Performance of recombinant whole-cell-catalyzed reductions in Deep-Eutectic-Solvents – Aqueous mixtures.

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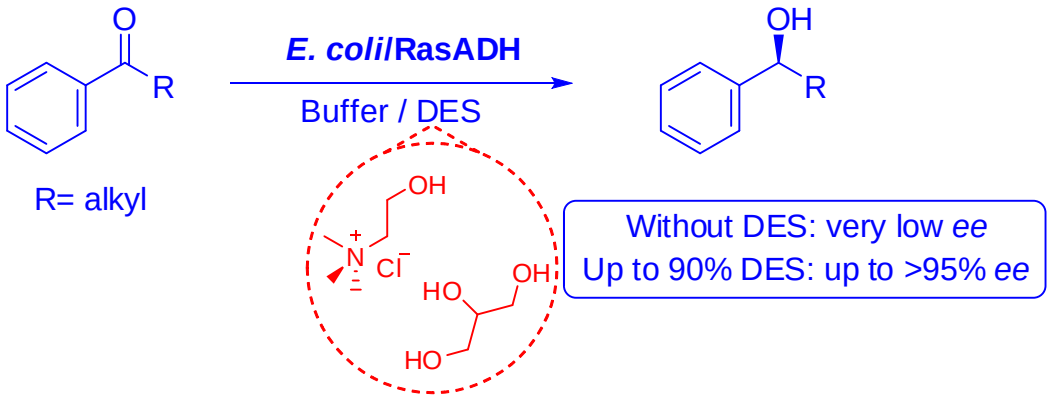
GRAPHICAL ABSTRACT



Summary

Deep-eutectic-solvents are cost-effective non-hazardous solvents that may be used in biocatalysis as non-conventional media, enabling biotransformations with industrially-sound high substrate loadings. Based on promising prognoses, this paper successfully explores the use of whole-cell overexpressing oxidoreductases as biocatalysts for the stereoselective reduction of ketones in different DES-aqueous media solutions. Enzymes like *Ralstonia* sp. ADH and horse liver ADH –overexpressed in *E. coli* –, remain active at different DES proportions (e.g. 80:20 DES:buffer v/v), enabling non-conventional biotransformations. Furthermore, enantiomeric excesses obtained for a broad range of aromatic substrates increase significantly when DES is added, providing a useful tool to combine high substrate loadings and improved enzymatic selectivities, while working with non-hazardous solvents. Different DES are successfully employed, giving hints on the potentiality that these emerging solvents may have.

Key-words: Alcohol dehydrogenases; Biocatalysis; Deep-Eutectic-Solvents, Medium Engineering;

1.- Introduction.

The use of oxidoreductases to afford optically active building blocks is currently a mature technology, with applications both at academic and industrial scale. Key-aspects of those processes, such as the need of high substrate loadings, efficient cofactor regenerating strategies, as well as the establishment of effective overexpression systems, have been adequately tackled over the last years.[1] In this respect, the set-up of highly efficient and creative cofactor regeneration systems has been sorted out with outstanding results so far, comprising both free, isolated enzymes, and whole-cells,[2] as well as natural and non-natural cofactors.[3] Likewise, different concepts related to medium engineering – e.g. use of organic co-solvents or biphasic systems to enhance substrate loadings – have been in-depth assessed as well. Herein, apart from more *classic* aqueous-based systems, several research groups have shown that oxidoreductases (lyophilized whole-cells overexpressing alcohol dehydrogenases, ADHs) can actually catalyze stereoselective processes in water-free systems (e.g. solvent-free or organic solvents), thus enabling biocatalytic oxido-reductions in non-conventional media, and opening new possibilities for these enzymes.[2-4] Last but not least, the identification of novel useful catalytic performances for these biocatalysts – e.g. imine reductases[5] –, provides even a stronger potential for future sustainable solutions in enzyme catalysis.

From the perspective of neoteric, non-hazardous and environmentally-friendly solvents, Deep Eutectic Solvents (DES) have recently appeared as a promising alternative for many chemical applications.[6] DES are typically formed by the combination of a quaternary ammonium salt (e.g. choline chloride) and hydrogen-bond donor molecules (HBDs) such as polyols, carboxylic acids, amines, etc. The

formation of a deep eutectic mixture is caused by disruption of the crystalline structures of the individual components, hence lowering the lattice energy, and leading to liquids at room temperature.[6,7] Among the emerging DES applications, their use as solvents for biocatalytic reactions has been envisaged,[8] and examples covering hydrolases,[9] lyases[10] and oxidoreductases[11] have been successfully reported over the last years. Depending on the biocatalyst type, different amounts of water are often needed to maintain enzymes in their optimal active form. In any case, useful DES-aqueous mixtures, e.g. DES containing 10% v/v water, can be applied, leading to rather water-saturated neoteric solvents with (still) large dissolving capacity for industrially-sound high substrate loadings. Given that DES are non-hazardous, biodegradable and cost-effective, the emerging strategies are promising for future practical applications in biocatalysis.

With regard to oxidoreductases, reported examples in DES have focused on wild-type whole-cells (baker´s yeast, *Acetobacter* sp.). DES exert a beneficial effect on the whole-cell biocatalysis, with significant improvements in stereoselectivity, e.g. when using baker´s yeast.[11c] Being a wild-type cell containing several oxidoreductases – most of them overexpressed at analogous low levels[1a] –, the observed improvements were ~~suggested to occur due to~~ attributed to the inhibition of some of these enzymes by DES, while others would remain fully active in such DES-aqueous mixtures.

For an applied-driven biocatalysis, the use of recombinant overexpressing whole-cells appears as a powerful option, as the desired enzyme is then present at high loadings within the cell. By means of these highly optimized biocatalysts, high productivities have been reported in asymmetric synthesis, even at industrial scale,

using micro-aqueous solutions, solvent-free processes, as well as biphasic systems to enhance the substrate loadings.[4,12]

Considering that point, and the potential importance that DES may have for future (bio)catalytic processes, the use of recombinant whole-cells overexpressing oxidoreductases in DES-aqueous mixtures is assessed in this work for the first time.

2.- Results & Discussion.

As prototypical DES for the oxidoreductase-catalyzed processes, choline choride – glycerol (1:2 mol:mol) was chosen,[7a] as such system has been shown as beneficial for other biocatalytic processes, including (wild-type) whole cell biocatalysis.[8-11] In a first set of experiments, different ADHs overexpressed in *E. coli* were screened, using 2-octanone, benzaldehyde or propiophenone as substrates, in combination with alcohols as ancillary substrates to regenerate the cofactor (ethanol for benzaldehyde, and propan-2-ol for the ketones). From that preliminary screening, three ADHs displayed outstanding activities at different DES-buffer proportions, namely ADHs from *Thermoanaerobacter ethanolicus* (TeSADH),[13] horse liver ADH (HLADH)[14] and *Ralstonia* sp. ADH (RasADH).[15] Results are depicted in Figure 1.

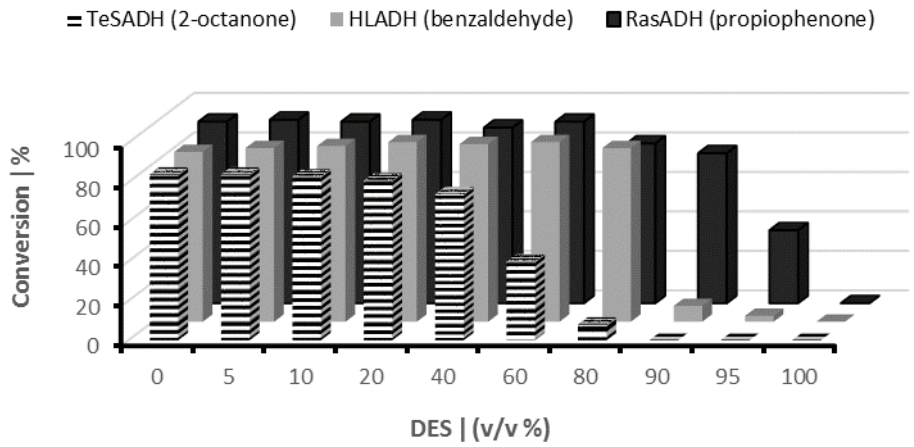


Figure 1. Redox performance in DES-aqueous mixtures of TeSADH (using 2-octanone), HLADH (using benzaldehyde) and RasADH (using propiophenone). Reaction conditions: 3 U enzyme, 30 μL alcohol (0.39 mmol propan-2-ol; 0.51 mmol ethanol in case of HLADH), 3 μL carbonyl compound (0.02–0.03 mmol), 60 μL phosphate buffer 50 mM pH 7.5 with cofactor (10 mM), 540 μL 1:2 ChCl / glycerol DES – phosphate buffer 50 mM pH 7.5, 24 h, 30 °C, total volume: 630 μL.

As it can be observed, the three whole-cell overexpressing ADH systems displayed high biocatalytic reduction conversions at different DES-aqueous buffer proportions. Remarkably, RasADH remained virtually fully active at 60-70% DES (v/v) and even at 95% DES (v/v) almost half of the activity was still observed. At very high proportions, conversions dropped drastically, suggesting the deactivation of the biocatalyst by high concentrations of DES together with low water quantities. Thus, in addition to other wild-type whole cells reported in the literature,[11] recombinant cells overexpressing ADHs may also be used in these DES-buffer neoteric mixtures, that can certainly broaden the applicability of these systems in non-hazardous unconventional media. The other two ADHs (TeSADH and HLADH) displayed high-

to-moderate activities at different DES-buffer proportions. The significant observed difference of activities in DES-buffer media – depending on the overexpressed enzyme –, provides support to the whole-cells remaining integrity in DES.[16,17] Experiments performed with free enzymes led actually to very low conversions (e.g. 12% vs. 90% in the case of HLADH), presumably due to deactivation of the biocatalyst by the DES outside the whole-cell. Therefore, cell integrity secures the stability of the enzyme to conduct practical biocatalysis.

Given its outstanding performance, overexpressed RasADH was chosen as the biocatalyst for further reactions in DES-buffer media. In a subsequent set of different experiments ancillary co-substrates (ethanol, propan-1-ol and propan-2-ol) were assessed in the DES-aqueous mixtures, using propiophenone as model substrate for RasADH. In this case, not only activities, but also stereoselectivities were evaluated at different DES-buffer proportions. Results are depicted in Figure 2.

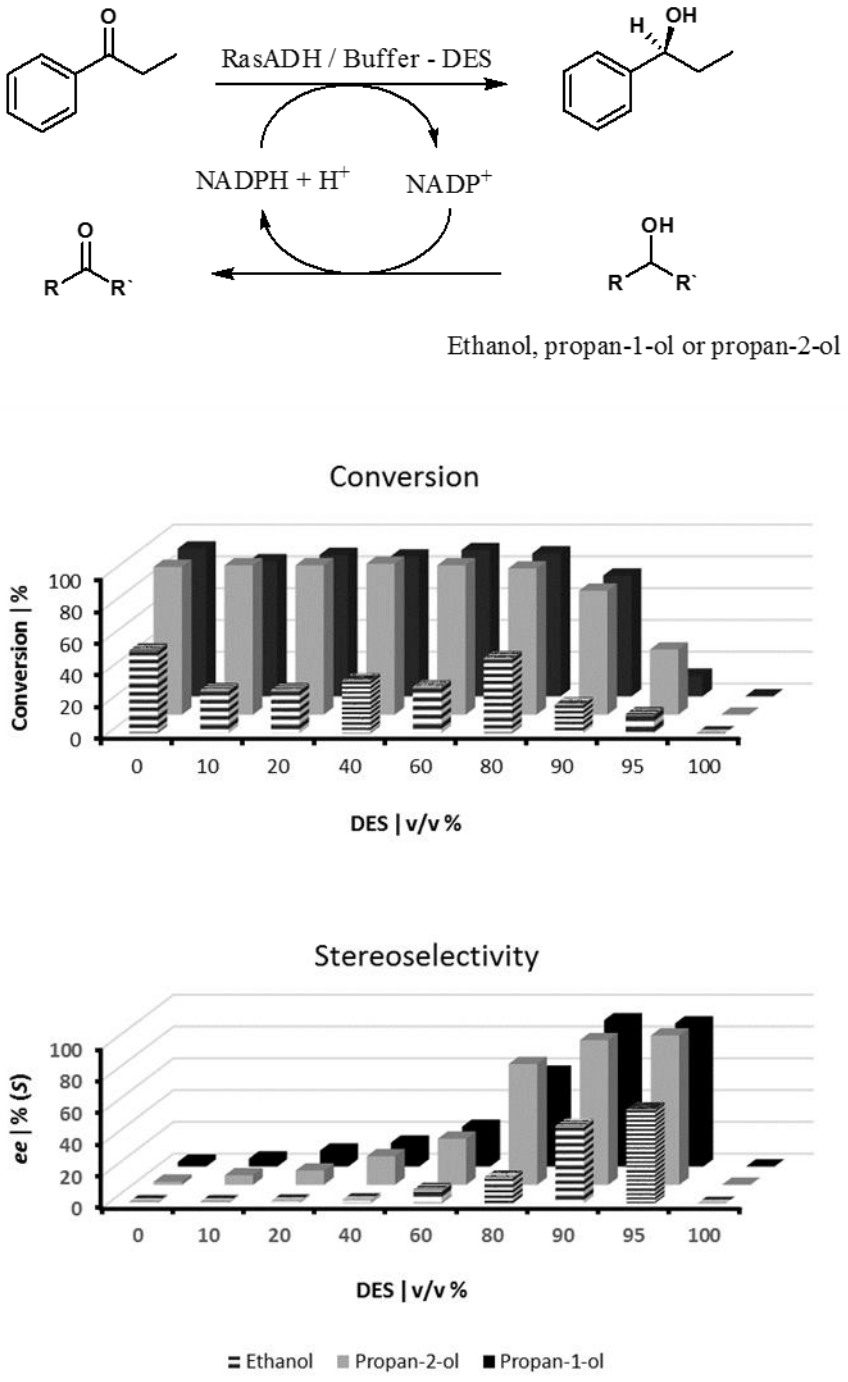


Figure 2. Performance of RasADH for the bioreduction of propiohenone in DES-aqueous mixtures using different ancillary substrates to regenerate the cofactor. Above: Enzyme activities; Below: Stereoselectivities. Reaction conditions: 3 U *E. coli*/RasADH, 30 µL alcohol (0.39 mmol propan-1-ol and propan-2-ol; 0.51 mmol ethanol), 3 µL propiophenone (0.02 mmol), 60 µL phosphate buffer 50 mM pH 7.5

with $NADP^+$ (10 mM) , 540 μL 1:2 ChCl / glycerol DES – phosphate buffer 50 mM pH 7.5, 24 h, 30 °C, total volume: 630 μL.

With regard to conversions (Figure 2, above), they varied significantly at different DES-buffer proportions, but also depending on the ancillary co-substrate. Thus, ethanol led to the lowest conversions irrespective of the amount of DES, an effect that must be obviously related to the selectivity of RasADH to this substrate, and not to the non-conventional media applied. On the other hand, both propan-1-ol and propan-2-ol led to similar results, showing again the stability of RasADH in different DES-buffer media.

Quite remarkably, a more interesting trend was obtained from the stereoselectivity point of view (Figure 2, below). Thus, a significant improvement of the enantiomeric excess was observed at higher DES proportions. In the case of propan-1-ol and propan-2-ol as ancillary substrates, excellent *ees* of >90% (enantiomeric ratios of > 95:5) were achieved, starting from virtually racemic production in the absence of DES for that enzyme and substrate (Figure 2 below). The observed improvement of the stereoselectivity is consistent with previous cases using wild-type whole cells.[11]

Stimulated by these results, the substrate range scope of RasADH in DES-buffer mixtures was evaluated. RasADH is a recently explored biocatalyst, that bears promising substrate range, specially covering hindered ketones, which makes it an attractive biocatalyst for practical applications.[4b,15,18] In this case, different more or less sterically hindered aryl-ketones were assessed at several DES-buffer proportions. Results are depicted in Figure 3.

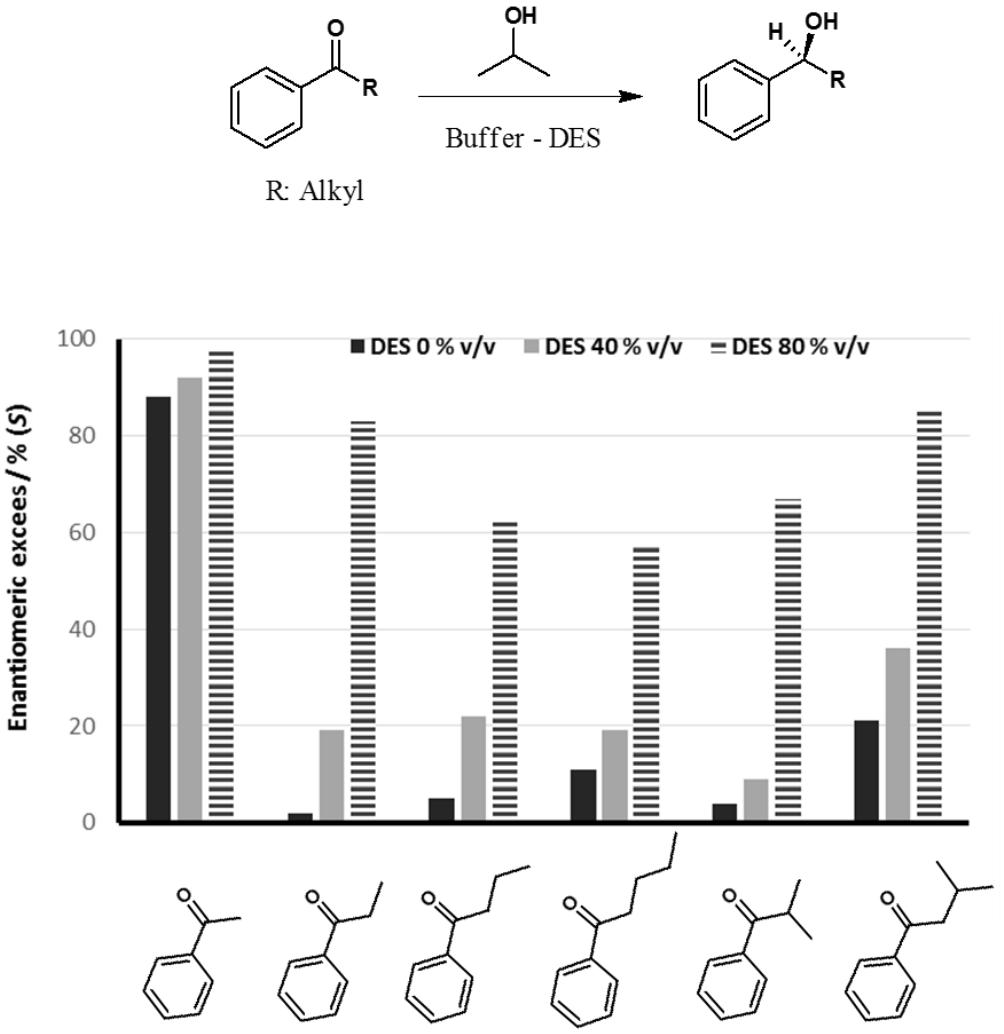


Figure 3. Stereoselectivity observed in the aryl substrate scope of RasADH at different DES-buffer proportions. Reaction conditions: 3 U *E. coli*/RasADH, 30 μL propan-2-ol (0.39 mmol), 3 μL ketone (0.02-0.03 mmol), 60 μL phosphate buffer 50 mM pH 7.5 with $NADP^+$ (10 mM) , 540 μL 1:2 ChCl / glycerol DES – phosphate buffer 50 mM pH 7.5, 24 h, 30 °C, total volume: 630 μL.

The observed enzymatic conversions remained high in the overall DES-buffer media range (not shown). Gratifyingly, the stereoselectivity improvement was significantly observed in all tested substrates (Figure 3). Especially in bulky substrates the effect was notorious, as the enantiomeric excess was improved from low optical

purities in pure buffer to moderate-to-high values (*ee´s* 60-90%; enantiomeric ratios of 80:20 to 95:5) at larger DES proportions. Hence, the combination of an inexpensive and non-hazardous non-conventional media to dissolve bulky substrates – such as DES –, together with protein engineering to further improve enzyme performances, may become a powerful tool for future practical biocatalysis.

Triggered by the outstanding observed results in terms of stereoselectivity improvement with DES, further experiments were conducted by using different choline-chloride-based DES with different HBDs, namely using glycerol (at 1.5 and 2 equiv), urea (2 equiv), and ethylene glycol (2 equiv). Results comprising conversion and stereoselectivity are summarized in Figure 4.

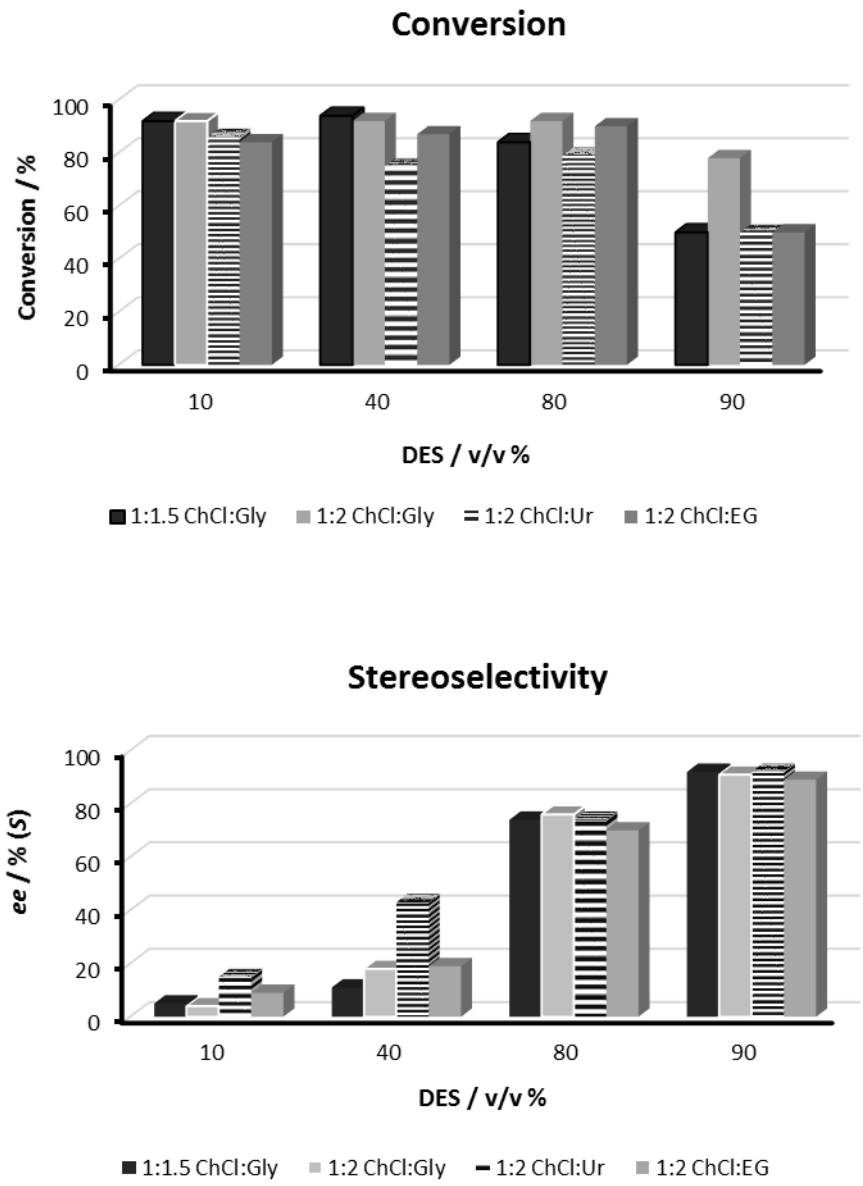


Figure 4. Performance of RasADH for the bioreduction of propiohenone using different DES mixed with buffer at several proportions. Above: Enzyme activities; Below: Stereoselectivities. Conditions: 3 U *E. coli*/RasADH, 30 µL propan-2-ol, 3 µL propiophenone, 60 µL phosphate buffer 50 mM pH 7.5 with $NADP^+$ (10 mM), 540 µL DES – phosphate buffer 50 mM pH 7.5, 24 h, 30 °C, total volume: 630 µL.

As it can be observed (Figure 4), the improvement in the stereoselectivity was general for all DES assayed, reaching excellent stereopreferences at DES proportions of 90% (v/v), albeit often at lower conversions due to enzyme deactivation. As a compromise, DES-buffer 80-20 (v/v) appeared to be optimal for these processes, from a practical perspective. Another aspect to be considered was whether the observed improvement was due to DES as structure or as separate components dissociated in the aqueous media. Recent literature has shown that DES would retain their structure in aqueous solutions.[19] Herein, experiments with low proportions of glycerol or choline chloride (10% v/v) did not lead to significant improvements in the enzymatic performance. Aspects such as the intrinsic glycerol viscosity or the low solubility of choline chloride in buffer must be considered as well. In this respect, the synergistic combination of both components in a DES is the proper way to use them in biocatalysis, as low viscosities, high substrate solubilities, and enhanced enzymatic activities may be reached.

To further investigate the effect of DES in the stereoselectivity improvements, experiments using hexane as cosolvent, and "cosolvent-free" solutions (that is, using a more concentrated buffer system, as the remnant cosolvent volume was not fulfilled) were conducted, and the stereoselectivity explored. For comparison, results of ChCl:Gly (1:2) are included. Results are depicted in Figure 5.

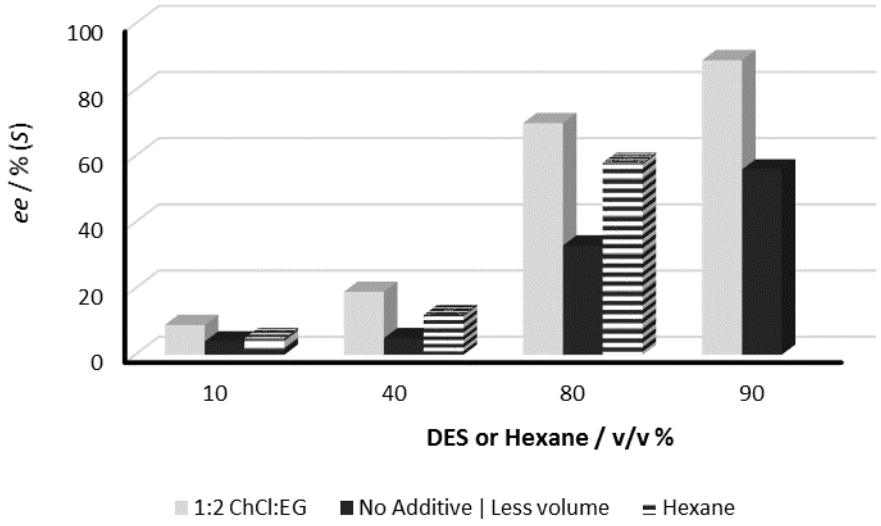


Figure 5. Comparison of stereoselectivity improvement using DES ChCl:Gly (1:2), hexane or no additives (less volume). Reaction conditions: 3 U *E. coli*/RasADH, 30 µL propan-2-ol, 3 µL propiophenone, 60 µL phosphate buffer 50 mM pH 7.5 with $NADP^+$ (10 mM) , 540 µL cosolvent – phosphate buffer 50 mM pH 7.5, 24 h, 30 °C, total volume: 630 µL. In case of "no additive": 3 U *E. coli*/RasADH, 30 µL propan-2-ol, 3 µL propiophenone, 60 µL phosphate buffer 50 mM pH 7.5 with $NADP^+$ (10 mM) , 540 µL cosolvent – phosphate buffer 50 mM pH 7.5, 24 h, 30 °C. Total volume: 600 µL.

Interestingly, both the use of less-volume batch, or hexane as cosolvent led to improvements in the stereoselectivity. In the case of no-additive, an increase in the substrate concentration may (kinetically) lead to better stereoselectivities. Albeit the effect of DES in stereoselectivity was much pronounced than for hexane, observed results suggested that the improvement of stereoselectivity may be related to internal interaction of cosolvent (either hexane or DES compositions) within the enzyme active site. Analogous results have been recently observed and modelled for thiamine-

dependent lyases and co-solvents,[20] as well as when ionic liquids are added as performance additives.[8a] In any case, the use of non-hazardous DES offers not only better outcomes (higher stereoselectivities), but also more environmentally-friendly conditions for biocatalysis using recombinant whole-cells.

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3.- Conclusions.

Herein the use of whole-cell overexpressing oxidoreductases as biocatalysts in DES-aqueous media has been reported for the first time. Different ADHs have proven to remain active at high DES concentrations (80:20 DES-buffer v/v, for instance choline chloride – glycerol DES), and furthermore, significant improvements in the enantiomeric excesses of the reactions have been observed for a broad range of aromatic substrates. Results demonstrate that it is possible to employ these enzyme-friendly and non-hazardous neoteric solvents – with the ability of dissolving high substrate loadings –, with recombinant whole-cells. The ease of DES production and its biodegradability confers further promising features for their practical implementation in biocatalyzed processes.

4.- Experimental Section.

Chemicals & Biocatalysts.- All chemicals were purchased from Sigma-Aldrich and used without further purification. With regard to biocatalysts, *E. coli* strains to overexpress the RasADH from *Ralstonia* sp., SyADH from *Sphingobium yanoikuyae*, and TeSADH from *Thermoaerobacter ethanolicus* were kindly provided by Prof. Wolfgang Kroutil (University of Graz). *E. coli* strain overexpressing horse liverADH

was provided by Prof. Martina Pohl (*Forschungszentrum* Jülich GmbH). The activity of the overexpressed biocatalysts is approximately 0.3 U/mg for propiophenone (RasADH and SyADH), 2-octanone (TeSADH), and benzaldehyde (HLADH).

DES formation.- The components were directly mixed together and stirred at 60 °C until a clear solution was obtained (15-60 min). After cooling down to room temperature, the formed DES could be used directly.

Standard reaction with oxidoreductases and DES.- For HLADH. 15 mg of *E. coli*/HLADH were added into an Eppendorf vial and dissolved in 420 µL phosphate buffer 50 mM pH 7.5 and a solution containing 60 µL phosphate buffer 50 mM pH 7.5 and NAD^+ (10 mM). Then, 120 µL 1:2 ChCl / glycerol DES were added to the mixture as well as benzaldehyde (3 µL) and ethanol (30 µL). The reaction was shaken (300 rpm) for 24 h at 30 °C. The aqueous phase was extracted with ethyl acetate (2 x 500 µL). After separation and drying over sodium sulfate, the organic layer was analyzed *via* GC (HP-1 column).

For RasADH. 15 mg of *E. coli*/RasADH were added into an Eppendorf vial and dissolved in 420 µL phosphate buffer 50 mM pH 7.5 and a solution containing 60 µL phosphate buffer 50 mM pH 7.5 and $NADP^+$ (10 mM). Then, 120 µL 1:2 ChCl / glycerol DES were added to the mixture as well as propiophenone (3 µL) and propan-2-ol (30 µL). The reaction was shaken (300 rpm) for 24 h at 30 °C. The aqueous phase was extracted with ethyl acetate (2 x 500 µL). After separation and drying over sodium sulfate, the organic layer was transferred to another Eppendorf vial for derivatization. DMAP (1.0 mg, 8.2 µmol) and acetic anhydride (30 µL, 0.32 mmol) were added and the vial was shaken for 1 h at 40 °C. The reaction was quenched by the addition of water (500 µL). After extraction of the aqueous phase with ethyl acetate (2 x 500 µL), the combined organic layers were dried over sodium sulfate and

filtered. The product solution was analyzed *via* GC (Agilent CP-ChiraSil-Dex-CB column).

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