



MASTERARBEIT | MASTER'S THESIS

Titel | Title

Establishment of a biocatalytic cascade in *Synechocystis* sp.
PCC6803

verfasst von | submitted by

Christian Walzl BSc

angestrebter akademischer Grad | in partial fulfilment of the requirements for the degree of
Master of Science (MSc)

Wien | Vienna, 2024

Studienkennzahl lt. Studienblatt | Degree
programme code as it appears on the
student record sheet:

UA 066 834

Studienrichtung lt. Studienblatt | Degree
programme as it appears on the student
record sheet:

Masterstudium Molekulare Biologie

Betreut von | Supervisor:

Florian Rudroff

Acknowledgements

First, I would like to thank Florian Rudroff for welcoming me so warmly into the group and for the possibility to work on such an interesting topic. Further thanks go to Marko Mihovilovic, Christian Stanetty and Michael Schnürch for their input and for creating a positive work environment.

Furthermore, I would like to thank Julia “Großgrundbesitzerin” Jodlbauer for supervising my work and countless other things: her calm demeanour, her infectious good mood (even the COVID precautions did not aid in containment), her wits and her generally positive mindset against which my fatalism could hardly compete. Her inputs regarding my work were always much appreciated, which I do not just mention to save me from a woman who knows how to use bow and arrow. Julia if you read this in your mansion in front of a cozy fire with Paul by your side remember to throw a housewarming party so I too get to enjoy your luxurious lifestyle (if only for a day). Speaking of luxurious, I would also like to express my gratitude to Lydia “Lüdlü” Suchy, whose facial expressions range from “Vogue cover model” over “Uncle Sam wants you” all the way to “expressions you see in a Jim Carrey movie” and thus consistently elevate a comical situation from mediocrity to hilarity. Whilst discussing hilarity one must, of course, also speak of Thomas “heute ist Feiertag???” Rohr, someone so in love with what he does, that he frequently shows up to work although that day is a holiday. Judging by his work ethic you guessed right, he is indeed German, a fact on which I blame his extremely dry dad jokes, which have no business being as entertaining as they are. But dry dad jokes turn out to be a very competitive field, in which Michael “Steininger” Steinacher also excels. Sitting simultaneously with both at a table is truly an experience. Helping me to experience the marvel of chemistry, I would like to thank Stefan “Gyrosstakis” Giparakis and Astrid “Aschtrid” Schiefer in the hope that the hit “Schiefern” will live on long beyond my time in the lab. I also wish Stefan “Ze män” Zeman all the best, hoping that Verena Scheibelreiter will be a bit more careful in future “Jijitzn” sessions, the man knows why he prefers training with me. Staying fit is also a big theme for Frederik “Freddyrick” Mortzfeld, who not only sponsors my fitness center visits, but often, in combination with Maximilian “Da Wutschah” Wutscher, nullifies them with a few beers, much to my delight. The dynamic duos Nina “El Nino” Biedermann with Johanna Templ and Viktor “Big boy” Savic with Dominik Schnalzer have my gratitude for discussions about life and putting up with my humour. Next, I would like to apologize to Richard “Richy Rich” Fried, who may be delighted to hear that I now own fitting pants for my suit, meaning I will not turn up looking like a child in a grown man’s suit to his next wedding. Thanks also to the many other members of the lab: Simon Leonhartsberger, Katharina Schlögl, Eleni Papaplioura, Farooq Zia, Björn Bielec, Christoph Suster, Nanditha Narayanan, Raheleh Pourkaveh, Jorge Gonzalez Rodriguez, Hubert “Hubsi” Kalas and Sebastian “Hetschi” Heckl. I tried to keep this to a single page, you know I appreciate you.

Now it is time to thank my family and friends for their never-ending support in whatever I do and the humour with which they express it. I sincerely do not take it as granted.

Ich möchte meinem Vater an dieser Stelle noch einmal explizit auf Deutsch danken, er hat es sich verdient.

Table of Contents

1. Abbreviations	4
2. Abstract	7
3. Deutsche Zusammenfassung	8
4. Introduction	9
4.1. Cyanobacteria	9
4.2. Biocatalysis	10
4.2.1. Biocatalytic cascade reactions	11
4.2.2. Biocatalysis in <i>Synechocystis</i> sp. PCC6803	13
4.3. Designing enzymatic cascades	14
4.4. Challenges of protein expression in whole cell approaches	15
4.5. Challenges of biocatalytic <i>in vivo</i> cascades	15
4.6. Redox reactions and enzymes used in biocatalysis	16
4.6.1. Alcohol dehydrogenases (ADHs)	17
4.6.2. Enoate reductases (EREDs)	18
4.6.3. Baeyer-Villiger monooxygenases (BVMOs)	19
5. Scope of this Thesis	21
6. Results and Discussion	22
6.1. Assessing the impact of a GFP tag on the functioning of enzymes used in the cascade	22
6.1.1. Level T cloning and transformation in <i>E. coli</i>	22
6.1.1.1. Discussion	25
6.1.2. Biotransformation of sfGFP tagged versus untagged enzymes	26
6.1.2.1. Discussion	30
6.2. Generation of cascade	31
6.2.1. Construct design	31
6.2.2. Cloning and transformation	31
6.2.2.1. Level 0	32
6.2.2.2. Level 1	35
6.2.2.3. Level T	36
6.2.2.4. Discussion	38
6.2.3. Transformation of <i>Synechocystis</i> spp. PCC6803	38
6.2.3.1. Discussion	39
6.3. Biotransformations utilizing the generated strains	40
6.3.1. Pilot biotransformations in <i>E. coli</i>	40
6.3.1.1. Discussion	44
6.3.2. Test biotransformation of LfSDR1M50 and cascade using substitute substrates	44
6.3.2.1. Discussion	46
6.3.3. Assessment of cascade in <i>Synechocystis</i>	47
6.3.3.1. Discussion	51
6.3.4. Attempt at isolating the unknown substance from whole cell <i>Synechocystis</i>	52
6.3.4.1. Discussion	55
7. Conclusion and Outlook	55
8. Materials and Methods – General Preparations	57
8.1. Antibiotic stock solutions	57
8.2. Stock solutions for use in biotransformations	57
8.3. Further stock solutions	58
8.4. Standard media and buffers for <i>Synechocystis</i> spp. PCC6803	58
8.5. Standard media and buffers for <i>Escherichia coli</i>	61
9. Materials and Methods – General Procedures	65
9.1. Organism invariant methods	65

9.1.1. Dilution of Primers	65
9.1.2. Agarose Gel Electrophoresis.....	65
9.1.3. SDS-PAGE	66
9.1.4. Sample preparation for Gas chromatography	66
9.1.5. Preparation of Calibration curves for GC/FID.....	67
9.1.6. GC/MS	68
9.1.7. Enzymes	69
9.2. <i>Synechocystis</i> spp. PCC6803	70
9.2.1. Cultivation conditions.....	70
9.2.2. Liquid culture inoculation.....	70
9.2.3. Optical density measurements	70
9.2.4. Cryostocks.....	70
9.2.5. Transformation.....	71
9.2.6. Colony PCR	71
9.2.7. Biotransformation	73
9.2.8. SDS-PAGE	74
9.2.9. Isolation of product and attempted isolation of unidentified side product/metabolite from whole cells	74
9.3. <i>Escherichia coli</i>	75
9.3.1. Cultivation conditions.....	75
9.3.2. Overnight culture	75
9.3.3. Preparation of cryostocks.....	75
9.3.4. Optical density measurements	75
9.3.5. Preparation of RbCl competent cells	76
9.3.6. Transformation of chemically competent cells.....	76
9.3.7. Isolation and quantification of plasmid DNA from cells.....	76
9.3.8. Purification and quantification of PCR amplified DNA from Agarose gel.....	77
9.3.9. DNA construct verification via Sequencing	77
9.3.10. Colony PCR	77
9.3.11. Biotransformation	78
9.3.11.1. Autoinduction	78
9.3.11.2. Induction via IPTG	79
9.3.12. SDS-PAGE	80
9.3.13. Determination of protein content using the BCA assay.....	80
9.3.13.1. Calibration.....	80
9.3.13.2. Measurements	82
9.3.14. Cloning.....	82
9.3.14.1. Cloning and Mutagenesis for transfer into Lv.0 constructs	82
9.3.14.2. Cloning for transfer into Lv.1 constructs	84
9.3.14.3. Cloning for transfer into Lv.T constructs	87
10. Appendix	91
10.1. Genetic Sequences	91
List of Primers.....	97
10.2. NMR Spectra	98
11. References	103

1. Abbreviations

%	percent
+CTRL	Positive control
°C	degree Celsius
μE	Microeinstein
μg	Microgram
ADH	Alcohol dehydrogenase
AIM	Autoinduction medium
APS	Ammonium persulfate
BCA	bicinchoninic acid assay
bp	Base pair
BVMO	Baeyer-Villiger monooxygenase
CB	Construct, more info see Table 67
CBG	sfGFP tagged version of CB
C-C bond	Carbon-carbon bond
C=C bond	Carbon-carbon double bond
CFE	Cell-free extract
CM10	<i>Synechocystis</i> spp. PCC6803 Δflv1 strain
CO	Construct, more info see Table 68
COG	sfGFP tagged version of CO
Conc.	concentration
cPCR	Colony PCR
-CTRL	Negative control
Da	Dalton
dH ₂ O	Distilled H ₂ O
DHAP	Dihydroxyacetone phosphate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Doublestranded DNA
<i>E. coli</i>	<i>Escherichia coli</i>
e.g.	“exempli gratia” (for example)
EDTA	Ethylenediaminetetraacetic acid
ERED	Enoate reductase
FAD	Flavin adenine dinucleotide
FBA	Flux-balance-analysis
FMN	Flavin mononucleotide
g	Gram
g	gram
Ga	Giga-annum (10 ⁹ years)
GC	Gas chromatography
GC/MS	Gas chromatography/ mass spectrometry

GOI	Gene of interest
h	hour
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
iPrOH	Isopropyl alcohol
IPTG	Isopropyl β -d-1-thiogalactopyranoside
kb	kilobasepairs
kDa	kilodalton
KO	knockout
kV/cm	Kilovolt per centimetre
l	litre
LacI	Lac repressor
LB	Construct, more info see Table 63
LB	Lysogeny broth
LBG	sfGFP tagged version of LB
LO	Construct, more info see Table 64
LOG	sfGFP tagged version of LO
Lv.	Level
LYC	Construct, more info see Table 69
M	Mol per litre
m	Metre
mbar	millibar
mg	milligram
MHz	megahertz
min	Minute
ml	millilitre
mM	millimolar
mm	millimetre
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP ⁺	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
N-free	Nitrogen-free
nm	Nanometre
NMR	Nuclear magnetic resonance
OD	Optical density
OYE	Old yellow enzyme
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction

pH	Potential of hydrogen
PMSF	phenylmethanesulfonyl fluoride
PSI	Photosystem I
PSII	Photosystem II
RBS	Ribosome binding site
RNA	Ribonucleic acid
rpm	Rotations per minute
rRNA	Ribosomal RNA
rRNA	Ribosomal RNA
RT	Room temperature
S	svedberg
s	Second
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	Sodium dodecyl sulfate
sfGFP	Superfolder green fluorescent protein
SOC-medium	Super optimal broth medium with catabolite repression
spp.	species
ssDNA	Single strand DNA
<i>Synechocystis</i>	<i>Synechocystis</i> spp. PCC6803
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEMED	Tetramethylethylenediamine
TLC	Thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
U	unit
UV	Ultraviolet
V	Volt
v/v	Volume per volume
W	Watt
w/v	Weight per volume
WR	Working reagent
xg	Times gravity
YB	Construct, more info see Table 65
YBG	sfGFP tagged version of YB
YO	Construct, more info see Table 66
YOG	sfGFP tagged version of YO

2. Abstract

Sustainability is an ever more important criterion which future industrial processes will have to consider. Present host organisms for the generation of value-added chemicals are heterotrophic. A possible approach to make the chemical industry more sustainable would thus be the substitution of these heterotrophic hosts with autotrophic ones, advancing from CO₂ release to CO₂ capture. Recently cyanobacteria, such as *Synechocystis* spp. PCC6803, are emerging as such hosts. To put these new hosts into practice, a biocatalytic cascade for the conversion of carveol to ϵ -carvolactone was introduced into *Synechocystis* spp. PCC6803. Substituting carveol with 2-cyclohexen-1-ol, a different product, namely ϵ -caprolactone became accessible. Both products could be used to produce polymers. Assessing the production of ϵ -caprolactone more closely revealed a yield of 50% and a specific activity of 5.45 units/g of cell dry weight. This could already be improved to 9.92 units/g of cell dry weight using a strain with higher NADPH availability. This productivity is, yet, significantly lower than what is needed for industrial applications, but nonetheless remains optimizable and serves as a proof of concept.

3. Deutsche Zusammenfassung

Nachhaltigkeit ist ein immer wichtiger werdendes Kriterium, das zukünftige industrielle Prozesse berücksichtigen werden müssen. Gegenwärtig verwendete Wirtsorganismen für die Herstellung von Chemikalien mit Mehrwert sind heterotroph. Eine mögliche Herangehensweise die chemische Industrie nachhaltiger zu machen wäre daher das Austauschen jener heterotrophen mit autotrophen Wirtsorganismen, was an Stelle von CO₂ Freisetzung CO₂ Bindung ermöglichen würde. Neuerdings sind Cyanobakterien, wie *Synechocystis* spp. 6803, als solche Wirtsorganismen im Kommen. Um diese neuen Wirtsorganismen zu nutzen, wurde eine biokatalytische Kaskade ausgehend von Carveol zu ϵ -Carvolacton in *Synechocystis* spp. PCC6803 eingeführt. Durch das Austauschen von Carveol mit 2-Cyclohexen-1-ol konnte ein anderes Produkt, nämlich ϵ -Caprolacton erschlossen werden. Beide Produkte können zu Polymeren weiterverarbeitet werden. Eine nähere Betrachtung der Produktion von ϵ -Caprolacton zeigte, dass eine Ausbeute von 50% und eine spezifische Aktivität von 5,45 units/g Zelltrockengewicht erreicht werden können. Dies konnte bereits zu 9,92 units/g Zelltrockengewicht verbessert werden, indem ein Stamm mit erhöhter NADPH Verfügbarkeit verwendet wurde. Diese Werte liegen, noch, weit unter den für eine industrielle Anwendung benötigten. Sie sind jedoch weiter optimierbar und dienen als Machbarkeitsnachweis.

4. Introduction

4.1. Cyanobacteria

Phototrophs are generally of critical importance for the functioning of ecosystems, due to their importance in the fixation of CO₂¹. This fixation not only aids in lowering atmospheric CO₂ concentrations², which in turn cools the planet³, but also fuels primary production which heterotrophic organisms depend on.

Cyanobacteria present an ancient and monophyletic group of oxygen producing photoautotrophs, so called oxygenic photoautotrophs, for which fossilized evidence exists from between 2 – 3.5 Ga ago⁴. The main characteristic of cyanobacteria is arguably their dual photosystem, unique among prokaryotes, with the ability to use H₂O as an electron donor⁵, which provides them with a basically infinite supply of electrons, given the high abundance of H₂O on earth. Using light, they fixate CO₂ mainly through the reductive pentose phosphate pathway and store excess as glycogen⁶, whereas under dark conditions the glycogen reserves are used for cellular respiration via the oxidative pentose phosphate pathway⁷. Some cyanobacteria are also able to fixate N₂, which necessitates the protection of the nitrogenase enzyme from O₂, which they achieve in different ways, leading to morphological diversity. Some form filaments with heterocysts (cells specialized for N₂ fixation), whereas others such as *Crocospaera* manage to fix molecular nitrogen unicellularly⁸.

As all cyanobacteria are oxygenic phototrophs, they are the most likely culprit for the great oxygenation event ca. 2.4 Ga ago⁹, in which the formerly reductive atmosphere of earth changed to an oxidative one¹⁰, which had major implications for life on earth¹¹. Additionally, they are thought of as the predecessors of chloroplasts which are seen in all phototrophic eukaryotes¹². Given the great impact they have had in the past, what are the current effects of cyanobacteria on life on earth? Some cyanobacteria have significant effects through secondary metabolites which can be toxic to humans as well as fish and other organisms¹³. Other secondary metabolites however, show antibacterial properties and could thus be promising candidates for the development of antibiotics in the future¹⁴.

Furthermore, cyanobacterial blooms have great impacts on the bodies of water in which they occur, altering countless parameters of these ecosystems¹⁵. Cyanobacterial blooms gained further relevance in recent times¹⁶, due to the high eutrophication of waterbodies through agricultural pollution¹⁷. In addition to the above, they continue their vital role as primary producers until today and maybe become even more important in the near future¹⁸. Through their ability to fixate N₂, cyanobacteria are also implicated in the natural productivity of rice fields, produce of which is a staple food for billions of people worldwide¹⁹. There they present a viable alternative to chemical fertilizers, which, in recent years, have come under evermore scrutiny²⁰, in part due to the environmental impacts that the leaching of these chemical fertilizers into bodies of water has, as was described above.

Cyanobacteria are, however, also directly accessible to humans as a source of food, as the genera *Arthrospira* spp. show, which are marketed under the commercial name of *Spirulina*²¹. Used as a

source of food, it boasts a high protein content of about sixty percent and also contains vitamin B12²², which is highly sought after in vegetarian and vegan diets²³.

In recent years, cyanobacteria also came into focus when used as so-called cellular factories where they, through genetical engineering, are used for the generation of molecules of interest²⁴.

4.2. Biocatalysis

At the end of the 20th century, the chemical industry came under evermore scrutiny due to the amount of waste it generated. Up to this point, the guiding principles that needed to be maximized were reaction efficiency and selectivity²⁵. However, with rising awareness also came a paradigm shift that pushed the optimality criteria towards waste prevention, instead of remediation after the waste had been generated²⁶. The guiding principles of the new approach towards “green chemistry²⁷” reflect this change in mentality. Enzymes present a renewable, biodegradable alternative to conventional catalysts used in organic synthesis, enabling milder reaction conditions (lower temperature, pH closer to neutral, in aqueous solution) and thus reducing pollution, partly due to simpler waste management. Additionally, the use of enzymes does not necessitate the usage of protection groups and protection steps as is often the case with less regio- or chemoselective conventional catalysts²⁸. In the pharmaceutical and food industry for example, contaminations with metals, as found in many of the conventionally used catalysts, are not tolerable²⁹ due to the detrimental effect many metals have on human health³⁰.

Biocatalysis, the use of biocatalysts (most commonly enzymes) to generate value added products from substrates³¹, is becoming more widespread as methods for the engineering of proteins (which most enzymes are) are refined. Additionally, recent developments in many other fields can be employed in the pipeline of enzyme optimization³². Arguments against the use of enzymes were, for example, their often narrow substrate scope as well as their perceived high cost and delicateness³³. Progress made in genomics as well as molecular biology, among other fields, weakened these key counterarguments against the use of enzymes as catalysts over time³⁴. Directed evolution, for example, enables researchers to modify and refine an enzyme to fit the task at hand³⁵. In a directed evolution approach, the enzyme is mutated randomly and the mutants are screened for improved qualities such as better stability at high temperatures³⁶. The importance of directed evolution for the field of enzyme engineering is probably best illustrated by the Nobel Prize for Chemistry 2018 which was awarded in part “*for the directed evolution of enzymes*”³⁷. A different tactic is represented by rationale design, in which various parameters are considered and based on this data, an attempt is made to optimize the enzyme via key mutations³⁸. Both concepts, however, are not mutually exclusive but have rather been combined into semi-rationale design in which both prediction and random mutagenesis are employed³⁹. This, in sum, means that researchers no longer only have the natural pool of enzymes available to them but can modify enzymes towards defined goals⁴⁰. Noteworthy parameters accessible through enzyme engineering include a broadening of substrate scope⁴¹ as well as the introduction of non-canonical amino acids into the protein. The inclusion of non-canonical amino acids enables access to entirely new types of reactions, as well as further optimizations regarding e.g., stability⁴².

Possible products of reactions catalysed by engineered enzymes are diverse and plentiful. They range from the bulk production of chemicals for industrial use, such as acrylamide⁴³, to the production of pharmaceuticals used in the treatment of HIV⁴⁴.

However, some challenges remain. For instance, carbon-carbon (C-C) bond formation is essential to the field of organic synthesis and is traditionally achieved through a myriad of non-biocatalytic methods^{45–47}. Addressing C-C bond formation biocatalytically has long been described as a major challenge³³. More recently, strides were made towards creating C-C bonds via enzymes such as aldolases and other lyases⁴⁸ and although there are already industrial applications for these enzymes⁴⁹, they face issues regarding their donor substrate dihydroxyacetone phosphate (DHAP). DHAP is expensive as well as unstable and it would thus be desirable to substitute it with a different donor substrate⁵⁰.

Expensive cofactors also plague other biocatalytic approaches such as when enoate reductases are used. Their cofactor nicotinamide needs to be present in stoichiometric amounts for them to be able to reduce C=C bonds⁵¹. A solution for this problem may be found in light-mediated nicotinamide recycling, which grants access to other hydride donors for the reduction of flavin mononucleotide (FMN) enabling more cost-effective cofactor alternatives⁵². Of course, the problem can also be partially solved by designing a redox neutral cascade, in which the cofactors get recycled. Biocatalytic cascades will be further explored in the following chapter. All in all, the field of biocatalysis not only sees practical application in e.g., the pharma industry, and is taught in some high school classes⁵³, showcasing its importance, but furthermore continues to evolve in many aspects as demonstrated by the introduction of new types of reactions to its repertoire⁵⁴ and the refinement of already existing ones⁵¹.

4.2.1. Biocatalytic cascade reactions

A one-pot reaction is a reaction in which the reaction vessel is not exchanged. This approach saves time and, through minimization of isolation processes of intermediates, also optimizes energy and resource usage⁵⁵.

According to Tietze and Beifuss, the term cascade is, in a catalytical context, used to refer to “...a process involving two or more consecutive reactions in which subsequent reactions result as a consequence of the functionality formed by bond formation or fragmentation in the previous step”⁵⁶. They differentiate it from another type of one-pot reaction, the consecutive reaction, in which “...another reagent, mediator, or catalyst is added after the first transformation without isolation of the first formed product; the subsequent reaction steps then lead to the final product.” Using this definition, a cascade is a one-pot reaction to which no further component is added after the initial reaction setup.

Advancements in biocatalysis, as already discussed in 4.2, made it easier to create multi-enzyme cascades instead of single-step reactions. There are generally two different ways in which a multi-enzymatic cascade will be realized: *in vitro* or *in vivo*.

In vitro approaches use either purified enzymes or simply lysed cells which expressed the enzyme, the so-called cell-free extracts (CFE). Advantages of the CFE compared to the purified approach are the already present cofactors e.g., NADPH, which are often needed for correct en-

zyme function⁵⁷, as well as the lower cost due to the skipping of enzyme isolation procedures⁵⁸, as depicted in Figure 1. On the flipside, there are far more factors at play than in a strictly controlled environment as provided by using purified compounds, which potentially complicates troubleshooting. For an overview of CFE's see Alissandratos, 2020.⁵⁹ The use of purified enzymes is also advantageous in regards to product isolation, which may be easier due to the absence of non-reaction related compounds, especially if the enzymes are immobilized to a matrix⁶⁰.

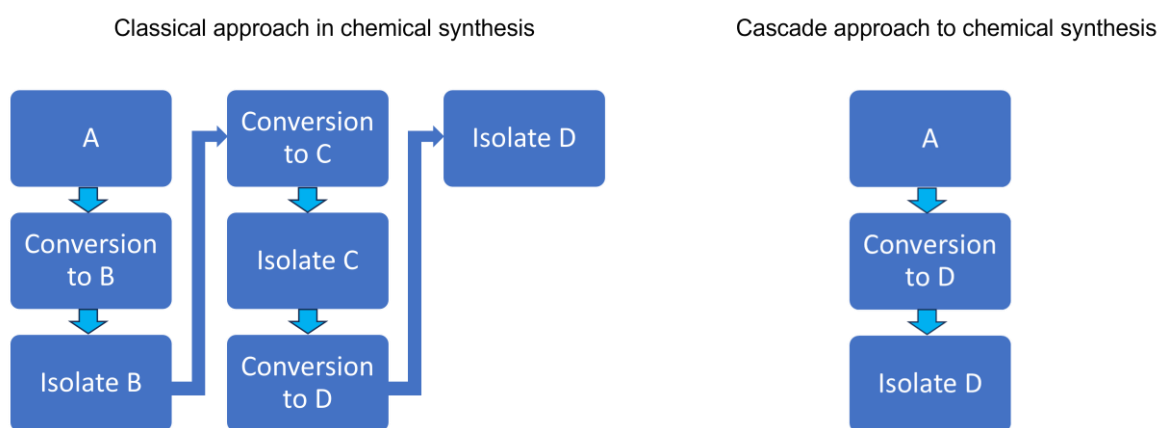


Figure 1: Comparison between a classical approach versus a cascade approach to synthesize a compound D. Steps necessary to go from compound A to compound D via the intermediates B and C are fewer in the cascade, due to cancellation of unnecessary isolation steps.

In vivo, also called whole-cell, approaches on the other hand, use the living host as their reaction vessel. This has advantages when it comes to cofactor recycling, because the cofactors are recycled endogenously within the cells, lowering cost⁶¹. However, as cells are enclosed by a lipid bilayer, some substrates may face problems diffusing into the cell. Furthermore, if the product is not shuttled out of the cell, or passively diffuses out, this can complicate product isolation⁶². Additionally, transport of product outside the cell can aid in product formation as it reduces product concentration in the reaction environment⁶³. Another factor to consider is cellular toxicity not only of substrate and product⁶⁴, but also by arising intermediates. The immense complexity of a living cell can further complicate troubleshooting and outcome prediction. Recently, *in silico* methods such as metabolic modelling are becoming increasingly prevalent to address the latter issue⁶⁵. Whole-cell approaches also need considerably more time commitment due to the complexity of genomic engineering/protein expression. To sum up, *in vitro* approaches offer advantages in product isolation, complexity, and shorter lead times, whereas whole-cell approaches offer cofactor recycling and milder reaction environments for the enzymes.

4.2.2. Biocatalysis in *Synechocystis* sp. PCC6803

Due to the rising interest in a more sustainable economy, cyanobacteria are increasingly explored as cellular factories in biocatalytic applications. *Synechocystis* sp. PCC6803, simply *Synechocystis* hereafter, is a cyanobacterium and was the first phototrophic organism whose genome was fully sequenced⁶⁶. This, in addition to its natural transformability and the possibility to grow in the dark, enabling easier assessments of photosystem I (PSI) and photosystem II (PSII) mutants⁶⁷, made it a widespread phototrophic model organism⁶⁸. As such a well-established model organism, it was then, and is now, used in a variety of biocatalytic research, reaching from the production of biofuels such as butanol⁶⁹ to the hydroxylation of testosterone⁷⁰. But why even prefer cyanobacteria over more established cellular factories such as the classical heterotrophs *Escherichia coli* (*E. coli*) and *Saccharomyces cerevisiae* (*S. cerevisiae*)? Phototrophs such as *Synechocystis* have the advantage that they fixate atmospheric CO₂, which is currently a sought-after quality. Sequestration of CO₂ could help mitigate climate change⁷¹ and the acidification of the oceans which threatens many marine animals⁷². In contrast, heterotrophs do not fixate CO₂, indeed many of them generate it through their primary metabolism. Only sequestering CO₂ would already be helpful, but further valorisation is possible via genetically engineered *Synechocystis* as mentioned above. Their metabolism also provides advantages complimentary to those offered by commonly used heterotrophs. For instance, their NADPH/NADH ratio is higher and O₂ becomes less of a limiting factor. In heterotrophs O₂ often becomes limiting to the organism if it is also used in the biocatalytic reaction because the host metabolism and the reaction compete for it. In *Synechocystis* however O₂ is unlikely to become limiting because under light conditions it is a metabolic end product.

Therefore, *Synechocystis* is best chosen for biocatalytic reactions that need NADPH and O₂. Despite these significant advantages, there are still remaining challenges. The main bottlenecks in current cyanobacteria cultivation approaches are insufficient lighting conditions due to self-shading of cyanobacteria at the high cell densities required for catalytic efforts as well as a considerably slower duplication time when compared to the competitors mentioned above. Another disadvantage currently lies in the comparatively low gene expression levels. In sum, these shortcomings are currently prohibiting a large-scale, industrial use of cyanobacteria due to their low yields. An in-depth review of cyanobacteria for biocatalysis was written by Jodlbauer *et al.*, 2021⁷³.

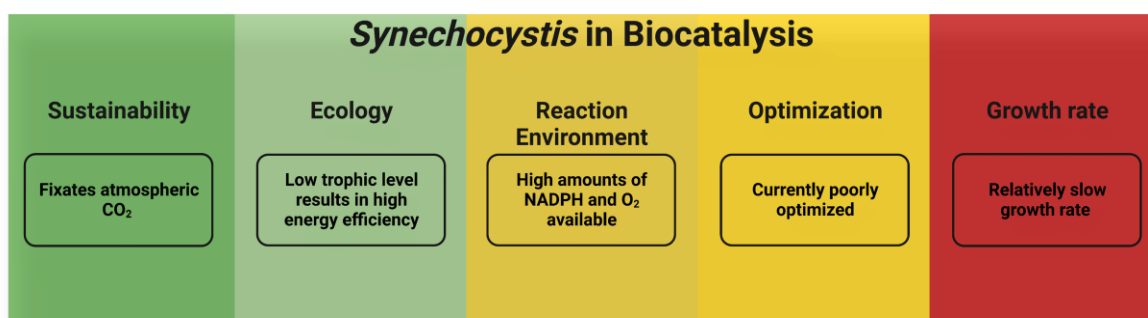


Figure 2: Advantages and disadvantages of biocatalysis in *Synechocystis* sp. PCC6803.

4.3. Designing enzymatic cascades

First, one needs to have a product in mind which should be produced. The next step in creating a cascade is through retrosynthesis, which means starting from the product and deconstructing it until arriving at a substrate that is commercially available and then finding enzymes that catalyse the steps taken, but from the direction of substrate to product. Alternatively, one can employ “forward design”, which means starting with a substrate and looking for possible enzymatic reactions and then do the same again for the product of this first conversion, until arriving at a desirable compound⁷⁴. Basically “forward design” is often more of a blind approach as all one knows at the start is the first substrate, whereas starting retrosynthesis one knows the product.

The design of enzymatic cascades is a complex process, due to the many variables involved compared to singular enzymatic reactions. For instance, the pH as well as the temperature at which the different enzymes operate must be compatible between the different enzymes in order to successfully develop a one-pot approach. Next, a choice needs to be made regarding the host, if the cascade is to be realized *in vivo*. Probably the most common host is *E. coli*, due to its fast growth and the relatively inexpensive nutrients it requires. Another advantage is the huge body of work that has been done in *E. coli*, as its genetics are well researched^{75,76} and there is plenty of additional data on metabolism⁷⁷, proteome⁷⁸ et cetera. After electing the host organism, genes encoding the enzymes of choice are to be introduced. Here one needs to make an important choice, either selecting an approach that integrates the genes into the genome of the host, offering greater genetic stability, or introducing a genetic vector, most commonly a plasmid into the host⁷⁹. One advantage of plasmids is the relative ease with which they can be introduced into most organisms and even be shuttled between different hosts e.g., between *E. coli* and *S. cerevisiae*⁸⁰. This shuttling is often taken advantage of to clone in a relatively fast reproducing organism such as *E. coli* and then shuttle the finished plasmid into the final host, who often displays slower growth. There are also different approaches when it comes to the organisation of the genes on a sequence. One can either organize them as a polycistronic operon (all genes share a single promoter and terminator), as a pseudo-operon (all genes have their own promoter but have a common terminator) or as multiple monocistronic operons (each gene with its own promoter and terminator). A good review on operons was written by Osbourn *et al.*, 2009⁸¹. Nowadays there are many different techniques available to anyone wanting to clone a genetic construct. Among these are Golden Gate⁸²/Modular cloning (MoClo⁸³), Gibson assembly⁸⁴, and sequence- and ligation-independent cloning (SLIC⁸⁵). When using a genomic integration approach or to knockout genes in the host e.g., to eliminate background interference, CRISPR can be of use⁸⁶. Another important choice is which medium to use, for example if it should provide for cellular growth or not. There are cases in which it is fruitful to choose a resting cell medium⁸⁷, however this may not always be the case⁸⁸. A brief review on biocatalysis with emphasis on whole cells and the relevant associated methods was written by Caralho *et al.*, 2017⁸⁹.

4.4. Challenges of protein expression in whole cell approaches

Heterologous protein expression puts a high metabolic burden on the host cell, seen as the introduced proteins are often overexpressed and, if introduced via plasmids, additional resources are diverted towards DNA replication⁹⁰. The latter is the case because a common, easy way to control the amount of protein expression is through gene copy number. However, this strategy for the regulation of protein expression is imprecise and not always available. For instance, in *Syn-echocystis* high copy number shuttle plasmids available to cloning are still scarce⁹¹ and therefore shuttle vectors with a low copy number have to be used⁹². The expression of heterologous enzymes is also not trivial due to the different host metabolites with which the newly introduced enzymes are now confronted, which can lead to a number of problems⁹³.

It should be kept in mind that protein expression is also dependent on gene expression, which in itself is quite complex^{94,95} and will not be discussed here, except for context dependency, which means that the neighbouring sequences can have an effect on how well a particular gene is expressed⁹⁶. One parameter affecting protein expression are ribosome binding sites (RBS's), which are located upstream of the start codon and are reported to have an over 10,000-fold impact on protein expression⁹⁷. The RBS affects how fast the ribosome is recruited to the RBS and how efficiently translation can be initiated⁹⁸. In prokaryotes, they often contain the Shine-Dalgarno sequence which binds to the anti-Shine-Dalgarno sequence present on the 16S rRNA of the ribosome and thus facilitates ribosome binding. However, more recently several Shine-Dalgarno independent mRNA's have been described⁹⁹. For an extensive review on translation in prokaryotes see Rodnina, 2018¹⁰⁰.

In cyanobacteria, the tools for regulating proteins via *in silico* predictions are not there yet¹⁰¹, compared to *E. coli* where an array of RBS calculating tools are available^{102–104}. This leaves one with the option of, preferably high-throughput, screening, as RBS performance has been shown to differ significantly, not only interspecifically, but also for different genes¹⁰⁵ or try to guesstimate a RBS that could perform well based on literature.

4.5. Challenges of biocatalytic *in vivo* cascades

As 4.4 should illustrate, control of protein expression presents quite a challenge in single enzymatic approaches, which of course is then also the case for cascades. Probably the first thing that comes to mind discussing *in vivo* cascades, are cross reactivities between the different enzymes and the substrates e.g., an enzyme can use multiple substrates from the cascade and thus generate unwanted side products, or the host's enzymes can accept a substrate of the cascade and generate side products⁹³. Furthermore, the reaction conditions between the different enzymes need to be compatible, optimally in a way in which every enzyme operates at the same optimum regarding pH and temperature. The different optima should be kept in mind when later assessing bottlenecks, as it might be the case that a protein is well expressed but can still not perform its function adequately due to inappropriate conditions¹⁰⁶, in which case upregulation of expression would hardly be of use. If the flux through the cascade is disturbed by reactions with enzymes from the host background (through the siphoning off of cofactors), a KO of the genes encoding these enzymes might be feasible¹⁰⁷. More recently, it became possible for some host organisms to, *in silico*, perform a flux-balance-analysis (FBA) to potentially address the issue of generating lethal

mutants by the KO of these genes and to simulate how well the flux through the cascade will be improved¹⁰⁸. If further information about the fluxes is desired, their distribution can be determined via ¹³C metabolic flux analysis, and if the introduced cascade is coupled to the hosts carbon metabolism, further insight about the specific flux in the cascade can be gained¹⁰⁸. Moreover, coenzymes and cosubstrates need to be available in sufficient quantities inside the cell to ensure optimal enzymatic activity. For instance, if one designs a three-step cascade, which only consists of reductive steps, each one using up one NADPH, the host organism might run into problems supplying enough reductive power. Cascade design can alleviate this issue by keeping the difference of oxidation and reduction as close to zero as possible. Furthermore, steps could be taken to make more NADPH available in the host by eliminating existing electron sinks¹⁰⁹ or by boosting production through metabolic engineering¹¹⁰. The toxicity of intermediates also plays a role in some cascades. This can be addressed, for example, by choosing a faster enzyme for the conversion of the toxic intermediate into the next compound in sequence, so that the amount of that specific, toxic compound at any moment inside the cell is minimized¹¹¹. Another way to shield the host cell from damage by toxic intermediates or to facilitate the expression of enzymes not compatible with the intracellular environment is to spatially separate the cascade from the rest of the cell e.g. via artificial intracellular compartments¹¹² or by restriction of enzyme expression to already present ones, e.g. peroxysomes¹¹³.

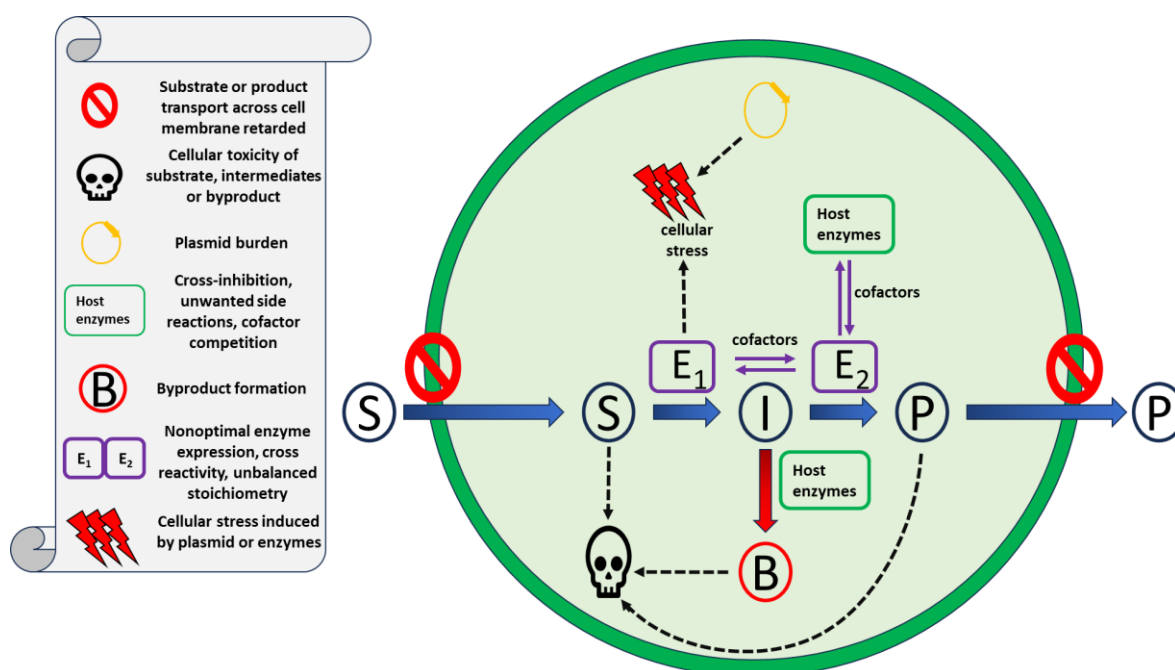


Figure 3: Challenges in a whole-cell biocatalytic cascade. The substrate S is converted to product P or byproduct B via intermediate I.

4.6. Redox reactions and enzymes used in biocatalysis

Redox reactions are highly present in the essential chemical processes of life such as in photosynthesis or the Krebs-cycle and could thus be seen as the motor of life. However, they are not only of fundamental importance in biology, but also in synthetic chemistry¹¹⁴. To give an overview over all redox reactions used in biocatalysis would go beyond the scope of this thesis, thus only the ones of importance to this thesis will be introduced. However, a good overview can be gained by familiarizing one with Blank *et al.*, 2010¹¹⁵.

4.6.1. Alcohol dehydrogenases (ADHs)

Alcohol dehydrogenases are oxidoreductases which reversibly catalyse the selective reduction of aldehydes and ketones to primary and secondary alcohols¹¹⁶. Nature offers a wide array of different alcohol dehydrogenases¹¹⁷, in a multitude of different organisms ranging from bacteria all the way to mammals. They can serve quite different metabolic purposes, such as detoxification (or accidental toxification¹¹⁸) of alcohols and xenobiotics or involvement in signalling pathways and hormone regulation¹¹⁹. An important feature for the classification of dehydrogenases is the presence or absence of the Rossmann-fold, which enables binding of nicotinamide-based coenzymes. Along with features such as protein chain length, distinct sequence motifs, mechanistic features, and structural comparisons this enabled further classification of ADHs into a system of aldo-ketoreductases, short-, medium-, and long-chain dehydrogenases/reductases¹²⁰. Currently ADHs are widely used in biocatalysis, as they are combinable with many other bio- and chemocatalysts and are, as most enzymatic approaches, especially useful in generating products of high enantiopurity. They are used in processes that yield products such as amines, amino alcohols, lactones and others¹²¹. The stereochemistry of the reaction using ADHs depends mostly on the substrate¹²². ADHs generally follow Prelog's rule¹²³ (see Figure 4), however there are exceptions¹²⁴. Recently Guo *et al.* used ADHs to access all possible stereoisomers of carveol, starting with either *R*-carvone or *S*-carvone. LfSDR1, a glucose 1-dehydrogenase originating from *Limosilactobacillus fermentum*, exhibited Prelog- as well as anti-Prelog preference, depending on which mutated version was used, showing that the same enzyme may can, with minor alterations, access multiple stereoisomers¹²⁴.

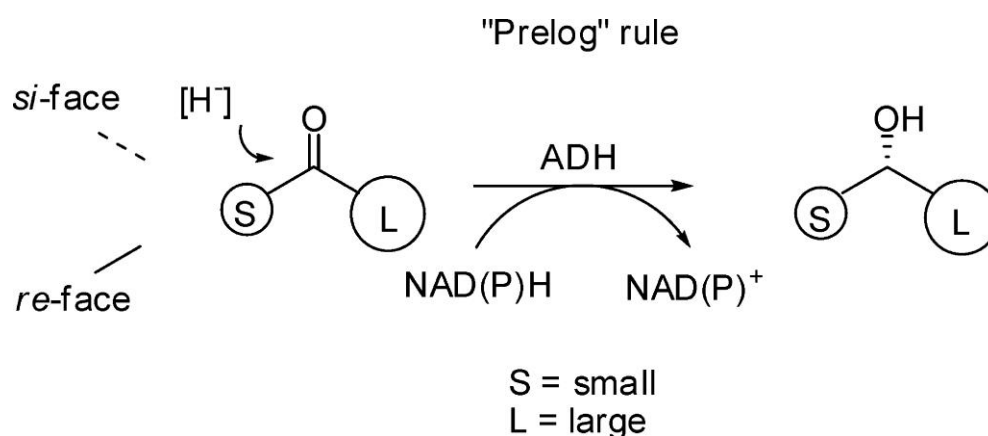


Figure 4: If L has priority over S, the depicted case yields a product of *S*-configuration (Taken from Hall *et al.*, 2011¹²⁵)

4.6.2. Enoate reductases (EREDs)

EREDs are ubiquitous in nature and play a part in e.g. the production of fatty acids and in morphine synthesis¹²⁶, where they stereoselectively reduce activated C=C bonds. The asymmetric reduction variant of this reaction is of special interest to chemists to access *trans*-hydrogenated compounds, which otherwise may be difficult to produce¹²⁷. The mechanism of this *trans*-hydrogenation through ene-reductases is well understood (see Figure 5: Mechanism of EREDs.). The hydride, derived from the reduced flavin cofactor, stereo selectively attacks the β -carbon of the α,β -unsaturated substrate, while a proton is added on the opposite side onto the α -carbon from a tyrosine residue (the proton is ultimately derived from the solvent). The reaction mostly takes place with relative *trans*-stereospecificity¹²⁸. Most enoate reductases are, in contrast to most ADHs, quite unselective regarding their nicotinamide cofactor¹²⁷, leading to possibilities in the application of synthetic nicotinamide mimics¹²⁹.

Probably the best studied enoate reductases belong to the old yellow enzyme family and allow for a wide range of substrates, with increasing structural knowledge enabling further molecular insight into crucial factors determining activity and stability. The enoate reductase from *Saccharomyces carlsbergensis* was the first flavin containing enzyme to be isolated¹³⁰. It is also known as old yellow enzyme (OYE) and gave name to a whole family of enzymes. The OYE family contains many different enzymes, amongst which is YqJM from *Bacillus subtilis*¹³¹. YqJM is well described¹³², a crystal structure is available¹³³, and is used in a multitude of studies^{134–136}, especially in cyanobacterial hosts¹³⁷. In cyanobacteria the possibility of a knockout (KO) of an electron sink, to increase NADPH availability for YqJM was recently realized by Assil-Companiononi *et al.* and yielded an increase in catalytic activity¹⁰⁹.

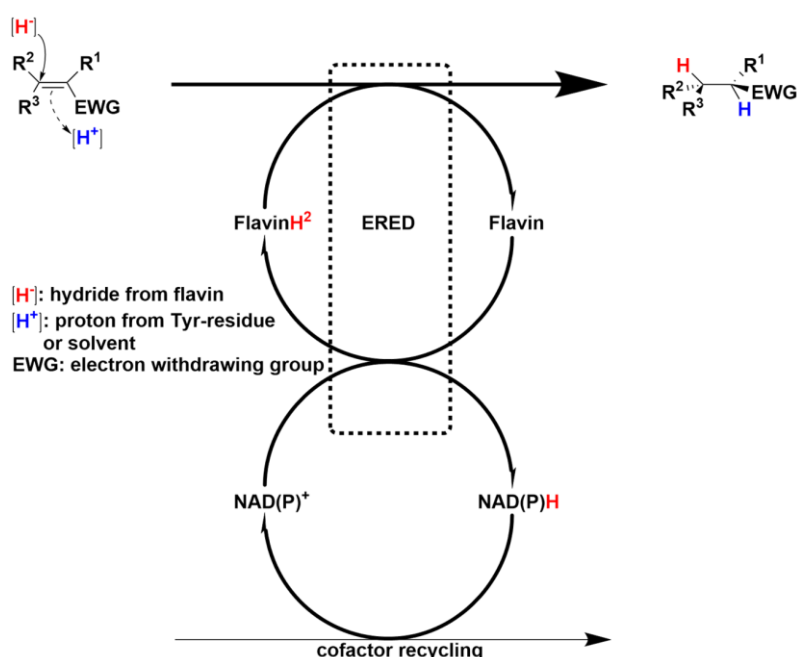


Figure 5: Mechanism of EREDs.

4.6.3. Baeyer-Villiger monooxygenases (BVMOs)

The Baeyer-Villiger oxygenation reaction was first chemically described in 1899 by Adolf Baeyer and Victor Villiger, who oxidized a ketone to the corresponding lactone¹³⁸. Since then, the Baeyer-Villiger oxygenation has become widespread in organic synthesis, due to its tolerance of many different functional groups and its general versatility regarding oxidants as well as substrates. However, said versatility is relative as all available oxidants are peroxy acids, which necessarily generate quite harsh reaction conditions. Optimization of the chemical reaction however is still ongoing, mainly regarding the environmental friendliness of the reaction. Choosing the traditional, chemical instead of an enzymatic route generates a lot of waste and the reactants presently used are often difficult to handle. A good overview of the chemical Baeyer-Villiger oxygenation and possible routes making it “greener” was given by ten Brink *et al*, 2004¹³⁹. Due to the above-mentioned limitations of chemical Baeyer-Villiger oxygenation, there has been exploration towards an enzymatic approach, which promises milder reaction conditions using a way less reactive oxidant, namely O₂. The enzymes catalysing this reaction are the Baeyer-Villiger monooxygenases (BVMOs). BVMOs have different functions in their natural hosts and are hypothesized to already been present in the last universal common ancestor¹⁴⁰. They may be involved in primary as well as in secondary metabolism, allowing growth on atypical carbon sources such as cyclic ketones or taking part in the synthesis of secondary metabolites, respectively¹⁴¹. Used as biocatalysts, BVMOs help accessing compounds that are not easily produced by chemical means¹⁴². Presently two different types of BVMOs are known. They are either FAD and NADPH dependent (Type I) or they require FMN and NADH (Type II)¹⁴³. Cyclohexanone monooxygenase (CHMO), a Type I BVMO, was isolated from *Acinetobacter* sp. in 1976¹⁴⁴ and represents the best studied BVMO¹⁴⁵. The catalytic mechanism of CHMO_{Acineto} is known and depicted in Figure 6. At the start of the cycle the enzyme bound FAD is present in its oxidized form. Now NADPH reduces the FAD and complexes with the enzyme. This FAD-enzyme-NADP⁺ complex is then oxidized again via the binding of molecular oxygen to FAD, which yields flavin peroxide. The Criegee-intermediate is subsequently formed via the nucleophilic attack of 4a-flavin peroxide on the α -carbon of the ketone. The Criegee-intermediate then rearranges generating the lactone and flavin hydroxide. Next water is eliminated from flavin hydroxide and NADP⁺ as well as the product are released, closing the cycle¹⁴⁶. CHMO_{Acineto} was successfully implemented in a cascade by Oberleitner, 2013 leading from carveol to carvolactone in *E. coli*¹⁴⁷. Carvolactone is of interest due to its ability to polymerize after ring opening and could thus be functionalized as a biorenewable polyester¹⁴⁸. Most BVMOs exhibit poor stability, even at temperatures as low as room temperature they may become inactive within minutes. This is exceptionally unfortunate if they are to be used industrially, where conditions are often significantly harsher¹⁴⁹. For this reason, substantial effort has been directed towards development of more stable versions. For example, Mansouri *et al.*, 2022 developed CHMO_{Acineto} variants which showed significant increases in stability as well as activity and FAD binding¹⁵⁰.

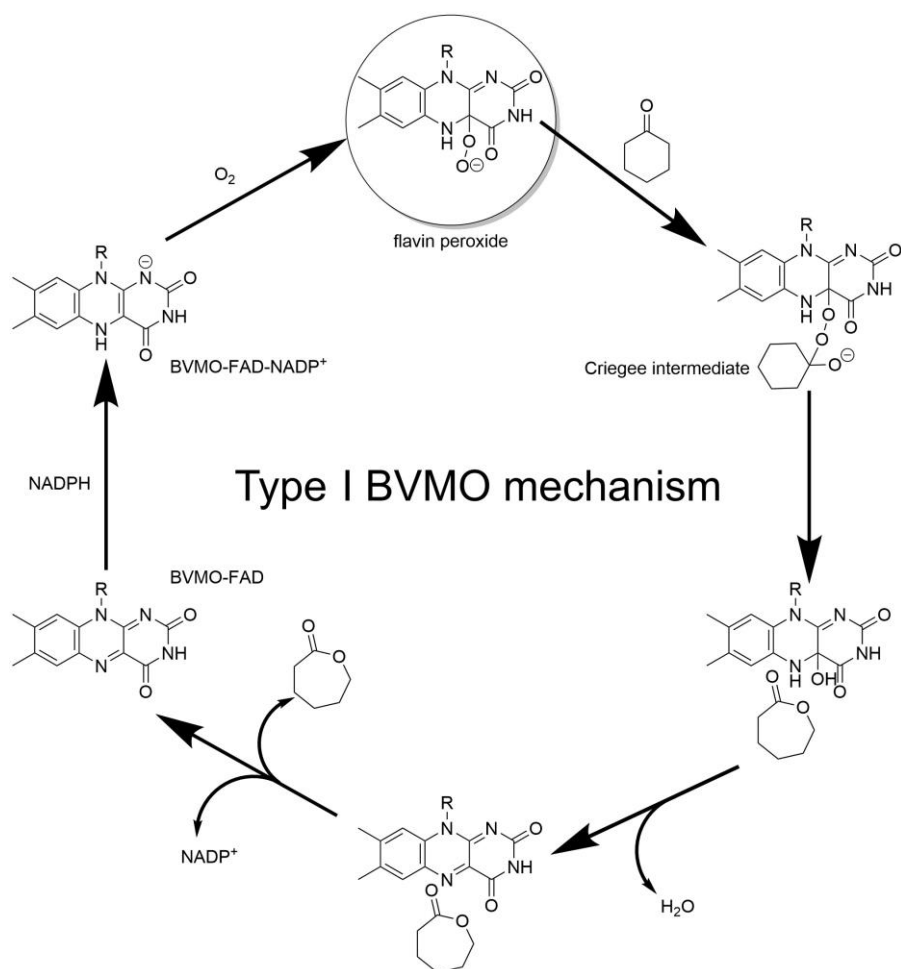


Figure 6: Mechanism of CHMO.

5. Scope of this Thesis

The aim of this thesis was to generate an artificial cascade in *Synechocystis* spp. PCC6803. The cascade includes a modified alcohol dehydrogenase (LfSDR1M50)¹²⁴, an enoate reductase (YqjM) and a modified Baeyer-Villiger monooxygenase (CHMO_{Acineto}S7¹⁵¹). The enzymes will be co-expressed to generate an *in vivo* cascade from Carveol (1a) to Carvolactone (4a) as depicted in Figure 7. Carveol (1a) can be generated from Limonene which in turn can be extracted from orange peel waste of the citrus industry¹⁵². This enables sustainable sourcing of the substrate for the cascade. Carvolactone (4a) in turn is a promising building block for polymers¹⁵³ or for the synthesis of various natural products. Having a closer look at the cascade, the reason of choosing cyanobacteria as hosts for this particular cascade becomes immediately obvious. Not only does the cascade profit from the molecular oxygen generated by photosynthesis through the Baeyer-Villiger monooxygenase, but it also utilizes the abundant NADPH for the reduction steps. All in all, this would serve as a proof of concept, that cyanobacteria may be used as hosts for cascades to further enhance the possible sustainability of such reactions. To achieve this goal, the genetic constructs containing the sequences that encode the respective enzymes were to be planned and generated. This was achieved via the CyanoGate toolkit in combination with the MoClo toolkit, both of which are based on Golden Gate cloning and follow the BioBrick standard (see 6.1). The cloning steps were done in *E. coli* due to its faster generation time compared to *Synechocystis* spp. PCC6803. This necessitated the use of a shuttle vector as the final construct backbone, so that the construct was viable in both organisms. Genetic sequencing (Sanger sequencing) was used to ensure that the relevant genes on the constructs were not mutated. Next (see 6.3), the generated strains were assessed via biotransformations, in which substrate was added and after some time samples were taken to determine (via gas chromatography) how fast and if the substrate was converted. Due to better accessibility a substitute substrate was used for the cascade (see 6.1 and 6.3.2). The cascade was shown to work in *Synechocystis* spp. PCC6803 (see 6.3.3). Finally, the results had to be summarized and contextualized (see 7).

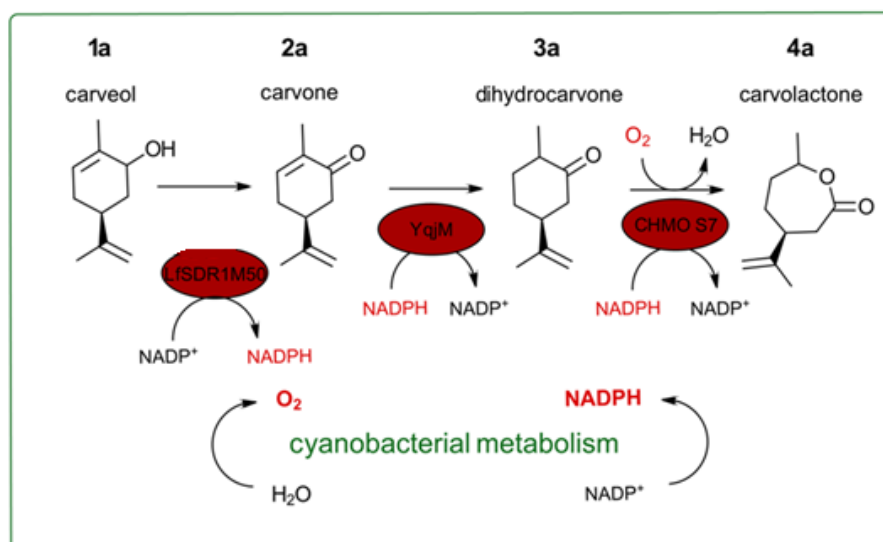


Figure 7: Schematic of the finished cascade including relevant cofactors.

6. Results and Discussion

6.1. Assessing the impact of a GFP tag on the functioning of enzymes used in the cascade

The following work was done as part of a collaboration with Julia Jodlbauer¹⁵⁴ and presents a sideproject for the author. The intent was to compare the activity of the enzymes used in the cascade with and without a C-terminally added sfGFP-tag.

The enzymes were:

- LfSDR1M50, a modified glucose 1-dehydrogenase originating from *Limosilactobacillus fermentum*
- YqJM, an enoate reductase originating from *Bacillus subtilis*
- CHMO_{Acineto}S7, a modified cyclohexanone monooxygenase originating from *Acinetobacter* sp.

More details about individual components can be found in chapters 9.1.7 and 10.

This was of interest because the sfGFP-tag enables quick sorting of cells differing in expression levels of the tagged protein and thus allows to rank e.g., different promoters according to expression level for this specific enzyme. This could, in the future, be used to quickly find strong RBSs (ribosome binding sites) and strong promoters. This work was performed mainly during the same time as that in 6.2, meaning that some of the insights generated that are used later in this thesis (mainly the use of a substitute cascade, Figure 8) were already utilized in this chapter despite being first described in detail in 6.3.2.

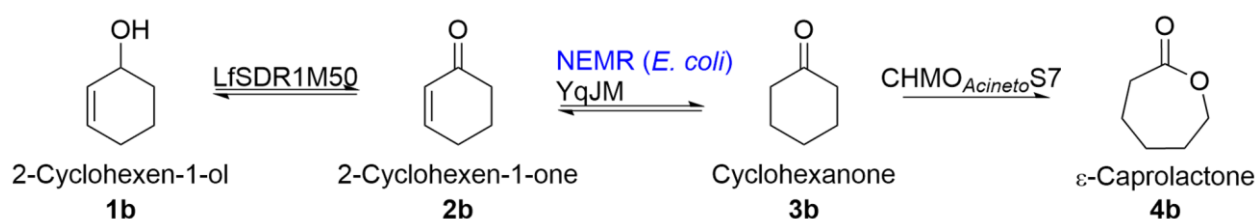


Figure 8: Substitute substrates and planned course of cascade. Note that again NEMR is reported to act like YqJM in *E. coli*¹⁰⁷.

6.1.1. Level T cloning and transformation in *E. coli*

All constructs were transformed into BL21(DE3) for protein expression as well as into NEB_stable for long term storage. Constructs coding for untagged enzymes needed to be cloned from existing LV.1 parts described in 6.2.2.2. The same promoters, plasmids and RBS's were used as in the Lv. T cloning of the cascade (see 6.2.2.2 and 6.2.2.3). Initially it was planned to benchmark the RBS chosen from preliminary results via a comparison to a widely used RBS in cyanobacteria (RBSS1 see 10.1), however as the evaluation of said results was delayed, it was decided to be out of scope for this work, due to time concerns. The relevant constructs, however,

were already cloned and transformed into *E. coli* (Figures 9-12) as well as some into *Synechocystis* (not shown). They are listed as “benchmark” in 10.1. The other constructs were termed “optimal”, however as the preliminary results from Julia Jodlbauer were fully evaluated, none of the used RBS’s were actually the best, but were rather mediocre to good, except for the RBS used for LfSDR1M50 which was slightly worse than mediocre¹⁵⁴. Nonetheless, the naming scheme as XB or XO for the untagged and XBG and XOG where X is the first letter of the enzyme, will be kept due to its brevity (e.g. YqJM with the “optimal” RBS and without sfGFP tag will be called YO).

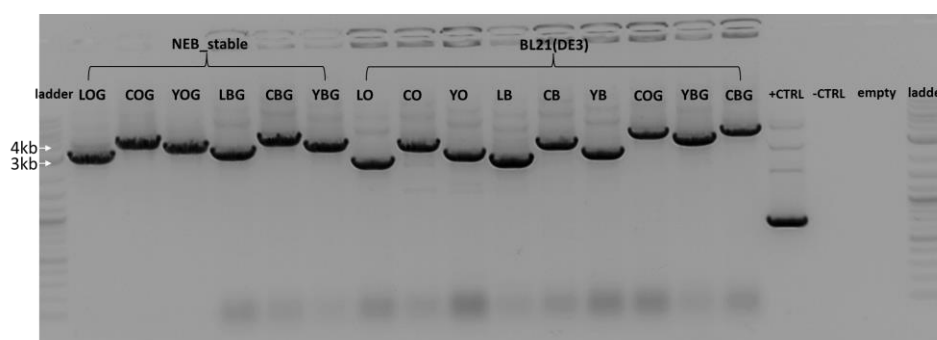


Figure 9: Colony PCR of diverse colonies resulting from transformation. Positive control was pPMQAK1-T Y25F and negative control had dH₂O instead of template. Expected fragment sizes can be seen in **Table 1**. Picture was edited in Microsoft Powerpoint for increased visibility (Brightness -40%, Contrast 50%, Sharpness 30%).

Table 1: Important properties of gene constructs

Name	ORF Length [bp]	Analysis level (pJJ059 + pJJ060) [bp]	length T [bp]	Estimated protein weight [kDa]
LO	744	2384		26.3
YO	1017	2762		37.6
CO	1632	3327		61
LB	744	2384		26.3
YB	1017	2657		37.6
CB	1632	3272		61
LOG	1485	3125		54.2
COG	2373	4030		88.9
YOG	1758	3484		65.4
LBG	1485	3125		54.2
CBG	2373	4013		88.9
YBG	1758	3398		65.4
LYC	-	5480		-

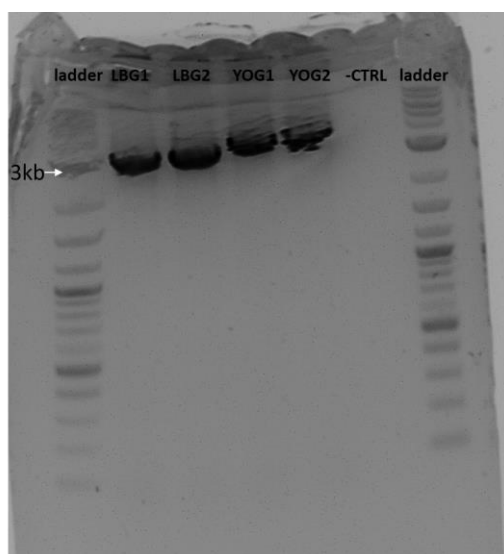


Figure 10: Colony PCR of diverse colonies of BL21(DE3) resulting from transformation. The first colony of YOG was chosen for further work. Negative control had dH₂O instead of template. Expected fragment sizes can be seen in **Table 1**. Picture was edited in Microsoft Powerpoint for increased visibility (Brightness -40%, Contrast 50%, Sharpness 30%).

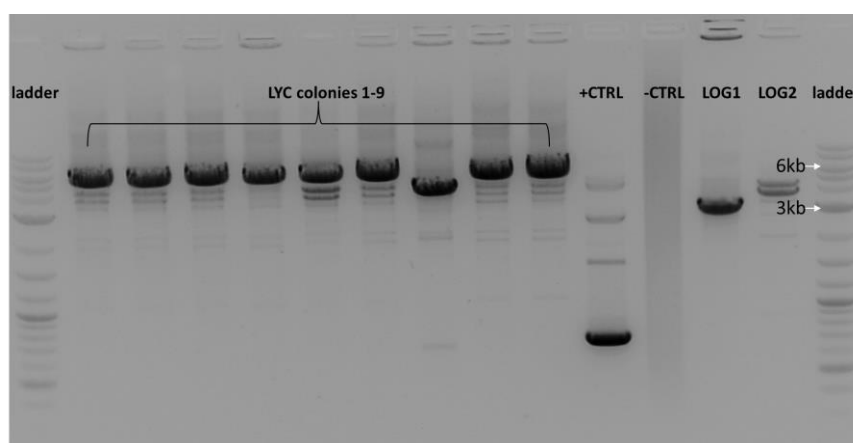


Figure 11: Colony PCR of diverse colonies of BL21(DE3) resulting from transformation. The first colony of LOG was chosen for further work as was the 4th colony of LYC. Positive control was pPMQAK1-T Y25F and negative control had dH₂O instead of template. Expected fragment sizes can be seen in **Table 1**. Picture was edited in Microsoft Powerpoint for increased visibility (Brightness -40%, Contrast 50%, Sharpness 30%).

The sfGFP tagged constructs were sequenced (for correctness of insert) prior to the transformation into BL21(DE3) by Julia Jodlbauer and as the constructs generally seemed to be quite stable, they were not sequenced again after successful transformation into BL21(DE3). The same approach was taken for the non-tagged constructs, which were first transformed into NEB_stable, where the plasmid was then isolated and sequenced, followed by transformation into BL21(DE3).

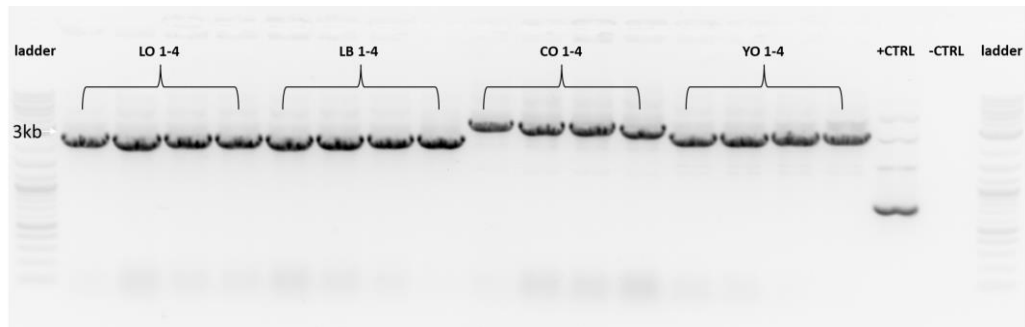


Figure 12: Colony PCR of diverse colonies of NEB_stable resulting from transformation. The 1st colony of LO was chosen for further work as was the 4th colony of CO and the 2nd of YO. Positive control was pPMQAK1-T Y25F and negative control had dH₂O instead of template. Expected fragment sizes can be seen in **Table 1**.

The constructs displayed no mutations in the analysed regions, except for CO which had a possible mutation in a non-coding region (Figure 13).

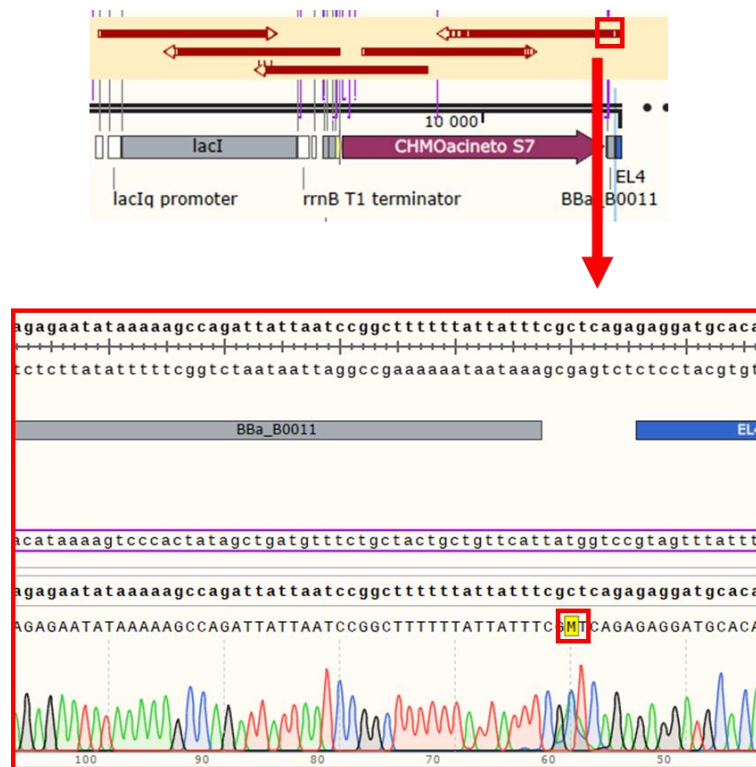


Figure 13: Excerpt from Sanger sequencing of CO showing a possible mutation from T → C.

6.1.1.1. Discussion

The cloning of each construct worked well, as was expected. The possible mutation revealed by sequencing CO was deemed acceptable, as it seemed to be a silent mutation in a non-coding region and could thus likely be ignored. If certainty about this particular position would be needed, another round of sequencing with a primer more optimized for this particular position would surely provide it. All in all, cloning and transformation succeeded in generating the desired strains, which could be compared in the next step.

6.1.2. Biotransformation of sfGFP tagged versus untagged enzymes

Biotransformations were done using the generated *E. coli* BL21(DE3) strains due to the shorter generation time compared to *Synechocystis*. The methodology was the same as described in 6.3.3, except shortening of the assay time after substrate addition. The sfGFP tag affected the different enzymes and their activities with different intensity.

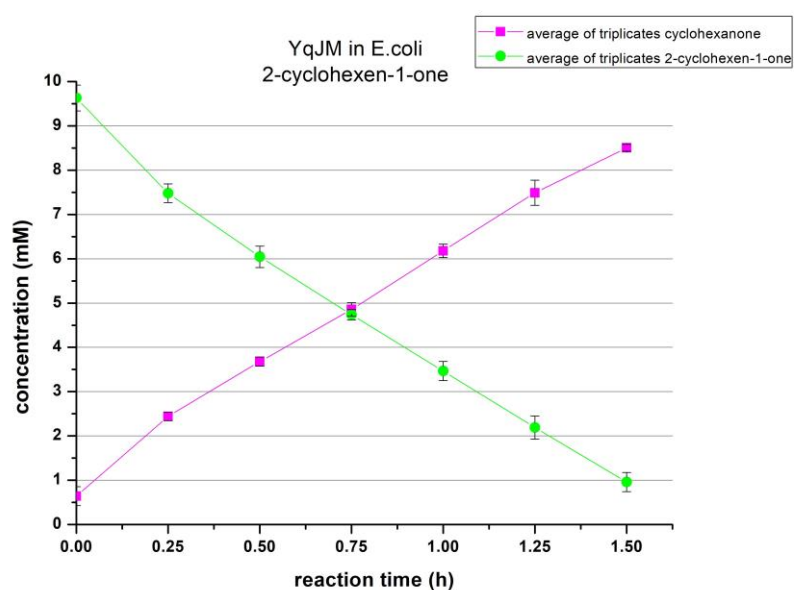


Figure 14: Biotransformation result of YO (nomenclature see 6.1.1) in *E. coli* BL21(DE3) using 2-cyclohexen-1-one (2b) as substrate.

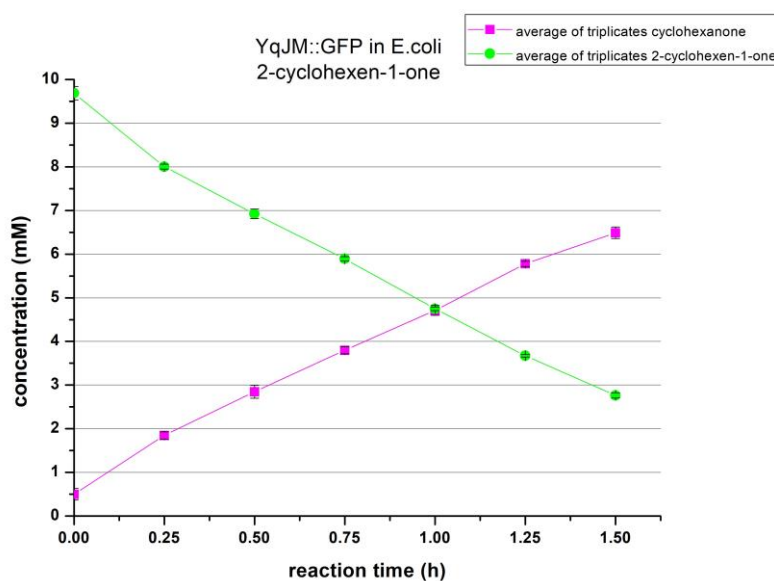


Figure 15: Biotransformation result of YOG (nomenclature see 6.1.1) in *E. coli* BL21(DE3) using 2-cyclohexen-1-one (2b) as substrate.

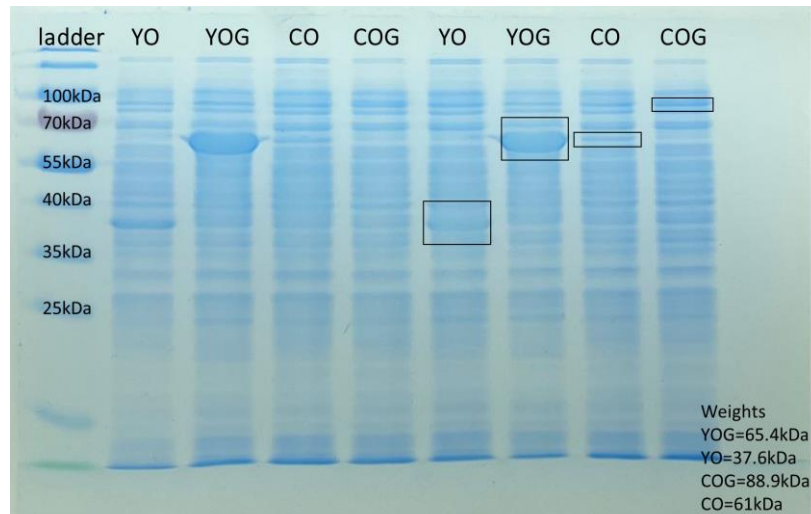


Figure 16: SDS PAGE showing soluble fraction of proteins. Total protein amount was 15 μ g per lane as determined via BCA assay. The black rectangles indicate positions of protein expression. Lanes without rectangles were from a different experiment.

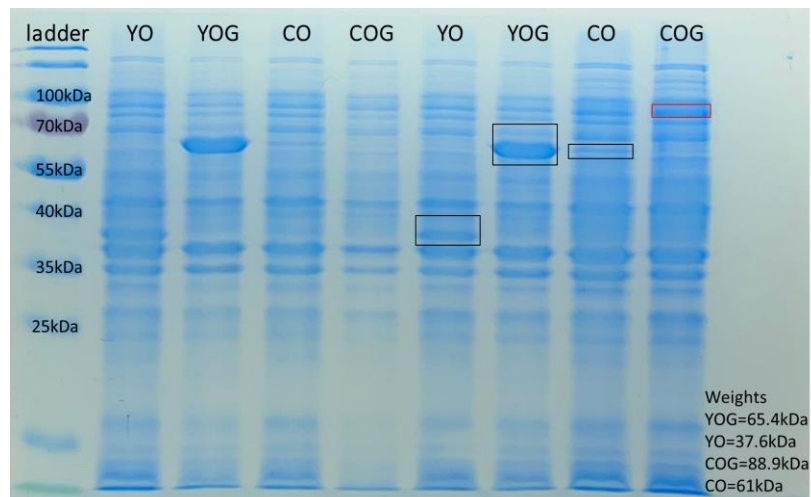


Figure 17: SDS PAGE showing insoluble fraction of proteins. Total protein amount was 15 μ g per lane as determined via BCA assay. The black rectangles indicate positions of protein expression. The red rectangle indicates a position where the expression could not be confirmed by eye and is thus uncertain. Lanes without rectangles were from a different experiment.

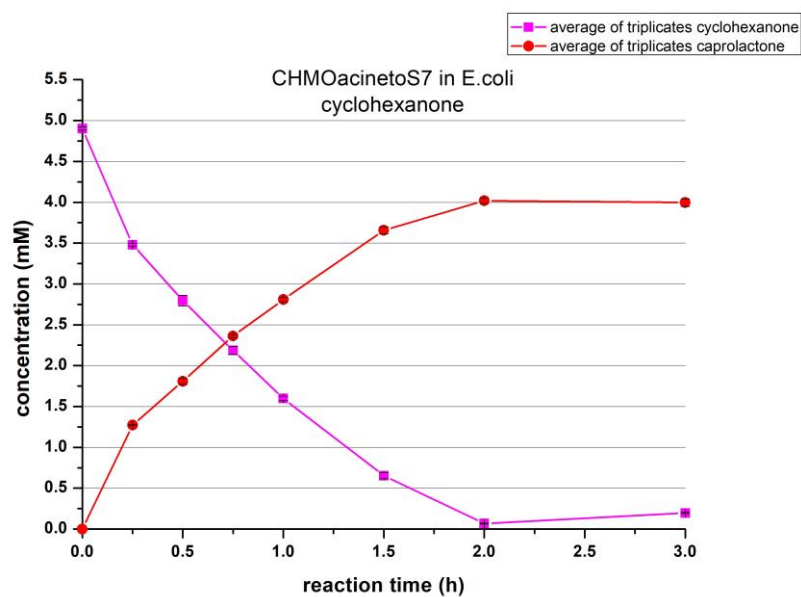


Figure 18: Biotransformation result of CO in *E. coli* BL21(DE3) using cyclohexanone (3b) as substrate.

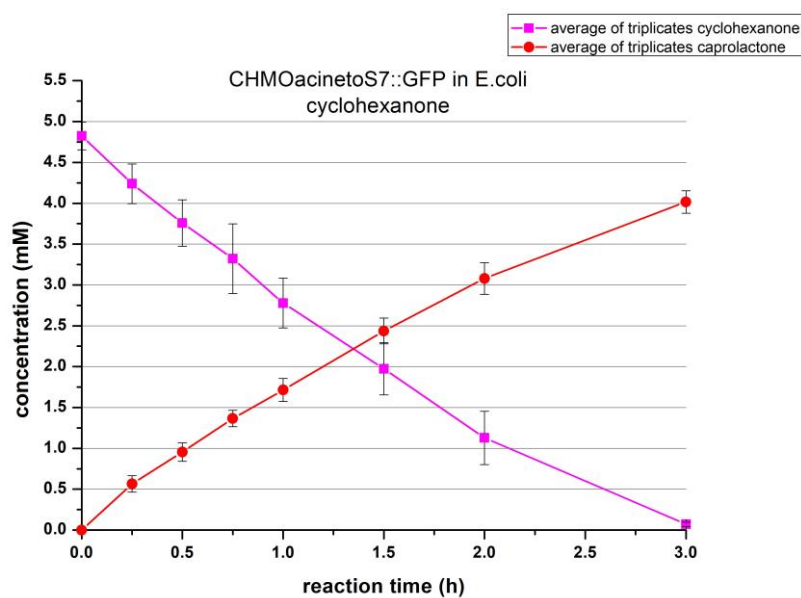


Figure 19: Biotransformation result of COG in *E. coli* BL21(DE3) using cyclohexanone (3b) as substrate.

The expression of YO, YOG and CO, COG worked well enough to confirm all of them via SDS PAGE (be it only in the soluble fraction) with the naked eye (Figure 16 and Figure 17). Both sfGFP tagged strains also displayed lower activity compared to their untagged counterparts (Table 2 and Figure 14 vs. Figure 15 and Figure 18 vs. Figure 19).

Table 2: Comparison of catalytic activity of enzymes in recombinant *E. coli* BL21(DE3), with and without sfGFP. Table taken with permission from Julia Jodlbauer. For strain nomenclature see 6.1.1.

Strain	Substrate	Product	Activity [U/OD10] ^a	Remaining activity [%]
LO	2-cyclohexen-1-ol (1b)	2-cyclohexen-1-one (2b)	47 ^b	100
LOG	2-cyclohexen-1-ol (1b)	2-cyclohexen-1-one (2b)	11.2 ^b	23.8
YO	2-cyclohexen-1-one (2b)	cyclohexanone (3b)	110 ± 4.1	100
YOG	2-cyclohexen-1-one (2b)	cyclohexanone (3b)	82.7 ± 2.3	75.2
CO	cyclohexanone (3b)	caprolactone (4b)	75.5 ± 4.8	100
COG	cyclohexanone (3b)	caprolactone (4b)	36.4 ± 2.8	48.2

^a the activity was calculated by amount of product formed (mM) within 30 minutes per culture of OD₆₀₀ = 10. The mean and standard deviation of 3 biological replicates is shown.

^b due to side reactions, for LfSDR1M50, the activity was calculated by change of 2-cyclohexen-1-ol (1b) (mM) within 30 minutes per culture of OD₆₀₀ = 10. Only one biological replicate is shown.

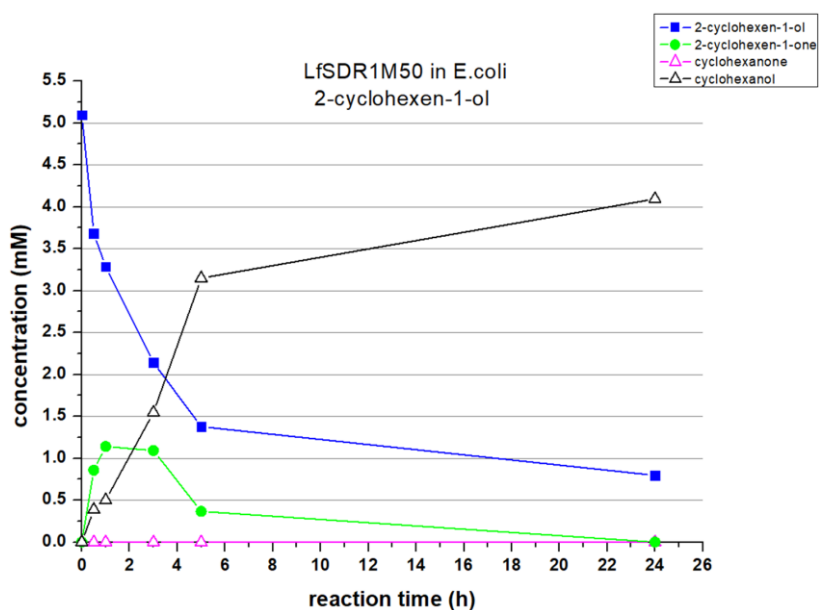


Figure 20: Biotransformation result of LO in *E. coli* BL21(DE3) using 2-cyclohexen-1-ol (1b) as substrate.

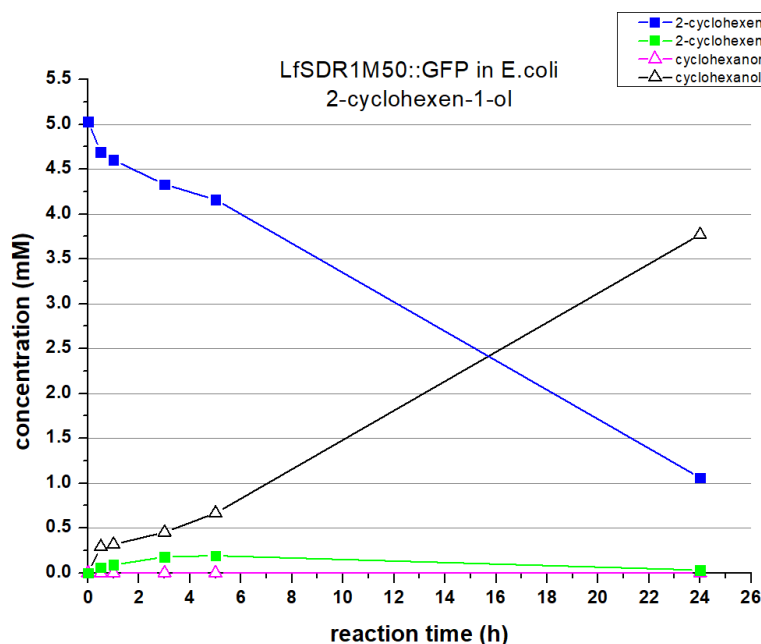


Figure 21: Biotransformation result of LOG in *E. coli* BL21(DE3) using 2-cyclohexen-1-ol (1b) as substrate.

LfSDR1M50 suffered the most from the addition of a sfGFP tag with a reduction in activity of roughly 76.2% (Table 2 and Figure 20 vs. Figure 21). The conversion to cyclohexanol seen in Figure 20 and Figure 21 is a consequence of innate EREDs in *E. coli*¹⁰⁷ which further convert 2-cyclohexen-1-one (2b) to cyclohexanone (3b) which can in turn be acted upon again by LfSDR1M50. The three assessed recombinant strains all show a clear reduction in biocatalytic activity following tagging with sfGFP, however the severity of the reduction varies strongly depending on the specific enzyme.

6.1.2.1. Discussion

Our results show that tagging our enzymes will not be viable in settings where output must be maximized (e.g. industrial applications), as the strains in which the enzymes were tagged all perform worse regarding substrate conversion. It is thus probably better to assess the best expression in a GFP tagged strain and once the best genetic construct is found, use the construct without a tag. The question if and how much the linker region and sfGFP tag affect the determined optimality of a specific genetic element e.g., a promoter, remains. It is for instance conceivable that the optimal promoter for a GFP tagged YqJM differs from the one for an untagged YqJM. The activity of LfSDR1M50 could be higher because each molecule of enzyme can now act not only on 2-cyclohexen-1-ol (1b) but also again on cyclohexanone (3b), resulting in a worse stoichiometry. Conversely, the reduction in activity from YqJM to GFP tagged YqJM is likely underestimated due to the innate EREDs present in *E. coli*¹⁵⁵. All in all, the comparison between the different strains showed that a sfGFP tag clearly results in lower catalytic activity.

6.2. Generation of cascade

6.2.1. Construct design

The first step in realizing the planned cascade was to choose a cloning method and design the constructs within the restrictions of the chosen cloning method. The cloning method chosen was the CyanoGate toolkit in combination with the MoClo toolkit, both of which are based on Golden Gate cloning and follow the BioBrick standard. The reason for choosing those methods was that they were already well established in cyanobacteria both in the literature^{156–159} and in our lab (unpublished). It was decided to ultimately arrange the genes on a single plasmid in the style of multiple monocistronic operons, each with its own promoter, ribosome binding site (RBS) and terminator. This configuration was partly necessitated due to the syntax of the chosen cloning method, which gets considerably more complicated when trying to design, for example, a pseudo-operon configuration. On the other hand, it was also practical to have the design this way, because the final construct arises from the constructs before it, which in this case were already fully expressible in *E. coli* and could thus, in case of complications, be tested individually, facilitating troubleshooting. All cloning steps were first planned *in silico* using SnapGene software.

To build the cascade the following enzymes were selected:

- LfSDR1M50, a modified glucose 1-dehydrogenase originating from *Limosilactobacillus fermentum*
- YqJM, an enoate reductase originating from *Bacillus subtilis*
- CHMO_{Acineto}S7, a modified cyclohexanone monooxygenase originating from *Acinetobacter* sp.

More details about individual components can be found in chapters 9.1.7 and 10.

6.2.2. Cloning and transformation

Cloning necessitated three steps to yield the final plasmid, namely Level 0, Level 1, and Level T cloning (see Figure 22). In Level 0 all the components (see 9.3.14 for details about construct assembly) were cloned into individual vectors which prepared their overhangs according to the syntax of the used method. These level 0 vectors were then used to clone into Level 1 constructs which in this case already yielded constructs which could express our enzymes in *E. coli*. However, to combine them all in a single construct, another cloning step (Level T) was needed. This final construct was now expressible in *E. coli* as well as in *Synechocystis*. Components were chosen according to internal experience in the lab (promoter and terminator) as well as preliminary experimental data (RBS and RiboJ).

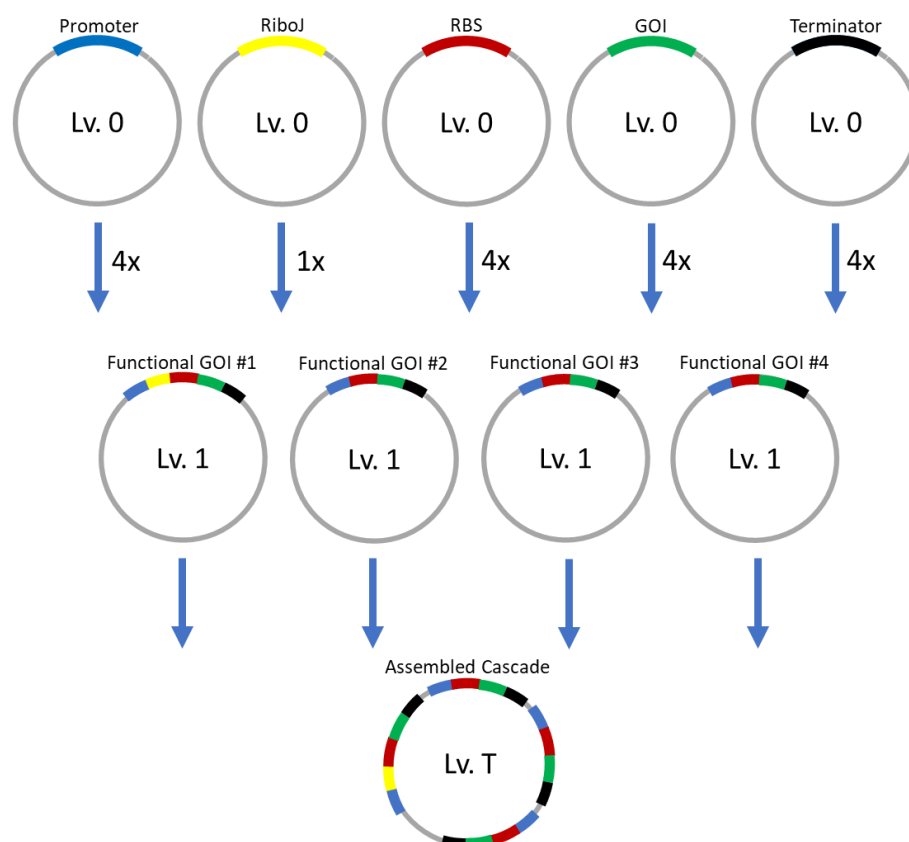


Figure 22: Schematic of cloning strategy starting from Lv.1 and parts used to generate final Lv. T construct. Note that there are four constructs at Lv.1, one for each enzyme used in the cascade plus another containing the LacI gene to enable expression control of our genes via IPTG (or lactose).

6.2.2.1. Level 0

The goal of Lv. 0 cloning was to introduce each genetic component that will be needed for later assembly of the cascade into Lv. 0 vectors, as to conform them to the syntax that is predefined by the MoClo cloning technique. LfSDR1M50 was already available in the lab in a suitable vector and did not have to be cloned for Lv. 0. YqJM and CHMO_{Acineto}S7 were edited via PCR, yielding the fragments with the right overhangs and stop codons (Figure 24 and Figure 25). The resulting fragments were loaded onto an agarose gel for gelelectrophoresis where they were shown to have approximately the expected size (Figure 23). They were then excised and cleaned up via a gel extraction kit and used for cloning into the Lv. 0 vector. The other components such as promoters and terminators were already available in suitable Lv.0 constructs from either the MoClo or the CyanoGate kits or had already been made by our group.

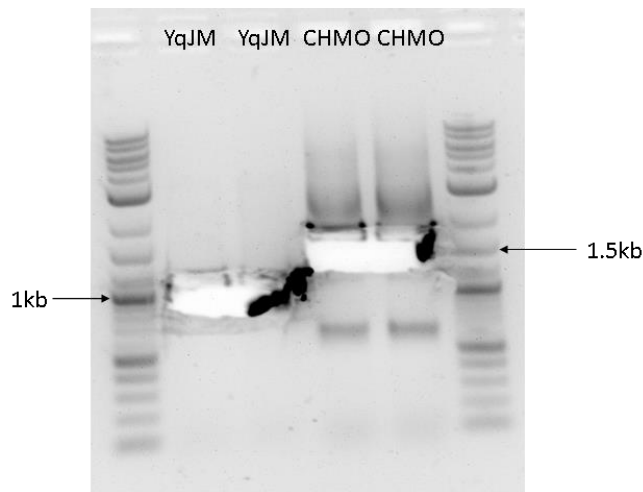


Figure 23: Gelelectrophoresis of YqJM and CHMO_{AcinetoS7} PCR fragments. Gel picture was taken after fragment excision. Expected fragment sizes were YqJM = 1058bp and CHMO_{AcinetoS7} = 1673bp

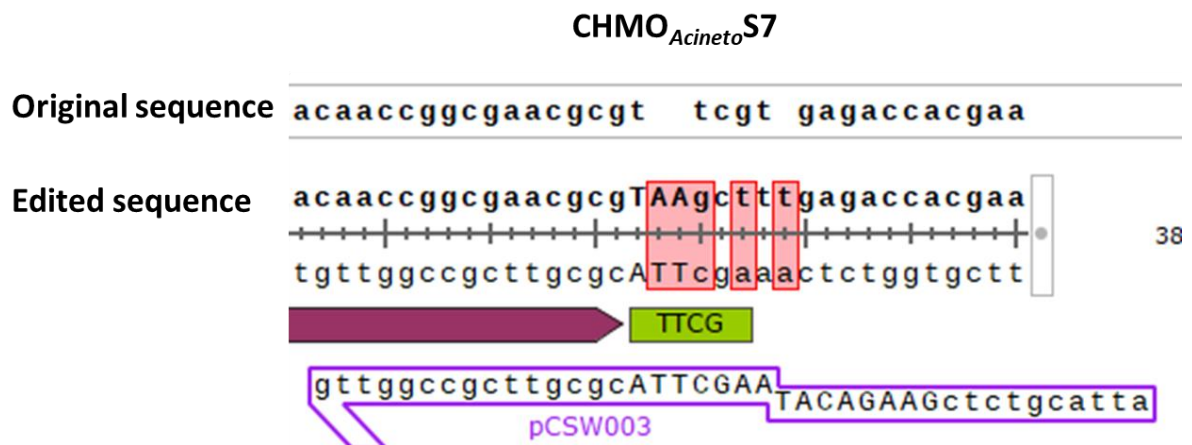


Figure 24: Original sequence of 3' end of CHMO_{AcinetoS7} (upper) vs. edited sequence (lower). Differences marked in red, the primer used is purple. From 5' to 3' end: the first two insertions introduce a stop codon (TAA) which is followed by the motif GCTT which is important for syntax reasons. The last insertion (T) is needed for proper spacing of the restriction enzyme BsaI, which would otherwise cut too far upstream.

YqJM

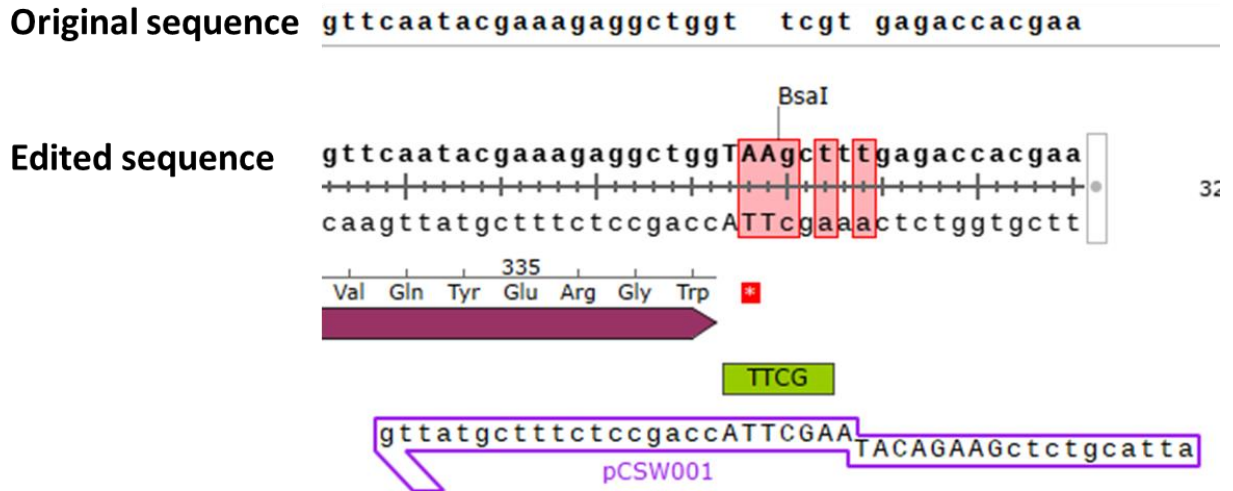


Figure 25: Original sequence of 3' end of YqJM (upper) vs. edited sequence (lower). Differences marked in red, the primer used is purple. From 5' to 3' end: the first two insertions introduce a stop codon (TAA) which is followed by the motif GCTT which is important for syntax reasons. The last insertion (T) is needed for proper spacing of the restriction enzyme BsaI, which would otherwise cut too far upstream.

The product of the cloning reactions was used to transform *E. coli* (DH5 α). The colonies were then assessed for potential candidates via colony PCR (Figure 26). All assessed colonies were potential candidates according to colony PCR but only two colonies each were chosen for sequencing. Sequencing revealed that all candidates contained the correct sequences and thus they were frozen at -80°C until further use.

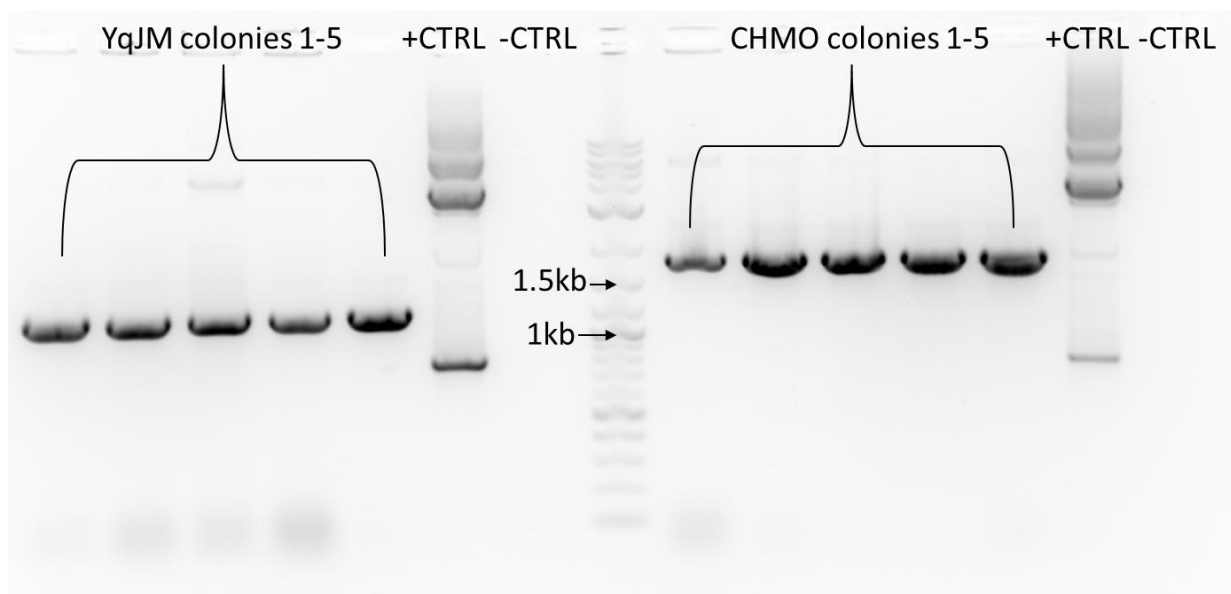


Figure 26: Results of colony PCR of Lv.0 cloning of YqJM and CHMO_{AcinetoS7}. All colonies were potentially correct, but only colonies 1 and 2 (YqJM) and 4 and 5 (CHMO_{AcinetoS7}) were sent to sequencing. Positive control was pICH41308 vector. Negative control had dH₂O instead of template. Expected sizes were YqJM = 1194bp and CHMO_{AcinetoS7} = 1809bp.

6.2.2.2. Level 1

The aim of the Lv. 1 cloning was to assemble the genetic components in an orderly fashion into functional genetic units. Each of these functional units was made up by a promoter, a ribosome binding site, the respective gene of interest, and a terminator. The YqJM construct additionally had the RiboJ genetic insulator downstream its promoter. This time, the product of the cloning reactions was used to transform *E. coli* (NEB_stable), which were subsequently assayed via colony PCR. Proceeding with Level 1 cloning, the construct for YqJM was easily generated, however a practical mistake prevented generating the other constructs (Figure 27). In a second attempt at cloning the constructs for CHMO_{Acineto}S7 and LfSDR1M50 could be generated (Figure 28). Candidates from all constructs were sequenced and shown to be correct (colonies 1 and 2 for LfSDR1M50, 4 and 6 for CHMO_{Acineto}S7 and 6 for YqJM).

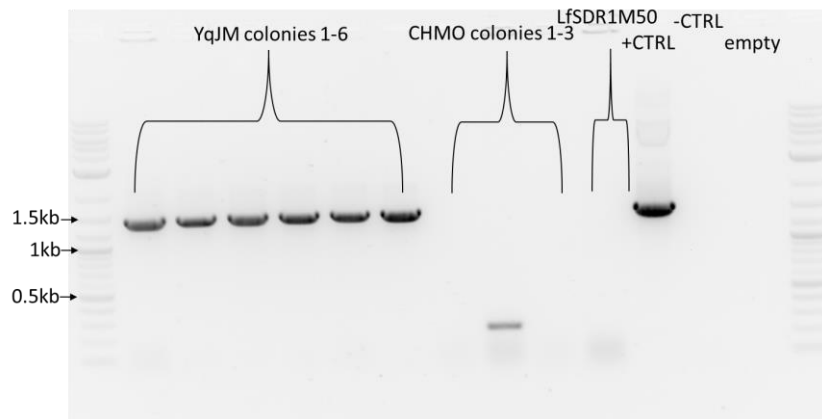


Figure 27: Results of colony PCR of Lv.1 cloning of YqJM and CHMO_{Acineto}S7 and LfSDR1M50. Expected sizes were YqJM = ca. 1.5kb, CHMO_{Acineto}S7 = ca. 2kb and LfSDR1M50 = ca. 1.1kb. Positive control was pICH47742 LacI, negative control had dH₂O instead of template. CHMO and LfSDR1 did not work due to a practical mistake made.

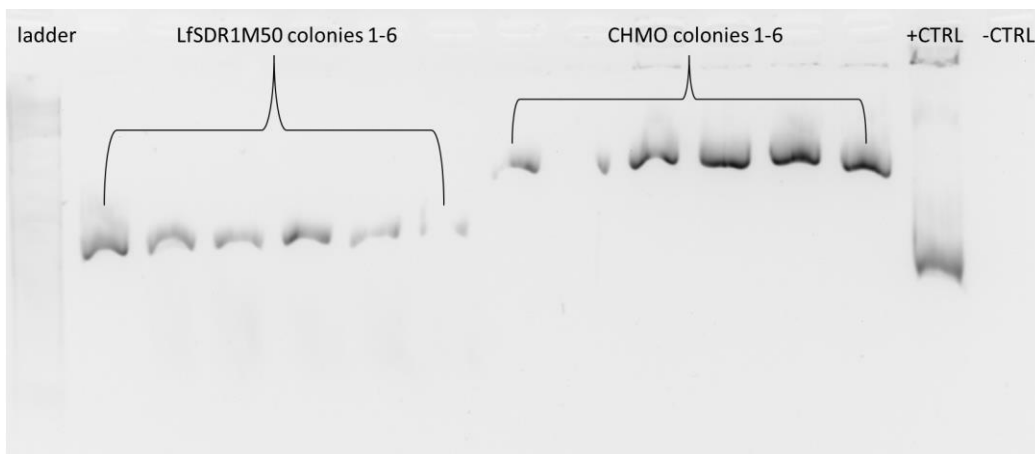


Figure 28: Results of colony PCR of Lv.1 cloning of CHMO_{Acineto}S7 and LfSDR1M50. Expected sizes were CHMO_{Acineto}S7 = ca. 2kb and LfSDR1M50 = ca. 1.1kb. Positive control was pICH47761, negative control had dH₂O instead of template. The suboptimal look of the gel is due to taking dH₂O instead of TAE buffer.

6.2.2.3. Level T

Reaching the final cloning step, all that was left to do was to assemble the cascade from the individual functional genetic units generated in Lv. 1. The resulting product of the cloning reactions was again used to transform *E. coli* (NEB_stable), which were then assayed via colony PCR. Level T cloning necessitated the use of dummies as well as endliners, which were included in either the MoClo or the CyanoGate kits. YqJM, CHMO_{Acineto}S7 and LfSDR1M50 were, together with LacI, cloned into the vector pPMQAK1-T Y25F. The mutation of this vector (Y25F) is located in mobA, which gives better results when extracting the plasmid via alkaline lysis compared to non-mutated pPMQAK1-T (mobA nicks a DNA strand resulting in single strand DNA (ssDNA) which is prevented by the Y25F mutation)¹⁶⁰. Of the colonies picked for PCR, only one colony seemed to not be a candidate and colony 3 was chosen for sequencing (Figure 29). Sequencing confirmed that the cloning was successful (Figure 30). This construct will hereafter be referred to as LYC.

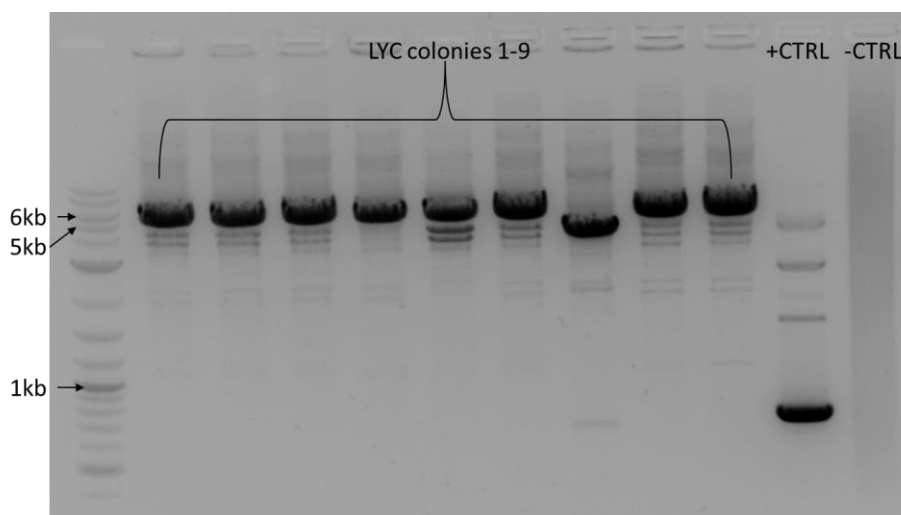


Figure 29: Results of colony PCR of Lv. T cloning. YqJM, CHMO_{Acineto}S7 and LfSDR1M50 were, together with LacI, cloned into pPMQAK1-T Y25F. Positive control was pPMQAK1-T Y25F and negative control had dH₂O instead of template. All colonies except 7 seem to be candidates and colony 3 was picked for sequencing analysis. Expected fragment size for LYC was 5480bp. Picture was edited in Microsoft Powerpoint for increased visibility (Brightness -40%, Contrast 60%).

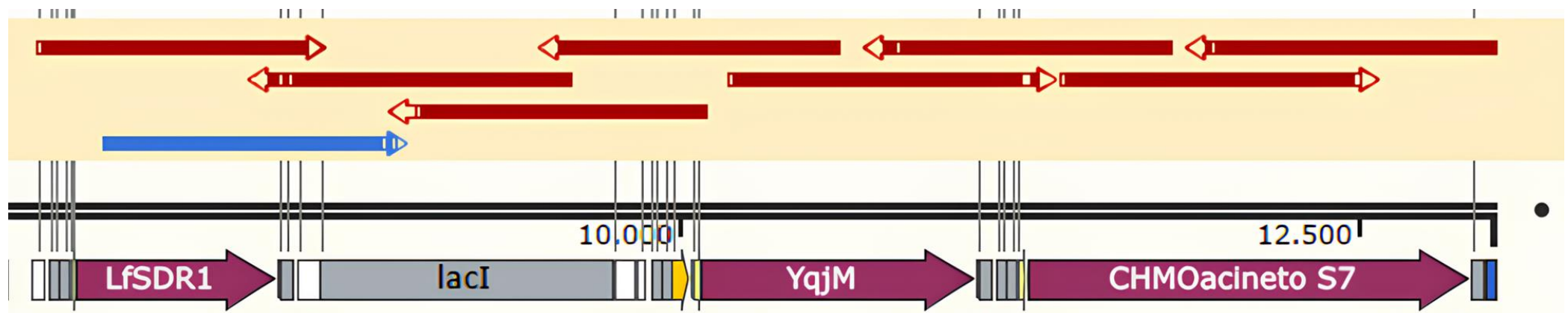


Figure 30: Sequencing results of the final construct containing all 3 GOI's. The arrows on top are coloured when matching the reference sequence. Mismatches can be observed at the beginning and end of the respective sequencing run, which is expected and thus the overlapping of different sequencings gives certainty. The very first upstream mismatch is well outside of LfSDR1M50 and its promoter (42bp upstream) and is thus, even if actually mutated, likely irrelevant for our intentions.

6.2.2.4. Discussion

Cloning worked exceptionally well in levels 0 and 1, where all colonies assayed via PCR were candidates. Level T cloning, on the other hand, necessitated a second round of cloning after the first one did not produce any positive colonies (assessed via PCR and sequencing). The second round, however, was very successful as shown above. All in all, cloning was swift and done with relative ease, speaking to the power of the used kits as well as Golden Gate cloning in general.

6.2.3. Transformation of *Synechocystis* spp. PCC6803

Transformation of the LYC construct into *Synechocystis* took considerably more time than the transformation in *E. coli*. This was not only due to the higher generation time of *Synechocystis* but also due to troubles with assaying the colonies via the PCR methods used for *E. coli*. After switching to another polymerase (from OneTaq® Quick-Load® DNA Polymerase to Q5 polymerase (NEB)), results could be improved in both consistency and signal strength (Figure 31 A and B vs. C). The construct was not only transformed into wildtype, but also into the *Synechocystis* spp. PCC6803 Δ flv1 strain, which will hereafter be referred to as CM10 (Figure 32).

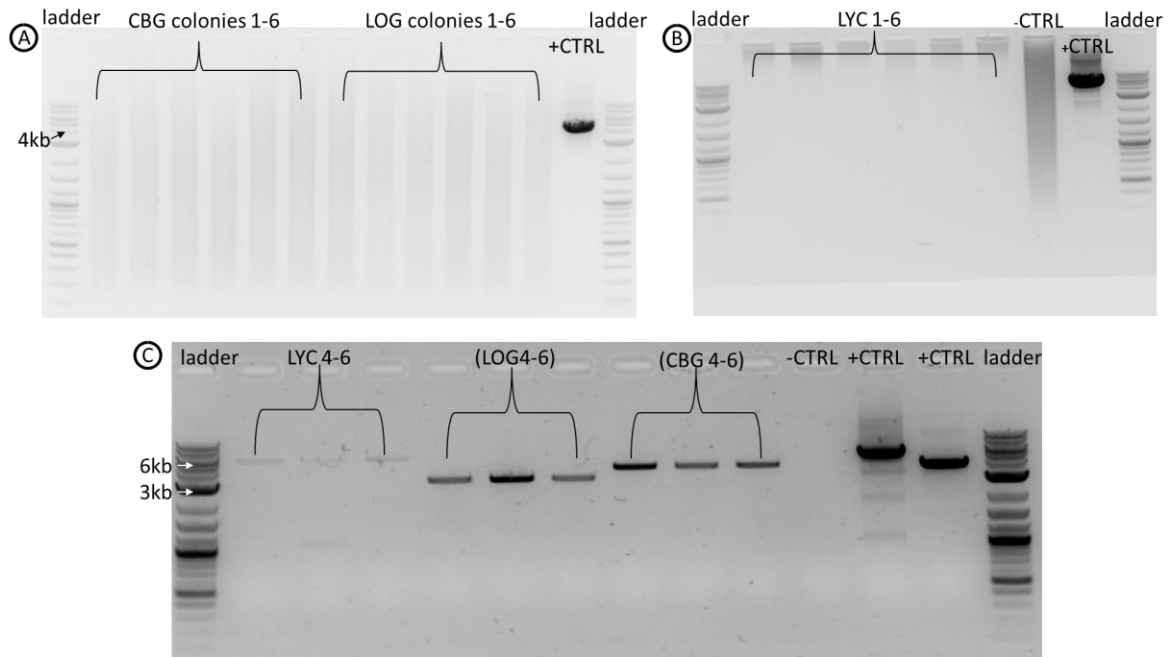


Figure 31: Exemplary colony PCR in *Synechocystis* using OneTaq® Quick-Load® DNA Polymerase and the same settings as for colony PCR in *E. coli*. **A:** CBG stands for CHMO_{Acineto}S7 fused to GFP and LOG for LfSDR1M50 fused to GFP. The positive control was isolated CHMO_{Acineto}S7 fused to GFP plasmid (4030bp). Expected fragment size was CBG = 4013bp and LOG = 3125bp. **B:** Using lysis buffer (TE (0,1% Triton-X100), pH 8). Negative control had dH₂O instead of template and positive control was CHMO_{Acineto}S7 fused to GFP (4030bp). LYC expected fragment size was 5480bp. **C:** Using Q5 polymerase and lysis buffer. The signal was clearly improved upon compared to A and B, where the PCR did not seem to work at all. Number 6 of the LYC colonies was chosen for further experiments. LOG stands for LfSDR1M50 fused to GFP and CBG for CHMO_{Acineto}S7 fused to GFP. Expected fragment size for LYC was 5480bp. Negative control had dH₂O instead of template, positive control 1 was isolated LYC plasmid (5480bp), and positive control 2 was CHMO_{Acineto}S7 fused to GFP (4030bp).

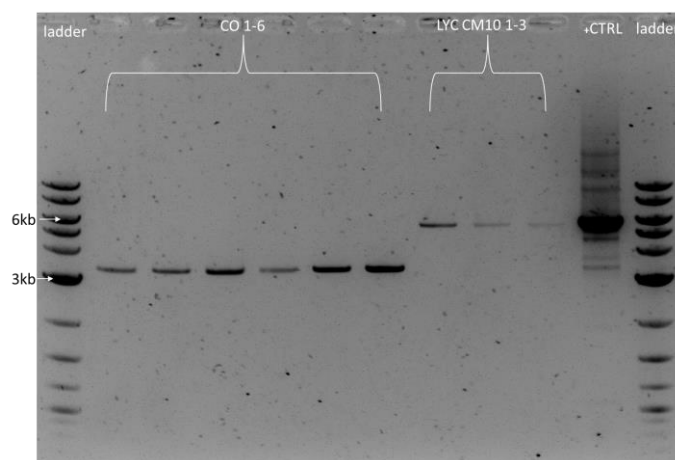


Figure 32: Colony PCR in *Synechocystis* using Q5 polymerase and lysis buffer. CM10 denotes the *Synechocystis* spp. PCC6803 Δ flv1 strain. CO stands for CHMO_{Acineto}S7 (3327bp). LYC CM10 had an expected fragment size of 5480bp. Positive control was isolated LYC plasmid (5480bp).

6.2.3.1. Discussion

Colony PCR of *Synechocystis* using OneTaq® Quick-Load® DNA Polymerase proved troublesome. Although not all PCR's using it were complete failures such as the ones depicted in Figure 31 (A and B), the success rate was much lower than what would be tolerable. First approaches to solve the problem were repeating the transformation and PCR, but with a growing sample size of colonies that were not selected by the antibiotic plates, suspicion rose. A first try at improving colony PCR conditions was the use of a lysis buffer (Table 19), to increase the likelihood of cell lysis for easier DNA access. The use of the buffer resulted in no visible increase of signal (Figure 31 B). Next the polymerase was exchanged (Figure 31 C) in combination with the use of lysis buffer, which solved the problem. The reason for this remained elusive. Transformation into CM10 worked as well as for wildtype. Transformation into CM10 was done because it was at hand in the lab and would make for an interesting first approach at improving the electron flow of the cascade.

6.3. Biotransformations utilizing the generated strains

The generated cascade strain and the LfSDR1M50 strain were assessed in *E. coli*, where a few preliminary checks were performed to assure the proper function of LfSDR1M50 and of the whole cascade before assessment in *Synechocystis*, again due to the faster growth of *E. coli*. Reasons for checking LfSDR1M50 separately are described in 6.3.2. In *Synechocystis*, the final function was determined in addition to the evaluation of a first attempt at optimization.

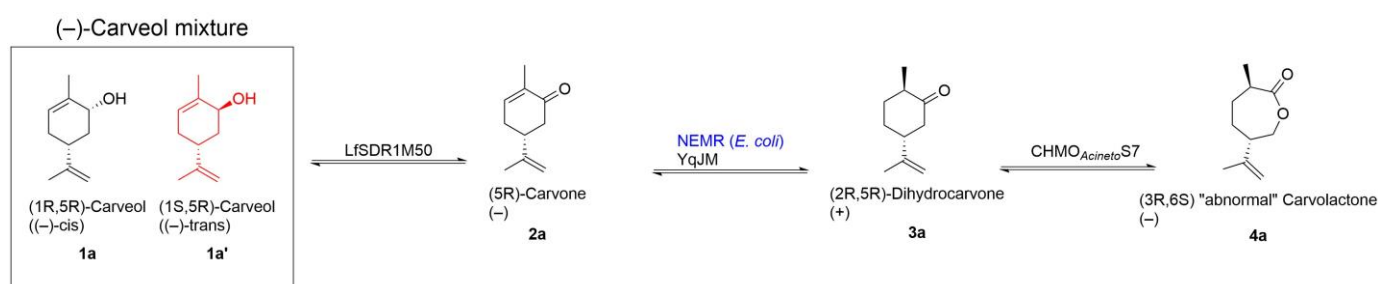


Figure 33: Substrates and course of the planned cascade. **1a'** is marked red because though it is part of the used substrate mixture, it is not accepted by LfSDR1M50. NEMR (marked blue) is, just like YqJM, part of the old yellow enzyme family and catalyses the same reaction as YqJM. It is native to *E. coli*¹⁵⁵ and is not reported to be present in *Synechocystis*.

6.3.1. Pilot biotransformations in *E. coli*

The whole cascade was pilot screened in *E. coli* to see if LfSDR1M50 worked when used together with the other enzymes. To do so, two different methods to induce protein expression were tried, namely either autoinduction or the induction via IPTG. The difference between autoinduction and IPTG induction is that in autoinduction, a specific amount of glucose is added to the medium which will be used up after a certain amount of time. After the glucose is used up, the primary carbon source in the medium is lactose, which is now transported inside the cells and converted to allolactose. Allolactose on the other hand leads to the release of the lac repressor (LacI) which then enables expression of genes formerly blocked by this repressor, such as all the enzymes used in this thesis. Conversely, IPTG induction works by adding IPTG, an allolactose analogue, to the culture at a certain cell density, requiring more manual labour. Protein expression seemed not to work, via autoinduction (Figure 35). The absence of conversion of carveol (1a) during autoinduction was still useful data as it confirmed that no other enzyme that naturally occurs in *E. coli*, accepts carveol (1a) as substrate (Figure 34) After determining that autoinduction did not work as it should, it was decided to just switch to IPTG induction and simultaneously do an additional test run with carvone (2a) instead of carveol (1a) as a substrate, leaving out the first step of the cascade (Figure 37), and thus enabling the elimination of LfSDR1M50 as the troublemaker in the cascade. The induction via IPTG however, still did not lead to an enzyme amount for LfSDR1M50 that could be detected via SDS PAGE (Figure 38). Yet, GC analysis revealed that the cascade worked, starting from carveol (1a) (Figure 36) as well as from carvone (2a) (Figure 37). As carvolactone (4a) is not easily available and using it as a reference substance

is thus difficult, the absence of educt combined with the buildup of a substance with a higher retention time than all the other intermediates of the cascade (Figure 39 and Figure 40) had to be used as a proxy for functionality. Buildup of another substance was detected (Figure 39 and Figure 40), however due to the lack of quantification for the suspected carvolactone (4a) product, it could not be assessed if this was a side product arising from the cascade or if it was buildup of a substance involved in *E. coli* metabolism which could for example be upregulated due to a stress response of the cells.

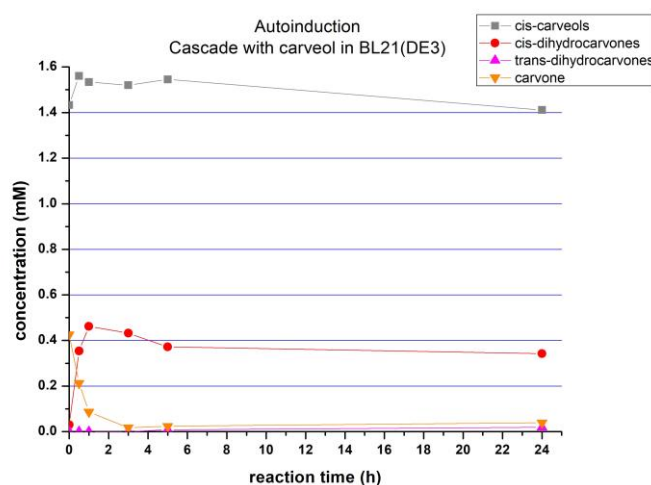


Figure 34: Biotransformation using the autoinduction method for the conversion of 5mM of a (–)-carveol mixture (*trans:cis* of 2:1.4). Only the stereoisomer available for transformation via LfSDR1M50 is shown in grey. The gap to 5mM is explained in part by the other stereoisomer. However, it is not enough to explain the whole difference, which is likely to have been due to the age of the used mixture and the volatility of carveol, as there was only 3.4mM of (–)-carveol to begin with (*trans*-carveol (1a') not shown above).

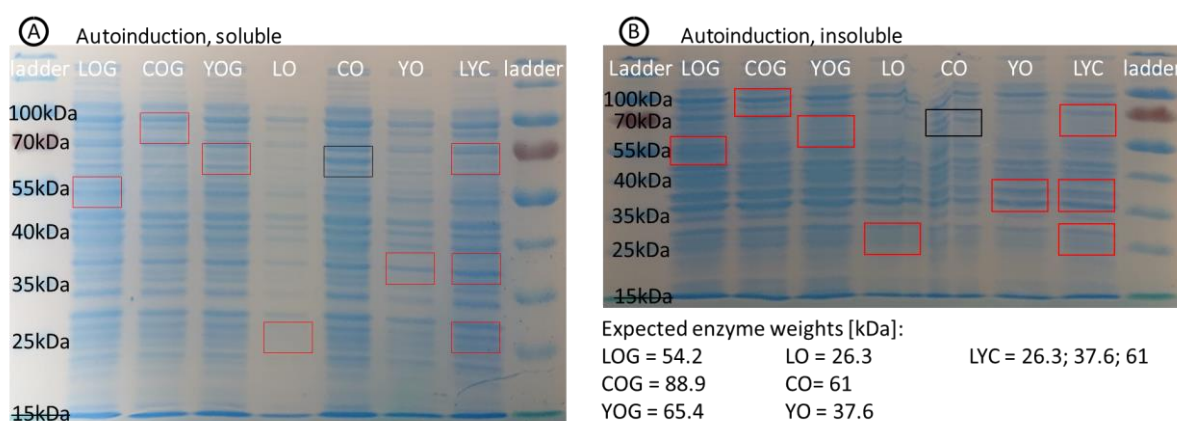


Figure 35: SDS PAGES of the soluble (A) and insoluble (B) fraction of BL21(DE3) transformed with the respective genetic constructs. The method for induction of protein expression was autoinduction. Boxes indicate where the respective protein should run. If the box is red, no band indicating expression of the respective protein could be found. A black box indicates an identifiable expression of the respective protein. As can be seen, there was no detectable expression of any of our enzymes except for CHMO_{Acinetobacter}S7. The 15kDa band did not migrate to completion and is thus struck through. The acronyms are to be deciphered as follows: The first letter is identical with the first letter of the enzyme, the second letter describes the RBS used and the third letter, if present, designates a GFP tag (details see 1).

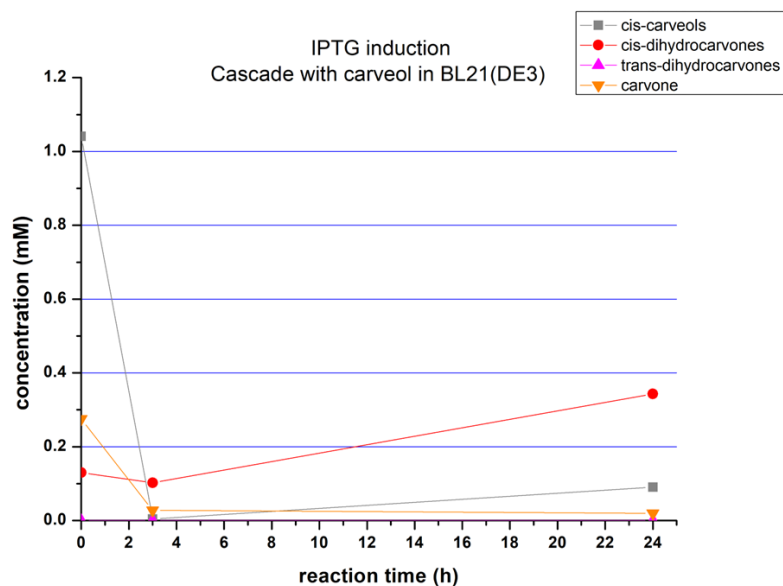


Figure 36: Biotransformation using the IPTG method of induction for the conversion of 5mM of a (–)-carveol mixture. Only the stereoisomer available for transformation via LfSDR1M50 is shown in grey. The gap to 5mM is explained in part by the other stereoisomer. However, it is not enough to explain the whole difference, which is likely to have been due to the age of the used mixture and the volatility of carveol. Measurement timepoints were reduced to three to save resources, as this should be enough to determine if the cascade works or not.

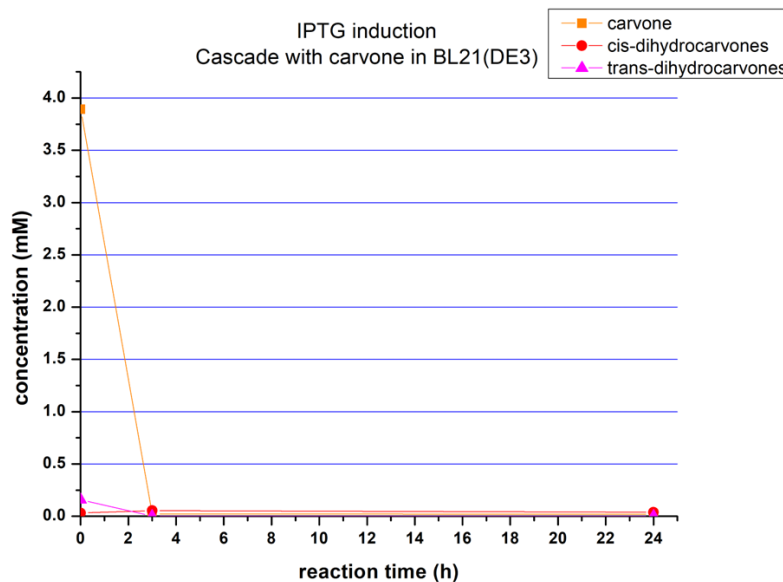


Figure 37: Biotransformation using the IPTG method of induction for the conversion of 5mM of (–)-carvone (2a). The difference to 5mM is explained by suboptimal mixing following substrate addition and impurities in the stock solution. *Trans*-dihydrocarvones (3a) are immediately generated showcasing the speed of YqJM. Measurement timepoints were reduced to three to save resources, as this should be enough to determine if the cascade works or not.

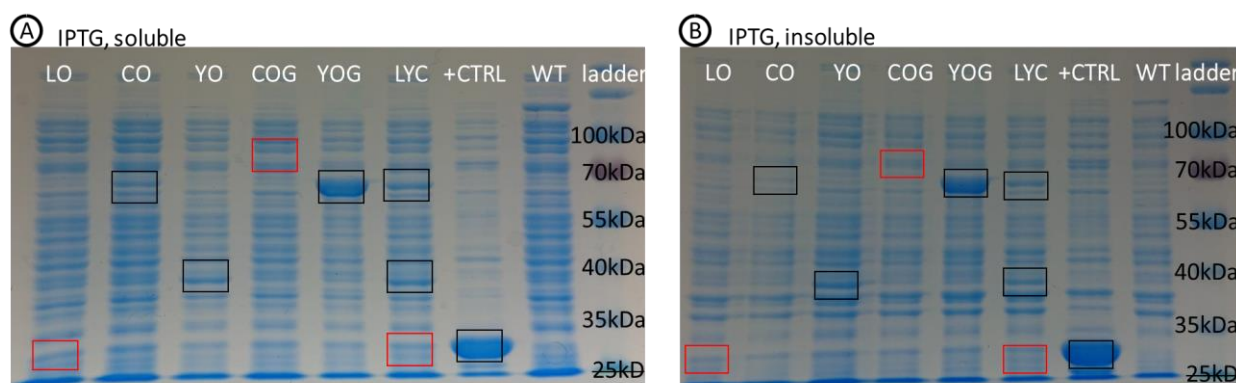


Figure 38: SDS PAGEs of the soluble (A) and insoluble (B) fraction of BL21(DE3) transformed with the respective genetic constructs. The method for induction of protein expression was IPTG. Boxes indicate where the respective protein should run. If the box is red, no band indicating expression of the respective protein could be found. A black box indicates an identifiable expression of the respective protein. As can be seen, the enzyme expression was still lacklustre as LfSDR1M50 and the GFP tagged CHMO_{Acineto}S7 could both not be found. The 25kDa band did not migrate to completion and is thus struck through. The acronyms are to be deciphered as follows: The first letter is identical with the first letter of the enzyme, the second letter describes the RBS used and the third letter, if present, designates a GFP tag (details see 1).

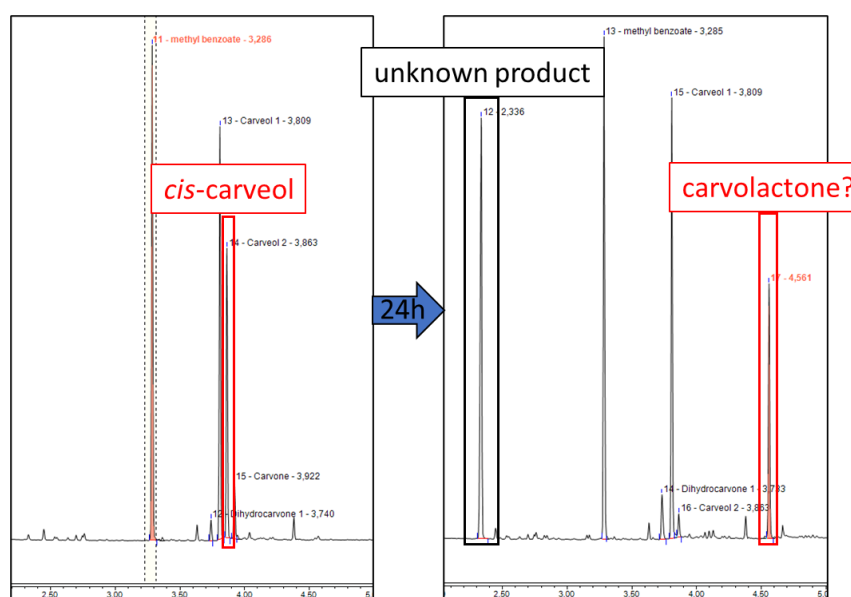


Figure 39: Achiral gas chromatography results showing substrate concentrations before and after 24h of biotransformation using the IPTG method of induction for the conversion of 5mM of a (-)-carveol mixture. Methyl benzoate was used for calibration purposes. Only *cis*-carveol (1a) changes concentration, *trans*-carveol (1a') appears unaffected. After 24h two new peaks appear, one at 2.336 which is either a sideproduct or a metabolite and the other at 4.561 which is assumed to be carvolactone (4a).

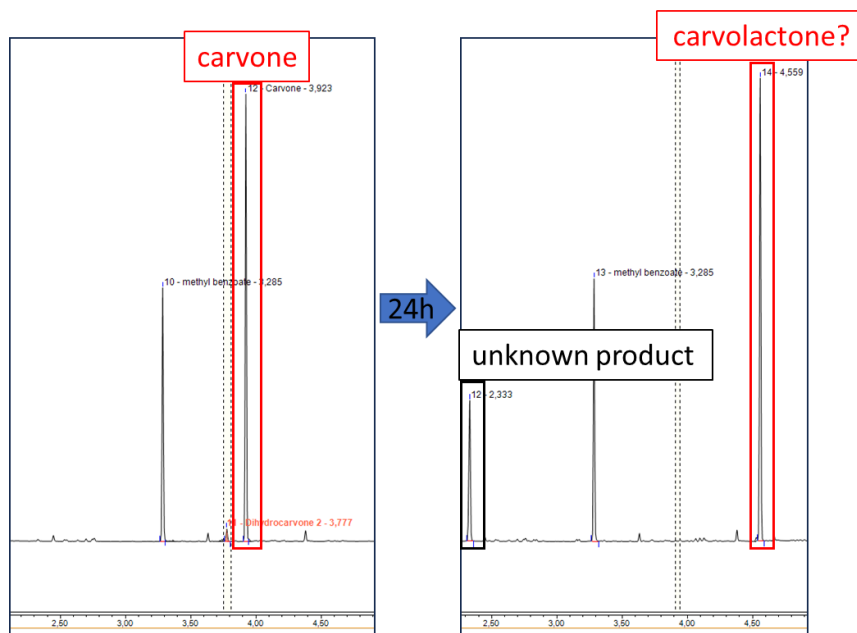


Figure 40: Achiral gas chromatography results showing substrate concentrations before and after 24h of biotransformation using the IPTG method of induction for the conversion of 5mM of (–)-carvone (2a). Methyl benzoate was used for calibration purposes. After 24h two new peaks appear, one at 2.333 which is either a sideproduct or a metabolite and the other at 4.559 which is assumed to be carvolactone (4a).

6.3.1.1. Discussion

The small pilot screening gave good evidence that LfSDR1M50 worked together with the other enzymes. It also showed that there might be a side product, however this might not be the case in *Synechocystis*. It furthermore highlighted the necessity of finding substitute substrates for the cascade, due to the complex synthesis of the appropriate carvolactone (4a) for calibration of the GC analysis, without which the amount of product cannot be assessed. The next step was thus to find a substitute substrate of less stereochemical complexity and thus better commercial availability to make the cascade more easily quantifiable.

6.3.2. Test biotransformation of LfSDR1M50 and cascade using substitute substrates

A substitute that would likely work was found in 2-cyclohexen-1-ol (1b) (Figure 41). 2-cyclohexen-1-ol (1b) was already used as a substitute in other cascades utilizing alcohol dehydrogenases, which also aimed at producing carvolactone (4a)¹⁰⁷. The intermediates were also already known to be accepted by YqJM and CHMO_{Acineto}¹⁶¹. Thus, two things that had to be assessed was if LfSDR1M50 accepts 2-cyclohexen-1-ol (1b) and if it converts it *in vivo* to 2-cyclohexen-1-one (2b) when there is no additional enzyme altering the reaction equilibrium in this direction. So far, LfSDR1M50 had only be described to catalyse the opposite reaction direction and only *in vitro*¹²⁴.

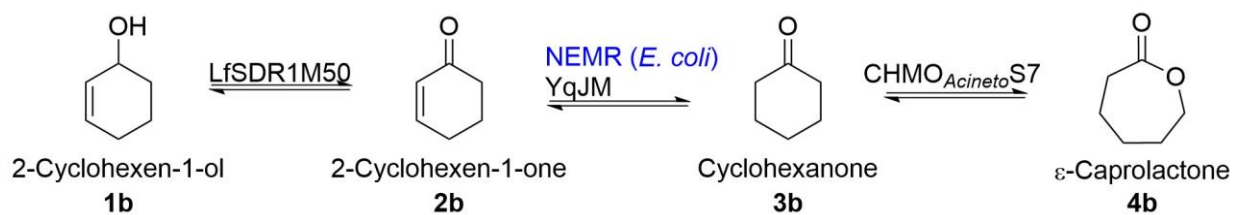


Figure 41: Substitute substrates and planned course of cascade. Note that again NEMR is reported to act like YqJM in *E. coli*¹⁰⁷.

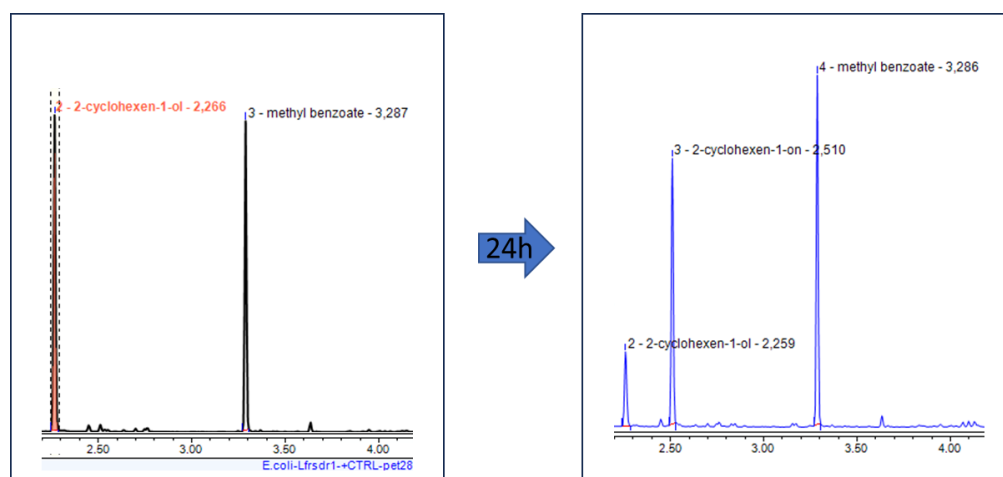


Figure 42: Achiral gas chromatography results showing substrate concentrations before and after 24h of biotransformation using the IPTG method of induction for the conversion of 5mM of 2-cyclohexen-1-ol (1b). Methyl benzoate was used for calibration purposes. LfSDR1M50 was introduced via pET28a(+).

The generation of 2-cyclohexen-1-one (2b) from 2-cyclohexen-1-ol (1b) worked (Figure 42). Next the whole cascade was tested, starting with substrate as well as with both intermediates (Figure 43). Curiously, the cascade seemed to partially stop at cyclohexanone (3b) every time.

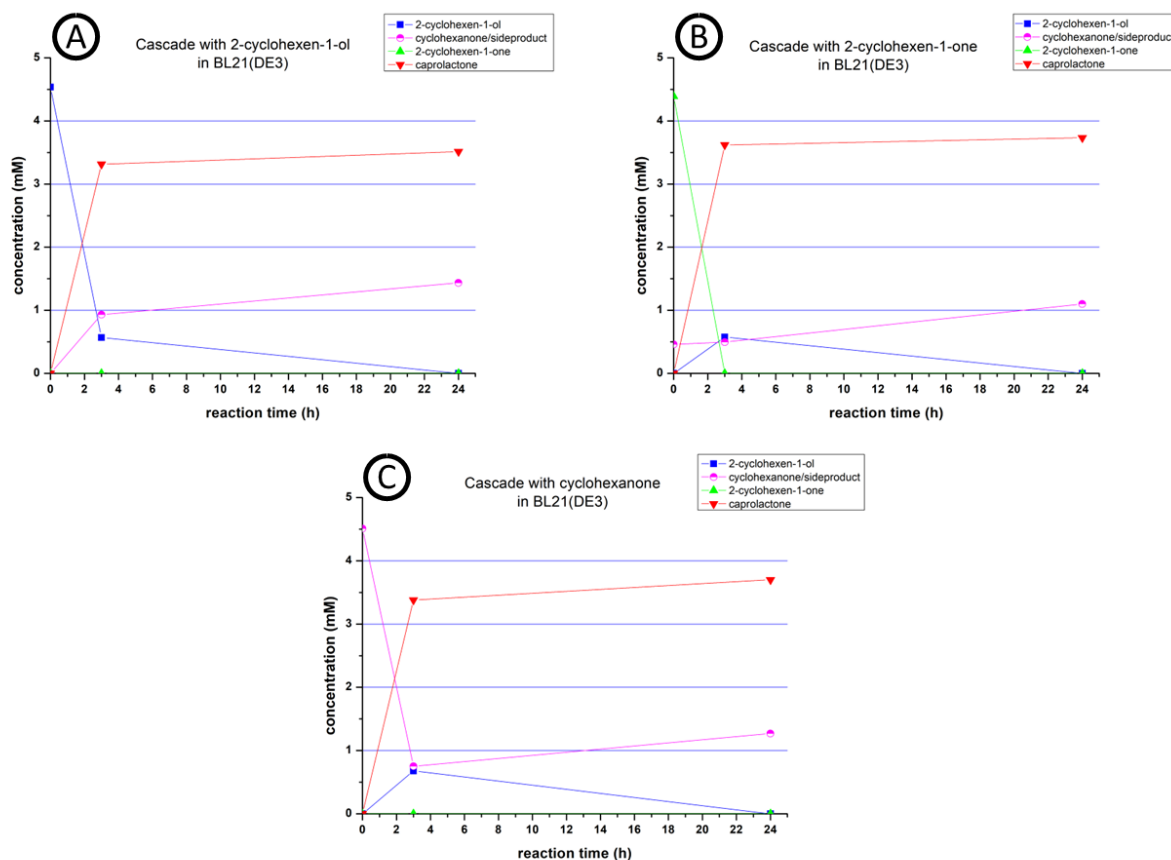


Figure 43: Biotransformation results of substitute substrates in *E. coli* BL21(DE3) via achiral GC. (A) Biotransformation using 2-cyclohexen-1-ol (1b) as starting substrate. (B) Biotransformation using 2-cyclohexen-1-one (2b) as starting substrate. (C) Biotransformation using cyclohexanone (3b) as starting substrate.

6.3.2.1. Discussion

The cascade seemingly stopping at cyclohexanone (3b) was unexpected because it did not stop at this step when using (–)-carveol mixture or carvone (2a) as demonstrated in Figure 36 and Figure 37. This led to the assumption that there may be a side product or newly generated metabolic compound that has the same retention time on our achiral GC column as cyclohexanone (3b), (this indeed appears to be the case, even though exact identification of the substance causative for this peak remained elusive, as further discussed in 6.3.4) which was to be tested by running future samples on an achiral GC column as well as on a chiral one.

6.3.3. Assessment of cascade in *Synechocystis*

The cascade was assessed via biotransformations with biological triplicates in CM10 as well as in the wildtype *Synechocystis* strain. Conversion of the cascades was determined by chiral or achiral GC, as it turned out that on the chiral GC one enantiomer of 2-cyclohexen-1-ol (1b) and 2-cyclohexen-1-one (2b) were indistinguishable from one another. Conversely, the achiral GC could not discern between cyclohexanone (3b) and an unidentified sideproduct/metabolite. 2-cyclohexen-1-one (2b) concentration remained below detectable levels as achiral GC analysis confirmed, attesting to the efficiency of YqJM, and enabling the conclusion that LfSDR1M50 prefers one 2-cyclohexen-1-ol (1b) enantiomer over the other. The conversion of 2-cyclohexen-1-ol (1b) to caprolactone (4b) worked in wildtype *Synechocystis*, however loss, likely due to a potential side product, was substantial as yield was only about 50% after 24 hours with still a substantial amount of substrate left. After 48 hours the substrate was further diminished but the amount of product stayed the same (Figure 44).

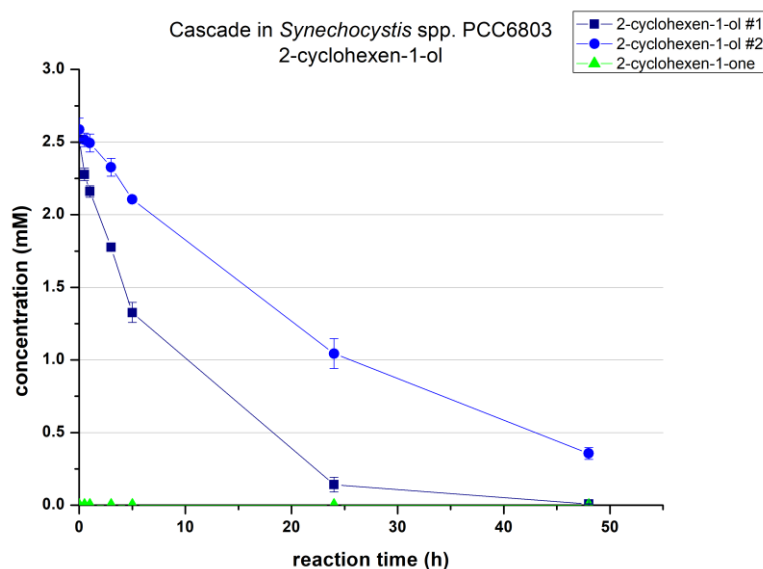


Figure 44: Different acceptance of 2-cyclohexen-1-ol (1b) enantiomers by the cascade strain.

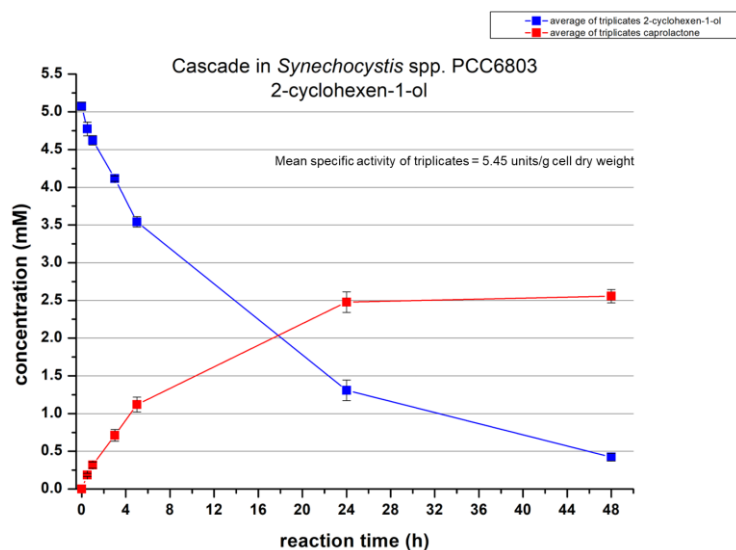


Figure 45: Conversion of 2-cyclohexen-1-ol (1b) to caprolactone (4b) as measured via chiral and achiral GC in wildtype *Synechocystis*.

The CM10 strain showed significantly faster conversion and had no substrate left after 48 hours. On the other hand, the overall yield stayed about the same (Figure 46). A direct comparison between the first few hours of CM10 and wildtype strain is shown in Figure 47.

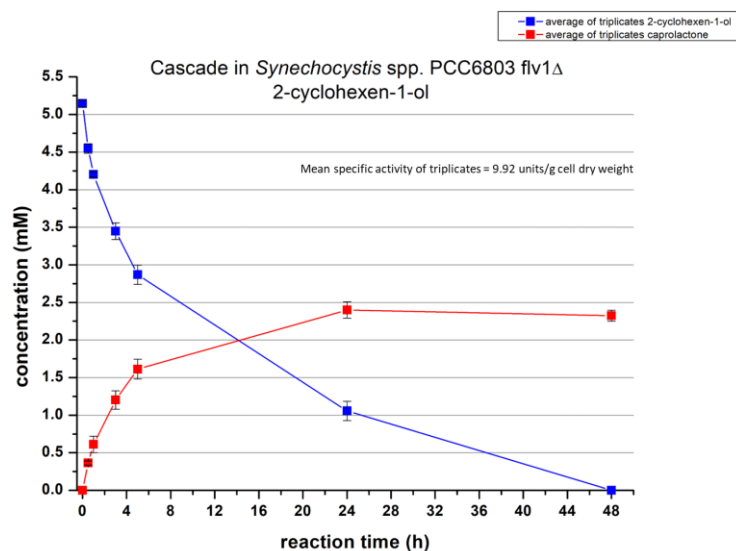


Figure 46: Conversion of 2-cyclohexen-1-ol (1b) to caprolactone (4b) as measured via chiral and achiral GC in CM10 *Synechocystis*.

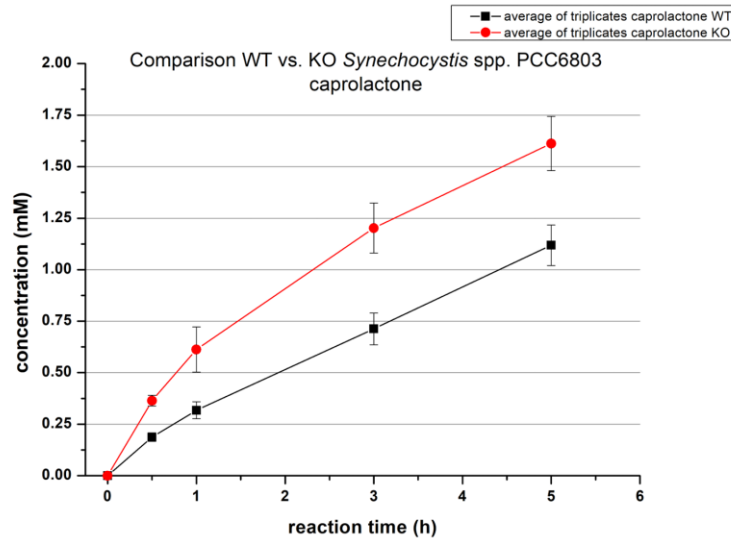


Figure 47: Direct comparison of the first few datapoints from Figure 30 and Figure 31 regarding cascade product. KO is the flv1 knockout.

Protein expression of the sampled strains was also checked, as shown in Figure 48. Expression could not be made visible via SDS PAGE except for YqjM.

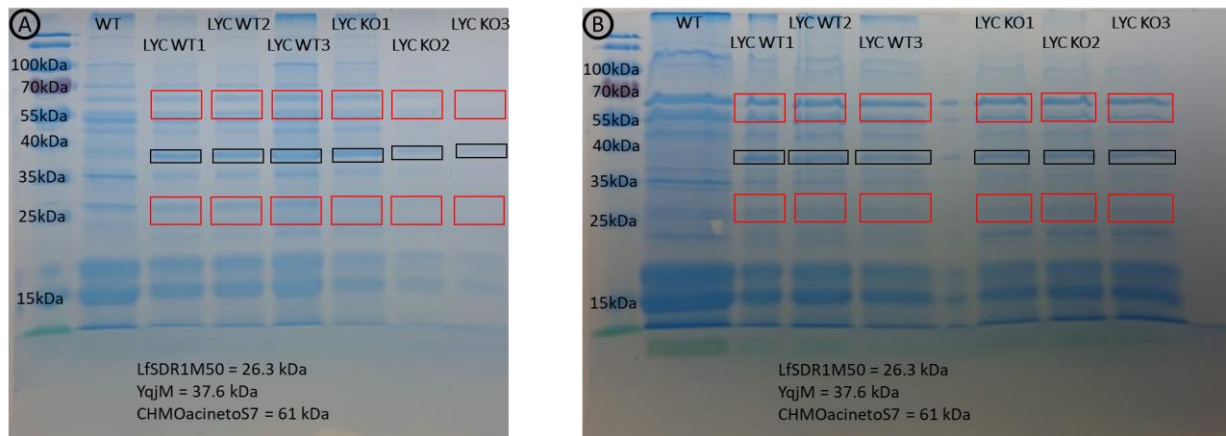


Figure 48: SDS PAGE showing (A) soluble fraction of proteins and (B) insoluble fraction of proteins. The black rectangles indicate positions of protein expression. The red rectangles indicate where protein should be expressed. Note that all samples lack visible expression of LfSDR1M50 and CHMO_{Acineto}S7 in both A and B.

A quick look at the growth of the different strains also revealed a slight reduction in cell amount (measured via optical density at 750nm as a proxy) of LYC after 72h which was further amplified in CM10 when compared to wildtype *Synechocystis* (Figure 49). Looking at the specific growth rates however, the WT strain grows slower than the LYC WT strain, which could be due to measurement inaccuracy (the WT strain was not measured in triplicates due to facility limitations at the time). The specific growth rate of LYC WT is higher than that of LYC CM10 (see Figure 49).

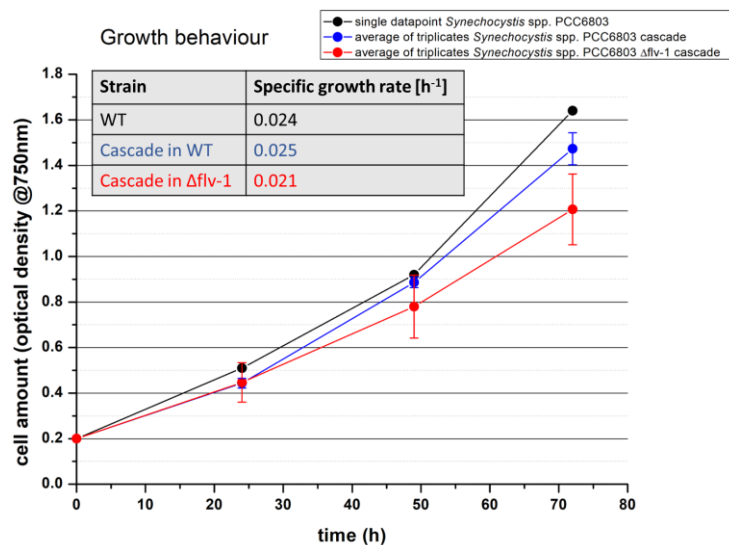


Figure 49: Growth comparison between the different *Synechocystis* strains used for the cascade and wildtype *Synechocystis* over 72 hours. Specific growth rates were calculated from the data between 24h and 72h as this was part of the exponential phase of cell growth. The specific growth rate (μ) was calculated according to the following formula: $\mu = \ln(X-X_0)/t$, where X_0 is the initial OD750 and X is the OD750 after the chosen time interval and t is the time interval (here it was 48h).

GC/MS analysis of the unknown side product/metabolite revealed a mass of 94 or 96 Dalton (Figure 50).

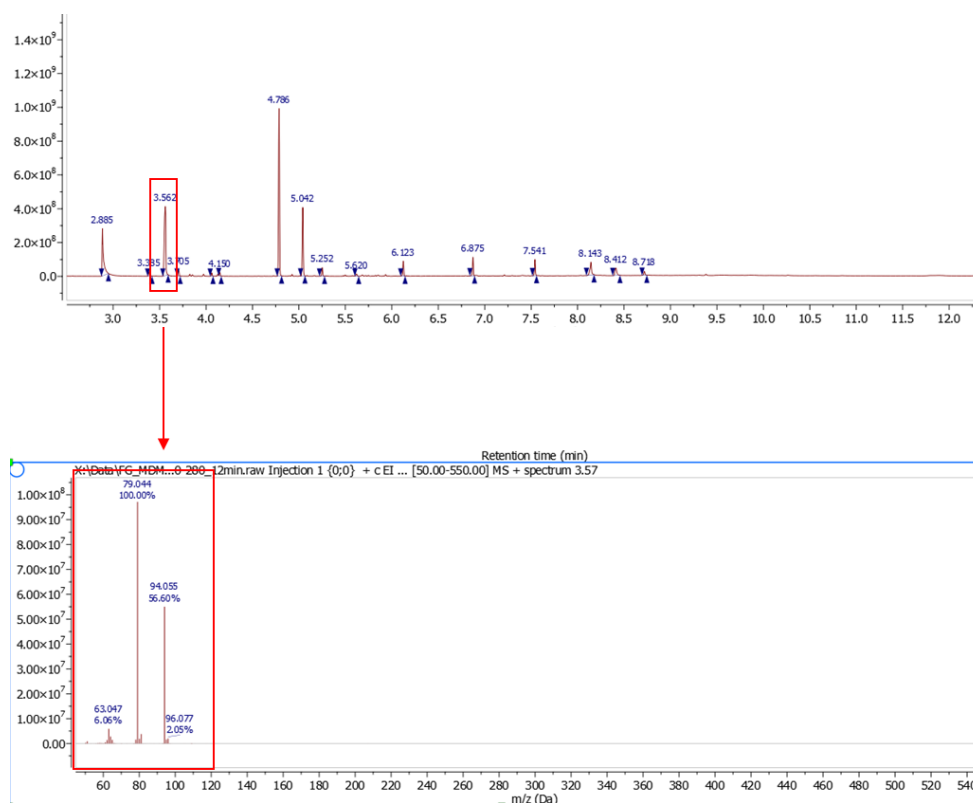


Figure 50: Achiral GC/MS measurement of a 48h sample of cascade in CM10. The upper graph depicts the GC measurement and the lower one the MS measurement. The probable side product is marked red. Possible mass of the substance is either 96 or 94 Da.

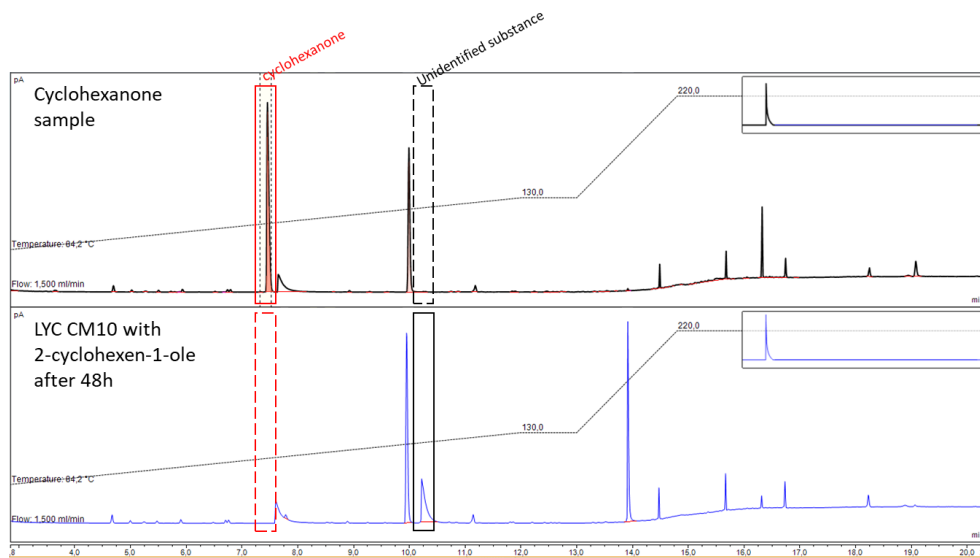


Figure 51: Comparison of chiral GC results of a calibration sample of cyclohexanone (3b) (top) and a LYC CM10 sample 48h after 2-cyclohexen-1-ol (1b) addition (bottom). It is apparent that there is no cyclohexanone (3b) in the lower sample (dashed red rectangle). The suspected side product is indicated by the black rectangle.

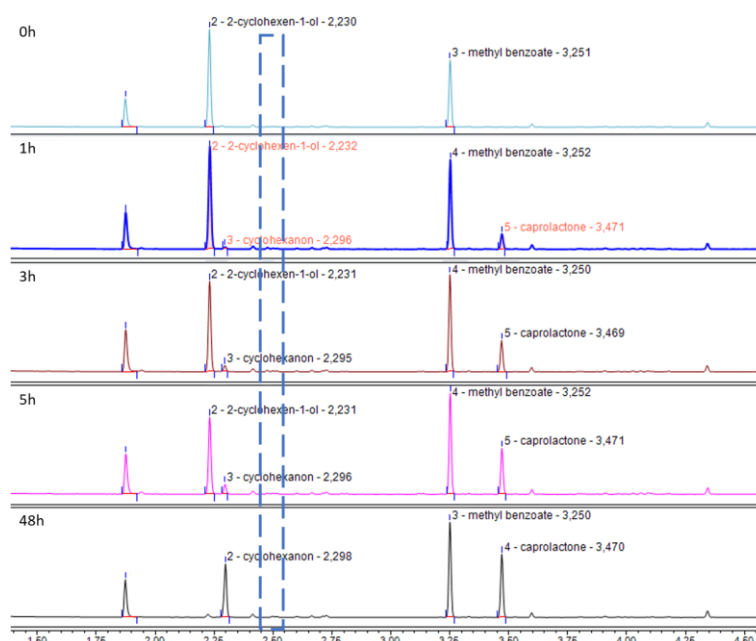


Figure 52: Representative achiral GC data of different timepoints of LYC CM10 biotransformation with 2-cyclohexen-1-ol (1b). Timepoints from top to bottom: 0h, 1h, 3h, 5h, 48h. Absence of 2-cyclohexen-1-one (2b) is marked by the dashed blue rectangle.

6.3.3.1. Discussion

The low expression of LfSDR1M50 and CHMO_{Acineto}S7 was expected as it was also seen to be the case in *E. coli*. Yet, the low expression did not seem to be an obstacle at the relatively low concentrations at which the cascade was tested. Nonetheless future improvements in the expression of LfSDR1M50 and CHMO_{Acineto}S7 would still be desirable.

The choice of loading samples not only onto the achiral but also the chiral GC revealed an unknown side product/metabolite in *Synechocystis*. A known side product generated when capro-

lactone (4b) is present in *Synechocystis* is 6-Hydroxyhexanoic acid¹⁶². However, this thus not fit with the GC/MS results, which revealed a mass of 94 or 96 Dalton which is far too low to be 6-Hydroxyhexanoic acid. Cyclohexanone (3b) would be a perfect fit with exactly 96 Dalton, and the achiral GC measurements even would confirm as much but the chiral GC results show that there is clearly no cyclohexanone (3b) present (Figure 51). Another compound with a weight of 94 Dalton is phenol, which is structurally quite similar to 2-cyclohexen-1-one (2b). Achiral GC data however shows that the unknown side product/metabolite was unlikely to be generated from 2-cyclohexen-1-one (2b), as the concentration of it remained below detection threshold for the entirety of the experiment, speaking to the efficiency of YqJM compared to the other enzymes in the cascade (Figure 52), with which the conversion to phenol would have to compete. It is thus unlikely to be the case. As the answer to our question seemed to be non-trivial, the next attempt was to identify the unknown substance via isolation and subsequent NMR analysis. Another way to at least discern between a metabolite and a side product would have been to use isotopic labeling of our substrate and see if the mass according to GC/MS changes. It was decided against due to the price and delivery time of the 2-cyclohexen-1-ol (1b) combined with the lesser potential insight offered by this approach.

6.3.4. Attempt at isolating the unknown substance from whole cell *Synechocystis*

Four liquid cultures (150ml each) of LYC wildtype were made. Cultures should reach OD750 of 1-1.5. After 4 days this was the case (Table 3).

Table 3: Measured OD750 of LYC wildtype cultures after 4 days

Culture	OD750 measured
#1	1.5
#2	1.3
#3	1.7
#4	1.55

Protein production was induced via IPTG. Growth of the cultures was checked 24h after induction (Table 4).

Table 4: Measured OD750 of LYC wildtype cultures 24h after induction

Culture	OD750 measured
#1	1.8
#2	1.7
#3	2.2
#4	2.3

48 hours after induction the OD750 was measured again (Table 5) and a 75ml culture with a final OD750 of 7.8 was made for biotransformation (aim was an OD750 of about 10 but due to the higher volume than usual it was decided that a lower density is acceptable and should aid in limiting self-shading of cells). Added 750µl of 0.5M 2-cyclohexen-1-ol (1b) in DMSO for a final concentration of 5mM. Proceeded with the biotransformation as described in 9.2.7. One sample was taken after 26h to check the progress via GC, which showed that some side product had already formed. After 46 hours another sample was taken and measured via chiral and achiral GC, which showed generation of the unknown substance (Figure 53).

Table 5: Measured OD750 of LYC wildtype cultures 48h after induction

Culture	OD750 measured
#1	2.6
#2	2.4
#3	2.7
#4	2.5

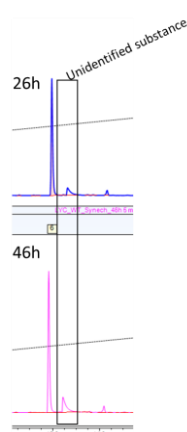


Figure 53: Chiral GC results of the LYC wildtype biotransformation. 26 hours (upper) and 46 hours (lower) after addition of substrate. The black rectangle indicates the unknown side product.

The attempt at isolation was started. First, we wanted to use CH₂Cl₂ as a solvent instead of the hitherto used ethyl acetate due to the lower boiling point of CH₂Cl₂ which could be advantageous due to the unknown boiling point of our side product. This was done via thin layer chromatography (TLC) using a Al₂O₃ matrix. CH₂Cl₂:petrolether (1:1) had indeed a comparable solubility to ethyl acetate:petrolether (1:1) (Figure 54).



Figure 54: TLC for solubility comparison between either CH_2Cl_2 (DCM) or ethyl acetate (EAc) mixed 1:1 with petrolether. Staining was done via KMnO_4 dip with subsequent heat gun treatment.

After extraction (described in 6.3.4) the evaporation was done at 40°C at 600mbar initially which was switched at a later point to 200mbar for ca. 10 minutes to remove residual CH_2Cl_2 . In total ca. 120mg were extracted which was stored at -20°C overnight. Since two kinds of substances were still discernable by eye, a GC (chiral and achiral) was made of both (one white and solid, the other orange/yellow and a liquid). The side product was only present in the liquid phase, whereas the product was present mainly in the liquid but had a measurable concentration in the solid phase (Figure 55).

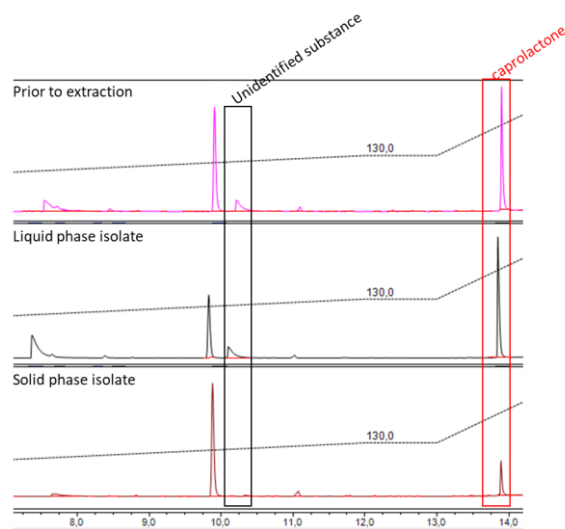


Figure 55: Chiral GC of the sample prior to extraction (top), as well as the liquid (middle) and solid (bottom) phases isolated via extraction. The black rectangle denotes the unknown side product, and the red rectangle shows caprolactone (4b).

Next the 120mg were loaded on a silica column for separation. To do so, a pentane:diethylether mixture (2:1) was employed as solvent as it was determined via TLC to have good solubility for our compounds. A total of 24 fractions were taken and each one checked via TLC. The end was determined after all three spots discernible via TLC were taken. The fractions containing the

same compound were combined and the solvent was again evaporated. This left three different compounds (Table 6). These were sent to the NMR.

Table 6: Weight of isolated compounds

Compound number	Weight [mg]
1	5.8
2	3.2
3	20.8

The NMR spectra of compounds #1 and #2 failed to give any insights because there were only solvent peaks detectable of the pentane:diethylether used for the column as well as the CDCl_3 used as solvent for the NMR. The spectrum of #3 on the other hand confirmed that caprolactone (4b) was produced (relevant NMR data see literature¹⁶³ and 10.2).

6.3.4.1. Discussion

The attempt at isolation of the unknown sideproduct/metabolite failed. A probable cause for the failure is the very low amount of produced substance. Generally, LYC CM10 had shown a higher production of the substance, but due to the slower growth of this strain it was decided against, and LYC wildtype was used instead. Other approaches to reach more production could be longer biotransformation of up to 72h, as the sought after substance is produced relatively late and still sees significant increase between 24h and 48h. Paired with a larger culture for biotransformation and using an actual OD750 of 10 and not of 7.8, this may yield enough substance to be analysed via NMR. Yet not all was in vain, as the NMR gave definitive proof of the production of epsilon-caprolactone (4b). The isolation of 20.8mg of product (with minor impurities of about 2%) from ca. 42.7mg of possible product, resulted in a yield of roughly 50%. As the main goal of this thesis was reached, there were no further attempts at elucidating the unknown substance.

7. Conclusion and Outlook

In summary our cascade in *Synechocystis* worked, as shown via a substitute substrate, with a yield of about 50% over 3 steps. With this number one must keep in mind that the lactone may undergoes ring opening, which was not factored into our results. The first attempt at optimization through a mutated host which has more NADPH available (CM10) was also a success as the mean specific activity could nearly be doubled when compared to wildtype (Figure 45 vs. Figure 46). Further improvements can likely be made by increasing the expression of LfSDR1M50 and CHMO_{Acineto}S7, as both had no visible expression when assessed via SDS PAGE (Figure 48). This could be done via optimization of the genetic parts involved (e.g. stronger promoters). Furthermore, as carveol (1a) can be synthesized from limonene, attempts could be made at integrating a (di)oxygenase into the cascade, as shown by Oberleitner *et al.*, 2017¹⁵². Cyanobacteria would again offer abundant molecular oxygen as it is a metabolic end product and thus the dis-

turbance of cellular homeostasis would likely be minimal. Very recently, it was also shown that productivity may be enhanced via the addition of a lactonase, converting ϵ -caprolactone (4b) to 6-hydroxyhexanoic acid¹⁶⁴.

A different approach would be to switch host organism to a different, faster growing cyanobacterium. *Synechococcus* spp. PCC11901 would come to mind, as it was already shown in our lab (yet unpublished results by Thomas Rohr) to be competitive with other heterotrophs such as *E. coli* when it comes to the number of workdays needed until a biotransformation can be started, due to its high growth rate and possible cell density¹⁶⁵. This would be very welcome, as one could either transition faster to expression in cyanobacteria or even eliminate the need for a shuttle vector altogether, giving access to constructs more optimized for cyanobacterial expression. A further step could be the genomic integration of the GOI's, improving genetic stability and lessening the burden on the cell through the omission of plasmid maintenance. Here the expression may be additionally upregulated via gene dosage.

Additionally, yield may be enhanced by possible improvements in cultivation. As mentioned before, self-shading is still an ever-present problem when cultivating bigger batches of cyanobacteria, which would need to be addressed. Furthermore, as our cascade uses a reductive equivalent more than it produces, the reductive power provided by the cell may still be too low, as indicated by the better performance of the CM10 strain. One possible solution would be to supply the cells with lower levels of CO₂ during biotransformation. This at first sounds unintuitive, but fixing CO₂ is another big sink of reductive power in cyanobacteria, which links inorganic carbon availability to reductive power¹⁶⁶.

Further investigations should be taken to analyse the unidentified side product/metabolite present in *Synechocystis* biotransformations. One approach to decipher if it is a genuine side product and thus siphons yield from the cascade would be to use isotope labelled substrates which are commercially available at least in the case of the substitute substrates and subsequently do a biotransformation with GC/MS analysis and see if the fragmentation pattern shows an increase in molecular mass corresponding to the used isotope. If that is not the case, the origin of the unknown substance would have to be metabolic. If it is the case, the side product would have to be isolated and identified, likely in an approach similar to the one described in this thesis but with the improvements described in 6.3.4.1. When identified, the cause would have to be determined and if a host enzyme is causative, a gene knockout may be tried.

The sfGFP tagging of the three enzymes used in the cascade was shown to have a negative influence on the potential biocatalytic activity of the cells. This decrease in biocatalytic activity differed strongly between different enzymes ranging from a 25% reduction in YqJM to a 75% reduction in LfSDR1M50, with CHMO_{Acineto}S7 being in between.

8. Materials and Methods – General Preparations

Materials and solutions used for the cultivation of *E. coli* as well as those used for *Synechocystis* spp. PCC6803 were sterilized either by autoclaving (220°C until elevated pressure, followed by 120°C for 20 minutes using a WMF pressure cooker Cromargan stainless steel) or via filtration (sterile syringe filter, 0.22µm cellulose acetate, avantor by VWR).

8.1. Antibiotic stock solutions

All antibiotic stock solutions were sterilized by filtration and subsequently stored at -20°C.

Table 7: Antibiotic stock solutions

Antibiotic	Stock conc. [mg/ml]	Working conc. [µg/ml]
Kanamycinsulfate in dH ₂ O	50	50
Chloramphenicol in abs. EtOH	10	10
Gentamycinsulfate in dH ₂ O	2.5	2.5
Streptomycinsulfate in dH ₂ O	125	125
Ampicillin (sodium salt) in dH ₂ O	100	100

8.2. Stock solutions for use in biotransformations

All stock solutions for biotransformations were kept in brown glasses at room temperature.

Table 8: Stock solutions for biotransformations

Compound	Stock conc.
(–)-carveol mixture	0.5 M (DMSO)
carvone (2a)	0.5 M (DMSO)
dihydrocarvone (3a)	0.5 M (DMSO)
2-cyclohexen-1-ol (1b)	0.5 M (DMSO)
2-cyclohexen-1-one (2b)	0.5 M (DMSO)
cyclohexanone (3b)	0.5 M (DMSO)
caprolactone (4b)	0.5 M (DMSO)

8.3. Further stock solutions

These solutions were kept at -20°C.

Table 9: Further stock solutions

Compound	Stock conc.
IPTG in dH ₂ O	1 M
PMSF in abs. <i>i</i> PrOH	0.1 M

8.4. Standard media and buffers for *Synechocystis* spp. PCC6803

Media were stored at room temperature and in the dark. The solvent was dH₂O unless stated otherwise. If stored for longer periods of time, 4°C was chosen instead.

100 x BG11

Autoclaved for sterility.

Table 10: 100 x BG11

Compound	Conc.
CaCl ₂ x 2 H ₂ O	3.60 g/l
Citric acid	0.60 g/l
NaNO ₃	149.58 g/l
MgSO ₄ x 7 H ₂ O	7.49 g/l
0.25 M Na ₂ -EDTA (pH 8)	0.56 ml/l

100 x Trace elements

Filtered for sterility.

Table 11: 100 x Trace elements

Compound	Conc. [mg/100ml]
CaCl ₂ x 2H ₂ O	360
Citric Acid x H ₂ O	63
Boric Acid	27.8
MnCl ₂ x 4H ₂ O	11.3
ZnSO ₄ x 7H ₂ O	2
Na ₂ MoO ₄ x 2H ₂ O	3.9
CuSO ₄ x 5H ₂ O	0.7
Co(NO ₃) ₂ x 6H ₂ O	0.5
FeCl ₃ x 6H ₂ O	16.1
Na ₂ EDTA x 2H ₂ O	59.5

1 M HEPES, pH 7.8

The pH was adjusted using NaOH and the solution was subsequently autoclaved.

Table 12: 1 M HEPES, pH 7.8

Compound	Conc. [g/l]
HEPES	238.31

50 x Na₂CO₃

Filtered for sterility.

Table 13: 50 x Na₂CO₃

Compound	Conc. [g/l]
Na ₂ CO ₃	9.5

YBG11, pH 7.5

The pH was adjusted prior to autoclaving using HCl. Trace elements and Na₂CO₃ were added after the solution cooled down from autoclaving.

Table 14: YBG11, pH 7.5

Compound	Conc.
NaNO ₃	1.4900 g/l
MgSO ₄ x 7H ₂ O	0.0740 g/l
K ₂ HPO ₄	0.0305 g/l
Na ₂ CO ₃ (50x)	2 ml/l
Trace elements (100x)	10 ml/l
HEPES 1 M	either 10 ml/l (for 10mM final conc.) or 50 ml/l (for 50mM final conc.)

BG11 Agar plates

After adding 6g of Bacto Agar to 780ml dH₂O and autoclaving, the agar was cooled to be just hot enough to be handled without burning oneself. Then the following solutions (sterilized via filtering unless claimed otherwise) were added:

Table 15: BG11 Agar plates

Solution	Volume added [μl]
BG11 (100x), autoclaved	8000
K ₂ HPO ₄ (30mg/ml)	800
Na ₂ CO ₃ (50x)	1684
Fe ₂ NH ₄ citrate (6mg/ml)	800
Trace elements (100x)	800
1 M HEPES, pH 7.8, autoclaved	8000
Respective antibiotic (if applicable)	800

Finally, the plates (plates used were “TC Dish 100, Standard” from Sarstedt) were poured, cooled until solidifying and stored at 4°C until usage.

1mM HEPES, pH 7.5

The pH was adjusted using NaOH and the solution was subsequently autoclaved.

Table 16: 1mM HEPES, pH 7.5

Compound	Conc. [g/l]
HEPES	0.24

TBS-buffer, pH 7.5

The buffer does not need to be sterile and was stored at 4°C.

Table 17: TBS-buffer, pH 7.5

Compound	Conc. [g/l]
Tris	21.10
NaCl	8.76

TBS-buffer with 1mM PMSF, pH 7.5

The buffer does not need to be sterile and was stored at 4°C.

Table 18: TBS-buffer with 1mM PMSF, pH 7.5

Compound	Conc.
Tris	21.10 g/l
NaCl	8.76 g/l
0.1M PMSF in abs. <i>i</i> PrOH	10 ml/l

TE (0.1% Triton-X100), pH 8

This buffer was used for better cell lysis and was adjusted to pH 8 using HCl and afterwards autoclaved.

Table 19: TE (0.1% Triton-X100), pH 8

TE-buffer 0.1% (v/v) Triton-X100, pH 8 (500ml)	
0.2370g EDTA Dinsodiumsalt x 2H ₂ O	
0.6057g Tris	
0.4940 ml Triton-X100	
494ml dH ₂ O	

8.5. Standard media and buffers for *Escherichia coli*

LB-Miller medium

Autoclaved and stored at room temperature.

Table 20: LB-Miller medium

Compound	Conc. [g/l]
Bacto-peptone	10
Yeast extract	5
NaCl	10

SOC medium, pH 7

Glucose and MgCl_2 were added after pH adjustment and autoclaving.

Table 21: SOC medium, pH 7

Compound	Conc.
Bacto-tryptone	20 g/l
Yeast extract	5 g/l
KCl	0.19 g/l
40% (w/v) glucose, sterile filtered	9 ml/l
1 M MgCl_2 , sterile filtered	10 ml/l

LB-Agar plates

After autoclaving and cooling down to be just hot enough to be handled without burning oneself, the respective antibiotic was added and the plates were poured (petri dishes, 94x16mm).

Table 22: LB-Agar plates

Compound	Conc. [g/l]
Bacto-peptone	10
Yeast extract	5
NaCl	10
Agar No.1	15

Composition of Bacterial Autoinduction Medium (AIM)

After adding bacto-peptone, yeast extract and NaCl, LB-0.8G and LB-5052 were autoclaved. NPS (20x) was autoclaved as well. 5052 (50x) was sterile filtered.

Table 23: Composition of Bacterial Autoinduction Medium (AIM)

LB-0.8G (400ml)	LB-5052 (400ml)	NPS (20x) (400ml)	5052 (50x) (250ml)
4g bacto-peptone	4g bacto-peptone	26.4g $(\text{NH}_4)_2\text{SO}_4$	62.50g glycerol
2g yeast extract	2g yeast extract	54.4g KH_2PO_4	6.25g glucose
4g NaCl	4g NaCl	56.8g Na_2HPO_4	25g α -lactose
0.4ml 1M MgSO_4	0.4ml 1M MgSO_4		
8ml 40% (w/v) glucose	8ml 5052 (50x)		
20ml NPS (20x)	20ml NPS (20x)		

N-free M9 medium

N-free M9 salts (5x) were autoclaved. N-free M9 medium was prepared with sterile components (including autoclaved dH₂O) and thus did not need further sterilization.

Table 24: N-free M9 medium

N-free M9 salts (5x)	N-free M9
2.5g NaCl	20ml N-free M9 salts (5x)
55.4g Na ₂ HPO ₄ x 5H ₂ O	2ml glucose (20% w/v) sterile filtered
15g KH ₂ PO ₄	20µl MgSO ₄ (1M, autoclaved)
	10µl CaCl ₂ (1M, autoclaved)
Concentrations on 1000ml dH ₂ O, autoclaved	Fill up to 100ml using sterile dH ₂ O

50 x TAE buffer for gelelectrophoresis

To 600ml of dH₂O add:

Table 25: 50 x TAE buffer for gelelectrophoresis

Component	Conc.
Tris	242 g/l
Glacial acetic acid, pure	57.1 ml/l
0.5M EDTA, pH 8	100 ml/l

Buffers for chemocompetent *E. coli*

Both buffers were filtered for sterility after being filled up to the nominal volume with dH₂O. Note that most protocols adjust the pH of both buffers, however I was not aware of this.

Table 26: Buffers for chemocompetent *E. coli*

RF1-buffer (250ml)	RF2-buffer (100ml)
3.02g RbCl	0.12g RbCl
1.57g MnCl ₂	0.21g MOPS
0.74g KOAc	0.83g CaCl ₂
0.28g CaCl ₂	15g glycerol
37.5g glycerol	

50mM Tris-HCl, pH 7.5

This buffer does not need to be sterile and was adjusted to pH 7.5 using HCl.

Table 27: 50mM Tris-HCl, pH 7.5

50mM Tris-HCl, pH 7.5 (500ml)
3.0275g Tris
Filled up to 500ml using dH ₂ O

Reagents for SDS-PAGE**Table 28: Reagents for SDS-PAGE**

30% (w/v) acrylamide (100ml)	10% (w/v) APS (10ml)
29.2g acrylamide	1g APS
0.8g N', N'-bis-methylene acrylamide	
Dissolved in 100ml dH ₂ O, filtered, kept at 4°C in the dark	Dissolved in dH ₂ O, aliquoted 1ml each and stored at -20°C

Buffers for SDS-PAGE

Sample buffer (6x) was stored at 4°C. The pH-adjustments of resolving and stacking gel buffers were made using HCl.

Table 29: Buffers for SDS-PAGE

10x SDS running buffer (1l)	Resolving gel buffer (250ml, pH 8.8)	Stacking gel buffer (50ml, pH 6.8)	Sample buffer (6x) (25ml)
30.3g Trizma [®] base	46.2g Tris	15.15g Tris	1.47g Tris-HCl
144g glycine	10ml 10% (w/v) SDS stock	10ml 10% (w/v) SDS stock	1.2ml Glycerol
10g SDS			1.50g SDS
			2.25ml beta-mercaptoethanol
			7.5mg bromphenol blue
			Fill up to 25ml using dH ₂ O

9. Materials and Methods – General Procedures

9.1. Organism invariant methods

9.1.1. Dilution of Primers

Primers obtained from Sigma-Aldrich were centrifuged (1min, 16160xg using a SIGMA tabletop centrifuge 1-14). They were then diluted to 100 μ M using nuclease-free dH₂O and put to 55°C for 20min with 600rpm (using Biometra TS1 Thermoshaker Analytik Jena). They were then vortexed (IKA[®] Vortex 4 basic) and stored as stock solutions at -20°C. From these stock solutions working solutions were made with a concentration of 10 μ M using nuclease-free dH₂O, which were also stored at -20°C.

9.1.2. Agarose Gel Electrophoresis

If not stated otherwise, 1% (w/v) agarose gel was prepared. For this, 1.7g of agarose (or 0.6g) were dissolved in 170ml (or 60ml) TAE buffer in the microwave, until the solution was clear. It was then cooled to be hot enough to be handled without burning of the hands and 17 μ l SYBR Safe DNA stain by Thermos Fisher Scientific was added. The gel was then poured and left to solidify. The electrophoresis chamber was filled with TAE buffer and the gel positioned and loaded. Depending on if the sample already contained loading dye (as for PCR OneTaq[®] Quick-Load[®] DNA Polymerase samples) or not (as for Q5 High-Fidelity DNA Polymerase from NEB) Loading dye was added 5:1 (6x Purple DNA loading dye from NEB). For analytical purposes 25 μ l of sample were combined with 5 μ l of loading dye and then loaded. For preparative purposes, 50 μ l of sample were combined with 10 μ l of loading dye and loaded. In the cases where no loading dye was needed, the sample was directly loaded. As a reference, 6 μ l DNA marker (1kb plus DNA ladder from NEB) was loaded. The electrophoresis was run at 120V (80V) for 30-60min depending on how much separation should be achieved. A picture was made of the gel visualizing the DNA under UV light (UVP UVsolo touch, Analytik Jena).

9.1.3. SDS-PAGE

Polyacrylamide gels (12% w/v) (0.75mm thickness) were cast for SDS-PAGE.

Table 30: Recipe for two polyacrylamide gels (12% w/v) for SDS-PAGE

Resolving gel	Stacking gel
3.2ml dH ₂ O	2.2ml dH ₂ O
2.5ml resolving gel buffer	1.3ml stacking gel buffer
4.2ml 30% (w/v) acrylamide	0.5ml 30% acrylamide
50µl 10% (w/v) APS	30µl bromophenol blue (w/v)
8µl TEMED	25µl 10% (w/v) APS
	8µl TEMED

The above amounts to 2 gels.

After the addition of APS and TEMED the solution was shortly shaken by hand and the resolving gel was poured immediately into the chambers. A layer of *i*PrOH was added on top to get a nicely uniform border between stacking and resolving gel. After ca. 20min the gel had solidified and the *i*PrOH was discarded. The stacking gel was then prepared, shaken and immediately poured on top of the resolving gel. Then the comb was stuck into the gel, and it was left to solidify. In cases where the gel was not needed immediately, it was wrapped in a wet paper towel, put in a box and stored at 4°C. For the specifics of sample preparation in *E. coli* and *Synechocystis* see 9.3.12 and 9.2.8, respectively. All 20µl of samples were loaded onto the gel. As a reference, 10µl of SDS ladder by Thermo Fisher (Prod.# 26616) was loaded. Electrophoresis was performed at 80V for 20min and then at 120V for 50-70min depending on the weight of the protein of interest (bigger protein means shorter duration). Afterwards, the gels were stained using a microwave staining technique. As a first step, the stacking gel was removed, the gels put into a box where they were covered with dH₂O and incubated at 750W in a microwave for 1min. The gels were slightly shaken by hand for ~2min, after which the old dH₂O was discarded and new one filled in. After incubating in the microwave at 500W for 1min, and shaking again for 2min, the dH₂O was again discarded and the staining solution (Invitrogen™ SimplyBlue™ SafeStain, LC6065) was applied. After incubating for ~45sec at 350W in the microwave, the gels were shaken at room temperature for ~5min. The staining solution was removed and the gels were destained overnight in dH₂O.

9.1.4. Sample preparation for Gas chromatography

For each sample the following was prepared: 1.5ml Eppendorf tube containing 300µl ethylacetate with 1mM methyl benzoate, 1.5ml Eppendorf tube containing some Na₂SO₄ (filled ca. 3mm high), GC vial with 200µl inlet and cap; Each sample was prepared by taking 150µl of sample and adding it to the Eppendorf tube containing ethylacetate with 1mM methyl benzoate. After vortexing (IKA® Vortex 4 basic) for ~30sec and centrifugation (16160xg in SIGMA tab-

letop centrifuge 1-14) the organic phase (~300µl) was transferred into the Eppendorf tubes containing Na₂SO₄ which were then likewise vortexed and centrifuged. Ca. 200µl of supernatant was taken up, filled into the prepared GC vials with inlets and the vials were sealed.

9.1.5. Preparation of Calibration curves for GC/FID

Calibration curves were generated by diluting the respective compounds in the respective reaction buffers. For this, 0.5M stock solutions were used to make triplicates of 0.5 - 6mM concentration. These were then treated as described for samples in 9.1.4. Achiral (column was Rxi®-5Sil MS by Restek®) GC (Trace1300 by Thermo Scientific) was employed, using the method “A_80_40r_280_7.1min_with Gas saver mode ON” (Table 31). The retention times of the respective compounds for this are depicted in Table 32. The Chiral (column was BGB®178) GC (Trace1300 by Thermo Scientific) was done using the method “80_2min_5r_130_1min_50r_220_6.20min_1,5mlmin_1ul – Copy” (Table 33). The retention times of the respective compounds for this are listed in Table 34.

Table 31: Method used for achiral GC/FID (“A_80_40r_280_7.1min_with Gas saver mode ON”)

Temperature	Timepoint [min]	Ramp [°C/min]	Hold time [min]
-	0	-	
80	1	40	1
280	7	-	1
-	7.1	-	

Table 32: Retention times of compounds for achiral GC

Compound	Retention time [min]
2-cyclohexen-1-ol	2.237
2-cyclohexen-1-on	2.478
caprolactone	3.494
cyclohexanone	2.285
methyl benzoate	3.273
dihydrocarvone 1	3.746
dihydrocarvone 2	3.782
carveol 1	3.820
carveol 2	3.868
carvone	3.927

Table 33: Method used for chiral GC/FID (“80_2min_5r_130_1min_50r_220_6.20min_1,5mlmin_1ul – Copy”)

Temperature	Timepoint [min]	Ramp [°C/min]	Hold time [min]
-	0	-	
80	2	5	2
130	13	50	1
220	21	-	6.2

Table 34: Retention times of compounds for chiral GC

Compound	Retention time [min]
2-cyclohexen-1-ol #1	8.286
2-cyclohexen-1-ol #2	8.577
2-cyclohexen-1-on	8.590
caprolactone	13.913
cyclohexanol	7.760
cyclohexanon	7.420
methylbenzoate	9.980

9.1.6. GC/MS

GC/MS was used with samples which were prepared as described in 9.1.4. The GC was a Trace1300 by Thermo Scientific with a ISQ Single Quadrupole Mass Spectrometer by Thermo Scientific. The column was a Rxi®-5Sil MS by Restek®.

Table 35: Method used for GC/MS (“method60-280_12min”)

Initial temperature	60°C
Initial hold time	2min
Ramp rate	35°C/min
Ramp final temperature	280°C
Ramp hold time	4min

9.1.7. Enzymes

Table 36: Enzyme characteristics

Enzyme	Enzyme class	Primary host	Reference	Gene size	Amino acids	Experimental molecular mass
YqJM	Enoate reductase	<i>Bacillus subtilis</i>	UniProt: NP_390263.1	1017 bp	338	37.6 kDa
LfSDR1M50 (LfSDR1V186A/G92F/E141L/D146V/K206L)	Alcoholdehydrogenase	<i>Limosilactobacillus fermentum</i>	NCBI: WP_015638890 Edit according to Guo <i>et al.</i> , 2018 ¹²⁴	744 bp	247	26.3 kDa
CHMO _{Acineto} S7 (CHMO _{Acineto} G14A/N336E/M400I/Q451K/T453A/V454E/Q473R/N477E)	Baeyer-Villiger monooxygenase	<i>Acinetobacter</i> sp.	Variant L3-4 from Mansouri <i>et al.</i> , 2022 ¹⁵⁰	1632 bp	453	61 kDa

9.2. *Synechocystis* spp. PCC6803

9.2.1. Cultivation conditions

Unless stated otherwise, all liquid cultures of *Synechocystis* spp. PCC6803 were kept under the following conditions:

Table 37: Culture conditions for 6.1. *Synechocystis* spp. PCC6803

Temperature [°C]	30
Orbital shaking [rpm]	120
Light [$\mu\text{E m}^{-2} \text{s}^{-1}$]	50

The CO₂ level was the ambient atmospheric level. The shaker was a Thermoshake from Gerhardt with a Planon Frameless 600x300 CCT from Osram to regulate light levels.

The cultures on plate were kept under the same conditions but without shaking. The *Synechocystis* spp. PCC6803 Δflv1 strain was provided by Yagut Allahverdiyeva.

9.2.2. Liquid culture inoculation

Liquid cultures were either inoculated from cryostocks or by picking a single colony from a plate. If picking a single colony with sufficient size was not possible, the smaller single colony was streaked out on a new BG-11 plate and grown to sufficient size. The liquid culture was then inoculated. If a liquid culture was made from another liquid culture, the new culture was inoculated to an OD750 of 0.05.

9.2.3. Optical density measurements

Optical density was determined at 750nm in a cuvette (SEMI-MICRO greiner bio-one) using a NanoDrop (NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific).

9.2.4. Cryostocks

For each *Synechocystis* strain cryostocks were prepared. Cryostocks were stored at -80°C. Always made 2x 1ml with OD750 of 9. One of which was aliquoted into 5 Eppendorf tubes with 200 μl each which could directly be used for liquid culture. The other one was kept as backup.

To reach an OD750 of 9, the appropriate amount of culture was centrifuged (1122xg, 2min, SIGMA tabletop centrifuge 1-14). Then 1ml of YBG-11 (5 % v/v DMSO) was added to each Eppendorf tube and the cells were resuspended, aliquoted and frozen.

9.2.5. Transformation

First a preculture was made by inoculating 30ml YBG11 (10mM HEPES), in sterile 100ml baffled Erlenmeyer flasks. After 3 days 80ml YBG11 (10mM HEPES) were prepared in a sterile, baffled 250ml Erlenmeyer flask and inoculated using the preculture to an OD750 of 0.05 - 0.07. After another 2 days the OD750 was again measured and the culture diluted into 2 separate flasks, one containing 80ml of OD750 = 0.25 and the other 80ml of with final OD750 = 0.3. The next day OD750 was measured again and the cells were harvested by centrifugation (3282xg, 10min, 4°C in either SIGMA 6k15 or SIGMA 3k30), where the supernatant was discarded. The cells were then washed 3 times in 10ml ice-cold HEPES (1mM, pH 7.5), after each wash they were centrifuged again, and the supernatant was discarded. The cells were then resuspended in the remaining ice-cold HEPES (OD750 ~ 30). The volume was calculated like this: number transformation reactions + 3 backup reactions * 100µl (volume needed for a single transformation). The backup reactions were there in case of a short circuit of the electroporation device. Sterile, pre-cooled Eppendorf tubes containing 100µl of cells were prepared. Then, individually, 1µg of the respective plasmid for transformation was added and it was mixed gently by pipetting. The cells were then immediately transferred to pre-cooled electroporation cuvettes (450µl volume, 2mm gap width by Carl Roth) and electroporated (2500V, 5ms (12.5kV/cm) using PEP[®] ECM399 by BTX). Immediately added 1ml of YBG11, gently mixed by pipetting and transferred to 50ml YBG11 in a 100ml sterile, baffled Erlenmeyer flask. After incubating for 4-6h under standard conditions, the cells were harvested via centrifugation (3282xg, 10min) and the supernatant was discarded. The cells were resuspended in the remaining YBG11 and spread on BG11 plates with the respective antibiotic, using inoculation loops. Plates were incubated at $20\mu\text{E m}^{-2} \text{ s}^{-1}$, 30°C for about a week. If colonies were visible by then, the light intensity was switched to standard conditions. As soon as the colonies were of appropriate size, they were broadly restreaked onto BG11 plates with the respective antibiotic to get more cells for colony PCR. If colony PCR confirmed that the construct was indeed in the cells, they were taken into liquid culture and from there frozen as cryostocks.

9.2.6. Colony PCR

The OneTaq[®] Quick-Load[®] DNA Polymerase (M0509) from NEB which was used for cPCR in *E. coli* did not function reliably for cPCR in *Synechocystis*. Thus, Q5 polymerase (NEB) was used instead for most cPCR's. Sample preparation included taking a good amount of cells (until the buffer was visibly green) and resuspending them in 100µl TE (0.1% Triton-X100), pH 8 in an sterile Eppendorf tube. The samples were then boiled at 95°C for 10 minutes (in a Biometra TS1 Thermoshaker Analytik Jena) followed by 5 minutes of cooling on ice and 5 minutes of centrifugation (16162xg, 5min, Sigma tabletop centrifuge 1-14). After that, 0.75µl of supernatant were used as template for the cPCR. If needed later, the sample was frozen at -20°C. The PCR parameters were set according to the manual provided by the manufacturer.

Table 38: Temperature program Q5 polymerase

Temperature [°C]	Time [seconds]	Step
98	30	Initial denaturation
98	8	Denaturation
59	20	Annealing
72	30/kb	Extension
72	120	Final extension
4	indefinite	Hold

The steps Denaturation, Annealing and Extension were cycled 30 times.

Some of the constructs however, were verified using the OneTaq® Quick-Load® DNA Polymerase from NEB. Namely YqJM::GFP in wildtype *Synechocystis*.

Table 39: Temperature program OneTaq® Quick-Load® DNA Polymerase

Temperature [°C]	Time [seconds]	Step
94	300	Initial denaturation
94	30	Denaturation
46	30	Annealing
68	330	Extension
68	300	Final extension
4	indefinite	Hold

The steps Denaturation, Annealing and Extension were cycled 30 times.

Table 40: Colony PCR reaction mix for the OneTaq® Quick-Load® DNA Polymerase

Compound	Amount [µl]
Forward Primer (pJJ059)	0.5
Reverse Primer (pJJ060)	0.5
OneTaq® Quick-Load® Reaction Buffer	5
dNTP mix (10mM each)	0.5
OneTaq® Quick-Load® DNA Polymerase	0.25
Nuclease-free water	18.25

Table 41: Colony PCR reaction mix for Q5 High-Fidelity DNA Polymerase

Compound	Amount [µl]
Forward Primer (pJJ059)	1.25
Reverse Primer (pJJ060)	1.25
Q5 Reaction Buffer	5
dNTP mix (10mM each)	0.5
Q5 High-Fidelity DNA Polymerase	0.25
Nuclease-free water	16.55

All PCR reactions were prepared on ice. The colony PCR reactions were made by preparing a master mix, in which the DNA template was absent. In the next step 25µl of the master mix were combined with the 0.75µl of the template (for preparation see above) in a PCR tube (250µl) and put in a thermocycler (Biometra TAdvanced Twin Analytik Jena). Where applicable, a positive control was run which contained the respective plasmid used for transformation. Regardless of the concentration of the plasmid, 0.5µl was used as template for the positive control. The PCR reactions were analysed using a 1% (w/v) agarose gel as described in 9.1.2.

9.2.7. Biotransformation

The first preculture was made in 30ml YBG11 medium (10mM HEPES), supplemented with the respective antibiotic, in 100ml sterile, baffled Erlenmeyer flasks and incubated for 5 days under standard conditions. The OD750 was measured and the culture diluted to a final OD750 of 0.2 in 30ml YBG11 medium (10mM HEPES), supplemented with the respective antibiotic, in 100ml sterile, baffled Erlenmeyer flasks and incubated for 3 days under standard conditions for the second preculture. For the main culture, 100ml YBG11 medium (10mM HEPES), supplemented with the respective antibiotic, in 250ml sterile, baffled Erlenmeyer flasks was inoculated to an OD750 of 0.05 – 0.07 and incubated for 3 days under standard conditions. The day of induction the OD750 was measured again and 1mM IPTG (final concentration) was added to the samples, which were incubated for another 2 days under standard conditions. Then the OD750 was measured again and the cells were harvested (total OD750 of 25, which was done because later they are resuspended in 2.5ml to get an OD750 of 10) by centrifugation (10min, 5000xg, roomtemperature in either SIGMA 6k15 or SIGMA 3k30). The rest of the cultures was harvested the same way and the pellet was stored at -20°C for later analysis via SDS-PAGE. Continuing with the biotransformation, the cells were resuspended in 2.5ml YBG11 (50mM) to a final OD750 of ca. 10. Then 2ml were taken and transferred to 25ml glass vials with screwable lids. The remaining 0.5ml were used to measure the actual OD750 of the biotransformations. Eppendorf tubes with 300µl ethylacetate, supplemented with 1mM methylbenzoate were prepared which were used to quench the reactions at the respective timepoints. The substrate was added to the 2ml cell suspension to a final concentration of 5mM from a 0.5M stock. The samples were incubated at 30°C, 120rpm, 150 µE m⁻² s⁻¹. Samples were then taken at the respective timepoints. The 0h timepoint was taken prior to incubating, which necessitated a strong shaking of the reaction vial by hand in order to mix the substrate well with the cell suspension.

Specific activity of each sample was calculated as follows:

$$x \cdot 1000 / ((y/a) \cdot t) = \text{units/g cell dry weight}$$

where

x = (amount of product measured [mM] after 30 minutes) – (amount of product measured [mM] after 0 minutes)

y = optical density [750nm]

a = gram of cell dry weight per litre

t = time [minutes]

The time was always 30 minutes since this was the time interval between the first two measurement points which were used for the calculations as described above.

9.2.8. SDS-PAGE

The cell pellet mentioned in 6.1.7. was washed in cold 10ml TBS buffer (pH 7.5) and again centrifuged (10min, 5000xg, 4°C). The pellet was resolved again 2ml TBS buffer supplemented with 1mM PMSF in 15ml Greiner tubes. The cells were disrupted using a sonicator (Bandelin Sonoplus HD4100 with TS106 tip) for 6min. 30sec. total (5x15 sec., 50% duty cycle intercalated with 1 min. off duty). The samples were constantly kept on ice. The opened cell suspensions were then centrifuged again (10min, 5000xg, 4°C). The supernatant (soluble fraction) was transferred to a fresh 1.5ml Eppendorf tube. The cell pellet (insoluble fraction) was resuspended in 0.5ml TBS supplemented with 1mM PMSF. Insoluble and soluble fraction were mixed 1:5 with 6x SDS sample buffer respectively and denatured for 10min at 99°C in an Biometra TS1 Thermoshaker by Analytik Jena. 30µl were loaded per sample on SDS-PAGE. Unlike in *E. coli* the protein content was not normed on 15µg of protein with a BCA assay. This was due to the low amounts of protein generally present in *Synechocystis*. The BCA assay was tried once which confirmed this notion. Further proceeded as described in 9.1.3.

9.2.9. Isolation of product and attempted isolation of unidentified side product/metabolite from whole cells

Four 150ml liquid cultures of LYC wildtype were made from liquid cultures used in 6.3.3. After four days protein production was induced by adding 150µl of 1M IPTG. The OD750 was measured 24 hours after induction and again 48 hours after induction, which was when the biotransformation was started (procedure see 9.2.7 except that 75ml of culture with OD750 of 7.8 were taken here) using 2-cyclohexen-1-ol (1b) as substrate. The attempt at product isolation was made 46 hours after the substrate had been added. First a solvent with a preferably low boiling point had to be determined, due to the unknown boiling point of the product which would later be purified via evaporation of the solvent. The solubility was checked in comparison to ethyl acetate which was used as a solvent before. This was done via thin layer chromatography (TLC). Now the whole culture was centrifuged (5min, 14000xg, RT). Next, the aqueous phase was decanted and 1:3 of solvent added. The whole mixture was transferred to a separation funnel and thoroughly shaken. The organic phase was collected, and the aqueous phase was again poured into the funnel with 1:3 of solvent. In total there were three such extraction steps. A TLC was done of the aqueous phase to see if there was any product left. If there is none left one can proceed, else extract until there is none left. The cells were centrifuged, afterwards the supernatant was taken, the cells again resuspended in solvent and centrifuged again. All supernatants were poured together in the separation funnel and Brine was added to get a clearer separation between the phases. The organic phase was taken and dried using MgSO₄. The MgSO₄ was filtered out and the solvent was evaporated using a Rotavapor R210 from Büchi. A flask was weighed in the mean-

time. The product was transferred to the weighted flask and stored at -20°C overnight. Made a GC of the isolated substances to check if the wanted ones are there. Next, made a solubility test by TLC again to check if substances were soluble in the pentane:diethylether solvent we would use. The column used for separation should always have a silicagel:substrate ratio of 70:1. The column was made, and fractions taken. TLC was made of every fraction to check for products. Fractions were combined in a way that, according to TLC, they were pure. The solvents were evaporated again with the Rotavap and the samples sent to NMR in CDCl₃ solution. Results were analysed using MestreNova software.

9.3. *Escherichia coli*

9.3.1. Cultivation conditions

Standard conditions for the cultivation of *E. coli* were 37°C and 200rpm in a multitron standard by InforsHT. Cultures on plate were not shaken and cultivated in an INCU-line by VWR. The media, unless stated otherwise, was LB-Miller medium or LB-agar plates as described in 8.5. We used the strains BL21(DE3), NEB_stable and DH5 α .

9.3.2. Overnight culture

Overnight cultures were inoculated by either picking a single colony from a plate culture or from cryostock, where a small amount of still frozen material was scraped out and used to inoculate the overnight culture. The cultures were prepared as 12ml of LB-Miller medium, containing the respective antibiotics, in a 50ml falcon tube. Incubation was under standard condition for 12-24h.

9.3.3. Preparation of cryostocks

Each *E. coli* strain generated was preserved as a cryostock. Cryostocks were generated by mixing 0.5ml of *E. coli* culture with 0.5ml of 60% (v/v) glycerol in a cryogenic vial, which was then frozen and stored at -80°C.

9.3.4. Optical density measurements

Optical density of *E. coli* was measured at 590nm in a cuvette (SEMI-MICRO greiner bio-one) using a CO7500 Colorimeter “colourwave” by WPA.

9.3.5. Preparation of RbCl competent cells

Chemically competent cells were prepared on ice and under sterile conditions. A single colony of the desired *E. coli* strain was incubated in 4ml LB-Miller medium at 37°C at 200rpm overnight. Then, 100ml of fresh LB-Miller culture was inoculated with 1% (v/v) of the overnight culture and grown to an OD₅₉₀ of 0.35. The cells were then harvested by centrifugation (4000xg, 10min, 4°C in either SIGMA 6k15 or SIGMA 3k30) and resuspended in 1/5 volume of the main culture (20ml) in RF1 buffer. Cells were incubated for at least 15min in this buffer, centrifuged, and resuspended in RF2 buffer using 1/5 volume of the RF1 suspension. The now competent cells were dispensed in 100µl aliquots in 1.5ml Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C.

9.3.6. Transformation of chemically competent cells

1µl of plasmid DNA (with concentration of 50-100ng/µl) was added to 100µl of RbCl competent cells, which were then incubated on ice for 1h. Heat shock was performed at 42°C for between 30 to 60 seconds (using Biometra TS1 Thermoshaker Analytik Jena) and cells were subsequently put on ice for 2 minutes. To aid the cells in recovering, 0.5ml prewarmed (37°C) SOC medium was added and the cells were incubated with shaking at 37°C for 1h (using Biometra TS1 Thermoshaker Analytik Jena at 650rpm). In the next step, 50µl of the cells were plated on one half of a pre-warmed LB agar plate supplemented with the appropriate antibiotic and the rest was centrifuged (3615xg, 1min, SIGMA tabletop centrifuge 1-14). The supernatant was discarded, cells were resuspended in residual supernatant and plated on the other half of the plate. Plates were incubated overnight at 37°C.

9.3.7. Isolation and quantification of plasmid DNA from cells

For isolation of the plasmid DNA, the GeneJET Plasmid Miniprep Kit by Thermo Scientific was used according to the following, slightly adapted, enclosed instructions. An overnight culture was prepared (see 9.3.2). For high copy plasmids (e.g. the pICH – type plasmids from the MoClo Kit used for cloning) 4ml of overnight culture was used for DNA isolation. For low copy plasmids (e.g. pCAT.000 pPMQAK1-T Y25F) 6ml of overnight culture was used. All of the below was performed in a non-sterile environment at room temperature and all centrifugation steps were at 16160xg (SIGMA tabletop centrifuge 1-14) unless stated otherwise. Cells were harvested by centrifugation (2min, 6800xg) and the supernatant was discarded. The pellet was resuspended in 250µl of the Resuspension Solution. The cell suspension was transferred to an 1.5ml Eppendorf tube. Next, 250µl of the lysis solution were added and the suspension was mixed by inverting 4-6 times. Then, 350µl of Neutralization Solution was added and again mixed by inverting. Solution was centrifuged for 5 minutes. The supernatant (ca. 750µl) was transferred to the supplied GeneJET column by pipetting. The columns were centrifuged and the flow-through was discarded. Next, 500µl of the Wash Solution was added and centrifuged for 60 seconds. The

flow-through was discarded and the washing procedure repeated. After discarding the flow-through again, the column was centrifuged again for 1 minute and then heated to 50 °C for 5 minutes (using Biometra TS1 Thermoshaker Analytik Jena) to remove any leftover ethanol. The column was transferred to a fresh, sterile 1.5ml Eppendorf tube and 25µl nuclease-free, warm (50°C) water were used for elution. Elution was done by incubating 2 minutes with the nuclease-free water and then centrifuging for 2 minutes. The elution was repeated once, for a total of 50µl plasmid DNA eluate. The column was discarded, and the DNA quantified using a NanoDrop (NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific) in the measurement mode “dsDNA” using 1µl of the sample. The samples were stored at -20°C.

9.3.8. Purification and quantification of PCR amplified DNA from Agarose gel

Purification of DNA from agarose gels was done using the GeneJET gel extraction Kit by Thermo Scientific and according to the following, provided manual. All purification steps were performed at room temperature. All centrifugation steps were performed at 16160xg using a SIGMA tabletop centrifuge 1-14. Gel slices were excised using a scalpel and put into pre-weighed 1.5ml Eppendorf tubes. The weight of the slice was recorded. To visualize the DNA, it was shortly irradiated with UV-light using a UV Transilluminator 2000 by Bio-Rad. A 1:1 volume of Binding buffer was added to the gel slice (100µl per 100mg of gel slice). The gel mixture was incubated at 50-60°C for 10 minutes or until the gel slice was completely dissolved. The tube was repeatedly inverted to facilitate the melting process. It was then briefly vortexed. It was then transferred to the GeneJET purification column, centrifuged for 1 minute and the flow through discarded. Then, 700µl of Wash buffer were added and centrifuged for 1 minute and the flow-through was discarded. The column was then centrifuged again for 1 minute to remove residual wash buffer. The column was then transferred to a fresh, sterile 1.5ml Eppendorf tube and 45µl of nuclease-free water (50°C) was added, and again centrifuged for 1 minute. The column was discarded, and the DNA quantified using a NanoDrop (NanoDrop™ OneC Microvolume UV-Vis Spectrophotometer, Thermo Scientific) in the measurement mode “dsDNA” using 1µl of the sample. The samples were stored at -20°C.

9.3.9. DNA construct verification via Sequencing

Samples were prepared for Sanger sequencing (480 – 1200ng DNA in 12µl nuclease-free water + 3µl respective sequencing primer (10µM) = 15µl in total per sequencing reaction) in sterile 1.5ml Eppendorf tubes.

9.3.10. Colony PCR

For colony PCR OneTaq® Quick-Load® DNA Polymerase from NEB was used:

Table 42: Colony PCR reaction mix for OneTaq® Quick-Load® DNA Polymerase

Compound	Amount [μ l]
Forward Primer	0.5
Reverse Primer	0.5
OneTaq® Quick-Load® Reaction Buffer	5
dNTP mix (10mM each)	0.5
OneTaq® Quick-Load® DNA Polymerase	0.25
Nuclease-free water	18.25

All PCR reactions were prepared on ice. The colony PCR reactions were made by preparing a master mix, in which the DNA template was absent (Table 42). In the next step 25 μ l of the master mix were combined with a single colony which was picked with a pipette tip in a PCR tube (250 μ l) and put in a thermocycler (Biometra TAdvanced Twin Analytik Jena). Where applicable, a positive control was run. The PCR reactions were analysed using a 1% (w/v) agarose gel as described in 9.1.2.

Table 43: Temperature program for OneTaq® Quick-Load® DNA Polymerase

Temperature [$^{\circ}$ C]	Time [seconds]	Step
94	300	Initial denaturation
94	30	Denaturation
62 (Lv.0) 59 (Lv.1) 59 (Lv.T) 46 (Lv.T in ppCAT.000 or pPMQAK1-T Y25F)	30	Annealing
68	1min/kb	Extension
68	300	Final extension
4	indefinite	Hold

The steps Denaturation, Annealing and Extension were cycled 30 times.

9.3.11. Biotransformation

Biotransformations were always done in BL21(DE3).

9.3.11.1. Autoinduction

Made 12ml overnight culture with LB0.8G supplemented with respective antibiotic in 50ml Falcon tubes (Greiner). Inoculation was done from cryostocks or directly from plate culture. The overnight cultures were incubated at 37 $^{\circ}$ C with 275rpm. The next day main cultures were prepared using sterile, baffled flasks (200ml) with 50ml LB5052 with the respective antibiotic. They were inoculated with 0.2% (v/v) of the overnight culture. They were grown at 37 $^{\circ}$ C at 150rpm

for 4h and then the temperature was switched to 20°C for another 20h to facilitate protein expression. From here onwards, it was not worked sterile anymore. OD590 of each culture was measured. An OD590/ml of 25 was taken from each culture. The cells were then harvested by centrifugation (6000xg, 4°C, 15min using either SIGMA 6k15 or SIGMA 3k30). The rest of the cultures were harvested in the same way and frozen at -20°C for later SDS PAGE analysis. The cell pellet was washed with 5ml N-free M9 medium and pelleted again by centrifugation. It was then resuspended in 2.5ml N-free M9 medium. In the next step, 2ml of cell suspension were transferred into 25ml glass vials and the remaining 0.5ml were used to measure the final OD590. Then, 5mM of substrate was added to the glass vial containing the 2ml suspension, the vial shaken by hand, and the 0h measurement was taken. Further incubated at 20°C with 200rpm. The rest was done according to 9.1.4.

9.3.11.2. Induction via IPTG

Made 12ml overnight culture with LB-Miller supplemented with respective antibiotic in 50ml Falcon tubes (Greiner). Inoculation was done from cryostocks or directly from plate culture. The overnight cultures were incubated at 37°C with 275rpm. The next day main cultures were prepared using sterile, baffled flasks (200ml) with 50ml LB-Miller with the respective antibiotic. The OD590 of the overnight cultures was measured and the main cultures inoculated to an OD590 of ca. 0.05. The cultures were then grown at 37°C, 200rpm for about 90 minutes. The OD590 was measured, and the cells incubated again, until all cultures were in the range of OD590 of 0.6 – 0.8. Then protein production was induced by adding 1M IPTG to the cultures for a final concentration of 1mM IPTG. The temperature was then switched to the respective protein production temperature (Table 44) for 20h.

Table 44: Protein production temperatures chosen for the respective enzymes

Enzyme	Protein production temperature [°C]
LfSDR1M50	25
YqJM	25
CHMOacinetoS7	25
Complete cascade	25

From here onwards, there was no emphasis on sterile working conditions anymore. OD590 of each culture was measured. An OD590/ml of 25 was taken from each culture. The cells were then harvested by centrifugation (6000xg, 4°C, 15min using either SIGMA 6k15 or SIGMA 3k30). The rest of the cultures were harvested in the same way and frozen at -20°C for later SDS PAGE analysis. The cell pellet was washed with 5ml N-free M9 medium and pelleted again by centrifugation. It was then resuspended in 2.5ml N-free M9 medium. In the next step, 2ml of cell suspension were transferred into 25ml glass vials and the remaining 0.5ml were used to measure the final OD590. Then, 5mM of substrate was added to the glass vial containing the 2ml suspension, the vial shaken by hand, and the 0h measurement was taken. Further incubated at 25°C with 200rpm. The rest was done according to 9.1.4.

9.3.12. SDS-PAGE

The whole sample preparation was on ice under non-sterile conditions, starting from frozen cell pellet as described in 9.3.11. The pellet was resuspended in 1/10 volume of the main culture (5ml) in 50mM Tris-HCl (pH 7.5) and centrifuged again. The pellet was resuspended in 1/40 volume of the main culture (1.25ml) in 50mM Tris-HCl with 0.1mM PMSF. The cell suspension was repeatedly sonicated (9 cycles) for 5s (40% power) followed by 55s pause using a Bandelin Sonoplus HD4100 with a TS106 tip. To separate the soluble fraction (cell free extract) from the insoluble fraction the sample was centrifuged (14000xg, 4°C, 20min. using either SIGMA 6k15 or SIGMA 3k30). The supernatant (the soluble fraction) was transferred to 1.5ml Eppendorf tubes. The insoluble fraction was resuspended in 1ml Tris-HCl with 0.1mM PMSF. Both were either frozen at -20°C until further usage or kept on ice and protein content was assessed (as described in 9.3.13). Knowing the protein content, the amount to use per sample to reach 15µg protein was calculated. The calculated amount was taken, filled up to 10µl using Tris-HCl with 0.1mM PMSF and 10µl of SDS-buffer (2x) was added. The samples were denatured at 95°C for 5 minutes using a Biometra TS1 Thermoshaker Analytik Jena. The resulting 20µl were loaded onto the gel. For specifics about the gel and loading see 9.1.3.

9.3.13. Determination of protein content using the BCA assay

9.3.13.1. Calibration

The BCA assay was performed using a platereader (either an Anthos Zenyth 3100 or a Spark by Tecan). When the Anthos Zenyth 3100 was used the following calibration curve used (made by Lydia Suchy):

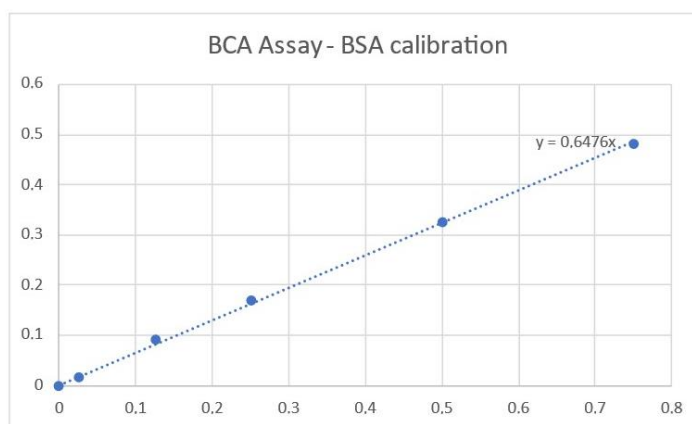


Figure 56: BCA assay calibration for Anthos Zenyth 3100. The calibration was done by Lydia Suchy in 50mM Tris-HCl (pH 7.5). Figure kindly provided by Lydia Suchy.

For the Spark by Tecan, the following calibration curve was made and used:

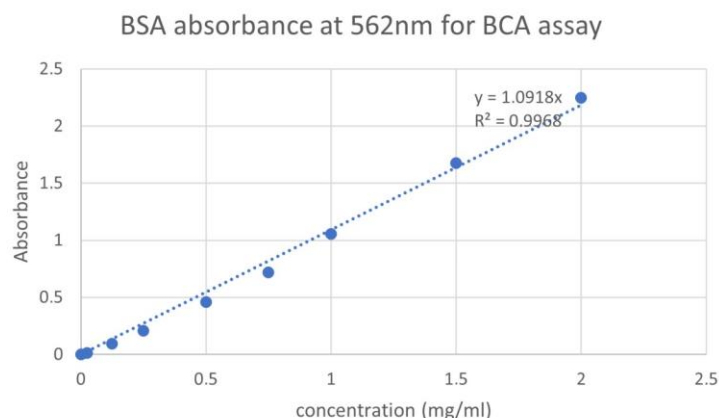


Figure 57: BCA assay calibration for Spark by Tecan. The calibration was performed according to the manual in 50mM Tris-HCl (pH 7.5) with 0.1mM PMSF.

For the BCA assay all probes had to be diluted to fit the linear range of the platereader. The dilution was 1:30 unless stated otherwise (0.83µl sample + 24.2µl Tris-HCl (pH 7.5) with 0.1mM PMSF). All else was done according to the manual of the following manual from the Pierce™ BCA Protein Assay Kit by Thermo Scientific.

Table 45: Dilution Table for the Pierce™ BCA Protein Assay Kit by Thermo Scientific

Vial	Volume of Diluent (µl)	Volume and Source of BSA (µl)	Final BSA Concentration (µg/ml)
A	0	300 of Stock	2000
B	125	375 of Stock	1500
C	325	325 of Stock	1000
D	175	175 of vial B dilution	750
E	325	325 of vial C dilution	500
F	325	325 of vial E dilution	250
G	325	325 of vial F dilution	125
H	400	100 of vial G dilution	25
I	400	0	0 = Blank

The contents of the Albumin Standard (BSA) ampule were diluted into several clean 1.5ml Eppendorf tubes. Table 45 was used to prepare the sets of protein standards that were used to create Figure 57. Three replicates were made and the results were averaged for each sample.

9.3.13.2. Measurements

Samples were measured in 96 well plates (cellculture microplate, 96 well, PS, F-bottom, clear, Cellstar®, TC, Lid with condensation rings, sterile, single packed by greiner bio-one). The lid was taken off prior to the measurements due to the condensation that would build up on it. All else followed the manual mentioned in 9.3.13.1:

Used the following formula to determine the volume of the Working reagent:

$(\#standards + \#samples) * (\#replicates) * (volume\ of\ WR\ per\ sample),$

where $\#replicates$ was always 3 and volume of WR per sample was always 200µl.

WR was prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B.

Each well contained 25µl of the respective sample. Then, 200µl of the WR were added to each well and the plate was thoroughly shaken on a plate shaker for approximately 30 seconds (1350rpm, Heidolph Titramax 1000). The plate was then incubated for 30 minutes at 37°C (IN-CU-line by VWR). The plate was put on room temperature for 5min to cool down and then the lid was taken off and the absorbance was measured at either 595nm (Anthos Zenyth 3100) or at 562nm (Spark by Tecan). For each sample measurement the blank was subtracted, the mean calculated. This blanked mean value was then multiplied with the dilution factor which was used for the measurement and finally it was divided by the slope of the respective calibration curve. As a result, the protein concentration in mg/ml was received. A protein amount of 15µg was desired for SDS-PAGE, thus the amount to take in µl was calculated via $(protein\ amount) / (protein\ concentration)$. For further steps regarding sample preparation for SDS-PAGE see 9.3.12.

9.3.14. Cloning

All cloning steps were performed in NEB_stable, except for the Lv.0 cloning, which was done in DH5α, due to the inherent streptomycin resistance of the NEB_stable strain. All cloning reactions were done with a vector:insert ratio of 1:3. Cloning was always started with 100ng of plasmid. Formula used to calculate the needed amount of insert: $[bp\ insert] / [bp\ vector] * 100ng * 3 = [ng\ of\ insert\ needed]$.

9.3.14.1. Cloning and Mutagenesis for transfer into Lv.0 constructs

YqJM as well as CHMO_{acineto}S7 had to be edited to give them stop codons as well as the right overhangs for further cloning (see Table 46). LfSDR1M50 could be kindly provided by Julia Jodlbauer because it was already in the appropriate configuration (pICH41308_LfSDR1M50) with a stop codon (TAA). To do so, a site directed mutagenesis via PCR was performed (Table 48 and Table 49), the resulting constructs analysed via gel electrophoresis (see 9.1.2) and purified for further use (see 9.3.8). The respective cloning reactions were then set up by calculating the amounts of insert and plasmid needed (Table 50, formula for calculations see 9.3.14). The reactions were then prepared on ice according to Table 51 and were put in a thermocycler (Bi-

ometra TAdvanced Twin Analytik Jena) according to Table 52. Next, 5µl of the result was taken and transformed into DH5α (further steps according to 9.3.6), after which colony PCR (9.3.10) was done to assess construct uptake. Afterwards the plasmid was isolated (according to 9.3.7) and sequenced (according to 9.3.9).

Table 46: Schematic overview of edits

Gene	Original overhangs	Edited overhang	Primer
CHMO _{acinetoS7}	AATG-gene-TTCG	AATG-gene-TAA-GCTT	pCSW002/pCSW003
YqJM	AATG-gene-TTCG	AATG-gene-TAA-GCTT	pJJ071/pCSW001

Table 47: PCR Reaction for mutagenesis of YqJM and CHMO_{acinetoS7}

Component	Amount [µl]
5x Q5 reaction buffer	10
10mM dNTP's	1
10µM Forward primer	2.5
10µM Reverse primer	2.5
Template DNA	1
Q5 High-Fidelity DNA Polymerase	0.5
Nuclease-free dH ₂ O	Fill to 50

Table 48: Program for mutagenesis PCR of YqJM

Step	Temperature [°C]	Time [seconds]
Initial denaturation	98	30
30 cycles	98	8
after 10 cycles switched annealing temperature	62 switch 72	20
	72	30
Final extension	72	120
hold	4	indefinitely

Table 49: Program for mutagenesis PCR of CHMO_{acinetoS7}

Step	Temperature [°C]	Time [seconds]
Initial denaturation	98	30
30 cycles	98	8
after 10 switched annealing temperature	68 switch 72	20
	72	50
Final extension	72	120
hold	4	indefinitely

Table 50: Overview of parts used in Lv.0 cloning

Genetic part	Concentration [ng/μl]	Length [bp]	Volume used [μl]
YqJM (insert)	99.2	1058	1.12
CHMO _{acineto} S7 (insert)	123.2	1673	1.41
pICH41308 (vector)	123.2	2845	0.811

Table 51: Reaction for Lv.0 cloning

Compound	Amount [μl]
T4 DNA ligase	1.5
BpiI	1
T4 DNA ligase buffer (10x)	2.5
Insert	See Table 50
Vector (pICH41308)	See Table 50
Nuclease-free dH ₂ O	Filled up to 25

Table 52: Reaction conditions for Lv.0 cloning

Step #	Time	Temperature [°C]
1	3min	37
2	90sec.	16
3	5min	50
4	10min	80
5	indefinitely	4

Steps #1 and #2 were cycled 25x.

9.3.14.2. Cloning for transfer into Lv.1 constructs

The cloning was started by isolation and quantification (9.3.7) of the respective plasmids for Level 1 cloning (see Table 53). All sequences were checked again for correctness via sequencing (9.3.9) of the part that will be used further for cloning. After calculation of the appropriate amounts of DNA for each construct (Table 54 - Table 59), the reactions were prepared on ice (Table 60) and were put in a thermocycler (Biometra TAdvanced Twin Analytik Jena) according to Table 61. Next, 5μl of the result was taken and transformed into NEB_stable (further steps according to 9.3.6), after which colony PCR (9.3.10) was done to assess construct uptake. Finally, the plasmids were isolated (according to 9.3.7) and sequenced (according to 9.3.9).

Table 53: Constructs used for LV.1 cloning

Name	Obtained from
pAGM1263_ <i>RiboJ</i>	Julia Jodlbauer ¹⁵⁴
pAGM1276_ <i>Rbs21</i>	Julia Jodlbauer
pICH41246_ <i>Rbsv32</i>	Julia Jodlbauer
pICH41246_ <i>RbsS5</i>	Julia Jodlbauer
pICH41308_ <i>LfSDR1M50</i>	Julia Jodlbauer
pICH41308_ <i>YqJM</i>	Selfmade
pICH41308_ <i>CHMO_{acineto}S7</i>	Selfmade
pICH47761	MoClo kit
pICH47732	MoClo kit
pICH47751	MoClo kit
pC0.062_ <i>BBa_B0011</i>	CyanoGate kit
pICH41233_ <i>Ptrc10</i>	Julia Jodlbauer

Table 54: Reaction for Lv.1 cloning of LfSDR1M50 “optimal”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41233_ <i>Ptrc10</i>	87	2322	1.8
pICH41246_ <i>RbsS5</i>	173.8	2273	0.9
pICH41308_ <i>LfSDR1M50</i>	185.2	2992	1.1
pC0.062_ <i>BBa_B0011</i>	127.1	2297	1.2
pICH47732	184.8	4356	0.5

Table 55: Reaction for Lv.1 cloning of LfSDR1M50 “benchmark”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41233_ <i>Ptrc10</i>	87	2322	1.8
pICH41246_ <i>RbsS1</i>	150.8	2273	1
pICH41308_ <i>LfSDR1M50</i>	185.2	2992	1.1
pC0.062_ <i>BBa_B0011</i>	127.1	2297	1.2
pICH47732	184.8	4356	0.5

Table 56: Reaction for Lv.1 cloning of YqJM “optimal”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41233_ <i>Ptrc10</i>	87	2322	1.8
pAGM1263_ <i>RiboJ</i>	207.2	2326	0.8
pAGM1276_ <i>Rbs21</i>	166	2280	0.9
pICH41308_ <i>YqJM</i>	261.7	3265	0.9
pC0.062_ <i>BBa_B0011</i>	127.1	2297	1.2
pICH47751	181.4	4356	0.6

Table 57: Reaction for Lv.1 cloning of YqJM “benchmark”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41233_ <i>Ptrc10</i>	87	2322	1.8
pICH41246_ <i>RbsS1</i>	150.8	2273	1
pICH41308_ <i>YqJM</i>	261.7	3265	0.9
pC0.062_ <i>BBa_B0011</i>	127.1	2297	1.2
pICH47732	184.8	4356	0.5

Table 58: Reaction for Lv.1 cloning of CHMO_{acinetoS7} “optimal”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41233_ <i>Ptrc10</i>	87	2322	1.8
pICH41246_ <i>Rbsv32</i>	151.8	2290	1
pICH41308_ <i>CHMO_{acinetoS7}</i>	223.4	3880	1.2
pC0.062_ <i>BBa_B0011</i>	127.1	2297	1.2
pICH47761	150	4356	0.7

Table 59: Reaction for Lv.1 cloning of CHMO_{acinetoS7} “benchmark”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41233_ <i>Ptrc10</i>	87	2322	1.8
pICH41246_ <i>RbsS1</i>	150.8	2273	1
pICH41308_ <i>CHMO_{aci-netoS7}</i>	223.4	3880	1.2
pC0.062_ <i>BBa_B0011</i>	127.1	2297	1.2
pICH47732	184.8	4356	0.5

Table 60: Reaction for Lv.1 cloning

Name	Amount [μl]
DNA	See Table 54 - Table 59
BsaI HFv2	1.5
T4 DNA ligase	1.5
T4 DNA ligase buffer (10x)	3
Nuclease-free dH ₂ O	Fill up to 30

Table 61: Reaction conditions for Lv.1 cloning

Step #	Time [min]	Temperature [°C]
1	5	37
2	5	16
3	5	60
4	indefinitely	4

Steps #1 and #2 were cycled 30x.

9.3.14.3. Cloning for transfer into Lv.T constructs

After calculation of the appropriate amounts of DNA for each construct (Table 63 - Table 69), the reactions were prepared on ice (Table 70) and were put in a thermocycler (Biometra TAdvanced Twin Analytik Jena) according to Table 71. Next, 5μl of the result was taken and transformed into NEB_stable (further steps according to 9.3.6), after which colony PCR (9.3.10) was done to assess construct uptake. Finally, the plasmids were isolated (according to 9.3.7) and sequenced (according to 9.3.9).

Table 62: Constructs used for LV.T cloning

Name	Obtained from
pICH41744	MoClo kit
LfSDR1M50 “benchmark” Lv.1	Selfmade
LfSDR1M50 “optimal” Lv.1	Selfmade
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	Julia Jodlbauer
pCAT.000 pPMQAK1-T Y25F	Julia Jodlbauer
pICH54011	MoClo kit
YqJM “benchmark” Lv.1	Selfmade
YqJM “optimal” Lv.1	Selfmade
pICH41766	MoClo kit
pICH54033	MoClo kit
pICH41780	MoClo kit
CHMO _{acineto} S7 “benchmark” Lv.1	Selfmade
CHMO _{acineto} S7 “optimal” Lv.1	Selfmade

Table 63: Reaction for Lv.T cloning of LfSDR1M50 “benchmark”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41744	232.9	3318	0.5
LfSDR1M50 “benchmark” Lv.1	280.2	5268	0.7
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	268.7	5656	0.8
pCAT.000 pPMQAK1-T Y25F	64.1	8255	1.6

Table 64: Reaction for Lv.T cloning of LfSDR1M50 “optimal”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41744	232.9	3318	0.5
LfSDR1M50 “optimal” Lv.1	152.7	5268	1.3
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	268.7	5656	0.8
pCAT.000 pPMQAK1-T Y25F	64.1	8255	1.6

Table 65: Reaction for Lv.T cloning of YqJM “benchmark”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41744	232.9	3318	0.5
YqJM “benchmark” Lv.1	149	5541	1.4
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	268.7	5656	0.8
pCAT.000 pPMQAK1-T Y25F	64.1	8255	1.6

Table 66: Reaction for Lv.T cloning of YqJM “optimal”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH54011	259.8	4368	0.6
YqJM “optimal” Lv.1	82.9	5627	2.5
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	268.7	5656	0.8
pCAT.000 pPMQAK1-T Y25F	64.1	8255	1.6

Table 67: Reaction for Lv.T cloning of CHMO_{acinetoS7} “benchmark”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41744	232.9	3318	0.5
CHMO _{acinetoS7} “benchmark” Lv.1	304	6156	0.7
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	268.7	5656	0.8
pCAT.000 pPMQAK1-T Y25F	64.1	8255	1.6

Table 68: Reaction for Lv.T cloning of CHMO_{acinetoS7} “optimal”

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH54011	259.8	4368	0.6
pICH54033	276	4368	0.6
pICH41780	252.2	3318	0.5
CHMO _{acinetoS7} “optimal” Lv.1	180.5	6173	1.2
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	268.7	5656	0.8
pCAT.000 pPMQAK1-T Y25F	64.1	8255	1.6

Table 69: Reaction for Lv.T cloning of the full cascade

Name	Concentration [ng/μl]	Length [bp]	Amount taken [μl]
pICH41780	252.2	3318	0.5
LfSDR1M50 “optimal” Lv.1	152.7	5268	1.3
YqJM “optimal” Lv.1	82.9	5627	2.5
CHMO _{acineto} S7 “optimal” Lv.1	180.5	6173	1.2
pICH47742_ <i>PlacIq_lacI_TrrnB</i>	268.7	5656	0.8
pCAT.000 pPMQAK1-T Y25F	64.1	8255	1.6

Table 70: Reaction for Lv.T cloning

Name	Amount [μl]
DNA	See Table 63 - Table 69
BpiI	1
T4 DNA ligase	1.5
T4 DNA ligase buffer (10x)	2.5
Nuclease-free dH ₂ O	Fill up to 25

Table 71: Reaction conditions for Lv.T cloning

Step #	Time [min]	Temperature [°C]
1	3	37
2	1.5	16
3	5	50
4	10	80
5	indefinitely	4

Steps #1 and #2 were cycled 25x.

10. Appendix

10.1. Genetic Sequences

In the following, genetic constructs and important genetic sequences for this thesis are summarized. Constructs containing sfGFP tags were made by Julia Jodlbauer.

Table 72: List of all backbones that were used in this work

Vector	Antibiotic resistance
pAGM1287	Streptomycin
pAGM1263	Streptomycin
pAGM1276	Streptomycin
pC0.062	Streptomycin
pICH41233	Streptomycin
pICH41246	Streptomycin
pICH41308	Streptomycin
pICH47761	Ampicillin
pICH47732	Ampicillin
pICH47751	Ampicillin
pICH47742	Ampicillin
pICH41744	Streptomycin
pICH54011	Ampicillin
pICH54033	Ampicillin
pICH41780	Streptomycin
pICH41766	Streptomycin
pET28a(+)	Kanamycin
pCAT.000 pPMQAK1-T Y25F	Ampicillin + Kanamycin

RBS 2 is originally RBS 21 from Taylor, G.M. *et al.* (2021)¹⁶⁷

The gene was obtained in plasmid pAGM1276.

TACGAAAGATTACGAAAGGAGGTTGGTT

RBS 13 is originally RBS S5 from Thiel, K. *et al.* (2018)¹⁶⁸

The gene was obtained in plasmid pICH41246.

TGTTTATGGAGGACTGACCTAG

RBS 19 is originally RBSv32 from Wang, B. *et al.* (2018)¹⁶⁹

The gene was obtained in plasmid 41246.

ATAACAAGATTTACCGTTCCCAAGAAGGAGGAACATAGC

RBS 10 is originally RBS S1 from Thiel, K. *et al.* (2018)¹⁶⁸

The gene was obtained in plasmid pICH41246.

CTAGAGTAGTGGAGGTTACTAG

RiboJ Sequence from Englund *et al.* 2018¹⁷⁰; first reported in literature for cyanobacteria by Taton 2014¹⁶⁰

The gene was obtained in plasmid pAGM1263.

AGCTGTCACCGGATGTGCTTTCCGGTCTGATGAGTCCGTGAGGACGAAACAGCCTC-
TACAAATAATTTTGTTTAA

PTRC10 promoter from CyanoGate kit (IPTG inducible)¹⁷¹

The gene was obtained in plasmid pICH41233.

TTGACAATTAATCATCCGGCTCGTATAATGTGTGGAATTGTGAGCGGA-
TAACAATTTACACATACTAGAG

lacIq promoter

The gene was obtained as plasmid pICH47742_*PlacIq_lacI_TrrnB*.

ACACCATCGAATGGTGCAAAACCTTTCGCGGTATGGCATGATAGCGCCCGGAAGA-
GAGTCAATTCAGGGTGGTGAAT

YqJM as described in 9.1.7

The gene was obtained in plasmid pAGM1287.

ATGGCCAGAAAATTATTTACACCTATTACAATTAAAGATATGACGTTAAAAAACCG-
CATTGTCATGTCGCCAATGTGCATGTATTCTTCTCATGAAAAGGAC-
GGAAAATTAACACCGTTCCACATGGCACATTACATATCGCGCG-
CAATCGGCCAGGTCGGACTGAT-
TATTGTAGAGGCGTCAGCGGTTAACCCTCAAGGACGAATCACTGACCAA-
GACTTAGGCATTTGGAGCGACGAGCATATTGAAGGCTTTGCAAACTGACTGAG-
CAGGTCAAAGAACAAGGTTCAAAAATCGGCATTCAGCTTGCCCATGCCGGAC-
GTAAAGCTGAGCTTGAAGGAGATATCTTCGCTCCATCGGCGATTGCGTTTGAC-
GAACAATCAGCAACACCTGTAGAAATGTCAGCAGAAAAAGTAAAAGAAAC-

GGTCCAGGAGTTCAAGCAAGCGGCTGCCCCGCGCAAAAGAAGCCGGCTTT-
GATGTGATTGAAATTCATGCGGCGCACGGATATTTAATTCATGAATTTTT-
GTCTCCGCTTTCCAACCATCGAACAGATGAATATGGCGGCTCACCTGAAAACCGC-
TATCGTTTCTTGAGAGAGATCATTGATGAAGTCAAACAAGTATGGGAC-
GGTCCTTTATTTGTCCGTGTATCTGCTTCTGACTACACTGATAAAGGCTTAGACATT-
GCCGATCACATCGGTTTTTGCAAAATGGATGAAGGAGCAGGGTGTGACTTAATT-
GACTGCAGCTCAGGCGCCCTTGTTACGCAGACATTAACGTATTCCTGGC-
TATCAGGTCAGCTTCGCTGAGAAAATCCGTGAACAGGCGGACATGGC-
TACTGGTGCCGTCGGCATGATTACAGACGGTTCAATGGCTGAAGAAATTCTG-
CAAAACGGACGTGCCGACCTCATCTTTATCGGCAGAGAGCTTTTGCGG-
GATCCATTTTTTGCAAGAACTGCTGCGAAACAGCTCAATACAGAGAT-
TCCGGCCCCTGTTCAATACGAAAGAGGCTGGTAA

LfSDR1M50 as described in 9.1.7

The gene was obtained in plasmid pAGM1287.

ATGGGACAGTTTGACAATAAGGTTGCCTTGGTTACCGGGGGAACGAAGGG-
GATTGGCTTAGCCATCGCCGAGCTGTTTTT-
GAAGGAAGGCGCCAAGGGGGTGGCCTTCACCGGTCGTCACGAGGAC-
GAAGGAAAAGCGGTTCAAGAACGCCTCGGTGAACGGTCTTTGTTTCATCACCCAA-
GACGTTTCCAAGGAAGAAGATTGGCAAAACGCCACCAAAGCCGTTGTT-
GACAAATTTGGGCAGCTTGATGCGATTGTCAACAACGCCG-
GAATTTTTACTCCGCTGGGGATCGAGGAAATGACGCTCGATCACTGGAAC-
CGCGAAATCGCCATCGATTTAACAGGGACGATGTTAGGTTGCAAGTAC-
GGGGTTAAAGCGATGAAGGAACATGGTGGCGCGATCGTCAACATTAG-
TTCGATCCTGGGGATGATCGGTGTGCCAACC GTTCCGGCCTACAAC-
GCTGCTAAGGGGGGCGTCCGTCTCCTACCAAGTCGGTAGCGCTTGAG-
TGTGCCGAAAAGGGTTACGCCATCCGCGTAAACTCGATTTACCCTGGGGCAATT-
GCCACGCCGCTGATCGATCACCTCGATGATGCGACCAAGCAATTCTACATCGAC-
CTGCACCCAATGGGCCGGCTGGGAAAGCCGGAAGAAGTGGCTAA-
GATGGCTGTCTTTGTTGCTTCCGATGGGGCCTCCTTTAGCACCGGCTCCGAGTTT-
GTTGTCGATGGGGGCTACACGGCCCAATAA

CHMO_{Acineto}S7 as described in 9.1.7

The gene was obtained in plasmid pAGM1287.

ATGAGCCAGAAGATGGACTTTGATGCGATTGTGATTGGTGCGGGTTTT-
GGTGGTCTGTATGCGGTGAAGAAGCTGCGTGACGAACTGGAGCTGAAAGTG-
CAGGCGTTTGACAAGGCGACCGATGTTGCGGGCACCTGGTACTGGAAC-
CGTTATCCGGGTGCGCTGACCGACACCGAAACCCACCTGTACTGCTATAGCTGGGA-
TAAAGAGCTGCTGCAGAGCCTGGAAATCAAGAAAAAGTATGTGCAAGGTCCGGAC-
GTTCGTAAATACCTGCAGCAAGTTGCGGAGAAGCACGATCTGAAAAAGAGC-

TATCAGTTTAAACACCGCGGTTCAAAGCGCGCACTACAACGAAGCGGAC-
 GCGCTGTGGGAAGTGACCACCGAATACGGCGATAAGTATACCGCGCGTTTCCTGAT-
 TACCGCGCTGGGTCTGCTGAGCGCGCCGAACCTGCCGAACATCAAAGGCATTAAC-
 CAGTTTAAGGGCGAGCTGCACCACACCAGCCGTTGGCCGGAC-
 GATGTGAGCTTCGAAGGCAAACGTGTGGGTGTTATCGGCACCGGTAGCAC-
 CGGCGTGCAAGTTATTACCGCGGTGGCGCCGCTGGCGAAGCACCTGAC-
 CGTTTTTCAGCGTAGCGCGCAATATAGCGTGCCGATCGGTAAC-
 GACCCGCTGAGCGAGGAAGATGTTAAAAAGATCAAGGACAACCTACGATAAGATTT-
 GGGATGGTGTGTGGAACAGCGCGCTGGCGTTTGGTCTGAACGAGAGCAC-
 CGTGCCGGCGATGAGCGTTAGCGCGGAGGAACGTAAAGCGGTTTTTCGA-
 GAAGGCGTGGCAGACCGGTGGCGGTTTCCGTTTTATGTTCGAAACCTTT-
 GGCGACATCGCGACCAACATGGAGGCGAACATCGAAGCG-
 CAAAACCTTCATTAAAGGCAAGATCGCGGAAATTGTGAAAGATCCGGCGATTGCG-
 CAGAAGCTGATGCCGCAAGACCTGTACGCGAAACGTCCGCTGTGCGA-
 TAGCGGTTACTATGAAACCTTCAACCGTGACAACGTGCGTCTGGAAGATGTTAAA-
 GCGAACCCGATCGTGGAGATTACCGAAAACGGCGTTAAGCTGGAGAAC-
 GGTGACTTTGTTGAACTGGATATGCTGATCTGCGCGACCGGTTTTGATGCGGTG-
 GATGGTAACTACGTTTCGTATGGACATTCAGGGCAAGAACGGTCTGGCGATTAAA-
 GATTACTGGAAAGAGGGTCCGAGCAGCTATATGGGTGTGACCGTTAACAAC-
 TACCCGAACATGTTTATGGTGCTGGGCCCCGAACGGTCCGTTACCAAC-
 CTGCCGCCGAGCATCGAGAGCCAAGTTGAATGGATCAGCGACACCATTAAA-
 TATGCGGAGGAAAACAACGTGGAGAGCATCGAAGCGACCAAAGAGGCGGAG-
 GAACAGTGGACCCGTACCTGCGCGGAGATTGCGGAAATGACCCTGTTTCCGAAA-
 GCGCAAAGCTGGATCTTTCGGCGCGAACATTCCGGGTAAAAAAGAACAC-
 CGTTTACTTCTATCTGGGCGGTCTGAAAGAATACCGTAGCGCGCTGGCGAACTG-
 CAAGAACCACGCGTATGAGGGTTTTTGACATCCAACCTGCAAC-
 GTAGCGACATTAAACAACCGGCGAACGCGTAA

**sfGFP (with N-terminal TEV site as linker) as used in YqJM::sfGFP and CHMO_{Aci-}
_{neto}S7::sfGFP**

AGAATTTGTATTTTCAGGGTGATATCAGCAAAGGAGAAGAAGTTTTCCTGAGTT-
 GTCCCAATTCTTGTTGAATTAGATGGTGTATGTTAATGGG-
 CACAAATTTTCTGTCCGTGGAGAGGGTGAAGGTGATGCTACAAACGGAAA-
 GCTTACCCTTAAATTTATTTGCACTACTGGAAAACCTGTTCCATGGCCAACAC-
 TTGTCCTACTCTCACTTATGGTGTTCATGCTTTTCCCGTTATCCGGATCAT-
 ATGAAACGGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAC-
 GCACTATATCTTTCAAAGATGACGGGACTTACAAGACGCGTGCTGAAGTCAAGTTT-
 GAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAA-
 GATGGAAACATTCTCGGACACAACTTGAGTACAACCTTAACTCACACAATGTATA-
 CATCACGGCAGACAAACAAAAGAATGGAATCAAA-
 GCTAACTTCAAATTCGCCACAACGTTGAAGATGGATCCGTTCAACTAGCAGAC-

CATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAAC-
CATTACCTGTGACACAATCTGTCCTTTTCGAAAGATCCCAACGAAAAGCGTGAC-
CACATGGTCCTTCTTGAGTTTGTAAGTCTGCTGCTGGGATTACACATGG-
CATGGATGAGCTCTACAAATAA

sfGFP (with N-terminal helix-forming linker) as used in LfSDR1M50::sfGFP

CTGAAGCTGCTGCTAAGGAAGCTGCTGCTAAGGCTAGCAAAGGAGAA-
GAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATT-
AGATGGTGATGTTAATGGGCACAAATTTTCTGTCCGTGGA-
GAGGGTGAAGGTGATGCTACAAACGGAAAGCTTACCCTTAAATTTATTTGCAC-
TACTGGAAACTACCTGTTCCATGGCCAACACTTGTCCTACTCTCAC-
TTATGGTGTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGG-
CATGACTTTTTCAAGAGTGCCATGCCCCGAAGGTTATGTACAGGAACGCACTA-
TATCTTTCAAAGATGACGGGACTTACAAGACGCGTGCTGAAGTCAAGTTT-
GAAGGTGATACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAA-
GATGGAAACATTCTCGGACACAACTTGAGTACAACTTTAACTCACACAATGTATA-
CATCACGGCAGACAAACAAAAGAATGGAATCAAA-
GCTAACTTCAAAATTCGCCACAACGTTGAAGATGGATCCGTTCAACTAGCAGAC-
CATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAAC-
CATTACCTGTGACACAATCTGTCCTTTTCGAAAGATCCCAACGAAAAGCGTGAC-
CACATGGTCCTTCTTGAGTTTGTAAGTCTGCTGCTGGGATTACACATGG-
CATGGATGAGCTCTACAAATAA

lacI

The gene was obtained as plasmid pICH47742_*PlacIq_lacI_TrrnB*.

ATGAAACCAGTAACGTTATACGATGTCGCAGAGTATGCCGGTGTCTCTTATCAGAC-
CGTTTCCCGCGTGGTGAACCAGGCCAGCCACGTTTCTGCGAAAACGCGG-
GAAAAAGTGGAAGCGGCGATGGCGGAGCTGAATTACATTCCCAACCGCGTGG-
CACAACAACCTGGCGGGCAAACAGTCGTTGCTGATTGGCGTTGCCACCTCCAG-
TCTGGCCCTGCACGCGCCGTCGCAAATTGTGCGGCGGAT-
TAAATCTCGCGCCGATCAACTGGGTGCCAGCGTGGTGGTGTGATGGTAGAAC-
GAAGCGGCGTCGAAGCCTGTAAAGCGGCGGTGCACAATCTTCTCGCGCAAC-
GCGTCAGTGGGCTGATCATTAACTATCCGCTGGATGACCAGGATGCCATTGCTGTG-
GAAGCTGCCTGCACTAATGTTCCGGCGTTATTTCTTGATGTCTCTGACCAGA-
CACCCATCAACAGTATTATTTTCTCCCATGAGGACGGTACGCGACTGGGCGTGGAG-
CATCTGGTCGCATTGGGTCACCAG-
CAAATCGCGCTGTTAGCGGGCCCATTAAGTTCTGTCTCGGCGCGTCTGCGTCTGGCT
GGCTGGCATAAATATCTCACTCGCAATCAAATTCAGCCGATAGCGGAACGG-
GAAGGCGACTGGAGTGCCATGTCCGGTTTTCAACAAAC-
CATGCAAATGCTGAATGAGGGCATCGTTCCCACTGCGATGCTGGTTGCCAAC-

GATCAGATGGCGCTGGGCGCAATGCGCGCCATTACCGAGTCCGGGCTGCGCGTT-
GGTGGCGATATCTCGGTAGTGGGATACGACGATACCGAGGACAGCTCATGTTA-
TATCCCGCCGTCAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAAC-
CAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGG-
CAATCAGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCTGGCGCCCAA-
TACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCAC-
GACAGGTTTCCCGACTGGAAAGCGGGCAGTGA

rrnB T1 terminator

The gene was obtained as plasmid pICH47742_*PlacIq_lacI_TrrnB*.

CAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTTCGTTTTATCTGTTGTTT-
GTCGGTGAACGCTCTC

BBa_B0011 terminator from CyanoGate kit¹⁷¹

The gene was obtained in plasmid pC0.062.

AGAGAATATAAAAAGCCAGATTATTAATCCGGCTTTTTTTATTATTT

List of Primers

The following is a list of all primers used in this thesis.

Table 73: Mutagenesis primers

Primer name	Primer usage	Primer sequence	Annealing temperature [°C]
pJJ071	Mutagenesis of YqJM	gaaacctgctGAAGACATAatggccagaaaattattacacc	61
pCSW001	Mutagenesis of YqJM	attacgtctcGAAGACATAAGCTTAccagcctctttcg-tattg	62
pCSW002	Mutagenesis of CHMO _{aci-neto} S7	gaaacctgctGAAGACATAatgagccagaagatggacttt-gatg	67
pCSW003	Mutagenesis of CHMO _{aci-neto} S7	attacgtctcGAAGACATAAGCTTAcgcggttcgccggttg	70

Table 74: Analysis primers

Primer name	Primer usage	Primer sequence	Annealing temperature [°C]
pJJ063	Lv.0 sequencing/PCR	cccaatacgcaaaccg	62
pJJ064	Lv.0 sequencing/PCR	gaaaagtgccacctgac	62
pJJ058	Lv.1 sequencing/PCR	caaattgacgcttagacaac	60
pJJ057	Lv.1 sequencing/PCR	gctcttttctcttaggtttacc	61
pJJ059	Lv.T sequencing/PCR	cgtatcacgaggcag	60
pJJ060	Lv.T sequencing/PCR	gcataacgcgaagtaatc	59
pJJ126	Lv.T sequencing	cgacatgacaatgcgg	-
pJJ137	Lv.T sequencing	ccatattcatctgttcgatgg	-
pJJ141	Lv.T sequencing	gattggcttagccatcg	-
pJJ123	Lv.T sequencing	cgcaccaatcacaatc	-
pJJ143	Lv.T sequencing	ctgcgtgacgaactgg	-
pJJ144	Lv.T sequencing	gcggttaataactgcacg	-
pJJ135	Lv.T sequencing	gtcatgtcgccaatgtg	-
pCSW004	Lv.T sequencing	gggtgggttttcttttcacc	-

10.2. NMR Spectra

Compounds of the substitute cascade were checked via ^1H NMR (400MHz) in CDCl_3 after they were bought. All spectra were measured in CDCl_3 and its characteristic solvent peak was present in all spectra at ca. 7.24 even if not depicted.

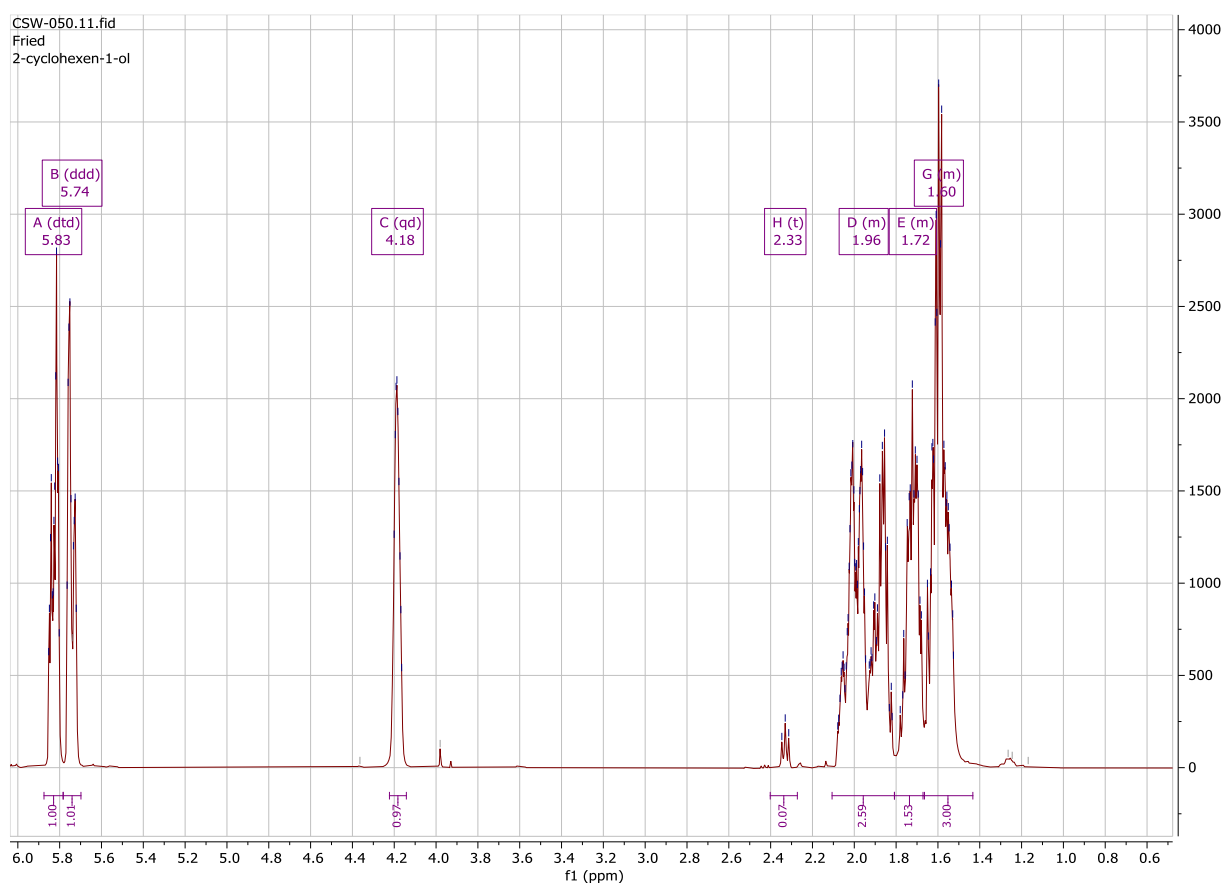


Figure 58: ^1H NMR (400MHz) spectrum of 2-cyclohexen-1-ol (1b).

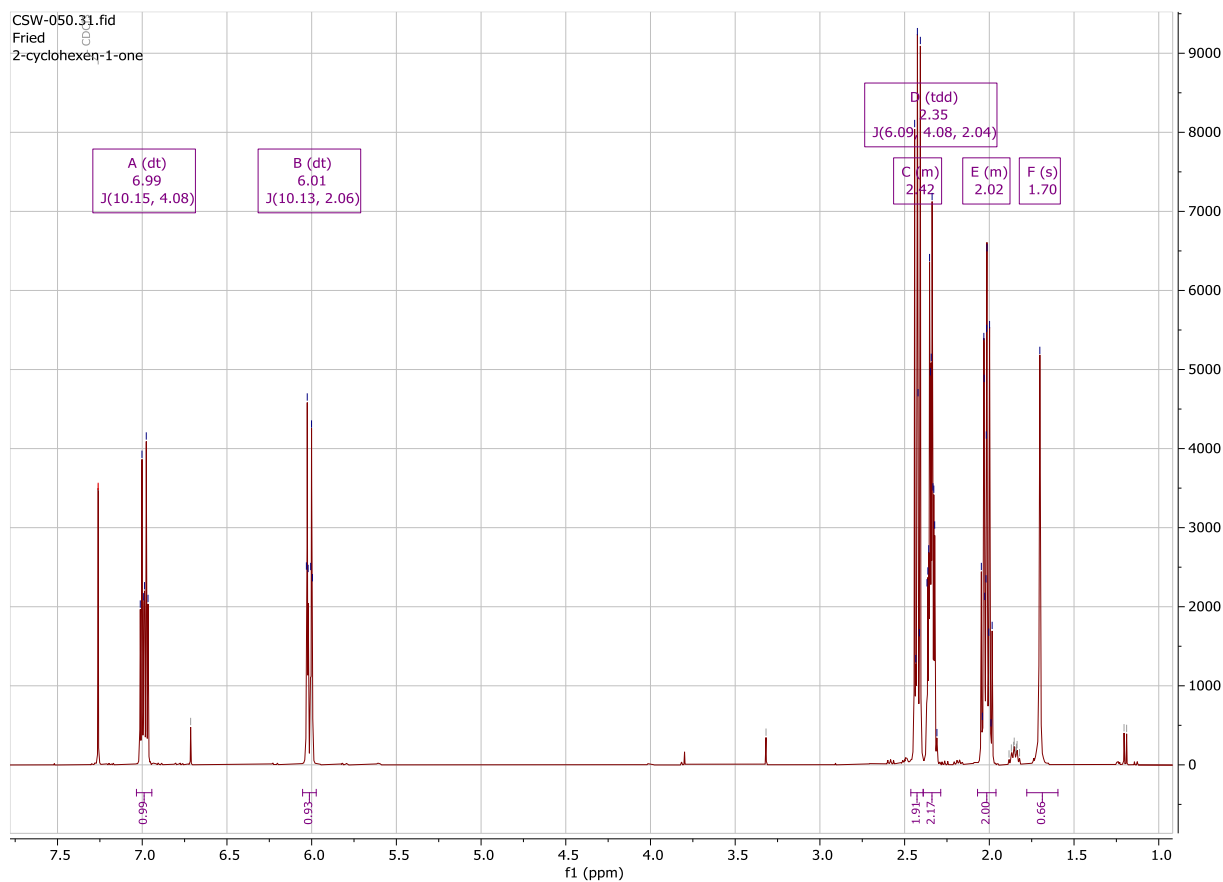


Figure 59: ^1H -NMR (400MHz) spectrum of 2-cyclohexen-1-one (2b).

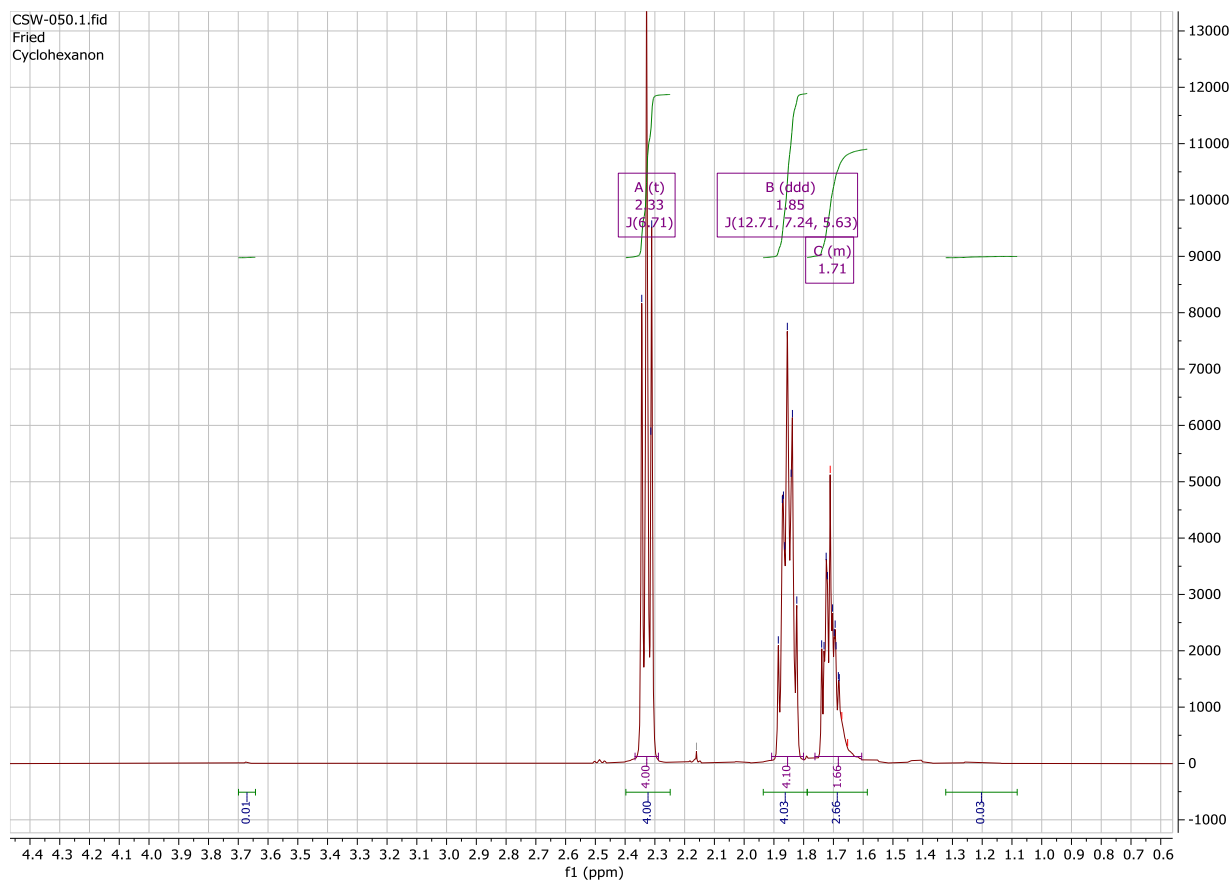


Figure 60: ^1H NMR (400MHz) spectrum of cyclohexanone (3b).

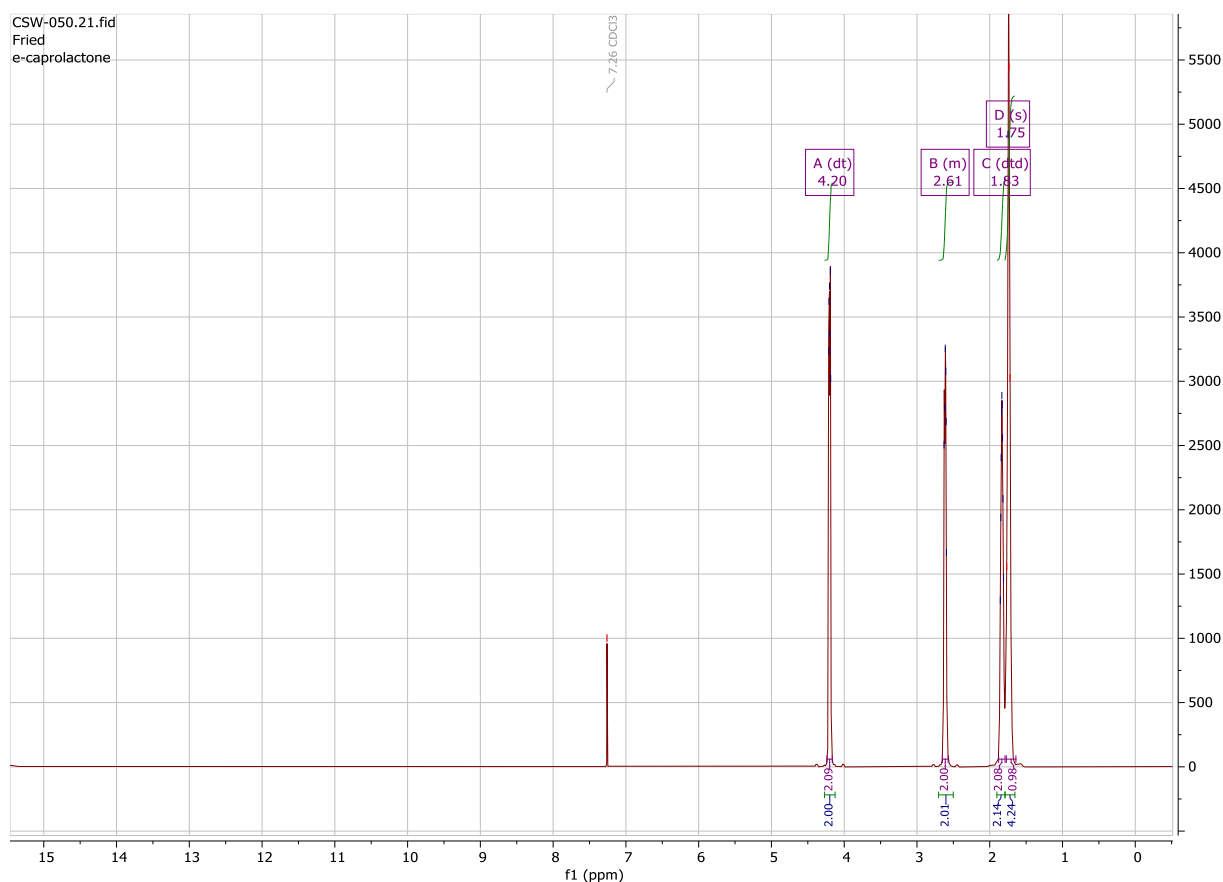


Figure 61: ¹H-NMR (400MHz) spectrum of epsilon-caprolactone (4b).

The spectrum of 2-cyclohexen-1-ol (1b) was according to literature¹⁷². The same was the case for 2-cyclohexen-1-one (2b)¹⁷³, cyclohexanone (3b)¹⁷⁴ and epsilon-caprolactone (4b)¹⁷⁵.

The ¹H-NMR spectra (600MHz in CDCl₃) of the isolated substances from 6.3.4 are below. Due to the use of pentane:diethylether as a solvent for the column, all of the below spectra have impurities related to these compounds.

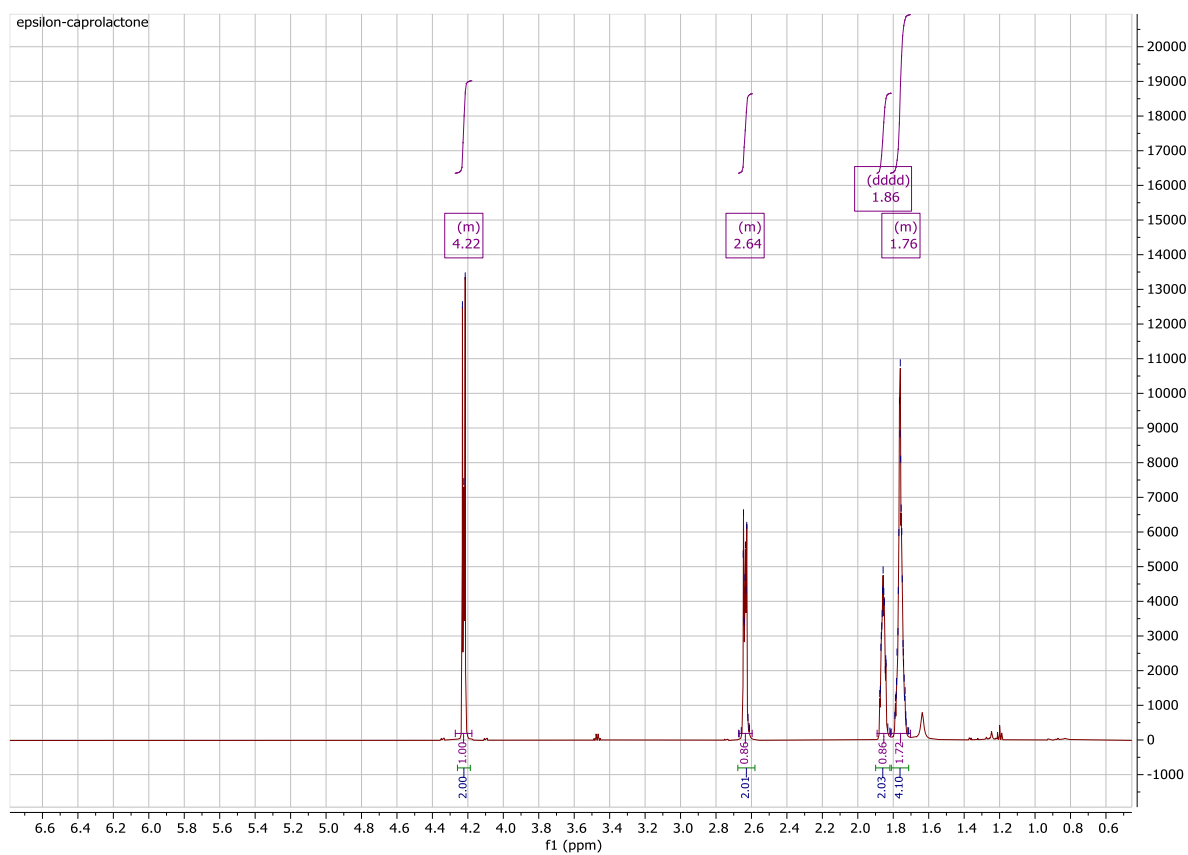


Figure 62: ¹H NMR (600MHz) spectrum of isolate #3 from biotransformation.

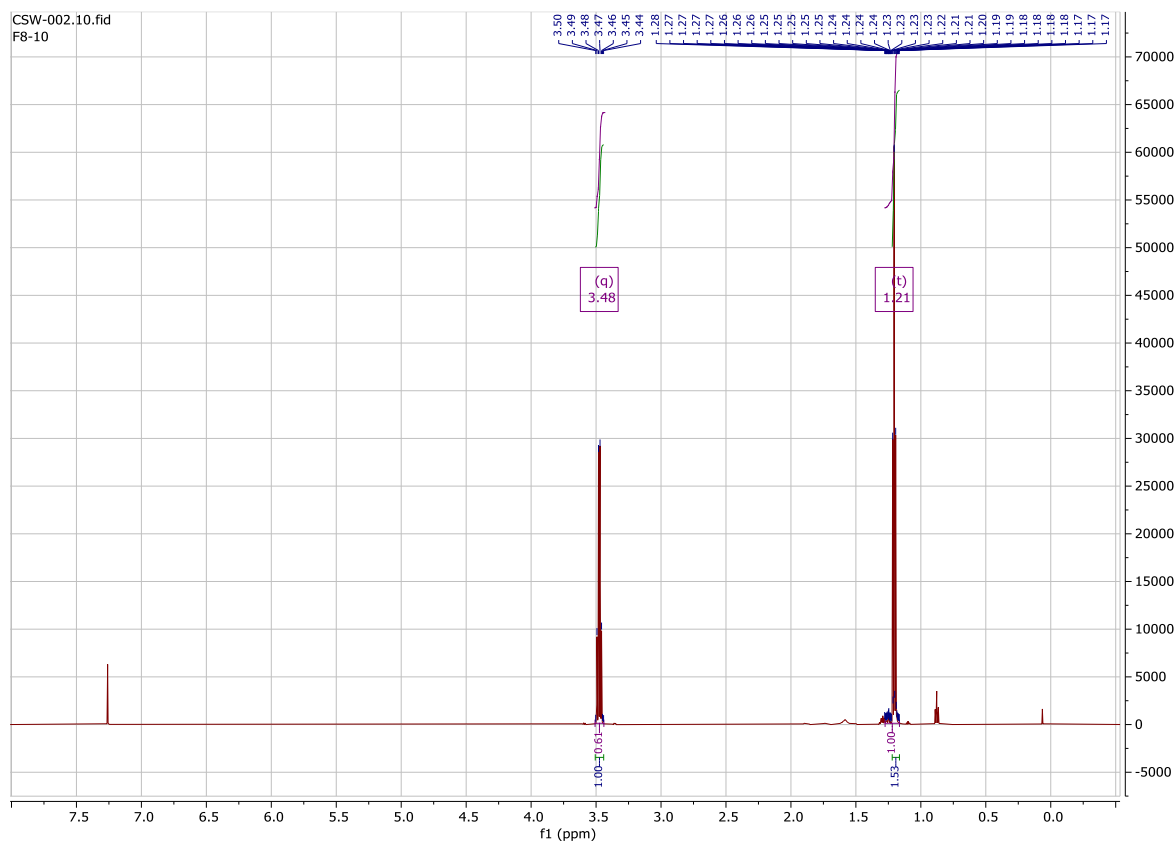


Figure 63: ^1H NMR (600MHz) spectrum of isolate #2 from biotransformation.

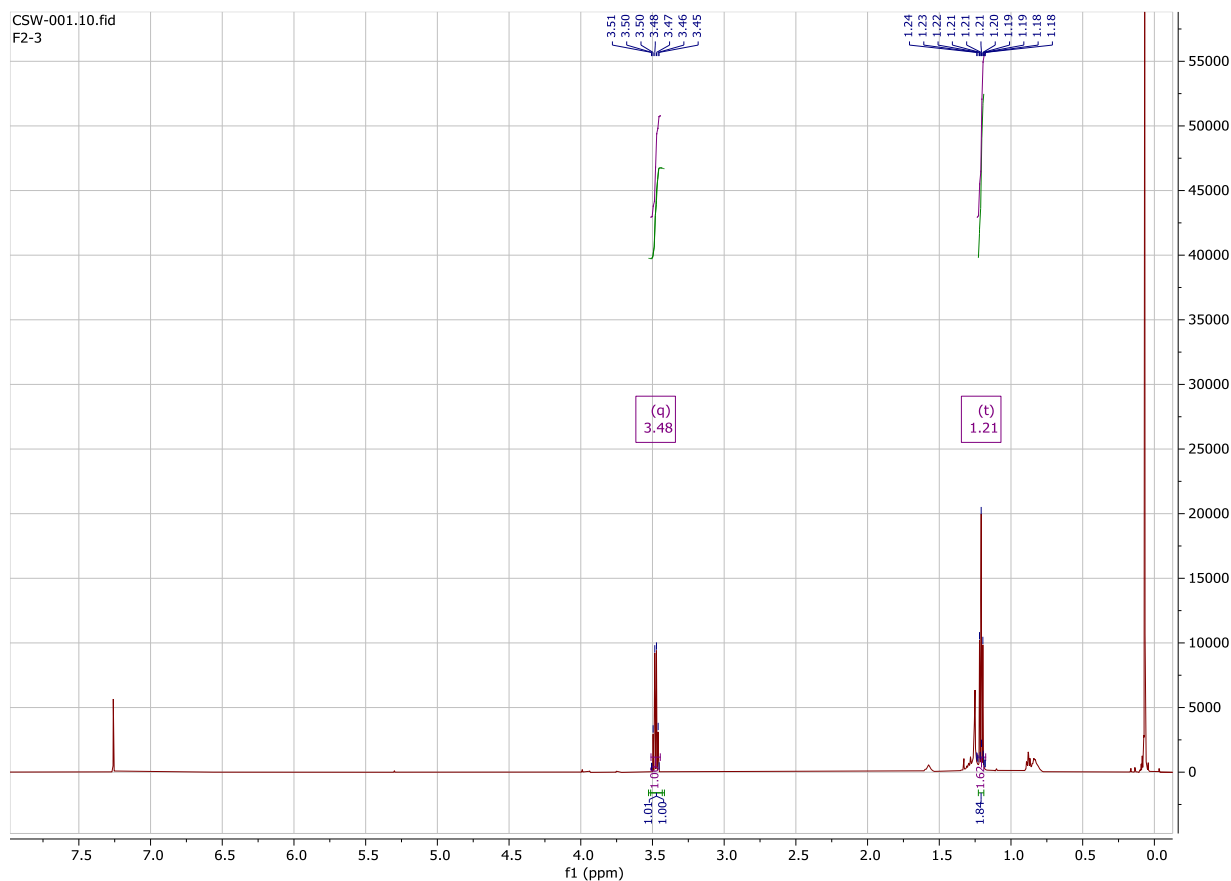


Figure 64: ^1H NMR (600MHz) spectrum of isolate #1 from biotransformation.

11. References

1. Stal, L. J. & Cretoiu, M. S. *The marine microbiome: An untapped source of biodiversity and biotechnological potential. The Marine Microbiome: An Untapped Source of Biodiversity and Biotechnological Potential* (2016). doi:10.1007/978-3-319-33000-6.
2. Planavsky, N. J. *et al.* Evolution of the structure and impact of Earth's biosphere. *Nat. Rev. Earth Environ.* **2**, 123–139 (2021).
3. Raven, J. A. The possible roles of algae in restricting the increase in atmospheric CO₂ and global temperature. *Eur. J. Phycol.* **52**, 506–522 (2017).
4. Garcia-Pichel, F. Cyanobacteria. in *Encyclopedia of Microbiology* 107–124 (Elsevier, 2009). doi:10.1016/B978-012373944-5.00250-9.
5. Castenholz, R. W. General Characteristics of the Cyanobacteria. *Bergey's Man. Syst. Archaea Bact.* 1–23 (2015) doi:10.1002/9781118960608.cbm00019.
6. Shinde, S. *et al.* Glycogen metabolism supports photosynthesis start through the oxidative pentose phosphate pathway in cyanobacteria. *Plant Physiol.* **182**, 507–517 (2020).
7. Smith, A. J. Modes of cyanobacterial carbon metabolism. *Ann. l'Institut Pasteur / Microbiol.* **134**, 93–113 (1983).
8. Zehr, J. P. Nitrogen fixation by marine cyanobacteria. *Trends Microbiol.* **19**, 162–173 (2011).
9. Olejarz, J., Iwasa, Y., Knoll, A. H. & Nowak, M. A. The Great Oxygenation Event as a consequence of ecological dynamics modulated by planetary change. *Nat. Commun.* **12**, 1–9 (2021).
10. Hodgskiss, M. S. W., Crockford, P. W., Peng, Y., Wing, B. A. & Horner, T. J. A productivity collapse to end Earth's Great Oxidation. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 17207–17212 (2019).
11. Ligrone, R. The Great Oxygenation Event. *Biol. Innov. that Built World* 129–154 (2019) doi:10.1007/978-3-030-16057-9_5.
12. Raven, J. A. & Allen, J. F. Genomics and chloroplast evolution: what did cyanobacteria do for plants? The endosymbiont hypothesis is mainstream. *Genome Biol.* **4**, 209 (2003).
13. Metcalf, J. S. & Codd, G. A. Cyanotoxins. in *Ecology of Cyanobacteria II* vol. 9789400738 651–675 (Springer Netherlands, 2012).
14. Swain, S. S., Paidesetty, S. K. & Padhy, R. N. Antibacterial, antifungal and antimycobacterial compounds from cyanobacteria. *Biomed. Pharmacother.* **90**, 760–776 (2017).
15. Havens, K. E. Cyanobacteria blooms: effects on aquatic ecosystems. *Adv. Exp. Med. Biol.* **619**, 733–747 (2008).
16. O'Neil, J. M., Davis, T. W., Burford, M. A. & Gobler, C. J. The rise of harmful cyanobacteria blooms: The potential roles of eutrophication and climate change. *Harmful Algae* **14**, 313–334 (2012).
17. Withers, P. J. A., Neal, C., Jarvie, H. P. & Doody, D. G. Agriculture and eutrophication: Where do we go from here? *Sustain.* **6**, 5853–5875 (2014).
18. Ullah, H., Nagelkerken, I., Goldenberg, S. U. & Fordham, D. A. Climate change could drive marine food web collapse through altered trophic flows and cyanobacterial proliferation. *PLoS Biol.* **16**, 1–21 (2018).
19. Sinha, R. P. & Häder, D.-P. Photobiology and Ecophysiology of Rice Field Cyanobacteria. *Photochem. Photobiol.* **64**, 887–896 (1996).
20. Mishra, U. & Pabbi, S. Cyanobacteria: A Potential Biofertilizer for Rice Upasana Mishra and Sunil Pabbi are at Centre for Conservation and Utilisation of Blue. *Resonance* 6–10 (2004).

21. Ciferri, O. Spirulina, the edible microorganism. *Microbiol. Rev.* **47**, 551–578 (1983).
22. Grosshagauer, S., Kraemer, K. & Somoza, V. The True Value of Spirulina. *J. Agric. Food Chem.* **68**, 4109–4115 (2020).
23. Pawlak, R., Parrott, S. J., Raj, S., Cullum-Dugan, D. & Lucas, D. How prevalent is vitamin B12 deficiency among vegetarians? *Nutr. Rev.* **71**, 110–117 (2013).
24. Jaiswal, D., Sahasrabuddhe, D. & Wangikar, P. P. Cyanobacteria as cell factories: the roles of host and pathway engineering and translational research. *Curr. Opin. Biotechnol.* **73**, 314–322 (2022).
25. Sheldon, R. A. & Woodley, J. M. Role of Biocatalysis in Sustainable Chemistry. *Chem. Rev.* **118**, 801–838 (2018).
26. Sheldon, R. A. Fundamentals of green chemistry: Efficiency in reaction design. *Chem. Soc. Rev.* **41**, 1437–1451 (2012).
27. Anastas, P. & Eghbali, N. Green Chemistry: Principles and Practice. *Chem. Soc. Rev.* **39**, 301–312 (2010).
28. Bell, E. L. *et al.* Biocatalysis. *Nat. Rev. Methods Prim.* **1**, 1–21 (2021).
29. Shaikh, I. R. Organocatalysis: Key Trends in Green Synthetic Chemistry, Challenges, Scope towards Heterogenization, and Importance from Research and Industrial Point of View. *J. Catal.* **2014**, 1–35 (2014).
30. Pizzol, M., Christensen, P., Schmidt, J. & Thomsen, M. Impacts of ‘metals’ on human health: A comparison between nine different methodologies for Life Cycle Impact Assessment (LCIA). *J. Clean. Prod.* **19**, 646–656 (2011).
31. Suchy, L. & Rudroff, F. Multi-Enzymatic Cascades In Vivo. in *Enzyme Cascade Design and Modelling* 49–63 (Springer International Publishing, 2021). doi:10.1007/978-3-030-65718-5_4.
32. Illanes, A., Cauerhff, A., Wilson, L. & Castro, G. R. Recent trends in biocatalysis engineering. *Bioresour. Technol.* **115**, 48–57 (2012).
33. Herries, D. Enzymes as catalysts in Organic Synthesis. *Biochemical Education* vol. 13 42 (1985).
34. Yi, D. *et al.* Recent trends in biocatalysis. *Chem. Soc. Rev.* **50**, 8003–8049 (2021).
35. Eijssink, V. G. H., GÅseidnes, S., Borchert, T. V. & Van Den Burg, B. Directed evolution of enzyme stability. *Biomol. Eng.* **22**, 21–30 (2005).
36. Wang, Y. *et al.* Directed Evolution: Methodologies and Applications. *Chem. Rev.* **121**, 12384–12444 (2021).
37. The Nobel Prize in Chemistry 2018. NobelPrize.org. Nobel Prize Outreach AB 2023. Mon. 12 Jun 2023. <https://www.nobelprize.org/prizes/chemistry/2018/summary/>.
38. Otten, L. G., Hollmann, F. & Arends, I. W. C. E. Enzyme engineering for enantioselectivity: from trial-and-error to rational design? *Trends Biotechnol.* **28**, 46–54 (2010).
39. Chica, R. A., Doucet, N. & Pelletier, J. N. Semi-rational approaches to engineering enzyme activity: Combining the benefits of directed evolution and rational design. *Curr. Opin. Biotechnol.* **16**, 378–384 (2005).
40. Ali, M., Ishqi, H. M. & Husain, Q. Enzyme engineering: Reshaping the biocatalytic functions. *Biotechnol. Bioeng.* **117**, 1877–1894 (2020).
41. Sheldon, R. A. & Brady, D. Broadening the Scope of Biocatalysis in Sustainable Organic Synthesis. *ChemSusChem* **12**, 2859–2881 (2019).
42. Zhao, J., Burke, A. J. & Green, A. P. Enzymes with noncanonical amino acids. *Curr. Opin. Chem. Biol.* **55**, 136–144 (2020).
43. Jiao, S., Li, F., Yu, H. & Shen, Z. Advances in acrylamide bioproduction catalyzed with *Rhodococcus* cells harboring nitrile hydratase. *Appl. Microbiol. Biotechnol.* **104**, 1001–1012 (2020).

44. Huffman, M. A. *et al.* Design of an in vitro biocatalytic cascade for the manufacture of islatravir. *Science* (80-.). **366**, 1255–1259 (2019).
45. Schäfer, H. J. Anodic and Cathodic CC-Bond Formation. *Angew. Chemie Int. Ed. English* **20**, 911–934 (1981).
46. Mahrwald, R. Organocatalytic methods for CC bond formation. *Drug Discov. Today Technol.* **10**, e29–e36 (2013).
47. Knappke, C. E. I. & Von Wangelin, A. J. 35 years of palladium-catalyzed cross-coupling with Grignard reagents: How far have we come? *Chem. Soc. Rev.* **40**, 4948–4962 (2011).
48. Brovetto, M., Gamenara, D., Saenz Méndez, P. & Seoane, G. A. C-C bond-forming lyases in organic synthesis. *Chem. Rev.* **111**, 4346–4403 (2011).
49. Muñoz Solano, D., Hoyos, P., Hernáiz, M. J., Alcántara, A. R. & Sánchez-Montero, J. M. Industrial biotransformations in the synthesis of building blocks leading to enantiopure drugs. *Bioresour. Technol.* **115**, 196–207 (2012).
50. Hartley, C. J. *et al.* Sugar analog synthesis by in vitro biocatalytic cascade: A comparison of alternative enzyme complements for dihydroxyacetone phosphate production as a precursor to rare chiral sugar synthesis. *PLoS One* **12**, 1–16 (2017).
51. Winkler, C. K., Faber, K. & Hall, M. Biocatalytic reduction of activated C[dbnd]C-bonds and beyond: emerging trends. *Curr. Opin. Chem. Biol.* **43**, 97–105 (2018).
52. Mifsud, M. *et al.* Photobiocatalytic chemistry of oxidoreductases using water as the electron donor. *Nat. Commun.* **5**, 1–6 (2014).
53. Hershlag, N., Hurley, I. & Woodward, J. A simple method to demonstrate the enzymatic production of hydrogen from sugar. *J. Chem. Educ.* **75**, 1270–1274 (1998).
54. Adams, J. P., Brown, M. J. B., Diaz-Rodriguez, A., Lloyd, R. C. & Roiban, G. D. Biocatalysis: A Pharma Perspective. *Adv. Synth. Catal.* **361**, 2421–2432 (2019).
55. Hayashi, Y. Pot economy and one-pot synthesis. *Chem. Sci.* **7**, 866–880 (2016).
56. Tietze, L. F. & Beifuss, U. International Edition in English Sequential Transformations in Organic Chemistry : *Angew. Chem., Int. Ed.* **32**, 131 (1993).
57. Zhao, H. & Van Der Donk, W. A. Regeneration of cofactors for use in biocatalysis. *Curr. Opin. Biotechnol.* **14**, 583–589 (2003).
58. Tufvesson, P., Lima-Ramos, J., Nordblad, M. & Woodley, J. M. Guidelines and cost analysis for catalyst production in biocatalytic processes. *Org. Process Res. Dev.* **15**, 266–274 (2011).
59. Alissandratos, A. In vitro multi-enzymatic cascades using recombinant lysates of *E. coli*: an emerging biocatalysis platform. *Biophys. Rev.* **12**, 175–182 (2020).
60. Di Cosimo, R., Mc Auliffe, J., Poulouse, A. J. & Bohlmann, G. Industrial use of immobilized enzymes. *Chem. Soc. Rev.* **42**, 6437–6474 (2013).
61. France, S. P., Hepworth, L. J., Turner, N. J. & Flitsch, S. L. Constructing Biocatalytic Cascades: In Vitro and in Vivo Approaches to de Novo Multi-Enzyme Pathways. *ACS Catal.* **7**, 710–724 (2017).
62. Chen, R. R. Permeability issues in whole-cell bioprocesses and cellular membrane engineering. *Appl. Microbiol. Biotechnol.* **74**, 730–738 (2007).
63. Yamada, S. *et al.* Effect of drug transporter genes on cysteine export and overproduction in *Escherichia coli*. *Appl. Environ. Microbiol.* **72**, 4735–4742 (2006).
64. Mariano, A. P., Qureshi, N., Filho, R. M. & Ezeji, T. C. Bioproduction of butanol in bioreactors: New insights from simultaneous in situ butanol recovery to eliminate product toxicity. *Biotechnol. Bioeng.* **108**, 1757–1765 (2011).
65. Covert, M. W. *et al.* Metabolic modeling of microbial strains in silico. *Trends Biochem. Sci.* **26**, 179–186 (2001).
66. Kaneko, T. *et al.* Sequence analysis of the genome of the unicellular cyanobacterium *synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and

- assignment of potential protein-coding regions (supplement). *DNA Res.* **3**, 185–209 (1996).
67. Kaneko, T. & Tabata, S. Complete Genome Structure of the Unicellular Cyanobacterium *Synechocystis* sp. PCC6803. *Plant Cell Physiol.* **38**, 1171–1176 (1997).
 68. Gao, L. *et al.* Toward the complete proteome of *Synechocystis* sp. PCC 6803. *Photosynth. Res.* **126**, 203–219 (2015).
 69. Tian, X., Chen, L., Wang, J., Qiao, J. & Zhang, W. Quantitative proteomics reveals dynamic responses of *Synechocystis* sp. PCC 6803 to next-generation biofuel butanol. *J. Proteomics* **78**, 326–345 (2013).
 70. Mascia, F. *et al.* Light-driven hydroxylation of testosterone by *Synechocystis* sp. PCC 6803 expressing the heterologous CYP450 monooxygenase CYP110D1. *Green Chem.* **24**, 6156–6167 (2022).
 71. Salek, S. S., Kleerebezem, R., Jonkers, H. M., Witkamp, G. J. & Van Loosdrecht, M. C. M. Mineral CO₂ sequestration by environmental biotechnological processes. *Trends Biotechnol.* **31**, 139–146 (2013).
 72. Guinotte, J. M. & Fabry, V. J. Ocean acidification and its potential effects on marine ecosystems. *Ann. N. Y. Acad. Sci.* **1134**, 320–342 (2008).
 73. Jodlbauer, J., Rohr, T., Spadiut, O., Mihovilovic, M. D. & Rudroff, F. Biocatalysis in Green and Blue: Cyanobacteria. *Trends Biotechnol.* **39**, 875–889 (2021).
 74. Wu, S. & Li, Z. Whole-Cell Cascade Biotransformations for One-Pot Multistep Organic Synthesis. *ChemCatChem* **10**, 2164–2178 (2018).
 75. Mizoguchi, H., Mori, H. & Fujio, T. *Escherichia coli* minimum genome factory. *Biotechnol. Appl. Biochem.* **46**, 157 (2007).
 76. Lukjancenko, O., Wassenaar, T. M. & Ussery, D. W. Comparison of 61 Sequenced *Escherichia coli* Genomes. *Microb. Ecol.* **60**, 708–720 (2010).
 77. McCloskey, D., Palsson, B. & Feist, A. M. Basic and applied uses of genome-scale metabolic network reconstructions of *Escherichia coli*. *Mol. Syst. Biol.* **9**, 1–15 (2013).
 78. Han, M.-J. & Lee, S. Y. *The Escherichia coli Proteome: Past, Present, and Future Prospects*. *Microbiology and Molecular Biology Reviews* vol. 70 (2006).
 79. Englaender, J. A. *et al.* Effect of Genomic Integration Location on Heterologous Protein Expression and Metabolic Engineering in *E. coli*. *ACS Synth. Biol.* **6**, 710–720 (2017).
 80. Hill, J. E., Myers, A. M., Koerner, T. J. & Tzagoloff, A. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. *Yeast* **2**, 163–167 (1986).
 81. Osbourn, A. E. & Field, B. Operons. *Cell. Mol. Life Sci.* **66**, 3755–3775 (2009).
 82. Engler, C., Kandzia, R. & Marillonnet, S. A one pot, one step, precision cloning method with high throughput capability. *PLoS One* **3**, (2008).
 83. Weber, E., Engler, C., Gruetzner, R., Werner, S. & Marillonnet, S. A modular cloning system for standardized assembly of multigene constructs. *PLoS One* **6**, (2011).
 84. Gibson, D. G. *et al.* Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* **6**, 343–345 (2009).
 85. Li, M. Z. & Elledge, S. J. SLIC: A method for sequence- and ligation-independent cloning. *Methods Mol. Biol.* **852**, 51–59 (2012).
 86. Doudna, J. A. & Charpentier, E. The new frontier of genome engineering with CRISPR-Cas9. *Science* (80-.). **346**, (2014).
 87. Julsing, M. K., Kuhn, D., Schmid, A. & Bühler, B. Resting cells of recombinant *E. coli* show high epoxidation yields on energy source and high sensitivity to product inhibition. *Biotechnol. Bioeng.* **109**, 1109–1119 (2012).
 88. Kiss, F. M., Lundemo, M. T., Zapp, J., Woodley, J. M. & Bernhardt, R. Process development for the production of 15 β -hydroxycypoterone acetate using *Bacillus megaterium* expressing CYP106A2 as whole-cell biocatalyst. *Microb. Cell Fact.* **14**, 1–13

- (2015).
89. de Carvalho, C. C. C. R. Whole cell biocatalysts: essential workers from Nature to the industry. *Microb. Biotechnol.* **10**, 250–263 (2017).
 90. Bentley, W. E., Mirjalili, N., Andersen, D. C., Davis, R. H. & Kompala, D. S. Plasmid-encoded protein: the principal factor in the ‘metabolic burden’ associated with recombinant bacteria. *Biotechnology Bioengineering*, 1990. *Biotechnol Bioeng* **102**, 1284–97; discussion 1283 (1990).
 91. Jin, H., Wang, Y., Idoine, A. & Bhaya, D. Construction of a shuttle vector using an endogenous plasmid from the cyanobacterium *synechocystis* sp. PCC6803. *Front. Microbiol.* **9**, 1–13 (2018).
 92. Huang, H. H., Camsund, D., Lindblad, P. & Heidorn, T. Design and characterization of molecular tools for a synthetic biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Res.* **38**, 2577–2593 (2010).
 93. Rudroff, F. Whole-cell based synthetic enzyme cascades—light and shadow of a promising technology. *Curr. Opin. Chem. Biol.* **49**, 84–90 (2019).
 94. Platt, T. and the Regulation of Gene Expression. *Annu. Rev. Biochem.* (1986).
 95. Arraiano, C. M. Post-transcriptional control of gene expression: bacterial mRNA degradation. *World J. Microbiol. Biotechnol.* **9**, 421–432 (1993).
 96. Ellis, T., Adie, T. & Baldwin, G. S. DNA assembly for synthetic biology: From parts to pathways and beyond. *Integr. Biol.* **3**, 109–118 (2011).
 97. Farasat, I. *et al.* Efficient search, mapping, and optimization of multi-protein genetic systems in diverse bacteria. *Mol. Syst. Biol.* **10**, 731 (2014).
 98. Kierzek, A. M., Zaim, J. & Zielenkiewicz, P. The Effect of Transcription and Translation Initiation Frequencies on the Stochastic Fluctuations in Prokaryotic Gene Expression. *J. Biol. Chem.* **276**, 8165–8172 (2001).
 99. Chang, B., Halgamuge, S. & Tang, S. L. Analysis of SD sequences in completed microbial genomes: Non-SD-led genes are as common as SD-led genes. *Gene* **373**, 90–99 (2006).
 100. Rodnina, M. V. Translation in prokaryotes. *Cold Spring Harb. Perspect. Biol.* **10**, 1–22 (2018).
 101. Sun, T. *et al.* Toolboxes for cyanobacteria: Recent advances and future direction. *Biotechnol. Adv.* **36**, 1293–1307 (2018).
 102. Seo, S. W. *et al.* Predictive design of mRNA translation initiation region to control prokaryotic translation efficiency. *Metab. Eng.* **15**, 67–74 (2013).
 103. Na, D. & Lee, D. RBSDesigner: Software for designing synthetic ribosome binding sites that yields a desired level of protein expression. *Bioinformatics* **26**, 2633–2634 (2010).
 104. Espah Borujeni, A. & Salis, H. M. Translation Initiation is Controlled by RNA Folding Kinetics via a Ribosome Drafting Mechanism. *J. Am. Chem. Soc.* **138**, 7016–7023 (2016).
 105. Reeve, B., Hargest, T., Gilbert, C. & Ellis, T. Predicting translation initiation rates for designing synthetic biology. *Front. Bioeng. Biotechnol.* **2**, 1–6 (2014).
 106. Daniel, R. M., Danson, M. J., Eienthal, R., Daniel, R. M. & Eienthal, R. Erratum: The temperature optima of enzyme: A new perspective on an old phenomenon (Trends in Biochemical Science). *Trends Biochem. Sci.* **26**, 401 (2001).
 107. Oberleitner, N. Biocatalytic cascade reactions towards the production of valuable fine chemicals. (Technische Universität Wien, 2016).
 108. Bayer, T., Milker, S., Wiesinger, T., Rudroff, F. & Mihovilovic, M. D. Designer Microorganisms for Optimized Redox Cascade Reactions - Challenges and Future Perspectives. *Adv. Synth. Catal.* **357**, 1587–1618 (2015).
 109. Assil-Companiononi, L. *et al.* Engineering of NADPH Supply Boosts Photosynthesis-Driven Biotransformations. *ACS Catal.* **10**, 11864–11877 (2020).

110. Lee, W. H., Kim, M. D., Jin, Y. S. & Seo, J. H. Engineering of NADPH regenerators in *Escherichia coli* for enhanced biotransformation. *Appl. Microbiol. Biotechnol.* **97**, 2761–2772 (2013).
111. Song, J. W., Seo, J. H., Oh, D. K., Bornscheuer, U. T. & Park, J. B. Design and engineering of whole-cell biocatalytic cascades for the valorization of fatty acids. *Catal. Sci. Technol.* **10**, 46–64 (2020).
112. Jiang, Y., Sun, Q., Zhang, L. & Jiang, Z. Capsules-in-bead scaffold: A rational architecture for spatially separated multienzyme cascade system. *J. Mater. Chem.* **19**, 9068–9074 (2009).
113. Grewal, P. S., Samson, J. A., Baker, J. J., Choi, B. & Dueber, J. E. Peroxisome compartmentalization of a toxic enzyme improves alkaloid production. *Nat. Chem. Biol.* **17**, 96–103 (2021).
114. Monti, D., Ottolina, G., Carrea, G. & Riva, S. Redox reactions catalyzed by isolated enzymes. *Chem. Rev.* **111**, 4111–4140 (2011).
115. Blank, L. M., Ebert, B. E., Buehler, K. & Bühler, B. Redox biocatalysis and metabolism: Molecular mechanisms and metabolic network analysis. *Antioxidants Redox Signal.* **13**, 349–394 (2010).
116. An, J., Nie, Y. & Xu, Y. Structural insights into alcohol dehydrogenases catalyzing asymmetric reductions. *Crit. Rev. Biotechnol.* **39**, 366–379 (2019).
117. Jörnvall, H., Höög, J. O. & Persson, B. SDR and MDR: Completed genome sequences show these protein families to be large, of old origin, and of complex nature. *FEBS Lett.* **445**, 261–264 (1999).
118. Becker, C. E. Methanol poisoning. *J. Emerg. Med.* **1**, 51–58 (1983).
119. Kavanagh, K. L., Jörnvall, H., Persson, B. & Oppermann, U. Medium- and short-chain dehydrogenase/reductase gene and protein families: The SDR superfamily: Functional and structural diversity within a family of metabolic and regulatory enzymes. *Cell. Mol. Life Sci.* **65**, 3895–3906 (2008).
120. Gröger, H., Hummel, W., Borchert, S. & Krauß, M. *Reduction of Ketones and Aldehydes to Alcohols. Enzyme Catalysis in Organic Synthesis, Third Edition* vol. 2 (2012).
121. de Gonzalo, G. & Paul, C. E. Recent trends in synthetic enzymatic cascades promoted by alcohol dehydrogenases. *Curr. Opin. Green Sustain. Chem.* **32**, 100548 (2021).
122. Faber, K. *Biotransformations in Organic Chemistry*. (Springer Berlin Heidelberg, 2004). doi:10.1007/978-3-642-18537-3.
123. Prelog, V. Specification of the stereospecificity of some oxido-reductases by diamond lattice sections. *Pure Appl. Chem.* **9**, 119–130 (1964).
124. Guo, J. *et al.* Stereodivergent Synthesis of Carveol and Dihydrocarveol through Ketoreductases/Ene-Reductases Catalyzed Asymmetric Reduction. *ChemCatChem* **10**, 5496–5504 (2018).
125. Hall, M. & Bommarius, A. S. Enantioenriched compounds via enzyme-catalyzed redox reactions. *Chem. Rev.* **111**, 4088–4110 (2011).
126. Barna, T. *et al.* Crystal structure of bacterial morphinone reductase and properties of the C191A mutant enzyme. *J. Biol. Chem.* **277**, 30976–30983 (2002).
127. Stuermer, R., Hauer, B., Hall, M. & Faber, K. Asymmetric bioreduction of activated C=C bonds using enoate reductases from the old yellow enzyme family. *Curr. Opin. Chem. Biol.* **11**, 203–213 (2007).
128. Winkler, C. K., Tasnádi, G., Clay, D., Hall, M. & Faber, K. Asymmetric bioreduction of activated alkenes to industrially relevant optically active compounds. *J. Biotechnol.* **162**, 381–389 (2012).
129. Paul, C. E. & Hollmann, F. A survey of synthetic nicotinamide cofactors in enzymatic

- processes. *Appl. Microbiol. Biotechnol.* **100**, 4773–4778 (2016).
130. Chaparro-Riggers, J. F., Rogers, T. A., Vazquez-Figueroa, E., Polizzi, K. M. & Bommarius, A. S. Comparison of three enoate reductases and their potential use for biotransformations. *Adv. Synth. Catal.* **349**, 1521–1531 (2007).
 131. Fitzpatrick, T. B., Amrhein, N. & Macheroux, P. Characterization of YqjM, an old yellow enzyme homolog from *Bacillus subtilis* involved in the oxidative stress response. *J. Biol. Chem.* **278**, 19891–19897 (2003).
 132. Pesic, M., Fernández-Fueyo, E. & Hollmann, F. Characterization of the Old Yellow Enzyme Homolog from *Bacillus subtilis* (YqjM). *ChemistrySelect* **2**, 3866–3871 (2017).
 133. Kitzing, K. *et al.* The 1.3 Å crystal structure of the flavoprotein YqjM reveals a novel class of old yellow enzymes. *J. Biol. Chem.* **280**, 27904–27913 (2005).
 134. Hall, M. *et al.* Asymmetric bioreduction of C=C bonds using enoate reductases OPR1, OPR3 and YqjM: Enzyme-based stereocontrol. *Adv. Synth. Catal.* **350**, 411–418 (2008).
 135. Grau, M. M. *et al.* Photoenzymatic reduction of C=C double bonds. *Adv. Synth. Catal.* **351**, 3279–3286 (2009).
 136. Pesic, M. *et al.* Multienzymatic in situ hydrogen peroxide generation cascade for peroxygenase-catalysed oxyfunctionalisation reactions. *Zeitschrift für Naturforsch. - Sect. C J. Biosci.* **74**, 101–104 (2019).
 137. Malihan-Yap, L., Grimm, H. C. & Kourist, R. Recent Advances in Cyanobacterial Biotransformations. *Chemie-Ingenieur-Technik* **94**, 1628–1644 (2022).
 138. Baeyer, A. . V. V. Einwirkung der Caro'sohen Reagens auf Ketone. *Ber. Dtsch. Chem. Ges.* **32**, 3625–3634 (1899).
 139. ten Brink, G. J., Arends, I. W. C. E. & Sheldon, R. A. The Baeyer-Villiger reaction: New developments toward greener procedures. *Chem. Rev.* **104**, 4105–4123 (2004).
 140. Mascotti, M. L., Lapadula, W. J. & Ayub, M. J. The origin and evolution of Baeyer - Villiger Monooxygenases (BVMOs): An ancestral family of flavin monooxygenases. *PLoS One* **10**, 1–16 (2015).
 141. Tolmie, C., Smit, M. S. & Opperman, D. J. Native roles of Baeyer-Villiger monooxygenases in the microbial metabolism of natural compounds. *Nat. Prod. Rep.* **36**, 326–353 (2019).
 142. Kamerbeek, N. M., Janssen, D. B., Van Berkel, W. J. H. & Fraaije, M. W. Baeyer-Villiger Monooxygenases, an Emerging Family of Flavin-Dependent Biocatalysts. *Adv. Synth. Catal.* **345**, 667–678 (2003).
 143. Ch, A. M. *et al.* A Cold-Active Flavin-Dependent Monooxygenase from *Janthinobacterium svalbardensis* Unlocks Applications of Baeyer – Villiger Monooxygenases at Low Temperature. (2023) doi:10.1021/acscatal.2c05160.
 144. DONOGHUE, N. A., NORRIS, D. B. & TRUDGILL, P. W. The Purification and Properties of Cyclohexanone Oxygenase from *Nocardia globerulea* CL1 and *Acinetobacter* NCIB 9871. *Eur. J. Biochem.* **63**, 175–192 (1976).
 145. Bučko, M. *et al.* Baeyer-Villiger oxidations: biotechnological approach. *Appl. Microbiol. Biotechnol.* **100**, 6585–6599 (2016).
 146. Kayser, M. M. 'Designer reagents' recombinant microorganisms: new and powerful tools for organic synthesis. *Tetrahedron* **65**, 947–974 (2009).
 147. Oberleitner, N. *et al.* An enzymatic toolbox for cascade reactions: A showcase for an in vivo redox sequence in asymmetric synthesis. *ChemCatChem* **5**, 3524–3528 (2013).
 148. Lowe, J. R., Martello, M. T., Tolman, W. B. & Hillmyer, M. A. Functional biorenewable polyesters from carvone-derived lactones. *Polym. Chem.* **2**, 702–708 (2011).
 149. Fürst, M. J. L. J., Boonstra, M., Bandstra, S. & Fraaije, M. W. Stabilization of cyclohexanone monooxygenase by computational and experimental library design. *Biotechnol. Bioeng.* **116**, 2167–2177 (2019).

150. Mansouri, H. R. *et al.* Mutations Increasing Cofactor Affinity, Improve Stability and Activity of a Baeyer-Villiger Monooxygenase. *ACS Catal.* **12**, 11761–11766 (2022).
151. Mansouri, H. R. *et al.* Mutations Increasing Cofactor Affinity, Improve Stability and Activity of a Baeyer – Villiger Monooxygenase. (2022) doi:10.1021/acscatal.2c03225.
152. Oberleitner, N. *et al.* From waste to value-direct utilization of limonene from orange peel in a biocatalytic cascade reaction towards chiral carvolactone. *Green Chem.* **19**, 367–371 (2017).
153. Knight, S. C., Schaller, C. P., Tolman, W. B. & Hillmyer, M. A. Renewable carvone-based polyols for use in polyurethane thermosets. *RSC Adv.* **3**, 20399–20404 (2013).
154. Jodlbauer, J. A high-throughput strategy for the optimization of gene expression in cyanobacteria to improve whole-cell biocatalysis. (Technical University Vienna, Austria, 2024).
155. Mueller, N. J. *et al.* The substrate spectra of pentaerythritol tetranitrate reductase, morphinone reductase, N-ethylmaleimide reductase and estrogen-binding protein in the asymmetric bioreduction of activated alkenes. *Adv. Synth. Catal.* **352**, 387–394 (2010).
156. Arthur, G. & Gale, R. Generating Novel Molecular Tools to Manipulate Gene Regulation in Cyanobacteria. (The University of Edinburgh, 2021). doi:http://dx.doi.org/10.7488/era/1643.
157. Nagel, R. A bridge between kingdoms: Introduction of a golden gate-based tool kit for cyanobacteria. *Plant Physiol.* **180**, 10–11 (2019).
158. Gale, G. A. R., Schiavon Osorio, A. A., Puzorjov, A., Wang, B. & McCormick, A. J. Genetic modification of cyanobacteria by conjugation using the cyanogate modular cloning toolkit. *J. Vis. Exp.* **2019**, 1–17 (2019).
159. Gale, G. A. R., Wang, B. & McCormick, A. J. Evaluation and Comparison of the Efficiency of Transcription Terminators in Different Cyanobacterial Species. *Front. Microbiol.* **11**, (2021).
160. Taton, A. *et al.* Broad-host-range vector system for synthetic biology and biotechnology in cyanobacteria. *Nucleic Acids Res.* **42**, 1–16 (2014).
161. Peters, C. Kopplung von Oxidoreduktasen in synthetischen Enzymkaskaden. *Math. Fak. PhD*, (2015).
162. Tüllinghoff, A. *et al.* Maximizing Photosynthesis-Driven Baeyer–Villiger Oxidation Efficiency in Recombinant *Synechocystis* sp. PCC6803. *Front. Catal.* **1**, 1–14 (2022).
163. Markiton, M., Chrobok, A., Matuszek, K., Seddon, K. R. & Swadzba-Kwasny, M. Exceptional activity of gallium(III) chloride and chlorogallate(III) ionic liquids for Baeyer–Villiger oxidation. *RSC Adv.* **6**, 30460–30467 (2016).
164. Tüllinghoff, A., Djaya-Mbissam, H., Toepel, J. & Bühler, B. Light-driven redox biocatalysis on gram-scale in *Synechocystis* sp. PCC 6803 via an in vivo cascade. *Plant Biotechnol. J.* 1–10 (2023) doi:10.1111/pbi.14113.
165. Włodarczyk, A., Selão, T. T., Norling, B. & Nixon, P. J. Newly discovered *Synechococcus* sp. PCC 11901 is a robust cyanobacterial strain for high biomass production. *Commun. Biol.* **3**, (2020).
166. Holland, S. C., Kappell, A. D. & Burnap, R. L. Redox changes accompanying inorganic carbon limitation in *Synechocystis* sp. PCC 6803. *Biochim. Biophys. Acta - Bioenerg.* **1847**, 355–363 (2015).
167. Taylor, G. M., Hitchcock, A. & Heap, J. T. Combinatorial assembly platform enabling engineering of genetically stable metabolic pathways in cyanobacteria. *Nucleic Acids Res.* **49**, (2021).
168. Thiel, K. *et al.* Translation efficiency of heterologous proteins is significantly affected by the genetic context of RBS sequences in engineered cyanobacterium *Synechocystis* sp. PCC 6803. *Microb. Cell Fact.* **17**, 1–12 (2018).

169. Wang, B., Eckert, C., Maness, P. C. & Yu, J. A Genetic Toolbox for Modulating the Expression of Heterologous Genes in the Cyanobacterium *Synechocystis* sp. PCC 6803. *ACS Synth. Biol.* **7**, 276–286 (2018).
170. Englund, E., Shabestary, K., Hudson, E. P. & Lindberg, P. Systematic overexpression study to find target enzymes enhancing production of terpenes in *Synechocystis* PCC 6803, using isoprene as a model compound. *Metab. Eng.* **49**, 164–177 (2018).
171. Vasudevan, R. *et al.* Cyanogate: A modular cloning suite for engineering cyanobacteria based on the plant moclo syntax. *Plant Physiol.* **180**, 39–55 (2019).
172. Petsi, M., Orfanidou, M. & Zografos, A. L. Organocatalytic epoxidation and allylic oxidation of alkenes by molecular oxygen. *Green Chem.* **23**, 9172–9178 (2021).
173. Rossi-Fernández, L., Dorn, V. & Radivoy, G. A new and efficient methodology for olefin epoxidation catalyzed by supported cobalt nanoparticles. *Beilstein J. Org. Chem.* **17**, 519–526 (2021).
174. Guo, T., Gao, Y., Li, Z., Liu, J. & Guo, K. Cyclopropenium-Activated DMSO for Swern-Type Oxidation. *Synlett* **30**, 329–332 (2019).
175. Nicklaus, C. M. *et al.* Ruthenium/1,1'-bis(diphenylphosphino)ferrocene-catalysed Oppenauer oxidation of alcohols and lactonisation of α,ω -diols using methyl isobutyl ketone as oxidant. *Adv. Synth. Catal.* **355**, 2839–2844 (2013).