Green Chemistry

COMMUNICATION

A designer natural deep eutectic solvent to recycle the cofactor in alcohol dehydrogenase-catalysed processes

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Deep eutectic solvents (DES) represent nowadays a sustainable alternative to traditional organic solvents in (bio)transformations. Herein, the use of a solvent composed by an aqueous buffer and choline chloride: glucose (1.5:1 mol/mol) is proposed, a natural DES (NADES) serving as both cosolvent and efficient system to recycle the nicotinamide cofactor. Thus, glucose from NADES served as co-substrate required for several alcohol dehydrogenases to reduce different prochiral ketones, and also helped to solubilise the organic compounds to develop effective biotransformations at higher substrate concentrations.

The application of oxidoreductases (EC.1) in organic synthesis has gained maturity in the last decades due to their high levels of activity and selectivity displayed in redox processes.[1] Particularly alcohol dehydrogenases (ADHs, EC.1.1.1.x) represent a sustainable alternative to non-enzymatic methods for the production of optically active alcohols from racemic and prochiral carbonylic compounds.[2] This class of redox catalyst requires the use of a cofactor, β-nicotinamide adenine dinucleotide, which exists in a phosphorylated ($NADP^+$) and non-phosphorylated (NAD^+) oxidised form, or in their reduced versions [NAD(P)H]. These cofactors can mediate as electron acceptors/donors in either oxidation or reduction processes. Since the use of cofactors in stoichiometric amounts is hampered due to inhibition effects and economic hurdles, the employment of cofactor recycling systems is compulsory for the development of efficient and economic feasible redox transformations.[3] For the case of reductive reactions, this normally implies the coupling of a second enzymatic reaction typically mediated by glucose dehydrogenase (GDH)[4] or formate dehydrogenase (FDH) at expenses of a sacrificial cosubstrate, glucose or formate, respectively, in the so-called coupled-enzyme approach. Other methodologies involve the use of chemical, electrochemical or photochemical transformations,[5] or the use of cheaper synthesised nicotinamide cofactor mimetics in stoichiometric amounts.[6]

Deep eutectic solvents (DES) have recently appeared as a new generation of ionic liquids (ILs).[7] A DES is typically composed by a quaternary ammonium salt acting as hydrogen bond acceptor (HBA, i.e. choline chloride), and a hydrogen bond donor (HBD, such as glycerol, urea or a sugar), so the intermolecular interactions between its components provide specific properties for these neoteric solvents. Their straightforward preparation by simply mixing a HBA and a HBD to form a liquid, represents an excellent waste-free process providing a significant advantage in comparison with the preparation of traditional ILs. Moreover, the possibility of utilising natural and degradable compounds is also highly valuable from an environmental point of view.

In this context, natural deep eutectic solvents (NADES) are defined as a mixture of two or more compounds that are generally plant based primary metabolites (organic acids, sugars, alcohols, amines and amino acids), that interact via intermolecular forces and liquefy if combined in specific molar ratios.[8] The NADES concept dates from 2011 and it created great expectations in the context of Green Chemistry as they are less toxic and more environmentally friendly than ionic liquids (ILs) and traditional metal-based DES.

Interestingly, the use of (NA)DES as green (co)solvents in enzymatic transformations has rapidly gained attention,[9] including their application to redox processes.[10] In this context, the development of bioreductions using ADHs appears as a robust technique for the asymmetric synthesis of alcohols using aqueous-(NA)DES mixtures, where mainly choline chloride: glycerol (ChCl:Gly) has been employed as cosolvent.[11] In some cases, other HBDs have also been used, the choice of glucose being scarcely reported and in all cases for whole cell-mediated biotransformations.[12] Herein, we have focused on the development of efficient global reduction processes by combining an overexpressed ADH for the reduction of prochiral ketones accomplished with an appropriate cofactor recycling system, considering in this case glucose

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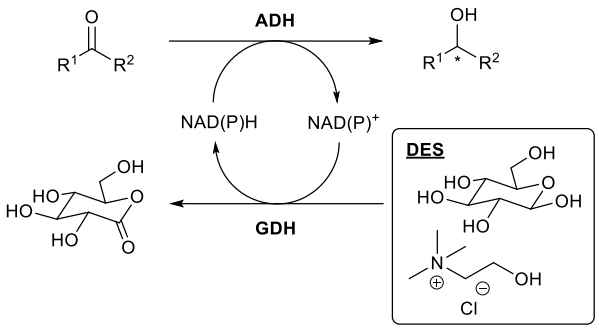
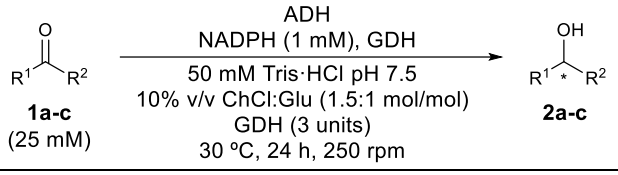
dehydrogenase (GDH), which allows the cofactor reduction by transforming D-glucose into D-glucono-1,5-lactone that later spontaneously hydrolyses in the reaction medium towards the formation of D-gluconic acid. For that reason, we decided to investigate the use of D-glucose as HBD in a designer NADES with an efficient solution for: (a) Better solubility of the lipophilic substrates; and (b) Suitable cofactor regeneration system (Scheme 1).



Scheme 1 Use of a designer deep eutectic solvent formed by D-glucose and ChCl to recycle the nicotinamide cofactor [NAD(P)H] in alcohol dehydrogenase-catalysed processes.

For this study, commercially available GDH-105 from Codexis Inc. was selected for cofactor recycling, and the behaviour of different made in house overexpressed NADPH-dependent alcohol dehydrogenases (ADHs) was studied. We used the (*R*)-selective one from *Lactobacillus brevis* (LbADH)[13] and the (*S*)-selective ADHs from *Thermoanaerobacter ethanolicus* (TeSADH),[14] *Thermoanaerobacter* sp. (ADH-T),[15] *Sphingobium yanoikuyae* (SyADH)[16] and *Ralstonia* sp. (RasADH).[17] To check the enzyme activity in the presence of NADES, the model substrate for each ADH was added in 25 mM concentration, ideal conditions for this class of redox enzymes, and commercial GDH-105 was used as coupled-enzyme (3 units). From ChCl:Glu mixtures, the 1.5:1 mol/mol was selected in order to have a significant amount of glucose for cofactor recycling purposes without the detriment of using a highly dense and viscous NADES at considerable glucose concentrations, which would make it difficult to handle.[18] 10% v/v of the NADES formed by choline chloride (ChCl) and glucose (1.5:1 mol/mol) was added into the reaction medium (50 mM Tris·HCl pH 7.5, 1 mM NADPH). Finally, 10 mg of the lyophilised ADH were resuspended and the reactions were incubated at 30 °C for 24 h. The results from the bioreduction experiments are shown in Table 1.

Table 1 Bioreduction of ketones using ADHs in aqueous medium and 10% v/v of NADES ChCl:Glu (1.5:1 mol/mol) as cosolvent.[a]



Entry	ADH	Substrate	Conv. (%)[b]	*ee* (%)[b]
1	Lb	Acetophenone (**1a**)	>99	>99 (*R*)
2	TeS	2-Octanone (**1b**)	86	92 (*S*)
3	T	2-Octanone (**1b**)	99	>99 (*S*)
4	Sy	Propiophenone (**1c**)	81	>99 (*S*)
5	Ras	Propiophenone (**1c**)	>99	>99 (*S*)

[a] See the ESI file for detailed reaction conditions and protocols. [b] Conversion and enantiomeric excess values were determined by GC analysis. The absolute configuration of the alcohol appears in brackets.

Gratifyingly, complete conversions and selectivities were attained for LbADH, ADH-T and RasADH in the bioreduction of acetophenone, 2-octanone and propiophenone, respectively (entries 1, 3 and 5), while TeSADH (entry 2) and SyADH (entry 4) also led to high conversions and good to excellent enantiomeric excess. As a result of their remarkable performances, we decided to delve into bioreductions catalysed by LbADH, ADH-T and RasADH using this designer NADES as both co-solvent and co-substrate.

As one could argue regarding the integrity of the NADES in aqueous medium at low concentrations, we decided to increase its concentration in order to study this effect. Edler and co-workers recently showed that using DES in water at >50% (w/w) concentrations,[19] the nanostructure of the eutectic solvent is perfectly retained (see also NMR experiments in the ESI, Figures S2-S8). Therefore, we increased the proportion of NADES employed in the biotransformation up to 90% v/v[20] (see Fig. 1).

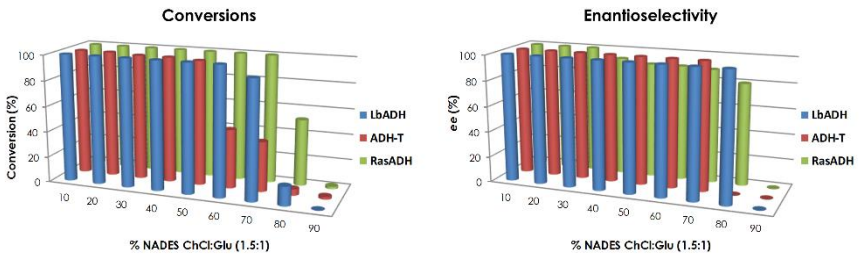


Fig. 1 Effect in activity and selectivity using different amounts of NADES ChCl:Glu (1.5:1 mol/mol) in the bioreduction of acetophenone (LbADH), 2-octanone (ADH-T) and propiophenone (RasADH). Numerical values are given in the ESI.

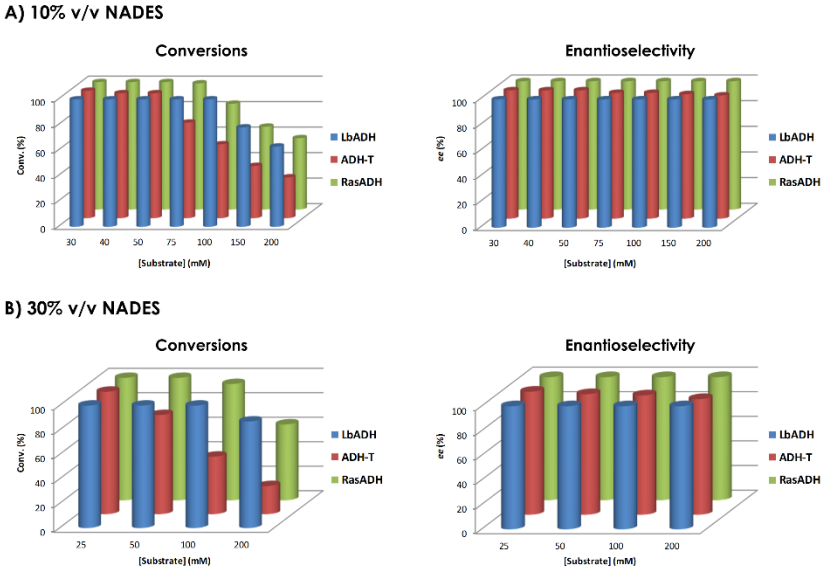


Fig. 2 Effect in activity and selectivity using different amounts of NADES ChCl:Glu (1.5:1 mol/mol) at variable substrate concentrations in the bioreduction of acetophenone (LbADH), 2-octanone (ADH-T) and propiophenone (RasADH). Numerical values are given in the ESI.

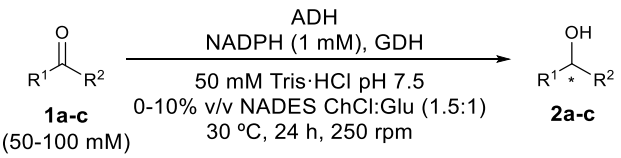
Gratifyingly, LbADH and ADH-T revealed perfect results in terms of conversion and enantioselectivity at high NADES contents. On the one hand, LbADH catalyzed quantitatively the bioreduction of acetophenone even at 60% v/v NADES concentration, maintaining complete selectivity even at 80% v/v of NADES (>99% *ee*). On the other hand, RasADH led to perfect conversions until 70% v/v of NADES in the bioreduction of propiophenone, but a drop in the selectivity was observed when using a proportion equal or higher than 40% v/v of NADES (93% *ee*). Interestingly, this surprising change in the stereoselectivity has been reported for several authors when using different alcohol dehydrogenases (*e.g.*, RasADH),[11b] and it is usually associated with the inhibition of other biocatalytic activities present in the enzyme preparation or due to protein conformational changes.[10]

To show the applicability of this methodology, the use of higher substrate concentrations was explored, ranging from 30 to 200 mM (Fig. 2) under similar reaction conditions to those previously reported at 25 mM. First of all, 10% v/v of NADES was employed (Fig. 2A) and different trends were observed. On the one hand, ADH-T and RasADH catalysed the complete reductions of 2-octanone and propiophenone up to 50 mM, yielding both alcohols in enantiopure form. ADH-T displayed lower activities at 75 mM substrate concentration (75% conversion) with a slight decrease in the selectivity (96-98% *ee* at 75-200 mM), while RasADH led to 83% conversion at 100 mM, to later significantly decrease the activity although the selectivity was optimum in all cases. On the other hand, LbADH was capable of transforming up to 100 mM of acetophenone into enantiopure (*R*)-1-phenylethanol with complete conversion.

Having in mind that glucose is employed as co-substrate, so its concentrations decreased with the advance of the reaction, higher amounts of NADES were employed (30% v/v of NADES) at different ketone concentrations (25-200 mM, Fig. 2B). Remarkably, the three ADHs worked with excellent levels of selectivity, and although the activity of ADH-T was worse in comparison when using 10% v/v of NADES, LbADH and RasADH acted in a similar manner than in the reaction with 10% NADES.

The best substrate concentration *vs* conversion ratio for each enzyme was selected in order to compare the effect of our system regarding the standard glucose/GDH system typically employed (Table 2). Using the same amount of glucose for all the bioreductions, the NADES/GDH system was superior (even entries) to the biotransformations performed by just adding glucose/GDH (odd entries), especially for the bioreduction of acetophenone with LbADH (entries 1 and 2). While this effect remains unclear, there are reports that have described that choline chloride can exert a stabilizing effect in the structure of biomolecules such proteins[21] or DNA.[22]

Table 2 Bioreduction of ketones using ADHs with glucose as co-substrate in pure buffer or using a 10% v/v ChCl:Glu mixture as cosolvent at 30 °C and 250 rpm for 24 h.[a]



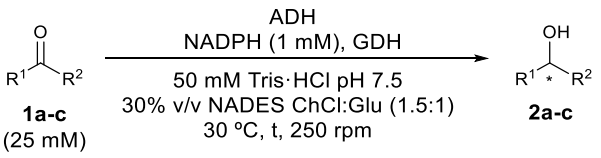
Entry	NADES (%)	ADH-Substrate[b]	Conv. (%)[c]	*ee* (%)[c]
1	0	Lb and **1a** (100 mM)	59	>99 (*R*)
2	10	Lb and **1a** (100 mM)	>99	>99 (*R*)
3	0	T and **1b** (50 mM)	89	>99 (*S*)
4	10	T and **1b** (50 mM)	98	>99 (*S*)
5	0	Ras and **1c** (100 mM)	61	>99 (*S*)
6	10	Ras and **1c** (100 mM)	83	>99 (*S*)

[a] See the ESI file for detailed reaction conditions and protocols. [b] The ketone concentration is shown in brackets, while the glucose concentration is 240 mM. [c] Conversion and enantiomeric excess values were determined by GC analysis. The absolute configuration of the alcohol appears in brackets.

Selected bioreduction processes were monitored within the time, trying to find optimal conditions for the selective bioreduction of the ketones (Table 3). 30% v/v NADES, 25 mM ketone concentrations and 30 °C were fixed, achieving full conversions for each substrate at 1 h (acetophenone, LbADH, entry 2), 1.5 h (2-octanone, ADH-T, entry 5) or 20 min (propiophenone, RasADH, entry 8).

Also, the recyclability of the enzyme and NADES (30% v/v) was subjected to study (Fig. 3). After the first transformation was completed, the reaction mixture was extracted in high yields with EtOAc (upper phase), showing full conversions and excellent selectivities, and the aqueous medium formed by the NADES and the enzymes (ADH and GDH, bottom phase) was employed for running a second biotransformation by simply adding the corresponding ketone **1a**-**c**. Unfortunately, a significant activity loss was observed (10-56% conversion), which was dramatic for LbADH (Fig. 3, left) and ADH-T (Fig. 3, middle).

Table 3 Conversions *vs* time of alcohol dehydrogenase-catalysed bioreductions using 30% v/v NADES ChCl:Glu (1.5:1).[a]



Entry	ADH	Substrate	t (min)	Conv. (%)[b]	*ee* (%)[b]
1	Lb	**1a**	30	70	>99 (*R*)
2	Lb	**1a**	60	>99	>99 (*R*)
3	T	**1b**	30	82	>99 (*S*)
4	T	**1b**	60	95	>99 (*S*)
5	T	**1b**	90	>99	>99 (*S*)
6	Ras	**1c**	10	97	>99 (*S*)
7	Ras	**1c**	15	98	>99 (*S*)
8	Ras	**1c**	20	>99	>99 (*S*)

[a] See the ESI file for detailed reaction conditions and protocols. [b] Conversion and enantiomeric excess values were determined by GC analysis. The absolute configuration of the alcohol appears in brackets.

There are two possible reasons to explain this inactivation effect. On the one hand, the use of a great amount of an organic solvent such as EtOAc could lead to this effect. On the other hand, D-glucono-1,5-lactone is produced through the glucose oxidation that later spontaneously hydrolyses to D-gluconic acid as by-product, decreasing the pH of our aqueous system (Tris·HCl 50 mM pH 7.5). In order to test the influence of these two parameters in the enzyme activity, firstly pentane was used instead of EtOAc to extract the product (Fig. 4, blue bars), and later the Tris·HCl buffer 200 mM pH 7.5 was also employed instead of the previous 50 mM buffer (Fig. 4, red bars) in order to see if a higher buffer concentration could balance out the production of D-gluconic acid without altering the pH of the buffer. The results are shown in Fig. 4, observing significant benefits when using pentane and the 200 mM Tris HCl buffer.

Interestingly, the use of pentane already led to better results allowing a successful first recycling with LbADH (Fig. 4 left, 97% conversion) and RasADH (Fig. 4 right, 99% conversion). In order to explore the possible enzyme deactivation due to the extracting solvent, additional experiments (Fig. 4, green bars) were carried out by filtering the ADH after the bioreduction, to later extract the reaction medium and perform the next reuse. In this case only slight improvements were found for LbADH (Fig. 4, left), while not significant changes for the recycling of ADH-T (Fig. 4, middle) and RasADH (Fig. 4, right). When the pH value of the reaction medium after a biotransformation was measured, it was found 3.5 for those performed in the Tris·HCl buffer 50 mM, while a value around 4.9-5.0 was attained in the ones carried out at a 200 mM buffer concentration due to the release of D-gluconic acid. For that reason, pH was readjusted up to 7.5 before each recycling experiment after enzyme filtration and extraction with pentane (Fig. 4, purple bars), finding in this manner a great improvement, especially when using LbADH (98% conversion and 98% *ee* after 5 cycles, Fig. 4 left) and RasADH (98% conversion and >99 *ee* after 8 cycles, Fig. 4 right and ESI).

Finally, in order to demonstrate the applicability of the method we set up some semi-preparative biotransformations (100 mg of ketone) finding results in accordance with those obtained at analytical scale. Full conversions into the enantiopure alcohols were reached, producing (*R*)-1-phenylethanol in 78% isolated yield with LbADH (150 mg of the lyophilised preparation and 100 mM ketone concentration), (*S*)-2-octanol in 85% yield using ADH-T (350 mg of the lyophilised preparation, 50 mM) and (*S*)-1-phenyl-1-propanol in 89% yield with RasADH (150 mg of the lyophilised preparation, 100 mM), after extractions with ethyl acetate.

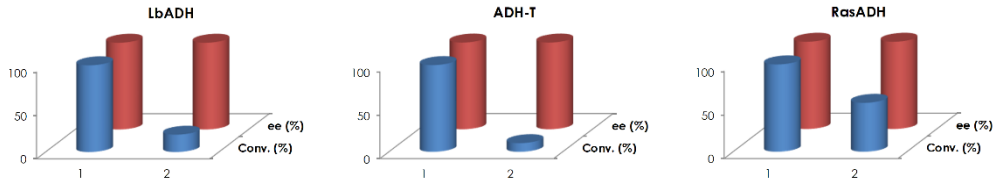


Fig. 3 Recycling of the enzyme and 50mM buffer-NADES (30% v/v) mixture after extraction with EtOAc in the bioreductions of acetophenone with LbADH (left), 2-octanone with ADH-T (middle) and propiophenone with RasADH (right). Numerical values are given in the ESI.

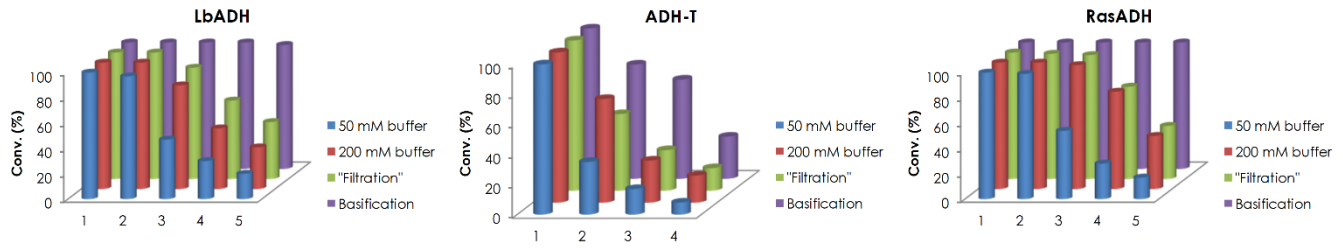


Fig. 4 Recycling of the enzyme and NADES (30% v/v) after extraction with pentane in the bioreductions of acetophenone with LbADH (left), 2-octanone with ADH-T (middle) and propiophenone with RasADH (right): A) Blue bars are reactions carried out in 50 mM buffer, extracted with pentane and reusing the NADES-buffer plus the enzyme; B) Red bars are reactions carried out in 200 mM buffer, extracted with pentane and reusing the NADES-buffer plus the enzyme; C) Green bars are reactions carried out in 200 mM buffer, filtering the enzyme, extracting with pentane and reusing the NADES-buffer plus the enzyme; D) Purple bars are reactions carried out in 200 mM buffer, filtering the enzyme, extracting with pentane, readjusting the pH of the NADES-buffer mixture up to 7.5 and reusing the NADES-buffer plus the enzyme. Numerical values are given in the ESI.

For these semi-preparative transformations, we performed a simple quantification of the *E*-factor[23] to obtain an overview of the environmental impact of this methodology. Hence, the EATOS tool[24] was used focusing on the relevance of the reaction conditions regarding the reagents, catalysts and solvents employed (excluding water), and taking into account the waste generated. As can be seen in the ESI (Figure S1), the values obtained were between 236-286. While these numbers are still high, it can be seen that the highest percentage of these numbers come from the solvents employed, in particular from EtOAc used to extract the final products. Since at big scale the recycling of organic solvents is a common applied technique, we are sure that these numbers could be further optimised.

Overall, a ChCl:Glu mixture has been used as a designer natural DES applied to ketone bioreduction transformations using five different ADHs. The combination of an aqueous buffer system with the NADES in up to 50% v/v ratio has provided two main advantages. On one hand, the presence of glucose provides the co-substrate for the GDH-catalysed reaction for the nicotinamide cofactor recycling. On the other hand, the bioreductions were run at higher substrate concentrations in comparison with the buffer system employing glucose/GDH, and the development of practical protocols in terms of excellent conversions were possible in up to 100 mM concentration with excellent selectivities. After optimisation of the reaction conditions, the bioreductions were carried out in semi-preparative scale finding that the use of pentane as extracting organic solvent presented great advantages for enzyme recycling in comparison with ethyl acetate, traditional solvent used in these work-up procedures, as pentane preserve better from enzyme inactivation. It is also worth mentioning that the use of concentrated buffers (i.e. 200 mM Tris HCl buffer) highly improved the enzyme activity of ADHs in these reactions, since D-gluconic acid is formed as co-product in the cofactor recycling reaction, dramatically changing the pH in the presence of the more diluted buffer with the reaction course advance.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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