Supporting Information

A Deep Eutectic Solvent Thermomorphic Multiphasic System for Biocatalytic Applications

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Experimental Procedures

General information All solvents, reactants, and starting materials were received from commercial suppliers in the highest available purity (Sigma-Aldrich, VWR) and used as received. Ultrapure water (UPW, 18.2 MΩ·cm) was produced with a Milli-Q® Synthesis system by Millipore Corporation (now Merck Millipore, Darmstadt, Germany) and used throughout this study. Mass fraction (m/m) of the DES were always calculated for monophasic system conditions. As biocatalyst, recombinant horse liver alcohol dehydrogenase (HLADH) expressed in E. coli was purchased from Sigma-Aldrich (SKU: 55689-100MG) and used throughout this study. All experiments were carried out under atmospheric conditions if not stated otherwise. For tempering and shaking of the reaction vessels, a MKR 23 thermoshaker from Hettich (The Netherlands) was used.

Potassium phosphate buffer solution (KPi buffer, 50 mmol·L⁻¹, pH 7.5). 0.641 g dipotassium hydrogenphosphate (K₂HPO₄) and 0.180 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 100 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 7.5.

KPi buffer, 100 mmol·L⁻¹, pH 7.5. 1.281 g dipotassium hydrogenphosphate (K₂HPO₄) and 0.360 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 100 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 7.5.

KPi buffer, 500 mmol·L⁻¹, pH 7.5. 6.405 g dipotassium hydrogenphosphate (K₂HPO₄) and 1.800 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 100 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 7.5.

KPi buffer, 50 mmol·L⁻¹, pH 6.0. 0.601 g dipotassium hydrogenphosphate (K₂HPO₄) and 2.932 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 500 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 6.0.

KPi buffer, 100 mmol·L⁻¹, pH 6.0. 0.241 g dipotassium hydrogenphosphate (K₂HPO₄) and 1.173 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 100 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 6.0.

KPi buffer, 200 mmol·L⁻¹, pH 6.0. 0.482 g dipotassium hydrogenphosphate (K₂HPO₄) and 2.346 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 100 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 6.0.

KPi buffer, 50 mmol·L⁻¹, pH 7.0. 0.467 g dipotassium hydrogenphosphate (K₂HPO₄) and 0.315 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 100 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 7.0.

KPi buffer, 50 mmol·L⁻¹, pH 8.0. 0.814 g dipotassium hydrogenphosphate (K₂HPO₄) and 0.044 g potassium dihydrogenphosphate (KH₂PO₄) were dissolved in UPW in a 500 mL volumetric flask and, if necessary, the pH was adjusted with potassium hydroxide to pH 8.0.

Preparation of the deep eutectic solvent DES (LID:OA). The DES was prepared according to a modified literature description,[1] and lidocaine (LID, 234.34 g mol⁻¹) and oleic acid (OA, 282.46 g mol⁻¹) in a mole fraction of 1:1 were used. A sealed 20 mL screw cap vessel with nitrogen headspace was applied to prevent the oleic acid of any possible oxidation side reactions, and as an example, 6.01 g (21.3 mmol) of LID and 4.99 g (21.3 mmol) of OA were dissolved in UPW in a 100 mL volumetric flask, yielding a light brown and highly viscous liquid (see also Figure 1). The eutectic point is estimated to −60 °C according to the literature.[1][2]

Determination of the temperature dependent binodal of DES with UPW or with buffer solutions. To investigate the LCST phase behavior of the DES with water, 20 samples with mass fractions from wDES = 5-100% (m/m) within 5% (m/m) steps with UPW were prepared in GC vials to a final mass of one gram. All samples were prepared by mass on a fine scale and afterwards shaken at 1200 rpm and the temperature was stepwise increased within 0.5 K starting from 15 °C with an equilibration time of 5 minutes for each temperature. The experiment was continued until all samples had become a two-phase system (the liquid was turbid).

Determination of the influence of proteins towards the binodal curve. To investigate a possible influence of proteins towards the LCST phase behavior of the DES with KPi buffer, bovin serum albumin (BSA) was used as a model protein. A system of DES and 100 mmol·L⁻¹ KPi (pH 7.5) in a mass fraction of wDES = 50% was used with BSA concentrations from 0.024–1.563 mg·mL⁻¹. The vessels were shaken, tempered, and investigated as mentioned above. To test the influence of the enzyme HLADH towards the cloud point temperature of a system composed of DES and 50 mmol·L⁻¹ KPi (pH 6.0) with a mass fraction of wDES = 50%, 0.400 mg·mL⁻¹ of HLADH were added and the resulting cloud point temperature was compared to the system without the addition of enzyme.
Determination of the influence of educts, products, and cofactors towards the binodal curve. To investigate a possible influence of the substrate benzaldehyde and the product benzyl alcohol towards the LCST phase behavior, a system of 50 mmol·L⁻¹ KPi (pH 6.0) with a mass fraction of \( w_{\text{DES}} = 50\% \) was used with concentrations of \( (10, 25, 50, 100 \text{ and } 200) \text{ mmol·L}^{-1} \) of either the substrate or the product. The possible influence of the cofactor NADH was tested with concentrations of \( (5, 10, 25, 50, \text{ and } 62.5) \text{ mmol·L}^{-1} \) of NADH. The vessels were shaken, tempered, and investigated as mentioned above.

Protein distribution studies. To analyze the distribution of proteins in the upper and the lower phase of a 30 °C-heated two-phase system, SDS polyacrylamide gel electrophoresis was used. One gram of a system of DES and 50 mmol·L⁻¹ KPi (pH 6.0) in a mass fraction of \( w_{\text{DES}} = 50\% \) was prepared and 0.25 mg BSA (800 mg·L⁻¹) was added. Afterwards, the sample was cooled to 18 °C and shaken at 1200 rpm forming a one-phase, homogenous, system. Then, the samples were heated to 30 °C and equilibrated for 15 minutes without stirring generating a clear two-phase system. A sample of each phase layer was collected and analyzed via SDS polyacrylamide gel electrophoresis. PageRulerTM prestained protein ladder and GenScript SurePAGE, Bis-Tris,10 x 8 gel at 140 V were used. 30 mL InstantBlue Coomassie Protein Staining was added to visualize the protein bands. Additionally, samples of the upper, DES-enriched phase and of the lower, aqueous (AQ)-enriched phase were analyzed without the addition of protein as a reference.

Product and educt distribution studies. To analyze the distribution of benzaldehyde (substrate) and benzyl alcohol (product), a system composed of DES and 50 mmol·L⁻¹ KPi (pH 6.0) in a mass fraction of \( w_{\text{DES}} = 50\% \) was used. In total of 1 mL volume, 25 mmol·L⁻¹ of either benzaldehyde or benzyl alcohol were diluted at 20 °C. Then, the system was heated to 45 °C for five minutes and afterwards, the samples were settled at 30 °C for 24 h. A sample was taken from the lower, AQ-enriched phase and extracted with DCM/n-dodecane 1:1 (v/v). The vial was centrifuged for one minute at 13400 rpm to promote the phase separation and the lower, DCM/n-dodecane phase was then analyzed with the GC. The upper, DES-enriched phase was diluted in DCM/n-dodecane 1:1 (v/v) and analyzed with the GC.

Biocatalytic Reaction in buffer 19 mL of KPi (50 mmol·L⁻¹, pH 6.0) was transferred to a reactor vessel tempered with a RE630S thermostat from Lauda (Lauda-Königshofen, Germany). The substrate benzaldehyde was added to a concentration of 25 mM in a total volume of 20 mL. A sample was taken as reference (see below) and then, one mL of horse liver alcohol dehydrogenase stock solution (8000 mg·L⁻¹ in KPi, 50 mmol·L⁻¹, pH 6.0) was added to start the reaction (final enzyme concentration was 400 mg·L⁻¹). Three different temperatures were evaluated (20 °C, 25 °C and 30 °C) and 200 µL-samples were taken at definite time intervals in triplicates. The AQ samples were extracted with the same volume of DCM/n-dodecane, centrifuged and measured with the GC.

Biocatalytic DES-TMS reaction system. For the biocatalytic reaction, DES was combined with 50 mmol·L⁻¹ KPi buffer (pH 6.0) in a mass fraction of \( w_{\text{DES}} = 50\% \). Ten g DES and 9 mL KPi (50 mmol·L⁻¹, pH 6.0) were transferred to a tempered reactor vessel. 51 µL benzaldehyde (25 mM) and 0.66 g NADH (50 mM) were added. The solution was cooled to 20 °C and stirred yielding a one-phase system. The reactor was then heated to 45 °C for 5 min and then cooled to 30 °C without stirring yielding a clear two-phase system. After 24 h settling, samples were taken from each phase of the clear two-phase system. A 200-µL sample was taken from the lower, AQ-enriched phase and extracted with 200 µL of DCM/n-dodecane 1:1 (v/v). The vial was centrifuged for one minute at 13400 rpm to promote the phase separation and the lower, DCM/n-dodecane phase was then analyzed with the GC. Additionally, 200 µL of the upper, DES-enriched phase was diluted into 200 µL DCM/n-dodecane 1:1 (v/v) and analyzed with the GC. The system was then cooled to 20 °C (one-phase system), and 1 mL HLADH solution was added (8000 mg·L⁻¹ stock and 400 mg·L⁻¹ final concentration). After 24 h reaction time, the system was heated to 45 °C for 5 min, and subsequently cooled to 30 °C. After 24 h, the two phases completely separated, and samples were taken from the upper and the lower phase as mentioned above.

Reuse of Biocatalyst within the system DES-TMS. After the biocatalytic reaction was carried out (see above), the enzyme was recycled by removing the AQ-enriched phase while discarding the DES phase. The reactor was cleaned, and the volume and mass of the AQ-enriched phase was determined and transferred back to the reactor. New DES was added in same mass as the AQ-enriched phase which varied for each recycling. Benzaldehyde (10 mmol·L⁻¹) was added to the new system and the system was again tempered to 20 °C yielding a one-phase system. This procedure was then repeated three times.

Gas chromatography (GC). A Nexis GC-2030 gas chromatograph from Shimadzu (Japan) with a flame ionization detector (FID) equipped with a CP-Chirasil-Dex CB column (CP7502, NLR0663300, 25 m × 0.25 mm within a coating thickness of 0.25 µm) from Agilent was used for analytics. Carrier gas helium (purity: 99.9999%) with a flow rate of 0.67 mL·min⁻¹ was used for all measurements. Temperatures of the injector and detector were set to 275 °C and 250 °C, respectively. Temperature program: 3 min at 130 °C for equilibration, followed by hold time for 12 min at 130 °C after injection, followed by a heating rate of 30 °C·min⁻¹ to 190 °C and a hold time for 5 min. The sample was injected within split mode with a split ratio of 100 and a purge flow of 3 mL·min⁻¹. Two different calibration methods were used. For the calibration of the samples from the DES phase, the neat substrate and product were diluted in a solution of dichloromethane (DCM) with 5 mmol·L⁻¹ of n-dodecane as internal tracer substance and measured with the GC. For the samples being extracted from the AQ layer, the neat substrate and product were first diluted in 50 mmol·L⁻¹ KPi (pH 6.0) and then extracted with DCM/n-dodecane. The vials were centrifuged for one minute at 13400 rpm to promote the phase separation and the lower, DCM/n-dodecane phase was then analyzed with the GC.
Author Contributions

L.-E. Meyer conceived the conception and design of this work. Method development was carried out by L.-E. Meyer. Experimental work was carried out by M. B. Andersen and L.-E. Meyer. L.-E. Meyer and S. Kara were responsible for supervision, L.-E. Meyer was responsible for validation, data curation, and visualization. S. Kara was responsible for funding acquisition and project administration. L.-E. Meyer wrote the original draft, and L.-E. Meyer and S. Kara reviewed and edited the final draft. All authors agreed with the final version of the submitted manuscript.

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