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Comparison of catalysis by haloalkane dehalogenases in aqueous solutions of a deep eutectic and organic solvents

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Haloalkane dehalogenases catalyze the hydrolytic cleavage of carbon-halogen bonds in diverse halogenated hydrocarbons and are attractive catalysts for sustainable biotechnologies. However, their use in industrial processes is limited due to the poor water solubility of their substrates and the tendency of the substrates to undergo abiotic hydrolysis. Here we systematically and critically compare the performance of three haloalkane dehalogenases, DbjA, DhaA and LinB, in aqueous solutions of the deep eutectic solvent ethaline, its components (ethylene glycol and choline chloride), and two organic solvents (methanol and acetone). Each of the solvents had different effects on the activity of each enzyme. Haloalkane dehalogenase DhaA was found to be the most tolerant of ethaline, retaining 21% of its reference activity even in solutions containing 90% (v/v) of ethaline. However, dissolution in 75% (v/v) ethylene glycol, 50% (v/v) methanol, or 25% (v/v) acetone caused almost total loss of DhaA activity. In contrast, the activities of DbjA and LinB were higher in ethylene glycol than in ethaline, and moreover the activity of DbjA was 1.5 times higher in 50% (v/v) ethylene glycol than in pure buffer. Interestingly, the enantioselectivity of 2-bromopentane hydrolysis catalysed by DbjA increased more than 4-fold in the presence of ethaline or ethylene glycol. Our results demonstrate that ethylene glycol and an ethylene glycol-based deep eutectic solvent can have beneficial effects on catalysis by haloalkane dehalogenases, broadening their usability in "green" biotechnologies.

Introduction

The past two decades have witnessed a major drive to increase the efficiency of biotransformation by performing enzyme catalysis in the presence of organic solvents. The use of aqueous solutions containing water-miscible organic solvents can increase the solubility of water-immiscible reactants and suppress undesired hydrolysis reactions.[1,2] However, organic solvents are the main sources of volatile organic compound emissions produced during biotransformations.[3–5] Ionic liquids (ILs) have been introduced as green and sustainable alternatives to hazardous organic solvents. They have a number of useful properties, including non-flammability, high solvation ability and negligible vapour pressure.[6–8] Unfortunately, their high cost and challenging synthesis together with a lack of relevant toxicity data prevent their utilization on larger scales.[9–11]

A promising alternative to ILs was introduced in 2003 when Abbot et al. reported that a mixture of (2-hydroxyethyl)trimethylammonium (choline) chloride (ChCl), also known as vitamin B_4, with urea in a molar ratio of 1:2 has a low melting point.[12] The term deep eutectic solvent (DES) was introduced to denote mixtures of ammonium salts with hydrogen bond donors whose freezing points are significantly depressed relative to those of their isolated components such that they remain liquid at ambient temperatures. It was subsequently demonstrated that various hydrogen bond donors, including alcohols, carboxylic acids, and urea derivatives form mixtures with similar properties when combined with ChCl in appropriate proportions.[10,13] DESs share many properties with ILs.[14] Their negligible vapour pressure under ambient conditions enables the direct distillation and separation of volatile components under mild conditions, which is energy-efficient and good for fragile compounds.[15] Moreover, they are straightforward and inexpensive to prepare, do not require extensive purification, and are expected to be more environmentally friendly than ILs.[16] They also exhibit unusual solvation properties that are strongly influenced by hydrogen bonding. Any molecule capable of forming hydrogen bonds is likely to be highly soluble in these mixtures.[17] DESs are miscible with protic solvents but not generally with aprotic ones. Aprotic solvents such as ethyl acetate or diethyl ether can therefore be used to extract molecules of lower polarity from DESs, allowing the DES to be reused. Moreover, because DESs are miscible with water, the addition of water to DES-based reaction mixtures often causes organic products to either form a separate layer or to precipitate, enabling their isolation by filtration.[18] To date, there have been relatively few published studies on the dissolution of organic compounds in DESs. Morrison and co-workers investigated the dissolution of several poorly soluble drugs in ChCl:urea and ChCl:malonic acid and found them to be up to 20 000 times more soluble in these DESs than in pure water.[19] Linberg and co-workers reported that the highest achievable concentrations of certain epoxides in various DES solutions were 1.5 times higher than those for phosphate buffer.[20]

DESs have been used as green solvents for several industrial processes including biodiesel purification, polymer synthesis, and gas absorption.[21–24] However, only a few studies have examined the use of DESs as media for biocatalytic reactions. A pioneering report on biocatalysis in DESs was published in 2008 by Gorke et al.[25] These authors showed that whereas enzymes are usually denatured by polar organic co-solvents such as methanol or acetone, they can exhibit good catalytic activity in DESs of similar polarity. This is noteworthy because the DESs contain strong hydrogen bond donors, which are often responsible for denaturing enzymes. In particular, the conversion of styrene oxide by epoxide hydrolases was enhanced by a factor of 20 when using ChCl:glycerol as a co-solvent.[25] Zhao and co-workers studied protease activation in a glycerol-based DES. Cross-linked subtilisin exhibited high activity and selectivity in ChCl:glycerol (1:2) containing 3% (v/v) water.[26] A range of new deep eutectic mixtures have recently been

described. For instance, a combination of choline acetate and glycerol proved to be significantly less viscous than previous DESs while also being highly compatible with lipases.[27,28] This DES was successfully used as a medium for the *Candida antarctica* lipase B-catalyzed transesterification of Miglyol® oil with methanol,[27] giving a yield that was 1.5 times higher than that achieved in pure glycerol. Unfortunately, only a few studies have directly compared the effects of conventional organic solvents and DESs on the same enzymes, making it difficult to perform reasonable comparative evaluations of the two solvent types as media for biocatalysis. Comparative studies using both DESs and organic solvents as media for enzyme-catalyzed reactions would therefore provide extremely valuable data.

The aim of the work presented herein was to determine how DESs affect the structure, stability, activity, and enantioselectivity of three haloalkane dehalogenases (HLDs): DbjA from *Bradyrhizobium japonicum* USDA110,[29,30] DhaA from *Rhodococcus rhodochrous* NCIMB13064,[31] and LinB from *Sphingobium japonicum* UT26.[32] Most HLDs are enzymes of microbial origin that catalyse the hydrolytic cleavage of carbon-halogen bonds in haloalkanes, yielding an alcohol, a proton, and a halide ion. They can convert a wide spectrum of substrates including halogenated alkanes, cycloalkanes, alkenes, amides, ethers, alcohols, ketones and cyclic dienes.[33–35] Many of these compounds are hazardous environmental pollutants and some are chemical warfare agents. In addition, some halogenated substrates and alcoholic products are valuable building blocks in organic and pharmaceutical synthesis, making HLDs attractive for both biodegradation and biocatalysis.[36–40] Most HLD substrates are hydrophobic compounds and therefore require the use of co-solvents for solubilisation in primarily aqueous media. The eutectic solvent of choice for our study was a 1:2 molar mixture of ChCl and ethylene glycol that is known as ethaline. This mixture was selected due to its relatively low viscosity and known compatibility with hydrolases.[27,41] The characteristics of the studied HLDs were also examined in the presence of the individual constituents of ethaline – ethylene glycol and ChCl – as well as two organic co-solvents with different physico-chemical properties: the protic solvent methanol and the aprotic acetone. To the best of our knowledge, this is the first systematic study to critically compare a DES and its constituent compounds to conventional organic liquids as co-solvents for biocatalysis.

Results and discussion

Structure of HLDs

Circular dichroism (CD) spectroscopy was used to investigate the secondary structures of LinB, DhaA and DbjA in various reaction media. The spectra of enzymes in the presence of ethaline, ethylene glycol, ChCl, methanol and acetone were compared to those measured under native conditions (50 mM potassium phosphate buffer, pH 7.5). The spectra of HLDs measured in pure buffer exhibited two negative features at 208 and 222 nm, indicating the presence of α-helices (Fig. 1).[42] Ethaline was found to be the most HLD-friendly co-solvent in terms of its ability to preserve a native-like enzyme structure. The secondary structures of all studied enzymes remained unchanged in the presence of ethaline up to a concentration of 50% (v/v). Interestingly, while LinB retained its native structure above this concentration, the CD spectra of DhaA and DbjA changed significantly when the concentration of ethaline was raised to 75% (v/v).

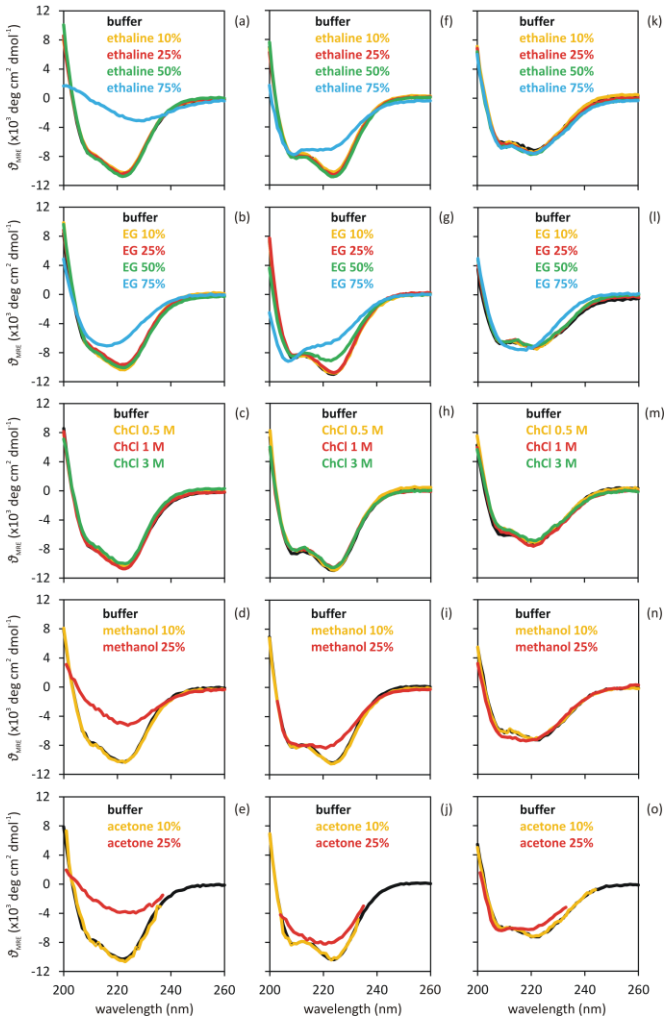

Fig. 1 CD spectra of DbjA (a-e), DhaA (f-j), and LinB (k-o) in the presence of various concentrations of ethaline, ethylene glycol (EG), choline chloride (ChCl), methanol and acetone, respectively, measured at 37 °C and pH 8.6.

The secondary structures of the studied enzymes did not change noticeably when dissolved in solutions of 0.5, 1 or 3 M ChCl. However, the other component of ethaline, i.e. ethylene glycol, had a much more significant impact on their conformational stability than did ethaline itself. Specifically, the addition of 75% (v/v) ethylene glycol caused significant changes in the CD spectra of every tested enzyme. Moreover, structural changes in DhaA were apparent at an ethylene glycol concentration of only 50% (v/v) (Fig. 1). This result supports the previously reported hypothesis that the hydrogen bond network between DES components decreases their denaturation potential.[27,41] However, it is also important to note that there is significant hydrogen bonding between the DES and water in DES-water mixtures.[17] Gutierrez and co-workers reported that for DESs based on choline chloride and urea, the water content of the system should be around 50% (v/v) in order to establish a simple aqueous solution of the two individual components.[43] However, this observation may not hold for mixtures containing different hydrogen bond donors.

Both methanol and acetone proved to be strong protein

denaturants, inducing noticeable changes in CD spectra of the studied HLDs whenever they accounted for ≥ 25% of the solvent mixture (v/v). This is consistent with previous reports on the tendency of water-miscible organic solvents to promote protein unfolding and deactivation.

Thermal stability of HLDs

The thermal stability of DbjA, DhaA and LinB was measured in the absence and presence of various concentrations of ethaline, ethylene glycol, ChCl, methanol and acetone (Table 1). All of the enzymes exhibited a higher degree of thermal stability in ethaline than in any of the organic co-solvents, including ethylene glycol. The melting temperatures (T_m) of LinB, DhaA and DbjA in 75% (v/v) ethaline were 53, 41 and 37 °C, respectively. Conversely, in solutions containing an equivalent volume of ethylene glycol, all of the enzymes had T_m values of less than 30 °C (Table 1). In keeping with our previous findings,[44] methanol and acetone had significantly more pronounced adverse effects on the thermal stability of the studied enzymes than ethaline or ethylene glycol (Table 1). These co-solvents caused conformational changes in the enzymes even at 10 °C when present at concentrations of ≥ 25% (v/v). This effect may be due to the tendency of hydrophilic organic solvents to strip water from the enzyme surface and disrupt native non-covalent interactions, especially at elevated temperatures.[45,46]

Table 1. The melting temperatures (T_m) of DbjA, DhaA and LinB in the presence of various concentrations of ethaline, ethylene glycol, choline chloride, methanol, and acetone determined by monitoring the ellipticity at 221 nm. Each T_m was measured in three independent experiments, with standard errors of less than 3 %.

Solvents	c (v/v)	T_m (°C)		
		DbjA	DhaA	LinB
Buffer	-	54	49	47
Ethaline	10% (v/v)	54	49	50
	25% (v/v)	52	51	52
	50% (v/v)	51	52	56
	75% (v/v)	37	41	53
Ethylene glycol	10% (v/v)	52	48	47
	25% (v/v)	51	47	47
	50% (v/v)	47	39	46
	75% (v/v)	29	-[a]	29
Choline chloride	0.5 M	53	48	49
	1 M	52	50	50
	3M	51	53	55
Methanol	10% (v/v)	51	45	43
	25% (v/v)	42	35	39
	50% (v/v)	30	-[a]	-[a]
	75% (v/v)	-[a]	-[a]	-[a]
Acetone	10% (v/v)	45	42	41
	25% (v/v)	34	29	32
	50% (v/v)	-[a]	-[a]	-[a]
	75% (v/v)	-[a]	-[a]	-[a]

[a] Value not determined due to the structural changes observed at 20 °C.

Interestingly, the T_m of LinB increased from 47 °C in glycine buffer to 56 °C in 50% (v/v) ethaline. A higher thermal stability implies the possibility of performing reactions at higher temperatures, which is useful in practical applications. On the other hand, such stabilization may not be entirely beneficial because it is usually associated with enzyme rigidification, which can reduce enzyme activity.[47] The different effects of ethaline and its component, ethylene glycol, suggest that the higher thermal stability of enzymes in ethaline may be linked to the presence of ChCl. In keeping with this suggestion, chloride salts have previously been shown to stabilize HLDs (unpublished data). As shown in Table 1, the thermal stabilization effect of ChCl was similar to that of ethaline, supporting this hypothesis.

The remarkable stability of enzymes in DESs has been discussed previously. Choi et al. have hypothesized that the formation of natural DESs from chemicals that are present at high concentrations in living cells may contribute to the survival of living organisms under extreme conditions.[48]

Catalytic activity of HLDs

To assess the effects of ethaline, its components, and selected organic solvents on enzyme activity, the hydrolysis of 1-iodohexane catalyzed by LinB, DhaA and DbjA was studied in aqueous media containing the studied co-solvents at various concentrations (Fig. 2). A reaction temperature of 37 °C was chosen on the basis of two criteria: (a) the optimal activity of HLDs,[36] and (b) the relatively low viscosities of DESs at this temperature.[49] 1-iodohexane was selected as a model substrate because all of the studied enzymes have good catalytic efficiencies for this compound and because of its poor solubility in water. The latter property is important because many industrial applications also use quite hydrophobic substrates, necessitating the use of organic co-solvents to increase their solubility in the reaction media. The highest achievable concentration of 1-iodohexane in water is about 0.1 mM (Fig. S1a in ESI†). For comparative purposes, the addition of 50% (v/v) of ethaline, ethylene glycol and methanol increased its concentration in the reaction solution to 0.6, 0.7 and 1.2 mM, respectively.

The different co-solvents had different effects on the activities of the studied HLDs even though they all belong to the same enzyme family (Fig. 2a). The most ethaline-tolerant enzyme studied in this work was DhaA, followed by DbjA and then LinB. In all cases, the enzymes' activity in unadulterated buffer solution was used as a benchmark. DhaA retained 70% of its reference activity (i.e. its activity in phosphate buffer solution) in a reaction mixture containing 75% (v/v) ethaline and exhibited detectable activity in mixtures containing ≥ 90% (v/v) ethaline. Remarkably, it even exhibited modest activity (4.4 nmol min^{-1} mg^{-1}) in a reaction mixture containing only 5% (v/v) water. This value corresponds to around 1% of the reference activity (Fig. S2 in ESI†). Surprisingly, increasing the water content of the system to 10% (v/v) was sufficient to restore significant enzyme functionality: under these conditions, the activity of DhaA was 21% of that seen in unadulterated buffer solution. This clearly demonstrates the importance of water in the dehalogenase reaction. These results are very important because they demonstrate for the first time that HLD-catalyzed reactions can be performed in almost anhydrous reaction media. This may have important implications in terms of the enzymes' behaviour.

Surprisingly, the individual components of ethaline had significantly more pronounced adverse effects on the activity of DhaA than ethaline itself. The level of DhaA activity observed in 75% (v/v) ethylene glycol was less than 5% of the reference value, and a 2.5-fold reduction in activity was observed in the presence of 3 M ChCl. This corresponds roughly to the activity observed in 50% (v/v) ethaline. These results are consistent with those presented by Gorke et al., who reported that *Candida antarctica* and *Candida rugosa* lipases were more stable in a DES than in its individual constituents.[25] Despite the presence of strong hydrogen bond donors and halides, which might inhibit the proteins, the tested lipases showed good catalytic activity in DESs. This seems to support the proposal that hydrogen bonding between the DES components reduces the chemical potential of the mixture relative to that of its

components and thus prevents the denaturation and inactivation of the enzymes.[27,41] The productivity of DhaA was clearly higher in ethaline than in ethylene glycol: its volumetric productivity in 75% (v/v) ethaline was 88 mg/L/h whereas that in 75% (v/v) ethylene glycol was only 2 mg/L/h (Fig. S3 in ESI†). Thus, although ethaline is less effective at solubilizing the substrate than ethylene glycol, the enzyme is much more tolerant of high ethaline concentrations than it is of ethylene glycol concentrations. Consequently, the maximum achievable substrate concentration in DES solutions that retain high enzyme activity will be higher than that achieved in aqueous ethylene glycol solutions that permit similarly high activity.

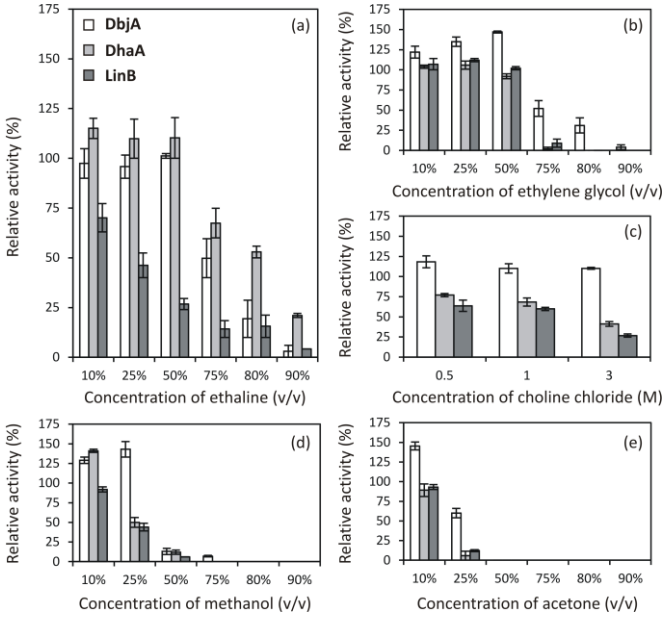

Fig. 2 The relative activities of DbjA, DhaA and LinB measured in the presence of various concentrations of a) ethaline, b) ethylene glycol, c) choline chloride, d) methanol, and e) acetone at 37 °C and pH 8.6. Relative activities are expressed as a percentage of the specific activity in glycine buffer (pH 8.6, 37 °C). The specific activities (in μmol s^{-1} mg^{-1} of enzyme) of DbjA, DhaA and LinB in glycine buffer were 0.0233, 0.0150 and 0.0442, respectively.

In contrast to the situation observed for DhaA, DbjA and LinB both had higher activities in ethylene glycol solutions than in ethaline. Interestingly, the activity of DbjA was 1.5 times higher in the presence of 50% (v/v) ethylene glycol than in pure buffer, but no such activation was observed in solutions of the DES, which contains ethylene glycol. As shown in Fig. 2c, ChCl did not affect the initial activity of DbjA even at the highest tested concentration (3 M). It is likely that this tendency for enzyme activity to be less affected by DESs than by their constituents also explains the reduction in ethylene glycol's ability to activate DbjA when it is present as a component of ethaline.[27,41] In the case of LinB, ethylene glycol did not affect enzymatic activity until its concentration exceeded 50% (v/v). However, the activity of LinB in 50% (v/v) ethaline was less than 30% of the reference activity observed in the unadulterated buffer. Subsequent evaluations of LinB activity in the presence of ChCl revealed that the decrease in enzyme activity observed in various concentrations of ethaline correlated with the decrease observed in the presence of the corresponding amount of ChCl.

For comparative purposes, the activities of the studied HLDs were also assayed in the presence of methanol and acetone. In contrast with the situation for ethaline and ethylene glycol, all of the enzymes were only slightly active in methanol and acetone (Fig. 2d, e), losing almost all of their activity in solutions with organic solvent concentrations in excess of 25% (v/v). These results are in good agreement with previous observations that unlike DESs, polar organic co-solvents often inactivate proteins.[2,3,44] Gorke et al. reported that replacing an aqueous buffer solution with 25% (v/v) ChCl:glycerol increased the rate of epoxide hydrolase-catalyzed hydrolysis of styrene oxide by up to a factor of 20 whereas replacing the DES with an equivalent quantity of DMSO or acetonitrile reduced the enzyme's activity 2-6 fold.[25,41] Similar observations have been reported for lipases,[17,27,41,50] esterases,[41] and proteases,[26] suggesting that DESs are more viable reaction media for biotransformation reactions than traditional organic solvents.

Due to the large variability in reaction system components, it is difficult to identify the exact effects of various co-solvents on enzyme activity. The most commonly assumed explanation for enzyme inactivation in the presence of co-solvents is structural unfolding.[3,51–53] This mechanism of inactivation may also be applicable to many of the systems studied in this work.

Enantioselectivity of DbjA

It is widely recognized that in addition to their effects on enzyme activity, different solvents and reaction media can have different effects on enzyme enantioselectivity.[1] We therefore investigated the effects of ethaline, its components, methanol, and acetone on the kinetic resolution of racemic 2-bromopentane catalysed by the haloalkane dehalogenase DbjA. The well-tolerated co-solvents ethaline and ethylene glycol were added to the reaction mixture at a concentration of 50% (v/v), while the more denaturing co-solvents methanol and acetone were added at concentrations of 25% (v/v) and 10% (v/v), respectively. Choline chloride was added to a final concentration of 1 M. As in the activity assays, enzyme enantioselectivity was measured at 37 °C in order to decrease the viscosity of the tested DES. As reported previously, DbjA exhibited high enantioselectivity towards 2-bromopentane (*E*-value > 130) at 20-25 °C and its enantioselectivity decreased sharply as the temperature increased.[29,54] At 37 °C, i.e. the temperature at which DbjA exhibits optimal activity, its enantioselectivity was reduced more than 3-fold (Fig. 3d). Surprisingly, the addition of 50% (v/v) ethaline into the reaction mixture significantly increased the enantioselectivity of DbjA, raising its *E*-value from 53 to >200. Visual inspection of progress curves demonstrated that this increase was accompanied by no substantial consumption of the non-preferred (*S*)-enantiomer even though the preferred (*R*)-enantiomer was completely converted (Fig. 3a).

As was observed for ethaline, the conversion of the preferred enantiomer (*R*)-2-bromopentane was almost unchanged in the presence of the tested organic co-solvents. However, the consumption of the unpreferred (*S*)-enantiomer was negligible in the presence of ethylene glycol, partially reduced (relative to the situation observed in unadulterated buffer solution) in the presence of methanol, and unchanged by the presence of acetone (Fig. 3b, e, f). Consequently, the *E*-value achieved with ethylene glycol was comparable to that seen with ethaline whereas the use of methanol as a co-solvent provided only a 1.4-fold improvement in *E* and acetone had no effect on the enantioselectivity of DbjA.

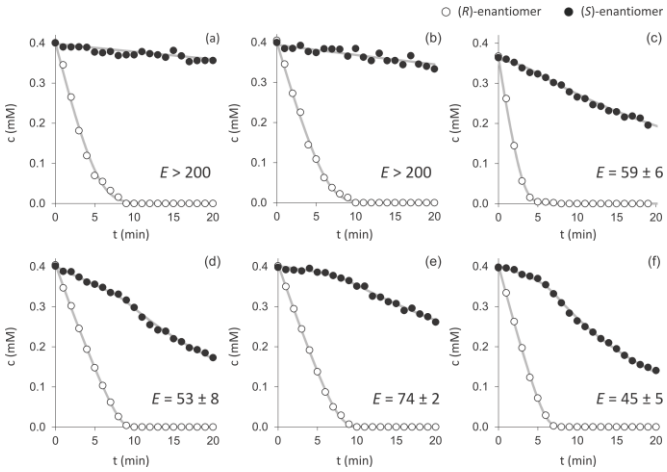

Fig. 3 Kinetic resolution of 2-bromopentane catalyzed by DbjA in a) ethaline 50% (v/v), b) ethylene glycol 50% (v/v), c) choline chloride 1 M, d) glycine buffer, e) methanol 25% (v/v), and f) acetone 10% (v/v) measured at 37 °C and pH 8.6.

It is clear that ethylene glycol is responsible for the increase in DbjA enantioselectivity in the presence of ethaline because choline chloride alone had no effect on *E* (Fig. 3c). The water solubility of 2-bromopentane is limited, but it increased by a factor of 3 in the presence of 50% (v/v) ethylene glycol and by a factor of 2 in the presence of an equivalent concentration of ethaline (Fig. S1b in ESI†). Additionally, the non-enzymatic hydrolysis of 2-bromopentane was heavily suppressed in ethaline (Fig. S4 in ESI†). This competitive hydrolysis reaction is non-stereoselective and reduces the enantioselectivity of the overall process. Taken together, these results clearly indicate that media containing ethylene glycol or an ethylene glycol-based DES increase the efficiency of DbjA in the production of optically pure compounds.

In the past, changes in enzyme enantioselectivity induced by organic co-solvents have generally been attributed to three mechanisms: (i) the potential for co-solvent molecules to interfere more significantly with the orientation or transformation of one enantiomer in the enzyme's active site than with the other, (ii) interactions between co-solvent molecules and the enzyme's surface or active site that lead to conformational changes, or (iii) co-solvent-induced shifts in the racemic temperature.[55–57] All three proposed mechanisms for the modification of enantioselectivity by organic solvents may be applicable to the hydrolysis of 2-bromopentane catalysed by DbjA.

Experimental section

Synthesis of ethaline

Ethaline was synthesized using a simple thermal mixing procedure.[12] The ChCl/ethylene glycol derivative was prepared by heating a 1:2 molar mixture of ChCl and ethylene glycol at 80 °C until a colourless homogenous liquid was formed (~ 2 h). This mixture was then gradually cooled to room temperature.

Protein expression and purification

The His-tagged LinB, DhaA and DbjA enzymes were over-expressed in *Escherichia coli* BL21 cells, as described previously.[30,58,59] Proteins were purified on a Ni-NTA Superflow Cartridge (Qiagen). His-tagged enzymes were bound to the resin in an equilibrating buffer (20 mM potassium phosphate buffer, pH 7.5 containing 0.5 M sodium chloride and 10 mM imidazole), while unbound and weakly bound proteins were washed away. The His-tagged enzymes were then eluted using a buffer containing 300 mM imidazole. The active fractions were pooled and dialyzed overnight against a 50 mM potassium phosphate buffer (pH 7.5) and then stored at 4 °C. Protein concentration was determined using the Bradford reagent (Sigma-Aldrich). The purity of the purified proteins was checked by SDS-PAGE.

Circular dichroism spectroscopy

CD spectra of enzymes were acquired at 37 °C using a Chirascan spectropolarimeter (Applied Photophysics) equipped with a Peltier thermostat. Data were collected from 200 to 260 nm, at 100 nm/min with a 1 s response time and 2 nm bandwidth. Spectra were recorded using a 0.1 cm quartz cuvette containing 0.25 mg/ml enzyme in 50 mM phosphate buffer (pH 7.5) and a defined amount of ethaline, ethylene glycol, ChCl, methanol or acetone. All of the spectra shown in the paper are averages of 5 individual scans and have been baseline corrected. CD spectra were expressed in terms of the mean residue ellipticity (Θ_{MRE}), calculated using the following equation:

$$\Theta_{MRE} = (\Theta_{obs} \,.\, M_w \,.\, 100) / (n \,.\, c \,.\, l)$$

where Θ_{obs} is the observed ellipticity in degrees, M_w is the protein's molecular weight, n is the number of residues, l is the cell path length, c is the protein concentration and the factor of 100 originates from the conversion of molecular weight to mg/dmol.

Thermal denaturation

Each protein's thermal unfolding in the presence of an appropriate amount of ethaline, ethylene glycol, ChCl, methanol or acetone was studied by monitoring its ellipticity at 222 nm over a temperature range of 20 to 80 °C, with a 0.1 °C resolution, at a heating rate of 1 °C/min. The recorded thermal denaturation curves were roughly normalized to represent signal changes between approximately 1 and 0, and fitted to sigmoidal curves using the software Origin 6.1 (OriginLab Corporation). The T_m was then determined based on the midpoint of the normalized thermal transition.

Activity assay

HLD activity was assayed at 37 °C in a reaction volume of 15 ml consisting of 100 mM glycine buffer (pH 8.6), 5 µl of the substrate (1-iodohexane), and an appropriate amount of ethaline, ethylene glycol, ChCl, methanol, or acetone. The reaction was initiated by adding the enzyme to a final concentration of 0.2 µM and monitored by periodically withdrawing samples from the reaction mixture. The samples were analysed using a Trace GC 2000 gas chromatograph (Finnigen) equipped with a flame ionization detector and a DB-FFAP capillary column (J&W Scientific). The enzyme's dehalogenating activity was quantified as the rate of product formation over time. Each activity was measured in 3-5 independent replicate experiments and the quoted activities are the means of these replicate experiments as a percentage of the mean activity observed in unadulterated buffer solution.

Productivity assay

The volumetric productivity of DhaA was assayed at 37°C in a reaction volume of 25 ml consisting of 100 mM glycine buffer (pH 8.6), 10 µl of substrate (1-iodohexane) and 75% (v/v) ethaline or

ethylene glycol, corresponding to the highest concentrations of these two co-solvents that yielded detectable DhaA activity. The reaction was monitored for 100 min by periodically withdrawing samples from the reaction mixture, which were analysed using a 6890N gas chromatograph (Agilent) equipped with a flame ionization detector.

Solubilization of halogenated compounds

The solubilization of model halogenated compounds (1-iodohexane, 2-bromopentane, 2-bromohexane and 2-bromoheptane) was investigated in water, ethaline, ethylene glycol and methanol. Solubility was determined by saturating a given solvent with a test compound at 37 °C for 1 hour while being agitated. Undissolved material was allowed to sink to the bottom of the flask and samples were carefully taken from the surface of the solution. The concentration of each halogenated compound in each saturated solution was determined using a 6890N gas chromatograph (Agilent) equipped with a flame ionization detector.

Enantioselectivity measurements

Kinetic resolution experiments were performed at 37 °C. The racemic substrate was added at a final concentration of 1 mM to a reaction vessel containing a total volume of 25 ml of glycine buffer (100 mM, pH 8.6) and a defined amount of ethaline, ChCl, ethylene glycol, methanol or acetone. The enzymatic reaction was initiated by adding the enzyme at a final concentration of 1 μM. The progress of each reaction was monitored by periodically withdrawing samples from the reaction mixture and analyzing them using a Hewlett-Packard 6890 gas chromatograph (Agilent) equipped with a flame ionization detector and a Chiraldex G-TA chiral capillary column (Alltech). The enantiomeric ratio was calculated using the following equation:

$$E = (k_{cat,R} / K_{m,R}) / (k_{cat,S} / K_{m,S})$$

where k_{cat}/K_m represents the specificity constant.[60] To estimate *E*-values, equations describing competitive Michaelis-Menten kinetics were fitted by numerical integration to time courses of changes in substrate concentrations obtained from kinetic resolution experiments using MicroMath Scientist for Windows (ChemSW).

Conclusions

This study represents the first published example of HLD-catalysed reactions conducted in aqueous DES solutions, which are significantly "greener" than traditional organic co-solvent systems. The performance of biocatalytic reactions using three HLDs – DbjA, DhaA and LinB – in solutions of the DES ethaline was critically compared to that of the same reactions performed in the presence of the individual components of ethaline (ChCl and ethylene glycol) as well as two representative conventional organic solvents - methanol and acetone**.** The activities of the studied HLDs responded differently to the different co-solvents. However, all of the HLDs clearly tolerated ethaline and ethylene glycol significantly better than methanol and acetone. Ethylene glycol was found to be the best co-solvent for DbjA, giving high activity and enantioselectivity. However, DhaA was most active in ethaline solutions. The excellent compatibility of ethaline with DhaA was demonstrated by the enzyme's enhanced thermal stability in this solvent, and by its retention of detectable catalytic activity even at very high ethaline concentrations (≥ 90% v/v). None of the ever tested solvents allowed enzymes to retain activity when used at such high concentrations. This finding will make it possible to perform HLD-catalysed reactions in low-water media, which may provide valuable information on HLD behaviour. It was also demonstrated that ethylene glycol and ethylene glycol-based DESs can improve the solubility of poorly water-soluble halogenated hydrocarbons and suppress their unwanted non-enzymatic hydrolysis. Although the solubilisation potential of DESs is lower than that of methanol and acetone, they can be used at much higher concentrations than traditional organic solvents without causing enzyme deactivation. This more than compensates for their lesser ability to solubilise organic compounds on a mole-for-mole basis. These observations together with the expected acceptability of DESs from environmental and pharmaceutical points of view should significantly enhance the usability of HLDs in industrial biotechnology.

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Notes

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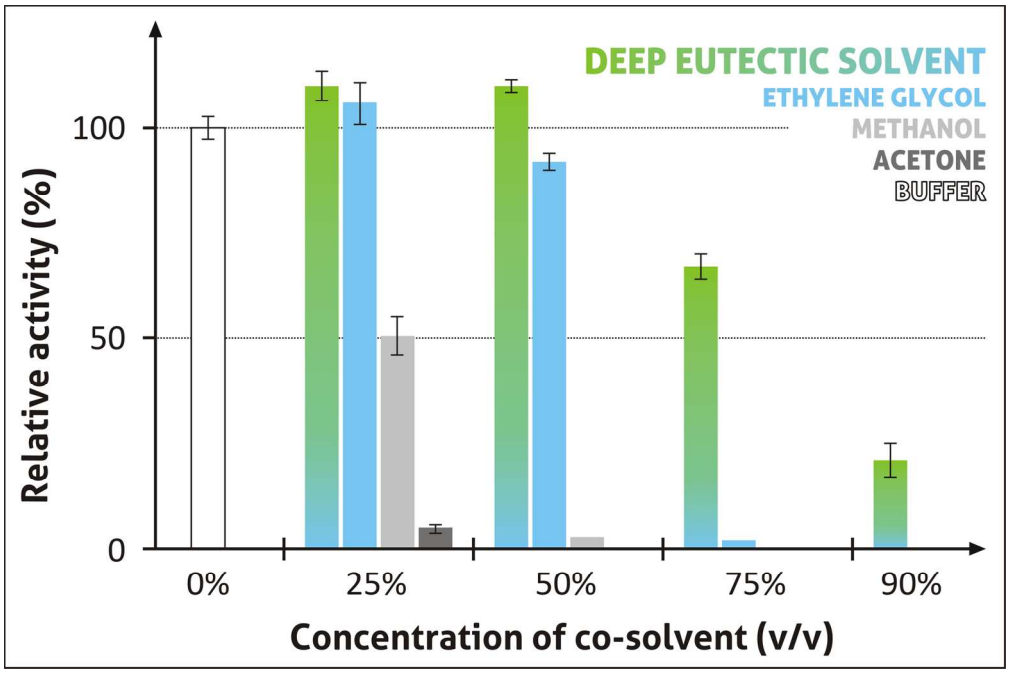

Excellent compatibility of ethaline with dehalogenase DhaA demonstrated by retention of its activity at high ethaline concentration (≥ 75% v/v).
157x104mm (300 x 300 DPI)