SUPPORTING INFORMATION

Impact of deep eutectic solvents (DESs) and individual DES components on alcohol dehydrogenase catalysis: Connecting experimental data and molecular dynamics simulations

Jan Philipp Bittner,[†a] Ningning Zhang,[†b] Lei Huang,[b] Pablo Domínguez de María,[c] Sven Jakobtorweihen,[*a,d] Selin Kara[*b]

[a] Institute of Thermal Separation Processes
Hamburg University of Technology
Eißendorfer Straße 38, 21073 Hamburg (Germany)
E-mail: jakobtorweihen@tuhh.de

[b] Biocatalysis and Bioprocessing Group, Department of Biological and Chemical Engineering
Aarhus University
Gustav Wieds Vej 10, 8000 Aarhus (Denmark)
E-mail: selin.kara@bce.au.dk

[c] Sustainable Momentum, SL.
Av. Ansite 3, 4-6, 35011, Las Palmas de Gran Canaria, Canary Islands (Spain)

[d] Department for Chemical Reaction Engineering
Hamburg University of Technology
Eißendorfer Straße 38, 21073 Hamburg (Germany)

[†] Both authors contributed equally to this work and are ordered alphabetically.

TABLE OF CONTENTS

1. Deep eutectic solvents (DESs) investigated in this study

Table S1. The DESs investigated in this work.

DESs			Properties (25 °C)		
Name	**HBA**	**HBD**	ρ **g mL^{-1}**	η **mPa·s**	a_{W} **-**
ChCl-Gly (1:2) *A*	ChCl 1	Gly 2	1.185 ± 0.018	342.8	0.030
ChCl-Gly (1:2) *B*	ChCl 1	Gly 2	Same components as *A*; Prepared by simply mixing without heating and stirring.		
ChCl-Gly (1:9)	ChCl 1	Gly 9	1.271 ± 0.007	553.6	0.028
ChCl-EG (1:2)	ChCl 1	EG 2	1.115 ± 0.000	42.6	0.066
EACl-Gly (1:1.5)	EACl 1	Gly 1.5	1.181 ± 0.009	268.2	0.063

2. Conversion of volume fractions and mole fractions for DES-water mixtures

In terms of molecular dynamics (MD) simulations, the experimentally used volume fractions (vol.%) need to be transferred to ion-based mole fractions (mol/mol). A corresponding equation used for the conversion can be found in Table S2, whereby, the mole fraction x_i is defined based on the ionic species (**Equation 1**):

$$x_i = \frac{n_i}{n_{\mathrm{cation}^+} + n_{\mathrm{Cl}^-} + n_{\mathrm{HBD}} + n_{\mathrm{W}}} \qquad \textbf{(Eqn. 1)}$$

Table S2. Conversion between volume fractions (vol. %) and mole fractions of water (mol/mol, x_{W}) used in this work.

Mole fraction [x_{W} **(mol/mol)**]	**ChCl-Gly (1:2) Volume fraction [vol.% H_2O]**	**ChCl-EG (1:2) Volume fraction [vol.% H_2O]**	**EACl-Gly (1:1.5) Volume fraction [vol.% H_2O]**
0	0	0	0
0.1	2.9	3.3	3.6
0.2	6.2	7.1	7.8
0.3	10.2	11.6	12.7
0.4	15.0	16.9	18.5
0.5	20.9	23.4	25.3
0.6	28.4	31.5	33.7
0.7	38.2	41.6	44.2
0.8	51.4	55.0	57.6
0.9	70.4	73.4	75.3
1	100	100	100

3. Preparation of lyophilized purified HLADH

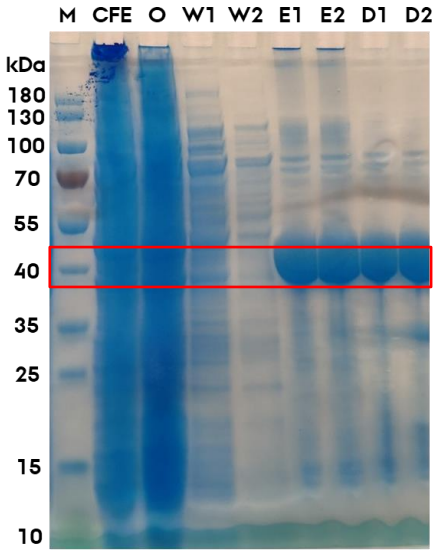

Figure S1. SDS-PAGE analysis of purified HLADH. CFE: cell free extract, O: outflow fraction, W: wash fraction, E: elution fraction, D: dialyzed elution fraction. MW of HLADH is around 40 kDa (red box).

4. Determination of the specific activity of HLADH for cyclohexanone reduction

4.1 Assay procedure No. 1

10.4 µL cyclohexanone and 4.4 µL 1,4-butanediol were dissolved in the corresponding reaction media in 1.5 mL GC vials and kept at room temperature overnight for equilibration (Table S3, S4). The stock of NAD^+ (20 mM) and freshly lyophilized purified HLADH (20 mg/mL) were prepared in Tris-HCl (50 mM, pH 7.5) and incubated at 25 °C for 30 minutes. The reactions were started by adding 50 µL HLADH and NAD^+ stock solution into the equilibrated reaction media (DES-water mixtures with various water contents of 0 – 100 vol.%) in 1.5 mL GC vials and incubated at 25 °C and 1200 rpm.

The final system had a total volume of 1.0 mL containing 100 mM cyclohexanone, 50 mM 1,4-butanediol, 1 mg/mL HLADH, 1 mM NAD^+ and various water contents. Each reaction was performed in duplicate. Samples were taken at definite times (2 – 6 min) when product yields were less than 10% and analyzed with GC. Figure 1b in the main text was generated by following this procedure.

4.2 Assay procedure No. 2

Solution of NAD^+ (20 mM) and lyophilized purified HLADH (20 mg/mL) was prepared in Tris-HCl (50 mM, pH 7.5) (as stock 1). Different procedures were followed to prepare the solution, to refer to the different preparation procedures they were denoted with ChCl-Gly (1:2) *A* and *B*.

For ChCl-Gly (1:2) *A* with 40 vol.% water, 600 µL ChCl-Gly *A*, 10.4 µL cyclohexanone, and 4.4 µL 1,4-butanediol were dissolved in 350 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 950 µL and kept at room temperature overnight for equilibration (as stock 2).

For ChCl-Gly (1:2) *B* with 20 vol.% water, 0.409 g choline chloride, 428 µL glycerol, 10.4 µL cyclohexanone, and 4.4 µL 1,4-butanediol were dissolved in 350 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 950 µL and kept at room temperature overnight for equilibration (as stock 3).

For ChCl-Gly (1:2) *B* with 40 vol.% water, 0.32 g choline chloride, 335 µL glycerol, 10.4 µL cyclohexanone, and 4.4 µL 1,4-butanediol were dissolved in 350 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 950 µL and kept at room temperature overnight for equilibration (as stock 4).

For ChCl-Gly (1:2) *B* with 60 vol.% water, 0.204 g choline chloride, 214 µL glycerol, 10.4 µL cyclohexanone, and 4.4 µL 1,4-butanediol were dissolved in 350 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 950 µL and kept at room temperature overnight for equilibration (as stock 5).

For ChCl-Gly (1:9) with 20 vol.% water, 800 µL ChCl-Gly (1:9), 10.4 µL cyclohexanone, and 4.4 µL 1,4-butanediol were dissolved in 150 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 950 µL and kept at room temperature overnight for equilibration (as stock 6).

For ChCl with 40 vol.% water, 0.723 g choline chloride, 10.4 µL cyclohexanone, and 4.4 µL 1,4-butanediol were dissolved in 350 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 950 µL and kept at room temperature overnight for equilibration (as stock 7).

For Gly with 40 vol.% water, 600 µL glycerol, 10.4 µL cyclohexanone, and 4.4 µL 1,4-butanediol were dissolved in 350 µL Tris-HCl (50 mM, pH 7.5) reach the final volume of 950 µL and kept at room temperature overnight for equilibration (as stock 8).

Then 950 µL stock 2/3/4/5/6/7/8 was mixed with 50 µL stock 1 in 1.5 mL GC vials and the final reaction system had a total volume of 1 mL with 100 mM cyclohexanone, 50 mM 1,4-butanediol, 1 mg/mL HLADH, 1 mM NAD^+ and 20/40/60 vol.% H_2O. Duplicate reactions were performed at 25 °C and 1200 rpm. Samples were taken at specific times (product yields < 10%) and analyzed with GC. Figures 2b, 3 in the main text and Figure S3 in supporting information were generated by following this procedure.

Table S3. Components of HLADH-catalyzed reaction in ChCl-Gly mixtures with various water contents.

Vol. water content [v/v] or vol.%	ChCl-Gly [µL]	ChCl [g]	Gly [µL]	Tris-HCl [µL]	HLADH & NAD^+ stock [µL]	CHO [µL]	1,4-BD [µL]	HLADH [mg]	NAD^+ [mg]	n_{H2O} [mmol]	n_{ChCl} [mmol]	n_{Gly} [mmol]
0%	1000	0	0	0	0	10.4	4.4	1	0.77			
1.36%	986.4	0	0	13.6	0	10.4	4.4	1	0.77			
5%	950	0	0	0	50	10.4	4.4	0	0			
10%	900	0	0	50	50	10.4	4.4	0	0			
12.5%	875	0	0	75	50	10.4	4.4	0	0			
15%	850	0	0	100	50	10.4	4.4	0	0			
20%	800	0	0	150	50	10.4	4.4	0	0	11.11	2.928	5.855
30%	700	0	0	250	50	10.4	4.4	0	0			
40%	600	0	0	350	50	10.4	4.4	0	0	22.22	2.196	4.391
ChCl + 40%	0	0.723	0	350	50	10.4	4.4	0	0	22.22	5.178	0
Gly + 40%	0	0	600	350	50	10.4	4.4	0	0	22.22	0	8.216
50%	500	0	0	450	50	10.4	4.4	0	0			
60%	400	0	0	550	50	10.4	4.4	0	0	33.33	1.464	2.928
70%	300	0	0	650	50	10.4	4.4	0	0			
80%	200	0	0	750	50	10.4	4.4	0	0			
90%	100	0	0	850	50	10.4	4.4	0	0			
100%	0	0	0	950	50	10.4	4.4	0	0			

Table S4. Components of HLADH-catalyzed reaction in ChCl-EG and EACl-Gly mixtures with various water contents.

Vol. water content [v/v] or vol.%	ChCl-EG or EACl-Gly [µL]	Tris-HCl [µL]	HLADH and NAD^+ stock [µL]	CHO [µL]	1,4-BD [µL]	HLADH [mg]	NAD^+ [mg]
0%	1000	0	0	10.4	4.4	1	0.77
5%	950	0	50	10.4	4.4	0	0
10%	900	50	50	10.4	4.4	0	0
20%	800	150	50	10.4	4.4	0	0
30%	700	250	50	10.4	4.4	0	0
40%	600	350	50	10.4	4.4	0	0
50%	500	450	50	10.4	4.4	0	0
60%	400	550	50	10.4	4.4	0	0
70%	300	650	50	10.4	4.4	0	0
80%	200	750	50	10.4	4.4	0	0
90%	100	850	50	10.4	4.4	0	0
100%	0	950	50	10.4	4.4	0	0

5. Catalytic performance of HLADH for cinnamaldehyde reduction

12.6 µL cinnamaldehyde and 4.4 µL 1,4-butanediol were dissolved in DES-water mixture [800 µL ChCl-Gly (1:2) or ChCl-Gly (1:9) with 100 µL water] in 1.5 mL GC vials. The stock of NAD^+ (10 mM) and freshly lyophilized purified HLADH (50 mg/mL) were prepared in Tris-HCl (50 mM, pH 7.5) and incubated at 25 °C for 30 minutes. The reactions were started by adding 100 µL HLADH and NAD^+ stock solution into the equilibrated reaction media and incubated at 25 °C and 1200 rpm. The final system had a total volume of 1.0 mL containing 100 mM cinnamaldehyde, 50 mM 1,4-butanediol, 5 mg/mL HLADH, and 1 mM NAD^+. Each reaction was performed in duplicate. Samples were taken at definite times and analyzed with GC while specific activity was calculated based on the initial reaction rates when the product yields were less than 10%. Figure 4 in the main text was generated by following this procedure.

6. Gas chromatography analysis

Aliquots samples (50 µL) from each reaction system were taken at definite time intervals and mixed with 250 µL of ethyl acetate (2 mM methyl benzoate as the internal standard). After centrifuging (13,000 rpm; 1 min) and separating the two phases, the EtOAc layer was dried with anhydrous $MgSO_4$. All reaction components were then analyzed by gas chromatography (GC) and the methods were developed with ß-DEX 120 column (30 m x 0.25 mm x 0.25 µm, Supelco® Analytical, USA; catalogue reference: 24304) and CP-Chirasil-DEX CB column (25 m x 0.25 mm x 0.25 µm, Agilent Technologies, USA, item no.: AGCP7502I5). Peaks were identified by standards while the final products were quantified by GC calibration curves. The details could be seen in Table S5 and Table S6.

Table S5. Details of GC method used for cyclohexanone reduction analysis in this study.

Heating program			Column	Components	t_R [min]
Rate [°C/min]	*T* [°C]	Hold [min]	ß-DEX 120 30 m x 0.25 mm x 0.25 µm	CHO	13.440
-	70	5		CHL	14.397
20	140	7		GBL	15.074
20	160	4		MB	17.463
20	220	0		1,4-BD	18.604

T(Injector): 250 °C; Detector: FID; *T*(Detector): 250 °C; Carrier gas: He; Pressure: 0.366 bar; Column flow: 0.4 mL/min; Total flow: 11.4 mL/min; Split ratio: 30.

CHO: cyclohexanone, CHL: cyclohexanol, GBL: γ-butyrolactone, MB: methyl benzoate, 1,4-BD: 1,4-butanediol.

Table S6. Details of GC method used for cinnamaldehyde reduction analysis in this study.

Heating program			Column	Components	t_R [min]
Rate [°C/min]	*T* [°C]	Hold [min]	CP-Chirasil-DEX CB, 25 m x 0.25 mm x 0.25 µm	GBL	4.123
-	130	10		MB	5.559
30	190	5		1,4-BD	8.827
				CinH	12.411
				CinOH	13.891

T(Injector): 275 °C; Detector: FID; *T*(Detector): 250 °C; Carrier gas: He; Pressure: 0.707 bar; Column flow: 0.67 mL/min; Total flow: 70.8 mL/min; Split ratio: 100.

GBL: γ-butyrolactone, MB: methyl benzoate, 1,4-BD: 1,4-butanediol, CinH: cinnamaldehyde, CinOH: cinnamyl alcohol.

7. Determination of the half-life time of HLADH

7.1 Assay procedure No. 1

The half-life times of HLADH were determined by incubating 500 µL of 1.0 mg/mL purified HLADH at 60 °C in DESs and DES-water mixtures with various water contents of 0 – 100 vol.%. The corresponding incubation media was put in 1.5 mL plastic tubes and kept at room temperature overnight for equilibration (Table S7, S8). 25 µL HLADH solution (20 mg/mL) was mixed with the above-equilibrated solution and was incubated at 60 °C. Aliquot samples were taken at specific time intervals and the residual activities were measured in 1 mL reaction system (Tris-HCl [50 mM, pH 7.5], 50 mM cyclohexanone 0.2 mg/mL heated HLADH samples, 0.1 mM NADH) with photometer for 1 min at 25 °C. Half-life times were determined from plots of the natural logs of residual activities versus the incubation time and calculated based on Equation 2. Each reaction was performed in duplicate. Figure 1a in the main text was generated by following this procedure.

7.2 Assay procedure No. 2

Solution of lyophilized purified HLADH (20 mg/mL) was prepared in Tris-HCl buffer (50 mM, pH 7.5) (as stock 1).

For ChCl-Gly (1:2) *A* with 40 vol.% water, 300 µL ChCl-Gly *A* was dissolved in 175 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 475 µL and kept at room temperature overnight for equilibration (as stock 2).

For ChCl-Gly (1:2) *B* with 40 vol.% water, 0.16 g choline chloride, 167 µL glycerol was dissolved in 175 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 475 µL and kept at room temperature overnight for equilibration (as stock 3).

For ChCl with 40 vol.% water, 0.362 g choline chloride was dissolved in 175 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 475 µL and kept at room temperature overnight for equilibration (as stock 4).

For Gly with 40 vol.% water, 300 µL glycerol was dissolved in 175 µL Tris-HCl (50 mM, pH 7.5) to reach the final volume of 475 µL and kept at room temperature overnight for equilibration (as stock 5).

Then 25 µL stock 1 was mixed with 475 µL stock 2/3/4/5 in 1.5 mL microcentrifuge tubes. The final system had a total volume of 500 µL with 1 mg/mL HLADH, 40% H_2O (v/v), and was incubated at 60 °C. The samples were taken at aliquot time points ranging from 0 to 24 h and the residual activities were detected at 25 °C as mentioned above (7.1). An exponential decrease function including the deactivation constant k_{DES} has been fitted to the residual activities. The half-life times ($t_{1/2}$) were then calculated based on Equation 2. Figure 2a in the main text and Figure S2 in supporting information was generated by following this procedure.

$$\tau_{1/2} = \frac{\ln 2}{k_{\mathrm{DES}}} \qquad \textbf{(Eqn. 2)}$$

$\tau_{1/2}$*:* Half-life time [min]

k_{DES}*:* Deactivation constant [min^{-1}]

Table S7. Components of HLADH stability assay in ChCl-Gly mixtures with various water contents.

Vol. water content [v/v] or vol.%	ChCl-Gly *A* [µL]	ChCl [g]	Gly [µL]	Tris-HCl [µL]	HLADH stock [µL]	HLADH [mg]	n_{H_2O} [mmol]	n_{ChCl} [mmol]	n_{Gly} [mmol]
0%	500	0	0	0	0	0.5			
5%	475	0	0	0	25	0			
10%	450	0	0	25	25	0			
20%	400	0	0	75	25	0			
30%	350	0	0	125	25	0			
40%	300	0	0	175	25	0	11.11	1.107	2.214
ChCl+Gly+40%	0	0.16	167	175	25	0	11.11	1.146	2.287
ChCl + 40%	0	0.362	0	175	25	0	11.11	2.593	0
Gly + 40%	0	0	300	175	25	0	11.11	0	4.108
50%	250	0	0	225	25	0			
60%	200	0	0	275	25	0			
70%	150	0	0	325	25	0			
80%	100	0	0	375	25	0			
90%	50	0	0	425	25	0			
100%	0	0	0	475	25	0			

Table S8. Components of HLADH stability assay in **ChCl-EG** and **EACl-Gly** mixtures with various water contents.

Vol. water content [v/v] or vol.%	ChCl-EG or EACl-Gly [µL]	Tris-HCl [µL]	HLADH stock [µL]	HLADH [mg]
0%	500	0	0	0.5
5%	475	0	25	0
10%	450	25	25	0
20%	400	75	25	0
30%	350	125	25	0
40%	300	175	25	0
50%	250	225	25	0
60%	200	275	25	0
70%	150	325	25	0
80%	100	375	25	0
90%	50	425	25	0
100%	0	475	25	0

8. Comparison of catalytic performance of HLADH in ChCl-Gly A and B

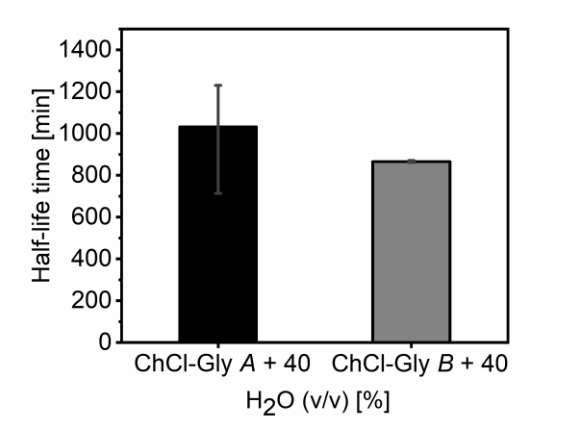

Figure S2. Comparison of half-life time of HLADH in ChCl-Gly (1:2) *A* and ChCl-Gly (1:2) *B* with 40 vol.% water at 60 °C.

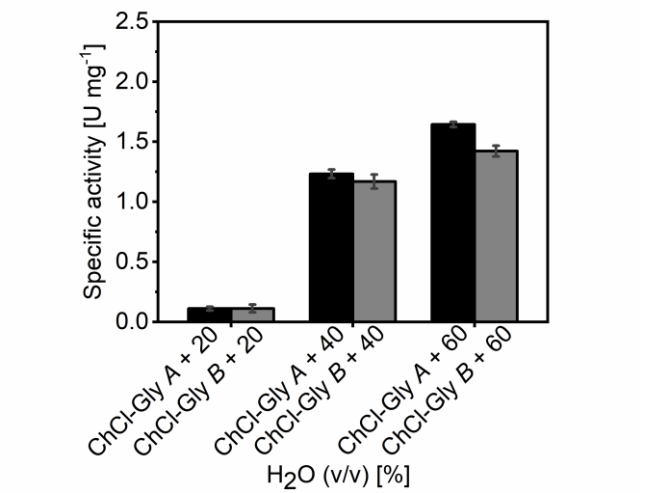

Figure S3. Comparison of specific activity of HLADH-catalyzed reduction in ChCl-Gly (1:2) *A* and ChCl-Gly (1:2) *B* with 20, 40, and 60 vol.% water.

9. Thermodynamic water activity and viscosity of DESs and DES-water mixtures

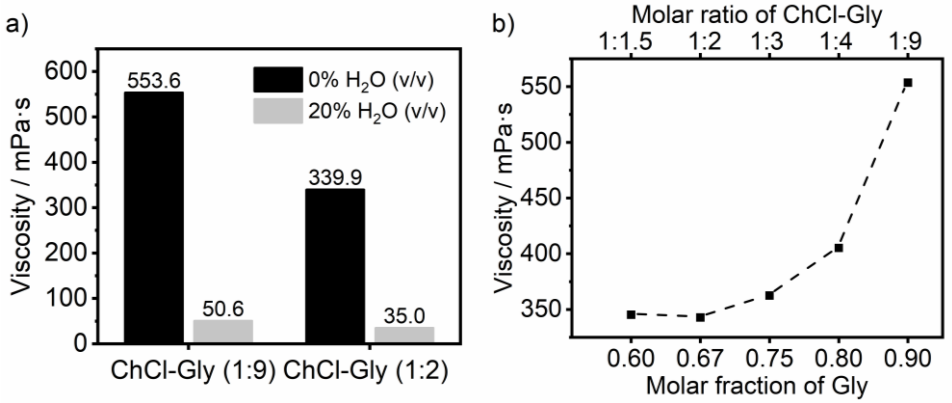

Figure S4. Experimentally measured viscosity of (a) ChCl-Gly (1:2) and ChCl-Gly (1:9) as pure DESs and in mixtures with 20 vol.% water and (b) ChCl-Gly with different molar ratio of ChCl:Gly.

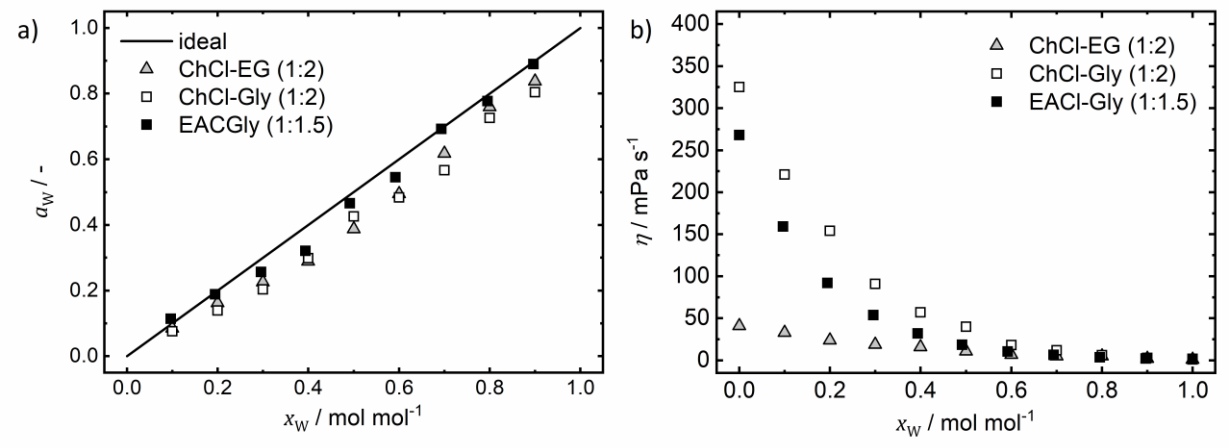

Figure S5. (a) Experimentally determined thermodynamic water activity of mixtures with ChCl-Gly (1:2), ChCl-EG (1:2), and EACl-Gly (1:1.5) in dependency of the water mole fraction (x_W). (b) Experimentally measured viscosity of ChCl-Gly (1:2), ChCl-EG (1:2), and EACl-Gly (1:1.5) mixtures with water in dependency of the water mole fraction (x_W). The activity and viscosity values for ChCl-Gly (1:2) and ChCl-EG (1:2) were taken from Bittner *et al*. [*J. Chem. Theory Comput.* 2021].

10. Catalytic performance of HLADH for cinnamaldehyde reduction

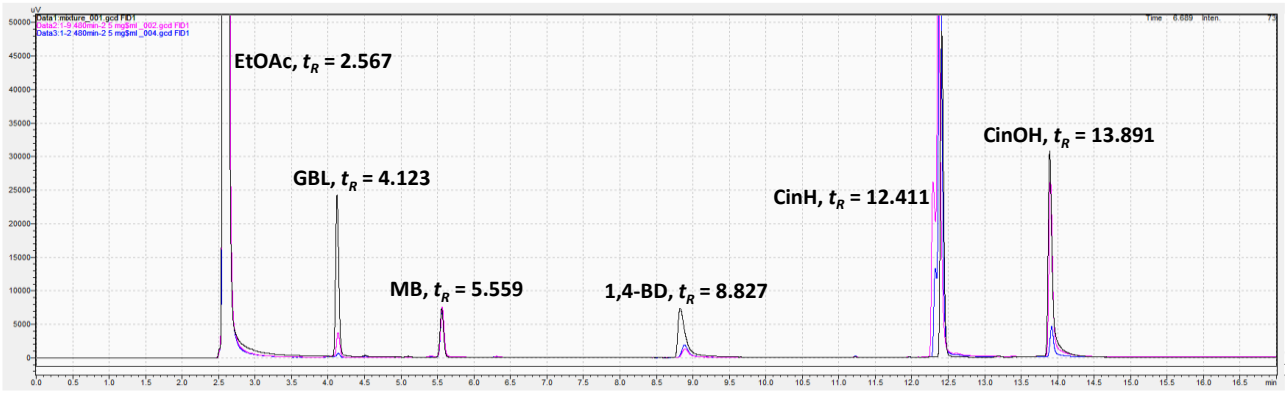

Figure S6. Representative GC chromatogram of components in cinnamaldehyde reduction system. (Black: mixture of all standards; blue: reaction in ChCl-Gly (1:2) with 20 vol.% water after 8 h; pink: reaction in ChCl-Gly (1:9) with 20 vol.% water after 8 h).

11. Summary of the investigation of individual components of ChCl-Gly

Table S9. Influence of individual components of ChCl-Gly on properties of the solvent e.g. water activity (a_W), enzyme catalysis (specific activity and half-life time of HLADH) and its properties in the MD simulations (flexibility in terms of RMSF, hydration, and solvation of DES molecules).

Entry	Composition	x_W	a_W	Specific activity of HLADH	Half-life time of HLADH	RMSF	Hydration layer	Solvation layer of glycerol	Solvation layer of choline	Solvation layer of chloride
		[-]	[-]	[U/mg]	[min]	[Å]	[-]	[-]	[-]	[-]
40% (v/v) water										
1	ChCl	0.807	0.547	0.53	277	0.824	1094	-	226	101
2	ChCl-Gly (1:2)	0.716	0.633	1.23	1033	0.696	599	410	94.7	21.9
3	Gly	0.729	0.656	6.55	3466	0.775	624	554	-	-
20% (v/v) water										
4	ChCl-Gly (1:2)	0.486	0.408	0.17	47.4	0.606	380	456	136	40.0
7	ChCl-Gly (1:9)	0.917	0.524	0.36	-	0.639	432	566	37.6	14.4
8	water	1	1	8.05	575	1.18	1554	-	-	-

12. Phase diagram of eutectics at different glycerol mole fractions

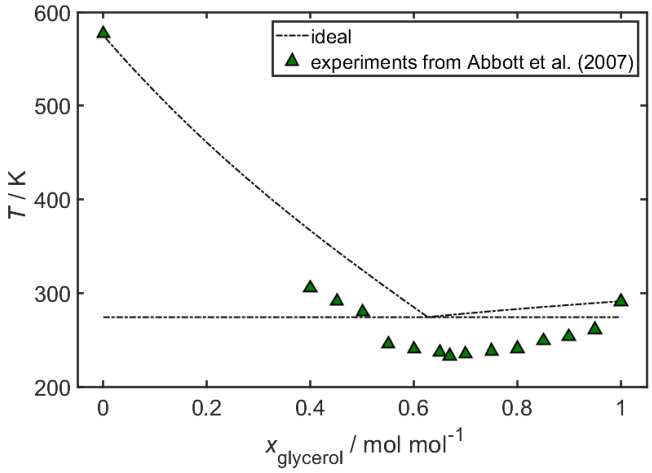

Figure S7. Phase diagram of eutectics at different glycerol mole fractions. ChCl: Gly (1:1.5, X_{Gly} 0.6 mol mol^{-1}; 1:2, X_{Gly} 0.66 mol mol^{-1}; 1:9, X_{Gly} 0.9 mol mol^{-1}) shows DES characteristics. Abbott et al. *Green Chem.,* 2007, 9, 868–872; DOI: 10.1039/B702833D.