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Research Article
doi.org/10.1002/cctc.202500986

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# Rational Design of Hydrophilic Deep Eutectic Solvents to Outperform Oxidoreductase Activity in Aqueous Media

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*In memory of Prof. Iván Lavandera García, our beloved and dear colleague and friend*

Outperforming biocatalysis through nonconventional media opens new avenues in synthesis, from process intensification to industrial integration. While hydrophobic deep eutectic solvents (DESs) based on fatty acids have emerged as a promising landscape for oxidoreductases, the potential of hydrophilic DESs to rival or surpass aqueous systems remains largely underexplored. Building on findings that glycerol aids alcohol dehydrogenase (ADH) catalysis and choline chloride hinders it, this study designs enzyme-friendly glycerol-based DESs using betaine and sarcosine at varied ratios and water contents. Consistently, a higher glycerol fraction (mol:mol, 1:8) contributed to a stronger stabilizing effect on ADHs, and betaine emerged as the most favorable component, followed by sarcosine and choline chloride. Enzyme thermostability improved in Bet-Gly and Sar-Gly, though activity was lower in all hydrophilic DESs; Bet-Gly (1:8) with 80% buffer showed the best performance. Encouragingly, the enzyme's specific activity for the cyclohexanone reduction outperformed that observed in the pure buffer, and the optimal eutectic conditions of Bet-Gly (1:8) and Sar-Gly (1:8) with 60 vol.% buffer outperformed the pure buffer system in cinnamaldehyde reduction (a more industrially-sound reaction). This study advances DES research and aids in the design of DESs for redox biocatalysis in hydrophilic media.

## 1. Introduction

Establishing biocatalysis in nonconventional (nonaqueous) conditions—particularly when using more sustainable solvents—has become an essential working line toward *Green Chemistry*, as reaction media account for the most significant waste production and plays a pivotal role beyond being a mere reaction vehicle at the same time.[1] Solvents may serve as (de)solubilizers for reaction components while providing a favorable reaction environment for catalysts.[2] Moreover, many solvents feature as reaction mediators and exert synergistic effects (e.g., (de)activation of catalysts),[3] thereby creating a significant impact on substrate specificity, enzyme kinetics, and even selectivity.[4] Biocatalysis has proven to be efficient beyond traditional aqueous conditions, with outstanding examples in nonaqueous media,[5] including organic solvents, new generations of master solvents such as ionic liquids (ILs), and supercritical fluids.[5b,6] The recognized environmental concern associated with solvents has stimulated research toward biomass-based solvents,[7] eco-friendly solvents,[8] and even solvent-free systems.[9]

Deep eutectic solvents (DESs) are recognized as a neoteric class of (potentially) greener solvents for enzymatic catalysis.[10] DESs are mixtures of hydrogen bond donors (HBDs) and hydrogen bond acceptors (HBAs), displaying low melting temperatures at a specific molar ratio.[11] In some cases, due to the nonideal behavior of DESs, expanding the molar ratios can still result in eutectic mixtures.[12] Furthermore, DESs are highly tuneable given the large (biogenic) reserves of DES components[11a,13] and adjustable molar ratios, indicating the large customizability of "designer" DESs for specific biocatalytic purposes. Importantly, DESs can be designed to be biocompatible and nontoxic, as well as with mild (bio)degradation after use,[14] making them attractive for sustainable chemistry.[15] As a downside, however, DESs often exhibit high viscosities (~7–86,800 mPa·s versus 0.89 mPa·s of water at room temperature), which can be (partly) overcome by adding water to form DES-water mixtures, or by increasing temperatures (with some challenges for biocatalysts).[12,16]

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*Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.202500986*

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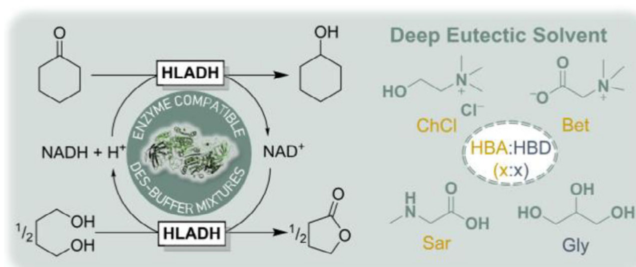
Selected references[17] show that DESs have been explored in a variety of biotransformations, including reactions catalyzed by hydrolases (EC3),[17b,17c] oxidoreductases (EC1),[17d,17e] lyases (EC4),[17f,17g] and transaminases (EC2),[17h,17i] demonstrating their potential. In particular, redox biocatalysis in DESs is an attractive approach because reductions/oxidations account for one-third of the reported enzymatic transformations.[18] The use of DESs for biocatalysis holds potential to solve one of the most problematic issues when using water as reaction media—the low substrate loadings often associated—due to their high solubilizing capacity for hydrophobic compounds. In certain cases, DESs have been designed as bi-functional systems, with components serving both as solvents and substrates, particularly in cofactor regeneration setups (e.g., choline chloride/glucose,[19] and choline chloride/1,4-butanediol).[20] However, enzyme kinetics and (thermo)stability can be significantly altered in the presence of nonaqueous media, especially the enzyme's affinity to its reactants ($K_M$). Thus, careful characterization and media design are often necessary to find useful reaction media. Herein, to rationally design suitable DESs for redox biocatalysis, a comprehensive understanding of the reactions in DES-enriched reaction systems is essential. However, studies on oxidoreductase kinetics in DESs have been scarce, with few notable examples, such as the kinetics of formate dehydrogenase in betaine-based DESs.[21]

Over the last decades, enzyme-compatible DESs have been designed, ranging from glycerol-based hydrophilic ones to other more hydrophobic combinations based on fatty acids.[22] From these studies, hydrophobic DESs outperformed kinetics in buffer, enabling high substrate loadings and conversions.[22] However, identifying hydrophilic DESs that can compete with aqueous conditions has traditionally been more challenging, with only a few notable examples.[23] To broaden those hydrophilic DES options, and based on our previous work,[12] herein, the impact of several hydrophilic glycerol-based DESs containing three hydrogen bond acceptors, choline chloride (ChCl), betaine (Bet), and sarcosine (Sar) (i.e., ChCl-Gly, Bet-Gly, and Sar-Gly), on oxidoreductase kinetics and catalytic performance, is assessed. In particular, the osmolytes betaine and sarcosine, derived from plants and deep-sea fish, respectively, are protein-stabilizing and may thus serve as promising ingredients for DES.[24] Moreover, our previous work demonstrated that a higher glycerol content promoted enzyme stability, whereas choline chloride was deleterious.[12] Besides, glycerol is biocompatible, low in toxicity, and widely used as a stabilizer for microorganisms and proteins.[25] Thus, apart from just replacing choline chloride—in the quest for enzyme-compatible and biogenic HBAs—different glycerol proportions (up to 8-surplus) are considered. For comparisons with previous works, horse liver ADH (HLADH) was used as a model enzyme, and the reduction of cyclohexanone (CHO) coupled with the oxidation of 1,4-butanediol (1,4-BD) to $\gamma$-butyrolactone (GBL) for cofactor regeneration was used as a prototypical reaction (Scheme 1). Subsequently, the best conditions were applied for a more industrially-sound substrate cinnamaldehyde (CinH) to afford cinnamyl alcohol (CinOH).




**Scheme 1.** Reduction of cyclohexanone to cyclohexanol catalyzed by horse liver alcohol dehydrogenase (HLADH) in deep eutectic solvents comprising glycerol as hydrogen bond donor (HBD) and three hydrogen bond acceptors (HBA): choline chloride (ChCl), betaine (Bet), and sarcosine (Sar) at varying molar ratios. Use of smart co-substrate to regenerate cofactor.[12]

## 2. Results and Discussion

### 2.1. Design and Characterization of Deep Eutectic Solvents

As stated above, our previous work demonstrated the detrimental effect of choline chloride and the beneficial effect of higher glycerol content on ADHs.[12] Thus, our rationale was to explore biogenic HBAs that could be more enzyme-compatible, and hence, protein-stabilizing kosmotropic osmolytes, such as betaine and sarcosine, were selected for comparison with the deleterious ChCl-Gly.[24] Two molar ratios, 1:2 and 1:8, were set for the DESs, i.e., the mainly used molar ratio with a low melting temperature and the reported molar ratio range with a potential more substantial stabilizing effect for enzymes.

The DES containing sarcosine (Sar-Gly, 1:1 and 1:2) required water during preparation to form a stable, homogeneous mixture, consistent with the literature.[24] In contrast, a molar ratio of 1:8 resulted in a very stable DES (Sar-Gly, 1:8) without the addition of water, which has not been reported previously. To delve deepen on this, Sar-Gly DESs were formed at other molar ratios (1:3, 1:4, 1:6, and 1:8). All of them led to stable homogeneous eutectic solutions without water (but not the Sar-Gly with less glycerol fraction, like 1:2 and 1:1), suggesting a different behavior at different ranges for the formation of DESs. To gain a broader understanding of the features of the selected DESs, a comprehensive characterization was conducted regarding their physicochemical properties (e.g., density, dynamic viscosity, and dynamic water activity) (Table 1). Overall, the three hydrophilic DESs displayed higher densities than pure water (as expected), with very low water activity (when freshly prepared and sealed stored) within a range of 0.18–0.30 due to the hygroscopic properties of DES components.

Fluid viscosity quantifies resistance to flow and is directly governed by the strength and nature of molecular interactions, including hydrogen bonding, electrostatic forces, van der Waals interactions, ion sizes, and the presence of void volumes.[26] As observed (Table 1), the viscosities of pure DESs remained high, except when some water was added (e.g., Sar-Gly 1:1 with 29 wt.% $H_2O$). Subsequently, the viscosity of the DESs and DES-buffer mixtures was examined at different temperatures (Figure 1). Expectedly, all DESs and DES-water mixtures exerted


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**Table 1.** Physicochemical properties of the pure DESs at room temperature in this study.

| DES | HBA | HBD | Molar Ratio (HBA:HBD) | $\rho$ [g·mL$^{-1}$] | $\eta$ [mPa·s] | $a_W$ [-] | Water Content [wt.%] |
|---|---|---|---|---|---|---|---|
| ChCl-Gly | ChCl | Gly | 1:2 | 1.185 ± 0.018[b)] | 328.0 ± 7.1 | 0.030[b] | 1.203 ± 0.002 |
| ChCl-Gly | ChCl | | 1:8 | 1.239 ± 0.003 | 539.0 ± 24.0 | 0.023 ± 0.001 | 1.684 ± 0.025 |
| Bet-Gly | Bet | | 1:2 | 1.153 ± 0.073 | 2286.7 ± 72.3 | 0.018 ± 0.001 | 0.771 ± 0.047 |
| Bet-Gly | Bet | | 1:8 | 1.243 ± 0.012 | 1130.0 ± 56.6 | 0.028 ± 0.012 | 0.245 ± 0.021 |
| Sar-Gly | Sar | | 1:1[a)] | 1.209 ± 0.002 | 48.2 ± 0.5 | 0.501 ± 0.130 | 26.813 ± 0.104 |
| Sar-Gly | Sar | | 1:8 | 1.285 ± 0.002 | 1355.0 ± 21.2 | 0.025 ± 0.013 | 0.205 ± 0.009 |

[a)] Including 29 wt.% $H_2O$ added during the preparation.
[b)] Data were taken from our previous study[12] for comparison.

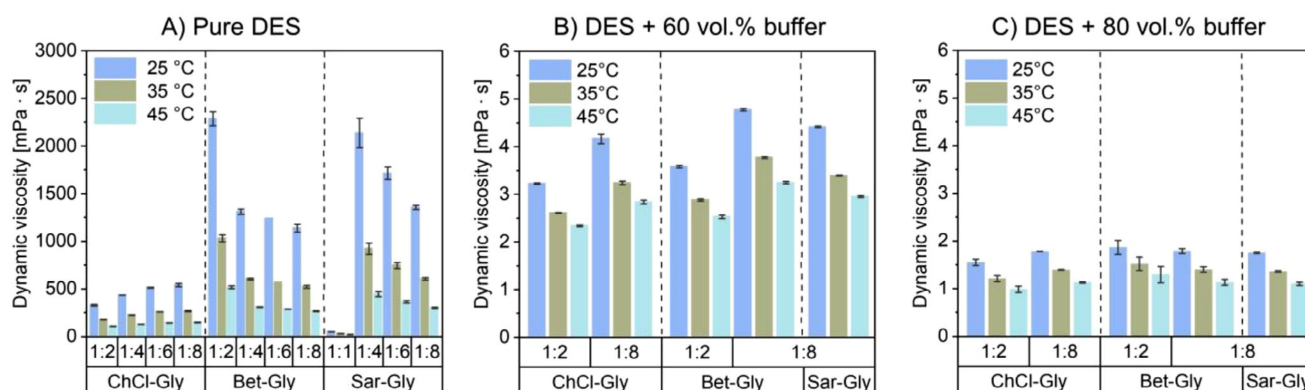


**Figure 1.** Dynamic viscosity of A) pure DESs, DES-buffer mixtures with B) 60 vol.%, and C) 80 vol.% buffer (Tris-HCl, 50 mM, and pH = 7.5) at different temperatures. Sar-Gly (1:1) is prepared with 29 wt.% $H_2O$ added during the preparation.

decreased viscosity at higher temperatures (25–45 °C). In the case of pure DESs (Figure 1A), betaine-DESs showed higher viscosity than ChCl-Gly, which decreased with the increase of glycerol fraction, especially for the molar ratios of 1:2 and 1:4. Intriguingly, Bet-Gly (1:8) displayed two times lower viscosity (1130 mPa·s) than that of the 1:2 molar ratio (2286.7 mPa·s), resulting in a different behavior from the ChCl-Gly one, where the 1:8 composition resulted in more viscous solvent. In contrast, Sar-Gly (1:1) was prepared with inherent water (29 wt.%) to form a homogeneous solution,[24] while the other molar ratios (1:3, 1:4, 1:6, and 1:8) did not require water addition. Therefore, Sar-Gly (1:1) displayed very low viscosity. For the other molar ratios, Sar-Gly exhibited comparable high viscosity to Bet-Gly and a similar trend with increasing temperature. The viscosity differences among ChCl-Gly, Bet-Gly, and Sar-Gly arise from their distinct molecular interactions. For ChCl–Gly, increasing glycerol fractions (from 1:2 to 1:8) enhances hydrogen bonding, thus raising viscosity. In contrast, betaine and sarcosine are zwitterions with carboxylate groups (Scheme 1); and excess glycerol disrupts their structured ionic hydrogen bond networks, acting more as a diluent and thus reducing viscosity.[26] Water addition is widely recognized as a tool to reduce the high viscosity of DESs. When 60 vol.% buffer was added, all mixtures showed significantly reduced viscosity of 3–5 mPa·s (Figure 1B), while adding 80 vol.% buffer led to a viscosity (around 1.5 mPa·s) comparable to water (0.89 mPa·s) at 25 °C (Figure 1C). Additionally, they all decreased with increasing temperature, reaching approximately 1 mPa·s at 45 °C.

Furthermore, considering all the potential factors that could influence enzyme performance, pH is one of the utmost aspects as it significantly affects enzyme stability and catalytic efficiency. The reported acidity of pure DESs promoted the pH analysis of DES-buffer mixtures. For example, all the DES-buffer mixtures with an 80% water content yielded very similar pH values as the pure buffer system (Tris-HCl, 50 mM, and pH = 7.5). These results indicate that, at higher water contents, pH values are more determined by the pH of the buffer and less influenced by DES components, which overall provides a suitable environment for enzyme catalysis (Table 2).

### 2.2. Thermostability of HLADH in DES-Buffer Mixtures

Once DESs were characterized, the behavior of HLADH therein was assessed, starting with its thermostability. The melting temperature ($T_m$) of the purified HLADH (SDS-PAGE result see Figure S1) was determined with NanoDSF (melting curves see Figures S2–S4). As shown in Figure 2, the deleterious effect of ChCl on the thermostability was evident, as expected from the literature.[12] In contrast, hydrophilic DESs containing betaine or sarcosine led to higher $T_m$ values at ≥20 vol.% buffer content compared to the pure buffer system. Notably, the Bet-Gly (1:8) resulted in the best option, exhibiting the most substantial stabilizing effect on HLADH, with a comparable $T_m$ to the pure buffer systems at a 10% buffer content. These results empha-


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**Table 2.** The pH values of various DES-buffer mixtures at room temperature.

| DES | Molar Ratio | DES with 60 vol.% Buffer | DES with 80 vol.% Buffer |
|---|---|---|---|
| ChCl-Gly | 1:2 | 7.60 ± 0.01 | 7.56 ± 0.01 |
| ChCl-Gly | 1:8 | 7.54 ± 0.01 | 7.50 ± 0.02 |
| Bet-Gly | 1:2 | 7.70 ± 0.01 | 7.54 ± 0.01 |
| Bet-Gly | 1:8 | 7.57 ± 0.02 | 7.47 ± 0.03 |
| Sar-Gly | 1:1[a] | 7.59 ± 0.06 | 7.58 ± 0.03 |
| Sar-Gly | 1:8 | 7.41 ± 0.01 | 7.45 ± 0.01 |

[a] Including 29 wt.% $H_2O$ added during the preparation.

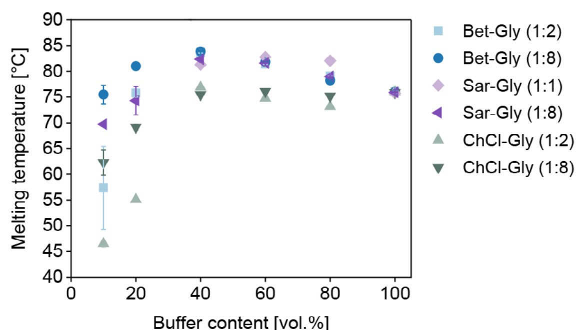


**Figure 2.** Melting temperature ($T_m$) of purified HLADH in various DESs and DES-buffer mixtures. Sar-Gly (1:1) is prepared with 29 wt.% $H_2O$ added during the preparation. The error bars represent the standard deviation of triplicate experiments.

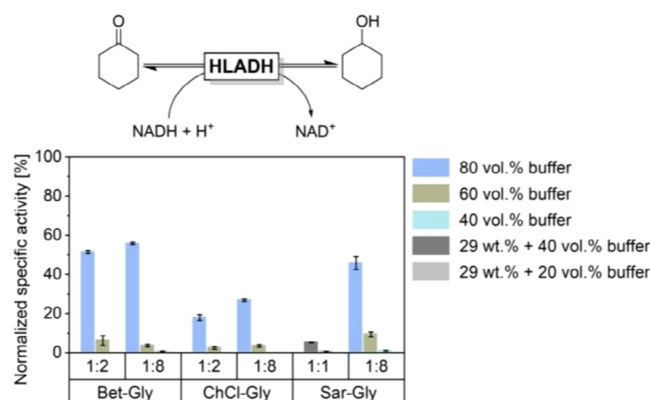


**Figure 3.** Normalized specific activity of HLADH toward cyclohexanone reduction in DES-buffer mixtures with varying buffer contents of 80, 60, and 40 vol.%. Specific activity is normalized for each system, referring to the pure buffer system (as 100%). Sar-Gly (1:1) is prepared with 29 wt.% $H_2O$ added during the preparation. Reaction conditions: the 1 mL buffer system (Tris-HCl, pH = 7.5, and 50 mM) or DES-buffer system contained 50 mM CHO, 0.1 mM NADH, and a defined amount of purified HLADH, and was monitored at 340 nm, at 25 °C for 1 min. The error bars represent the standard deviation of triplicate experiments.

size the potential of rationally selecting DES components, such as HBAs, which can be both enzyme-compatible and biogenic at the same time.

## 2.3. Specific Activity and Kinetics of HLADH in DES-Buffer Mixtures

As thermostability resulted in positive outcomes with the analyzed hydrophilic DES, specific enzyme activities and Michaelis–Menten kinetics were subsequently assessed. Before the assay via photometer, the spectral analysis of DES-buffer systems was confirmed not to interfere with the cofactor absorbance at 340 nm (Figure S5). To identify the optimal buffer content, the enzyme's specific activity was examined in the three DESs with 80, 60, and 40 vol.% buffer, while referring to the pure buffer system (enzyme specific activity recognized as 100%). As shown in Figure 3, in general, the enzyme activity was suppressed by the presence of DESs. Specifically, at molar ratios of 1:2, reduced activity was observed for all buffer contents, with a significant decrease at 60 vol.% and no detectable activity at 40 vol.%. A similar trend was observed for the 1:8 molar ratio, with enhanced enzyme activity in all cases, as well as slight activity at 40 vol.% buffer. These results further evidence the beneficial effect of glycerol[12] and the potential of betaine over sarcosine and choline chloride. Still, the inferior enzyme activity compared to the pure buffer system indicates an overall detrimental effect of DES on enzyme performance, in line with our previous findings[12] and somewhat inconsistent with the observed thermostability results (Figure 2).

Thermostability (expressed with $T_m$ values) refers to the enzyme's ability to maintain its structural integrity and resist denaturation under specific temperature conditions. In contrast, enzyme activity depends not only on the enzyme's structural state but also on some external factors, such as the surrounding environment (e.g., pH, substrate concentrations, and solvent conditions). Therefore, apart from those two aspects, studying enzyme kinetics is essential to gain a deeper understanding of this discrepancy between structural stability and functional performance. To this end, the kinetic analysis of HLADH toward the model substrate cyclohexanone (CHO) was conducted under optimal conditions of DESs with 80 vol.% buffer, while referring to the pure buffer system (Figure 4).

The enzyme displayed similar kinetic trends in DES-buffer mixtures as the pure buffer system, but with reduced activity. Higher activity was obtained in Bet-Gly, followed by Sar-Gly and ChCl-Gly. The three DESs with higher glycerol content led to relatively higher $V_{max}$ and thus higher $k_{cat}$ (Table 3), even comparable to the pure buffer in Bet-Gly (1:8). The lowest $V_{max}$ (6–8 times lower than pure buffer) was obtained with ChCl-


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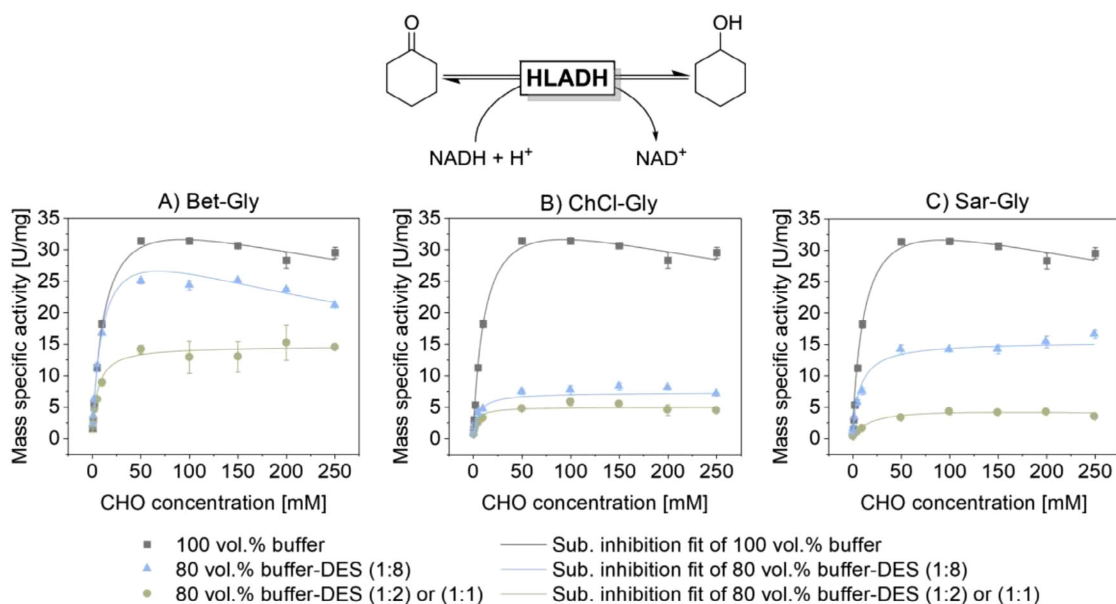


**Figure 4.** Kinetics of purified HLADH toward cyclohexanone (CHO) reduction in buffer and DES with 80 vol.% buffer: A) Bet-Gly, B) ChCl-Gly, and C) Sar-Gly. Sar-Gly (1:1) is prepared with 29 wt.% $H_2O$ added during the preparation, and an additional 40 vol.% buffer added afterwards. Reaction conditions: The 1 mL buffer system (Tris-HCl, pH = 7.5, and 50 mM) or DES-buffer system contained 0.5–250 mM CHO, 0.1 mM NADH, and a defined amount of purified HLADH, and was monitored at 340 nm at 25 °C for 1 min. The error bars represent the standard deviation of triplicate experiments.

**Table 3.** Summarized kinetics of the purified HLADH toward cyclohexanone reduction in various DES-buffer (with 80 vol.% buffer) systems.

| System | $V_{max}$ [µmol min$^{-1}$ mg$^{-1}$] | $K_M$ [mM] | $k_{cat}$ [s$^{-1}$] | $k_{cat}$ / $K_M$ [mM$^{-1}$ s$^{-1}$] |
|---|---|---|---|---|
| Pure buffer | 40.22 ± 1.35 | 12.68 ± 0.57 | 26.68 | 2.11 |
| Bet-Gly (1:2) | 14.66 ± 0.58 | 5.34 ± 0.61 | 9.72 | 1.82 |
| Bet-Gly (1:8) | 34.78 ± 2.22 | 10.54 ± 1.30 | 23.07 | 2.19 |
| ChCl-Gly (1:2) | 4.94 ± 0.29 | 3.46 ± 0.38 | 3.28 | 0.95 |
| ChCl-Gly (1:8) | 7.24 ± 0.47 | 5.21 ± 1.00 | 4.81 | 0.92 |
| Sar-Gly (1:1)[a] | 5.05 ± 0.79 | 18.83 ± 5.01 | 3.35 | 0.18 |
| Sar-Gly (1:8) | 15.40 ± 0.32 | 7.84 ± 0.72 | 10.22 | 1.30 |

[a] Sar-Gly (1:1) is prepared with 29 wt.% $H_2O$ added during the preparation and an additional 40 vol.% buffer added afterward.

Gly (1:2), once again demonstrating the incompatibility of the choline chloride-based DESs. When it comes to substrate affinity, all DES-buffer systems resulted in lower $K_M$ values, i.e., improved affinity, except for Sar-Gly (1:1). Given the large water solubility of cyclohexanone (~800 mM), the improved substrate affinity is attributed to enhanced accessibility of the substrate and possible structural changes of the enzyme. However, the presence of DESs caused suppressed enzyme activity, especially for ChCl-Gly, which could be due to the altered solvation and structure of the enzyme as previously reported.[12] For example, for ChCl-Gly with an 80 vol.% buffer, there are still choline chloride molecules attached to the enzyme surface, which have been shown to suppress enzyme activity.[12] The different kinetic values underscore the importance of identifying and choosing enzyme-friendly DES components. Overall, among the investigated systems, the optimal DES system is Bet-Gly (1:8) with an 80% buffer, resulting in a high $k_{cat}$ of 23.07 s$^{-1}$ and a reduced $K_M$ of 10.54 mM.

## 2.4. Enzymatic Cascade with Cyclohexanone in DES-Buffer Mixtures

Based on the Michaelis–Menten kinetic studies (see above), it became clear that glycerol-containing DES with a molar ratio of 1:8 is more enzyme-friendly, not only with choline chloride but also with other HBAs (betaine and sarcosine), and enables a trade-off in viscosity when blended with water. Therefore, the activity of HLADH was further evaluated in the cofactor-regenerating cascade reaction, which involved reducing cyclohexanone (CHO) while coupling with the oxidation of 1,4-butanediol (1,4-BD) in DES-buffer mixtures (Figure 5), as compared to the pure buffer system. The progressive curves were recorded for 24 h with close monitoring of the initial reaction rates by GC analysis (methodology details are provided in Table S1 and Figure S6). Regarding the long-term reaction course of the cascade reaction (Figure S7), HLADH showed reduced activity at lower buffer contents of 10 and 20 vol.% for all three


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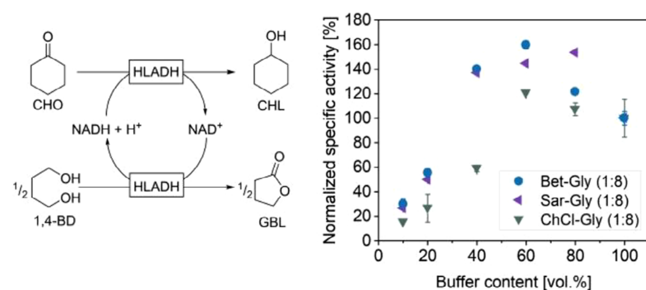


**Figure 5.** Normalized specific activity of HLADH toward cyclohexanone (CHO) reduction in the cascade reaction in DES (1:8)-buffer mixtures containing varying buffer contents (10, 20, 40, 60, and 80 vol.%). Specific activity is normalized for each system, referring to the pure buffer system (as 100%). Reaction conditions: The 1 mL pure buffer system (Tris-HCl, pH = 7.5, and 50 mM) or DES-buffer system contained 100 mM CHO, 50 mM 1,4-BD, 1 mM NADH, and a defined amount of purified HLADH (~6 U/mL), and proceeded at 1000 rpm, 25 °C. The error bars represent the standard deviation of duplicate experiments.

DESs. Specifically, Bet-Gly (1:8) with buffer contents of ≥ 40 vol.% led to similar reaction trends and yields as the pure buffer system, which was also observed for Sar-Gly (1:8) with buffer contents of ≥ 60 vol.%. Conversely, in the case of ChCl-Gly (1:8), HLADH afforded almost no product at 10 vol.% buffer, and highly reduced yields at 40–80 vol.%. Based on that yield analysis, Bet-Gly and Sar-Gly are more favorable than ChCl-Gly. Hence, rational design enables the identification of components with more promising prognoses.

Moreover, when it comes to the specific activity based on the initial reaction rate (when the yield is ≤ 10% within a linear progress range), the preference of HLADH to the DESs involving betaine and sarcosine is clearly reflected. The specific activities of HLADH in Bet-Gly (1:8) and Sar-Gly (1:8) were comparable across all the water contents (10–60 vol.%), in contrast to the reduced activity of ChCl-Gly (1:8) (Figure 5). Notably, HLADH demonstrated even superior performance in Bet-Gly and Sar-Gly with buffer contents of 40–80 vol.% compared to the pure buffer system, which is also consistent with the analogous yields shown in Figure S7. In the presence of ChCl-Gly, the enzyme displayed lower reaction efficiency at 10–40 vol.% buffer, whereas a higher buffer content (60–80 vol.%) led to a slightly higher specific activity. The combined results demonstrated Sar-Gly and Bet-Gly are more enzyme-compatible at specific buffer contents (≥40 vol.%) compared to ChCl-Gly and the pure buffer system, which further confirmed the effectiveness of replacing detrimental ChCl.[12]

## 2.5. Application of Optimal Hydrophilic DES-Buffer Mixtures for Cascade with Cinnamaldehyde

In a previous study, we demonstrated how ADHs can efficiently perform oxidoreductions in hydrophobic DES.[22] In this section, we will apply the same reaction in the hydrophilic DES as assessed in our previous study, now using Bet-Gly (1:8) and Sar-Gly (1:8) for the cascade using cinnamaldehyde (CinH) to produce a more valuable product of cinnamyl alcohol (CinOH)

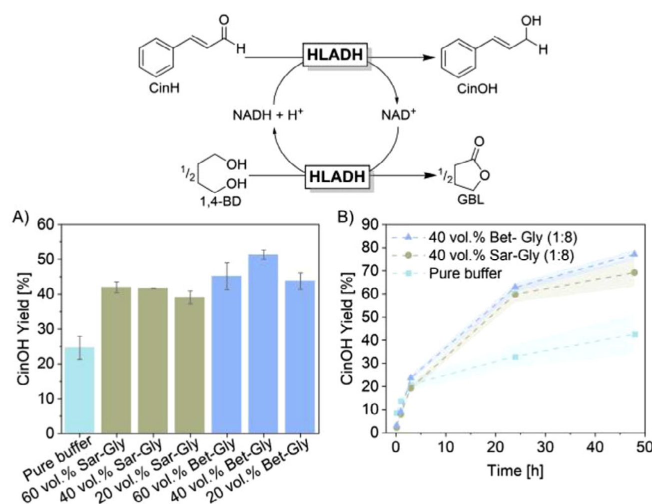


**Figure 6.** The cascade reaction using cinnamaldehyde (CinH) as a substrate. A) 24 h-Yield of cinnamyl alcohol (CinOH) in DES-buffer mixtures with 20–60 vol.% Bet-Gly (1:8) and Sar-Gly (1:8). B) Progress curves (48 h) of CinOH formation in DES-buffer mixtures with 60 vol.% buffer. Reaction conditions: The 1 mL pure buffer system (Tris-HCl, pH = 7.5, and 50 mM) or DES-buffer system contained 50 mM CinH, 25 mM 1,4-BD, 1 mM NADH, and a defined amount of purified HLADH (~10 U/mL), and proceeded at 1000 rpm and 25 °C. The error bars represent the standard deviation of triplicate experiments.

(Figure 6A). For this, the optimal conditions of the two DESs with varying buffer contents (40–80 vol.% buffer corresponding to 20–60 vol.% DESs) were first tested, considering the different solubilities of the two substrates, cyclohexanone and cinnamaldehyde, in comparison to the pure buffer system. The reaction was evaluated by GC (methodology details are provided in Table S2 and Figure S8). Notably, when performing the reaction in DES-buffer systems with varying water contents, higher yields were achieved compared to the pure buffer system (24% yield after 24 h). Specifically, the yields remained similar for Sar-Gly across all water contents, whereas Bet-Gly with 60 vol.% buffer produced the highest yield of 52% (Figure 6B), consistent with the trends observed in thermostability and specific activity in the cascade cyclohexanone reduction. The superior performance could be due to the improved long-term enzyme stability, as enzyme aggregation was observed at the very beginning in the pure buffer system, but not in the DES-buffer systems.

Overall, the results are both promising and compelling. Further experiments with progressive curve analysis demonstrated rapid reaction efficiency at the early stages (Figure 6 and Figure S9). From 3 h to 24 h, the reaction proceeded significantly faster in both DES-buffer systems compared to the pure buffer, indicating a long-term stabilizing effect of DESs on the enzyme. In contrast, protein aggregation was observed in a pure buffer system at an early stage, probably due to the adverse effects exerted by insoluble substrate. Ultimately, the yields in the DES-buffer mixtures were nearly double of those observed in the pure buffer system, demonstrating that it is feasible to design hydrophilic DESs that can work with oxidoreductases as well.


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## 3. Conclusion

The use of organic solvents as nonconventional media in biocatalysis has been developed over the last decades. A common understanding has been the need to use hydrophobic solvents (with high logP values) to avoid the total withdrawal of water from enzymes. As classic solvents cannot be tailored, most of the reported work in biocatalysis in nonaqueous media has been performed in hydrophobic organic solvents (logP values range from 2 to 4), with few remarkable outliers (e.g., MTBE, CPME, and 2-MeTHF).[27] Significantly, this can change with neoteric solvents, as they can be potentially tuned to be both enzyme-compatible and hydrophilic at the same time. To pave the way for that, this work studied alcohol dehydrogenase catalysis in glycerol-based DESs with other enzyme-compatible components like sarcosine and betaine. These two DESs (Bet-Gly and Sar-Gly) were prepared at different molar ratios, ranging from 1:1 to 1:8, to understand their physicochemical properties. A homogeneous eutectic solution was observed at a molar ratio of 1:2 for betaine and glycerol. Instead, the molar ratio from 1:3 on for sarcosine and glycerol led to a homogeneous solution at room temperature. Interestingly, the viscosity of these glycerol-based DESs showed different trends, with Bet-Gly and Sar-Gly showing lower viscosity at higher glycerol fraction. In contrast, for ChCl-Gly, a higher glycerol fraction (1:8) led to a more viscous system than the counterpart of 1:2.

Concerning the enzyme performance in DES-buffer systems, the thermostability ($T_m$ values) was enhanced at ≥20 vol.% buffer and peaked at 40 vol.% buffer content, following the order of Bet-Gly and Sar-Gly, while ChCl-Gly displayed no beneficial effect at the same water contents. Yet, the enzyme's specific activity was suppressed and relieved by gradually adding buffer. At 80 vol.% buffer of most DES-buffer mixtures, the enzyme showed a reduced $K_M$ as well as $k_{cat}$, but revealed comparable efficiency in Bet-Gly (1:8) to the buffer system. When proceeding with the optimal molar ratio of 1:8, the enzyme exhibited a highly enhanced specific activity and comparable yields at specific buffer concentrations. For Bet-Gly and Sar-Gly, optimal performance was observed at 40–80 vol.% buffer, while ChCl-Gly required 60–80 vol.%, with all systems peaking at 60 vol.% buffer. When applying the optimal conditions of Bet-Gly (1:8) and Sar-Gly (1:8) containing 60 vol.% buffer, the cinnamaldehyde reduction yielded higher production of cinnamyl alcohol compared to the pure buffer system. In contrast, in the aqueous system, enzyme aggregation occurred at a very early stage, confirming the long-term stabilizing effect of certain DES on enzymes. Overall, this work showcases that the systematic design and optimization of DES may lead to solvents that can be enzyme-compatible and useful for biocatalysis in hydrophilic non-conventional media.

Looking ahead, exploring the impact of DESs on enzyme stereoselectivity represents a compelling direction, especially for asymmetric synthesis. Additionally, the integration of carbonyl and alkene reductions in multienzyme cascade systems, such as combining ADHs with ene-reductases, offers a promising path toward streamlined and sustainable biocatalytic transformations. These future avenues can further unlock the potential of eutectic media for sustainable and selective synthesis.

## Acknowledgments

The authors acknowledge the financial support from the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG) under grant no. 391127961 and from the DFG Research Unit (FOR 5730) DESMOL2PRO under grant no. 528814716. The project has received funding from the EU's Horizon Europe Doctoral Network Program under the Marie Skłodowska-Curie grant agreement no. 101072731.

## Conflict of Interests

The authors declare no conflict of interest.

## Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Keywords:** Alcohol dehydrogenases · Hydrophilic deep eutectic solvents · Kinetics · Redox biocatalysis · Thermostability

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Manuscript received: June 15, 2025
Revised manuscript received: July 16, 2025
Accepted manuscript online: July 21, 2025
Version of record online: ■■, ■■


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