been used in numerous enzymatic reactions [11–16].

Studies have shown that certain DES can maintain enzyme activity by stabilizing their secondary structures [16–18] primarily through the formation of H-bonds between the OH-groups of the HBD and the amino acids of the enzyme [19]. This stabilizing property of DES is also reflected in their strong thermostabilizing capacity, which can enable enzymatic reactions at elevated temperatures, leading to higher product yields. The thermostabilizing effect of some DES has already been demonstrated in several studies with enzymes such as laccase [20,21], lipase [10,22], cutinase [23], and cellulase [24]. Despite the high potential of DES in enzymatic reactions, previous studies have also shown that the interactions between DES and enzymes are highly complex and that the results for one enzyme cannot simply be generalized to another enzyme or reaction. Therefore, a more detailed investigation of specific DES-enzyme interactions is required.

In this study, we selected lipase as a model enzyme because lipases are among the most widely used biocatalysts in research and industry, with diverse applications including detergent formulations, biofuel synthesis, ester synthesis, food processing, pharmaceuticals, the leather and paper industries, flavor enhancement, biosensor development, agrochemicals, cosmetics, perfumery, and bioremediation [25–27]. The commercially available lipase Novozym 435 is immobilized, which enables easy removal or replacement of the reaction medium. We tested various DES and DES formulations – differing in HBA and HBD types, HBA:HBD ratios, and water content – for their activating and thermostabilizing effects on lipase enzyme compared to aqueous buffer. We also examined the effects of different buffer compositions and pH values, as

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well as the timing of water addition (before or after DES formation), to assess their influence on the system's thermal stability. More importantly, we compared the effects of DES to those of aqueous solutions of their individual components. The role of individual components in lipase stability was further investigated through molecular docking calculations, which identified the strongest interactions between the individual components and the enzyme. The results of this study will enhance our understanding of how DES can affect enzyme activity. This knowledge will enable the selection of the most suitable DES or DES formulation for specific enzymes, leading to significant benefits such as higher reaction productivity.

2. Materials and methods

2.1. Materials

Lipase B from *Candida antarctica* immobilized on Lewatit VP OC 1600 macroporous (average pore diameter ~ 15 nm (≈150 Å)) acrylic resin carrier beads (Novozyme 435; Sigma-Aldrich); 5,5′-Dithio-bis-(2-nitrobenzoic Acid) (DTNB; 98 % purity; Sigma-Aldrich); 2,3-Dimercapto-1-propanol tributyrate (BAL; 97 % purity; Sigma-Aldrich); Trisma® Base (Sigma-Aldrich); Choline chloride (ChCl; 98 % purity; Sigma-Aldrich); Betaine (Bet; 98 % purity; Sigma-Aldrich); Urea (U; 99.5 % purity; Sigma-Aldrich); Glycerol (G; 98–101 % purity; Pharmachem); Sorbitol (Sorb; 98 % purity; Sigma-Aldrich); Xylitol (Xyl; 99 % purity; Sigma-Aldrich); Ethylene glycol (EG; 99 % purity; Sigma-Aldrich); Glucose (Glu; 99.5 % purity; Merck); Sucrose (Suc; 99.5 % purity; Merck); Fructose (Fru; 99 % purity; Merck); Potassium carbonate (K_2CO_3; 99 % purity; Sigma-Aldrich); Menthol (Menth; 99 % purity; Sigma-Aldrich); Butanol (But; 99.9 % purity; Honeywell); Oxalic acid (OxA; 98 % purity; Sigma-Aldrich); Disodium phosphate (Na_2HPO_4; 99 % purity; Merck),); Dimethyl sulfoxide-d6, 99.9 atom % D, contains 0.03 % (*v*/v) TMS (DMSO-d_6; 99 % purity; Sigma-Aldrich); Chloroform-*d*, 99.8 atom % D (CDCl3; 99 % purity; Sigma-Aldrich).

2.2. DES preparation

Most of the DES or their components were selected based on literature data demonstrating stabilizing or activating effects [20,21,28–32]. Others were chosen based on additional factors, such as acidic or alkaline H-bond donors and hydrophobic components. The selected DES and the molar ratios of their components were: ChCl:G (1:2), ChCl:G (1:1), ChCl:U (1:2), ChCl:Sorb (1:1), ChCl:Xyl (1:1), ChCl:EG (1:2), Bet:G (1:2), Bet:Sorb:water (1:1:1), ChCl:Glu (1:1), ChCl:Glu (5:2), ChCl:Glu:water (1:1:1), ChCl:Suc (4:1), ChCl:Fru:water (5:2:5), K_2CO_3:G (1:4), Menth:But (1:2) and ChCl:OxA (1:2). The DES components were mixed in the appropriate molar ratios and heated to 80 °C until a clear liquid formed. Afterwards, a selected amount of water (0, 5, 10, 25 or 75 wt%) was added to the mixture and mixed thoroughly. Aqueous solutions of the individual components ChCl, Bet, U, and G, with either 25 or 75 wt% water, were also prepared.

Finally, the order of preparation of the DES solution (i.e. the timing of water addition) was tested. In these experiments, ChCl:U (1:2) DES was prepared with 10 or 25 wt% water, with the water added either at the beginning (before DES formation) or at the end (after DES formation).

Note: Water was always added after the DES was formed unless otherwise specified (i.e.in the timing of water addition experiments).

2.2.1. NMR spectroscopy

^{1}H NMR measurements were performed on a Bruker AVANCE NEO 600 MHz NMR spectrometer. For analysis of the individual pure compounds, samples were dissolved in DMSO-d_6. In contrast, spectra of the prepared DES were acquired using an NMR insert filled with $CDCl_3$.

2.3. Lipase activity reactions

The lipase activity protocol followed the BAL-DTNB method described by Lombard et al. [33]. All reactions were carried out with 2 mg of lipase enzyme *per* 1 mL of reaction solution. Each 1 mL reaction solution contained 50 μL of 20 mM BAL, 50 μL of 20 mM DTNB, and 900 μL of Tris/HCl buffer (pH 8). The enzyme was incubated for 30 min at 37 °C. Samples were then diluted 10-fold with Tris/HCl buffer and centrifuged at 165,000 rcf for 2 min to remove solid particles. The supernatant (200 μL) was transferred to a flat-bottomed microtiter plate and absorbance was measured at 412 nm using a UV–vis spectrophotometer (Synergy H1, BioTek). A molar absorption coefficient of 14,200 M^{-1} cm^{-1} was used to quantify the concentration of reaction products released. Lipase activity was calculated based on Eq. (1):

$$U/L = \left(A \times f_{dil} \times V_{rxn} \times 10^6\right)/\varepsilon_{412nm} \quad (1)$$

where, A is the absorbance of the sample, f_{dil} is the dilution factor (10-fold), V_{rxn} is the sample volume (0.2 mL), 10^6 is the conversion factor from M to μM, and ε_{412nm} is the DTNB molar absorption coefficient. Lipolytic activities are expressed in units; 1 unit represents 1 μmol of substrate hydrolyzed *per* minute under the experimental conditions.

Since it was previously shown that DES can affect the extinction coefficient of a compound [20], and since our reaction solution was sensitive to pH and temperature (as indicated by a noticeable color change), the incubations of the enzyme in different media (DES, buffers, water) and at different temperatures were performed separately, i.e. the lipase activity measurements were not performed in DES and other media. The general protocol for all reactions was as follows: 2 mg of enzyme was incubated in 200 μL of the selected medium at a given temperature for 30 min. Then, 1 mL of ultrapure distilled water was added and mixed well to remove the medium. The samples were centrifuged for 2 min at 165,000 rcf, the supernatant was removed and the enzyme was washed again with 1 mL of water and centrifuged. The supernatant was then removed, and 1 mL of reaction solution was added to the enzyme. All experiments were performed in duplicate, unless otherwise stated, in which case three replicates were used. The average values and with standard deviations are reported.

Note: To confirm that remnants of DES in and around the carrier beads after the washing steps did not affect the absorbance of the BAL-DTNB reaction solution, carrier beads with heat-inactivated enzyme were incubated in selected DES at 80 °C, then washed and incubated in reaction solution according to our protocol. The results showed no deviation in absorbance.

2.3.1. pH and temperature dependence of lipase

To determine the pH range of this lipase, 2 mg of the enzyme was incubated in 200 μL McIlvaine buffer with a pH of 3–8 or in distilled water (pH 6). The incubation temperature was set to 30 °C, and the incubation time was 30 min. For the temperature dependence tests, 2 mg of enzyme was incubated in 200 μL Tris/HCl buffer (pH 8) at temperatures ranging from 30 to 90 °C for 30 min. The experiments were conducted once with three replicates, and the average values with standard deviations are reported. The results are presented relative to the highest value.

2.3.2. Activity of lipase in DES

The effect of DES on lipase activity was measured at two temperatures, 30 and 80 °C. A total of 200 μL of 90 wt% DES (10 wt% water) was added to 2 mg of enzyme and mixed to ensure the enzyme was completely covered with DES and did not remain in an air pocket at the bottom of the microcentrifuge tube. The mixture was then incubated at either 30 °C or 80 °C. Subsequently, the five DES with the highest thermostabilizing activity were prepared with 5, 25, 50, and 75 wt% water and tested at 80 °C. Additionally, these DES with 10 wt% water were also tested at other temperatures (30–80 °C) as well as for longer incubation times (up to 24 h) at 80 °C. Lipase was also incubated in

water, McIlvaine buffer at pH 5 and 8, and in aqueous solutions of selected individual DES components, i.e. 25 and 75 wt% ChCl, Bet, G, and U, at 30 °C and 80 °C. To test the effect of water addition timing, ChCl:U (1:2) DES with 10 or 25 wt% water was used. Water was added to the DES either at the beginning, before the DES was fully formed, or at the end, after the DES was fully formed. The reaction was carried out at 80 °C. In all reactions, Tris/HCl buffer at pH 8 was used as a control, and the results were expressed as relative activity. The experiments were performed in two to three independent experiments with three replicates each, and the average values along with standard deviations are reported. The results are given relative to the control (Tris/HCl).

2.3.3. Molecular docking

Molecular docking simulations were performed to investigate interactions between individual components (ChCl, Bet, U, G, Sorb, and Xyl), as well as Tris and its protonated form (Tris+), with lipase. To determine the possible stabilizing effects of individual components, we performed molecular docking simulations on the surface of the entire enzyme (*Candida antarctica* lipase B, PDB ID 1TCA [34]). The grid box was defined to encompass the whole enzyme, centered at the enzyme's center of mass, with 126, 112, and 126 equidistant grid points in the x, y, and z dimensions respectively, and a spacing of 0.464 Å between points. Additionally, another set of docking simulations was performed, in which the same compounds were docked to the enzyme's active site. In this case, the grid box was centered at the catalytic triad Ser105-His224-Asp187 center of mass, with 40 equidistant grid points and a spacing of 0.375 Å in each direction. These calculations were performed to obtain the components' affinity for binding to the lipase's active site.

In both sets of molecular docking simulations, 1000 molecular docking runs were performed for each individual component using the Lamarckian genetic algorithm [35]. In each run, the maximum number of evaluations was 2,500,000 and the maximum number of generations was set to 27,000. During the molecular docking calculations, the enzyme was treated as rigid, while the non-hydrogen-containing bonds of the DES components were considered flexible. The structures of the individual component-enzyme complexes obtained from each docking run were ranked according to their binding free energy and clustered based on their RMSD, using a tolerance of 2.0 Å. Molecular docking simulations were performed using the AutoDock4.2 software package [36].

3. Results and discussion

3.1. Activation and thermostabilization of lipase in different DES mixtures

First, we determined the temperature and pH stability of the enzyme. The temperature range was tested from 30 to 90 °C. Fig. 1a and Table S1 show a rapid decrease in activity as temperature increased, with only minor activity at 80 °C and almost none at 90 °C. Since some measurable activity remained at 80 °C, this temperature was selected for the thermostabilization studies. For pH, the enzyme showed relatively high activity at all tested pH values (3–8) (Fig. 1b and Table S1). Although the optimum was at pH 5, the enzyme retained approximately 70 % and 80 % of its activity at pH 3 and 8, respectively. The enzyme activity in water (approximately pH 6) was similar to that in buffer at pH 6, indicating that, at least at 30 °C, the medium type (McIlvaine buffer or water) has no significant effect.

The effects of DES on enzyme activity were tested at 30 °C and 80 °C and compared with Tris/HCl buffer (pH 8) and pure water (Fig. 2 and Table S2). As in the thermostabilization experiments in DES performed by Khodaverdian et al. [20], the enzyme was first incubated in DES at the selected temperature, after which the DES was removed and the substrate mixture was added. Therefore, the actual enzymatic reaction did not occur in the DES. Although the enzyme retained relatively high activity at pH 8 in Tris/HCl buffer (Fig. 1b), the thermostabilizing effect

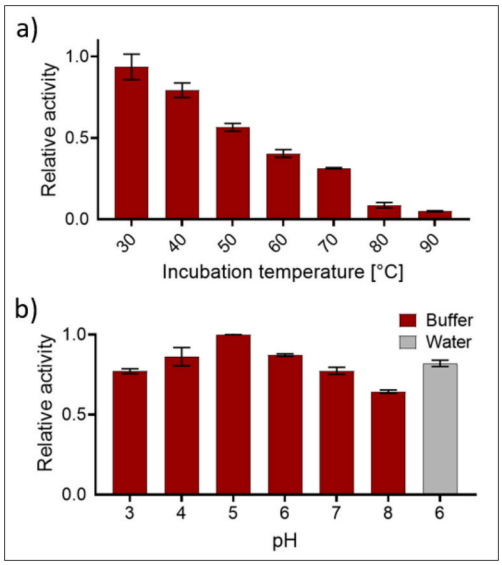

Fig. 1. Temperature (a) and pH (b) range of lipase. The relative activity is calculated based on the highest value.

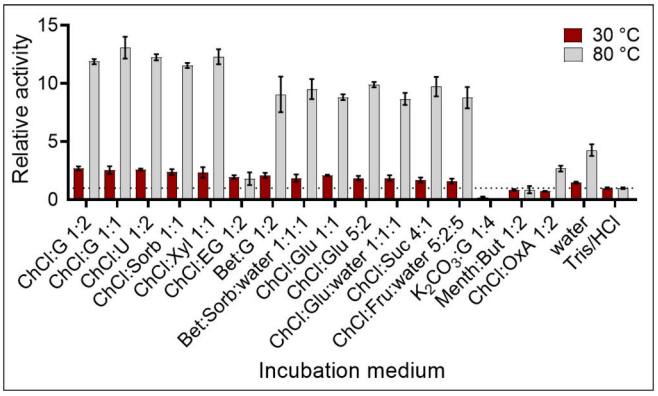

Fig. 2. Relative activity of lipase in 90 wt% DES (10 wt% water) and water compared to Tris/HCl buffer at 30 °C and 80 °C. The relative activity is calculated based on the activity in Tris/HCl buffer.

of pure water (pH 6) was significantly greater, with a relative activity of 4.3. Despite this, we chose to compare the effects of DES with Tris/HCl buffer rather than water, as buffers are commonly used in enzymatic reactions to maintain pH stability. In addition, Tris/HCl is the reaction buffer used in the enzyme activity measurements. Fig. 2 and Table S2 show that most of the DES tested had a notable activation effect on the enzyme at 30 °C, although they were not present during the actual enzymatic reaction. The highest relative activities were measured for ChCl:G (1:2), ChCl:G (1:1) and ChCl:U (1:2), with 2.7-, 2.6-, and 2.6-fold increases over buffer activity, respectively. The DES that did not exhibit activating properties and even showed minor to notable inhibitory effects were K_2CO_3:G (1:4) (relative activity 0.2), Menth:But (1:2) (relative activity 0.9) and ChCl:OxA (1:2) (relative activity 0.7). At 80 °C, the effects of DES were even more pronounced (Fig. 2 and Table S2). Except for ChCl:EG (1:2) (relative activity 1.8), K_2CO_3:G (1:4) (relative activity 0), Menth:But (1:2) (relative activity 0.9) and ChCl:OxA (1:2) (relative activity 2.7), all DES strongly thermostabilized the enzyme, with activity at least 8.7-fold higher than that of buffer. The best DES were ChCl:G (1:1), ChCl:U (1:2), and ChClXyl (1:1) with relative activities of 13.1, 12.3, and 12.3, respectively.

In addition, lipase activity was tested across the entire temperature range from 30 °C to 80 °C in 5 selected DES (see Section 3.2 for selection

justification). The results in Fig. S1 show different mechanisms among the selected DES. Most exhibit the highest stabilization at 70 °C, which is moderately reduced at 80 °C. Only Bet:G (1:2) showed the highest stabilization at 60 °C, with a notable decrease at higher temperatures.

As explained earlier in this section, the relative enzyme activity in DES was calculated using Tris/HCl buffer, even though water had a stronger effect. At 30 °C, the effect of water on enzyme activity is most likely due to its slightly acidic pH (pH 6), which is more favorable than the alkaline conditions of Tris/HCl (pH 8). This beneficial pH effect was even more pronounced at higher temperatures, increasing the difference between water (which provided better thermostabilization) and Tris/HCl buffer. Thus, the difference in enzyme activity and stability between DES and buffer would probably be smaller if a buffer with a more optimal pH were used. By using a buffer with a less favorable pH, we demonstrated that DES as a reaction medium can be advantageous in situations where a particular reaction requires conditions that are suboptimal for a specific enzyme.

The choice of HBA and HBD, the HBA:HBD ratio, water addition (dilution), and pH are factors known to influence the effect of DES [19,21,37]. This is also reflected in our results. For HBA, the relative activity decreased from 11.9 (ChCl:G 1:2) to 9.1 (Bet:G 1:2) when ChCl was replaced with Bet in combination with G. When K_2CO_3, a very alkaline compound, was used, the positive effects (activation at 30 °C and thermostabilization at 80 °C) were completely abolished. The difference between the stabilization mechanism of ChCl and Bet is also evident in the results showing stabilization over the entire temperature range (Fig. S1). These results show that Bet:G reaches its peak of stabilization at 60 °C, with a steep decrease afterward, whereas stabilization with ChCl:G increases steadily and begins to decrease only after 70 °C. In contrast to our results with lipase regarding ChCl and Bet HBAs, two other groups showed that aqueous ChCl-based DES performed poorly, and that replacing ChCl with Bet in combination with different HBDs greatly improved the thermostabilization of laccase [20,21]. However, it should be emphasized that lipases and laccases are two very different enzyme classes (hydrolases vs oxidoreductases) and thus perform best under distinct medium conditions, making direct comparison impossible. Additionally, Varriale et al. [38] tested the thermostability of different laccase variants in Bet-based DES and demonstrated that the effects of DES are highly specific. This proves that even within the same enzyme class, different variants and sources significantly influence the results.

Among the HBDs, G, U, Sorb, and Xyl performed best in combination with ChCl, followed by the sugar-based HBDs Glu, Suc and Fru. This trend is observed at both 30 °C and 80 °C. Studies have shown that the OH-groups of HBDs in DES form H-bonds with enzyme amino acids, making the enzyme structure more rigid and stable, which prevents the protein from unfolding at higher temperatures [19–21]. A higher proportion of OH-groups, and consequently more H-bonds, has been associated with a stronger thermostabilizing effect [19,21]. G, Sorb and Xyl all have a high number of OH-groups (three or more), which can stabilize the protein structure via H-bond formation. Although the sugar-based HBDs also have a high number of OH-groups, their efficiency is likely lower due to their higher viscosity, which hinders mass transfer [39]. The enzyme is immobilized on carrier beads, and our additional experiment has shown that even with an aqueous buffer (Tris/HCl), the shape of the carrier such as e.g. whole beads or powdered beads, affects mass transfer and subsequent enzyme activity kinetics (Fig. S2). Therefore, it would be even more difficult for a viscous solvent, such as DES, to penetrate the beads to reach and stabilize the enzyme bound within. This is supported by a study by Durand et al. [40], which showed that grinding immobilized lipase carrier beads was essential for good lipase activity in DES. However, if working with a non-immobilized enzyme, higher viscosity would be advantageous for thermostabilization, as Guo et al. [24] showed with cellulase and pectinase enzymes. ChCl:U also showed a high thermostabilizing effect, although U does not contain OH-groups and the H-bonds between ChCl and U are therefore formed via the NH_2-groups of U [41]. Interestingly, it has been shown that the H-bonds with the NH_2-groups of urea are responsible for the denaturation of proteins [42], but in a DES formulation they seem to have the opposite effect. Regarding the biocompatibility of DES components, Cao et al. [10] have shown, using the example of the enzyme laccase, that in addition to the amount of OH- groups, the length of the carbon chain can also be advantageous, while the presence of carboxyl groups and C—C double bonds can have a negative effect on enzyme activity.

OxA as an HBD in ChCl-based DES showed slight inhibitory effects at 30 °C (relative activity 0.7) compared to the buffer, likely due to the acidic nature of OxA, which may cause alterations in enzyme structure and potentially lead to unfolding [43]. This destabilizing effect of pH would be expected to intensify with the addition of another destabilizing factor, such as high temperature. However, at 80 °C, ChCl:OxA actually demonstrated some thermostabilizing effects compared to the buffer, suggesting that pH-dependent stabilization or destabilization mechanisms may be temperature-dependent. When EG (HBD) was combined with ChCl (HBA), enzyme activity increased at 30 °C, but only minimal thermostabilization was observed at 80 °C. Similarly, ChCl:EG DES showed minimal thermostabilizing capacity for a laccase enzyme; however, exchanging ChCl HBA for Bet (Bet:EG) resulted in a marked increase in thermostabilization [21]. On the other hand, EG combined with ChCl has proven to be a good thermostabilizing medium for enzymes such as cellulase and pectinase [24,43]. Regarding HBA:HBD ratio, Delorme et al. [21] tested various Bet:Xyl ratios and observed a significant effect on the thermostability of laccase. Results indicate that higher ratios of Bet compared to Xyl have a more positive effect on laccase relative activity, yet ratios higher than 2:1 Bet:Xyl lead to the opposite effect. Similarly, our results show that a 1:1 ChCl:G ratio had better thermostabilizing effects compared to a 1:2 ratio.

The effects of the reaction medium on enzyme activity depend on interactions between the solvent and the amino acids on the enzyme's surface or at the active site [10,19]. This explains why the same DES can have varying effects on different enzymes or enzyme variants. These interactions may also explain the poor performance of Menth:But, as the more hydrophobic nature of both compounds likely limits their ability to bind efficiently to the enzyme, thus preventing stabilization. This has been corroborated by other authors [10], who have shown that the half-life of lipase is highly influenced by the hydrophobic or hydrophilic character of the DES. In the presence of hydrophilic DES, lipase half-life was enhanced almost 10-fold depending on the DES studied compared to the buffer, whereas for hydrophobic DES, the system performed much worse. However, this was also shown to depend on the class of enzyme, as for β-glucosidase, only the hydrophobic DES performed slightly better than the buffer.

Previous research on enzymes such as laccase, lipase, and β-glucosidase has shown that DES can significantly enhance reaction kinetics [20,28,44]. However, in our study, the activation effects of most DES at 30 °C were unexpected, as the DES had been removed before the addition of the reaction mixture, meaning that the reaction itself did not occur in a DES medium. A possible explanation for these results is that a small amount of DES remained bound to the enzyme, particularly inside the carrier beads, even after washing, which may have stabilized the enzyme and subsequently increased its activity. Although these DES are highly water-miscible, the viscosity difference between DES and water requires some agitation to facilitate mixing. We observed a noticeable swelling of the carrier beads after incubation in various DES media, indicating some penetration of the media into the beads despite their hydrophobic nature. Therefore, if DES did indeed penetrate the carrier beads, a brief washing with water (without extended incubation and vigorous shaking) may not have fully removed it, leaving some DES bound to the enzymes and thereby stabilizing and activating them. This feature could be beneficial when increased enzyme activity is desired but the enzyme's reaction cannot occur in a DES medium, such as when the substrate is sensitive to DES or its components, or when a high-

viscosity DES would hinder mass transfer and negatively impact reaction kinetics.

Our findings, together with those from previous studies, clearly demonstrate that the use of DES in enzymatic reactions is a complex subject. The characteristics of the chosen DES (such as HBA and HBD selection and ratio, water content, viscosity, and hydrophobicity) must be considered, as well as the reaction conditions, including temperature and pH. However, the most crucial factor may be the type or variant of the enzyme, as interactions between its amino acids, both on the surface and in the active site, and the DES ultimately determine the outcome of this interaction.

3.2. Effect of water contents on the thermostabilizing effects of DES

One important factor influencing DES features, is the amount of added water (dilution) [31,37,44]. We selected the 5 DES that (i) had the highest thermostabilizing effects, (ii) featured different HBAs (ChCl or Bet), (iii) could be formed without water and (iv) did not solidify at room temperature: ChCl:G (1:2), ChCl:U (1:2), ChCl:Sorb (1:1), ChCl:Xyl (1:1), and Bet:G (1:2). The DES were prepared with 5, 10, 25, 50, or 75 wt% of water and their thermostabilizing effects were tested at 80 °C, comparing them to buffer and pure water. As shown in Fig. 3a (and Table S2), all DES demonstrated their highest thermostabilizing effects with 10 wt% water, with relative activities ranging from 9.1 (Bet:G 1:2) to 12.3 (ChCl:U 1:2). Notably, ChCl:U (1:2) also showed similarly high activity with 25 wt% water, with a relative activity of 12.1. The addition of 75 wt% water caused the most significant decrease in the thermostabilizing effect of the DES; however, the relative activity remained higher than that of water, confirming the thermostabilizing effect of DES. The lowest and highest values were for Bet:G 1:2 and ChCl:Xyl 1:1, with relative activity of 5.1 and 7.1, respectively. ChCl:U (1:2) was the only DES where the addition of 75 wt% water reduced thermostabilizing effects (relative activity of 2.6) to below that of water.

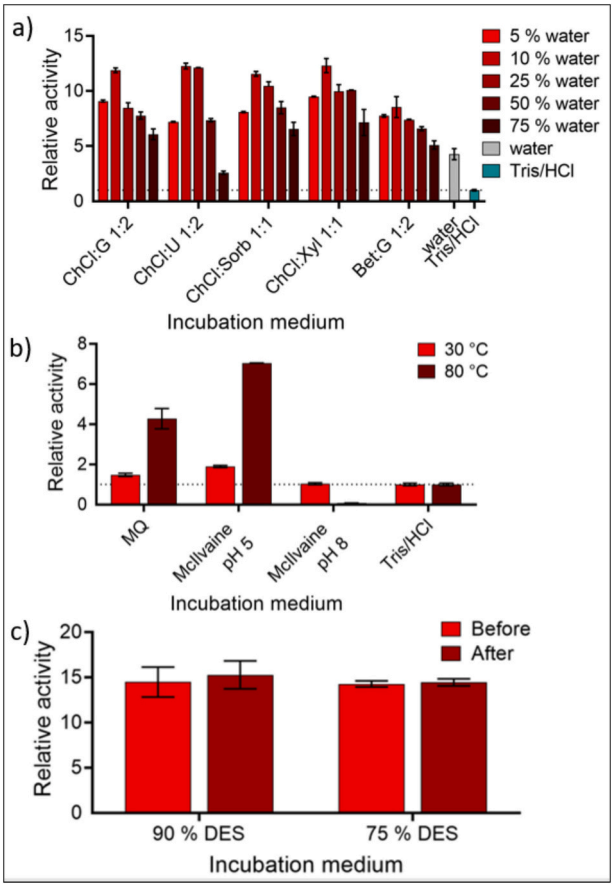

Fig. 3. Relative activity of lipase in different dilutions of selected DES (a) and buffers (b), and the effect of water addition time on the thermostabilizing capacity of 90 wt% and 75 wt% ChCl:U DES (c).

Given the substantial difference in enzyme activity between pure water and Tris/HCl, we also examined whether medium composition and/or pH influence enzyme activity at 30 °C and 80 °C. We tested pure water, Tris/HCl at pH 8, and McIlvaine buffer at pH 8 and pH 5. McIlvaine buffer was chosen for its wide pH range, allowing us to use the same buffer composition at with different pH values. pH 5 was selected as our results showed it to be the optimum pH for the lipase enzyme used in our study (Fig. 1a and Table S1). As expected, both the medium and its pH had a significant effect on enzyme activity, particularly at 80 °C (Fig. 3b and Table S3). At 30 °C, the effects on enzyme activity appeared to be due only to pH. In the mildly acidic media, water (pH 6) and McIlvaine buffer at pH 5, relative enzyme activity was 1.5 and 1.9, respectively, compared to the alkaline Tris/HCl. In McIlvaine buffer at pH 8, the relative activity was comparable to Tris/HCl despite their different compositions. However, at 80 °C, both pH and medium composition had notable effects. Lower pH resulted in higher thermostabilizing effects with relative activities of 5.6 and 7 for water and McIlvaine buffer at pH 5, respectively. In contrast, alkaline pH (pH 8) and medium composition played a significant role, resulting in a relative activity of less than 0.1 in McIlvaine buffer at pH 8.

A previous microwave irradiation study demonstrated that the organization of the DES network differs depending on when water is added: at the beginning, before the DES forms, at the end, after the DES is established; or if it is present in the crystalline structure of the compound. The latter scenario allowed for a considerably faster heating rate than the other DES compositions due to stronger hydrogen bonding, following the trend ChCl:OxA dihydrate (intrinsic 13.5 wt% water) > ChCl:OxA + 13.5 wt% water added at the beginning > ChCl:OxA + 13.5 wt% water added at the end > individual DES compounds [45]. We prepared a DES with 10 wt% and 25 wt% water, adding the water either at the beginning or the end. The selected DES was ChCl:U (1:2), as it demonstrated some of the highest thermostabilizing effects, which were nearly identical for both water amounts. Fig. 3c (and Table S4) illustrates that, in contrast to the microwave irradiation study indicating differences in the DES network based on the timing of water addition [45], this factor did not influence the thermostabilizing capacity of our selected DES.

The amount of water added to DES (DES dilution) is a factor previously shown to influence the effects of DES [21]. Even small amounts of water can affect the polarity and viscosity of the DES solution, as well as the rigidity and strength of the H-bond network [45,46]. Here, we performed ^{1}H NMR spectroscopy of two DES in the absence and presence of water, as well as their individual components to shed light on the hydrogen bond network. Thus, ChCl:G (1:2) was selected as one of the most performant systems, and ChCl:U (1:2) was chosen as the system with greater deviations in enzymatic activity upon water addition. Fig. S3 shows that upon DES formation in the absence of water, there are downfield shifts of the proton resonances located within or near hydrogen-bonding sites, hence confirming interactions between the HBA (choline chloride) and the HBDs (glycerol/urea). For ChCl:G (1:2), the addition of 10 wt% water induces further downfield shifts, consistent with additional hydrogen bonding, while the water resonance appears at ~4.4 ppm. At 75 wt% water, the strong water signal dominates the region around 4.8 ppm, partially obscuring glycerol and choline chloride resonances. Nevertheless, the remaining signals (2 and 3) still exhibit a clear displacement, corroborating the strengthening and reorganization of the hydrogen-bonding network in the highly hydrated system. A similar behavior is observed for ChCl:U (1:2), where progressive water addition results in systematic shifts of both urea and choline chloride resonances, again consistent with enhanced hydrogen-bonding interactions and restructuring of the DES-water network. Overall, at low water concentrations, the water resonance appears at ~4.4 ppm,

indicating its inclusion in a strong hydrogen-bonding network, characteristic of DES systems. At higher water concentrations, the resonance of water protons shifts downfield towards ~4.75 ppm, coinciding with the chemical shift of pure water protons. This suggests that at 25 wt% DES and 75 wt% water, the system behaves more like a salt solution in water rather than a DES. Consequently, lipase activity will mainly depend on the influence of the individual DES components, as discussed in detail in the next section.

Research has shown that hydration of DES is linked to changes in protein conformation and stability [47] and that even the presence of 4 wt% water can significantly affect reaction kinetics [31,44]. However, adding too much water disrupts the DES network, leading to DES-water and water-water interactions [2]. But how much water is too much? One study found that the ChCl:U (1:2) DES network remains intact at 42 wt% water but dissociates at 51 wt% [48]. Other studies have shown that DES containing up to 90 wt% water (10 % DES) still significantly affects reaction kinetics and thermal stabilization [20–22,28,49,50], suggesting that the presence of HBD and HBA components in aqueous solution may still produce synergistic effects despite the (partial) disruption of the DES network. Furthermore, one study demonstrated that Bet:Xyl (2:1) DES exhibited the most potent thermostabilizing effect on laccase at 75 wt% water [21]. Overall, this indicates that general conclusions cannot be made; instead, in each case, authors need to study the behavior of individual aqueous solutions of the HBA and HBD and compare them to the respective aqueous solutions of DES.

Our results show that the impact of water amount depends on the specific DES formulation, as different HBA:HBD combinations exhibit different H-bond network strengths. Additionally, at high water amounts, where the solution resembles an aqueous mixture of DES components rather than a "true" DES, the effect depends on the individual DES components. In some cases, the HBA and HBD still act synergistically (ChCl:G, ChCl:Sorb, and ChCl:Xyl with 75 wt% water), while in others, the inhibitory effect of one component dominates (ChCl:U with 75 wt% water, due to the denaturation effect of U).

Additionally, the DES containing 10 wt% water were tested for their long-term stability effects, which are crucial for relevant (industrial) enzyme applications. Lipase was incubated in DES or Tris/HCl buffer for 0.5, 1, 3, 5, and 24 h at 80 °C. The results (Fig. S4) show only minimal residual activity after 0.5 h and a complete loss of activity after 5 h in Tris/HCl buffer. In ChCl:U (1:2), activity decreased from 37,000 U/L to 16,000 U/L (43 % residual activity) after 5 h, and was completely lost after 24 h. In contrast, the other DES (ChCl:Sorb (1:1), ChCl:Xyl (1:1) and Bet:G (1:2)) maintained high and stable enzyme activity throughout the entire 24 h incubation process at 80 °C, highlighting their potential for industrial use.

3.3. Effect of individual DES components

To confirm that the thermostabilizing effects were indeed due to DES, we also tested the thermostabilizing effects of individual DES components (Fig. 4 and Table S5). The reference DES were ChCl:G, ChCl:U, and Bet:G, chosen to screen different HBAs and HBDs. We prepared 25 wt% aqueous solutions (75 wt% water) of ChCl, U, G, and Bet, and 75 wt% aqueous solutions (25 wt% water) of ChCl, U, and G. Unfortunately, 75 wt% Bet could not be used as that amount of Bet did not completely dissolve in water. Additionally, 75 wt% U required heating to at least 80 °C to remain in liquid form. The results in Fig. 4 show that aqueous solutions of ChCl and Bet both exhibit high thermostabilizing effects. However, the results also demonstrate that the relationship between HBAs and HBDs is quite complex. The relative activity in ChCl with 25 wt% water was 10.7 and dropped to 5.1 with 75 wt% water. In contrast, U showed inhibitory effects on enzyme activity at both concentrations, with relative activity equivalent to that of Tris/HCl buffer at 75 wt% water and lower at 25 wt% water. Comparing these results to the respective DES (ChCl:U 1:2), it is evident that at 25 wt% water, the inhibitory effects of U are not only nullified by the thermostabilizing

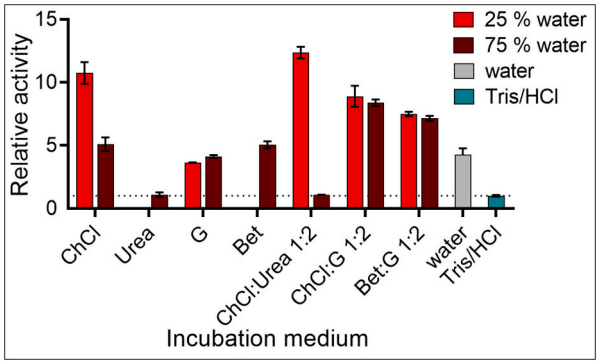

Fig. 4. Effect of DES and their individual components, with different water content, on thermostabilization of lipase at 80 °C.

effects of ChCl, but also suggest a possible synergistic effect between the two components. The relative activity of G with 25 wt% water (3.6) is slightly lower than with 75 wt% water (4.2), both comparable to the activity in pure water (4.2). This suggests minor inhibitory effects of G at 25 wt% water. Although the relative activity of ChCl:G (1:2) DES with both water amounts was similar (8.9 and 8.4), the effects of the individual components appear to differ. At 25 wt% water, G inhibited the thermostabilizing effects of ChCl, reducing the relative activity from 10.7 (ChCl) to 8.9 (ChCl:G 1:2). However, at 75 wt% water, the addition of G to ChCl increased the relative activity from 6.0 (ChCl) to 8.3 (ChCl:G 1:2). For Bet at 75 wt% water, strong thermostabilization effects were also observed, with a relative activity of 5.0. Similarly to the ChCl:G mixture with 75 wt% water, the addition of G to Bet resulted in synergistic effects, increasing the relative activity of the Bet:G mixture to 7.1.

Previous studies have shown that Bet can prevent the thermal denaturation of citrate synthase [51] and laccase [20,21]. Although we could not test higher Bet concentrations due to solubility constraints, our results also demonstrated the stabilizing effects of Bet (with 75 wt% water). In contrast, Toledo et al. [19] showed that in aqueous solutions of ChCl-based DES, the ChCl ion likely causes denaturation of laccase. These inhibitory effects of ChCl on laccase have also been reported by other groups [20,21]. Additionally, Romano et al. [23] tested the thermostabilizing effects of aqueous solutions of ChCl and Bet on cutinase, and showed strong inhibitory effects from both. These findings contrast with our results for lipase, which show significant thermostabilizing effects of aqueous ChCl and, to a slightly lesser degree, Bet. This again highlights the importance of enzyme type in determining the effects of specific DES and/or their individual components. Furthermore, our findings emphasize the critical role of HBA and HBD selection, especially at higher DES dilutions, where the DES network may partially or completely dissociate, as corroborated by the NMR spectra (Fig. S3), making the effects of individual components more pronounced.

3.4. Molecular docking

For all individual component–enzyme complexes, clusters with either a population over 10 % or the three most populated clusters were considered. The binding poses of the two HBAs, Bet and ChCl, were well defined, as the three (four for Bet) most populated clusters accounted for 96.4 % (Bet) and 81.2 % (ChCl) of all binding poses. Notably, all three binding poses of ChCl were located near the enzyme's substrate binding site, forming 2H-bonds each with the backbones of Ala275 and 278 (cluster 1), the backbone of Ala225 and the sidechain of Glu188 (cluster 2), and the backbone and the sidechain of Asp223 (cluster 3; Fig. 5b and Table 1). While ChCl forms H-bond interactions with residues near the catalytic triad, it does not bind inside the substrate-binding site and therefore does not compete with the substrate. These interactions between ChCl and lipase could provide a stabilizing effect and help retain the enzyme's activity even at higher temperatures. In contrast, the most

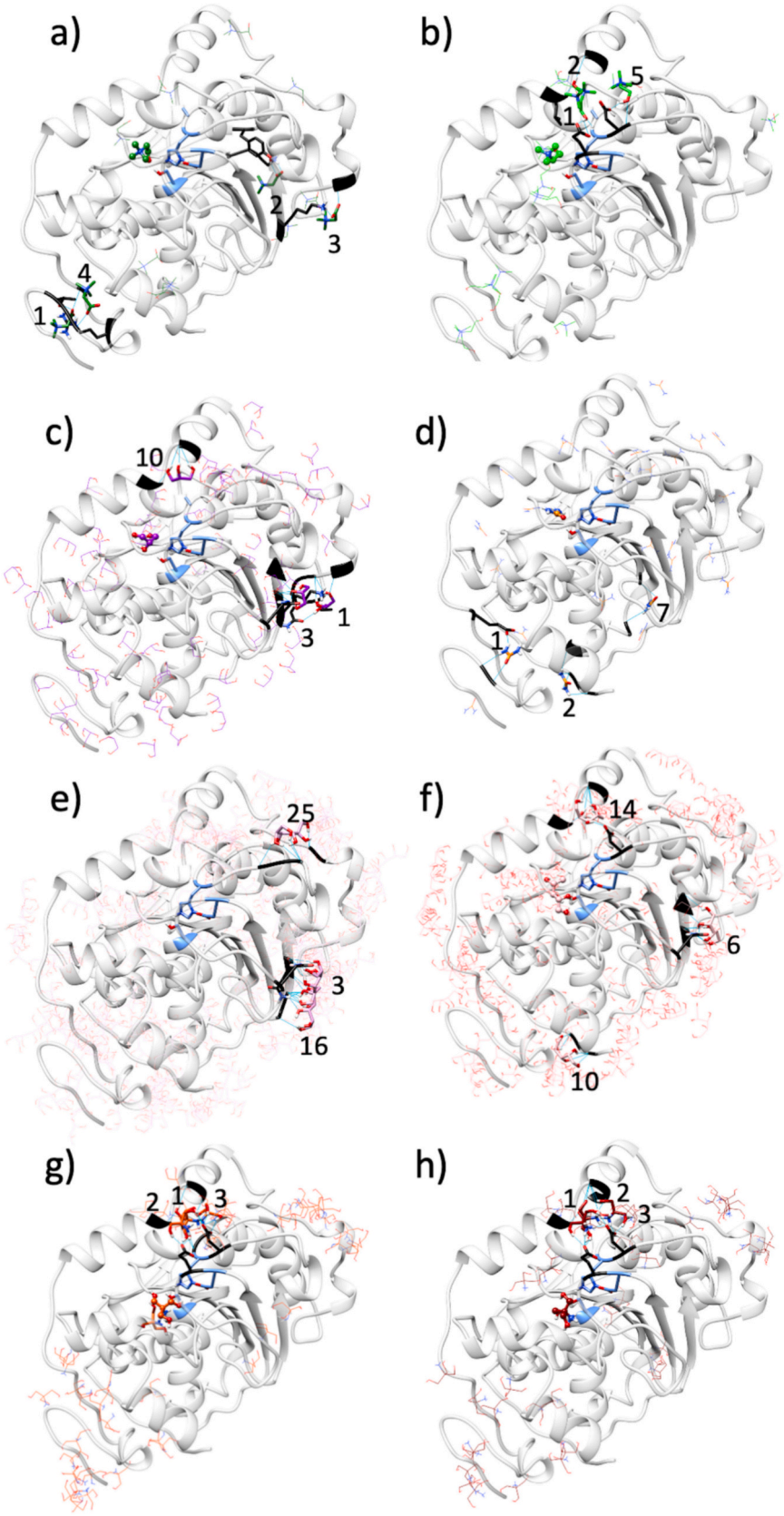

Fig. 5. Docking results to the enzyme surface are shown in sticks representation with the three or four highest populated clusters shown as non-transparent (and marked with the cluster ranking) and the remaining structures as transparent and the results to the active site are shown in balls and stick representation. The catalytic triad residues (Ser105-His224-Asp187) are colored in blue. Oxygen atoms are red, nitrogen atoms blue, and polar hydrogen atoms are white, while non-polar ones are not shown due to clarity reasons. Lipase B residues interacting with DES are colored in black and hydrogen bonds are shown as turquoise lines. a) Bet (green), b) ChCl (light green), c) G (purple), d) U (orange), e) Sorb (light violet), f) Xyl (pink), g) Tris (dark orange), and h) Tris+ (dark red).

Table 1
Results of molecular docking calculations for three or four (Betine) highest populated clusters.

Component	Total number of clusters	Cluster rank	Population of each cluster	Binding free energy of the representative structure [kcal mol^{-1}]	Number of hydrogen bonds between component and lipase B	Binding free energy of the representative structure to enzyme's substrate binding site [kcal mol^{-1}]
Betaine	13	1	12.9 %	−3.51	3	−2.08
		2	10.7 %	−3.21	2	
		3	45.2 %	−3.17	2	
		4	27.6 %	−3.13	1	
ChCl	17	1	51.2 %	−3.11	2	−2.01
		2	12.9 %	−3.05	2	
		5	17.1 %	−2.89	2	
Glycerol	84	1	6.4 %	−2.82	6	−2.35
		3	10.7 %	−2.58	6	
		10	9.3 %	−2.27	4	
Urea	28	1	20.2 %	−3.26	3	−2.47
		2	17.6 %	−3.11	4	
		7	17.0 %	−2.86	2	
Sorbitol	259	3	3.7 %	−2.84	7	no binding to the substrate binding site
		16	3.1 %	−2.49	7	
		25	2.8 %	−2.31	6	
Xylitol	138	6	6.7 %	−2.74	7	−1.46
		10	6.6 %	−2.46	5	
		14	4.0 %	−2.40	6	
Tris	50	1	43.1 %	−4.96	5	−2.40
		2	15.9 %	−4.26	4	
		3	17.5 %	−3.99	5	
Tris+	40	1	26.0 %	−4.07	6	−2.45
		2	22.8 %	−4.07	4	
		3	24.1 %	−3.73	5	

favorable binding poses of Bet were far from the substrate binding site, offering no direct stabilizing effect on the catalytic region; however, it can still stabilize the secondary structure of lipase in other parts of the protein. Namely, Bet interacts with the backbone of Leu297 and the sidechains of Arg302 and Thr310 (cluster 1), the sidechains of Lys32 and Tyr61 (cluster 2), the sidechain of Lys208 and the backbone of Ser250 (cluster 3), and the backbone of Arg309 (cluster 4; Fig. 5a and Table 1). The binding free energies of the representative structures of the most populated clusters of Bet (−3.17 kcal mol^{-1} for cluster 3)[1] were comparable to those of ChCl (−3.11 kcal mol^{-1} and -3.05 kcal mol^{-1} for clusters 1 and 2, respectively; Table 1).

Among the HBD components, the most localized binding poses were obtained for U, followed by G, Xyl, and Sorb. Specifically, the three most populated clusters for each HBD accounted for 54.8 % (U), 26.4 % (G), 17.3 % (Xyl), and 9.6 (Sorb) of all binding poses (Table 1). U behaved similarly to Bet, with the binding free energy of its most populated cluster being −3.26 kcal mol^{-1}; however, none of its binding poses were near the substrate-binding site. U preferentially interacted via H-bond formation with the sidechain of Glu294 and the backbone of Arg309 (cluster 1), the backbones of Ser3, Ser5, and Phe117 (cluster 2), and the backbones of Lys98 and Ser123 (cluster 7; Fig. 5d and Table 1). G exhibited weaker binding to lipase, with the lowest binding free energy of the representative structure of the most populated cluster being −2.82 (cluster 1 – forming H-bonds with the sidechains of Lys208, Thr245, and Gln237 and the backbones of Ala248 and Ser250) and − 2.58 kcal mol^{-1} (cluster 3 – forming H-bonds with the sidechain of Asn196 and the backbones of Asn209 and Gln211), and − 2.27 kcal mol^{-1} for the G's binding near the catalytic triad (cluster 10 – interacting via H-bonds with the backbones of Ala275 and Leu278; Fig. 5c and Table 1).

Sorb and Xyl exhibited no preferred binding site. The most populated cluster accounted for only 3.7 % and 6.7 % of all binding poses for Sorb and Xyl, respectively (Fig. 5e and f). Interactions between Sorb and Xyl and the enzyme were relatively weak (binding free energies were − 2.84 and − 2.74 kcal mol^{-1} for Sorb and Xyl, respectively) and were comparable to those of G. The most populated clusters of Sorb formed up to 7H-bonds with lipase B, namely with the sidechains of Asn196 and Asn209 and the backbones of Gly207 and Asn209 (cluster 3), the sidechain of Asn209 and the backbones of Asn206, Gly207, and Asn209 (cluster 16), and the backbone of Phe220, Val221, and Cys258 (cluster 25; Fig. 5e and Table 1). Similarly, up to 7H-bonds was observed in Xyl-lipase B complexes. They were formed with the sidechain of Asn196 and the backbones of Asn209 and Gln211 (cluster 6), the backbones of Ser4 and Ser5 (cluster 10); and the sidechain of Asp223 and the backbones of Ala275 and Leu278 (cluster14; Fig. 5f and Table 1).

G, Sorb, and Xyl formed the largest number of H-bonds with lipase, as expected, since they are rich in hydroxy groups and can form non-preferential interactions with any polar residues on the protein surface. This is also evident from the large number of clusters obtained in the molecular docking calculations. On the other hand, U, ChCl, and Bet form fewer H-bonds with the surface of lipase (up to 4, Table 1). Although G, Sorb, and Xyl form more H-bonds with lipase than U, ChCl, and Bet, their binding free energies are higher (less negative). Combined with the larger number of clusters obtained, this suggests that the binding of G, Sorb, and Xyl is weaker and more dynamic, while the binding sites of U, ChCl, and Bet are relatively more well-defined. Although the binding of G, Sorb, and Xyl is not as strong as that of the other components and lacks a well-defined binding site, their stabilizing function can possibly be explained by the formation of a structured solvation shell around the protein, thus protecting it from thermal denaturation.

Finally, docking of Tris and Tris+ to the surface of lipase resulted in the strongest binding among all components, with binding free energies of −4.96 and − 4.07 kcal mol^{-1} for Tris and Tris+, respectively. The three most populated clusters for each component accounted for 76.5 % (Tris) and 72.9 % (Tris+) of all binding poses (Table 1). Moreover, the binding poses of all highest populated clusters were similar to those of ChCl (Fig. 5g and h), suggesting that Tris could potentially stabilize the enzyme through formation of H-bonds with residues near the catalytic triad (Ser105-His224-Asp187). In the three most populated clusters, Tris formed H-bonds with sidechains of Glu188 and Asp223 and backbones

[1] While the binding free energies obtained from the molecular docking calculations usually do not agree well with the experimental data, they can still be used for ranking different binding poses and binding affinities for different substrates.

of Ala225, Ala275, and Leu278 (cluster 1), the sidechain of Glu188 and Asp223 and the backbone of Ala225 (cluster 2); and the sidechain of Asp223 and backbone of Ala274 (cluster 3). Tris+ formed H-bonds with the sidechains of Asp223 and Glu188 (cluster 1); the sidechain of Glu188 and the backbones of Ala275 and Leu278 (cluster 2); and the sidechain of Asp223 and the backbones of Ala274 and 275 (cluster 3; Fig. 5g and h and Table 1). However, because Tris functions as a pH buffer, its concentration under typical experimental conditions is usually too low to observe a measurable stabilizing effect.

The results of the molecular docking calculations indicate that, among all individual components, ChCl exhibits the highest stabilizing potential due to its preferred binding site near the enzyme's catalytic triad. Moreover, its binding free energy was among the most favorable of all the components studied. The preferred binding sites of all individual components were located outside the enzyme's substrate binding site (Fig. 5 and Table 1), suggesting they are unlikely to affect enzyme activity through competitive binding.

The present results suggest that the synergistic effect of the complete DES mixture on lipase thermostability arises from interactions between HBAs and HBDs with different regions of the protein, leading to combined thermostabilization that exceeds the sum of their individual contributions. While molecular docking calculations provide valuable insight into the stabilization capabilities of single components at the atomistic level, they can account only for single protein-small molecule interactions at a time. Therefore, more extensive simulations (e.g. molecular dynamics) of lipase solvated in various DES compositions and concentrations could offer a deeper understanding of the thermostabilization mechanisms.

4. Conclusions

In this study, we systematically explored the effects of various DES formulations and their water dilutions on the activation and thermostabilization of lipase. We tested different DES compositions at high (80 °C) and low (30 °C) temperatures to determine their effects on enzyme activity. A comprehensive comparison of whole DES and their individual components, revealed the importance of HBD and HBA selection and water dilution factor, resulting in either synergistic or inhibitory interactions. Comparing our results on lipase with studies using other enzymes showed distinct differences in the effects of DES components, emphasizing the need for enzyme-specific evaluations. While earlier studies focused on the bulk effects of DES, our molecular docking simulations identified the binding of choline near the catalytic triad as a probable atomistic mechanism for its thermostabilizing effects. Taken together, these findings highlight the importance of thoroughly understanding the enzyme-DES interaction for each specific enzyme system to design a targeted DES formulation appropriate for various biotechnological applications.

CRediT authorship contribution statement

Miša Mojca Cajnko: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Matic Pavlin:** Writing – original draft, Methodology, Data curation, Conceptualization. **Filipa A. Vicente:** Writing – review & editing, Conceptualization. **Blaž Likozar:** Supervision.

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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.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2025.149523.

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