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ASYMMETRIC BIOCATALYTIC REDUCTION OF CYCLIC IMINES: DESIGN AND APPLICATION OF A TAILOR-MADE WHOLE-CELL CATALYST

Nadine Zumbrägel,[a] Dennis Wetzl,[b] Hans Iding,[b] and Harald Gröger[a,*]

[a] Chair of Organic Chemistry I, Faculty of Chemistry, Bielefeld University, Universitätsstr. 25, 33615 Bielefeld, Germany; E-mail: harald.groeger@uni-bielefeld.de

[b] Process Research & Development, F. Hoffmann-La Roche Ltd., 4070 Basel, Switzerland

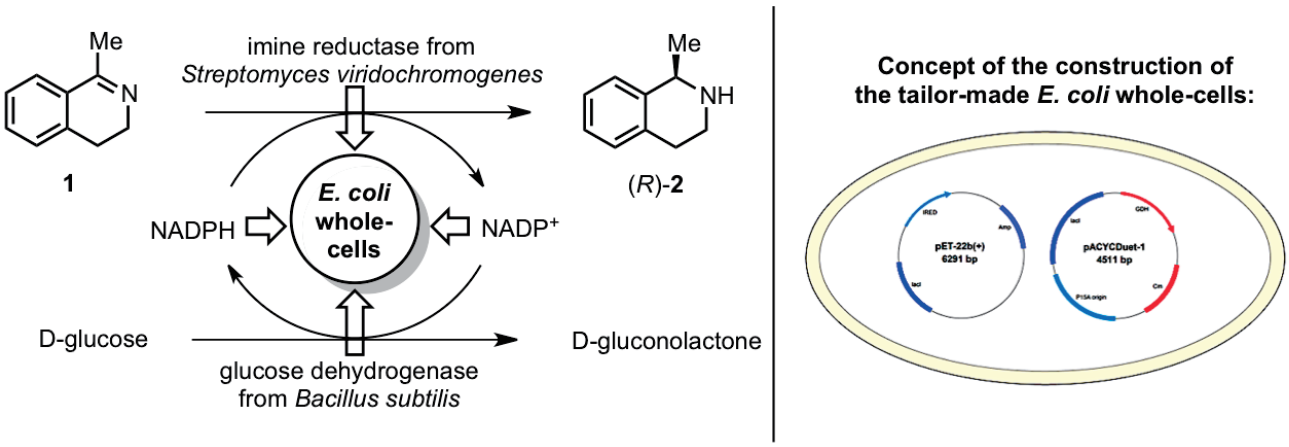
Abstract – The design of a recombinant whole-cell catalyst and its utilization in the asymmetric reduction of 1-methyl-3,4-dihydroisoquinoline chosen as a model substrate for cyclic imines is presented. As "designer cells" *E. coli* cells bearing the two enzymes imine reductase and glucose dehydrogenase (for *in situ*-cofactor recycling) were constructed, which turned out to be suitable for the asymmetric reduction of 1-methyl-3,4-dihydroisoquinoline at low biocatalyst loading of 2 g/L up to 10 g/L of lyophilized cells, leading to both high conversion and enantioselectivity of >99% ee of the resulting amine. The stoichiometric reducing agent is readily available D-glucose, and a proof of concept for running the reactions at elevated substrate concentration of up to 100 mM was demonstrated.

The asymmetric reduction of imines enables an efficient access towards chiral amines required for a range of applications in the pharmaceutical and agrochemical industries.[1,2] The most prominent example of large scale processes in this field is the production of the herbicide metolachlor through metal-catalyzed hydrogenation running on a multi-thousand tons scale.[3] Beside metal-catalyzed processes[2,3] also organocatalysis has been proven to represent an efficient way for imine reduction by means of Hantzsch esters as reagent for the reduction process.[4] In addition, recently enzymes have been demonstrated to be suitable catalysts for such transformations. By means of biocatalysis the reduction of in particular cyclic imines appears to have a high preparative potential due to the high stability of such heterocycles in aqueous medium (in contrast to acyclic imines which easily hydrolyze) and excellent enantioselectivities

Dedicated to Professor Dr. Masakatsu Shibasaki on the occasion of his 70th birthday

of >99% ee, which can be achieved in many of these cases.[5,6] However, so far such biotransformations are mostly carried out in diluted aqueous media and required high biomass loading. For example, reactions running at 5-20 mM are conducted with high (lyophilized) biomass loadings of 100 g/L.[6f] Exceptions are fed-batch processes in the presence of isolated enzymes for the reduction of 1-methyl-3,4-dihydroisoquinoline and 6-methyl-2,3,4,5-tetrahydropyridine, respectively, with an overall substrate input of 411 mM and 72 mM, respectively.[6d,g] Thus, bioprocess development in order to synthesize such amines with high efficiency, space-time-yield and enantioselectivity at low catalyst loading still remains a challenge. As whole-cells represent the most straightforward "formulation" of an enzyme being directly accessible from fermentation and due to the successful utilization of so-called "designer cells" for a variety of biotransformations in the past,[7] we became interested in developing a tailor-made recombinant whole-cell catalyst being suitable for asymmetric reduction of the C=N double bonds in cyclic imines even when being used at a low catalyst loading. In the following we report the design of such a whole-cell catalyst and its utilization in the asymmetric reduction of 1-methyl-3,4-dihydroisoquinoline (**1**) chosen as a model substrate for heterocycles bearing a C=N double bond.

The reaction principle of the biocatalytic reduction process for (cyclic) imines is based on the use of an imine reductase (belonging to the class of dehydrogenases) as a catalyst and NAD(P)H as a reducing agent (Scheme 1). Due to the high price of the cofactor NAD(P)H, which is oxidized in the reduction process to $NAD(P)^+$, its *in situ*-regeneration[8] is required, thus enabling the use of catalytic amount of this cofactor only. For this *in situ*-recycling we chose a glucose dehydrogenase, which can regenerate NAD(P)H through oxidation D-glucose into D-gluconolactone. Subsequent ring opening into D-gluconic acid, followed by neutralization with NaOH under formation of the carboxylate salt makes this reaction step (and thus, the whole reaction) irreversible. The attractiveness of this process results from the fact that

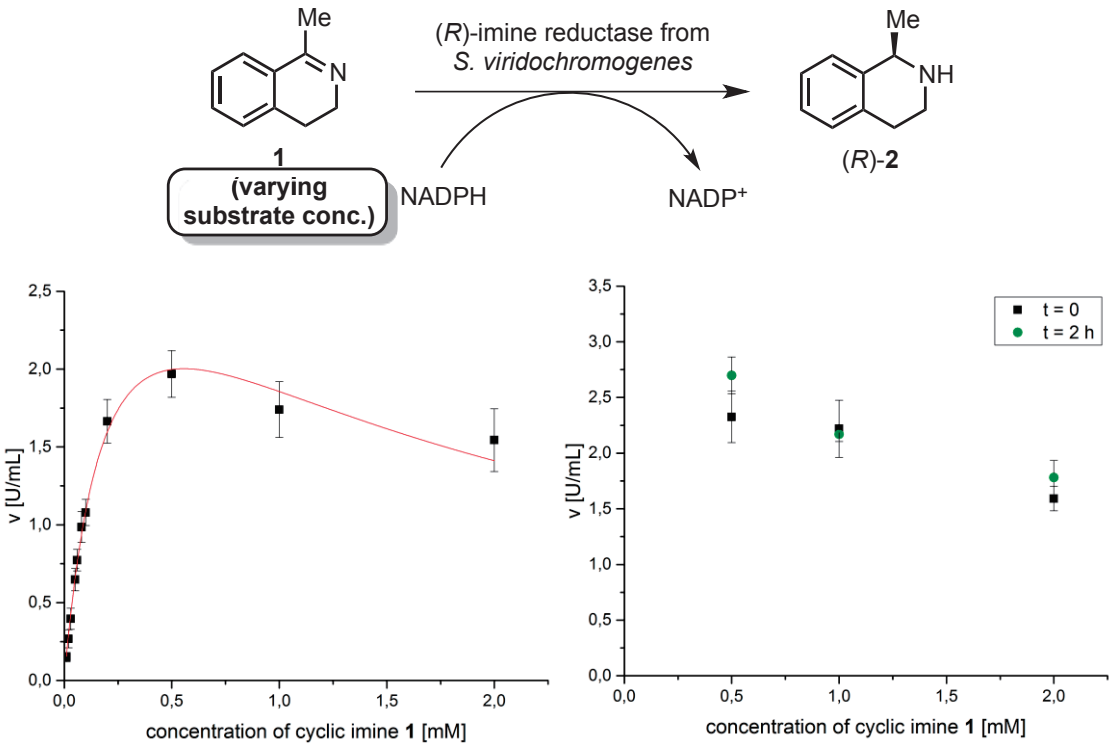


Scheme 1. Concept of asymmetric reduction of cyclic imine **1** and catalyst design

as a stoichiometric reducing agent, only cheap D-glucose is used. As the two enzymes being needed for this process are an imine reductase and a glucose dehydrogenase, we decided to construct an *E. coli* cell as a whole-cell catalyst, which overexpresses these two desired enzymes, thus making them available in the cell at a very high protein content (Scheme 1). Furthermore, such "designer cells" also contain the cofactor, thus avoiding or at least minimizing the addition of "external" amount of cofactor.

As enzymes we chose an (*R*)-enantioselective imine reductase from *Streptomyces viridochromogenes* reported to be suitable for this model substrate **1** (as well as for a range of other imines substrates),[6d] and a glucose dehydrogenase from *Bacillus subtilis*, which we recently used for *in situ*-cofactor regeneration within a C=C and C=O double bond reduction process.[9] Both genes encoding for these enzymes have been inserted on a different plasmid, thus obtaining after transformation an *E. coli* microorganism harbouring two plasmids with each of them encoding for one of the two enzymes. By means of this strategy, the amount of protein for each of these enzymes can be "fine-tuned".

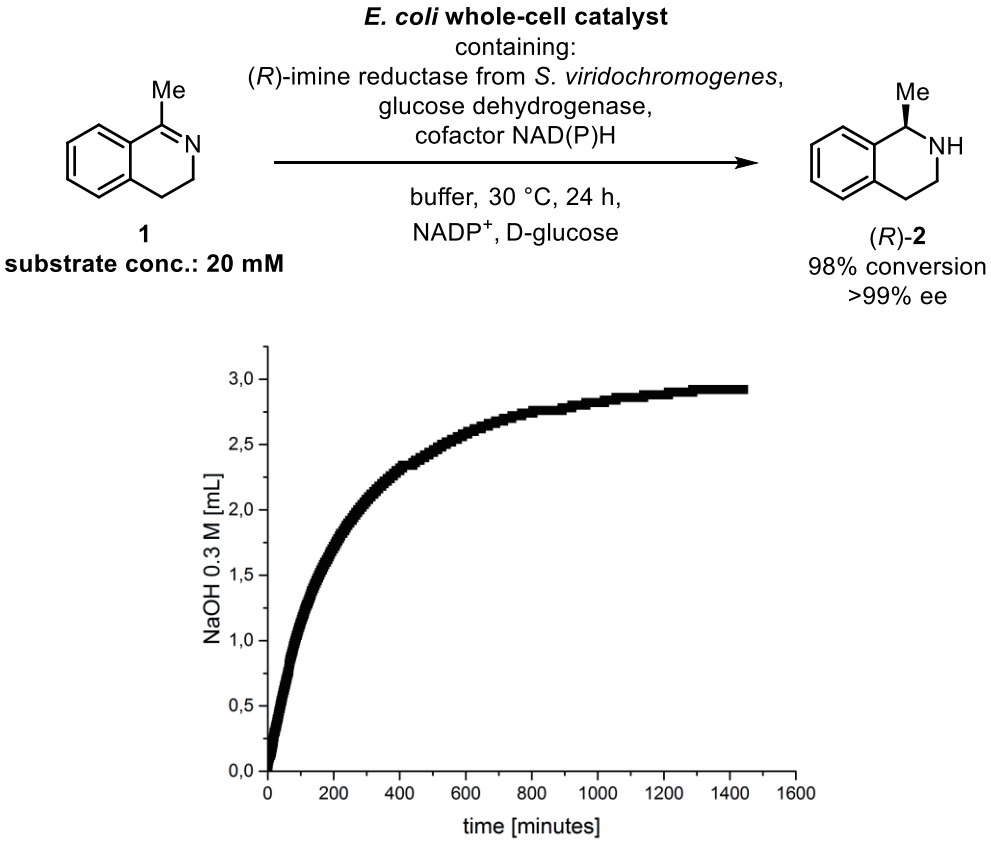
As a next step, we became interested in the characterization of the biocatalyst in terms of process stability and robustness when used in organic synthesis, addressing the question if there might be a negative impact on the enzyme by the substrate through inhibition or deactivation. Towards this end, we conducted spectrophotometric studies with the imine reductase as a purified enzyme, determining the enzyme



Scheme 2. Enzymatic properties of the imine reductase measured in a spectrophotometric assay

activity at different substrate concentrations. The results (Scheme 2, left side) indicate that there is no strong inhibition. In addition, a low K_m-value of only 0.225 mM has been found, which is beneficial for preparative synthesis (since even at low substrate concentration in the aqueous phase the maximum enzyme activity can be achieved). Furthermore, through treatment of the enzyme with the substrate **1** for different periods of time we also could get an insight into stability of the enzyme against the substrate. We found that there is no significant decrease of stability of the enzyme by the contact with the substrate (see Scheme 2, right side). A similar tendency was found when studying the impact of the product on the imine reductase (data not shown). Furthermore, we measured the specific activity of the imine reductase from *Streptomyces viridochromogenes* (being connected with a *his-tag* unit for simple purification) by means of a spectrophotometer activity test, which gave 0.7 U/mg of pure enzyme.

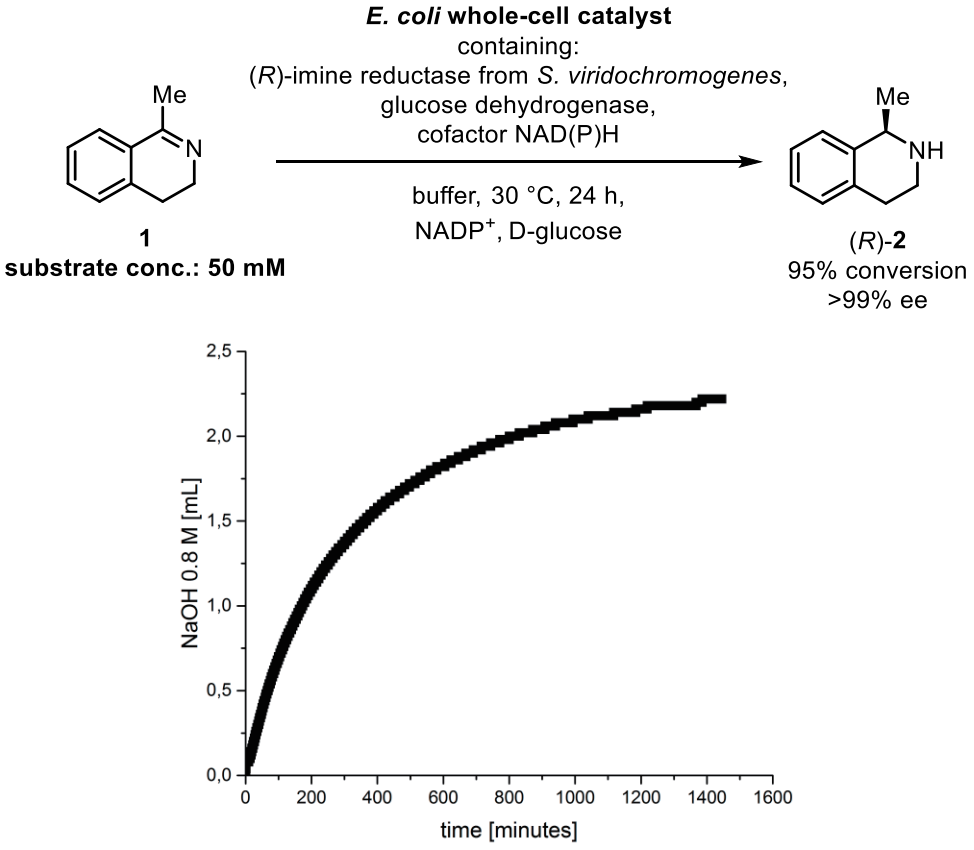
With the constructed whole-cell biocatalyst and the detailed characterization of the imine reductase in terms of activity and substrate as well as product inhibition in hand, next we focused on organic synthetic applications thereof. When utilizing this catalyst for the asymmetric reduction of 1-methyl-3,4-dihydroisoquinoline (**1**) at a substrate loading of 20 mM and a low catalytic loading of 2 g/L



Scheme 3. Whole-cell catalyzed asymmetric reduction of cyclic imine **1** at 20 mM substrate concentration (The NaOH consumption is used for neutralization of the formed gluconic acid, thus indicating the reaction course since per molecule of reduced imine one equivalent of gluconic acid is formed)

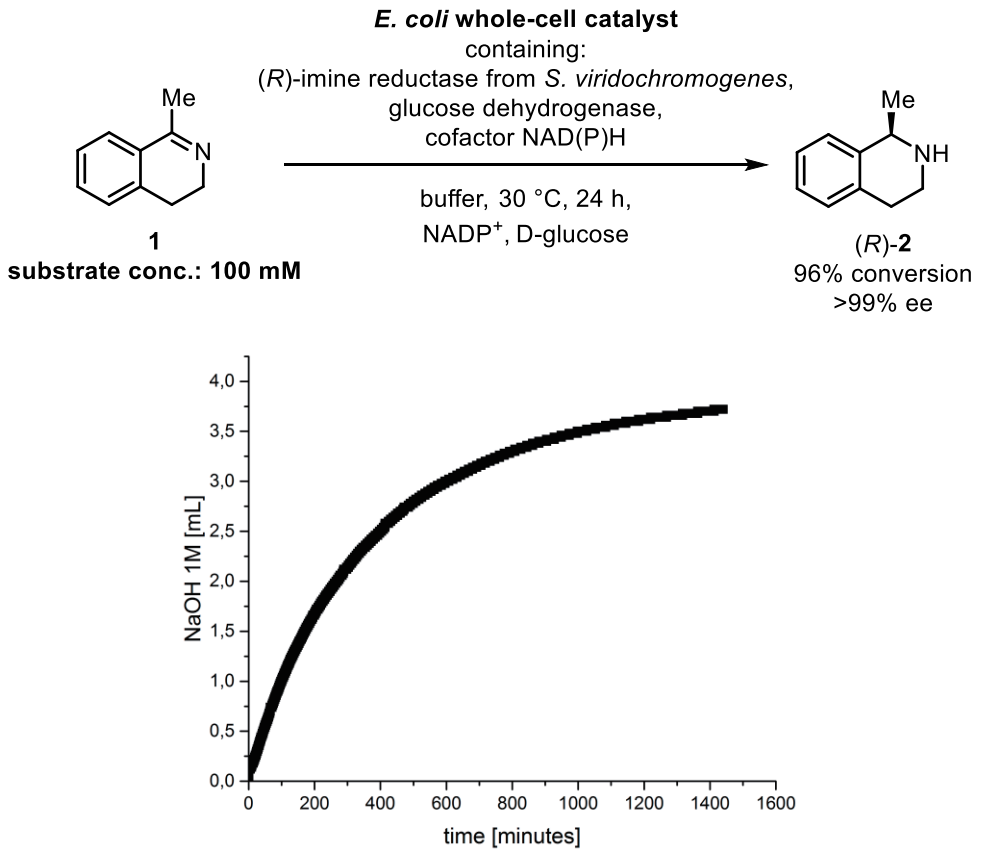
of biomass, we were pleased to find an efficient asymmetric reduction process leading to the desired cyclic amine (*R*)-**2** with both excellent conversion (98%) and enantioselectivity (>99% ee). This imine reduction process, which is shown in Scheme 3, has been conducted in aqueous media at pH 7 (which in earlier studies[10] was found as the pH optimum for the imine reductase from *Streptomyces viridochromogenes*), a maximum of 2% of methanol as cosolvent, a reaction temperature of 30 °C and a reaction time of 24 h on a 40 mL scale with D-glucose as (economically attractive and environmental friendly) reducing agent. In a cosolvent-screening methanol was found to be one of the preferred cosolvents for the designed biocatalyst and therefore was chosen as colsolvent for our synthetic studies.[10]

As a next step, we increased the substrate concentration to 50 mM and the biocatalyst loading to 5 g/L, in order to keep the ratio of catalyst loading per mmol of substrate constant (Scheme 4). It is noteworthy that even under such an elevated substrate concentration of 50 mM the corresponding amine (*R*)-**2** is formed with high conversion (95%) and enantioselectivity (>99% ee).



Scheme 4. Whole-cell catalyzed asymmetric reduction of cyclic imine **1** at 50 mM substrate concentration (The NaOH consumption is used for neutralization of the formed gluconic acid, thus indicating the reaction course since per molecule of reduced imine one equivalent of gluconic acid is formed)

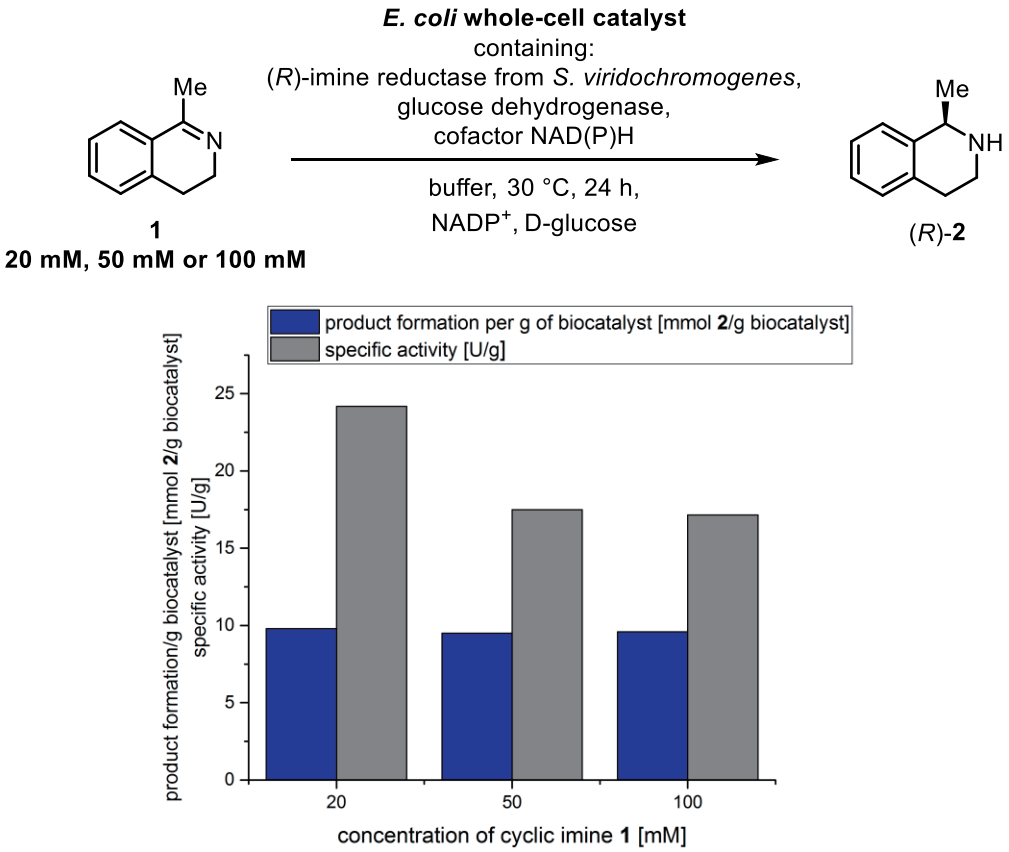
Furthermore, we increased the concentration of cylic imine **1** in a further biotransformation experiment up to 100 mM and kept the ratio of catalyst loading per mmol of substrate constant (Scheme 5). In detail, 10 g/L of lyophilized whole-cells were used at this substrate concentration of 100 mM of substrate **1**. We were pleased to find that also at this substrate concentration of 100 mM the desired biocatalytic reduction proceeds efficiently, resulting in the formation of the desired cyclic amine (*R*)-**2** with a conversion of 96% and an enantioselectivity of >99% ee. To the best of our knowledge, this represents one of the highest substrate concentrations for a cyclic imine reduction with an imine reductase as a biocatalyst. Again, D-glucose served as reducing agent and other reaction parameters such as reaction temperature, reaction time and buffer concentration remained the same.



Scheme 5. Whole-cell catalyzed asymmetric reduction of cyclic imine **1** at 100 mM substrate concentration (The NaOH consumption is used for neutralization of the formed gluconic acid, thus indicating the reaction course since per molecule of reduced imine one equivalent of gluconic acid is formed)

The whole-cell catalyzed asymmetric reduction of cyclic imine **1** running under comparable reaction conditions at the three substrate concentrations of 20, 50 mM and 100 mM, respectively, also enabled us to evaluate the catalytic efficiency of the biocatalyst in dependency on substrate concentration (Scheme 6). A valuable catalyst criterion for comparison is its initial catalytic activity (which can be calculated from

the initial reaction reaction rate in all biotransformations running at 20, 50 mM and 100 mM substrate concentration). Whereas at 20 mM of **1** a catalytic activity of 24.2 U/g was determined for the imine reductase from *Streptomyces viridochromogenes*, at an increased 50 mM substrate concentration a specific activity of 17.5 U/g of imine reductase biocatalyst was detected. At 100 mM a catalytic activity of 17.2 U/g was determined. Thus, in spite of being somewhat lower in the case of 50 and 100 mM all specific activities are in a similar range, indicating a tolerance of the biocatalyst towards substrate **1** also at higher substrate concentrations. A further interesting insight in the catalytic performance provides the comparison of the productivity, defined as mmol of formed product (*R*)-**2** per gram of recombinant whole-cell biocatalyst, at 20, 50 mM and 100 mM substrate concentration, respectively, after a reaction time of 24 h (Scheme 6). In case of 20 mM substrate concentration an amount of 9.8 mmol of product (*R*)-**2** was formed per gram of biocatalyst. It is noteworthy that when conducting the biotransformation at elevated 50 mM of **1**, a comparable productivity was found, which was nearly unchanged with 9.5 mmol of (*R*)-**2** per gram of biocatalyst. Even at further increased 100 mM substrate concentration, the productivity was still in the same range with 9.6 mmol of (*R*)-**2** per gram of biocatalyst. Thus, these results demonstrate the suitability of the whole-cell catalyzed reduction of cyclic imine **1** also for efficient biotransformations at increased substrate concentration of up to 100 mM.



Scheme 6. Comparison of catalytic properties of the whole-cell catalyst at 20, 50 and 100 mM of **1**

In summary, a recombinant whole-cell catalyst was designed and successfully utilized in the asymmetric reduction of 1-methyl-3,4-dihydroisoquinoline (**1**) chosen as a model substrate for cyclic imines. As "designer cells" *E. coli* cells bearing the two enzymes imine reductase and glucose dehydrogenase for *in situ*-cofactor recycling were constructed, which turned out to be suitable for the asymmetric reduction of cyclic imine **1** at low biocatalyst loading of 2 g/L up to 10 g/L, leading to both high conversion and enantioselectivity of >99% ee. The stoichiometric reducing agent is readily available D-glucose, and an initial proof of concept for running the reaction efficiently at an elevated substrate concentration of 100 mM was also shown, which represents a promising starting point for further process development of whole-cell catalyzed reduction of cyclic imine **1** towards processes at further increased substrate loading.

EXPERIMENTAL

Procedure for the Determination of the Enzyme Activities via Spectrophotometric Assay (related to the Experiments Shown in Scheme 2). The determination of the enzyme activities was carried out with a purified imine reductase from *Streptomyces viridochromogenes*[6d] by measuring the consumption of NADPH spectrophotometrically at 340 nm for 60 seconds. For this assay, 1-methyl-3,4-dihydro-isoquinoline (**1**, 0.01 – 2 mM) with a maximum of 4% MeOH as a co-solvent in KP_i buffer (100 mM, pH 7) and a final concentration of 0.25 mM NAD(P)H was used. The activities were measured at least three times and calculated according to the following equations:

$$\text{activity [U mL}^{-1}] = \frac{\Delta E}{\Delta t} \cdot \frac{v_T}{\varepsilon \cdot v_E \cdot d} \cdot f$$

(v_T = total volume; ε = extinction coefficient (here: 6.3 mL μmol^{-1} cm^{-1}); v_E = volume of enzyme solution; d = thickness of cuvette; f = dilution factor)

$$\text{specific activity [U mg}^{-1}] = \frac{\text{activity [U mL}^{-1}]}{\text{protein concentration [mg mL}^{-1}]}$$

Procedure for the Construction and Preparation of the Recombinant Whole-Cell Biocatalyst. An *Escherichia coli* strain BL21(DE3), which was used for expression, and pACYCDuet-1 vector were purchased from Novagen (Madison, USA). The whole-cell catalyst was constructed as a two-plasmid-system, harbouring the gene for the glucose dehydrogenase from *Bacillus subtilis* in a pACYCDuet-1 vector[9] and the gene for the imine reductase from *Streptomyces viridochromogenes* in a pET-22b(+) vector.[6d] A starting culture of *E. coli* BL21(DE3) carrying the two recombinant plasmids was cultivated over night at 37 °C in 10 mL LB medium, containing 80 μg mL^{-1} of carbenicillin and 28 μg mL^{-1} of chloramphenicol. The main culture in 600 mL TB medium, containing 80 μg mL^{-1} of

carbenicillin and 28 μg mL^{-1} of chloramphenicol, was inoculated with the starting culture to a final concentration of 1%. At an OD_{600} between 0.4 und 0.6, the production of recombinant protein was induced by addition of isopropyl-thio-β-D-galactoside (IPTG) to a final concentration of 0.5 mM. Cultures were shaken at 25 °C for 24 h and harvested by centrifugation (approx. 12.6 g of wet cells). For lyophilization of the cells a 50% cell suspension in water was used, and the resulting lyophilized cells (approx. 2.5 g) were stored in a freezer at -20 °C.

Procedure for the Asymmetric Biocatalytic Reduction of 1-Methyl-3,4-dihydroisoquinoline (1). The biotransformations on a preparative scale (40 mL) were performed in a pH stat apparatus flask with 20, 50 and 100 mM concentration of 1-methyl-3,4-dihydroisoquinoline (**1**), 2, 5 and 10 mg mL^{-1} of lyophilized whole-cell catalyst (added from 4, 10 and 20 mg mL^{-1} of cell suspension in KP_i buffer (pH 7, 50 mM)), 120 mM (in case of 20, 50 mM **1**) or 240 mM (in case of 100 mM **1**) of D-glucose, 0.1 mM of $NADP^+$ and 1% (in case of 50 mM **1**) or 2% (in case of 20, 100 mM **1**) of MeOH as cosolvent. The flask was equipped with the titration head and pH electrode of a pH stat apparatus and stirred at 30 °C. By addition of aqueous NaOH solution, the pH was kept stable at 7. The reaction was stopped by adding 2 mL of 32% NaOH solution and 30 mL of CH_2Cl_2. Phase separation was promoted by centrifugation. The organic phase was dried over magnesium sulfate. In all biotransformations conversion and enantiomeric excess was determined by analyzing the organic phase by means of a LC2000 *high performance liquid chromatography* (HPLC) system from Jasco (Easton, USA) with HPLC column Chiralpak IC from Daicel (supercritical CO_2:EtOH (Et_2NH) = 80:20 (0.02), 1 mL min^{-1}, 20 °C, 12 MPa, 213 nm). For isolation of the product, the organic phase was washed with HCl (2M, 3 x 10 mL). The pH of the resulting aqueous phase was adjusted to pH 13 and the aqueous phase was extracted with CH_2Cl_2 (3 x 30 mL). Subsequently, the organic phases were dried over magnesium sulfate and the solvent was evaporated *in vacuo*.

(*R*)-1-Methyl-1,2,3,4-tetrahydroisoquinoline ((*R*)-2):

(*R*)-1-Methyl-1,2,3,4-tetrahydroisoquinoline ((*R*)-**2**) was obtained as a brown oil.

^{1}H-NMR (500 MHz, $CDCl_3$): δ (ppm) = 7.11–7.17 (m, 3 H), 7.07–7.09 (m, 1 H), 4.11 (q, *J* = 6.7 Hz, 1 H), 3.26 (dt, *J* = 5.2, 12.5 Hz, 1 H), 3.02 (ddd, *J* = 4.7, 8.8, 12.5 Hz, 1 H), 2.84–2.91 (m, 1 H), 2.71–2.76 (m, 1 H), 1.82 (s, 1 H), 1.46 (d, *J* = 6.7 Hz, 3 H). NMR data are in accordance with literature data (ref. 6d).

HPLC analysis with chiral stationary phase: r_t = 15.8 min (HPLC column Chiralpak IC (Daicel), supercritical CO_2:EtOH (Et_2NH) = 80:20 (0.02), 1 mL min^{-1}, 20 °C, 12 MPa, 213 nm).

Determination of absolute configuration: α_D^{25} +73 (c 1.1; $CHCl_3$). Optical rotation is in accordance

with literature data (ref. 11). According to the *ired.biocatnet database* (ired.biocatnet.de) the imine reductase from *Streptomyces viridochromogenes* is indicated to be *R*-enantioselective.

Isolated yield: 85.6 mg, 73% (in case of 20 mM **1**), 169.8 mg, 58% (in case of 50 mM **1**), 339.6 mg, 58% (in case of 100 mM **1**).

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