catalysts

MDPI

Communication

Enzymatic Oxyfunctionalization Driven by Photosynthetic Water-Splitting in the Cyanobacterium *Synechocystis* sp. PCC 6803

Stefanie Böhmer [1], Katharina Köninger [1], Álvaro Gómez-Baraibar [1], Samiro Bojarra [1], Carolin Mügge [1], Sandy Schmidt [2], Marc M. Nowaczyk [3] and Robert Kourist [1,2,*]

1 Junior Research Group for Microbial Biotechnology, Ruhr-Universität Bochum, Universitätsstr. 150, 44801 Bochum, Germany; stefanie.boehmer@rub.de (S.B.); katharina.koeninger@rub.de (K.K.); Alvaro.Baraibar@rub.de (A.G.-B.); samiro.bojarra@rub.de (S.B.); Carolin.Muegge@rub.de (C.M.)

2 Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, 8010 Graz, Austria; s.schmidt@tugraz.at

3 Chair of Plant Biochemistry, Ruhr-Universität Bochum, Universitätsstr. 150, 44801 Bochum, Germany; Marc.M.Nowaczyk@rub.de

* Correspondence: kourist@tugraz.at; Tel.: +43-316-873-4072

Received: 8 July 2017; Accepted: 10 August 2017; Published: 17 August 2017

Abstract: Photosynthetic water-splitting is a powerful force to drive selective redox reactions. The need of highly expensive redox partners such as NADPH and their regeneration is one of the main bottlenecks for the application of biocatalysis at an industrial scale. Recently, the possibility of using the photosystem of cyanobacteria to supply high amounts of reduced nicotinamide to a recombinant enoate reductase opened a new strategy for overcoming this hurdle. This paper presents the expansion of the photosynthetic regeneration system to a Baeyer–Villiger monooxygenase. Despite the potential of this strategy, this work also presents some of the encountered challenges as well as possible solutions, which will require further investigation. The successful enzymatic oxygenation shows that cyanobacterial whole-cell biocatalysis is an applicable approach that allows fuelling selective oxyfunctionalisation reactions at the expense of light and water. Yet, several hurdles such as side-reactions and the cell-density limitation, probably due to self-shading of the cells, will have to be overcome on the way to synthetic applications.

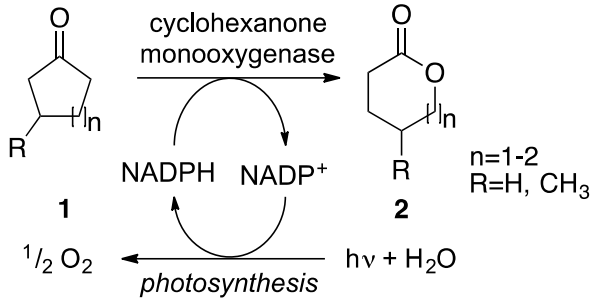
Keywords: photosynthesis; catalytic water-splitting; Baeyer–Villiger oxidation; cyanobacteria; recombinant enzyme expression

1. Introduction

With an increasing awareness of global warming and a foreseeable depletion of fossil resources, the demand for clean synthetic processes has soared. Redox transformations belong to the most important reactions in organic synthesis. Oxidoreductases, such as oxygenases, alcohol dehydrogenases, amine dehydrogenases, and ene-reductases selectively catalyse the introduction and modification of functional groups with often outstanding selectivity and under mild reaction conditions [1]. All these reactions require reduced electron donors, mostly nicotinamide cofactors. In synthetic applications, these external cofactors are recycled by using energy-rich organic molecules such as isopropanol or glucose as electron donors. Usually, only a small fraction of the electrons of these sacrificial cosubstrates are utilized, resulting in a poor atom efficiency. The limited stability of many oxidoreductases outside living cells and their often modular structure require their application as whole cells in biotransformations. Here, glucose is usually supplied as a sacrificial substrate for the cofactor-recycling of NADPH. The often-used glucose-6-phosphate dehydrogenase utilises only

two electron pairs from each glucose molecule. Degradation of the resulting 6-phosphogluconolactone in the catabolism leads to a significant formation of NADH and subsequent respiration. A typical example of the poor efficiency is in the recent synthesis of the polymer precursor ε-caprolactone in resting cells of *E. coli*, in which glucose had to be added in stoichiometric amounts [2]. Several alternative solutions for this challenge are currently under consideration. Coupling oxidative and reductive steps to redox-neutral enzyme cascades [3] is a very promising approach, and it allows for potential savings also in whole-cell biotransformations. However, this is not applicable for reactions without neutral redox balance or with decoupling, i.e., the need of an excess of redox cofactor. Autotrophic and chemolithotrophic metabolic organisms have recently received attention as they are capable of utilizing inorganic compounds as electron donors and thus would present a generally applicable method. Microalgae and cyanobacteria are already used in industry, and their cultivation is commercially viable [4]. Phototrophic microorganisms have been successfully engineered for the production of organic molecules such as 1-butanol, 2,3-butanediol and isopropanol, ethanol, and medium-chain fatty acids with titers up to several grams per litre [5,6]. A key factor for any synthetic application of cyanobacteria is the efficiency of light utilization [7]. Catalytic water splitting and photosynthetic electron transport (PET) between photosystems II and I use a total estimated 17% of the absorbed energy, resulting in a synthesis of the metabolites NADPH and ATP, with 12% total efficiency. The photosystem one (PS I) produces reduced ferredoxin (Fd_{red}). Two Fd_{red} reduce one molecule of NADPH via ferredoxin-NADPH reductase (FNR) [8]. Coupling whole-cell redox reactions to photosynthesis allows for the substitution of organic cosubstrates using water-splitting as the stoichiometric reductant [9,10].

The feasibility to recycle NADPH with photoautotrophic organisms has an outstanding potential to provide large amounts of the cofactor NADPH. The feasibility to recycle NADPH via photosynthesis by *Synechococcus elongatus* PCC7942 has been shown with endogenous alcohol dehydrogenases acting on aryl-aliphatic ketones [9,10]. With other cyanobacterial strains, the feasibility to reduce aldehydes [11] and phosphonates [12] was also shown. We have recently shown that this approach can be also extended toward recombinant enzymes by introducing a bacterial ene-reductase into the cyanobacterium *Synechocystis* sp. PCC6803. The reaction proceeded fast, and was not hampered by side-reactions [13]. We were interested whether the concept can be extended towards the utilization of NADPH for selective oxyfunctionalisation reactions, and if side-reactions and physiological side-effects might be a problem for a catalytic application. In order to test the performance of the system, one of the most widely used Baeyer–Villiger monooxygenases (BVMO), the NADPH-specific cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 was chosen as a model (Scheme 1) [2,14].



Scheme 1. Enzymatic Baeyer–Villiger oxidation coupled to recycling of the cofactor NADPH by photosynthesis.

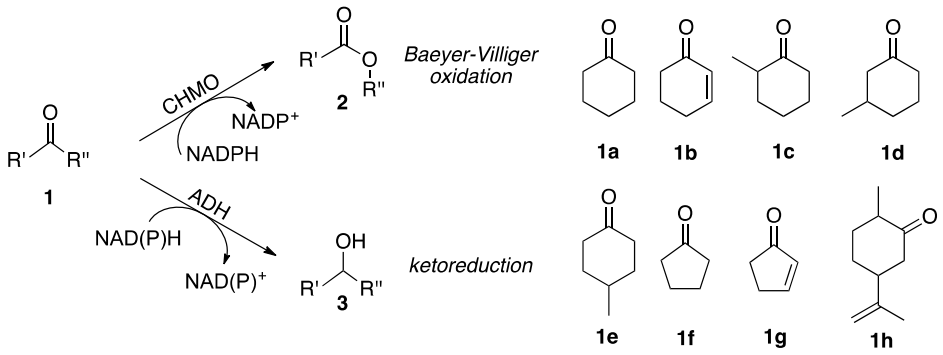
2. Results

2.1. Cloning and Expression of CHMO in Synechocystis sp. *PCC 6803*

The gene *chmo*, encoding the Baeyer–Villiger Monooxygenase with an N-terminal hexahistidine tag was inserted into a cassette under the light-induced promoter psbA2, and transformed into the naturally competent cyanobacterial cells. Homologous recombination occurs in the upstream and downstream region of the gene loci *slr0168*, resulting in a mutant carrying the gene and the resistance gene for kanamycin. As *Synechocystis* contains several hundred genome copies, we cultivated the mutant strain with increasing concentrations of the antibiotic kanamycin. After several cycles of segregation, a polymerase chain reaction showed the presence of the inserted cassette and the absence of the wildtype gene, which confirmed that the strain was homozygous (Figures S1 and S2). Soluble expression in *Synechocystis* was verified by SDS-PAGE analysis (not shown).

2.2. Substrate Spectrum and Side-Reactions

Growing *Synechocystis* cells harboring the CHMO were harvested in the exponential phase, separated by centrifugation and carefully resuspended in an aqueous medium (BG11) to a final optical density (at 750 nm) ranging from 2 to 15 and subjected to biotransformation reactions of the cyclic ketones 1a–g (Scheme 2) under constant illumination at 300 µE m^{-2}. After the addition of cyclohexanone 1a (15 mM), we were pleased to detect product formation of ε-caprolactone 2a within 24 h reaction time. However, the reaction did not proceed to complete conversion, and we observed the formation of a side-product, which was identified as cyclohexanol. Using wildtype cells without the recombinant BVMO, we did not observe any measurable oxidation of any of the ketones.



Scheme 2. Ketoreduction by endogenous alcohol dehydrogenases as side-reaction during the enzymatic Baeyer–Villiger oxidation in *Synechocystis* cells.

Ketoreduction by endogenous alcohol dehydrogenases [12], and the selective reduction of cinnamyl aldehyde in *Synechocystis* have been reported before [11]. Indeed, we also found that wildtype *Synechocystis* cells without the *chmo* gene catalysed the reduction in 1a and 1c–f to the corresponding alcohols. Previously, we used several cyclohexene derivatives similar to 1b as model compounds for the characterisation of the enereductase YqjM in *Synechocystis* [13] without observing ketoreduction. This is not surprising because wildtype *Synechocytis* cells did not reduce 1b. Interestingly, ketones 1f,g were not converted by the wildtype cells. A possible explanation is that the substrate spectrum of a strongly expressed alcohol dehydrogenases in *Synechocystis* does not include the unsaturated cyclic ketone 1b, and presents no activity towards the five-membered rings, such as 1f,g. 1b was not converted by cells harboring the CHMO. Using 1h as a substrate led to cell death within a few hours and is obviously toxic for *Synechocystis*. Interestingly, the ketoreduction proceeded faster in the *Synechocystis* wildtype than with the competing CHMO-reaction (Table 1), demonstrating the competing effect of both electrons sinks. We were pleased to find that cyclo-pentanone 1f was converted completely to δ-valerolactone 2f within 48 h without measurable alcohol formation. This opened the possibility to characterise the

Baeyer–Villiger oxidation in cyanobacteria without competing side-reactions. In biotransformation reactions with a substrate concentration of 5 mM, the methyl-substituted ketones 1c–e were converted with a specific activity between 2 to 5 U/g_{CDW} (Tables 1 and 2). This activity is a result of the Baeyer–Villiger oxidation and the competing ketoreduction. In the oxidation of cyclopentanone 1f, *Synechocystis* cells harbouring CHMO showed a specific activity of 2.3 U/mg (Table 2).

Table 1. Conversions for cyanobacterial ketoreduction by endogenous alcohol dehydrogenases with methylated cyclic ketone substrates.

Entry	Substrate	Side-Product Product	*Synechocystis* WT (%) [1]	*Synechocystis* Cyclohexanone Monooxygenase (CHMO) (%) [1]
1	1c	2c	0	48
		3c	35	23
2	1d	2d	0	72
		3d	16	8
3	1e	2e	0	82
		3e	47	8

[1] based on substrate consumption determined by gas chromatography. Reaction conditions: 5 mM substrate concentration, 24 h, 30 °C.

Table 2. Cyanobacterial Baeyer–Villiger Oxidation of cyclic ketones.

Entry	Substrate	Specific Activity [1] (U/g_{DCW} [2])	Ketoreduction (%)
1	1a	2.3 ± 0.05	50%
2	1b	n.c. [3]	-
3	1c	2.0 ± 0.09	30%
4	1d	2.94 ± 0.05	25%
5	1e	5.73 ± 0.02	<5%
6	1f	2.3 ± 0.06	0%
7	1g	n.c.	-
8	1h	n.c.	-

[1] based on substrate consumption determined by gas chromatography; [2] DCW: Dry cell weight; [3] n.c.: no conversion.

Within 48 h, 1f was completely converted to 2f. The formation of 2f proceeded continuously over a time course of two days. Moreover, cells could be re-cultivated after the biotransformation. This steady activity indicates that the biotransformation does not have a major negative effect on the viability of the cells.

Biotransformations at different substrate concentrations showed that the formation of 2f was faster at increasing substrate concentrations (Figure 1). At substrate concentrations beyond 5 mM, no further significant increase was observed, indicating saturation of the cells.

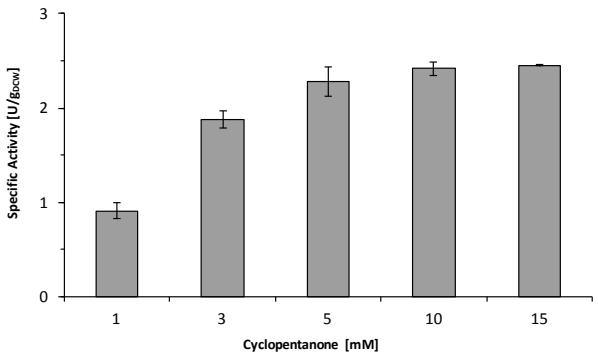


Figure 1. Biotransformation of 1f at different substrate concentrations by cells of *Synechocystis* sp. PCC6803 harboring CHMO under light of 300 µE m^{-2} and a cell concentration of 1.8 g/L.

2.3. Influence of the Light Availability

Figure 2 shows the time course of the Baeyer–Villiger oxidation of 1f. Cells in the absence of light showed only low conversions over 48 h (formation of approximately 0.4 mM 2f), which was attributed to the presence of storage sugars such as glycogen that provides energy for the synthesis of NADPH in catabolic pathways [15]. Addition of the photosynthesis inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) led to a measurable decrease of specific activity, underlining that photosynthesis is the major driving force for the reaction. This confirms the previous observation of a residual cofactor recycling either in darkness or in the presence of a photosynthesis inhibitor for biotransformations using endogenous alcohol dehydrogenases in *Synechocystis* [9].

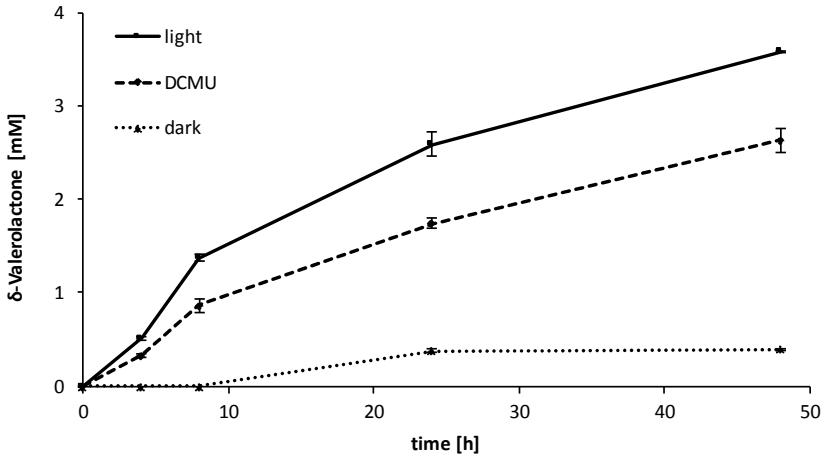


Figure 2. Biotransformation of cyclopentanone 1f (5 mM) by cells of *Synechocystis* sp. PCC6803 harbouring CHMO under light of 300 µE m^{-2} (solid line), under light in presence of the inhibitor DCMU (dashed line) and under darkness (pointed line).

Self-shading of the cells reduces their exposure to light and thus the photosynthetic activity. Therefore, we compared the reaction rate with different cell densities. While an increased amount of catalyst should lead to a higher activity, self-shading leads to lower activities at higher cell densities. Figure 3 shows the initial rates towards a solution of 1f at different cell densities. Above a cell density of 10, the initial rate does not increase, indicating a self-shading of the cells.

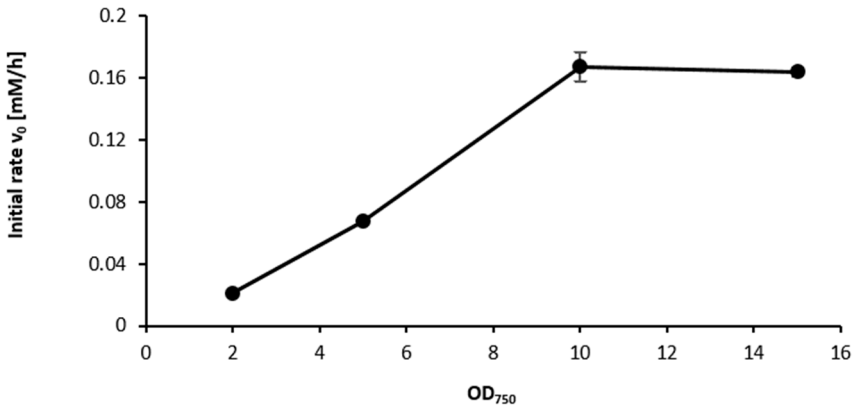


Figure 3. Biotransformation of a solution of cyclopentanone 1f (5 mM) by different amounts of *Synechocystis* sp. PCC6803 cells harbouring CHMO under light of 300 µE m^{-2}.

3. Discussion

A recombinant Baeyer–Villiger oxidase catalysed the oxidation of a series of cyclic ketones. The observation of a drastically reduced reaction rate under darkness demonstrates that the majority of the redox equivalents indeed originate from photosynthesis. This was sustained in a control experiment with a photosynthesis inhibitor that also decelerated the reaction, albeit to a lower extent. The specific reaction rates determined in this study of 2–5 U/g_{CDW} are similar to those achieved with the same enzyme in *E. coli* [2]. The possible self-shading of the cells, however, requires to operate at cell densities of a few g/L, which limits the resulting space-time yield.

It is known that *Synechocystis* harbours alcohol dehydrogenases that catalyse the reduction of ketones, which was recently exploited for the asymmetric synthesis of chiral phosphonates [12], and the selective reduction of cinnamyl aldehyde [11]. Hölsch et al. demonstrated that an alcohol dehydrogenase from *Synechococcus* sp. strain PCC7942 [16] shows a strong preference for NADPH. Therefore, exposure of the cells to high-intensity light supplies redox equivalents for heterologous enzymes on the one hand, but on the other hand also provides optimal conditions for alternative redox reactions, catalysed by host enzymes. We have shown that the percentage of undesired ketoreduction strongly depends on the substrate, however, full conversion of 1f to 2f was achieved with no detectable ketoreduction within 48 h. Notwithstanding, future research will show the possibility to inactivate endogenous alcohol dehydrogenases without impairing the integrity of the cells.

Cyanobacterial cells harbouring cyclohexanone monooxygenase showed specific activities in the range of 2–5 U/g_{CDW}. While this shows the sum of the Baeyer–Villiger oxidation and the ketoreduction, it indicates the amount of NADPH that was used for substrate conversion. Cyclopentanone was converted with a specific activity of 2.3 U/g_{CDW}. Expressing the ene-reductase YqjM in *Synechocystis* cells, we have previously obtained specific activities of up to 100 U/g_{CDW}, which clearly shows that the NADPH concentration provided by the cells can not be a limiting factor for the BVMO reaction [13]. While the cyanobacterial Baeyer–Villiger oxidation is substantially slower, we would like to emphasise that it is in the same order of magnitude as the same enzyme in *E. coli*, where 3.5 U/g_{CDW} were achieved [2].

Our results show the possibility to conduct oxyfunctionalization reactions in cyanobacteria harbouring recombinant oxidoreductases. This successfully shows that catalysts can be produced from carbon dioxide, water, salts, and light, and that the recycling of nicotinamide cofactors without the requirement of a stoichiometric addition of an organic cosubstrate is possible with this approach. While the specific activity is already comparable to heterotrophic organisms, the cell-density limitation requires a substantial increase in the view of synthetic applications. While the NADPH-metabolism is tightly regulated in heterotrophic organisms, cyanobacteria have overflow systems that protect them against fluctuating light.

4. Materials and Methods

4.1. General

All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany). The cell dry weight (CDW) was determined by lyophilising cell suspensions with different OD_{750} values. An OD_{750} of 10 corresponded to 1.8 g L^{-1} CDW.

4.2. Cloning and Transformation of Synechocystis

A plasmid containing CHMO (pET28a-His-CHMO) was kindly provided by Prof. Dr. Uwe Bornscheuer (Ernst-Moritz-Arndt-Universität Greifswald, Germany), inserted directly into the shuttle-vector SynRekB (Nowaczyk) to yield the vector SynRekB His-CHMO.

For homologous recombination, wildtype cells were cultivated into the exponential phase, and harvested at an optical density of OD_{750} = 2. The cells were resuspended in BG-11 medium (1 mL) and subjected to 3 aliquots of a solution containing the vector (100 µL, 5 µg). The cells were incubated at

30 °C for 6 h in darkness, and for 20 h at low-intensity light conditions (60 µmol photons m^{-2} s^{-1}). After incubation, the cells were plated out on agar plates containing the antibiotic kanamycin (50 µg mL^{-1}) for four weeks at 30 °C, under low-light conditions.

For segregation, 10 mL of a *Synechocystis* culture (OD_{750} = 2) were centrifuged (3800× *g*, 10 min, RT) and resuspended in 1 mL fresh BG11-medium enriched with glucose (5 mM). DNA (5 µg mL^{-1}) was added to 100 µL cell suspension. After regeneration for 5 h in darkness, the aliquots were illuminated under low-light conditions for an additional 24 h and subsequently plated onto BG11-plates containing kanamycin (50 µg mL^{-1}). Clones were transferred to plates with higher antibiotic concentration until full segregation was achieved.

4.3. Biotransformation Experiments

After cultivation, cells were concentrated by centrifugation (3800× *g*, 5 min). Subsequently, the OD_{750} was adjusted to 10 (or to another desired value) by diluting the cell suspension with fresh BG11 medium. The reaction was performed in 1.5 mL glass GC vials with a reaction volume of 1.5 mL or in reaction tubes of 15 mL. After addition of the respective substrate, the vials were placed at a constant distance of 3 cm from an LED lamp (10 W, 700 lm, coldwhite, MENGS®) providing 150 µE of light (Figure S3). The temperature was set to 30 °C by a water bath. Stirring was provided by magnetic bars inside each reaction vial (100 rpm). 250 µL samples were taken from the reaction vials at different time points, and were extracted with ethyl acetate (500 µL) containing 0.2% (*v*/*v*) octanol as injection standard. After extraction, the samples were centrifuged (17,000× *g*, 2 min), the organic phase dried with anhydrous $MgSO_4$, and transferred to a GC vial. Conversion rates were analysed by gas chromatography on a Shimadzu GC Plus 2010 device using a FS-Hydrodex-β-6TBDM column (Macherey-Nagel, Düren, Germany), with a method operating at an 80 °C isotherm, with an injection split of 1/20.

Supplementary Materials: The following data are available online at www.mdpi.com/2073-4344/7/8/240/s1, Figure S1: Confirmation of complete segregation; Figure S2: Transformation of *Synechocystis* cells by homologous recombination and segregation; Figure S3: Schematic representation (a) and picture (b) of the light-reactor used for the *Synechocystis* biotransformations on analytical scale.

Acknowledgments: The Ministry of Culture and Science of the Federal State of North Rhine-Westphalia (grant number PtJ-TRI/1411ng006) and the Austrian Centre of Industrial Biotechnology (grant number 12.101) are gratefully acknowledged for financial support.

Author Contributions: Robert Kourist, Sandy Schmidt and Marc M. Nowaczyk conceived and designed the experiments; Stefanie Böhmer, Samiro Bojarra, Carolin Mügge, Katharina Köninger and Álvaro Gómez-Baraibar conducted experiments and analysed the data; Robert Kourist, Álvaro Gómez-Baraibar and Sandy Schmidt wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

1. Hollmann, F.; Arends, I.W.C.E.; Buehler, K.; Schallmey, A.; Bühler, B. Enzyme-mediated oxidations for the chemist. *Green Chem.* **2010**. [CrossRef]
2. Schmidt, S.; Scherkus, C.; Muschiol, J.; Menyes, U.; Winkler, T.; Hummel, W.; Gröger, H.; Liese, A.; Herz, H.G.; Bornscheuer, U.T. An enzyme cascade synthesis of ε-caprolactone and its oligomers. *Angew. Chem. Int. Ed.* **2015**, *54*, 2784–2787. [CrossRef] [PubMed]
3. Kara, S.; Spickermann, D.; Schrittwieser, J.H.; Leggewie, C.; van Berkel, W.J.; Arends, I.W.; Hollmann, F. More efficient redox biocatalysis by utilising 1, 4-butanediol as a 'smart cosubstrate'. *Green Chem.* **2013**, *15*, 330–335. [CrossRef]
4. Abed, R.M.; Dobretsov, S.; Sudesh, K. Applications of cyanobacteria in biotechnology. *J. Appl. Microbiol.* **2009**, *106*, 1–12. [CrossRef] [PubMed]

5. Oliver, J.K.; Atsumi, S. Metabolic design for cyanobacterial chemical synthesis. *Photosynth. Res.* **2014**, *120*, 249–261. [CrossRef] [PubMed]
6. Formighieri, C.; Melis, A. A phycocyanin phellandrene synthase fusion enhances recombinant protein expression and β-phellandrene (monoterpene) hydrocarbons production in synechocystis (cyanobacteria). *Metab. Eng.* **2015**, *32*, 116–124. [CrossRef] [PubMed]
7. Nowaczyk, M.; Rexroth, S.; Rögner, M. *Biotechnology*; De Gruyter: Berlin, Germany, 2015.
8. Mutoh, R.; Muraki, N.; Shinmura, K.; Kubota-Kawai, H.; Lee, Y.-H.; Nowaczyk, M.M.; Rögner, M.; Hase, T.; Ikegami, T.; Kurisu, G. X-ray structure and nuclear magnetic resonance analysis of the interaction sites of the ga-substituted cyanobacterial ferredoxin. *Biochemistry* **2015**, *54*, 6052–6061. [CrossRef] [PubMed]
9. Nakamura, K.; Yamanaka, R. Light-mediated regulation of asymmetric reduction of ketones by a cyanobacterium. *Tetrahedron Asymmetry* **2002**, *13*, 2529–2533. [CrossRef]
10. Nakamura, K.; Yamanaka, R. Light mediated cofactor recycling system in biocatalytic asymmetric reduction of ketone. *Chem. Commun.* **2002**, 1782–1783. [CrossRef]
11. Yamanaka, R.; Nakamura, K.; Murakami, M.; Murakami, A. Selective synthesis of cinnamyl alcohol by cyanobacterial photobiocatalysts. *Tetrahedron Lett.* **2015**, *56*, 1089–1091. [CrossRef]
12. Górak, M.; Żymańczyk-Duda, E. Application of cyanobacteria for chiral phosphonate synthesis. *Green Chem.* **2015**, *17*, 4570–4578. [CrossRef]
13. Köninger, K.; Gómez Baraibar, Á.; Mügge, C.; Paul, C.E.; Hollmann, F.; Nowaczyk, M.M.; Kourist, R. Recombinant cyanobacteria for the asymmetric reduction of C=C bonds fueled by the biocatalytic oxidation of water. *Angew. Chem. Int. Ed.* **2016**, *55*, 5582–5585. [CrossRef] [PubMed]
14. Beier, A.; Bordewick, S.; Genz, M.; Schmidt, S.; van den Bergh, T.; Peters, C.; Joosten, H.J.; Bornscheuer, U.T. Switch in cofactor specificity of a baeyer–villiger monooxygenase. *ChemBioChem* **2016**, *17*, 2312–2315. [CrossRef] [PubMed]
15. Gründel, M.; Scheunemann, R.; Lockau, W.; Zilliges, Y. Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium *Synechocystis* sp. PCC 6803. *Microbiology* **2012**, *158*, 3032–3043. [CrossRef] [PubMed]
16. Hölsch, K.; Havel, J.; Haslbeck, M.; Weuster-Botz, D. Identification, cloning, and characterization of a novel ketoreductase from the cyanobacterium *Synechococcus* sp. strain PCC 7942. *Appl. Environ. Microbiol.* **2008**, *74*, 6697–6702. [CrossRef] [PubMed]