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Authors: Hanna Büchsenschütz, Viktorija Vidimce-Risteski, Bettina Eggbauer, Sandy Schmidt, Christoph Winkler, Jörg H Schrittwieser, Wolfgang Kroutil, and Robert Kourist

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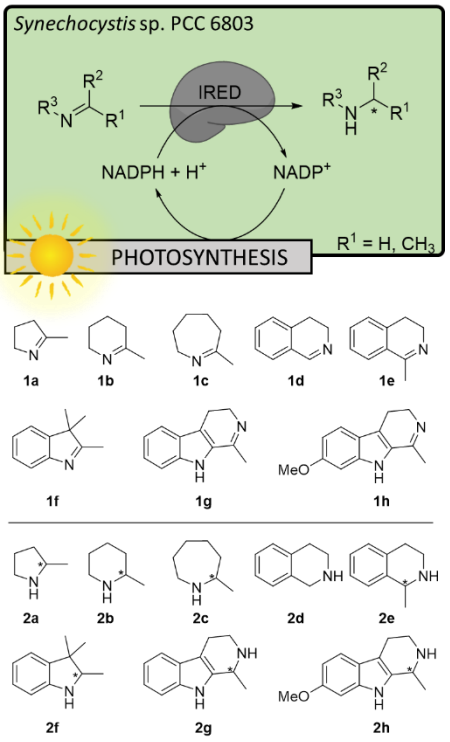
COMMUNICATION

Stereoselective biotransformations of cyclic imines in recombinant cells of *Synechocystis* sp. PCC 6803

Hanna C. Büchsenschütz,[#[b]] Viktorija Vidimce-Risteski[#],[[a]] Bettina Eggbauer,[[b]] Sandy Schmidt,[[b]] Christoph K. Winkler,[[a,c]] Joerg H. Schrittwieser,[[c]] Wolfgang Kroutil*[[a,c]] and Robert Kourist*[[a,b]]

Abstract: Light-driven biotransformations in recombinant cyanobacteria allow to employ photosynthetic water-splitting for cofactor-regeneration and thus, to save the use of organic electron donors. The genes of three recombinant imine reductases (IREDs) were expressed in the cyanobacterium *Synechocystis* sp. PCC 6803 and eight cyclic imine substrates were screened in whole-cell biotransformations. While initial reactions showed low to moderate rates, optimization of the reaction conditions in combination with promoter engineering allowed to alleviate toxicity effects and achieve full conversion of prochiral imines with initial rates of up to 6.3 mM h^{-1}. The high specific activity of up to 22 U g_{CDW}^{-1} demonstrates that recombinant cyanobacteria can provide large amounts of NADPH during whole cell reactions. The excellent optical purity of the products with up to >99 %*ee* underlines the usefulness of cyanobacteria for the stereoselective synthesis of amines.

Biocatalysis has emerged as important catalytic technology with significant contributions for the development of clean reactions.[1] A large number of biocatalytic processes employ oxidoreductases, which often require a stoichiometric supply of sacrificial organic cosubstrates for cofactor regeneration, which in turn results in a very poor atom economy. In this context, cyanobacteria have been receiving increasing attention as hosts for biotransformations to exploit photosynthetic water splitting for the supply of reductive enzymatic reactions with electrons. Beside the application of wildtype strains,[2–5] heterologous enzyme production is of main interest and has been greatly facilitated by the impressive progress in molecular biology of photoautotrophic organisms.[6–8] So far, ene-reductases, Baeyer-Villiger monooxygenases, carboxylic acid reductases, the AlkBGT-system and CYP450 monooxygenases have successfully been used in light driven biotransformations.[9–14] In particular, cells expressing the genes encoding for recombinant oxidoreductases showed reaction rates as high as 123 U g_{DCW}^{-1}.[10] In case of CYP450 from *Acidovorax* sp. CHX100, recombinant cyanobacteria exhibit higher specific activity than the corresponding heterotrophic *Pseudomonas taiwanensis* VLB120 with the same enzymes.[12,15] Cyanobacteria offer additional advantages such as the ease to extract the product from reaction solution[10] and the possibility of an *in situ* production of oxygen as cosubstrate for oxyfunctionalizations.[11,16] Despite a few encouraging examples, however, photoautotrophic organisms yet need to fulfill their promise. Besides efficiency, selectivity and robustness, a broad scope toward different substrates is crucial for the wide application of a biocatalyst.[1] Potential toxicity of substrates and products may have an immense influence on the system. Furthermore, the transport of substrate and product across cell membranes can restrict the application of whole-cell biocatalysts.[17] We were interested to which extent light-driven whole-biotransformations could be applied for the cofactor regeneration of imine reductases (IREDs) for the production of secondary amines. IREDs were discovered by Mitsukura *et al.* in *Streptomyces sp*. Since then, IREDs have rapidly assumed an important role as highly stereoselective catalysts for amine snythesis.[18–24] IREDs strictly require NADPH and are preferentially used in cellular systems, which makes them very suitable for application in photosynthesis-driven whole-cell biotransformations.



Scheme 1. Biotransformation of imines **1a**-**h** by recombinant IREDs in *Synechocystis* sp. PCC 6803. The required cofactor NADPH is regenerated by natural photosynthesis.

[a] Prof. Dr. Robert Kourist, Viktorija Vidimce-Risteski, Dr. Christoph Winkler, Prof. Dr. Wolfgang Kroutil
Austrian Centre of Industrial Biotechnology (ACIB GmbH),
Petersgasse 14/ Mozartgasse 12/II, 8010 Graz, Austria
E-mail: kourist@tugraz.at
[b] Prof. Dr. Robert Kourist, Dr. Sandy Schmidt, MSc. Hanna Büchsenschütz
Institute of Molecular Biotechnology
Graz University of Technology, NAWI Graz, BioTechMed,
Petersgasse 14, 8010 Graz, Austria
[c] Dr. Jörg H. Schrittwieser
Institute of Chemistry, Organic & Bioorganic Chemistry,
NAWI Graz, BioTechMed Graz, University of Graz
Heinrichstraße 28/II, 8010 Graz, Austria
# both authors contributed equally to this work.
Supporting information for this article is given via a link at the end of the document.

The simplicity of an imine reduction in cyanobacteria is shown in equation (1). Water is used as electron donor and molecular oxygen is released as side-product.

$$R\text{-}CH{=}NH\text{-}R' + H_2O \rightarrow R\text{-}CH_2\text{-}NH_2R' + \tfrac{1}{2}\, O_2 \qquad [1]$$

To investigate strengths and limitations of cyanobacterial imine reduction, we chose a set of eight substrates and three enzymes as representatives, namely IRED-A from *Streptomyces* sp. GF3587, IRED-J from *Kribbella flavida* DSM 17836 and IRED-M from *Saccharothrix espanaensis* ATCC 51144.[25] In a recent study comparing 15 IREDs, these enzymes proved to be particularly suitable for our approach.[25] IRED-A was most active on a variety of smaller imine substrates and exceled with stability toward high temperature, high substrate concentrations and its selectivity. IRED-J on the other hand was the most active (*S*)-selective IRED characterized by Velikogne *et al.* but also IRED-M showed high conversions and selectivites especially toward bulky imine substrates.[25]

Table 1. Specific activities ($A_{spec.}$) and conversions (*C*) for the light-driven imine reduction of substrates (S) **1a**-**1h** using three different recombinant *Synechocystis* strains. All heterologous IREDs are under the control of psbA2 promoter.

	P_{psbA2} IRED-A		P_{psbA2} IRED-J		P_{psbA2} IRED-M	
S	$A_{spec.}$[a] [U g_{CDW}^{-1}]	C[b] [%]	$A_{spec.}$[a] [U g_{CDW}^{-1}]	C[b] [%]	$A_{spec.}$[a] [U g_{CDW}^{-1}]	C[b] [%]
1a[c]	0.2 ± 0.1	18	0.6 ± 0.1	32	0.4 ± 0.3	19
1b[c]	2.7 ± 0.3	83	0.9 ± 0.2	21	n.c.	n.c.
1c[c]	0.6 ± 0.02	36	n.c.	n.c.	n.c.	n.c.
1d	0.2 ± 0.04	15	0.1 ± 0.03	6	0.2 ± 0.1	12
1e	0.2 ± 0.1	9	0.1 ± 0.04	9	n.c.	n.c.
1f	n.c.	n.c.	n.c.	n.c.	0.1 ± 0.04	6
1g	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.
1h	n.c.	n.c.	n.c.	n.c.	n.c.	n.c.

[a] determined from the substrate decay in the first 12 h using GC-FID analysis;

[b] determined from the substrate decay after 18-25 h using GC-FID analysis; exact time points are given in the supplements

[c] samples containing these substrates were derivatized before analysis

n.c = not converted.

Reaction conditions: 5 mL scale, 5 mM substrate, 150 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, 30 °C, 3.6 $g_{CDW}\ L^{-1}$, in BG11 (pH = 8)

Genes for all recombinant IREDs were integrated into the gene locus *slr0168* of the cyanobacterium *Synechocystis* sp. PCC 6803 (in the following *Synechocystis*) under the control of the light-inducible promoter P_{psbA2}. This native promoter is of moderate strength[10,26] and seemed to be very suitable to obtain a reasonable amount of soluble enzyme without overloading the folding machinery. Integration of the expression cassette into all genome copies of *Synechocystis* was verified by PCR (Supplementary Figure S1). While the enzymes showed activity in whole-cell biotransformations, protein bands for these enzymes could not be detected in SDS-PAGE (Supplementary Figure S2). This is not unusual for *Synechocystis*.[10,27] For an initial substrate screening, cells were concentrated to densities of 3.6 $g_{CDW}\ L^{-1}$ (correspond to OD_{750} = 15) and substrate conversion was monitored up to 25 h.

We were pleased to detect product formation for substrate **1a**-**e** in most cases (Table 1). No or very low product formation was detected for reactions with larger substrate **1f**-**h**. The low activity of IREDs in *Synechocystis* toward bulkier substrates demonstrates a clear difference to the activity of the same enzyme in *E. coli* [25] and might be attributed to the capacity of transport of substrates and products across the cell membrane. The measured conversions were low (≤ 36%) in all cases except for **1b** and IRED-A. Furthermore, cells changed their visible appearance which was not observed for analogous reactions with the ene-reductase YqjM from *B. subtilis*[10] or cyclohexanone monooxygenase from *A. calcoaceticus*[9] and is an indication for toxicity effects. The toxicity of some substrates such as **1g** was recognized by a color change to blue or brown of the cells during the reaction (Supplementary Figure S3). To investigate the extent of toxicity, cells used in biotransformations were streaked out on BG11-agar plates after different reaction time points. The vitality of cells treated with 5 mM **1b** or **2b** was reduced considerably within one hour while cells treated with 0.5 mM grew back after 24 h (Figure 1). Furthermore, toxicity experiments with all other imine substrates showed high toxicity of **1g**-**h**, moderate toxicity of **1b**-**e** and lowest toxicity of **1a** and **1f** (Supplementary Figure S4).

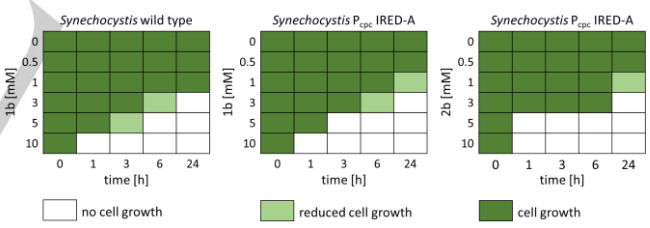


Figure 1. Growth of *Synechocystis* wild-type and a mutant expressing the gene of IRED-A in presence of different concentrations of **1b** and **2b**. Reaction conditions: 150 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, 30 °C, V_{total} = 1 mL, 3.6 $g_{CDW}\ L^{-1}$, in BG11 (pH = 8)

The substrate toxicity is an explanation why recombinant *Synechocystis* strains with the imine reductases show very different substrate spectra than observed for the same enzymes in *E. coli*. Although *Synechocystis* cells are viable in the presence low substrate or product concentrations, the toxicity reveals an intrinsic limitation for achieving high space-time-yields.

In the initial screen, the highest activities were observed with IRED-A and hence, this enzyme was chosen for further optimization. We expected an increase of expression levels of the recombinant IRED-A by changing the promoter psbA2 to the stronger promoter cpc.[28,29] Indeed, we were pleased to find that the effect of the promoter exchange was very pronounced and the recombinant *Synechocystis* strain with P_{cpc}_IRED-A converted

83 % **1b** already 3 h (Supplementary Figure S6). The effect of a stronger promoter is a clear indication that the intracellular concentration of the imine reductase is a limiting factor.

In subsequent experiments, we compared the specific rates of our P_{cpc}-IRED-A strain using different cell densities at a light intensity of $\mu mol_{photons}\ m^{-2}\ s^{-1}$ (Figure 2). For light-driven biotransformations, we expected constant specific activities when increasing cell densities until a point where self-shading of the cells reduces light-availability as we observed this trend for other oxidoreductases and cell densities above 2.4 $g_{CDW}\ L^{-1}$ per litre.[9] Very surprisingly, the activity of P_{cpc}-IRED-A towards **1b** was lowest when using 1.2 $g_{CDW}\ L^{-1}$ cells, constant between 2.4 and 4.8 $g_{CDW}\ L^{-1}$ cells and highest when 6 $g_{CDW}\ L^{-1}$ cells were used. In a reaction vessel with a diameter of 1 cm and at these high densities, self-shading is expected to reduce the reaction rate (Figure 2). This indicates that a higher cell density remediates toxicity effects on the cells. The increase of the cell density allowed a significant improvement of the productivity of up to 11.6 ± 1 U g_{CDW}^{-1}, which is comparable to activities obtained for ene-reductases,[10] Baeyer-Villiger monoogygenases,[9] AlkBGT[11,13] and CYP450 mono-oxygenases.[12]

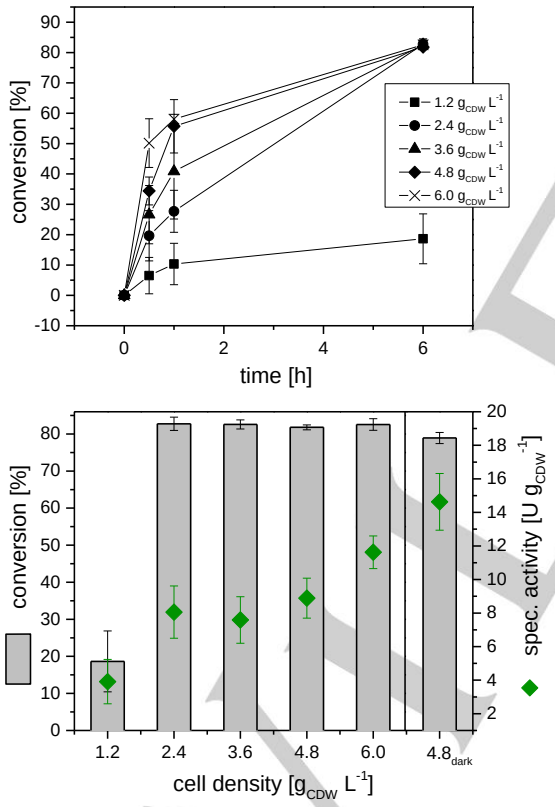


Figure 2. Conversion using recombinant *Synechocystis* harbouring P_{cpc}_IRED-A as biocatalyst at different cell densities. Top**:** Time course of the conversions; bottom: Specific activity from the same experiment (black dots) and total conversion after 6 h (bars). Standard deviation for three biological replicates is represented by error bars. Reaction conditions: 2 mL scale, 4 mM **1b**, 30 °C, 150 $\mu mol_{photons}\ m^{-2}\ s^{-1}$, in BG11 (pH = 8)

The control reaction with **1b** in darkness led to a specific activity in the same order of magnitude than the light-illuminated counterparts (Figure 2). This is not surprising as in case of the recombinant ene-reductase YqjM, cells could convert 5-7 mM in absence of light, while the conversion of a higher amount of substrate required illumination.[10] Accordingly, reactions under darkness with 8 mM **1a** showed some conversion, but with clearly lower activity and conversion compared to reactions under light (Supplementary Table S6). This can be attributed to the presence of storage carbohydrates that were produced during the cultivation under photoautotrophic conditions. As the toxicity of the imines prohibits increasing the substrate concentration above a few mM, it is difficult to differentiate whether NADPH-regeneration is fueled by photocatalytic water-splitting or the degradation of (photosynthetically produced) storage sugars. In both cases, however, the reaction is driven directly or indirectly by the photosynthetic system of *Synechocystis.*

Encouraged by the promising results for **1b** we were interested if the promoter exchange also increased activity of recombinant *Synechocystis* toward the other substrates, particularly for **1a** as this imine showed lowest toxicity in the plate assay. Indeed, 8 mM **1a** was completely converted within 3 h and the achieved specific activity of around 22 U g_{CDW}^{-1} was two magnitudes higher than that observed for P_{psbA2}-IRED-A (Table 2). Furthermore, activities were greatly improved for substrates **1c-f** with full conversion for **1c** and nearly full conversion of **1d** within 24 h. The results indicate that the amount of enzyme in the cell was the limiting factor in the initial screening. All biotransformations were performed in small scale batch modus, with shaking or stirring. In order to provide the cells with enough CO2, the head space inside the biotransformation tubes was even higher than 50 % of the total volume. Taking in consideration all these cultivation parameters, low product formations were attributed to the volatility of the products during biotransformations or a possible degradation of the product. The substrate scope of the whole-cell biocatalysts producing IRED-A with preference toward smaller imines is analogously obtained with purified enzyme or in *E. coli* as reported by Velikogne *et al*. [25]

We were pleased to find a high enantiomeric excess of up to 99% under optimized reaction conditions for compounds **2a**-**c** and **2f** as well as moderate *ee* for **2e**. Derivatization and volatility complicated the detection of 2a and 2b, therefore, ee% cannot be given with higher accuracy than >94%. The results nicely correspond to the selectivity of the isolated enzymes and in case of **2f** achieved purity was even higher (Supplementary Table S5).[25] The stereoselectivity of the recombinant cells underlines the usefulness of photosynthesis-driven biocatalysts for the synthesis of optical pure amines. The recombinant cyanobacteria fully reduced 8 mM **1a** within 3 h showing that our system is comparable to other systems which avoid organic cosubstrates,[17,30,31] and to biotransformations using hydrogen as electron donor such as the recently reported imine reduction coupled to a hydrogenase.[32] The main limitation of the reported system is the toxicity of substrates and products for *Synechocystis.* In principle, biphasic systems are applicable to cyanobacteria, [13] and it remains to be studied to which extent approaches such the one in micro-aqueous environment reported

by Maugeri *et al*. will be applicable to the imine reductases in cyanobacteria.[33] As the number of genetically amenable cyanobacterial strains is rapidly expanding, future research will focus on identifying strains less sensitive towards the toxicity of the reactants.

Table 2. Imine reduction of substrates **1a**-**1f** using P_{cpc}-IRED-A in recombinant *Synechocystis* The specific activity ($A_{spec.}$), the intial rate and the conversion (*C*) were calculated from the substrate depletion.

S	c(S) [mM]	$A_{spec.}$[a] [U g_{CDW}^{-1}]	T [h]	C[b] [%]	*ee* [%]	Product[c] [%]	Rate[a] [mM h^{-1}]
1a	4	19.5 ± 2.2	1 h 24 h	84 > 99	> 94 (*R*) > 94 (*R*)	82 65	4.8 ± 0.6
1a	8	21.8 ± 3.0	1 h 3 h 24 h	65 > 99 > 99	> 94 (*R*) > 94 (*R*)	63 88 66	6.3 ± 0.8
1b	4	8.9 ± 1.2	1 h 24 h	61 83	> 94 (*R*) > 94 (*R*)	49 81	2.6 ± 0.3
1c	5	5.5 ± 1.9	1 h 24 h	47 > 99	>99 (*R*) >99 (*R*)	N/A	1.4 ± 0.6
1d	4	10.2 ± 0.9	1 h 24 h	64 99	N/A	49 88	2.9 ± 0.3
1e	5	3.1 ± 1	1 h 24 h	18 94	71 (*R*) 63 (*R*)	13 67	0.9 ± 0.3
1f	5	1.6 ± 0.2	1 h 24 h	10 26	88 (*R*) 94 (*R*)	N/A	0.5 ± 0.1

[a] determined from the substrate decay after 0.5 h or 1 h

[b] determined from the substrate depletion

[c] product formation is based on product quantification by achiral GC-FID

Reaction conditions: 2 mL scale, 150 $\mu mol_{photons}$ m^{-2} s^{-1}, 30 °C, 3.6 g_{CDW} L^{-1}, in BG11 (pH = 8)

N/A = not analysed

In conclusion, we showed that recombinant cyanobacteria are promising hosts for imine reduction. Yet, high susceptibility of *Synechocystis* toward imine substrates and amine products is considered as main bottleneck for any synthetic application of the cyanobacterial imine reduction. Furthermore, dark control reactions indicated that the cofactor recycling might be largely fueled from storage compounds. The observed biotransformation is therefore not a light-driven reaction, but nevertheless does not need the addition of organic cosubstrates for cofactor regeneration. Our approach represents a simple system for highly selective imine reduction which we optimized by promoter exchange and increasing cell densities. Beside the excellent enantioselectivity, the highest specific activities of around 22 U g_{CDW}^{-1} and product formation rates of up to 6.3 mM h^{-1} for **1a** with full conversion within 3 h puts the use of photoautotrophs for cofactor recycling in line with other presently used systems and hosts and is a promising basis for further optimization.

Experimental Section

Detailed description to the used methods can be found in the supplements.

Cultivation of *Synechocystis* sp. PCC 6803
Synechocystis sp. PCC 6803 wildtype and mutant strains were cultivated under constant light intensity between 20-60 $\mu mol_{photons}$ m^{-2} s^{-1}, at 30 °C and 50 % humidity in BG11 or on BG11 agar plates. The medium for the mutants was supplemented with 50 µg mL^{-1} kanamycin. For biotransformations, cells were harvested at OD_{750} between 1 and 3 and concentrated to the respective density of the reaction.

Biotransformations using recombinant cells
All biotransformations (5 mL or 2 mL) with *Synechocystis* sp. PCC 6803 strains expressing heterologous IREDs harboring strains were performed in a photo-bioreactor at 30 °C, 140 rpm and with a light intensity of 150 $\mu mol_{photons}$ m^{-2} s^{-1}. The reaction was started by mixing BG11 medium (2.25 mL or 900 µL), substrate stock solution (100 mM in isopropanol or *N*,*N*-DMF, 250 µL or 100 µL) and cell suspension (OD_{750} = 30, 2.5 mL or 1 mL) in a glass vial (20 mL or 4 mL). The amount of concentrated cells was varied to obtain different ODs for biotransformations. Eight to ten sampling time points were spread over 40 h of biotransformation to monitor the reaction curve. Samples of the cell suspension (300 µl) were taken and the reaction was quenched by the addition of NaCl (1 spatula tip) and saturated Na_2CO_3 solution (200 µL). Till analysis, samples were frozen at -20 °C.

Biotransformations using wild-type cells
Control reactions with the wild type strain *Synechocystis* sp. PCC 6803 were performed in 1 mL total volume. Cell suspension (OD_{750} = 20, 500 µl), BG11 medium (450 µL) and substrate stock solution (100 mM in isopropanol or *N*,*N*-DMF, 50 µL) were mixed in glass vials (1.5 mL) and the biotransformations were performed at 30 °C in a photo-bioreactor with a light intensity of 150 $\mu mol_{photons}$ m^{-2} s^{-1}. Magnetic stirrers were used for stirring. After specific time points (0 h, 1 h, 6 h, 24 h) samples of the cell suspension (100 µL) were taken and immediately frozen at -20 °C.

Sample derivatization and extraction
Samples were extracted with equal volumes ethyl acetate (300 µL) containing acetophenone (2 mM) as internal standard and directly measured on GC FID and HPLC. Monocyclic amines **2a**, **2b** and **2c** were acetylated prior chiral GC analysis. Therefore, acetic anhydride (20 µL), saturated Na_2CO_3 solution (200 µL) and NaCl (1 spatula tip) were added to the organic phase and the suspension was mixed by inverting. After centrifugation (2 min, 13 000 *g*), the organic phase was dried with anhydrous K_2CO_3, vortexed briefly and centrifuged (2 min, 13 000 *g*). The organic phase of all other samples was dried on $MgSO_4$. Samples were analyzed on chiral GC FID.

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Keywords: biotechnology · enzyme catalysis · photobiocatalysis · photosynthesis · imine reductase

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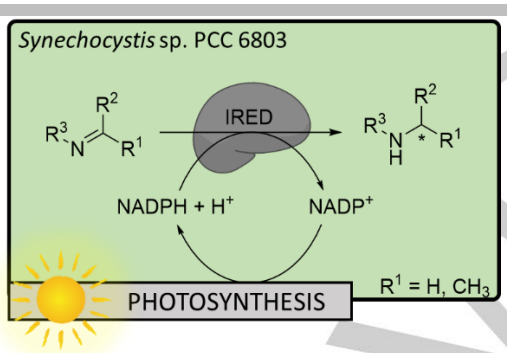
COMMUNICATION

Synechocystis sp. PCC 6803 harbouring recombinant IREDs can selectively reduce imines. With activities of up to 22 U g_{CDW}^{-1} and corresponding product formations of up to 6.3 mM h^{-1}, the system is comparable to other approaches avoiding the addition of organic cosubstrates for NADPH regeneration.



Hanna C. Büchsenschütz, Viktorija Vidimce-Risteski, Bettina Eggbauer, Sandy Schmidt, Christoph K. Winkler, Jörg H. Schrittwieser, Wolfgang Kroutil and Robert Kourist**

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