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Asymmetric reduction of ketones with recombinant *E. coli* whole cells in neat substrates

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The asymmetric reduction of ketones is performed by using lyophilized whole cells in neat substrates with defined water activity (a_w). Ketones and alcohols prone to be unstable in aqueous media can now be converted *via* biocatalysis.

Biocatalysis is receiving increasing attention as a powerful synthetic tool providing environmentally-friendly reaction conditions with high regio- and stereoselectivities.[1] However, a broader extension of industrial biocatalysis is still hampered due to several reasons, *e.g.* restricted enzyme availabilities, insufficient productivities and stabilities, time- and material-intensive work-ups in aqueous media—often leading to waste production—as well as costs related to catalysts and cofactors.[2] Low product concentrations resulting from a limited water solubility of many organic compounds can be, to some extent, enhanced by reaction engineering using organic co-solvents or water–organic biphasic systems.[3,4] On the other hand, process stability of biocatalysts can be increased and costs for catalysts and cofactors can be reduced by the use of whole cells rather than isolated enzymes.[5,6]

For the industrial performance of the asymmetric reduction of prochiral ketones by alcohol dehydrogenases the demand for expensive nicotinamide cofactors plays a particular role in cell-free systems.[7] Hence, the use of whole cells exhibits as a major advantage that cofactors can be intrinsically recycled *via* the coupling of a second substrate or by a second enzyme.[8] Few reduction reactions have been reported which work in water–organic mixtures showing exceedingly high substrate loadings and no or only little demand for external cofactors.[7,9,10] However, in such water-based systems emulsions may be formed, decreasing isolated yields and requiring tedious product separation. Moreover, the general applicability of the latter system is restricted when substrates and products with low stability in aqueous environments are involved.

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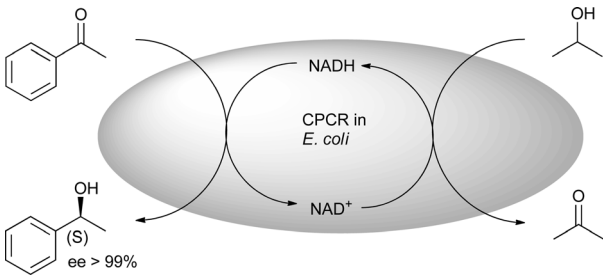

Fig. 1 Biocatalytic reduction of acetophenone using lyophilized *E. coli* cells with overexpressed carbonyl reductase in neat substrates with isopropanol-coupled cofactor regeneration.

Herein we show that *E. coli* whole cells, overexpressing a NADH-dependent carbonyl reductase, are able to perform enantioselective carbonyl reduction in quantitative yields in neat substrates without external cofactor addition (Fig. 1).

The reaction setup only requires a substrate, a co-substrate, and the biocatalyst at a defined water activity (see below). Despite its potentiality, there are only few examples using dried yeast cells in organic solvents,[11–14] as well as lyophilized *E. coli* overexpressing an alcohol dehydrogenase from *Rhodococcus ruber* in 99% (v/v) isopropanol.[15] In that latter example, however, cells displayed little catalytic activity, presumably due to cell rehydration before using them in the micro-aqueous system.[15]

For a model reaction, acetophenone as a substrate and isopropanol as an ancillary co-substrate for cofactor regeneration were used (Fig. 1). The catalyst was a NADH-dependent carbonyl reductase from *Candida parapsilosis* (CPCR)[16–18] overexpressed and directly applied in *E. coli* whole cells. In this highly non-natural environment the biocatalyst was able to effectively produce (*S*)-phenylethanol (ee > 99%; >98% conversion) with high productivities (300–500 g L^{-1}) (Fig. 2).[19] Compared to existing production processes, the developed reaction system has advantage in simplicity, economics (cheap starting material, no NADH addition, very limited waste production, high added value), scalability and high yields.[7,9,10,15] Moreover, a key feature is the straightforward work-up as the whole cells can easily be separated and product downstream requires only evaporating the co-substrate and the co-product.

A further study of the whole-cell-catalyzed reduction of acetophenone demonstrated a dependence of the initial

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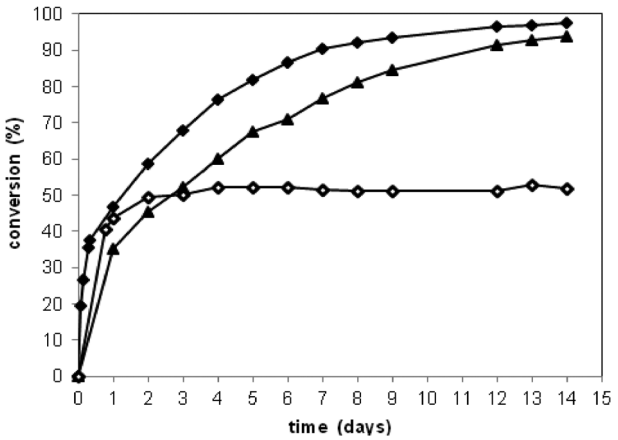

Fig. 2 Conversion of acetophenone by *E. coli* whole cells with overexpressed CPCR in neat substrates. Different volumetric ratios of isopropanol to acetophenone were set (50 : 50 ▲, 70 : 30 ◆◇). Closed symbols: daily removed acetone; open symbol: no acetone removal.

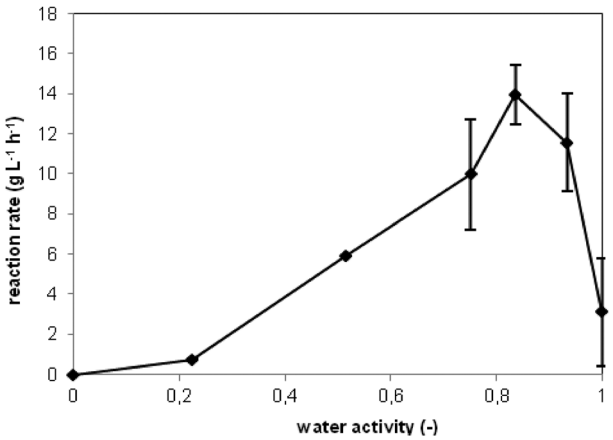

Fig. 3 Initial reaction rate of acetophenone in neat substrates employing *E. coli* whole cells with overexpressed CPCR. Dried cells and reaction mixtures were previously equilibrated to different water activities.[24,25]

reaction rate on the volumetric substrate : co-substrate ratio, indicating the underlying equilibrium conditions (Fig. 2).

Nevertheless, the conversion of acetophenone was always driven to completion, when acetone was intermittently removed from the reaction, and the enantioselectivity was always at maximum (ee > 99%).[20,21] Obviously, with a highly optimized process development these full conversions could be achieved in much shorter reaction times. Remarkably, after 14 days of operation in the system cells still displayed activity rendering this biocatalyst extremely stable.[22] This is consistent with the reported stable operation of *E. coli* cells harboring an alcohol dehydrogenase at high concentrations of isopropanol in aqueous media,[23] and can mainly be attributed to the enzyme preserving microenvironment of the host cell.[5] Inhibition or inactivation of the catalyst at elevated co-substrate concentrations could not be observed. Consequently, with this system it was possible to produce up to 500 g L^{-1} optically pure (*S*)-phenylethanol. Other substrates like 4-chloroacetophenone or aliphatic 2-butanone were converted in analogous systems as well.

The role of the water on the activity of the biocatalyst was analyzed in depth, since it is widely accepted that water plays a crucial role in biocatalysis in non-conventional media,[26] in lyophilized whole cells in gas–solid reactor systems,[27,28] and in whole cell biocatalysis in transesterification and *trans*-glucosylation reactions.[29,30] Lyophilized cells were equilibrated to different a_w values ranging from dry (a_w = 0) to wet (a_w = 1) and used in neat substrates (Fig. 3).[24,31] For low a_w values biocatalytic activity is increasing with water activity, which is in agreement with former findings.[27,28] The optimum water activity is around an a_w value of 0.84 (Fig. 3). Additionally, non-equilibrated wet cells were active but rapidly deactivated within few hours, whereas lyophilized cells remained stable for several days (data not shown).

As indicated previously, application of the herein described concept may be particularly beneficial for the asymmetric reduction of compounds exhibiting low stabilities in aqueous environments. For instance, propargylic ketone 3-butyn-2-one is unstable in aqueous media, shows thermal decomposition and, moreover, has been reported to deactivate enzymes and cells.[32–34] Notably, the corresponding (*S*)-3-butyn-2-ol is an important building block for anti-asthma drugs like 5-lipooxygenase inhibitors,[34] the potent β_2-adrenergic stimulant broxaterol[35] or the protease inhibiting hydroxyethylene dipeptide isosteres.[36] In virtue of this importance, the enzymatic production of (*S*)-3-butyn-2-ol has already been attempted *via* the asymmetric reduction of 3-butyn-2-one with alcohol dehydrogenases or *via* the enantioselective hydrolysis of the corresponding esters. To overcome substrate and product instability in aqueous media, either biphasic systems were applied, or the derivatized trimethylsilane-based compound was used as a substrate, albeit at the cost of adding further synthetic steps.[33–35,37]

So far, alcohol dehydrogenases displayed low-to-moderate enantioselectivities in monophasic systems (5–86%[32–35]), whereas the hydrolase-catalyzed ester hydrolysis led to high ee's, but with limited conversions at 50%.[38,39] To date, there is only one multi gram-scale preparation of (*S*)-3-butyn-2-ol using an alcohol dehydrogenase from *Pseudomonas aeruginosa* in a biphasic system with ee > 99%, though at low conversion (45.5%).[32]

In the herein developed solvent-free whole-cell system high enantioselectivities were achieved (ee = 99.9%; Fig. 4).[19] Data are at odds with the value reported by Schubert *et al.* for CPCR for 3-butyn-2-one (ee = 49%).[34] However, in the mentioned work, CPCR was used not in the recombinant form but as a crude extract from the *Candida* host. Hence, discrepancies might be explained by the acceptance of 3-butyn-2-one by yeast oxidoreductases other than CPCR which in turn may lower the ee, as it has been reported for other yeast systems like baker's yeast.[40]

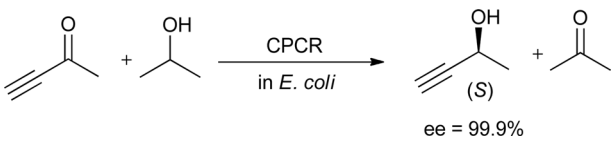

Fig. 4 Biocatalytic reduction of 3-butyn-2-one using lyophilized *E. coli* cells with overexpressed CPCR in the developed solvent-free system with isopropanol-coupled cofactor regeneration.

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According to the above-described findings that increasing the co-substrate concentration favors the product formation, a volumetric ratio 90 : 10 (isopropanol to 3-butyn-2-one) at optimum a_w was set up in this case. As proof-of-concept 67.6% conversion was achieved in 24 h, corresponding to 57.4 g L^{-1} of optically pure (*S*)-3-butyn-2-ol. This product concentration is already 2-fold higher than the so far reported maximum value (24.7 g L^{-1}).[32] Without acetone removal (hence, under non-optimized conditions), full conversion could be obtained within 120 h, yielding a product concentration of 87 g L^{-1}. This example notably points out that enzymatic reductions in neat substrates, while lacking bulk water, is very powerful in accessing optically pure alcohols, even if substrates and/or products are unstable in aqueous solutions. It can be expected that this will open new biocatalytic routes to the production of so far non- or hardly biocatalytically accessible building blocks.

In summary, lyophilized *E. coli* whole-cells with overexpressed carbonyl reductases perform enantioselective ketone reductions in neat substrates, producing enantiomerically pure alcohols in large amounts. The strategy is characterized by high cost-effectiveness (high added-value, no cofactor addition, simple work-up) and by an environmentally-friendly operation mode (largely diminished waste production, bio-based catalyst). A further notable point is the use of compounds in the reaction system that are unstable in aqueous environments. Together with inherent advantages of biocatalysis (high selectivity, high activity), the approach may represent a competitive alternative to classical chemical production of optically pure alcohols. Extension of the technology to other enzymes and substrates is currently under development and will be reported in due course.

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