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Whole-Cell Biocatalysis in Deep-Eutectic-Solvents/Aqueous Mixtures

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Whole-cell biocatalysis with the use of baker's yeast is demonstrated in different mixtures of water with deep eutectic solvents (DESs; choline chloride/glycerol, 1:2 mol/mol). Enantioselective ketone reduction is observed for long reaction times (>200 h), which suggests that the whole cells remain stable in these neoteric solvents. By changing the proportion of the DES added, a complete inversion of enantioselectivity is observed, from approximately 95% enantiomeric excess (*ee*) (*S*) in pure water to approximately 95% *ee* (*R*) in the pure DES. Presumably, some (*S*)-oxidoreductases present in baker's yeast are inhibited by DESs.

Biocatalysis has emerged in the last decades as a powerful option to conduct catalytic reactions typically under environmentally friendly conditions and with high selectivities.[1] Apart from the enzymatic (and whole-cell) performance in aqueous media, the setup of biocatalytic processes under non-aqueous conditions—the so-called non-conventional media—provides a practical framework to enhance the solubility of the substrate and product, whilst at the same time providing an enzyme-friendly working conditions. This has been known for decades for free enzymes, especially hydrolases, and in recent years whole cells have also started to be assessed under (bulk) water-free conditions, such as neat substrates[2] or ionic liquids[3] among other non-aqueous media.

In this area, ionic liquids have attracted some interest as novel (tailored) solvents for a broad number of applications.[3] Although the setup of solvent-free processes (if feasible) clearly appears as a superior alternative, in some cases the need to dissolve challenging substrates—or those with different polarities—make the choice of ionic solvents attractive. Yet, problems associated with the toxicological effects of many ionic liquids, as well as their (still) high price represent important hurdles for their further practical implementation.[3] To overcome these aspects, over the past decade the so-called deep eutectic solvents (DESs) have emerged as a new generation of ionic solvents with tailored properties, diminished toxicological features, and adequate biodegradability that are largely available at acceptable costs; consequently, a broad number of promising applications have already appeared in the open literature.[4] In a broad sense, DESs are formed if solid quaternary ammonium halide salts (e.g., choline chloride) are combined and gently heated with hydrogen-bond-donor molecules such as alcohols, amides, amines, and carboxylic acids.[4,5] On this basis, a broad number of biomass-derived compounds may be used to straightforwardly form novel tailored solvents. In comparison to more "classic" ionic liquids, DESs are composed of biodegradable and inexpensive materials.

Considering these aspects, some DESs have started to be assessed as tools for biocatalysis as well, either as solvents or as separative agents to overcome challenging workup procedures.[3,6,7] Examples comprise mainly hydrolases (lipases, proteases, and epoxide hydrolases) as robust and versatile enzymes already used for many applications.[1] For instance, the use of different biomass-derived DESs has found application for proteases in peptide synthesis, whereby the (frequent) need to dissolve substrates with different polarities makes the use of neoteric solvents attractive to reach high substrate loadings.[6a]

Apart from using free, isolated enzymes, the setup of whole-cell-catalyzed reactions enables simple processes with access to sophisticated chemistries.[8] Specifically, if cofactor-dependent enzymes (e.g., oxidoreductases) are considered, the possibility of in situ regenerating the cofactor upon establishing whole-cell biocatalysis represents an asset for its practical implementation. Typically, processes are set in aqueous solutions (emulsions) or in biphasic systems.[8] So far, just a few examples of whole-cell redox biocatalysis in virtually water-free[3] or micro-aqueous systems[9] have been reported. Another work showed the integrity of lyophilized *E. coli* whole cells in DESs,[10] but to our knowledge, no reports on whole-cell biocatalysis in DESs have been published so far and only examples of whole cells with ionic liquids mixtures have been reported.[3,11] Herein, the first studies in that line are presented.

As prototypical DESs, choline chloride/glycerol mixtures (1:2 mol/mol) were built. As whole cells, commercially available nonhazardous baker's yeast was used, for which many enantioselective reductions of ketones have been reported, catalyzed by one or more of the multiple redox enzymes present in its genome.[8,12] As a bench reaction, the enantioselective reduction of ethyl acetoacetate was selected (Scheme 1).

Considering that both whole cells and enzymes need a certain amount of water for their activity, in a first set of experiments different mixtures of the DES and water were assessed as reaction media for the desired process. A reaction conducted in pure water was included for comparison. The results are depicted in Figure 1.

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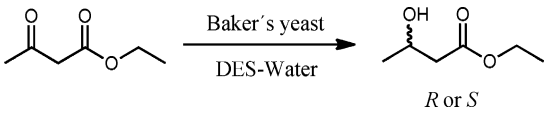

Scheme 1. Bench reaction for studies of baker's yeast as a reductive catalyst in different mixtures of DES/water.

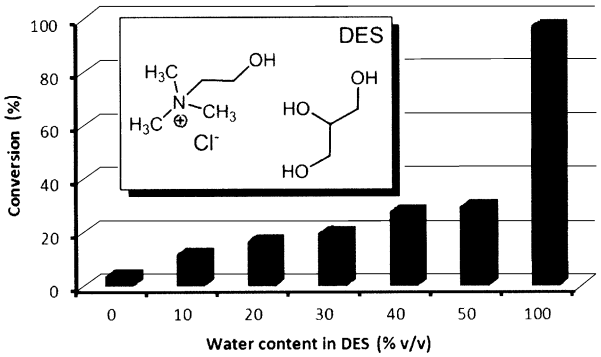

Figure 1. Conversion in the reduction of ethyl acetoacetate catalyzed by whole-cell baker's yeast. Conditions: ethyl acetoacetate (50 mM) and yeast (200 mg mL^{-1}) in choline chloride/glycerol DES (1:2 mol/mol) at room temperature for 72 h. Conversions were determined by ^{1}H NMR spectroscopy.

As can be observed, the presence of variable volumes of the DES in water led to much lower conversions at 72 h (range = 4–25% of product) than those achieved for oxidoreductions conducted in pure water (Figure 1). If neat DES was used (containing a remnant of ≈1 wt% water, determined by Karl-Fischer), a low conversion of approximately 5% at a reaction time of 72 h was reached. From this initial analysis, the conclusion might be that baker's yeast cells are actually not stable in different mixtures of DESs, and therefore, the observed inefficient biocatalysis might occur as a result of cell disruption and the denaturation of the enzyme(s). Triggered by this result, apparently inconsistent with the large stability and integrity of lyophilized cells reported in the literature,[10] and to study the biocatalytic behavior of these whole cells in the DES in more detail, conversions at different longer reaction times (72, 144, and 288 h) under the same reaction conditions were measured. The results are depicted in Figure 2.

As can be observed, at 10 and 50 vol.% water in the DES, the whole cells were still active in the enantioselective reduction at rather long reaction times (>200 h), considering that typical industrial biocatalysis is conducted in reaction times of up to 24–30 h. Thus, in agreement with the literature,[10] baker's yeast might maintain its integrity, and intracellular oxidoreductases still seem to be active after prolonged times. The observed low conversion rates should actually be related to the low concentration(s) of the enzyme(s) existing in the wild-type enzyme(s) within the whole cell(s). To envisage more practical applications, the use of recombinant cells overexpressing the desired enzyme(s) appears as a clear next step for this research line.[2]

To validate these hypotheses and to study the whole-cell biocatalysis in aqueous solutions of DESs further, in a subsequent step the enantioselectivity of the reaction was measured

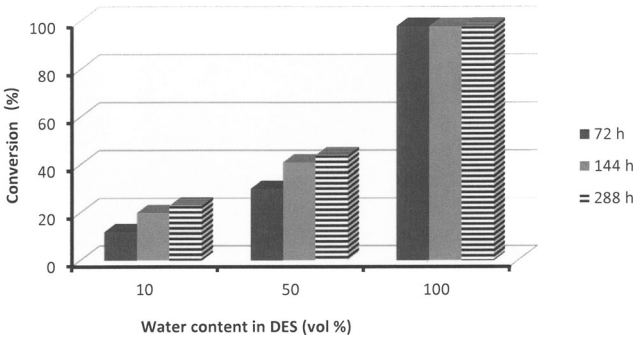

Figure 2. Conversion in the reduction of ethyl acetoacetate catalyzed by whole-cell baker's yeast at different reaction times and with variable amounts of the DES in water. Conditions: ethyl acetoacetate (50 mM) and yeast (200 mg mL^{-1}) in choline chloride/glycerol DES (1:2 mol/mol) at room temperature. Conversions were determined by ^{1}H NMR spectroscopy.

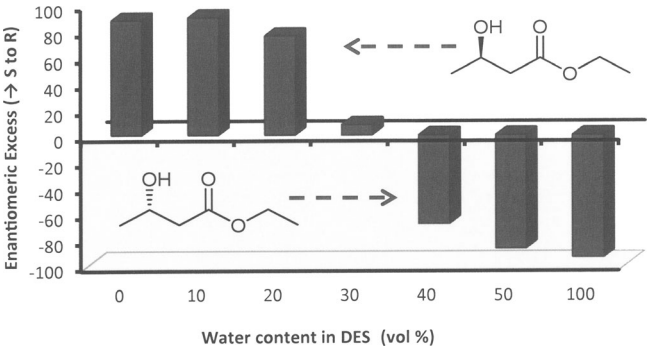

Figure 3. Enantioselectivity of the reductive reaction depending on the different water/DES mixtures set as reaction media. Conditions: ethyl acetoacetate (50 mM) and yeast (200 mg mL^{-1}) in choline chloride/glycerol DES (1:2 mol/mol) at room temperature for 72 h. Enantioselectivities were determined by gas chromatography.

at different proportions of the DES and water. The results are depicted in Figure 3.

Unexpectedly, the addition of different amounts of the DES to water led to complete inversion of the enantioselectivity displayed by the biocatalyst towards ethyl acetoacetate. In pure water (100% water, no DES), the baker's yeast whole cells displayed high stereoselectivity to the formation of the (*S*) enantiomer [≈95% enantiomeric excess (*ee*), Figure 3]. Conversely, by working in DES systems with some amounts of water (up to 20 vol% water in the DES), a high (*R*) enantioselectivity was observed (again ≈95% *ee*, Figure 3). Actually, a virtually racemic mixture was achieved at 30 vol% water in the DES. The explanation of this may be found in the complex mixture of oxidoreductases present in the baker's yeast genome, for which more than 50 enzymes with different enantioselectivities have been described.[8,12] Interestingly, analogous results were described in previous work dealing with medium-engineering approaches such as the modification of the substrate structure and/or substrate concentration, the use of different growth conditions in baker's yeast, and the addition of enzyme inhibitors in the fermentation.[13] In all of these cases, changes in the enantioselectivity of baker's yeast (as whole

cells) were created as a result of the inhibition—or even complete knock out—of several oxidoreductases, whereas others remained active. By using DESs, an explanation for the stereoselectivity inversion may be that some *S*-enantioselective enzymes are actually inhibited by DESs, whereas *R*-selective oxidoreductases (present at low concentrations, which explains the low conversions achieved in the pure DES, Figure 1) would stay as active biocatalysts within the whole cell.

In summary, the results reported in this communication suggest that DESs may actually become promising unconventional reaction media for whole-cell biocatalysis, in which (some) enzymes may be stable and active for synthetic purposes. The construction of recombinant hosts overexpressing robust enzymes for these systems (e.g., *R*-selective enzymes in this case) would enable the setup of industrially sound biotransformations in biomass-derived, nonhazardous neoteric solvents.

Experimental Section

Materials

All chemicals were used without any further purification. Choline chloride, glycerol, ethyl acetoacetate, and ethyl acetate were purchased from Sigma, and baker's yeast (from Deutsche Hefewerke GmbH) was acquired in a normal supermarket.

Synthesis of DESs

The quaternary ammonium choline chloride salt and glycerol were mixed together in a 1:2 mol/mol ratio in a 20 mL vial and heated up to 80 °C until a clear and homogenous liquid was formed (after ≈15 min).

Standard biocatalytic protocol in DES/aqueous mixtures

Baker's yeast (200 mg) was weighted into a G8 vial and dissolved in variable amounts of water (within a range of 0–1000 μL). The DES was added to the solution until a total volume of 1 mL was reached. Finally, ethyl acetoacetate (6.4 μL) was added to the mixture, which was stirred at room temperature at 300 rpm. After a precise interval of time, the reaction was stopped by adding water (2 mL), and the mixture was then extracted with ethyl acetate (3×2 mL). The organic phases were collected, and ethyl acetate was evaporated under reduced pressure.

Analytics

The conversions were determined by ^{1}H NMR spectroscopy. The *ee* values for ethyl 3-hydroxybutyrate were determined by a gas chromatograph equipped with a chiral Hydrodex (250 μm×0.250 μm×25 m) column and a flame ionization detector by using racemates as standards. The following temperature program was used for the analysis: 50 (0 min) to 160 °C (10 min) at a rate of 3 °C min^{-1}. The temperature of the injector was 250 °C, and the split ratio was 40:1. The carrier gas was hydrogen, and the flow rate was 2.0 mL min^{-1}.

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Keywords: biocatalysis · enantioselectivity · enzymes · reduction · sustainable chemistry

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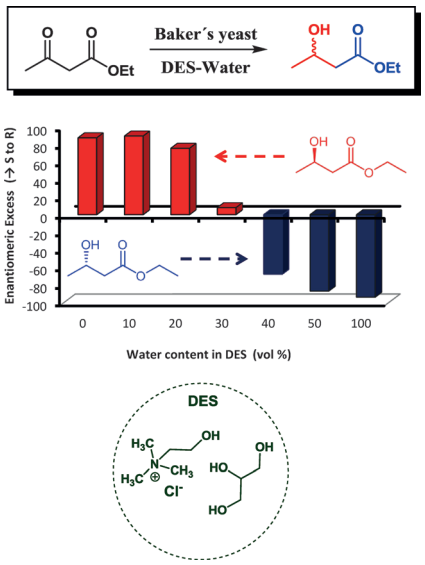

DESpicably good: Whole-cell biocatalysis with the use of baker's yeast is demonstrated in different mixtures of water with deep eutectic solvents (DESs). Enantioselective ketone reduction is observed for long reaction times (>200 h), which suggests that the whole cells remain stable in these neoteric solvents. By changing the proportion of the DES added, a complete inversion of enantioselectivity is observed.