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Application of Imine Reductases (IREDs) in Micro-Aqueous Reaction Systems

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Abstract: Here we present the applicability of different imine reductases (IREDs) in micro-aqueous reaction systems. Subjects of the study were the IREDs from *Streptomyces aurantiacus* (*Sa*IR), *Streptomyces* sp. GF3587 (RGF3587IR), *Streptomyces kanamyceticus* (*Sk*IR), *Streptomyces ipomoeae* 91-03 (*Si*IR), *Streptomyces* sp. GF3546 (SGF3546IR), and *Paenibacillus elgii* B69 (*Pe*IR). The IREDs were overexpressed in *Escherichia coli* (*E. coli*) cells and used directly after lyophilization. Several organic solvents and buffer amounts were screened for the reduction of the two substrates β-carboline harmane and 1-methyl-3,4-dihydroisoquinoline to the corresponding amines. Cyclopentyl methyl ether (CPME) proved to be the best solvent choice for the envisaged reduction. In addition, CPME is currently referred to as an environmentally benign solvent. Optimized reaction conditions were applied to 20 mM of the hardly water soluble substrates, leading to good conversions (up to 96%) and excellent enantiomeric excesses (>99%) in the best cases. The use of micro-aqueous reaction systems opens the way to further applications of IREDs with hardly water soluble substrates.

Keywords: imine reductases; imines; micro-aqueous reaction system; organic solvent; reduction; whole cells

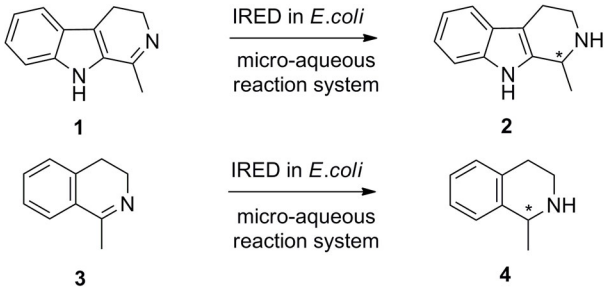
Chiral amines represent very important building blocks for the synthesis of biologically active pharmaceutical drugs, agro and fine chemicals, when high chemical and stereoisomeric purities are required. It is estimated that 40% of all pharmaceuticals contain chiral amine moieties.[1–3] Besides their application in pharmaceuticals and agrochemicals, optically pure amines, amino acids, and amino alcohols are frequently employed in chemical syntheses as chiral auxiliaries or resolving agents. A broad set of asymmetric catalytic methods has been developed including organocatalysis and transition metal catalysis, and intense efforts have been taken to increase both yields and stereoselectivities.[4] Recently published syntheses show the potential of biocatalytic methods as a valid alternative for the asymmetric synthesis of chiral amines. For many years the only relevant option has been the kinetic resolution of racemic amines using lipases.[5] Nowadays, enzymes from several classes such as transaminases, monoamine oxidases (MAO-N), amine dehydrogenases, and phenylalanine ammonia lyases are known to catalyze the production of chiral amines.[6–9] With the exception of MAO-N, all the above mentioned classes of enzymes generate primary amines.

Imine reductases (IREDs) represent a novel class of biocatalysts permitting asymmetric synthesis of secondary and tertiary amines by using nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor. Their discovery dates back to the last decade but a real breakthrough was made by Mitsukura and co-workers in 2011 when they purified and characterized the (*R*)-IRED from *Streptomyces* sp. GF3587.[10] They investigated the reduction of the 2-methyl-1-pyrroline (2-MPN), being able to gain 9.8 mM out of 10 mM of (*R*)-2-methylpyrrolidine [(*R*)-2-MP] with excellent an enantioselectivity of 99%. Two years later the same research group purified and characterized the (*S*)-IRED from *Streptomyces* sp. GF3546.[11] Therewith they broadened the substrate scope by testing 1-methyl-3,4-dihydroisoquinoline and 6,7-dimethoxy-1-methyl-3,4-dihydroisoquinoline and obtaining stereoselectivities higher than 90% in all cases. Just recently, Schrittwieser and co-workers published a thorough review on biocatalytic imine reductions collecting all results obtained in the last years utilizing IREDs.[3] Based on the protein sequences of the first characterized IREDs, more enzymes have been discovered *via* sequence homology[12] and an electronic library of about a thousand putative imine reductases, the *Imine*

Reductase Engineering Database (available on the webpage https://ired.biocatnet.de/), was generated.[13] Crystal structures of several IREDs were obtained[13–17] and possible reaction mechanisms proposed.[14,17] Several new substrates such as 5-, 6-, and 7-membered cyclic imines, 3,4-dihydroisoquinolines, 3,4-dihydro-β-carbolines, and 3*H*-indoles were tested, and good conversions and excellent stereoselectivities observed.[18–21] Besides the broad acceptance of cyclic imines (predominantly investigated because of their good stability in aqueous solutions), also acyclic imines were identified as substrates for IREDs-mediated catalysis by testing the $NADP^+$-dependent oxidation of several acyclic amines with different IREDs.[16] Another feature of IREDs, although at the moment only partially investigated, is their ability to reduce open-chain imines through a so-called reductive amination. This reaction was investigated for several ketones. Despite the fact that reaction rates were rather slow (potentially due to the hydrolytic instabilities of the intermediate imines), it was possible to access amines with good enantiomeric excesses representing a proof of principle of the great potential of IRED applications.[15,22]

It is frequently published that enzymes, apart from few exceptions (e.g., lipases), show highest activities in buffer, mimicking their natural environment. However, when hydrophobic or water unstable substrates (e.g., some acyclic imines) are involved, pure aqueous solutions are difficult to apply (especially when a second phase formation should be avoided) rendering the use of solubility enhancers essential. The application of biocatalysts in organic solvents,[23–25] micro-aqueous systems,[26–28] or in neat substrates,[29] might overcome these problems and in addition facilitate downstream processes. At the same time, overall enzyme activity might be reduced. Successful applications of whole cell biotransformations in micro-aqueous reactions systems have been lately published,[30–32] allowing substrate concentrations up to 0.5 M and simplified downstream processing. Whole cell catalyst formulation can alleviate the problem of reduced activity in the above-mentioned non-conventional media. The remaining cell envelope seems to protect the enzyme inside the cell to a certain extent guaranteeing enhanced stability. Moreover, by using whole cells, no time- and money-consuming purification is needed and, most importantly, expensive cofactor addition becomes obsolete as delivered by the cell, hence cutting overall production costs.[33]

We herein describe the potential of *E. coli* whole cells expressing IREDs operated in micro-aqueous reaction systems. The reductions of the hydrophobic β-carboline harmane (**1**) and 1-methyl-3,4-dihydroisoquinoline (**3**) to the corresponding amines (**2** and **4**) were used as test reactions (Scheme 1).



Scheme 1. Asymmetric reduction of the β-carboline (**1**) and isoquinoline (**3**) substrates to the corresponding amines (**2** and **4**) by using whole cells expressing IREDs in a micro-aqueous reaction system as model reactions.

In Table 1 the six selected IREDs are listed. The IREDs were overexpressed in *E. coli* cells and used in a lyophilized form without any further purification. D-Glucose was added to the reaction as co-substrate for cofactor regeneration using the glucose dehydrogenase (GDH) present in the cells.

Table 1. IREDs screened for the reduction of the imine substrates **1** and **3**. GI = GenInfo Identifier, NCBI database.

IRED	Organism	GI
*Sa*IR	*Streptomyces aurantiacus*[1]	514923777
RGF3587IR	*Streptomyces* sp. GF3587[2]	460838084
*Sk*IR	*Streptomyces kanamyceticus*[3]	123248375
*Si*IR	*Streptomyces ipomoeae* 91-03[2]	496688866
SGF3546IR	*Streptomyces* sp. GF3546[2]	460838082
*Pe*IR	*Paenibacillus elgii* B69[2]	498183793

In preliminary experiments, carried out to find suitable reaction conditions, the imine reductase from *Streptomyces aurantiacus* (*Sa*IR) was chosen as model enzyme. Aiming for the identification of the most suitable buffer for its later application in the micro-aqueous reaction system, four different buffer species with varying pHs were screened for the reduction of the imine substrate (**1**). The *Sa*IR catalyzed the reaction in all four buffers giving best performance in HEPES and TEA buffer at pH 7.5 and 10, respectively (Figure 1). As HEPES buffer pH 7.5 led to 99% conversion, this buffer was chosen for following experiments.

Further investigations were performed to identify the best organic solvent among seven selected ones with varying properties (e.g., different polarities) (Table 2). To restore the catalytic activity of the lyophilized cells, 10% (v/v) of 1 M HEPES buffer pH 7.5 was added to the reaction mixture. The high buffer concentration was chosen based on the results lately reported by our group[30] in which whole cells

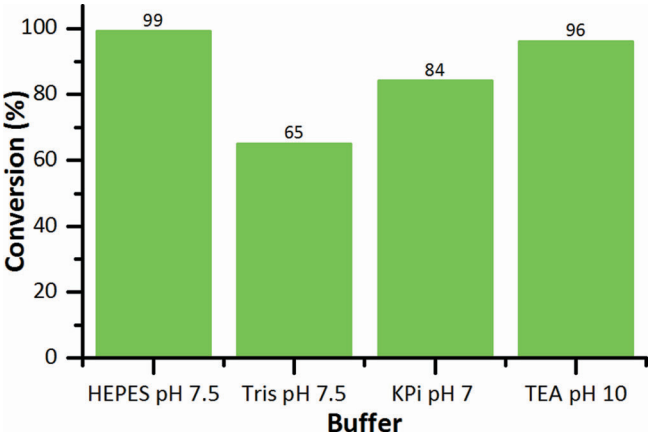


Figure 1. Preliminary screening of *Sa*IR with four different buffers for the reduction of the β-carboline harmane **1**. *Reaction conditions*: 50 mg of lyophilized *E. coli* cells overexpressing *Sa*IR, 5 mM of **1**, 50 mM of co-substrate (D-glucose), buffer concentration 100 mM, 2% (v/v) of dimethylformamide (DMF), total volume 500 μL, 25 °C, 16 h.

Table 2. Organic solvent screening for the reduction of **1** catalyzed by *E. coli* cells overexpressing the *Sa*IR.[a]

Organic solvent	Observed activity (conversion)
DMC	no
2-MTHF	no
EtOAc	no
MIBK	yes (34%)
CPME	yes (25%)

[a] *Reaction conditions*: 50 mg of lyophilized *E. coli* cells overexpressing the *Sa*IR, 5 mM of **1**, 50 mM of co-substrate (D-glucose), 10% (v/v) of 1 M HEPES buffer pH 7.5, total volume 500 μL, 25 °C, 8 h. MTBE = methyl *tert*-butyl ether; DMC = dimethyl carbonate; 2-MTHF = 2-methyltetrahydrofuran; EtOAc = ethyl acetate; MIBK = methyl isobutyl ketone; CPME = cyclopentyl methyl ether.

expressing the benzaldehyde lyase (BAL) from *Pseudomonas fluorescens* and the alcohol dehydrogenase from *Ralstonia* sp. (RADH), showed highest activities in micro-aqueous system in the presence of 1 M buffer concentration. Aiming to dissolve at least 20 mM of the substrate **1** in a micro-aqueous reaction system, two of the chosen solvents, specifically methyl *tert*-butyl ether (MTBE) and toluene, had to be excluded from the screening, due to the low solubility of the substrate (up to 10 mM) in these media. Among the other five organic solvents, the cells showed activity only in two of them, namely the methyl isobutyl ketone (MIBK) and the cyclopentyl methyl ether (CPME, Table 2). As mentioned above, the catalytic activity is often reduced when the enzyme is applied in unconventional media. The effect of organic solvents on the imine reduction activity has been further investigated using the imine 2-methyl-1-pyrroline. The data are reported in the Supporting Information. Besides this drawback, in sum enhanced substrate solubility and facilitated downstream processing can render such a system advantageous.

Once MIBK and CPME were selected, a more detailed study was conducted to determine whether both media are suitable for the micro-aqueous reaction system or if some limitations might favor one over the other one. In 1987 Yamane et al. introduced the concept of 'micro-aqueous' for the first time.[28,34] They proposed a model explaining how much water is bound to the protein depending on the use of water-miscible or water-immiscible solvents. In water-miscible solvents the excess of water will be dissolved, whereas in water-immiscible solvents it will remain as free water. Since the water bound to the protein is in equilibrium with free water molecules, for each system a specific amount of water molecules might cause cell clumping. In this work several amounts of 1 M HEPES buffer pH 7.5 [range of 5–15% (v/v)] were screened in micro-aqueous reaction systems with both MIBK and CPME. In all cases a monophasic system was formed, since the cells absorbed completely the buffer added to the reaction.

Clearly visible differences could be observed when the two organic solvents had been applied with varying buffer amounts in the micro-aqueous reaction system (Figure 2). At the beginning of the reaction, no cell clumping occurred and in both organic solvents the biocatalysts showed higher activity in the presence of increased buffer amounts. After 24 h, in vials containing MTBE higher percentages of buffer led to cell clumping, impeding good catalyst dispersion in the reaction media, making quantification of both substrate and product difficult. Although no substrate could be detected after 24 h anymore, it could not be excluded nor confirmed that cells are still active after 24 h due to the clumping problem. The product amount decreased from 8 to 24 h, correlating to the cell clumping occurring during this time. In fact, the mass balance was closed only at the beginning of the reaction, when cells were still freely dispersed. It can be assumed that both substrate and product either adhered directly to the cells or remained inside them when clumping occurs. In CPME, the cells, even with buffer concentrations up to 15% (v/v), remained well distributed and after 24 h the mass balance could still be closed. Two factors might influence the different behaviors of the systems. First, the solubility of water has been reported to be 2% (m/m) in MIBK and 0.3% (m/m) in CPME.[35,36] Therefore, MIBK adsorbs more water molecules from the air humidity in comparison to CPME. The effective higher concentration of water in the system might cause cell clumping. Second, depending on the

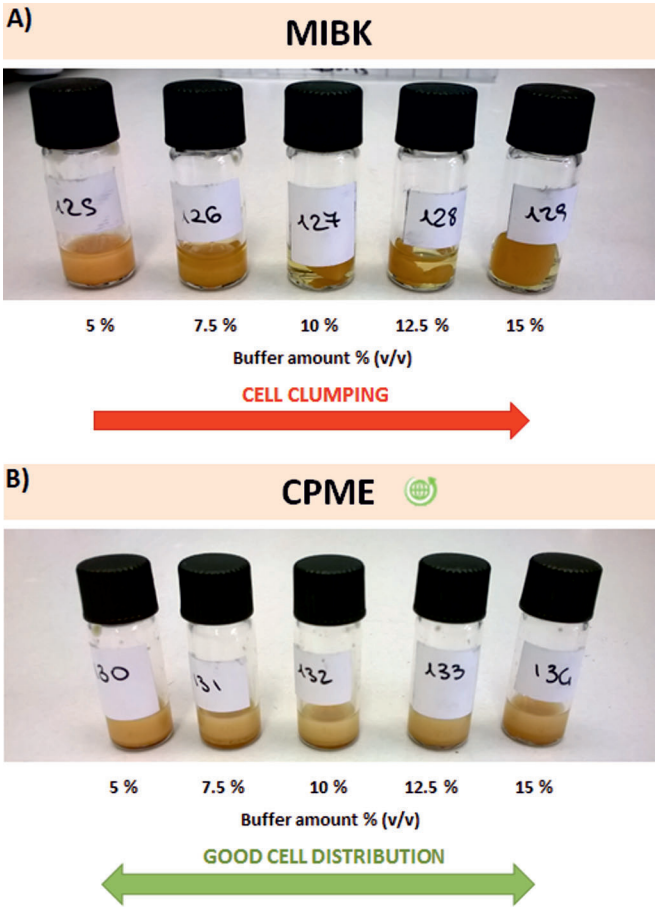


Figure 2. Screening of different buffer amounts in micro-aqueous reaction system using both MIBK (**A**) and CPME (**B**) as organic solvents. *Reaction conditions*: 50 mg of lyophilized *E. coli* cells overexpressing the *Sa*IR, 10 mM of **1**, 100 mM of co-substrate (D-glucose), 5–15% (v/v) of 1 M HEPES buffer pH 7.5, total volume 500 µL, 25 °C, 24 h.

organic solvent chosen, different interactions with the cell membrane can be expected, leading to morphological changes and aggregation.

Recently, CPME has been promoted as a new and alternative process medium to the classical ethereal solvents (e.g., diethyl ether, tetrahydrofuran, dioxane, 1,2-dimethoxyethane) due to its peculiar properties such as high boiling point (106 °C), low formation of peroxides, and relative stability under acidic and basic conditions.[35]

Results obtained applying the optimal solvent (CPME) and buffer (1 M HEPES buffer pH 7.5) to the reduction of **1** with *Sa*IR are shown in Figure 3. MIBK has been omitted due the impossibility of following the reaction satisfactorily. The reaction proceeded poorly with 5% (v/v) of buffer and slowly with 7.5%, but addition of 10–15% (v/v) of buffer led to very good results. The buffer amount of 10% was finally chosen for the optimized reaction set-up, representing a good compromise between minimum

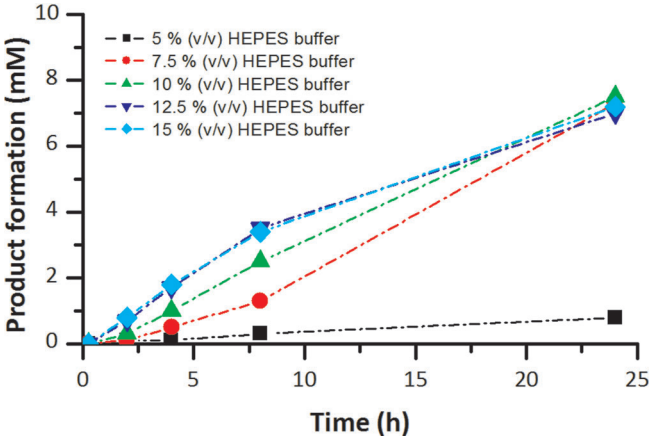


Figure 3. Screening of different buffer amounts in micro-aqueous reaction systems using CPME as organic solvent. *Reaction conditions*: 50 mg of lyophilized *E. coli* cells overexpressing *Sa*IR, 10 mM of **1**, 100 mM of co-substrate (D-glucose), 5–15% (v/v) of 1 M HEPES buffer pH 7.5, total volume 500 µL, 25 °C.

amount of water in the system and good catalytic activity.

The selected IREDs (Table 1) were tested under optimized reaction conditions [10% (v/v) of 1 M HEPES buffer pH 7.5 and CPME as organic solvent] in the presence of 20 mM of substrate concentration. In Table 3 the results for the reduction of both imine substrates **1** and **3** are summarized.

Moderate to excellent enantioselectivities were achieved confirming the observations by previous authors.[15–16,18] The IREDs from *Streptomyces aurantiacus* (*Sa*IR) and *Paenibacillus elgii* B69 (*Pe*IR) showed the best stereoselectivities for both substrates yielding in both cases enantiomeric excesses ≥99% of the (*S*)-product. With 96% conversion 1-methyl-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole (**2**) could be gained using *Sa*IR (Table 3). Best conversion for 1-methyl-1,2,3,4-tetrahydroisoquinoline (**4**) could be detected

Table 3. Reduction of the imine substrates **1** and **3** catalyzed by *E. coli* cells overexpressing the IREDs.[a]

	1		**3**	
IRED	Conv. %.	*ee* %	Conv. %	*ee* %
RGF3587IR	0	–	48	(*R*)-63
*Sk*IR	0	–	0	–
*Si*IR	0	–	54	(*R*)-46
*Sa*IR	96	(*S*)-99	48	(*S*)-99
SGF3546IR	36	(*S*)-99	67	(*S*)-94
*Pe*IR	71	(*S*)-99	51	(*S*)-99

[a] *Reaction conditions*: 50 mg of lyophilized *E. coli* cells overexpressing the IRED, 20 mM of substrate, 200 mM of co-substrate (D-glucose), 10% (v/v) of 1 M HEPES buffer pH 7.5 in CPME, total volume 500 µL, 25 °C, 24 h. Conv. = conversion.

with SGF3546IR. Although the IRED RGF3587 from *Streptomyces* sp. yielded lower conversion of **3** and an enantiomeric excess of 63%, the catalyst is an interesting candidate for future optimization as the (*R*) stereoisomer is gained, complementing the access to both possible products.

In summary, we have demonstrated the applicability of whole cells expressing IREDs in a micro-aqueous reaction system. Best reaction conditions in terms of optimal buffer species and amount, and suitable organic solvent, were applied to the reduction of the β-carboline harmane and 1-methyl-3,4-dihydroisoquinoline. Most of the screened IREDs were able to reduce both substrates to the corresponding amines in CPME by the addition of 10% (v/v) 1 M HEPES buffer pH 7.5. Excellent *ee* (99%) values were achieved by the IREDs from *Streptomyces aurantiacus* and *Paenibacillus elgii* B69 for both products. The results now open the possibility to expand the substrate scope of IREDs to more hydrophobic compounds such as complex heterocyclic aromatic molecules.

Experimental Section

Cloning and Expression of the IREDs

The gene sequences encoding for *Sa*IR and *Sk*IR, optimized as reported in the literature,[14,15] were ordered as synthetic genes from Thermo Fisher Scientific and cloned in pET28a (*Sk*IR) or pET22b (*Sa*IR). The genes of the other IREDs (RGF3587IR, *Si*IR, SGF3546IR and *Pe*IR) were purchased from Enzymicals AG (Greifswald, D) and obtained in vector pET28b. *E. coli* DH5α and BL21 (DE3) cells were used for cloning and expression of the recombinant protein. Recombinant cells were precultivated in lysogeny broth (LB) for 16 h at 37 °C with shaking at 150 rpm, transferred into auto-induction medium (AI-medium) and cultivated for another 48 h at 20 °C with shaking at 90 rpm. Finally, cells were harvested by centrifugation (4 °C at 7000 rpm for 30 min) and lyophilized.

Chemical Syntheses of Imine Substrates (1 and 3) and Corresponding Racemic Amines (3 and 4)

The substrate 1-methyl-4,9-dihydro-3*H*-pyrido[3,4-*b*]indole (**1**) was synthesized by adapting the reaction conditions reported by Espinoza-Moraga et al. and Santos et al.[37,38] The corresponding racemic amine 1-methyl-2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole (**2**) was synthesized according to Huber et al.[15] The substrate 1-methyl-3,4-dihydroisoquinoline (**3**) and the corresponding racemic amine 1-methyl-4,9-dihydro-3*H*-pyrido[3,4-*b*]indole were synthesized according to Huber et al.[15] as well (see chapter 3 in the Supporting Information for detailed reaction conditions).

Experimental Details for Bioreduction in Buffer

In a typical bioreduction in aqueous solution, 10 μL of DMF containing 250 mM of the imine substrate were added to 490 μL of buffer in a 1.5-mL siliconized vial containing 50 mg of lyophilized *E. coli* overexpressing the various IREDs and 50 mM of the co-substrate (D-glucose). The reaction was performed at 25 °C. After 16 h the reaction was stopped, basified with 10 M sodium hydroxide (NaOH) and centrifuged to remove the cells. The supernatant was extracted 3 times with 0.5 mL of ethyl acetate. The organic phases were collected and the solvent evaporated under vacuum. The sample was dissolved in 100 μL of ethyl acetate and conversions and stereoselectivities determined by normal phase chiral HPLC.

Experimental Details for Bioreduction in the Micro-Aqueous Reaction System

In a typical bioreduction in micro-aqueous reaction system, 450 μL of organic solvent containing 10 μmol of the imine substrate were transferred to a siliconized vial containing 50 mg of lyophilized *E. coli* overexpressing the IRED and 100 μmol of the co-substrate (D-glucose). 50 μL of 1 M HEPES buffer pH 7.5 were finally added and the reaction mixture performed at 25 °C. Samples were taken at specific time intervals, basified with 10 M NaOH, diluted 1/5 in the same organic solvent and centrifuged to remove the cells. Conversions and stereoselectivities were determined by normal phase chiral HPLC.

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