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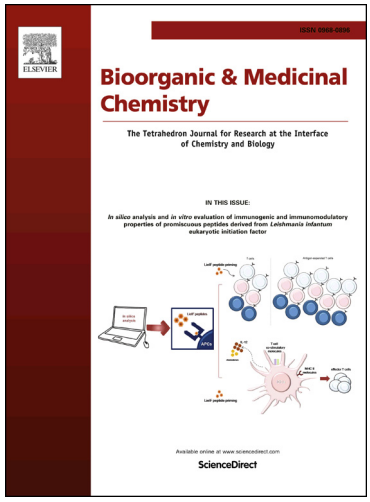

Enantioselective synthesis of amines *via* reductive amination with a dehydrogenase mutant from *Exigobacterium sibiricum*: Substrate scope and biocatalyst immobilization

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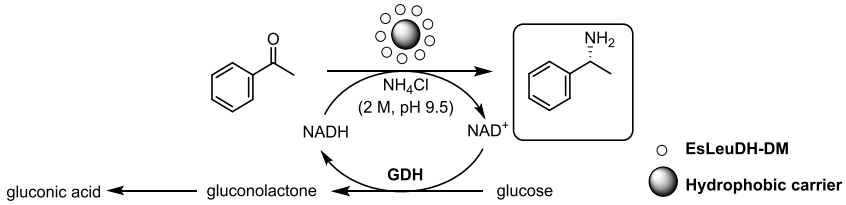

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Enantioselective synthesis of amines *via* reductive amination with a dehydrogenase mutant from *Exigobacterium sibiricum*: Substrate scope and biocatalyst immobilization

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ABSTRACT

In recent years, the reductive amination of ketones in the presence of amine dehydrogenases emerged as an attractive synthetic strategy for the enantioselective preparation of amines starting from ketones, an ammonia source, a reducing reagent and a cofactor, which is recycled *in situ* by means of a second enzyme. Current challenges in this field consists of providing a broad synthetic platform as well as process development including enzyme immobilization. In this contribution these issues are addressed. Utilizing the amine dehydrogenase EsLeuDH-DM as a mutant of the leucine dehydrogenase from *Exigobacterium sibiricum*, a range of aryl-substituted ketones were tested as substrates revealing a broad substrate tolerance. Kinetics as well as inhibition effects were also studied and the suitability of this method for synthetic purpose was demonstrated with acetophenone as a model substrate. Even at an elevated substrate concentration of 50 mM, excellent conversion was achieved. In addition, the impact of water-miscible co-solvents was examined, and good activities were found when using DMSO of up to 30% (v/v). Furthermore, a successful immobilization of the EsLeuDH-DM was demonstrated utilizing a hydrophobic support and a support for co-valent binding, respectively, as a carrier.

1. Introduction

Chiral amines serve as key building blocks for the preparation of pharmaceuticals and numerous methods for their enantioselective synthesis were developed.[1]Among them, for industrial scale applications biocatalysis plays a dominant role. [2-4] So far, lipase-catalyzed resolution of racemic amines is used on multi-thousand tons scale based on an acylation technology developed by BASF.[3] In addition, enzymatic transamination has been developed and proven as an efficient process for the synthesis of Sitagliptin by Merck and Codexis.[4] As a further alternative, Bommarius et al. demonstrated recently that altering an amino acid dehydrogenase through mutations led to an amine dehydrogenase (AmDH), thus providing a further alternative for enzyme catalyzed synthesis of enantiomerically pure amines.[5] Towards this end, mutation of two amino acids in the active site was carried out to create an access to such enzymes which showed activity towards a variety of ketones and enabled the synthesis of amines by means of an ammonium salt as an economical amino donor in combination with NADH as a cofactor. In general, the reduced cofactor can be regenerated *in situ* by means of a second enzyme, e.g., formate dehydrogenase or glucose dehydrogenase, and a suitable reducing agent such as, e.g., formate or glucose, as a co-substrate. This type of process, which only requires stoichiometric amount of the co-substrate whereas cofactor and enzymes are utilized in catalytic amounts, is shown in Figure 1. The use of cofactor regeneration systems is known for a long time for processes with amino acid dehydrogenase and alcohol dehydrogenase, and the option to use cheap and readily available co-substrates such as formate and glucose make such a process technology attractive also from an industrial perspective.

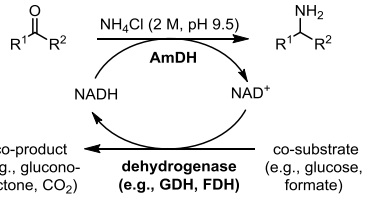


Figure 1: Reductive amination of ketones with *in situ*-cofactor recycling

So far, various sources for the design of amine dehydrogenases through mutagenesis have been used. The Bommarius, Turner and Mutti groups started from a leucine dehydrogenase from *Bacillus stereothermophilus* and a phenylalanine dehydrogenase from *Bacillus badius*. Furthermore Bommarius *et al*. developed a chimeric amine dehydrogenase mutant, consisting of this phenylalanine dehydrogenase and leucine dehydrogenase.[5] Moreover, a mutant of the phenylalanine dehydrogenase from *Rhodococcus sp*. M4 was established by the Li group and also used by the Mutti group[5,7] In 2015 Xu *et al.* described the application of the two-site mutation (K77S/N270L) towards a leucine dehydrogenase from *Exigobacterium sibiricum* (EsLeuDH-DM).[8] They examined a broad range of short-chain secondary aliphatic ketones and alkyl cyclic ketones, as well as acetophenone (**1**).[8] Current challenges in this field consists of providing a broad synthetic platform as well as process development including immobilization of biocatalysts. In continuation with our work on enzymatic enantioselective amine synthesis,[9] and inspired by the contributions on amine dehydrogenase described above,[5-8] we became interested in getting a detailed insight into the scope and limitations of this novel technology in terms of substrate scope, stability when using organic co-solvents and immobilization. For our study we chose the (*R*)-amine dehydrogenase EsLeuDH-DM[8] as a model enzyme.

2. Results and Discussion

2.1 Enzyme preparation, purification and characterization (kinetic data)

The initial step consisted in conducting the preparation of the (*R*)-amine dehydrogenase EsLeuDH-DM.[8] Although following in principle the procedure described in literature,[8] some changes were made. For example, expression was carried out at a lower temperature which enabled an improved overexpression leading to an activity of 170 (±0.050) mU/mg towards acetophenone (**1**) of crude extract compared to 0.10 U/mg reported in literature.[8] For protein purification a Ni^{2+}-NTA column was used in accordance to literature.[8] With higher salt-concentration *via* desalting the protein concentration could be raised to double amount. Despite of precipitation in both buffers, on ice an active enzyme could be isolated. After purification the K_M-value was measured, and for acetophenone (**1**) a K_M-value of 22.6 mM was observed (see Supplementary Material).

2.2 Substrate scope

With this recombinant biocatalyst in hand, the substrate scope was investigated *via* a spectrophotometric activity assay. The focus of our study was in particular on aromatic ketones as in previous work acetophenone was identified as suitable aromatic substrate.[8] For the first time it was examined if other types of aryl-substituted ketones with different substitution pattern are also accepted by the EsLeuDH-DM besides the "model substrate" acetophenone (**1**). The substrate concentration of this spectrophotometric assay was in a range of 5 mM and 20 mM. Taking into account the high K_M-value, the study at this substrate concentrations can give an initial general inside into a substrate tolerance but not necessarily an information about the maximum velocity, v_{max}.

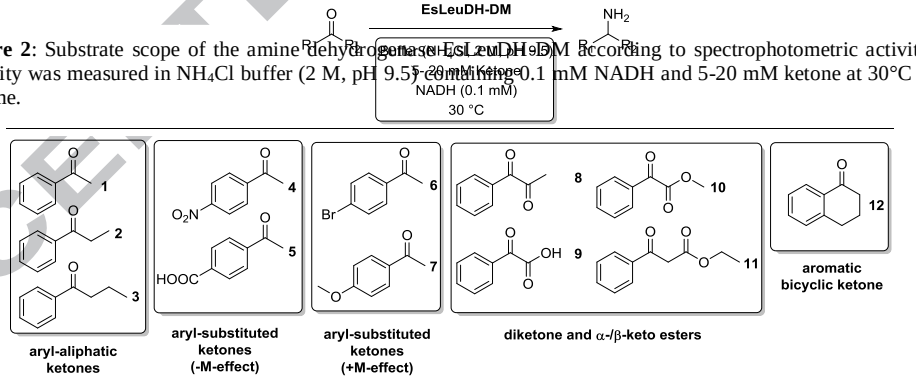
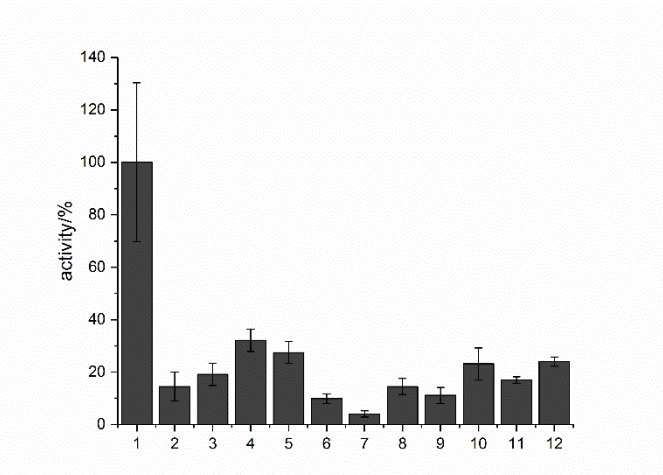


Figure 2: Substrate scope of the amine dehydrogenase EsLeuDH-DM according to spectrophotometric activity testing. Activity was measured in NH_4Cl buffer (2 M, pH 9.5) containing 0.1 mM NADH and 5-20 mM ketone at 30°C in a 1 mL volume.



Selected results of these spectrophotometric activity assay experiments, which gave an interesting insight into the substrate scope of EsLeuDH-DM, are shown in Figure 2. Although the enzymatic activity is highest for acetophenone (**1**), it is noteworthy that homologues with an enlarged alkyl side chain are tolerated as well with reasonable activity, e.g., butyrophenone (**3**). Furthermore, in general EsLeuDH-DM shows a higher activity for aromatic ketones with electron-withdrawing substituents (e.g., **4** and **5**) leading to a negative mesomeric effect (-M-effect). In contrast, lower activities were observed when using aromatic ketones with electron-donating substituents (e.g., **6** and **7**), which lead to a positive mesomeric effect (+M-effect). These results might be explained with the decreased electrophilic character of the carbonyl moiety in the latter case. In addition, the more bulky compounds α- and β-ketoesters **10** and **11**, respectively, as well as the bicyclic ketone **8**, gave reasonable activities. In contrast, a somewhat lower activity was found for the corresponding acid **9**. Besides a range of acylic ketones also the cyclic ketone **12** turned out to represent a suitable substrate.

2.3 Synthetic biotransformations and initial process development

Next we became interested to evaluate the synthetic potential of this method utilizing EsLeuDH-DM as a biocatalyst. For being considered as a practical method, prerequisites are a sufficient space-time-yield, a reasonable substrate loading as well as a smoothly proceeding work-up with an economical solvent consumption. Thus, a minimum substrate concentration of 50 mM was regarded to be desirable for such a synthesis on lab scale, whereas for larger scale applications substrate concentrations exceeding 500 mM would be advantageous. Accordingly, we conducted biotransformations running at elevated substrate concentration. As a substrate acetophenone (**1**) was used and in situ-cofactor regeneration was conducted by means of a glucose dehydrogenase (GDH) and D-glucose as a co-substrate. We confirmed that under the chosen reaction conditions (2 M NH_4Cl, pH 9.5) both cofactor forms NADH and NAD^+ remained stable (for details, see Supplementary Material). As a benchmark experiment a biotransformation at 20 mM substrate concentration was carried out which gave the desired amine with >98% conversion after a reaction time of 100 h. The enantiomeric excess was excellent with >99% ee (as determined for each sample taken for the biotransformation running at 20 mM). When increasing the substrate concentration to 50 mM of acetophenone (**1**) while maintaining the utilized absolute enzyme activity constant, we were pleased to find that the reductive amination also proceeded smoothly, leading to a conversion of 77% with formation of exclusively the desired (*R*)-amine (*R*)-**13** (Figure 3). In addition, increasing the enzyme amount by factor 2 led to a full conversion after 100 h even at 50 mM substrate concentration, thus indicating both a high biocatalyst stability as well as negligible inhibition concerns under these conditions. The reaction also proceeds at a further elevated substrate concentration of 100 mM, leading to a conversion of 43% after 100 h reaction time. This corresponds to a product formation of 43 mM, which is comparable to the biotransformation running at 50 mM substrate concentration. In this case, after 100 h a conversion of 77% was determined corresponding to a product formation of 39 mM. This indicates that increasing the substrate concentration does not led to (significant) substrate or product inhibition as well as to an elevated deactivation of the enzyme (although further studies and recording of kinetic courses would be needed to proof this hypothesis). Furthermore, there is a significant decrease in activity of the enzyme after about 30 h (see Supplementary Material), this could also be an explanation for the slowly conversion after 30 h reaction time.
In general and independent of the substrate concentration the biotransformations proceed with excellent enantioselectivity and gave the desired amine product with an *ee*- value of >99% in all cases.

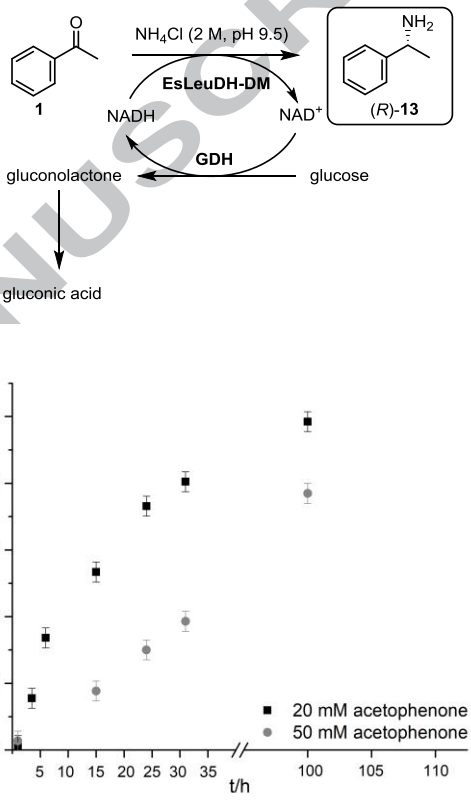


Figure 3: Reductive amination of acetophenone (**1**) with the amine dehydrogenase EsLeuDH-DM. Biotransformations were made with acetophenone **(1)** in NH_4Cl-buffer (2 M, pH 9.5), EsLeuDH-DM (10 U), glucose (100 mM), NAD^+ (1 mM) and GDH (18 U) in a total volume of 1 mL at 30°C; thus, the enzyme to substrate ratio was 500 and 200 U per mmol of **1** in case of 20 and 50 mM substrate concentration of **1**, respectively. The conversion is defined as the ratio of formed product amount related to the amount of used substrate (in %). Since no by-product was observed, this value corresponds to the ratio of substrate consumed in the reaction related to substrate used in the reaction.

2.4 Co-solvent screening

A further option in process development is the utilization of co-solvents. Taking into account the high K_M-value for model substrate **1** in combination with the (relatively) low water-solubility of hydrophobic ketones, we focused on the identification of suitable water-miscible solvents, which could be used as a co-solvent in combination with the buffer system. Such a solvent system would then enable an increase of substrate

concentration under homogeneous conditions as well as the option to operate at the maximum reaction rate, v_{max}. Therefore, the five water soluble co-solvents ethanol, isopropanol, methanol, acetonitrile and DMSO were selected and examined when being utilized in a volumetric amount of up to 30%(v/v). When studying their impact on the stability of the EsLeuDH-DM, in initial studies ethanol, isopropanol, acetonitrile and methanol led to a rapid loss of enzyme activity (data not shown). Thus, a more detailed investigation of the solvent impact on the stability was then carried out for DMSO (Figure 4).

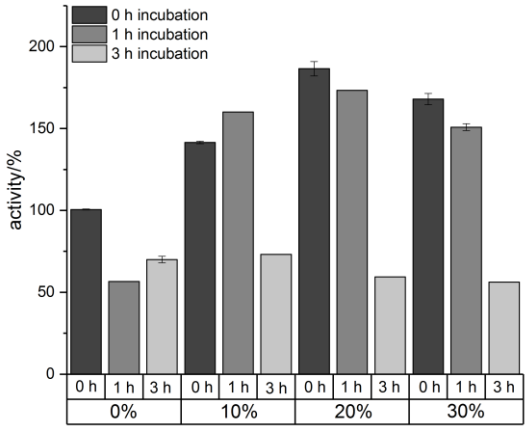


Figure 4: Stability of EsLeuDH-DM towards DSMO. Activity was measured in NH_4Cl buffer (2 M, pH 9.5) with a certain amount of DMSO containing 0.1 mM NADH and 20 mM **1** at 30°C in a 1 mL volume.

These results show that DMSO represents the best co-solvent when being utilized in a volumetric amount of up to 20%(v/v). Furthermore, the relative activity increased and the enzyme stability is comparable to the one when using EsLeuDH-DM without a co-solvent. However, for methanol as a co-solvent no activity was found after three hours independently of its volumetric amount (which was 10%, 20% or 30%). The utilization of 20%(v/v) of DMSO also enables a higher concentration of substrate being dissolved in the aqueous phase.

2.5 Immobilization of the biocatalyst

Besides optimizing the substrate concentration and other reaction parameters, also utilization of an heterogenized (bio-)catalyst can contribute to the attractiveness of a synthetic process. So far the immobilization of amine dehydrogenase have been rarely studied. This year, the Li group reported the immobilization of the phenylalanine dehydrogenase from *Rhodococcus sp*. M4 on magnetic nanoparticels *via* a his-tag, and the Wang group immobilized the amine dehydrogenase from *Bacillus badius* to polyethylene imine-titan nanoparticles.[10]

In our work, now for the first time two commercially available, well established and ready-to-use carriers were chosen to immobilize the EsLeuDH-DM. In detail, we selected a hydrophobic carrier from Lanxess® and a carrier for covalent binding from Sigma Aldrich® for this purpose. Furthermore, we chose this carriers to compare a carrier with and without covalent interactions, to evaluate the behavior of our enzyme on both supports. The carrier for covalent binding carries epoxy groups at the resin-type solid support (for ring-opening reactions with nucleophilic functional groups of the protein such as free amino groups resulting from Lys moieties), whereas the hydrophobic carrier forms non-polar, hydrophobic interactions with the enzyme. For immobilization both carriers were washed with buffer (hydrophobic carrier with 0.05 M KPi buffer, the epoxy carrier with 0.5 M KPi buffer). Afterwards the immobilization was performed for 18 h in the same buffer. We investigated the variation of the mass ratio of protein and carrier and therefore the effects on yield, loading and efficiency (see Supplementary Material).

The immobilization with the covalent carrier gave the heterogenized (*R*)-amine dehydrogenase with an immobilization efficiency of 48% and an amount of protein of 11.8 mg/g of solid support, whereas the hydrophobic carrier was obtained with an immobilization efficiency of 54% and a very high amount of protein of 63.2 mg/g support. Furthermore, no leaching of the enzyme was observed with both carriers. When using the hydrophobic carrier with the immobilized biocatalyst in a synthetic transformation, a conversion of 78% was achieved compared to 77% when utilizing the same amount of enzyme enzyme in free, non-immobilized form (Figure5). In addition, the enantioselectivity was studied for the biotransformation with the heterogenized biocatalyst and revealed for all taken samples (for details about the reaction times for taking the samples, see Figure 5) excellent enantiomeric excess of >99% for the resulting amine. To the best of our knowledge this heterogenized biocatalyst represents the first example of an immobilized EsLeuDH-DM.

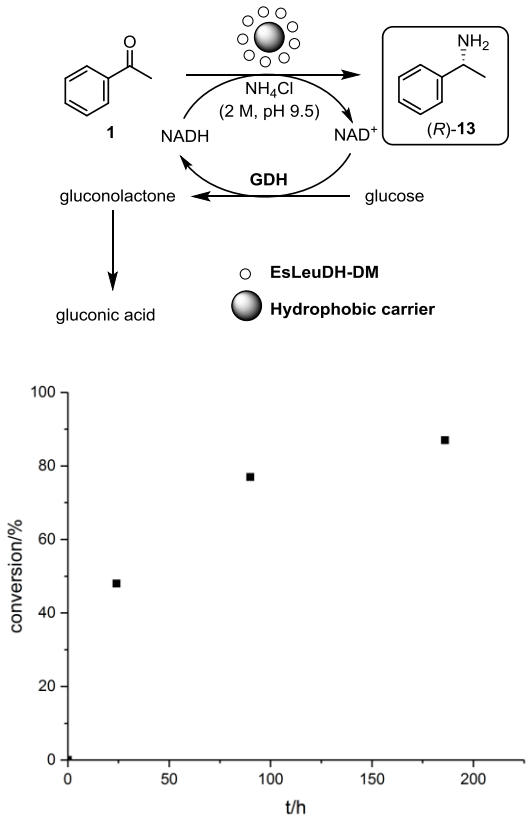


Figure 5: Biotransformation with the immobilized amine dehydrogenase EsLeuDH-DM. Biotransformations were made with acetophenone (**1**, 50 mM) in NH_4CL-buffer (2M, pH 9.5), immobilized EsLeuDH-DM on a hydrophobic carrier (3 g), glucose (50 mM), NAD^+ (1 mM) and GDH (22.5 U) in a total volume of 25 mL at 30°C for 100 h.

3. Summary and outlook

In conclusion, we characterized the recombinant amine dehydrogenase EsLeuDH-DM in terms of its substrate scope and studied its stability in dependency on the presence of water miscible co-solvents, the kinetic data as well as inhibition effects. We further demonstrated the suitability of this enzyme for synthetic purpose. For example, the desired reductive amination of acetophenone (**1**) proceeds with full conversion at an elevated substrate concentration of 50 mM. In addition, for the first time successful immobilization of the EsLeuDH-DM was demonstrated utilizing a hydrophobic support and a support for co-valent binding, respectively, as a carrier. Further process development in particular with increase of substrate loading and usage of co-solvent are currently in progress as well as a study on recyclability of the immobilized biocatalyst.

4. Experimental section

4.1 General

4.1.1 HPLC

For analytical HPLC a system of Jasco® was used. The system consisted of a degasser LC-Net II / ADC device, pumps (PU-2080 plus), a multiwavelength detector MD-2010 plus, an autosampler AS-2059-SF, a thermostat CO-2060 and a backpressure controller BP-2080. The supercritical carbon dioxide was cooled *via* cryostat from JulaboF250. The Chiralpak AD H® with a 5µm silica-gel column with 250 x 4.6 mm ID was used for the separation. A mixture of CO_2 and isopropanol in the ratio 95: 5 with a flow rate of 1.5 ml/min at 20 °C as a mobile phase were used.

4.1.2 GC

The gas chromatographic analyzes were carried out with the GC-2010 Plus from Shimadzu® using the autoinjector AOC-20i on the non-chiral Phenomenex® ZB-SMS column. The following temperature program was used: start at 90 °C, with 20 °C/min to 107 °C and 15 °C/min to 150 °C. For 1-phenylethylamine (**13**) a retention time of 2.5 min, and for acetophenone (**1**) a retention time 2.7 min was observed.

4.2 Construction of EsLeuDH-DM mutant

For the mutagenesis of EsLeuDH, the gene was isolated *via* innuPREP Plasmid Mini Kit® after cultivation of the *Exiguobacterium sibiricum*. The strain was purchased from DSMZ (DSM No. 17290). In the following, EsLeuDH gene was amplified *via* PCR. The following primers were used: 5'-GCGGCGTCA TATGGTTGAAACAAACGTAGAAGC -3', for NdeI restriction site and 3'-GCGCCAACTCGAGTTAACCGCGTGATCCTA AAATG -5' for XhoI restriction site. The mutation of the codon for K77S was performed by QuikChange®-PCR with 5'-CGTTTGGCAAAAGGCATGACGTATAGCAATGCGGCAG CCGG-3'and 5'- CCGGCTGCCGCATTGCTATACGTCATG CCTTTTGCCAAACG-3'. Mutation of the codon for N270L was performed by QuikChange® -PCR with 5'- GAAAATCATTGC CGGAGCAGCACTAAACCAACTCAAAGAAGATCGTC-3' and 3'-GACGATCTTCTTTGAGTTGGTTTAGTGCTGCTCCGGCA ATGATTTTC -5'. After construction of the first mutant, the PCR product was digested *via DpnI* (10 U) for 1.5 h at 37 °C and transformed into *E.coli* DH5α, after cultivation the plasmid was again isolated for the next round of mutation. After introduction of double mutation, EsLeuDH-DM was transformed into *E.coli* BL21(DE3).

4.3 Transformation of competent cells with plasmid-DNA

After digestion 10 µL Plasmid DNA was added to chemical competent cells (50 µL) and incubated for 30 minutes on ice. The cells were heated at 42°C for 90 seconds and incubated again for five minutes on ice. Afterwards 1 mL of LB media was added. The mixture was heated for three hours at 37°C and 800 rpm. Subsequently, the cells were cultured on LB agar plates with suitable antibiotic and incubated overnight at 37°C.

4.4 Protein expression and purification

TB medium with kanamycin (50 µg/mL) were inoculated with 1% overnight culture. The cultures were grown at 37 °C and 180 rpm. When the culture reached an OD of 0.5, cell cultures were induced with 200 µL of IPTG (1M). For expression temperature was reduced to 20°C. Afterwards cells were harvested (4000x g, 4 °C, 30 min). For purification cells were suspended in binding buffer (25 % cell suspension) and digested under ultrasound (3x 3 min, 5x10 cycles). The suspension was centrifuged at 20,000× g for 20 min. The pellet was discarded and the crude extract was used for further purification. The supernatant was loaded to the Ni^{2+}-NTA and eluted with 250 mM imidazole.

4.5 Enzyme activity assay

For the activity assay the oxidation of NADH to NAD^+ was measured by decrease in absorbance at 340 nm at 30°C. The enzyme activity is defined as µmolmin^{-1}. The extinction coefficient is 6.3 10^3 L mol^{-1} cm^{-1}. The reaction was performed in a 1 mL cuvette consisting of 980 µL buffer 2M ammonium chloride buffer, pH 9.5 with ketone (5-20 mM), 10 µL NADH (10 M, final concentration 0.1 mM) and 10 µL enzyme crude extract. The activity was measured with a V-630 UV/-vis spectrophotometer from Jasco®.

4.6 Kinetic constants

For determination of the *Michaelis-Menten* constant (K_M) **1** was used as a substrate. The activity was measured according to the enzyme activity assay with 0.1 mM NADH, but in a microtiter plate *via TecanReader*® at 30°C with a volume of 250 µL. The activity was examined at various substrate concentrations and a fixed enzyme amount.

*4.7 Biotransformation with acetophenone (**1**)*

1 (2.4mg, 0.02 mmol; 6.0 mg, 0.05 mmol; 11.3 mg, 0.09 mmol) were dissolved in ammonium chloride buffer (660 µL, 2 M, pH 9.5). EsLeuDH-DM (250 µL; 10 U), glucose (100 µL,1M, 100 mM final concentration), NAD^+ (20 µL, 50 mM, 1 mM final concentration) and GDH (20 µL, 18 U) were added and the mixture was heated to 30°C. The total volume of reaction mixture was 1 mL. At fixed times, samples were taken. The conversion was measured *via* gas chromatography. For measurement of the *ee*-value, the samples were acetylated with acetyl chloride (1.1 eq) and triethylamine (1.5 eq) in methylene chloride for one hour. The suspension was washed with hydrogen chloride (1:1, v/v). The solvent was removed *in vacuo*. Enantiomeric excess of the amine (*R*)-**13** was determined *via* HPLC. For one experiment **1** (6.0 mg, 0.05 mmol), EsLeuDH-DM (500 µL; 20 U), ammonium chloride buffer (410 µL, 3.2 M, pH 9.5), glucose (100 µL,1M, 100 mM final concentration), NAD^+ (20 µL, 50 mM, 1 mM final concentration) and GDH (20 µL, 18 U) were added and the mixture was heated to 30°C, the reaction was stopped after 100h. The conversion was measured *via* gas chromatography. For measurement of the *ee*-value, the samples were acetylated with acetyl chloride (1.1 eq) and triethylamine (1.5 eq) in methylene chloride for one hour. The suspension was washed with hydrogenchlorid (1:1, v/v). The solvent was removed *in vacuo*. Enantiomeric excess of the amine (*R*)-**13** was determined *via* HPLC.

4.8 Co-solvent screening

Into a microtiter plate 190/175/150 µL ammonium chloride buffer, 2 M, pH 9.5 with 20 mM **1**, 15 µL crude extract EsLeuDH-DM and water-soluble co-solvent (25/50/75 µL) were added. The suspension was incubated for 0/1/3 h and the assay was started by addition of 10 µL NADH (5 mM, final concentration of 0.2 mM). The activity was measured according to the enzyme activity assay.

4.9 Immobilization of the biocatalyst

4.9.1 Immobilization on hydrophobic carrier

The purchased carrier material was washed with KPi buffer (0.05 M, pH 7.0). The ratio of carrier to buffer was 1:1 (m/v). Subsequently, the washed carrier material was suspended in an enzyme solution (in KPi buffer (0.05 M, pH 7.0)) and shaken for 18 h at 20 °C and 80 rpm. The supernatant was removed with a pipette and the immobilizate was washed with KPi buffer (0.01 M, pH 7.0). The protein concentration in the supernatant was determined *via Bradford* assay for calculation of the immobilization yield.[11]

4.9.2 Immobilization on covalent carrier

The purchased carrier material was washed three times with KPi buffer (0.5 M, pH 7.0). The ratio of carrier to buffer corresponded to 1:1 (m/V). The washed immobilizate was then suspended in an enzyme solution in KPi buffer (0.5 M, pH 7.0) and shaken for 18 h at 20 °C and 80 rpm. The supernatant was removed with a pipette and the immobilizate was washed three times with KPi buffer (0.01 M, pH 7.0) and once with NaCl solution (0.5 M) in KPi buffer (0.01 M, pH 7.0). The protein concentration in the supernatant was determined *via Bradford* assay for calculation of the immobilization yield.[11]

4.9.3 Examination of leaching-process

For the examination of the leaching process the heterogenized catalyst was incubated in 1.3 M NH_4Cl-Buffer, 0.65 M NH_4Cl-buffer and H_2O for seven days. The protein concentration in the supernatant was determined *via Bradford* assay for calculation of the rate of enzyme leaching.[11]

4.10 Biotransformation with immobilized EsLeuDH-DM

1 (58 mg, 0.5 mmol) was dissolved in ammonium chloride buffer (8.1 ml, 2 M, pH 9.5), in a 25 ml *Erlenmeyer* flask. After addition of a glucose solution (1.25 ml, 1M, 50 mM final concentration), crude GDH extract (125 µL, 22.5 U) and an NAD^+ solution (10 M, 500 µl, 1 mM final concentration), the immobilized EsLeuDH-DM (3 g) was added to the suspension. Subsequently, the mixture was shaken for a total of 100 hours at 30 ° C. and 180 rpm. Samples were taken at various time, which were extracted with ethyl acetate (2x 500 µL), the conversion was determined *via* gas chromatography. Enantiomeric excess of the amine (*R*)-**13** was determined *via* HPLC according to the protocol described above in section 4.7.

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Supplementary Material

Supplementary Information for this article is available from the internet, see DOI: xxx.