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FULL PAPER

Deep Eutectic Solvents as Media in Alcohol Dehydrogenase-Catalyzed Reductions of Halogenated Ketones

Fatima Zohra Ibn Majdoub Hassani,[a,b] Saaid Amzazi,[a] Joseph Kreit,[a] and Iván Lavandera*[b]

Abstract: The application of deep eutectic solvents (DESs) in biotechnological processes has gained an outstanding relevance, as they can be used as greener media to obtain higher productivities and selectivities. In the present contribution, a eutectic mixture composed of choline chloride (ChCl): glycerol (1:2 mol/mol) has been used as a reaction medium in combination with Tris-SO_4 50 mM buffer pH 7.5, applied to the alcohol dehydrogenase (ADH)-catalyzed reduction of various carbonyl precursors of chiral halohydrins. These alcohols are key intermediates of biologically active compounds, and hence they are of industrial interest. In the presence of up to 50% v/v of DES, these biotransformations were achieved up to 300-400 mM of the α-halogenated ketone substrate, getting access to the final compounds with excellent conversions (usually >90%) and enantiomeric excess (*ee* >99%). Among the different ADHs tested, two stereocomplementary enzymes (*Lactobacillus brevis* ADH and *Rhodococcus ruber* ADH) afforded the best results, so both alcohol enantiomers could be obtained in all the studied examples. Selected bioreductions were scaled up to 250 mg and 1 g, demonstrating the potential that DESs can offer as media in redox processes for substrates with low solubility in water.

Introduction

In the last years, the synthesis of chiral halohydrins has received great attention due to their synthetic versatility and high reactivity. They have become privileged scaffolds to access biologically active derivatives. They are precursors of β-adrenoreceptor blockers[1] and also key intermediates of various active ingredients of medicaments that are currently commercialized. Examples of these compounds (Figure 1) include mirabegron[2] and ezetimibe,[3] used for treatment of overactive bladder and hypercholesterolemia, respectively.

Among the plethora of synthetic methods to synthesize halohydrins in enantioenriched form, probably the most employed is the reduction of the α-halogenated ketone precursors. Hence, the use of metals as catalysts has provided appreciable results,[4] but from an environmental point of view and selectivity, the utilization of enzymes is more desirable. In fact, in the recent literature, many examples can be found employing alcohol dehydrogenases (ADHs),[5] in order to obtain chiral halohydrins. These NAD(P)-dependent oxidoreductases have provided impressive results in terms of activity and selectivity under mild conditions. Thus, whole cells[6] and isolated or overexpressed enzymes[7] have been utilized for this purpose.

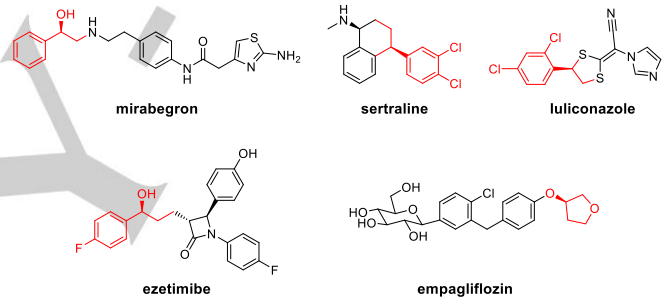

Figure 1. Examples of active ingredients of commercialized medicaments where halohydrins are key intermediates (the part of the molecule derived from the halohydrin appears in red).

Aqueous medium is the natural environment for these biocatalysts, but it has been discovered that they can work in non-conventional media containing organic solvents[8] or ionic liquids (ILs).[9] This can be advantageous for these enzymes due to the large hydrophobicity of the organic substrates, making feasible the employment of higher substrate concentrations and therefore, higher productivities. Related to this fact, new types of ionic liquids that are ecologically more convenient than the organic solvents, are the (natural) deep eutectic solvents (NA)DESs.[10] They are typically made of a hydrogen bond donor (HBD, such as glycerol, ethylene glycol or urea), and a quaternary ammonium salt acting as a hydrogen bond acceptor (HBA, *e.g.* choline chloride, ChCl). The intermolecular interactions between these components provide specific properties for these neoteric solvents. Among their advantages, it can be highlighted that they can easily be prepared from cheap precursors, and they are composed of natural, and therefore degradable, constituents. Their biocompatibility with redox enzymes has been well documented.[11] DESs have been mostly applied with (multienzymatic) whole cell systems to modify the stereoselectivity of bioreduction processes, due to the specific inhibition of some of the enzymes present in the cell.[12] Also, they

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Supporting information for this article is given via a link at the end of the document.

have been found as suitable media for preparations which overexpress ADHs,[13] and even for nicotinamide cofactor recycling purposes.[14]

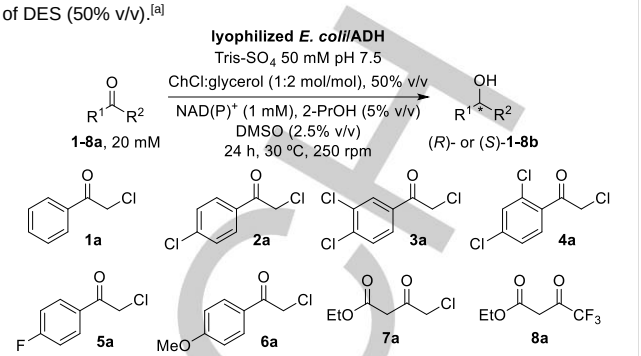
To the best of our knowledge, there is just one report that describes the bioreduction of an α-halogenated ketone using whole cells in a medium containing DESs.[15] Herein, we have focused on the development of efficient reduction processes by utilizing overexpressed ADHs to synthesize various enantiopure halohydrins, key precursors of biologically active compounds, studying the influence in the reaction mixture of a eutectic solvent composed of choline chloride and glycerol (1:2 mol/mol).

Results and Discussion

In the present study, we used lyophilized preparations of *E. coli* overexpressing the (*R*)-selective ADH from *Lactobacillus brevis* (LBADH)[16] and the (*S*)-selective ADHs from *Rhodococcus ruber* (ADH-A),[17] *Thermoanaerobacter ethanolicus* (TeSADH),[18] and *Thermoanaerobacter* sp. (ADH-T).[19] These preparations were employed due to their advantages regarding other enzymatic formulations in terms of higher stability and easy handling.[20] From a previous work in our research group,[13a] we already knew that the ChCl:glycerol (1:2 mol/mol) DES mixture was compatible with these ADHs. Also, this mixture presents an adequate viscosity, therefore no mass-transfer limitations were expected even at large DES concentrations in the reaction medium. Therefore, we focused on the bioreduction of 8 halogenated ketones (**1**-**8a**, Table 1), in the presence of this eutectic mixture at 20% and 50% v/v in Tris-SO_4 buffer[21] 50 mM pH 7.5 (Table 1 and Tables S2-S9 in the Supporting Information). In order to recycle the nicotinamide cofactor (1 mM), propan-2-ol was used as hydrogen donor in a "coupled-substrate" approach.[22] The reduction of α-halogenated carbonyl compounds is favored thermodynamically.[23] Therefore, it is not necessary to employ an elevated molar excess of the co-substrate to achieve quantitative conversions.

From the initial enzyme screening with substrate **1a** (Table S2 in the Supporting Information), it was clear that the best choices to accomplish the reduction processes were stereocomplementary ADH-A[17] and LBADH.[16] It must be noted that ADH-A afforded the (*R*)-alcohols while LBADH the (*S*)-antipodes. This is simply due to a change in the Cahn-Ingold-Prelog (CIP) priority of the alcohol substituents. The absolute configuration of these alcohols was assigned based on previous examples described in the literature and also by comparison of the GC retention times and optical rotation values (for more information, see Section 4 in the Supporting Information). As can be seen in Table 1, both ADHs catalyzed the bioreductions in the presence of a high amount of DES (50% v/v) in the reaction medium, providing the desired aromatic or aliphatic halohydrins (*R*)- or (*S*)-**1**-**8b** with excellent conversions and enantiomeric excess (*ee*). Due to the mildness of these processes, no by-products (*e.g.*, epoxides) were detected in the reaction mixtures.

Table 1. Bioreduction of halogenated ketones **1**-**8a** (20 mM) in the presence of DES (50% v/v).[a]



Entry	Ketone	ADH	Conv (%)[b]	*ee* (%)[b]
1	**1a**	ADH-A	>99	>99 (*R*)
2	**1a**	LBADH	>99	>99 (*S*)
3	**2a**	ADH-A	>99	>99 (*R*)
4	**2a**	LBADH	>99	>99 (*S*)
5	**3a**	ADH-A	>99	>99 (*R*)
6	**3a**	LBADH	>99	>99 (*S*)
7	**4a**	ADH-A	>99	>99 (*R*)
8	**4a**	LBADH	88	>99 (*S*)
9	**5a**	ADH-A	96	>99 (*R*)
10	**5a**	LBADH	95	>99 (*S*)
11	**6a**	ADH-A	>99	>99 (*R*)
12	**6a**	LBADH	>99	>99 (*S*)
13	**7a**	ADH-A	>99	>99 (*R*)
14	**7a**	LBADH	97	97 (*S*)
15	**8a**	ADH-A	>99	>99 (*R*)
16	**8a**	LBADH	>99	>99 (*S*)

[a] For reaction conditions, see the Experimental Section. [b] Measured by chiral GC. There is a change in the CIP priority.

Due to the fact that the substrate concentration in these reduction protocols was still modest (20 mM), we decided to increase it while maintaining the same amount of the biocatalyst and the cofactor in the experiments. We usually performed the bioreductions at 20% v/v and 50% v/v of DES, and compared the results with the reactions in plain buffer. As shown in Tables S2-S9 in the Supporting Information, in most cases the ketone concentration could be effectively increased up to 300-400 mM when employing 20% v/v of DES. However, slightly lower conversions were observed at these substrate concentrations in the presence of 50% v/v of DES. Meanwhile, the stereoselectivity of all these reactions remained elevated for both ADHs, so that the synthesis of both halohydrin enantiomers was feasible. For comparison, the same transformations were performed in plain buffer, obtaining in some cases comparable results, but for some reductions better conversions were achieved in the presence of

DES, as shown in Figure 2. We think that the cosolvent can be beneficial in terms of a better substrate solubility and therefore mass transfer. An advantage of employing lyophilized whole cells is that the cell walls are partially disrupted and therefore the substrate can be more available for the in-cell enzyme.

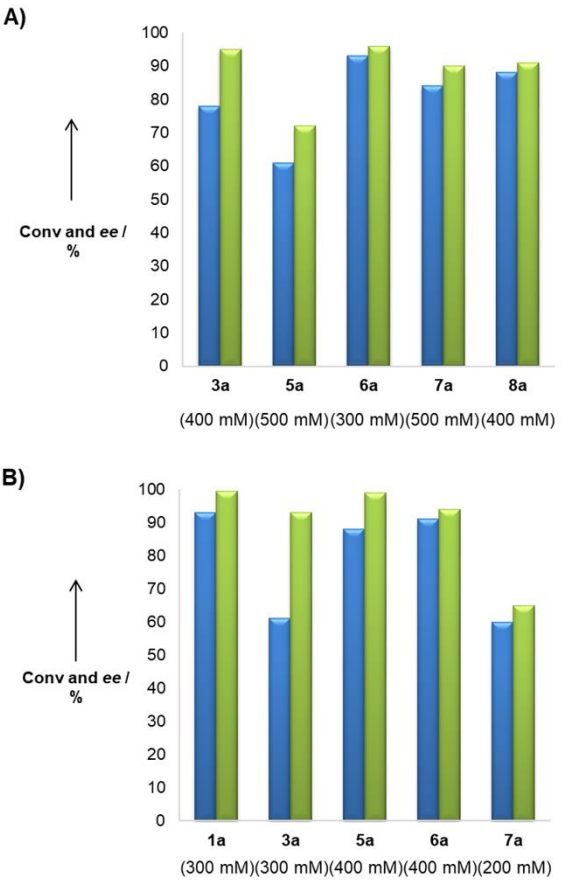
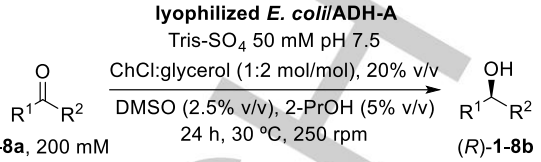


Figure 2. Comparison between conversions for some selected bioreductions with: A) *E. coli*/ADH-A; and B) *E. coli*/LBADH in plain buffer (blue bars) and in the presence of ChCl:glycerol (1:2 mol/mol) DES at 20% v/v (green bars). In all cases, the obtained *ee* values were >99%.

The cofactor is already present in the whole cell preparation, and although in lyophilized *E. coli* cells it can diffuse to the reaction medium, its external addition could not be of absolute necessity, as the biotransformation occurring favors the diffusion inside the cells. In a set of experiments, the bioreductions in the presence of DES were performed at 200 mM ketone concentration without adding the nicotinamide cofactor. While for NADP-dependent LBADH, the results were not satisfactory (data not shown), for NAD-dependent ADH-A, high conversions were achieved, especially when using 20% v/v of DES (Table 2 and Table S10 in the Supporting Information). This can be explained by the fact that there is a higher cytosolic concentration of NADH/NAD^+ with respect to NADPH/$NADP^+$ in *E. coli* cells.[24]

Table 2. Bioreduction of halogenated ketones **1-8a** (200 mM) with *E. coli*/ADH-A in the presence of DES (20% v/v) and without external addition of the cofactor NAD^+.[a]



Entry	Ketone	Conv (%)[b]	*ee* (%)[b]
1	**1a**	82	>99 (*R*)
2	**2a**	71	>99 (*R*)
3	**3a**	>99	>99 (*R*)
4	**4a**	97	>99 (*R*)
5	**5a**	>99	>99 (*R*)
6	**6a**	97	>99 (*R*)
7	**7a**	>99	>99 (*R*)
8	**8a**	97	>99 (*R*)

[a] For reaction conditions, see the Supporting Information. [b] Measured by chiral GC.

In order to demonstrate the applicability of this methodology, ADH-catalyzed transformations in the presence of DES were performed at a preparative scale (Figure 3). This way, 250 mg of target ketones **1-8a** and an extra experiment with substrate **4a** at 1 g-scale were performed to synthesize the corresponding enantiopure halohydrins. The products could easily be isolated after a liquid-liquid extraction protocol in high yields.

Conclusions

This contribution demonstrates that eutectic mixtures can improve the productivity in oxidoreductase-mediated transformations to synthesize relevant chiral halohydrins at high concentrations. Lyophilized *E. coli* whole cells overexpressing ADHs have been used in a mixture of aqueous buffer and DES (ChCl: glycerol 1:2 mol/mol) up to 50% v/v. This medium did not affect negatively the activity and selectivity of two stereocomplementary ADHs, namely *Lactobacillus brevis* ADH and *Rhodococcus ruber* ADH, obtaining better results in the presence of 20% v/v of the cosolvent. Hence, the bioreductions of various α-halogenated ketones could be performed up to 300-400 mM achieving high conversions (usually >90%) and excellent enantiomeric excess (>99%). The possibility of avoiding the external addition of the cofactor NAD in ADH-A-mediated reductions was also feasible, performing the reactions at 200 mM. These biotransformations were scaled up to 1 g giving access to a series of enantiopure halohydrins. Overall, the present study shows a good example where the combination of DESs with lyophilized whole cell systems overexpressing a biocatalyst can be beneficial in a biotransformation and shows the great potential that these cheap and environmentally friendly solvents can possess for biotechnological applications.

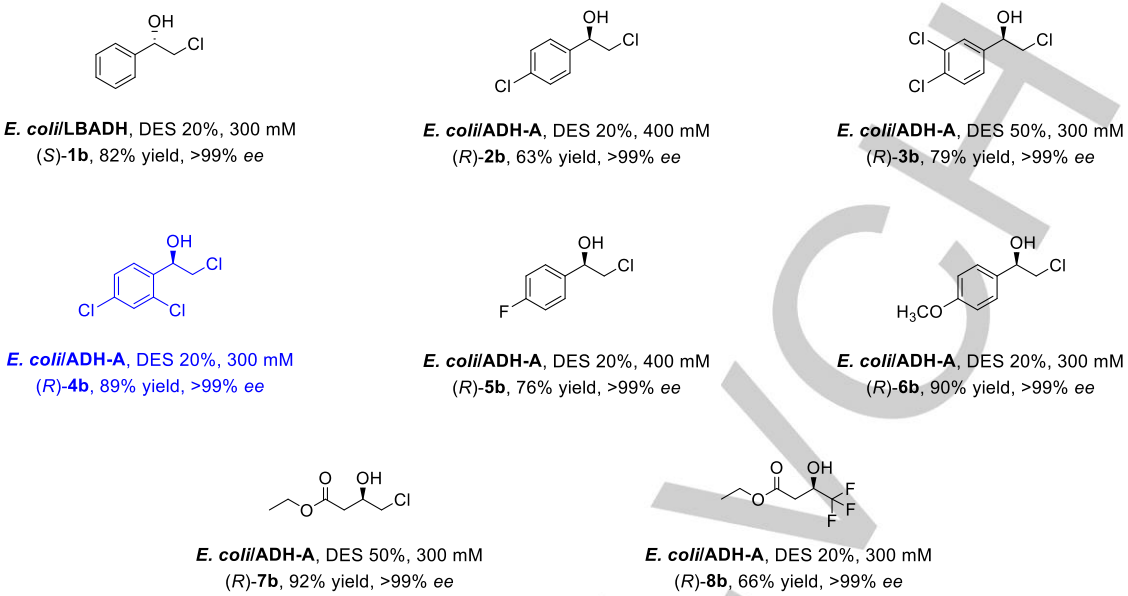


Figure 3. Preparative transformations at 250 mg (black color) and 1 g-scale (blue color) to synthesize enantiopure halohydrins in the presence of DES.

Experimental Section

General procedure for the enzymatic reduction of halogenated ketones in the presence of a deep eutectic solvent

In a 1.5 mL-Eppendorf vial, the reaction mixture (final volume: 0.6 mL), contains: 10 mg of the corresponding *E. coli*/ADH lyophilized cells, Tris-SO_4 buffer 50 mM pH 7.5, NAD(P)$^+$ (1 mM), DES (ChCl: glycerol 1:2 mol/mol, 20% or 50% v/v), propan-2-ol (5-15% v/v) and the halogenated ketone (**1-8a**, Table 1, 20-500 mM), dissolved in DMSO. The final amount of DMSO in the assay was between 2.5-5% v/v. The reduction reactions were carried out during 24 h, at 30 ºC in an orbital shaker at 250 rpm. The crude was extracted with ethyl acetate (2 x 500 µL). The organic layers were collected by centrifugation, combined, and dried over Na_2SO_4. Conversion rates and enantiomer excess of the halohydrins were determined by GC on a chiral column (see the Supporting Information). To determine the *ee* values for halohydrins **7b** and **8b**, an acetylation step was required. 4-Dimethylaminopyridine (DMAP, 1 mg) and acetic anhydride (30 µL) were mixed and then added to a volume of 200 µL of the extracted reaction crude solution. The mixture was shaken for 3 h at 30 °C, and then quenched with 0.5 mL water for 30 min. The acetylated alcohol was extracted with ethyl acetate (2 x 500 µL), and the combined organic phases were dried over Na_2SO_4, and subjected to GC analysis.

Scale-up of the bioreduction of 4a (1 g, 300 mM) in the presence of DES (20% v/v)

In an Erlenmeyer flask, 500 mg of *E. coli*/ADH-A were introduced. Then, 10.6 mL of Tris-SO_4 50 mM pH 7.5 buffer, 1.5 mL of the NAD$^+$ solution (10 mM in Tris-SO_4 50 mM pH 7.5) and 3.2 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 1.2 mL of propan-2-ol and 1 g of 2,2',4'-trichloroacetophenone were introduced. The flask was closed and the reaction was shaken for 24 h at 30 ºC in an orbital shaker at 250 rpm. The supernatant of the bioreduction mixture was collected by centrifugation and transferred into an extraction funnel. The deposited pellet was washed with ethyl acetate (30 mL) to extract the residual substrate and product in the Falcon tube by centrifugation, and it was pooled into the funnel with the previous organic phase. This organic solvent was washed with water (20 mL). The phases in the funnel were separately collected. The aqueous phase was again extracted with ethyl acetate (50 mL). This organic phase was combined with the previous one to yield a combined extract that was washed with water (10 mL, to eliminate the residual glycerol), collected in an Erlenmeyer flask, dried over Na_2SO_4 and filtered into a round-bottom flask. Na_2SO_4 was washed with ethyl acetate (10 mL). The solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**4b** (900 mg, 89% yield) in >99% *ee*.

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Keywords: deep eutectic solvents • alcohol dehydrogenases • bioreduction • selectivity • halohydrins

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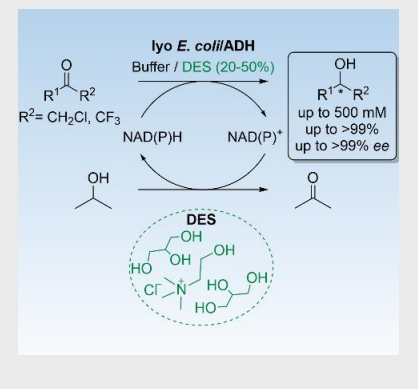
FULL PAPER

A deep eutectic solvent composed of choline chloride:glycerol (1:2 mol/mol) has been used as cosolvent in the alcohol dehydrogenase-catalyzed bioreduction of a series of α-halogenated ketones employing lyophilized *E. coli* cells overexpressing the enzyme. Various halohydrins were obtained at high concentration (up to 500 mM) with high conversions and enantiomeric excess



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Deep Eutectic Solvents as Media in Alcohol Dehydrogenase-Catalyzed Reductions of Halogenated Ketones