Enzyme Catalysis

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Enantioselective Reduction of Ketones with "Designer Cells" at High Substrate Concentrations: Highly Efficient Access to Functionalized Optically Active Alcohols**

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The cofactor-dependent asymmetric reduction of ketones catalyzed by alcohol dehydrogenases represents a valuable method for the synthesis of optically active alcohols.[1] For the

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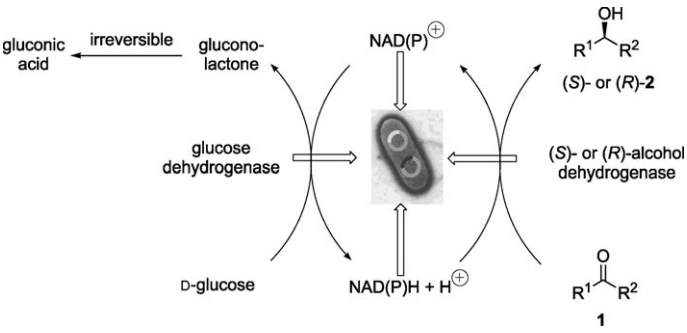
in situ regeneration of the cofactor, which is used in catalytic amounts owing to its high price, substrate-coupled[2] as well as enzyme-coupled[3] techniques are available. For the competitive application of this enzymatic technology on an industrial scale, in particular when one considers the high efficiency of the established metal-catalyzed hydrogenation as the benchmark,[4] it is essential to carry out biocatalytic reduction processes at high substrate concentrations and (preferably) in the absence of "external" cofactor. However, to date most of the biocatalytic syntheses based on the enzyme-coupled approach do not fulfill these requirements; they proceed with substrate concentrations of $<50\,g\,L^{-1}$, often even $<10\,g\,L^{-1}$. Accordingly, only a few examples have been reported that show a potential for technical applications or that already have been carried out on an industrial scale.[3,5]

One of these exceptions is the whole-cell-catalyzed reduction of ethyl 4-chloro-3-oxobutyrate developed by Shimizu et al., which proceeds at impressive substrate concentrations of 250 $g\,L^{-1}$ in a biphasic solvent system consisting of *n*-butyl acetate and an aqueous buffer solution (89% conversion, >99% *ee*).[3a,b] This reaction proceeds in the presence of a very low, catalytic amount of the expensive cofactor.[3a,b] In addition, Weuster-Botz et al. recently reported a new method based on the use of ionic liquids (which are, however, currently still expensive) with maximum substrate concentrations of $\approx 20\,g\,L^{-1}$ based on the total volume of solvents.[3c] The Matsuyama group described reductions with substrate concentrations of 30–50 $g\,L^{-1}$ in the presence of a recombinant whole-cell catalyst containing an alcohol dehydrogenase (ADH) and a formate dehydrogenase.[3d] One disadvantage of formate dehydrogenase used for the cofactor regeneration is its low specific activity of at most $\approx 10\,U\,mg^{-1}$ of purified enzyme, which leads to an unfavorable substrate/catalyst ratio.

In the following we report a simple, highly efficient, and broadly applicable process for the synthesis of optically alcohols complementing the previous processes. This process is characterized by the use of "designer cells" (with high overexpression of the required enzymes), which operate in pure aqueous media at high substrate concentrations of $>100\,g\,L^{-1}$ without an added "external" cofactor to furnish a broad range of (functionalized) *R* and *S* alcohols with conversions of typically >90% and enantioselectivities of >99% *ee* in most cases.

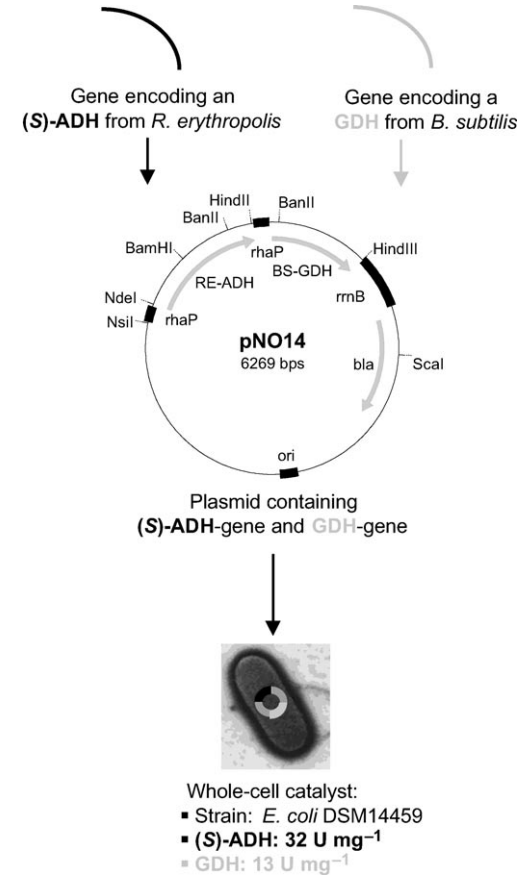
As we were interested in developing an efficient "tailor-made" whole-cell catalyst for the desired asymmetric reduction according to the process shown in Scheme 1, alcohol dehydrogenases (ADHs) with a high specific activity were chosen. An ADH from *Lactobacillus kefir* as an *R*-specific alcohol dehydrogenase[6a,b] and an ADH from *Rhodococcus erythropolis* as an *S*-specific alcohol dehydrogenase[6c,d] turned out to be particularly useful owing to their high specific activities, which exceed 1000 $U\,mg^{-1}$ for a range of substrates. These enzymes are also already available in recombinant form, which is required for technical applications.[6] Since an efficient whole-cell catalyst also relies on a cofactor-regenerating enzyme with high activity, glucose dehydrogenases with an activity of several hundred U per milligram of purified protein were chosen. The coexpression of the corresponding



Scheme 1. General reaction scheme of the target process.

alcohol dehydrogenase with a glucose dehydrogenase proceeded very successfully when the *E. coli* strain DSM14459 was used as a host organism.

We demonstrated this concept in the design of the *S*-enantioselective whole-cell catalyst (Scheme 2). A one-plasmid strategy was applied: genes that encode the alcohol dehydrogenase from *Rhodococcus erythropolis* and glucose dehydrogenase from *Bacillus subtilis*[6e] were ligated into one plasmid (pNO14c). The *E. coli* strain DSM14459 was used as a host organism. Both required enzymes were overexpressed and obtained with good activities of 32 $U\,mg^{-1}$ of crude



Scheme 2. Concept of the "designer bugs"-whole-cell catalysts (recombinant microorganisms overexpressing the genes of the desired enzymes).

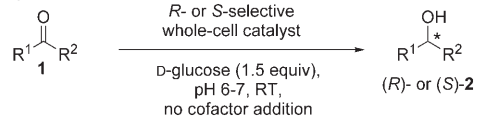
protein for the recombinant *S*-alcohol dehydrogenase from *R. erythropolis* [(*S*)-RE-ADH] and 13 U mg^{-1} of crude protein for the recombinant glucose dehydrogenase from *B. subtilis* (BS-GDH). The analoguous *R*-selective whole-cell catalyst was constructed in the same manner by using *E. coli* DSM14459 as host organism, but in this case two plasmids were used (pNO5c/pNO8c), which contain genes encoding the above-mentioned ADH from *L. kefir* [(*R*)-LK-ADH] and a glucose dehydrogenase from *Thermoplasma acidophilum* (TA-GDH),[6f] respectively.

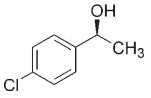
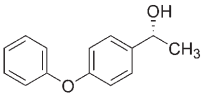
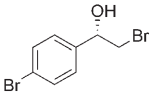
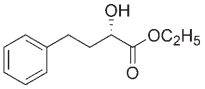
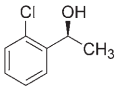
With respect to possible applications of these designer cells, we carried out the reaction in pure water or aqueous buffer solution. The solubility limit was exceeded and a second phase or an emulsion formed. This concept proved to be highly suitable for the envisioned asymmetric reductions, which also proceeded without addition of "external" cofactor. High conversion was obtained in the initial studies, which were carried out with the model substrate 4-chloroacetophenone (**1a**) at a concentration of 500 mM (78 g L^{-1}; Scheme 3). Interestingly, conversions of > 95 % were obtained with both the *R*- and *S*-selective whole-cell catalysts. A potential negative effect resulting from high substrate concentrations and subsequent destabilization caused by interactions at the extended phase interface does not occur or is negligible. In addition, the presence of an organic phase is expected to contribute significantly to cell-membrane permeability, which is essential for mass transfer.

Next, preparative conversions at high substrate concentrations were carried out with different types of substrates. Typically the substrate concentrations were 1.0 M or greater—significantly more than 100 g of substrate per L of reaction volume. The process operation was very economical and straightforward: Besides the substrate and glucose, only the whole-cell catalyst (as wet biomass) was needed to start the reaction; added cofactor was not required (or only in very small amounts in exceptional cases). The reaction with the model substrate **1a** proceeded at a substrate concentration of 1.0 M (156 g L^{-1}) in the presence of the *S*-selective whole-cell catalyst with a high conversion of 94 % and an excellent enantioselectivity of > 99.8 % *ee* (Table 1, entry 1). Addition of cofactor was not required.

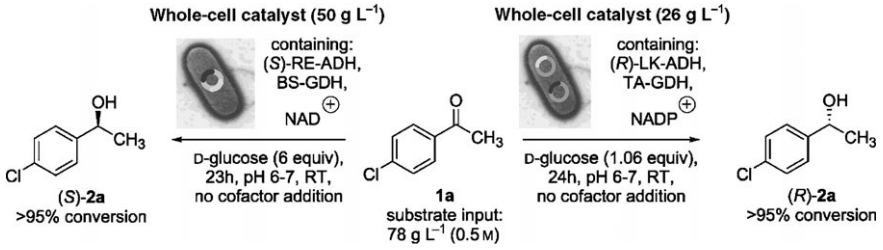
A range of further ketones were reduced to the corresponding *S*- and *R*-enantiomeric forms of alcohols **2**, and again added cofactor was not necessary. The reaction also proceeded with acetophenone-type substrates bearing sterically demanding substituents. The *R*-selective whole-cell catalyst was used to convert 4-phenoxyacetophenone (1.0 M; 212 g L^{-1}) into the desired (*R*)-**2b** with a conversion of > 95 % and an enantioselectivity of > 99.4 % *ee* (Table 1, entry 2). After simple extractive workup the product (*R*)-**2b** was obtained with high purity in 88 % yield.

Table 1: Preparative examples.



Entry	Product	Abs. config.[a]	Biocatalyst[b] (amount in g L^{-1})	Substrate conc. [g L^{-1}]	*t* [h]	Conv. [%]	*ee* [%]
1[c]		(*S*)-**2a**	(*S*)-ADH/GDH (52)	156	31	94	> 99.8
2		(*R*)-**2b**	(*R*)-ADH/GDH (52)	212	25	> 95	> 99.4
3		(*S*)-**2c**	(*R*)-ADH/GDH (52)	140	26	94	97
4		(*S*)-**2d**	(*S*)-ADH/GDH (51)	210	27	93	96
5		(*S*)-**2e**	(*S*)-ADH/GDH (48)	156	76	95	> 99.4

[a] Absolute configuration. [b] Used as wet biomass. [c] Glucose (3 equiv) was added.



Scheme 3. Initial conversions at substrate concentrations of 500 mM.

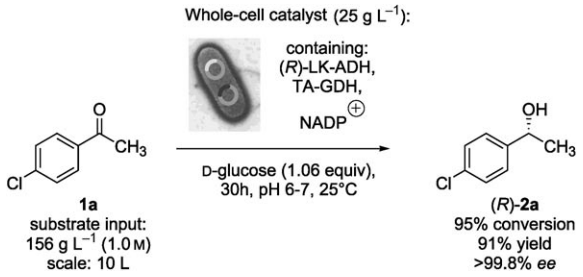
We also examined substrates bearing further functional groups, which thus allow subsequent transformations. α-Halogenated acetophenones are particularly interesting, as the corresponding alcohols of type **2c** may be converted under standard conditions into optically active epoxides, which are also of commercial interest. To test this, we examined the bioreduction of 2-bromo-1-(4-bromophenyl)ethanone. As the biocatalyst, the *R*-enantioselective strain *E. coli* DSM14459, containing the ADH from *L. kefir*, was chosen. The reaction proceeded highly efficiently at a substrate concentration of 0.5 M (140 g L^{-1}) providing the desired alcohol (*S*)-**2c** with a conversion of 94 % and an enantioselectivity of 97 % *ee* (Table 1, entry 3).

The enantioselective reduction of sterically demanding α-keto esters was also possible. Ethyl 4-phenyl-2-oxobutyrate

(1.02 M; 210 g L^{-1}) was transformed into (*S*)-**2d** with a conversion of 93% and an enantioselectivity of 96% ee (Table 1, entry 4). The biocatalyst in this case was the strain *E. coli* DSM14459 containing the *S*-enantioselective ADH from *R. erythropolis*.

Notably, the biocatalysts were also suitable for the reduction of *ortho*-substituted acetophenones. This type of substrate is often problematic in chemocatalytic reductions. The biocatalytic reduction with the *R*-selective whole-cell catalyst provided (*R*)-**2e** with a significantly decreased enantioselectivity of 90% *ee*; this reaction was also carried at a high substrate concentration of 156 g L^{-1}, and a conversion of >95% was obtained after a prolonged reaction time of 53 h. In contrast the analogous *S*-selective whole-cell catalyst led to the formation of (*S*)-**2e** with >99% *ee* and a conversion of 95%; however, an extended reaction time of 76 h was required (Table 1, entry 5). In the reduction reactions studied, which are also suitable for the conversion of purely aliphatic ketones, there were no limitations arising from the low solubility of the ketones in the aqueous reaction media.

The high reaction rates, conversions, and enantioselectivities along with the simple operation at high substrate concentrations allowed a fast scaleup of the process. As an example we tested the reduction of **1a** on a 10-L scale (Scheme 4). When the reaction was conducted at a substrate



Scheme 4. Application of the process on a 10-L scale.

concentration of 1.0 M (156 g L^{-1}) in the presence of the *R*-selective whole-cell catalyst, a conversion of 95% was observed after a reaction time of 30 h. Subsequent filtration (after the pH had been lowered and a filter aid added, in analogy to a related protocol[7] in order to avoid emulsion formation in downstream processing), extraction, and evaporation of solvent gave (*R*)-**2a** as a crude product in 91% yield with a purity of 95% and an excellent enantiomeric excess of >99.8% *ee* (Scheme 4). The reaction was carried out at a low biocatalyst concentration of 25 g of wet biomass per liter.

In summary, we have devised a practical biocatalytic reduction concept suitable for upscaling that is based on the use of a tailor-made whole-cell catalyst in a pure aqueous reaction medium and proceeds at >100 g L^{-1}. This methodology was used to furnish the desired (functionalized) optically active *R* and *S* alcohols with high conversions (>90%) and excellent enantioselectivities (>99% *ee* and partly even >99.8% *ee*). This process technology has been also established on a technical scale (after optimization), and the reduction of other substrates is being examined.

Experimental Section

General experimental protocol for the preparation of the whole-cell-catalyst strains: Chemically competent cells of *E. coli* DSM14459[8a] were transformed with the plasmid pNO14c (see Scheme 2) or the plasmids pNO5c and pNO8c (see the Supporting Information) according to the procedure described in reference [8b] (for the gene sequences of the plasmids, see the Supporting Information). The plasmid pNO14c encoded for an alcohol dehydrogenase from *R. erythropolis*[6c,d] and a glucose dehydrogenase from *B. subtilis*.[6e] These dehydrogenases genes were under the control of a rhamnose promotor.[8c] The plasmids pNO5c and pNO8c encoded for an alcohol dehydrogenase from *L. kefir* and a glucose dehydrogenase from *T. acidophilum*, respectively. Active cells were prepared, for example, by incubation of a single colony of *E. coli* DSM14459 (pNO14c) or *E. coli* DSM14459 (pNO5c, pNO8c) in 2 mL of an LB medium with antibiotics supplement (50 μg L^{-1} of ampicillin and also 20 μg mL^{-1} of chloramphenicol in the case of the *R*-selective whole-cell catalyst) with shaking (250 rpm) at 37 °C for 18 h. This culture was diluted 1:100 with fresh LB medium containing rhamnose (2 g L^{-1}) as the inductor, antibiotics supplement (50 μg L^{-1} of ampicillin and also 20 μg mL^{-1} of chloramphenicol in the case of the *R*-selective whole-cell catalyst) and 1 mM of $ZnCl_2$, and incubated with shaking (250 rpm) for 18 h at 30 °C. The cells were harvested by centrifugation (10000 g, 10 min, 4 °C), the supernatant was discarded, and cell pellets, which could be stored at −20 °C, were used in the biotransformation experiments.

General experimental protocol for the biocatalytic reduction of ketones: A Titrino reaction apparatus was filled with 20 mL of an aqueous phosphate buffer solution (0.2 M; adjusted to pH 7.0), the *R*- or *S*-enantioselective whole-cell catalyst of type *E. coli* DSM14459 [containing either (*S*)-RE-ADH or (*R*)-LK-ADH as well as a glucose dehydrogenase; cell concentration ≈50 g of wet biomass per liter (see Table 1)], D-glucose (typically 1.5 equiv based on the amount of ketone), and 20 mmol (0.5 M) or 40 mmol (1.0 M) of the corresponding ketone (see Table 1). Water was added until a volume of 40 mL was reached. The reaction mixture was stirred at room temperature for the set reaction time, and the pH was maintained at ≈6.5 by dosage of aqueous sodium hydroxide (5 M NaOH). After the reaction time given in Table 1 the conversion was determined by HPLC and NMR spectroscopy. The workup was carried out by lowering the pH to <3 with concentrated hydrochloric acid, adding 3.0 g of the filter-aid material Celite Hyflo Supercel to the reaction mixture, and then filtering. The filter cake was washed with methyl *tert*-butyl ether (3 × 50 mL), and the aqueous phase was extracted with the resulting organic fractions. The collected organic phases were dried over magnesium sulfate and concentrated to dryness to deliver the desired optically active alcohol in high purity, even as a crude product, of typically at least 93%. If necessary, the purity of the products can be further improved by means of standard purification methods such as, for example, distillation and chromatography.

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