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Recombinant Cyanobacteria for the Asymmetric Reduction of C=C Bonds Fueled by the Biocatalytic Oxidation of Water

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Abstract: *A recombinant enoate reductase was expressed in cyanobacteria and used for the light-catalyzed, enantioselective reduction of C=C bonds. The coupling of oxidoreductases to natural photosynthesis allows asymmetric syntheses fueled by the oxidation of water. Bypassing the addition of sacrificial cosubstrates as electron donors significantly improves the atom efficiency and avoids the formation of undesired side products. Crucial factors for product formation are the availability of NADPH and the amount of active enzyme in the cells. The efficiency of the reaction is comparable to typical whole-cell biotransformations in E. coli. Under optimized conditions, a solution of 100 mg prochiral 2-methylmaleimide was reduced to optically pure 2-methylsuccinimide (99 % ee, 80 % yield of isolated product). High product yields and excellent optical purities demonstrate the synthetic usefulness of light-catalyzed whole-cell biotransformations using recombinant cyanobacteria.*

The catalytic reduction of C=C bonds is one of the most important redox transformations in organic chemistry.[1] Oxidoreductases (i.e. enzymes that catalyze redox reactions) are attractive alternatives to the established chemical reduction catalysts, especially when highly selective transformations are desired.[2] Enoate reductases (ERs), for example, catalyze the highly selective *trans*-hydrogenation of (conjugated) C=C bonds and thereby ideally complement the chemical catalysts.[3] One shortcoming of ERs, however, is that they are not as environmentally benign as commonly perceived. Chemical reductions of C=C bonds by hydrogenation (i.e. addition of H_2 to the starting material under metal catalysis) occur with perfect atom efficiency. ER-catalyzed reductions require stoichiometric amounts of reductants such as glucose, formate, or 2-propanol, whose redox equivalents are delivered indirectly to the enzymes through reduced nicotinamide cofactors. As a consequence, ER-catalyzed reactions are generally far from atom efficient and equimolar amounts of by-products (gluconolactone, CO_2, or acetone) are formed.[4] The use of water as a stoichiometric reductant would solve these issues. Recently, several initial steps en route to water-driven redox biotransformations by water oxidation using inorganic catalysts have been reported.[5] Natural photosynthesis is a highly optimized catalytic system for the light-driven oxidation of water[6] and accounts for the majority of organic matter found on this planet. The coupling of enzymatic redox processes to the photosystem in photosynthetic cells circumvents the use of organic molecules as electron donors as well as the addition of costly redox mediators. Light-driven whole-cell biocatalysis offers the usual advantages of microbial biotransformations, such as the ease to produce the biocatalyst and cofactor in one pot. Photosynthesis is particularly suitable and effective for the regeneration of NADPH, which is difficult to supply in heterotrophic organisms.

Endogenous enzymes from cyanobacteria have already been utilized successfully for biotransformations, such as the functionalization of monoterpenes,[7] and the synthesis of chiral phosphonates.[8] However, the application of wild-type organisms offers only limited potential for optimization. At the same time, the tremendous progress made in genetic manipulation of enzyme expression makes the generation of recombinant cyanobacterial strains straightforward.[9] Engineered cyanobacteria thus have the potential to conduct multiple types of biotransformations with different classes of oxidoreductases. Most importantly, cyanobacteria are relatively simple and much easier to manipulate than more complex photoautotrophic green algae or plants. We have previously shown that biocatalysts can be efficiently expressed in the cyanobacterium *Synechocystis* sp. PCC 6803 and that the cell components do not interfere with enantioselective transformations.[10] The cyanobacterium contains up to 200 copies of its genome, which makes the functional expression of an heterologous gene in high copy number straightforward.[9] For the proof-of-concept of a photosynthetic biotransformation with recombinant oxidoreductases, we report here the highly enantioselective reduction of C=C bonds by recombinant *Synechocystis* sp. PCC 6803 cells harboring the NADPH-dependent enoate reductase YqjM from *Bacillus subtilis* using the model substrates **1a–7a** (Scheme 1).[11]

The gene of the enoate reductase YqjM was isolated by PCR from genomic DNA from *Bacillus subtilis* and integrated into the genome of *Synechocystis* sp. PCC 6803 under the control of the light-induced promoter psbA2. This promoter allows high expression levels and can be activated

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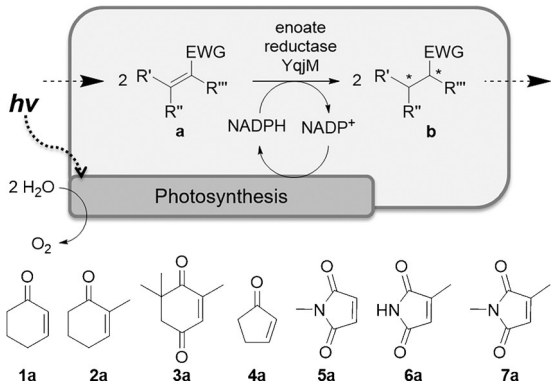

Scheme 1. Light-driven reduction of alkenes by enoate reductase YqjM using photosynthesis for cofactor regeneration in resting cells of the cyanobacterium *Synechocystis* sp. PCC 6803. EWG = electron-withdrawing group.

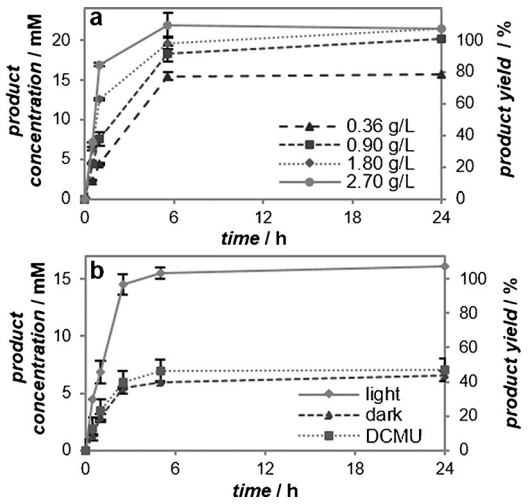

Figure 1. Formation of **7b** by asymmetric reduction of **7a** by ER YqjM expressed in *Synechocystis* sp. PCC 6803. a) Different cell densities (in $g\,L^{-1}$ cell dry weight). Substrate concentration: 20 mM. b) Conversion of **7a** under 150 μE light intensity and dark conditions and a light-exposed reaction with photosynthesis inhibitor DCMU at a substrate concentration of 15 mM and 1% ethanol (v/v). In all samples, the enantiopurity of the formed **7b** was >99% *ee*.

by high-intensity light.[10] Cyanobacterial cells harboring the ER YqjM showed a very promising conversion in the reduction of 2-methyl-*N*-methylmaleimide (**7a**) to (*R*)-2-methyl-*N*-methylsuccinimide (**7b**; Figure 1a). The data show a clear positive correlation between the cell density and conversion. In control experiments, *Synechocystis* cells without the recombinant gene did not show any measurable conversion (data not shown). The high optical purity of the formed **7b** (>99% *ee* in all experiments) is an additional indication that the reduction is clearly catalyzed by the enoate reductase. The cells were viable during a reaction time of 24 h. After that, high-intensity light of 300 μE led to a clear decrease in viable cells of the mutant overexpressing YqjM, which was not observed in the wild-type cells. As *Synechocystis* cells usually proliferate under light with a medium intensity of 150 μE, strategies for the induction of enzyme expression in cyanobacteria other than by induction with light,[8] and thus use of light with less intensity, can be expected to alleviate this limitation. The conversion rate of **7a** under visible light of 150 μE was significantly higher than that without light, thereby showing that the asymmetric synthesis of **7b** is indeed a light-dependent process (Figure 1b). The addition of the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) significantly reduced, but did not fully inhibit product formation. Similarly, some product formation (although at a markedly reduced rate) was observed under dark conditions. We attribute this observation to the production of carbohydrates by the cyanobacteria during their growth phase, which under "famine conditions" were consumed to regenerate NAD(P)H. Further experiments will be necessary to fully elucidate the underlying mechanism.

In any case, the reaction system allows full conversion without the need to add external sacrificial substrates or cofactors. Transformations carried out with different substrate concentrations of **7a** show the same initial rates, thus indicating saturation of the whole-cell catalyst until the diminishing substrate concentration slows down product formation (Figure 2a).

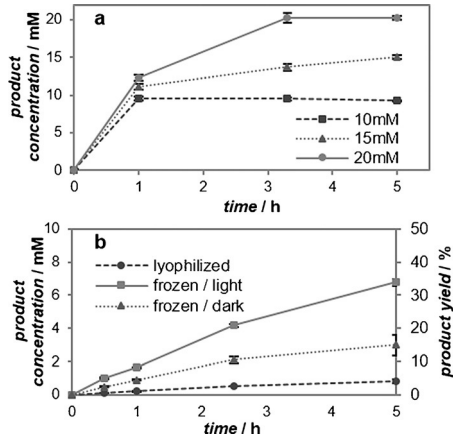

Figure 2. a) Reduction of **7a** at different concentrations. Cell density: 1.8 $g_{CDW}\,L^{-1}$. b) Stability of the cells towards different forms of storage. After dropping 1.8 $g\,L^{-1}$ cells into liquid nitrogen and storage at −20°C for 7 days, they showed activity towards a 20 mM solution of **7a**. The conversion rate in the presence of light (squares) was faster than in the dark (triangles), thereby indicating that the frozen cells retained the ability to perform photosynthesis.

To check the durability of the system, cyanobacterial cells were dropped into a solution of liquid nitrogen and stored at −20°C for 7 days. After being thawed, the cells catalyzed the stereoselective reduction of **7a** with a rate of 1.6 $mM\,h^{-1}$, thus partially retaining their initial activity (Figure 2b). The ease by which the catalysts can be stored underlines the practicability of the approach and the usefulness of cyanobacterial cells as catalysts.

Encouraged by these promising results, we further evaluated the substrate scope for the proposed photobiocatalytic reduction system (Table 1). We were pleased to see that a broad range of different starting materials was converted

Table 1: Light-catalyzed whole-cell biotransformation of different substrates using *Synechocystis* sp. PCC 6803 cells with recombinant enoate reductase YqjM at a cell density of 1.8 $g\,L^{-1}$.

Substrate	Substrate concentration [mM]	Time [h]	Specific activity[a,b] [$U\,g_{CDW}^{-1}$]	Max. conversion rate [$mM\,h^{-1}$][a]	Conversion[a] [%]	Space-time-yield [$g\,L^{-1}\,h^{-1}$]	Product concentration [$g\,L^{-1}$]	Cell productivity [$g\,g_{CDW}^{-1}$]
1a	15	4	39	4.1	70[d]	0.03	0.1	0.06
2a	15	24	21.1	2.2	42	0.03	0.71	0.4
3a	10	24	6.2	0.7	57	0.04	0.9	0.5
4a	15	24	25.6	2.7	99	0.5	1.2	0.72
5a	15	24	53.2	5.7	94	0.64	0.83	0.46
6a	20	3.3	90.9	9.6	99	0.67	2.24	1.24
6a	10[c]	1	123	10	99	1.1	1.1	0.62
7a	20	3	99.5	10.5	99	0.63	1.9	1.05

[a] Determined by gas chromatography. [b] Determined after 1 h. [c] 100 mg scale. [d] 0.5% formation of cyclohexanol was observed.

smoothly by the system. In several cases, depletion of the substrate was observed. A loss of up to 20% of the volatile substances **1b** and **2b** is likely due to evaporation. It is worth mentioning here that, with the exception of ketoisophorone **3a**, all the prochiral starting materials were converted with high enantioselectivity (Table 1). Comparative experiments with enantiopure levodione (*R*)-**3b**, however, showed that the product readily racemizes under the reaction conditions (data not shown).[3c]

Volumetric productivities of up to 10 $mM\,h^{-1}$ and product titers of up to 2 $g\,L^{-1}$ were obtained. Although this is still too low for industrial applications, it represents an encouraging starting point for further optimization. More importantly, the specific activity of the biocatalyst reached 100 $U\,g^{-1}$, thereby approaching activities required for industrial application. A preliminary calculation showed that the observed rate of product formation of 2 $mM\,h^{-1}$ with 1.8 $g_{CDW}\,L^{-1}$ corresponds to a turnover rate of 20000 s^{-1} per cell. A comparison of substrates **1a**–**3a** shows that small differences in the substrate structure have a strong impact on the specific activity. Increasing the space-time-yield and widening the substrate scope either by using different enoate reductases or by reaction engineering and enzyme engineering will be required to achieve a successful industrial application. His-tag purification enabled 8.4 mg enzyme to be purified from 1 g cells. The resulting total turnover number of 18750 per enzyme molecule indicates that the enantioselective reduction is indeed a catalytic process. To clarify the role of the amount of active enzyme, we compared the conversion of **6a** by two YqjM variants bearing different protein tags. The fusion of the N terminus of enoate reductases with different protein tags influences the folding and specific activity.[12] Cells with YqjM bearing a streptavidin-tag showed a specific activity towards **6a** of 12 $U\,g_{CDW}^{-1}$, while His-tagged YqjM showed a specific activity of 91 $U\,g_{CDW}^{-1}$. This comparison shows that the amount of active enzyme in the cell is an important factor for the activity of the biocatalyst.

Finally, we performed one photobiocatalytic reaction under semipreparative conditions. After three hours, a biotransformation of 100 mg **6a** resulted in 81 mg of optically pure (*R*)-**6b** (80% yield of isolated product, >99% *ee*).

The data show that the productivity of the photoautotrophic production system depends on three substrate-dependent factors, namely the activity of the overexpressed enzyme, the effectiveness of NADPH formation, and side reactions leading to the degradation of the substrate or product. The maximum rate of formation is clearly influenced by the enzyme concentration and its activity, the availability of the reduced cofactor NADPH, and the efficiency of the transport of the substrate and product across the cell membrane. Biotransformation experiments at different substrate concentrations show an initial maximum rate of product formation, thus indicating that the enzyme can work at full capacity under the optimized conditions.

At higher substrate concentrations, toxicity effects decrease the viability of the cells, which slows down the conversion. Furthermore, it is frequently observed in whole-cell biotransformations that the fed substrates and products are metabolized by the cellular metabolism. The targeted deletion of degrading pathways[13] or an optimization of the reaction conditions (as reported here for **6a**) can be used to circumvent this challenge.

To the best of our knowledge, this study presents the first example of the use of the cyanobacterial photosystem to fuel biotransformations with recombinant enzymes. Although visible-light-mediated photochemo-[14] and photobiocatalytic[5b,c,6a,15] reactions are a very promising concept, the strength of the reported approach is the simplicity and the ease of the production of the biocatalyst. In principle, the approach presented here can be extended to all oxidoreductases that use NADPH, NADH, or ferredoxin as the electron donor. The high conversion and enantioselectivity reported offer the possibility of using cofactor regeneration with the combination of water and light. The tremendous progress in the molecular biology of photosynthetic microorganisms provides the tools for fine-tuning the expression and thus further optimization. Despite very promising examples of the application of cyanobacteria as producers of organic molecules,[16] product titers are usually in the $mg\,L^{-1}$ range. In contrast, whole-cell photo-biotransformations are possible with several $g\,L^{-1}$. Although the product concentrations and space-time-yields reported here are rather low for industrial processes, the system has not yet been optimized. Standard molecular biology tools such as controllable promoters that are currently developed for *Synechocystis* offer considerable potential for improvement. Moreover, cyanobacteria utilize only a small part of the light energy because they prioritize "photosynthetic robustness", that is, protection strategies

against sudden changes in light availability. As these protection systems are not necessary for cultivations under controlled conditions, the photosynthetic electron pathways have considerable potential for streamlining, which will further increase the availability of reduced cofactors for synthetically useful biotransformations. The use of recombinant cyanobacteria as catalysts is a first step in this direction.

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