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Exploring different expression strategies for the optimization of the cyanobacterium *Synechocystis* sp. PCC 6803 as a host for biotechnological applications

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Abstract

In this study, the applicability of the cyanobacterium *Synechocystis* sp. PCC 6803 (further on: *Synechocystis*) as a host for whole-cell biocatalysis was explored. The advantage of using this unicellular organism for biotechnological applications, is its capability to produce the nicotineamide co-factor NADPH via the light-reaction of the photosynthesis. Reactions which use NADPH as a co-factor can be fuelled this way. In this study the ene-reductase YqjM from *Bacillus subtilis* was introduced in two different ways: via genomic integration at the gene locus *slr0168* or via a self-replicative pAWG_T5 plasmid. For the genomic integration, two different native *Synechocystis* promoters (*cpc* and *psbA2*) were used to optimize the expression of *YqjM* for the conversion of 2-methylmaleimid. In parallel, co-workers in Bochum investigated the NADPH concentration inside the cell during light exposure, darkness and by substrate addition. In presence of light and substrate, the NADPH steady state inside the cell was reduced for all recombinant strains. Substrate addition and switching of the light led furthermore to a faster decay of intracellular NADPH. The results strongly suggest a correlation of NADPH concentration and recombinant enzyme activity. The latter was investigated in this study, expecting highest activity for the self-replicative pAWG_T5_YqjM as the NADPH experiments were most promising.

The hypothesis was not proven in this study and both genomic integrative systems of YqjM showed a faster reaction rate than the pAWG_T5 plasmid (*cpc*: 76.8 U/g_{CDW} and *psbA2*: 65.5 U/g_{CDW} compared to 44.7 U/g_{CDW}). In addition, the effect of the cell concentration on the enzyme activity was also determined. It could be shown that with a smaller cell density a higher specific activity could be reached for all expression systems.

Furthermore, the potential of *Synechocystis* to perform cascade reactions was also explored. For this, the previously mentioned ene-reductase YqjM and the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 were combined to convert 2-methylcyclohexenone to 7-*O*-methyloxepanone. The cloning for a double integrative expression of both genes was performed. Furthermore, in an attempt to change the stereoselectivity of YqjM a double mutant was created, which however did not result in any conversion of the desired substrate.

Zusammenfassung

In dieser Studie wurde die Anwendbarkeit des Cyanobakteriums *Synechocystis* sp. PCC 6803 (fortan: *Synechocystis*) als Wirt für die Ganzzellbiokatalyse untersucht. Dieser einzellige Organismus ist von Vorteil für biotechnologische Anwendungen, da er die Herstellung des Nikotinamid-Cofaktors NADPH über die Lichtreaktion der Photosynthese ermöglicht. Dieses Merkmal macht die Kopplung mit heterolog exprimierten Enzymen, die NADPH als Cofaktor verwenden, attraktiv. In dieser Studie wurde die Ene-Reduktase YqjM aus *Bacillus subtilis* auf zwei verschiedene Arten exprimiert: über die genomische Integration am Genlocus *slr0168* oder über ein selbstreplizierendes pAWG_T5-Plasmid. Für die genomische Integration wurden zwei verschiedene native *Synechocystis*-Promotoren (*cpc* & *psbA2*) verwendet, um die Expression von YqjM für die Umwandlung von 2-Methylmaleimid zu optimieren. Parallel dazu untersuchten Kollegen aus Bochum die intrazelluläre NADPH-Konzentration bei Belichtung, Dunkelheit und Substratzugabe. In Gegenwart von Licht und Substrat war das intrazelluläre NADPH-Fließgleichgewicht für alle rekombinanten Stämme reduziert. Bei Substratzugabe und Abschalten des Lichts, kam es zu einem schnelleren Abfall der intrazellulären NADPH Konzentration. Diese Ergebnisse deuten stark auf eine Korrelation von NADPH-Konzentration und rekombinanter Enzymaktivität hin. Letzteres wurde in dieser Studie untersucht, wobei die höchste Aktivität für das selbstreplizierende pAWG_T5_YqjM System erwartet wurde, da die NADPH-Experimente am vielversprechendsten waren. Diese Hypothese konnte in dieser Studie nicht bewiesen werden. Beide genomisch-integrierte Systeme von *YqjM* zeigten eine schnellere Reaktionsrate als das pAWG_T5 Plasmid (*cpc*: 76,8 U/g_{CDW} und *psbA2*: 65,5 U/g_{CDW} im Vergleich zu 44,7 U/g_{CDW}). Zusätzlich wurde der Einfluss der Zellkonzentration auf die Enzymaktivität bestimmt. Es konnte gezeigt werden, dass eine geringere Zelldichte für alle Expressionssysteme zu einer höheren spezifischen Aktivität führt.

Zusätzlich wurde das Potential von *Synechocystis* untersucht, Kaskadenreaktionen durchzuführen. Hierzu wurde die zuvor erwähnte Ene-reduktase YqjM und die Cyclohexanonmonooxygenase (CHMO) von *Acinetobacter calcoaceticus* NCIMB 9871 zur Umsetzung von 2-Methylcyclohexenon verwendet. Die Klonierungen für eine doppel-integrative Expression beider Gene wurde in dieser Studie durchgeführt. Darüber hinaus wurde in einem Versuch, die Stereoselektivität von YqjM zu ändern, eine Doppelmutante erzeugt, die jedoch zu keiner Umwandlung des Substrats führte.

Abbreviations

µg	Microgram
µL	Microlitre
µm	Micrometre
µmol	Micromol
a.u	Arbitrary unit
ADHs	Alcohol Dehydrogenases
Ala	Alanine
approx.	Approximately
Arg	Arginin
asRNA	Antisense RNA
ATP	Adenosintriphosphate
B. subtilis	*Bacillus subtilis*
b6f	Cytochrome b6f complex
BVO	Baeyer-Villiger oxidation
C	Celsius
CDW	Cell dry weight
CHMO	Cyclohexenone monooxygenase
cm	Centimetre
Cm	Chloramphenicol
cPCR	Colony PCR
Cys	Cystein
ddH2O	Double distilled water
dNTPs	Desoxyrribonucleosidetriphosphate mix
dest.	Destilled
DMSO	Dimethyl sulofoxide
E. coli	Escherichia coli
EWG	Electron withdrawing group
EYFP	Enhanced yellow fluorescent protein
FAD	Flavin adenosine dinucleotide
Fd	Ferredoxin
FMN	Flavinmononucleotid
g	Gram

g	Relative centrifugal force compared to gravity
GC-FID	Gas chromatography with flame ionization detector
h	Hour(s)
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Kan	Kanamycin
kb	Kilobases
kV	Kilovolt
LB	*Lysogeny* broth
Lys	Lysin
m	Metre
M	Molar
mA	Milli Ampere
mg	Milligram
min	Minute(s)
mL	Millilitre
mm	Millimetre
mM	Millimolar (mmol mL^{-1})
mol	Mole(s)
ms	Millisecond(s)
NADPH	Nicotinamide adenine dinucleotide phosphate
No.	Number
OD	Optical density
ONC	Overnight culture
OYE	Old yellow enzyme
Pc	Plastocyanin
PCR	Polymerase chain reaction
g	gram(s)
PQ	Plastoquinone
PQH2	Plastoquinol
PS 1	Photosystem 1
PS 2	Photosystem 2
RBS	Ribosome binding site
rpm	Rounds per minute
RT	Room temperature

RuBisCO	Ribulose-1,5-bisphosphat-carboxylase/-oxygenase
s	Second(s)
SOC	Superoptimal broth with catabolite respression
Std	Standard
TAE	Tris base, acetic acid and EDTA
Tyr	Tyrosin
U	Enzyme unit(s)
V	Volt
v/v	Volume percentage
Val	Valin
vs.	Versus
w/w	Weight percentage
Wt	Wild type
YqjM	NADPH dehydrogenase from *Bacillus subtilis*

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1. Introduction

1.1 *Synechocystis* sp. PCC 6803

Cyanobacteria are often designated as “blue-green algae”, an anachronism, as electron microscopy revealed that due to their cell wall composition and ribosomal structure, they are clearly classified as prokaryotes and need to be classified as bacteria.[1] The misleading designation occurred because similar to algae and higher plants, but different to other phototrophic bacteria, cyanobacteria carry out oxygenic photosynthesis.[2] Due to the similarities of the cyanobacterial photosynthetic machinery to the one found in plants, cyanobacteria are an interesting research target for the investigation of oxygenic photosynthesis. As cyanobacteria grow much faster and their genetic system is much simpler to genetically modify compared to plants, it is worth to investigate this organism for biotechnological applications.[3] Today, the focus expanded and cyanobacteria are explored as hosts for autotrophic biotransformations. Here, *Synechocystis* sp. PCC 6803 (from now on referred to as *Synechocystis*) became one of the model organisms.[4–6]

1.1.1 Origin

Synechocystis was isolated from a freshwater lake in California and was firstly outlined by Stanier *et al*. The strain was firstly designated to the genus *Aphanocapsa*[1] but later on re-allocated to the genus *Synechocystis* as the cells do not form aggregates in culture. *Synechocystis* is able to perform oxygenic photosynthesis, as well as metabolise glucose.[7] *Synechocystis* was the first substrain which was fully sequenced and therefore, it was established as a model organism for cyanobacteria.[2] Throughout the years various substrains have evolved from the originally isolated *Synechocystis*. They vary in motility and glucose tolerance.[8]

1.1.2 Genome

The genome of *Synechocystis* consists of one circular chromosome of approx. 3.5 million bp with a GC content of 47.7 %.[3] Furthermore, *Synechocystis* contains three smaller and four larger plasmids.[9] The cyanobacterial genome includes varying copy numbers. This has been investigated by Zerulla *et al*. The authors showed that the genome copy number is dependent on cultivation conditions, as well as the growth state. At a cell density level of OD_{750} = 0.1 the copy number was 20 on average, whereas it contained 4 copies on average for an OD_{750} = 2.5. This

polyploidy was also discovered when varying the light intensity and phosphate concentration, resulting in higher copy numbers of the genome under low light intensities (53 copies) and high phosphate concentrations (35 copies).[8] Extremely large copy numbers of 218 and 142 have also been reported previously.[10] This large discrepancy was discussed by Zerulla *et al*. They concluded that both studies showed valid results and that this highlights the importance to consider the polyploidy of *Synechocystis*. [8]

1.1.3 Promoters

In this study three different promoters were used to drive the expression of the gene *YqjM*: the native *psbA2* and *cpc* promoter and the *T5* bacteriophage promoter.

1.1.3.1 *psbA2* promoter

The *psbA2* promoter is known to be a strong native promoter. The *psbA2* gene encodes for the D1 subunit of PS 2. The D1 subunit is degraded and replaced every 5 h to 20 min depending on light intensity.[11] As shown by Mohamed *et al*. *psbA2* and *psbA3* encode for the PS 2 protein, whereby *psbA2* controlled transcription makes up roughly 90 % of the total *psbA* transcript under standard cultivation conditions in *Synechocystis*. The two genes *psbA2* and *psbA3* are highly homologous, however, they differ in their 5' upstream region of the gene. The transcription start for *psbA2* was mapped -49 upstream of the gene and for *psbA3* -88. They do share an identical -35 region but differ in the -10 region.[12]

Mohamed and Jansson investigated the induceability of *psbA2* and *psbA3*. For both, more RNA was transcribed in high light conditions (1500 $\mu mol_{photons}/m^2$ for 20 h) compared to low light conditions (50 $\mu E/m^2$ for 48 h), suggesting that these promoters are light inducible.[13] Englund *et al*. showed that the length of the native 5' upstream region of the *psbA2* promoter had a large effect on the expression level of the enhanced yellow fluorescent protein (EYFP). The shortest version resulted in an expression level of EYFP that was approximately three times higher compared to longest version of the 5' upstream *psbA2* promoter region. In difference to the longer upstream region, the shortest version lagged the beginning of a regulatory antisense RNA (asRNA), which probably downregulates the expression of EYFP.[14]

1.1.3.2 *cpc* promoter

The *cpc* promoter lies upstream of the *cpcB* gene which encodes for the C-phycocyanin beta chain. This protein is part of the PBS which is responsible for the

light absorption in *Synechocystis*.[15] The *cpc* promoter sequence used in this study was adopted from Formighieri and Melis.[16]

Zhou *et al*. were the first to investigate the *cpc* promoter in more detail. The very strong cpc_{560} promoter has been found to contain 14 predicted transcription factor binding sites between 381 and 556 bp upstream and two predicted promoters from the start codon of the *cpcB* gene. These properties are speculated to give the *cpc* promoter its high strength. With this promoter an expression of the recombinant D-lactate dehydrogenase (from *E. coli* K12) resulted in 15 % of the total soluble protein content.[17] The regulation of the transcription factors is thought to be related to light, as the protein is responsible for the light harvesting. It has been shown that the *cpc* genes are down-regulated, when suspecting *Synechocystis* to high light intensities (300-500 $\mu mol_{photons}/m^2s$).[18] Till today, the exact mechanism of the transcription factors and the regulation of the *cpc* promoter remains unknown.

1.1.3.3 T5 promoter

The T5 promoter stems from the bacteriophage T5 and is able to recruit the bacterial RNA polymerase from *E. coli*.[19] However, there is a difference in the bacterial RNA polymerase to the cyanobacterial enzyme. The bacterial apoenzyme consists of five subunits: $\beta\beta'\alpha_2\omega$ and by binding the σ factor is able to initiate transcription at a promoter sequence. The cyanobacterial enzyme has the β' part split into two parts: γ and β'. It is still unknown to which extent this difference contributes to the transcript expression, especially when using non-native promoters.[20] This difference was discussed by Schyns *et al*. to result in varying transcription behaviours towards the P*lac* and P*lacUV5* promoter, when comparing the cyanobacterium *Calothrix* sp. PCC 7601 and *E.coli*.[21]

1.2 Photosynthesis

Photosynthesis describes a series of processes in which electromagnetic energy is converted to chemical energy which in turn is used for the biosynthesis of organic cell material.[22] As Martin *et al*. discussed, two types of photosynthesis can be distinguished: oxygenic and anoxygenic photosynthesis. Whereas the oxygenic photosynthesis consists of two reaction centres, the anoxygenic only harbours one.[23] The anoxygenic photosynthesis occurs at anaerobic conditions, in a non-cyclic mode, using inorganic electron donors such as hydrogen sulphide, hydrogen and ferrous ion as electron donors.[24] In contrast, the oxygenic photosynthesis uses the splitting of

water to gain electrons, as well as producing oxygen.[25,26] The process of oxygenic photosynthesis is found in cyanobacteria, algae and plants and is described in detail by Lea-Smith *et al*. At the thylakoid membrane water is split with the help of light captured from the phycobilisome (PBS) into protons and oxygen at the Photosystem 2 (PS 2). The redox centres necessary to perform these reactions at the protein complex, are mediated by the central transmembrane D1 and D2 subunits. The water oxidation itself occurs at a Mn_4O_5Ca cluster at the PS 2. This initiates the electron transfer to P_{680}. Two electrons are transferred for each molecule of water.[27] The electrons are transferred onto a plastoquinone (PQ) within the PS 2 along two protons. The reduced plastoquinol (PQH_2) then diffuses along the thylakoid membrane and transfers the electrons onto the cytochrome b_6f complex (b_6f). On the luminal side of the complex the PQH_2 is oxidized and the electrons can be transferred to either a plastocyanin (Pc) or the cytochrome c_6 complex. Both of these carrier molecules are able to transfer the electrons with similar kinetics to the photosystem 1 (PS 1). For *Synechocystis* the Pc system is however preferred. An excited electron is subsequently generated by the PS 1 via P_{700} and is further moved onto a ferredoxin (Fd) molecule. This in turn leads to the reduction of $NADP^+$ to NADPH via the ferredoxin-$NADP^+$ reductase (FNR). During this process the established proton gradient is used to drive ATP synthesis via the ATPase. In total this light reaction of the photosynthesis results in the production of oxygen, the reduction of $NADP^+$ and from the proton gradient a subsequent ATP production via the ATPase (Fig 1).[28]

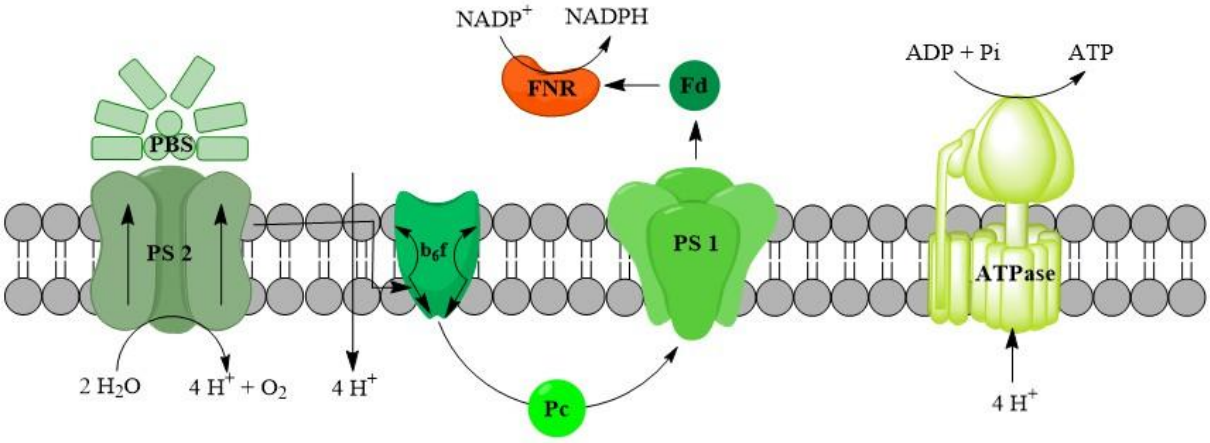

Fig 1: Photosynthesis in cyanobacteria: The light reaction of the photosynthetic pathway of cyanobacteria is depicted as a general scheme above. The electrons which are gained by the water splitting event at PS 2 are moved along the thylakoid membrane via a plastoquinol to the b_6f complex. The plastocyanin (Pc) is then able to transport the electrons to the PS 1 system. The electrons are further transported via ferredoxin to the FNR which reduces $NADP^+$ to NADPH. With the proton gradient which is built up during this process the ATPase is able to produce ATP from ADP and Pi. Image adapted from Hallenbeck.[27]

Photosynthesis is an important physiological process and cyanobacteria are among the few, which are capable of simultaneously performing oxygenic photosynthesis and respiration in the same compartment.[25]

1.3 Tools for genetic modification in *Synechocystis*

The genetic modification of *Synechocystis* can be achieved in several ways. Various protocols have been established to transfer exogenous DNA into the cyanobacterium, these include: natural transformation, electroporation and conjugation with autonomous or genome-integrative vectors.[29,30]

In the event of homologous recombination, a DNA exchange occurs between two extensive homologous sequences. Via the formation of a heteroduplex region, the DNA is then able to be transferred with the help of the RecBCD enzyme complex and RuvA, B and C.[31] The gene of interest is cloned into the expression cassette of a shuttle vector, e.g. SynRekB. This cassette is flanked by sequences homologous to the *slr0168* sequence. As *Synechocystis* contains multiple copies of its genome, segregation is needed to ensure the recombination occurs for all copies.[8]

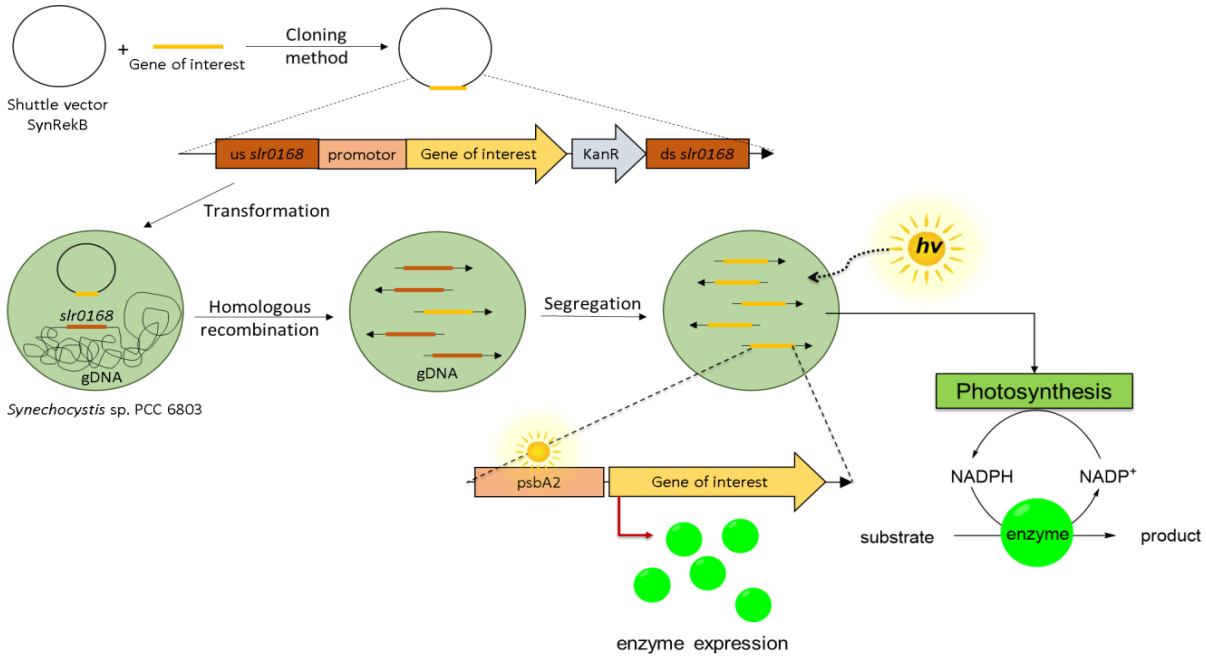

Fig 2: Homologous recombination as a mean for genetic modification in *Synechocystis*: The shuttle vector SynRekB was used for the expression of the gene of interest. The vector is constructed so that the expression cassette is flanked by homologous regions of the gene locus *slr0168*, which has been known as a neutral site.[3,32,33] Via homologous recombination the expression cassette is integrated into the host's genome. Due to the polyploidy of *Synechocystis*, a segregation process is necessary to ensure that all genomic copies contain the expression cassette. Image adapted from Hanna Büchsenschütz.

With the characterization of the *Synechocystis* genome neutral sites were also identified. The gene locus *slr0168* has been identified as a neutral site; even though it encodes for an unknown protein, no changes in growth or metabolism have been

observed, when it is disrupted and a heterologous gene is inserted. Therefore, it has been used as a neutral site for genomic integration into *Synechocystis*.[3,32,33] Additionally to this site, Pinto *et al*. searched for further neutral sites. With an unbiased systematic approach 16 neutral sites were identified. Two of these (N15 and N16) showed a similar growth pattern to the wild type when a heterologous gene was inserted, N8 showed a slightly higher growth rate compared to the parental strain when exposed to continuous light.[34]

A different approach of the genetic modification of *Synechocystis* is the transformation of wild type cells with self-replicative plasmids. *Synechocystis* and other cyanobacteria have been shown by Mermet-Bouvier *et al.* to be able to maintain plasmids with a RSF1010 replicon. The replication has been shown to be similar to *E. coli*, with approx. 12 plasmid copies per cell.[35]

Moreover, there is still a lot of room in terms of understanding the native genomic elements of *Synechocystis* for the exploitation of the host for biotechnological applications. By understanding these elements the expression of heterologous genes can be optimized specifically for *Synechocystis*. Through the optimization of genomic elements, *Synechocystis* could pose as a promising alternative to *E. coli*.

Only a few native ribosome binding sites (RBS) have been characterized so far according to their strength such as for the *psbA2* and *rbcL* genes.[14] In addition to this, the termination mechanism for gene expression is also not fully elucidated yet. Vijayan, Jain and O'Shea have postulated, that there is no Rho-dependent termination in *Synechocystis*. For this type of termination, a Rho-protein binds to the newly transcribed RNA and leads to the termination of the transcription. No such protein homologs have been found for *Synechocystis*. Furthermore, the authors showed, that a dip in minimum free energy occurs slightly before the transcript terminus. This suggests the formation of a stem-loop structure, which is typical for Rho-independent termination.[36] The only terminators which have been used so far in biotechnological processes in *Synechocystis* are the endogenous ribulose-1,5-bisphosphat-carboxylase/-oxygenase (RuBisCO) terminator and the *E.coli* terminator T_{rrnB}. In a recent study, Liu and Pakrasi explored more native elements of *Synechocystis* such as promoters, terminators and RBS. For promoters, they compared 9 new uncharacterized native promoters to 4 known ones which have been used in the cyanobacterium before (P_{cpc}, P_{trc10}, P_{rbcL}, P_{psbA2}). The three most highly active promoters for the expression of EYFP were the previously used P_{cpc},

P_{trc10} and the newly characterized $P_{sll1626}$. Additionally, they characterized 20 native RBS according to their strength, resulting in a RBS library, as well as a terminator library consisting of 8 terminators. For the RBS library, they used the same length for all RBS sequences. Some of the selected RBS did not show any activity, although they are known to work in their native environment. Liu and Pakrasi discussed that the length and environment of the RBS is crucial for its ability to work. Therefore this needs to be adjusted accordingly. The three strongest RBS were RBS-*psaF*; RBS-*ndhJ* and RBS-*psaL*. The three strongest terminator sequences included the T*psbC*, T*rrnB* and the T*apcC*.[37]

1.4 Biotransformations using *Synechocystis*

The review of Schmermund *et al*. deals in detail with light-driven catalysis. Various biotransformations, wild type reactions as well as reactions with recombinant enzymes, which have been performed with *Synechocystis*, are discussed.[38]

1.4.1 Wild type reactions

The reduction of cinnamaldehyde to cinammyl alcohol has been described as a wild type reaction for *Synechocystis* with a conversion of 98 % after four days.[39] Other cyanobacteria, such as *Synechococcus elongatus* PCC 7942 have shown to reduce various pentafuoroacetophenones, as well as α,α-difluoracetophenones.[40,41]

1.4.2 Reactions with recombinant enzymes

Apart from the wild type reactions, efforts have been made to introduce recombinant enzymes into *Synechocystis* to drive biotransformations.[4,5,30] A few examples are listed in Table 1.

Table 1: Biotransformation with recombinant enzymes using *Synechocystis*: some biotransformations with heterologously expressed enzymes are listed below.

Enzyme expressed	Substrate [mM]	Activity [U/g_{CDW} unless stated otherwise]	Conversion [%]	ee [%]	Reference
YqjM (*B. subtilis*)	Cyclohexenone (15)	39	70	-	[4]
	2-Methylcyclohexenone (15)	21.1	42	-	
	2,6,6-Trimethylcyclohexenone (10)	6.2	57	-	
	Cyclopentenone (15)	25.6	99	-	
	N-methylmaleimid (15)	53.2	94	-	
	2-methylmaleimid (20)	90.0	99	>99	
	2-methylmaleimid (10)	123	99	>99	
	2,N-dimethylmaleimid (20)	99.5	99	>99	
CHMO (*Acinetobacter calcoaceticus)*	various ketones, 4-Methylcyclohexanone (5) showed the highest specific activity	5.73	-	-	[5]
AlkBGT (*Pseudomonas putida*)	nonanoic acid methyl ester (10)	3.7 ± 0.5 mmol/min g_{CDW}	-	-	[6]
CYP450 (*Acidovorax* sp. CHX100)	Cyclohexane	26.3 ± 0.6 39.2 ± 0.7 (with in situ substrate supply)	-	-	[42]

1.5 Oxidoreductases

The enzyme database defines oxidoreductases as enzymes, which catalyze oxido-reductions. In these reactions one substrate is oxidized, whereas another substrate is concurrently reduced. The oxidoreductases are further subdivided depending on the chemical group they act upon.[43] This study deals with two oxidoreductases in

particular: the ene-reductase YqjM from *B. subtilis* and the monooxygenase CHMO from *Acinetobacter calcoaceticus* NCIMB 9871.

1.5.1 YqjM

The protein YqjM (Accession nr.: WP_003230377; EC: 1.6.99.1), was discovered to have similar biochemical properties to the homologous yeast Old Yellow Enzyme (OYE) and is involved in the oxidative stress response of *B. subtilis.*[44] It was found to have a FMN molecule bound non-covalently at the COOH end of the β-sheet. In contrast to members of the OYE family it is arranged in a homotetrameric fashion, consisting of dimers of catalytically dependent dimers. In addition to this difference, the conserved active site residues also differ. For YqjM these consist of Cys26, Tyr28, Lys109 and Arg336. This resulted in the creation of a new subfamily of OYE homologs. Cys26 is believed to act as a redox sensor, which then controls the redox potential of the FMN molecule when a substrate is present[45].Furthermore, Ala60 was also found to be in hydrogen bonding distance to the riboflavin cofactor. Both residues have been shown to be responsible for tuning the redox potential of said cofactor.[46]

YqjM is able to catalyze the reduction of C=C double bonds of α,β-unsaturated aldehydes and ketones. For this reduction of the double bond the riboflavin cofactor is oxidized. The regeneration of the cofactor is then performed at the expense of NAD(P)H (Fig 3).[47]

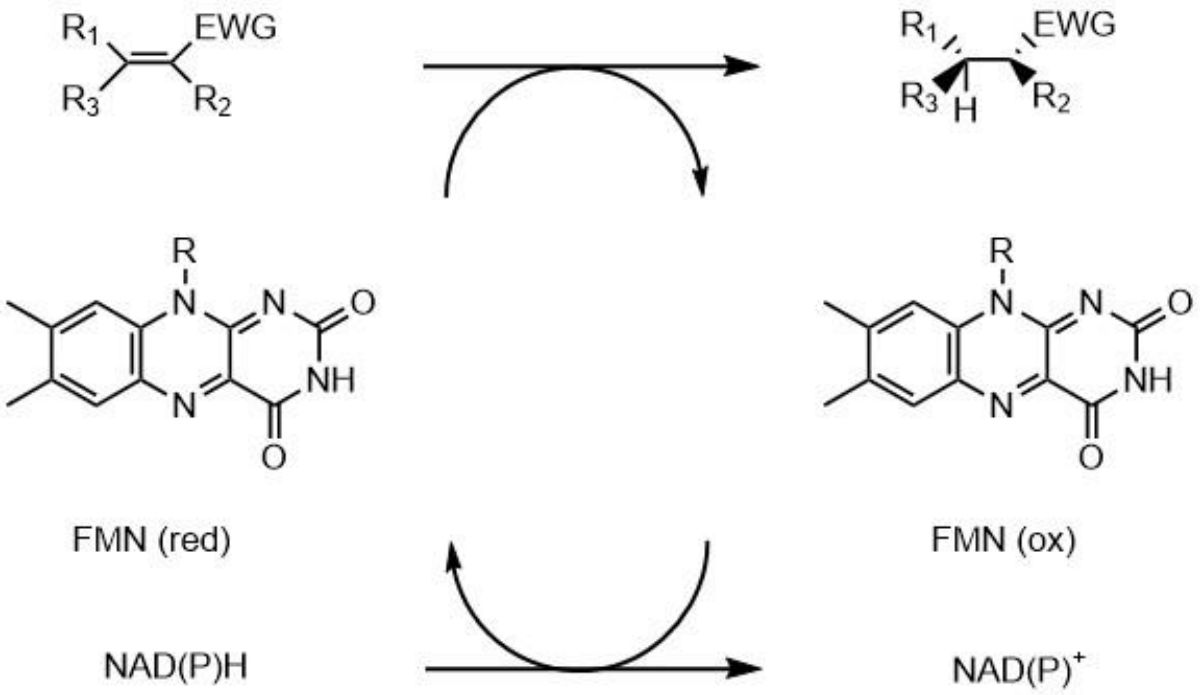

Fig 3: General reaction mechanism YqjM: The general mechanism of the ene-reductase YqjM is shown. The activated α,β- unsaturated substrate is reduced at the expense of FMN. In order to regenerate the reduced FMN from the oxidized version, NADPH is necessary. EWG: electron withdrawing group. Reaction scheme according to Durschein *et al*.[47]

The reaction catalyzed by YqjM resembles an asymmetric conjugate, Michael-type addition of a chiral hydride onto an enone. Therefore, non-activated C=C bonds are not able to be reduced.[48]

The phenomenon of uncoupling has been described previously for flavo enzymes in the extent of 5-10 % of the overall NAD(P)H consumption.[49] Pesic *et al*. reported this phenomenon for the ene-reductase YqjM as well. The reduced FMN cofactor can transfer the hydride either onto the ene-substrate or to molecular oxygen (see Fig 4). The latter reaction leads to the formation of hydrogen peroxide.[50]

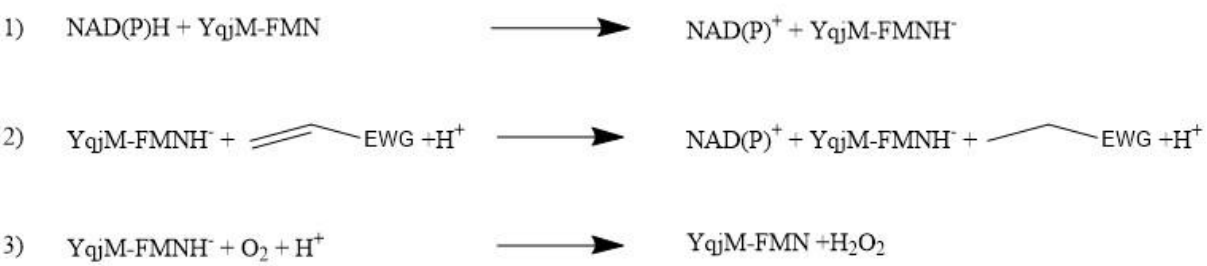

Fig 4: Uncoupling reaction of YqjM: 1) shows the reductive half reaction, where NAD(P)H is used to reduce the flavin cofactor. In the oxidative half of the reaction, the ene-substrate can either be reduced (2)) or in the presence of O_2 the enzyme can transfer a hydride and form hydrogen peroxide. Scheme adapted from Pesic *et al*.[50]

In an attempt to broaden the substrate scope and control the stereoselectivity of YqjM, Bougioukou *et al*. performed an iterative saturation mutagenesis on 20

residues. The model reaction which was used, was the biotransformation of 3-methylcyclohexenone to 3-methylcyclohexanone. They were able to show that two amino acid exchanges at C26G/A60V would lead to a shift in stereoselectivity towards the *S*-enantiomer with an approx. conversion of 80 %.[51]

Additionally, Nett *et al*. have created a novel engineering strategy, which can be applied to proteins from the same family. By the transference of previously altered residues, similar improvements can be seen when these are subsequently altered in family members. Working on this premise, they developed a scaffolding sampling strategy for the improvement of enzymes for biocatalysis.[52]

1.5.2 CHMO

Additionally to the biotransformation using YqjM for the reduction of activated double bonds, a part of this study also deals with the further oxidation via the cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* NCIMB 9871 to create a cascade reaction (Fig 5).

CHMO is naturally used by *A. calcoaceticus* as one of the steps to allow the bacterium to metabolise cyclohexanol and gain central metabolites from it, such as Acetyl CoA and 4-oxopentanoic acid.[53] The enzyme contains a flavin adenine dinucleotide cofactor (FAD) and is able to perform an asymmetric Baeyer-Villiger oxidation reaction (BVO).[54]

2 Aim

The aim of this study was to (a) compare two expression systems (genomic integration vs. self-replicative plasmid) in order to increase the reaction rate for the conversion of 2-methylmaleimid using the NADPH-dependent ene-reductase YqjM. For comparability of the systems, a promoter exchange was also done for the self-replicative plasmid, exchanging the *T5* promoter with the *cpc* promoter. An electroporation protocol should also be established to allow for a quick transformation of *Synechocystis* wild type cells.

(b) To determine the capability of *Synechocystis* to perform cascade reactions (Fig 5) by expressing two heterologous genes.

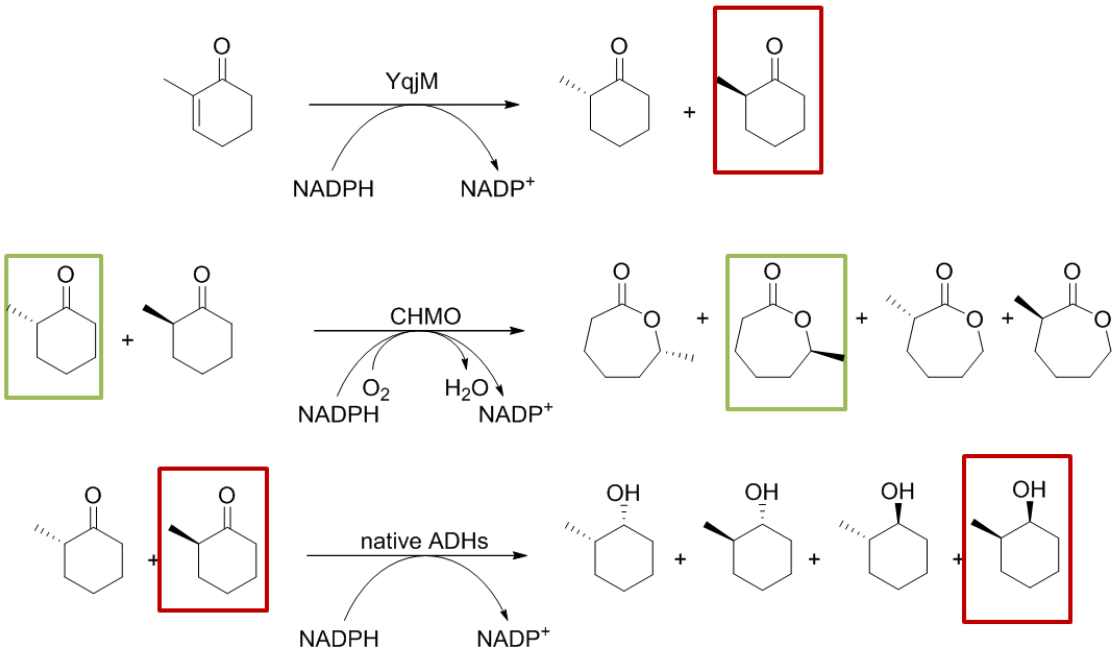

Fig 5: Cascade reaction: In the cascade reaction 2-Methylcyclohexenone is firstly reduced by YqjM to the ketone 2-Methylcyclohexanon, where the R-stereoisomer is preferred (indicated by the red box). In the second part of the cascade reaction the 2-Methylcyclohexanone is further reduced to its corresponding lactone by CHMO. The monooxygenase however preferably converts the *S*-steroisomer of the ketone (indicated by the green box). Due to native alcohol dehydrogenases (ADHs) the ketone is further reduced to its corresponding alcohol. Although the ADHs convert both stereoisomers, the *R*-ketone is preferred.

To study this, different plasmids were cloned to express *CHMO* and *YqjM* via a genomic double integration under the control of different promoters and integration at different neutral sites.

(c) To create an (*S*)-selective YqjM mutant, to increase the conversion of 2-methylcylohexanone to ε-caprolactone by CHMO.

3 Materials

3.1 Chemicals

All chemicals used in this study are listed in Appendix 9.1.

3.2 Strains

All strains used in this study are listed in Table 2. The transformation of *Synechocytis* with the suicide vectors SynRekB_cpc_YqjM and SynRekB_psbA2_YqjM to was previously done by Hanna Büchsenschütz.

Table 2: Strains: All strains used in this study are listed below. The purpose, as well as the description and origin is stated.

Strain	Purpose in this study	Description
E.coli Top 10	used for the transformation and amplification of cloning constructs	F- mcrA Δ(mrr-hsdRMS-mcrBC) ϕ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK λ- rpsL(StrR) endA1 nupG
Synechocystis sp. PCC 6803 wild type	Used for transformation (electroporation & natural transformation) with plasmids	geographical origin in California (USA) [1]; received from Paula Tamagnini (University of Porto)
Synechocystis sp. PCC 6803 cpc_YqjM	used to study whole-cell biotransformations with the ene-reductase YqjM	Integration of cpc_YqjM and a kanamycin resistance at the gene locus *slr0168*..
Synechocystis sp. PCC 6803 psbA2_YqjM	used to study whole-cell biotransformations with the ene-reductase YqjM	Integration of psbA2_YqjM and a kanamycin resistance at the gene locus *slr0168*..
Synechocystis sp. PCC 6803 pAWG_T5_YqjM	used to study whole-cell biotransformations with the ene-reductase YqjM	*Synechocystis* containing the self-replicative plasmid pAWG_T5_YqjM. Chloramphenicol resistance. Obtained from Marc Nowaczyk (Ruhr University Bochum, Germany)

3.3 **Primers**

The primers used in this study are listed in Table 3 with their name, purpose and sequence.

Table 3: Primers: The primers which were used during this study are listed below. The name, purpose and the 5' – 3' sequence is stated.

Name	**Purpose**	**Sequence (5'-3')**
Primer 71	QuikChange™ YqjM C26G	GTCATGTCGCCAATGGGCATGTATTCTTC
Primer 72	QuikChange™ YqjM C26G; cPCR YqjM constructs for double integration	CATGAGAAGAATACATGCCCATTGGCGAC
Primer 73	QuikChange™ YqjM A60V	GGACTGATTATTGTAGAGGTGTCAGCGG
Primer 74	QuikChange™ YqjM A60V	GTTAACCGCTGACACCTCTACAATAATC
P1_pSN15_AB_linear	Linearising pSN15K for pSN15C	GCGGGACTCTGGGGTTCGCTAG
P2_pSN15_AB-linear	Linearising pSN15K for pSN15C	GCGAAACGATCCTCATCCTGTC
Seq_Promoter	Sequencing QuikChange™ YqjM	CTATTCAATACACCCCCTAAGCTAG
P3_pSN15C_Cm_fw	Amplification of Cm insert for pSN15C	CTAGCGAACCCCAGAGTCCCGCTTACGCCC CGCCCTGCCAC
P4_pSN15C_Cm_rv	Amplification of Cm insert for pSN15C	GACAGGATGAGGATCGTTTCGCAGATCACTA CCGGGCGTATTTTTTG
pSN15K_Kan	cPCR pSN15C,	GTTAAAAAGGATCGATCCTCTA
pSN15_AB-R	cPCR pSN15C; Sequencing pSN15C	GTAAACTGGATGGCTTTCTTG

CpcYqjM_fw	Amplification cpc_YqjM for pAWG_cpc_YqjM	GCGGATAACAATTATGATAGATTCGAAGAGTCCCTGAATATCAAAATGG
CpcYqjM_rv	Amplification cpc_YqjM for pAWG_cpc_YqjM	CATTATTTGTAGAGCTCGAGATCTGCTTACCAGCCTCTTTCGTATTG
pAWG_fw_revised	Revised primers for pAWG linearization for pAWG_cpc_YqjM	GCAGATCTCGAGCTCTACAAATAATGAATTCG
pAWG_rv_revised	Revised primers for pAWG linearization for pAWG_cpc_YqjM	GAATCTATCATAATTGTTATCCGCTCACAAAGC
pSEVA_fw	cPCR pAWG_cpc_YqjM	GATTTGTCCTACTCAGGAGAGC
cpc_CHMO_fw	Amplification insert cpc_CHMO for double integration	GCAATTGGCGGCCGCTTCTAGAGCCCATTAGCAAGGCAAATC
cpc_CHMO_rv	Insert amplification cpc_CHMO for pSN15C_cpc_CHMO	GGACTGCAGCGGCCGCACTAGTTAGGCATTGGCAGGTTGC
P2_pSN15K_linear_rv	cPCR and sequencing CHMO & YqjM constructs for double integration;	GCGAAACGATCCTCATCCTGTC

	Sequencing CHMO & YqjM constructs double integration	
pAWG_Seq_fw	cPCR pAWG_cpc_YqjM; Sequencing pAWG_cpc_YqjM; cPCR CHMO constructs for double integration	CATTAATGCAGCTGGCACGACAG
cpc_YqjM_pSN15C_rv	Insert amplification psbA2_YqjM & cpc_YqjM for double integration (into pSN15C)	GGACTGCAGCGGCCGCACTAGTTACCAGCCTCTTTCGTATTG
psbA2_YqjM_fw	Insert amplification psbA2_YqjM for double integration (pSN15C_psbA2_YqjM)	GCAATTGGCGGCCGCTTCTAGAGCTAGACAGAATCCTTGCCC
cpc_YqjM_pSN15C_fw	Insert amplification cpc_YqjM for pSN15C_cpc_YqjM	GCAATTGGCGGCCGCTTCTAGAGGAAGAGTCCCTGAATATCAAAATGG
pSN15C_lin_fw	Linearising pSN15C for double integration	CTAGTGCGGCCGCTGCAG

	plasmids	
pSN15C_lin_rv	Linearising pSN15C for double integration plasmids	CTCTAGAAGCGGCCGCCAATTG
Seq_N15_fw	Sequencing CHMO & YqjM constructs for double integration	GCCCCATCAATTTGTCG

3.4 **Plasmids**

The plasmids used in this study are listed in Table 4.

Table 4: Plasmids: The plasmids used in this study are listed below. The corresponding purpose, antibiotic resistance and reference is also stated.

Name	**Purpose**	**Antibiotic Resistance**	**Reference**
pSN15K	vector backbone for pSN15C	kanamycin	Paula Tamagnini (IBMC, Portugal)
pSEVA351	amplification of chloramphenicol resistance for pSN15C	chloramphenicol	Paula Tamagnini (IBMC, Portugal)
pAWG_T5_YqjM	vector backbone for pAWG_cpc_YqjM	chloramphenicol	Marc Nowaczyk (Ruhr University Bochum, Germany)
pSN15C	created for further cloning experiments, including the double integration of YqjM and CHMO	chloramphenicol	this study
pAWG_cpc_YqjM	created for biocatalysis study using *Synechocystis*	chloramphenicol	this study

SynRekB_cpc_YqjM	amplification of cpc_YqjM insert for double integration and used to perform the QuikChange™ reactions for the YqjM double mutant (C26G/A60V)	kanamycin	Hanna Büchsenschütz, unpublished
SynRekB_cpc_CHMO	Amplification of cpc_CHMO insert for double integration	kanamycin	Hanna Büchsenschütz, unpublished
SynRekB_psbA2_YqjM	Amplification of psbA2_YqjM insert for double integration	kanamycin	[4]
SynRekB_psbA2_CHMO	Amplification of psbA2_CHMO insert for double integration	kanamycin	[5]
pSN15C_cpc_YqjM	created for the double integration of YqjM and CHMO in *Synechocystis*	chloramphenicol	this study
pSN15C_psbA2_YqjM	created for the double integration of YqjM and CHMO in *Synechocystis*	chloramphenicol	this study
pSN15C_cpc_CHMO	created for the double integration of YqjM and CHMO in *Synechocystis*	chloramphenicol	this study
pSN15C_psbA2_CHMO	created for the double integration of YqjM and CHMO in *Synechocystis*	chloramphenicol	this study
SynRekB_cpc_YqjM_C26G/A60V	created to study stereoselectivity effects of YqjM	kanamycin	this study

3.5 **LB agar plates**

The agar plates were prepared by adding 20 g/L LB and 15 g/L agar.

3.6 **LB medium**

20 g/L LB and dest. water used to create LB medium.

3.7 **BG11 medium**

The BG11 medium consisted of 5 mM HEPES/NaOH pH 8, 0.245 mM $CaCl_2$ x 2 H_2O, 0.189 mM Na_2CO_3, 0.0027 mM EDTA, 0.057 mM ammonium ferric citrate, 17.6 mM $NaNO_3$, 0.304 mM $MgSo_4$, 0.175 mM K_2HPO_4 and 1x BG-FPC (3.46 mM H_3BO_3, 3.1 mM citric acid, 0.92 mM MnCl x 4 H_2O, 0.06 mM $FeCl_3$ x 6 H_2O, 0.16 mM Na_2MoO_4 x 2 H_2O, 0.03 mM $CuSO_4$ x 5 H_2O, 0.08 mM $ZnSO_4$ x 7 H_2O).

3.8 **BG11 agar plates**

800 mL of BG11 medium was mixed with 1 mM of sodium thiosulfate and 15 g/L agar to create BG11 agar plates.

4 Methods

4.1 **FastCloning**

FastCloning[55] was used as the cloning technique and primers were designed as described by Li *et al*. PCR mix and program are described in Table 5. The protocol was adjusted according to the annealing temperature (Tm) of the primers and the size of the desired PCR product. The annealing temperature was estimated with the online Tm Calculator provided by ThermoFisher Scientific. (thermofisher.com) The amplification time was set according to the manufacturer's recommendation with 1 kb/30 s. All primers used for cloning are listed in Table 3.

Table 5 General PCR scheme: Components for the PCR were carefully mixed. The program was adjusted dependent on melting temperature Tm and size of the desired product.

PCR Mix		**PCR Program**		
Component	**Volume (µL)**	**Temperature (°C)**	**Time (min)**	**Cycle number**
5x HF Phusion Buffer	20	98	0:30	1x
dNTPs (10 mM)	2	98	0:10	30x
Forward Primer (10 µM)	5	Tm	0:15	
Reverse Primer (10 µM)	5	72	1 kb/30 s	
Template (20 ng)	X	72	5:00	1x
Phusion® Polymerase	1	4	∞	1x
ddH_2O	100 - 33 - X			

For the cloning of pAWG_cpc_YqjM the general PCR scheme (Table 5) was adapted for the linearization of the pAWG backbone, as the Pfu polymerase and a gradient PCR was used (Table 6). According to the manufacturer, the elongation time for the Pfu polymerase was estimated with 0.5 kb/min.

Table 6: pAWG vector backbone linearization: The linearization of the pAWG vector backbone was carried out as a gradient PCR using the Pfu polymerase. The volume of each reaction was 50 µL. Six different annealing temperatures were set.

PCR Mix (total volume: 6x50 µL)		**PCR Program**		
Component	**Volume (µL) for 6x50 µL**	**Temperature (°C)**	**Time (min)**	**Cycle number**
10x Pfu Buffer	30	98	0:30	1x
dNTPs (10 mM)	6	98	0:10	30x
Forward Primer (pAWG_fw_revised, 10 µM)	15	Gradient: 62, 62.7, 64.7, 67.3, 69.2, 70	0:15	
Reverse Primer (pAWG_rv_revised, 10 µM)	15	72	17:00	
Template (pAWG_T5_YqjM; 26.9 ng/µL)	11.2	72	5:00	1x
Pfu Polymerase (Promega, USA)	3	4	∞	1x
ddH_2O	219.8			

The success of the PCRs was confirmed by gel electrophoresis (described in 4.4). Afterwards the PCR reactions were digested with 1 µL *Dpn*I for 2 ½ hours at 37 °C, followed by 20 min of inactivation at 80 °C. Vector and insert in a ratio of 1:5 were transformed with chemo-competent *E. coli* TOP 10 cells (described in 4.5). Colony PCR (described in 4.2) was used to screen for the correct assembly of the cloning construct. Correct cloning was validated by sequencing (Microsynth AG, Switzerland).

4.2 **Colony PCR (cPCR)**

Colony PCR (cPCR) was used to investigate the *E. coli* TOP 10 clones from the FastCloning. The colonies were picked with a sterile toothpick and resuspended in 30 µL ddH_2O. Using the same toothpick, the colony was streaked on a masterplate containing the corresponding antibiotic. The re-suspended colonies were cooked for 10 minutes at 95 °C. After centrifugation for 2 min at 15.493 g, 5 µL of the supernatant was used as template for the colony PCR (cPCR). The PCR protocol is described in Table 7. The annealing time was adjusted dependent on the length of

the desired PCR product. According to the manufacturer of the HS-Tag-Mix, the Taq polymerase amplify 1 kb/min.

Table 7: cPCR general protocol: The PCR mix was prepared accordingly and the melting temperature (Tm) was calculated for each primer pair which was used for cPCR using the Tm calculator from ThermoFisher Scientific. The annealing time (Z) was calculated according to the amplified fragment length, assuming an amplification time of 1 kb/min for Taq-based polymerases.

PCR Mix		**PCR Program**		
Component	**Volume (µL)**	**Temperature (°C)**	**Time (min)**	**Cycle number**
HS-Taq-Mix	7.5	95	1:00	1x
Forward Primer (5 µM)	1.25	95	0:15	30x
Reverse Primer (5 µM)	1.25	Tm	0:15	
Template	5	72	Z	
		72	5:00	1x
		4	∞	1x

4.3 **Mutagenesis using QuikChange™**

In order to achieve the double amino acid exchange, two consecutive QuikChange™ reactions were performed. The primers were designed to achieve a cysteine to glycine exchange at position 26 (C26G) of YqjM and an alanine to valine exchange at position 60 (A60V). The QuikChange™ reactions were performed according to Table 8. For the A60V exchange a gradient QuikChange™ was performed, consisting of 6 temperature gradients.

Table 8: QuikChange™ reaction scheme: For the double amino acid exchange two consecutive QuikChange™ reactions were performed. For the A60V mutation 6 gradient QuikChange™ reactions were conducted.

Reaction Mix (total volume for one reaction: 50 µL)		**Program**		
Component	**Volume (µL)**	**Temperature (°C)**	**Time (min)**	**Cycle number**
Template (20 ng)	X	98	0:30	1x
fw Primer (10 µM)	1.25	98	0:10	30x
rv Primer (10 µM)	1.25	A60V: 60, 60.8, 63.2, 66.3, 68.6 and 69.5 C26G: 70.3	3:40	
Phusion Polymerase (2 U/µL)	1	72	Y	
5x Phusion Buffer	10	72	5:00	1x
dNTPs (10 mM)	1			
DMSO (3 v/v%)	1.5	4	∞	1x
ddH_2O	to final volume (50 µL)			

The successful QuikChange™ amplification was checked by gel electrophoresis (described in 4.4). The reaction mix was digested with *Dpn*I as described in 4.1. 5 µL of the QuikChange™ reaction was transferred into *E. coli* Top 10 cells as described in 4.5. The plasmid isolation was performed using the Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, USA). The double-mutant YqjM was verified via sequencing (Microsynth AG, Switzerland).

4.4 **Agarose gel electrophoresis**

Agarose gel electrophoresis was run with a 1 % agarose gel. 0.8 g agarose was dissolved in 80 mL 1x TAE Buffer (40 mM TRIS, 20 mM glacial acetic acid, 1 mM EDTA) and 4 µL GelGreen was added after letting it cool down to 60 °C. The electrophoresis was carried out for 35 min at 120 V and 400 mA. Detection was done via UV light using the G:BOX F3 gel documentation system (Syngene, United Kingdom).

4.5 Transformation of *E. coli* cells

4.5.1 Transformation of chemo-competent *E. coli* Top 10 cells

50 µL aliquots of chemo-competent cells were stored at -80 °C. For transformation, the cells were thawed on ice. 1 µL of the linearized vector backbone and 4 µL of the insert suitable for FastCloning were mixed and incubated with the competent cells on ice for 30 min. In the meantime, suitable antibiotic LB agar plates and LB-Soc medium were warmed up to 37 °C. The cell mixture was heat shocked for 42 s at 42 °C and afterwards placed on ice for 2 min. 300 µL LB-Soc medium was added to the competent cells and the sample was incubated for 1 h at 37 °C. 200 µL was plated on the pre-warmed antibiotic LB agar plate. The plates were incubated at 37 °C over night.

The antibiotic used for the selection process is dependent from the resistance encoded on the plasmid (Table 4).

4.5.2 Transformation of electro-competent *E. coli* Top 10 cells

5 µL of plasmid DNA was used for the transformation of electro-competent *E. coli* Top 10 cells. The plasmid DNA was de-salted for 30 min using a filter membrane and ddH_2O. After thawing 50 µL of electro-competent *E. coli* Top 10 cells on ice. The DNA was mixed with the cells and placed in an electroporation cuvette (2 mm gap distance). Using the Bio-Rad MicroPulser electroporator (Bio-Rad, USA) the cells were electroporated with the pre-programmed “Ec2” protocol (2.5 kV). 1 mL LB-Soc medium was added immediately after electroporation and the cells were regenerated for 1 h at 37 °C. 200 µL of cells were plated on antibiotic LB agar plates. The antibiotic used for the selection process is dependent on the resistance given by the plasmid (Table 4). The plates were incubated over night at 37 °C.

4.6 Overnight culture (ONC) preparation for *E. coli* Top 10 cells

For the ONC 5 mL LB medium was used and the appropriate antibiotic was added (50 µg/mL Kan; 25 µg/mL Cm) (Table 4).

4.7 Plasmid isolation from *E. coli* Top 10 cells

The plasmid isolation was performed with the GenJET Plasmid Miniprep kit from Thermo Fisher Scientific (Waltham, USA).

4.8 DNA concentration measurements

The DNA concentration was determined by spectral photometer analysis (Nanodrop^{TM} 2000/2000c, Thermo Fisher Scientific).

4.9 Glycerol stocks

E. coli glycerol stocks were prepared by mixing equal parts of ONC with 60 % (w/w) glycerol. The glycerol stocks were stored at -80 °C.

Synechocystis glycerol stocks were prepared by centrifuging 2 mL of a culture (OD_{750}= ~2) for 3 min at 13 000 rpm at room temperature (RT). The cell pellet was re-suspended in 750 µL BG11 and 250 µL 60 % glycerol (w/w). 200 µL aliquots were stored at -80 °C.

4.10 Cultivation of *Synechocystis*

Cells were renewed from glycerol stocks every two month. They were either grown on BG11 agar plates or cultivated in BG11 medium at 30 °C with 50 % humiditiy and under a light intensity of 60-150 $\mu mol_{photons}/m^2s$ (Witeg SWGC Growth chamber SmartLab illumination, Germany). Antibiotic was added accordingly: 50 µg/mL chloramphenicol (Cm) or 50 µg/mL kanamycin (Kan) (Table 2).

4.11 Transformation of *Synechocystis*

4.11.1 Electroporation

The protocol for the electroporation was adapted from Hoschek in order to transform wild type *Synechocystis* with the pAWG_cpc_YqjM plasmid.[30]

Electro-competent cells (*Synechocystis* wild type) were produced by harvesting 50 mL of cells with an OD_{750} = 0.5 – 1 at 3180 x g for 10 min. The cell pellet was washed three times with 10 mL 1 mM HEPES buffer (pH 7.5) and then re-suspended in 1 mL of the same HEPES buffer which was used for the washing steps. Cells were stored at -80 °C in 5 % (v/v) DMSO.

60 µL competent cells were transformed with 0.5 - 1 µg of (salt-free) DNA. These were electroporated with 12.5 kV/cm for 5 ms using electroporation cuvettes (2 mm gap distance) and immediately transferred into 50 mL of BG11 medium. After 24 h of cultivation at standard conditions (regeneration period) the cells were harvested at 3180 g for 10 min and re-suspended in 100 µL BG11. The cell suspension was then plated on a nylon membrane filter (GE Healthcare,USA; 0.45 µm, 47 mm diameter)

which was placed on a BG11 agar plate containing 10 µg/mL Cm. The following adaptions were implemented:

- Protocol was repeated with freshly made electro-competent cells
- Longer regeneration period (~ 50 h)
- Longer regeneration period (~ 74 h) and higher antibiotic concentration for the BG11 agar plates (50 µg/mL Cm)
- 2 µg DNA was used for the electroporation. Regeneration period was performed in the dark by wrapping the flask in paper. Cells were plated on 25 µg/mL Cm BG11 plates.

4.11.2 Natural transformation

Natural transformation was performed for the double integration of *YqjM* and *CHMO* into *Synechocystis*. *Synechocystis* cpc_YqjM and *Synechocystis* psbA2_YqjM were used for the transformation with each pSN15C_psbA2_CHMO and pSN15C_cpc_CHMO.

5 µg of plasmid was reduced into a volume of ca. 10 µL. The plasmid was heated for 10 minutes at 95 °C with closed lid. *Synechocystis* wild type cells were grown to an OD_{750} = 0.5 – 1 and concentrated to an OD_{750} of 2-3. Then, 500 µL of cells were added to the plasmid and incubated in darkness for 5-6 h at 30 °C. The suspension was plated onto an antibiotic free BG11 agar plate on top of a nylon filter membrane (GE Healthcare,USA; 0.45 µm, 47 mm diameter). The cells were incubated on the antibiotic free plate for 24 h at 30 °C. The filter was then placed on an antibiotic plate and incubated at 30 °C until colonies appeared. For the natural transformation of the double integrative *Synechocystis* two approaches were tested.

- Antibiotic plates with Kan/Cm (50 µg/mL/10 µg/mL) after regeneration on antibiotic-free BG11 agar plates.
- Antibiotic plates with Cm (10 µg/mL) after regeneration on antibiotic-free BG11 agar plates. When colonies appeared the filter membrane was transferred to 25 µg/mL and further onto 50 µg/mL Cm BG11 agar plates.

4.12 Biotransformation with different *Synechocystis* strains

4.12.1 Biotransformation (5 mL scale)

The *Synechocystis* strains used for the biotransformation (Table 2) were cultivated in 150 mL BG11 according to 4.10 till they reached an OD_{750}= 1.5-2. 2 mL were used to inoculate a new culture while the rest of the culture was centrifuged for 20 min at

2880 g at RT. The cell pellet was re-suspended in 1 mL BG11 and an OD_{750} of 20 was adjusted. 20 mM 2-methylmaleimid in BG11 was prepared as substrate stock. The biotransformation was carried out with 10 mM substrate concentration and a cell density of OD_{750}= 10 in a total volume of 5 mL in 20 mL GC vials (27.5 x 57 mm). The vials were placed in a 30 °C incubator at 120 rpm with a self-built light reactor emitting approx. 150 $\mu mol_{photons}/m^2s$ (self-built, Magdalena Wesseley, Fig 6).

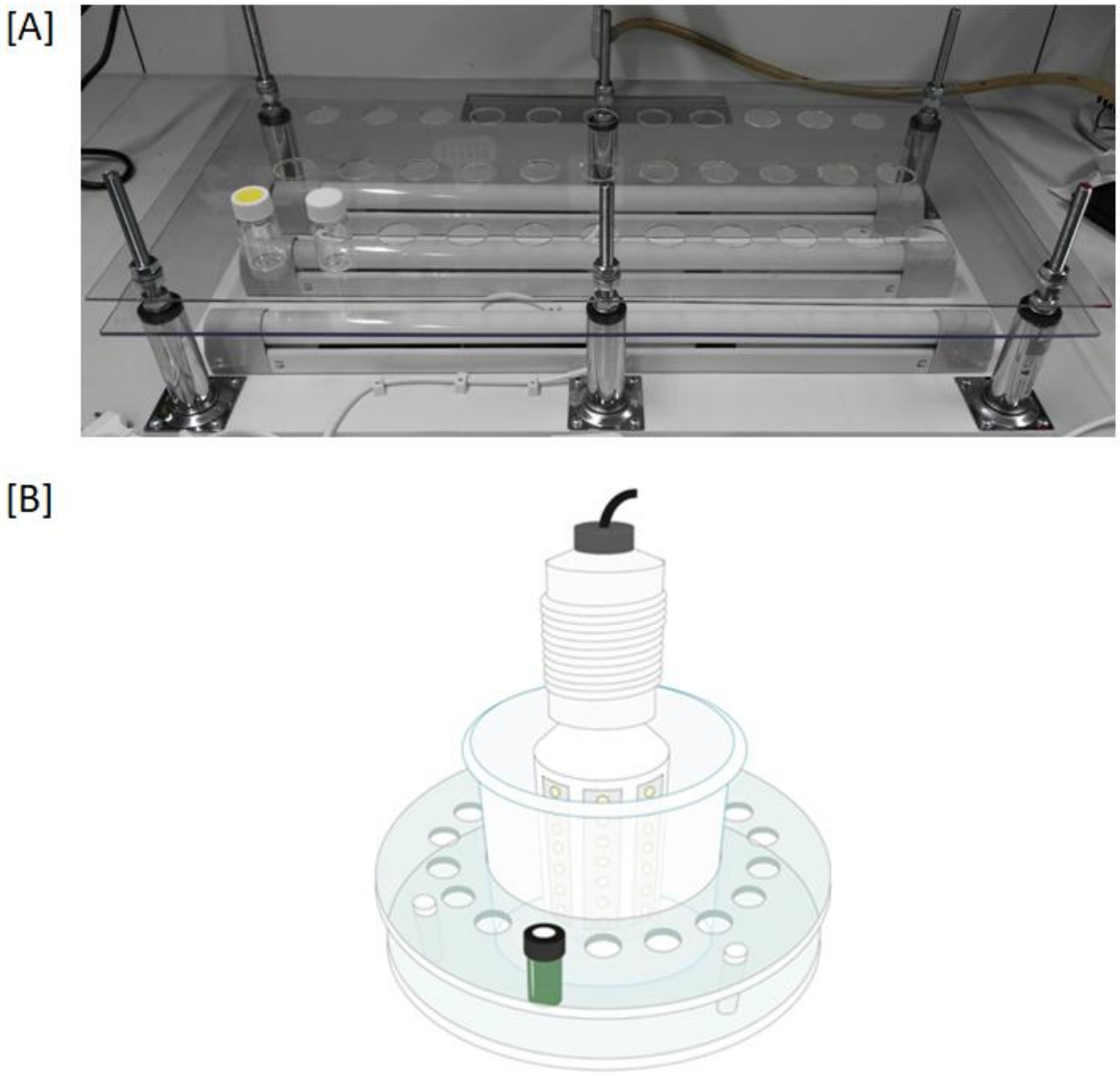

Fig 6: Light reactor: [A] shows the self-built light reactor by Magdalena Wessely, which was used for the 5 mL scale. [B] shows a schematic representation of the self-built light reactor which was used for the biotransformation experiments at 1 mL scale. Image was adapted from Katharina Köninger (Bochum, unpublished). Both emit light at approx. 150 $\mu mol_{photons}/m^2s$.

300 µL samples were taken at the time points 0, 10 min, 20 min, 40 min, 1 h, 3 h, 6 h and 24 h after reaction initiation. Samples were stored at -20 °C until they were extracted for GC analysis (see 4.13).

Biotransformations were performed in biological triplicates. Therefore, the *Synechocystis* strains were grown in three consecutive cultivations.

4.12.2 Biotransformation various ODs

In order to study the effects of varying cell densities on the biotransformation of 2-methylmaleimid the experimental set-up from 4.12.1 was adapted with different final OD_{750}= 5, 10, 15, 20. The experiment was run in a different light reactor emitting ~150 $\mu mol_{photons}/m^2s$ (self-built, Bochum, Fig 6) in a total volume of 1 mL in 1.5 mL GC vials (11.6 x 32 mm) at 30 °C. The cells were harvested at an OD_{750} of 0.5 to provide consistency to the NADPH decay assays from Bochum (Marc Nowaczyk *et al*., unpublished data). Mixing was ensured by magnetic stirrers. 200 µL samples were taken at the time points 0, 20 min, 40 min and 3 h after reaction start.

4.13 Analysis of biotransformation samples with gas chromatography (GC-FID)

The samples from the biotransformation were extracted 1:1 with ethylacetate containing 2 mM decanol as an internal standard. After proper mixing the organic phase with the sample, the emulsion was centrifuged for 2 min at 15493 g. The organic phase was transferred into a new Eppendorf tube containing a tip of a spatula $MgSO_4$ for drying. The suspension was vortexed and subsequently centrifuged for 5 min at 15493 g. 200 µL of the supernatant was transferred into a GC vial with inlet for analysis.

The samples were analyzed using a GC-FID (Shimadzu GC-2010 Plus) and a ZB-5 column (Zebron, film thickness: 0.25 µm, column length: 30 m, inner diameter: 0.32 mm). The calibration curve was done by Hanna Büchsenschütz. The method used for the detection of 2-methylmaleimid and the corresponding product 2-methylsuccinimid is shown in Table 9.

Table 9: GC-FID parameters: GC-FID (Shimadzu GC-2010 Plus) was used for the detection of the substrate 2-methylmaleimid and the product 2-methylsuccinimid. The parameters were set according to the table below. A ZB5 column (Zebron) was used.

Feature	Value
Injection volume	1 µL
Injection mode	Split
Temperature	230 °C
Carrier gas	N_2
Split ratio	20
Pressure	35 kPa
total flow	19.8 mL/min
Column flow	0.80 mL/min
Initial temperature column oven	100 °C
Temperature profile	100 °C (3 min hold time)
	30 °C/min rate
	310 °C (2 min hold time)
Total program time	12 min

4.14 Determination of the specific enzyme activity from GC analysis

In order to calculate the specific enzyme activity expressed in U/g_{CDW} the product concentration acquired from the GC analysis was plotted against the time. At the linear relationship between the two parameters a linear regression was calculated. From the slope (product concentration change per time) the units (µmol/min) were calculated. The correlation of dry cell weight and OD_{750} was experimentally determined by Viktorija Vidimce-Risteski (Formula 1).

Formula 1: Correlation cell dry weight per OD_{750}: experimentally determined by Viktorija Vidimce-Risteski

$$Cell\ dry\ weight\ (g)\ per\ OD_{750} = 0.24$$

This correlation was used to calculate the specific enzyme concentration in U/g cell dry weight (CDW).

5 Results

5.1 Comparison of a self-replicative plasmid system vs. genome integrative system for the biotransformation of 2-methylmaleimid using *Synechocystis*

For the biotransformation of 2-methylmaleimid to 2-methylsuccinimid two systems were compared: the genome integrative system and the self-replicative system. The self-replicative system consists of a pAWG plasmid and the ene-reductase YqjM under the control of a *T5* promoter. For the genome integrative system, YqjM was inserted into the gene locus *slr1068* via homologous recombination under the control of either the native *cpc* promoter or the native *psbA2* promoter. Köninger *et al*. tested seven substrates in total for the biotransformation in *Synechocystis* expressing the recombinant ene-reductase YqjM. In their study 2-methylmaleimid showed the highest specific activity (123 U/g_{CDW}).[4] For this reason 2-methylmaleimid was chosen as the standard substrate for the biotransformations this study. The product and substrate concentrations were determined by GC-FID. Fig 7 shows the verification of the distinct product and substrate peaks, as well as the internal standard peak (decanol) with the method described in 4.13.

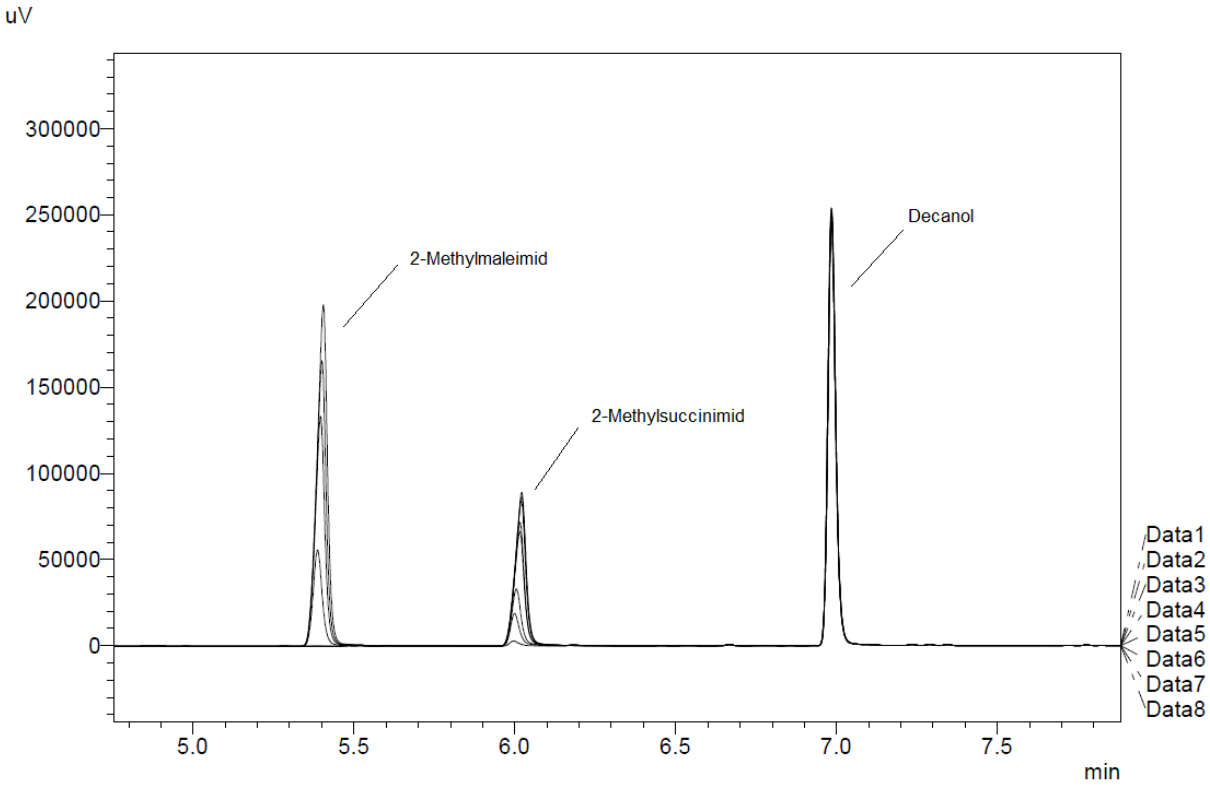

Fig 7: GC-FID substrate and product verification: The substrate (2-methylmaleimid) and product (2-methylsuccinimid) were able to be clearly distinguished using the method described in 4.13. Decanol was used as the internal standard and was found at a retention time of approx. 7.21 min. 2-methylmaleimid and 2-methylsuccinimid were found at approx. 5.64 min and 6.24 min respectively. Data 1-8 show the course of the biotransformation of 10 mM 2-methylmaleimid, for the time points 0, 10 min, 20 min, 40 min, 1 h, 3 h, 6 h and 24 h.

In Fig 8 the conversion over time ([A]) and the specific enzyme activity ([B]) are listed for the biotransformation of 2-methylmaleimid with all three expression systems. In biological triplicates this study was able to show that the conversion of 2-methylmaleimid with the ene-reductase YqjM was the slowest with the self-replicative pAWG_T5 plasmid system and the highest with the genome integrative *cpc* system (Fig 8 [A]). The *cpc* system reached full conversion after 1 h. The two remaining systems (*psbA2* and pAWG_T5), reached full conversion within 3 h. This trend was also reflected in the specific enzyme activity which was calculated using Formula 1. The genome integrative system showed an activity of 76.8 U/g_{CDW} and 65.5 U/g_{CDW} under the control of the *cpc* and *psbA2* promoter respectively. The self-replicative plasmid system reached an activity of 44.7 U/g_{CDW} (Fig 8 [B]). The data of both genomic integrative systems were coherent for the biological triplicates. However the self-replicative pAWG_T5 system showed a diverse enzyme activity for the biological triplicates. This is reflected in the large standard deviation of ± 14.3 U/g_{CDW}.

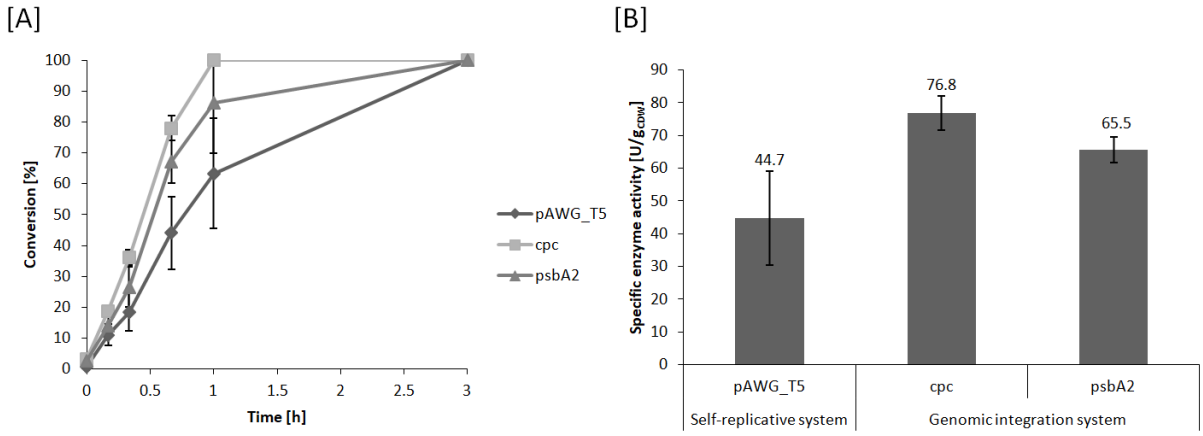

Fig 8: Comparison of different expression systems for the biotransformation of 2-methylmaleimid using *Synechocystis*: The biotransformation was carried out using 10 mM of 2-methylmaleimid as the substrate, cells with an OD_{750}: 10 were mixed 1:1 in a total volume of 5 mL. The reaction was carried out at a light intensity of ~ 150 $\mu mol_{photons}/m^2s$ and at 30 °C. Samples of 200 µL were taken at the start and 10 min, 20 min, 40 min, 1 h, 3 h, 6 h, 24 after the reaction was initiated. The samples were analyzed using a GC-FID and according the method described in 4.13. [A] shows the conversion in % of 2-Methylmaleimid into 2-Methylsuccinimid for the genomic integration with the *cpc* and *psbA2* promoter and the self-replicative pAWG_T5 plasmid. Full conversion was reached after 3 h for all constructs (data afterwards not shown). From the linear relationship between product formation and time the specific enzyme activity was calculated with the correlation of 0.24 g_{Cells}/OD_{750} ([B]). Standard deviation of three biological replicates is given by bars.

5.2 Influence of varying the cell concentration for the biotransformation of 2-methylmaleimid

Additionally, the effects of varying cell densities on the biotransformation of 2-methylmaleimid were investigated. Due to limited cultivation space, the reaction was performed in a total volume of 1 mL. Cell densities of OD_{750} of 5, 10, 15 and 20 which corresponds to 1.2, 2.4, 3.6 and 4.8 g_{CDW}/L, respectively, were investigated. The harvesting OD_{750} was lowered to 0.5 to ensure comparability between the NADPH decay assays performed by Nina Diczmons-Nowaczyk (Ruhr University Bochum, Germany) shown in Fig 15.

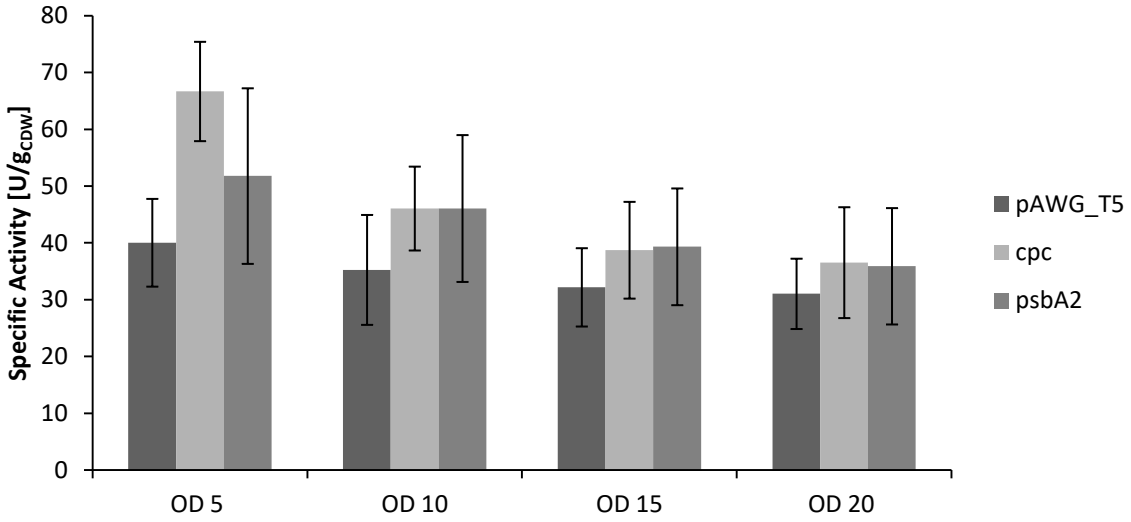

Fig 9: Biotransformation with various ODs: The biotransformation was repeated with varying cell densities (OD_{750}: 5, 10, 15 and 20) of *Synechocystis*. The experiment was conducted in a total volume of 1 mL using 10 mM of 2-methylmaleimid as a substrate. Samples of 200 µL were taken at the time points: 0, 20 min, 40 min and 3 h after addition of the substrate. The experiment was performed at a light intensity of ~ 150 $\mu mol_{photons}/m^2s$ and at 30 °C. No. of experiments: 3

All expression systems showed the highest specific activity for the smallest cell concentration (Fig 9; OD 5).The self-replicative pAWG_T5 system showed the lowest specific enzyme activity compared to both genome integrative systems for all investigated cell densities. The genome integrative system with the *cpc* and *psbA2* promoter showed similar activities for the expressed YqjM for OD 10, 15 and 20. For the smallest cell concentration the specific activity was the highest for the cpc system (Fig 9).

5.3 **Promoter exchange for the self-replicative pAWG plasmid system**

The data from Fig 8 showed that the cpc promoter leads to a higher specific enzyme activity compared to the self-replicative pAWG system. In order to fully compare these two systems, the T5 promoter was exchanged with the cpc promoter for the self-replicative pAWG plasmid. This exchange was done using the FastCloning method.[55]

For the linearization of the pAWG vector backbone the pAWG_T5_YqjM plasmid was used as template. A gradient PCR was performed to identify the best annealing temperature. 62 °C showed the best amplification result for the pAWG backbone linearization (Fig 10 [A]). The insert amplification of the cpc_YqjM was done with primers which were suitable for the FastCloning technique (Fig 10 [B]). In this technique, developed by Li *et al*. the primers are designed with a complementary

overhang to the linearized backbone. When transforming *E coli* Top 10 cells the nicks from the overhang is sealed by the bacterium, resulting in the closed circular plasmid.[55] After transformation with electro-competent *E. coli* Top 10 cells, a cPCR was performed. The linearized pAWG construct was used as a negative control for the cPCR and showed a band at 1.5 kb, whereas the pAWG_cpc_YqjM construct was expected to have a size of approx. 2 kb. The colonies 1, 4, 6, 13 and 16 displayed the correct fragment size (Fig 10 [C]). Sequencing confirmed the correct insertion of the *cpc* promoter for colony 1.

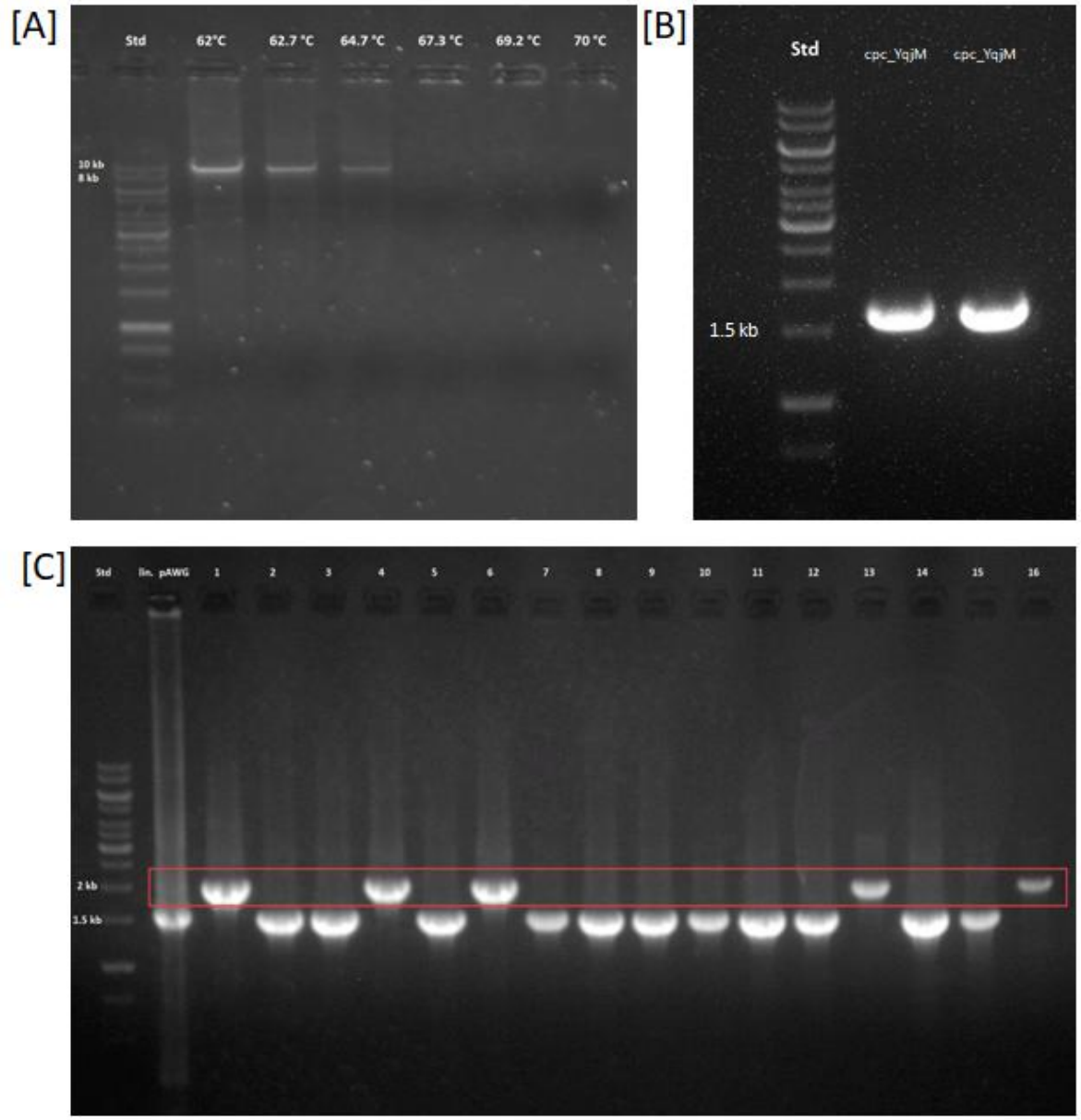

Fig 10: pAWG_cpc_YqjM FastCloning: [A] shows the gradient PCR which was performed for the linearization for the pAWG_T5_YqjM vector. The different annealing temperatures (62 °C, 62.7 °C, 64.7 °C, 67.3 °C, 69.2 °C and 70 °C) were compared. The linearized vector backbone was expected at around 8.7 kb. The fragment can be seen for the temperatures 62 °C, 62.7 °C and 64.7 °C. Std: GeneRuler 1kb. [B] shows the amplification of the insert cpc_YqjM. The fragment was expected at 1.6 kb. Std: GeneRuler 1kb ThermoScientific. [C] after pooling the three positive PCR reactions for the linearization of the pAWG plasmid ([A]), the vector was transformed with the amplified insert ([B]) into electrocompetent *E. coli* Top 10 cells. [C] shows the colony PCR which was performed after the transformation. The linearized pAWG backbone from [A] was used as a negative control. When the integration of the cpc promoter was successful the fragment was expected at around 2 kb. Colonies 1, 4, 6, 13 and 16 showed the correct size for the correct integration. Sequencing confirmed the successful pAWG_cpc_YqjM construct for colony 1 (Microsynth AG, Switherland). Std: GeneRuler 1kb ThermoScientific.

Wild type *Synechocystis* was then transformed with pAWG_cpc_YqjM via electroporation as mentioned in 4.11.1. However, no transformants were gathered. Various parameters were changed for the transformation of wild type *Synechocystis*. In a first attempt the regeneration period on antibiotic free BG11 plates was prolonged to approx. 50 h. In the second attempt the regeneration period was further prolonged to approx. 74 h with a subsequent transference of the cells onto a higher

antibiotic BG11 plate (50 µg/mL Cm). In the last attempt done in this study, a higher DNA concentration (2 µg) was transferred into electro-competent wild type *Synechocystis* cells, as well wrapping the flask for the regeneration in paper, to minimize light exposure. The cells were plated on antibiotic BG11 plates containing 25 µg/mL Cm. Despite all of these changes a successful transformation was not possible.

5.4 **Double integration of YqjM & CHMO into *Synechocystis***

In an attempt to optimize the cascade reaction, consisting of the ene-reductase YqjM and the monooxygenase CHMO (Fig 5), the two genes encoding for these enzymes should be integrated simultaneously into the *Synechocystis* genome. Therefore, one possibility is the integration of the genes into two different neutral sites. *Synechocystis* strains harbouring either cpc_YqjM or psbA2_YqjM at the neutral site *slr0168* were already available. The neutral site N15, discovered by Pinto *et al*, was chosen as second integration site for CHMO.[34] The plasmid allowing for the integration at N15 had to be adapted with a different antiobiotic cassette. This is necessary as two different genomic integration sites were chosen and hence two different antibiotic selection markers are necessary. Therefore, an exchange of the kanamycin resistance with a chloramphenicol resistance was performed, thus resulting in a pSN15C vector.

The pSN15C vector was created using the FastCloning method described by Li *et al*. [55] For the linearized vector backbone a pSN15K vector was used (Fig 11 [A]). The Cm resistance was obtained from the pSEVA351 plasmid. The insert was successfully amplified via PCR using primers, which were suited for the FastCloning technique (Fig 11 [B]). After transformation with electro-competent *E. coli* Top 10 cells, 16 colonies were picked which grew on Cm plates. A cPCR was conducted with these colonies, resulting in the successful integration of the Cm resistance into pSN15 in 7 colonies (Fig 11 [B]). Colonies 3, 6, 7 and 12 were sent to sequencing and all of these contained the correct Cm cassette.

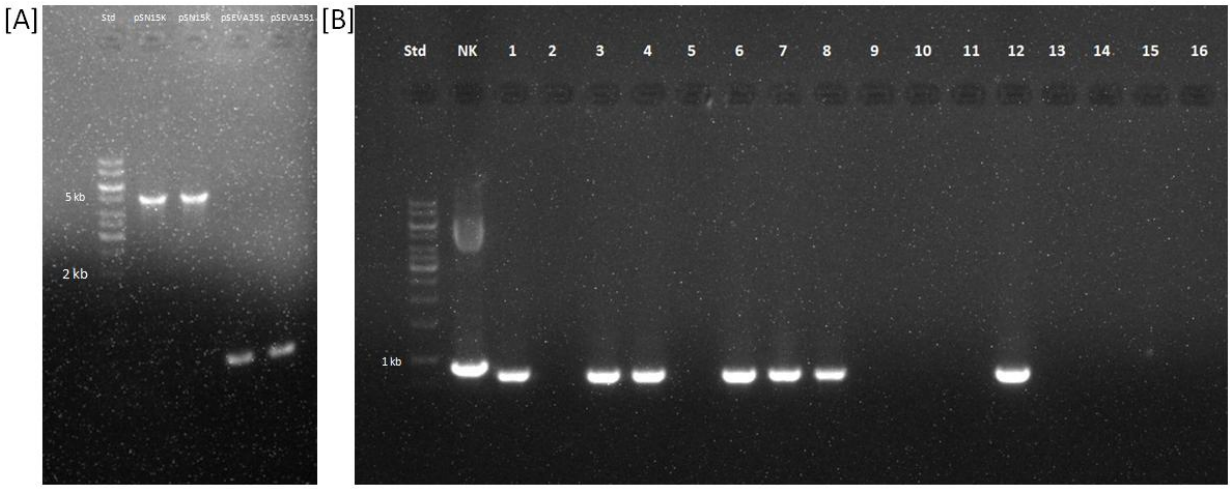

Fig 11: pSN15C Fast Cloning: [A] shows the amplification of the linearized vector backbone pSN15K without the kanamycin resistance (expected size at: 5 kb) and the chloramphenicol resistance amplification from pSEVA351 (expected size at: 720 bp). [B] shows the cPCR of 16 colonies which were picked after transformation into electro-competent *E. coli* Top 10 cells. NK: pSN15K. The expected band can be seen for colonies 1, 3, 4, 6, 7, 8 and 12. Std: GeneRuler 1kb (Thermo Fisher Scientific (Waltham, USA).

The pSN15C vector was then used to create various constructs for the double integration of *YqjM* and *CHMO* under the control of different promoters. Using the FastCloning method from Li *et al* pSN15C_cpc_CHMO and pSN15C_psbA2_CHMO were designed (Fig 12).[55]

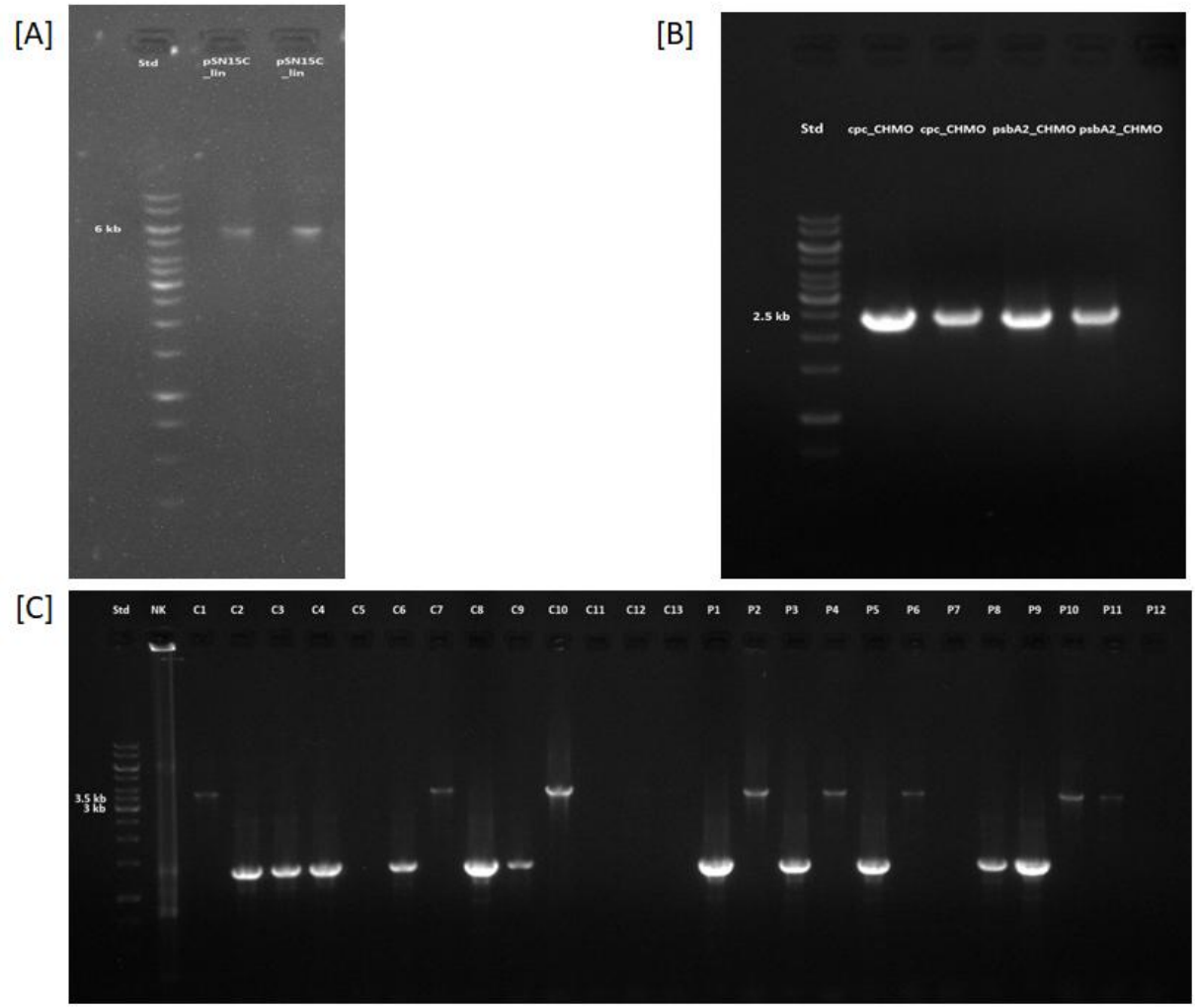

Fig 12 Cloning of pSN15C_cpc_CHMO & pSN15C_psbA2_CHMO: [A] shows the linearized vector pSN15C (expected size at around 6 kb). [B] shows the amplification of the cpc_CHMO and psbA2_CHMO insert with suitable primers for the FastCloning technique (expected size at around 2.5 kb). [C] shows the cPCR for pSN15C_cpc_CHMO (labelled C1 – C13) and pSN15C_psbA2_CHMO (P1 – P12). NK: negative control with pSN15C. For the pSN15C_cpc_CHMO construct clones C1, C7 and C10 displayed the expected band at around 3.5 kb. P2, P4, P6, P10 and P11 showed the corresponding band for the correct integration for the pSN15C_psbA2_CHMO construct. Std: GeneRuler 1kb (Thermo Fisher Scientific (Waltham, USA).

Sequencing confirmed the correct integration of the psbA2_CHMO construct into pSN15C for P2 and P10. The pSN15C_cpc_CHMO construct was confirmed for C7 and C10.

By transforming the *Synechocystis* strains cpc_YqjM and psbA2_YqjM with the newly created plasmids pSN15C_cpc_CHMO and pSN15C_psbA2_CHMO four double integrative strains were tried to be obtained: cpc_YqjM_psbA2_CHMO; cpc_YqjM_cpc_CHMO, psbA2_YqjM_cpc_CHMO and psbA2_YqjM_psbA2_YqjM (Fig 13).

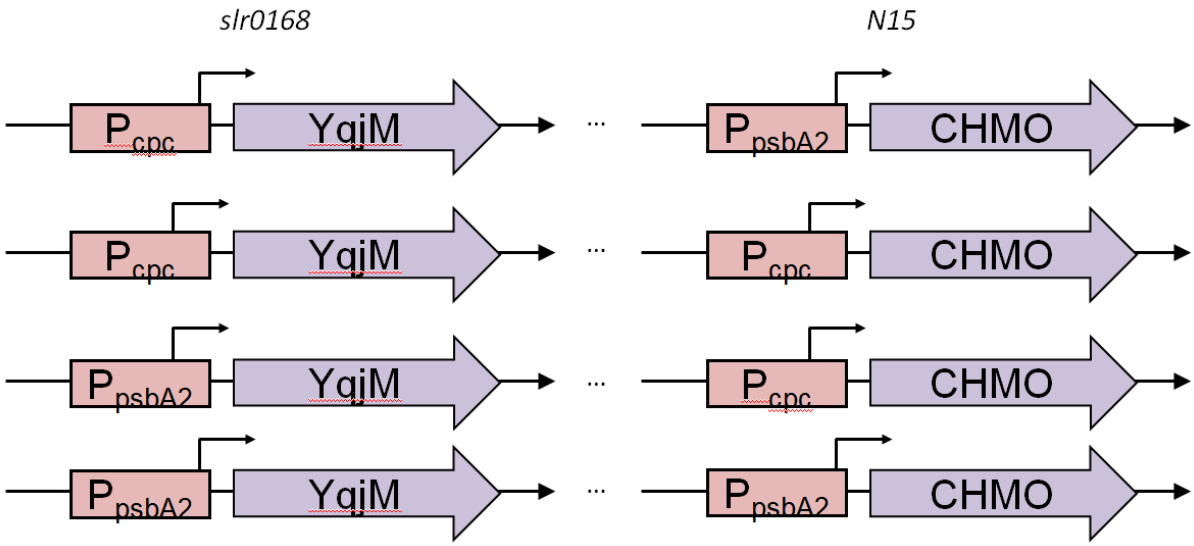

Fig 13: Double integration YqjM & CHMO: Schematic representation of the double integration of the ene-reductase YqjM and the monooxygenase CHMO. *YqjM* was either under the control of the *cpc* or *psbA2* promoter at the neutral site slr0168. CHMO was integrated via homologous recombination at the neutral site N15 under the control of either the *cpc* or the *psbA2* promoter. In total four constructs were obtained: cpc_YqjM_psbA2_CHMO, cpc_YqjM_cpc_CHMO, psbA2_YqjM_cpc_CHMO and psbA2_YqjM_psbA2_CHMO.

The transformation was carried out as mentioned in 4.11.2. However, no colonies were obtained. Various alterations to the transformation protocol were tested: light availability during the regeneration period, using double or single antibiotic treatment after the regeneration period and varying the concentration of the antibiotic after regeneration. None of these conditions resulted in the successful integration of the second cassette.

5.5 *S*-selective YqjM

For the cascade reaction (Fig 5) an *S*-selective YqjM would be beneficial to favour the conversion by CHMO to the corresponding lactone. Bougioukou *et al*. conducted a study involving the stereoselectivity of YqjM towards 3-methylcyclohexenone as a substrate.[51] According to this study, a double mutation at cysteine 26 to glycine and at alanine 60 to valine leads to a shift in stereoselectivity towards the *S*-enantiomer. Therefore, two consecutive QuikChange™ reactions were performed to create this double mutation. Sequencing confirmed that the exchange of the two amino acids was successful (Fig 14).

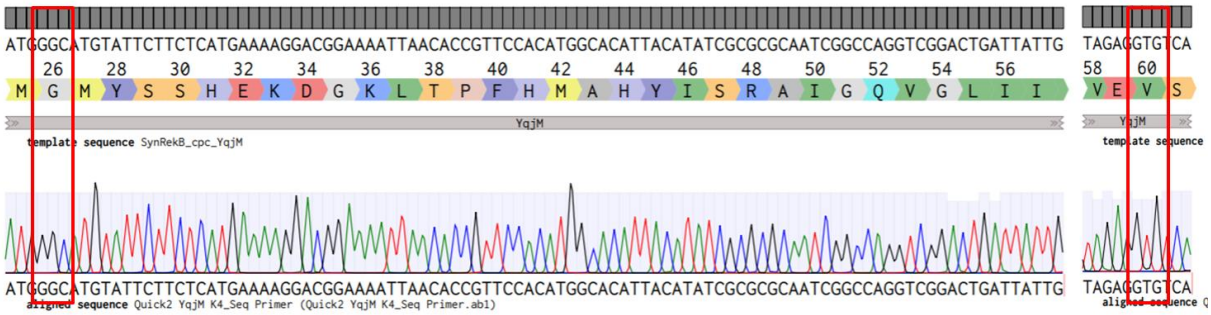

Fig 14 QuikChange™ YqjM: The two consecutive QuikChanges™ reactions for YqjM resulted in the exchange of cysteine to glycine at position 26 and from alanine to valine at position 60. Template sequence: SynRekB_cpc_YqjM (C26G/A60V). Aligned sequence: Quick2 YqjM K4_Seq Primer. This exchange was verified with Sanger sequencing (Microsynth AG, Switzerland).

Synechocystis wild type was transformed with the isolated plasmid. Biotransformations with the *S*-selective YqjM *Synechocystis* did not show any activity towards 2-methylcyclohexenone. However, in a control experiment with the achiral substrate cyclohexenone, activity was measured (biotransformations done by Hanna Büchsenschütz, data not shown).

6 Discussion

6.1 Comparison of a self-replicative plasmid system vs. genome integrative system for the biotransformation of 2-methylmaleimid using *Synechocystis*

In this study *Synechocystis* was used to explore its capability of performing biotransformations. The biggest advantage of this unicellular phototropic organism, is its ability to perform photosynthesis and therefore providing a large pool of NADPH, which can be redirected to biotransformations in need of said cofactor.[28] This was done by investigating different expression systems and their potential of increasing the specific activity of a homologous expressed gene. The gene used in this study encodes for the ene-reductase YqjM, which is able to reduce activated double bonds in a Michael-type fashion.[47,48] Köninger *et al*. have performed such a biotransformation with *Synechocystis* previously and showed that the fastest conversion (123 U/g_{CDW}) was reached with 2-methylmaleimid as a substrate for YqjM. In the study, YqjM was expressed under the control of the native *psbA2* promoter. The whole expression cassette was integrated into the neutral site *slr0168* of the genome.[4] Previous experiments have also indicated a relation between NADPH decay and the specific activity which was able to be reached by YqjM. The NADPH decay for *Synechocystis* expressing *YqjM* under the control of the cpc promoter was larger than the expressed gene under the control of the native *psbA2* promoter. This was reflected on the specific activity, where the cpc_YqjM construct showed a higher activity compared to the psbA2 construct. (unpublished data, Hanna Büchsenschütz; unpublished data, Nowaczyk *et al*.) Due to the polyploidy of *Synechocystis*, the genome integrative expression is a time intensive process as it takes time to allow for the full segregation of the strain.[8,10] Additionally, the system is limited to the amount of genome copies which are present inside the cell, as it integrates at a neutral site of the genome. In this study the self-replicative pAWG_T5_YqjM system was used, as it showed a faster NADPH decay when harboured by *Synechocystis* than the genome integrative systems mentioned previously (Fig 15, unpublished data, Nowaczyk *et al*.). In Fig 15 the NADPH decay assay for the genomic integration at *slr0168* with the *cpc* promoter (Fig 15 [A]) and the pAWG_T5_YqjM plasmid (Fig 15 [B]) can be seen. The black line corresponds to the intracellular NADPH concentration of the system without any substrate added.

The red line depicts the intracellular NADPH concentration once 1 mM of the substrate 2-methylmaleimid was provided. For the NADPH decay assay the light is switched off at the time point 0. From this point onwards *Synechocystis* does no longer produce NADPH via the oxidative photosynthesis.[28] It is notable that the steady state is higher without the substrate addition for the pAWG_T5 system, compared to the *cpc* system. Previous results from the comparison of the *cpc* and *psbA2* genomic integrative system, have suggested that there is a correspondence between NADPH decay and the recombinant enzyme activity. (Unpublished data, Nowaczyk et al.)

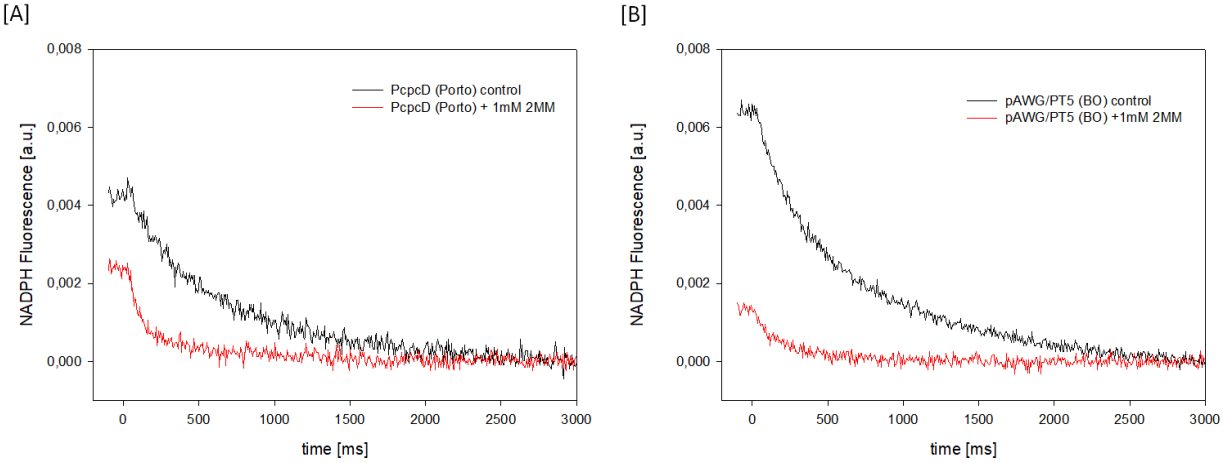

Fig 15: NADPH decay: The intracellular NADP level was measured from *Synechocystis* with genomically integrated cpc_YqjM [A] and Synechocystis containing the self-replicative pAWG_T5_YqjM system [B]. In order to measure the NADPH decay cells were exposed to actinic light. At the time point 0 the light was switched off and the decay of NADPH was tracked. The black line shows the control experiment with no substrate added. The red line depicts the addition of 1 mM 2-methylmaleimid. For this experiment cells were used at an OD_{750} = ~0.5. Unpublished data from Nowaczyk *et al*.

Working on this premise, it was expected that the pAWG_T5_YqjM system should show a faster conversion than the genome integrative systems. However, this could not be verified in this study (Fig 8). The fastest conversion was reached with the genome integrative system under the control of the *cpc* promoter, followed by the genome integrative system under the control of the *psbA2* promoter. The slowest conversion was reached by the self-replicative plasmid system (Fig 8 [A]). This also translated in the specific enzyme activity reached by the systems (Fig 8 [B]) where the activities of 76.8, 65.5 and 44.7 U/g_{CDW} were achieved respectively. It should be noted that the standard deviation of this experiment is quiet low for the genomic integrative system, however large for the self-replicative system. As these triplicates were produced over a time span of a couple of months, there could be issues with the stability of the plasmid, which results in the inconsistent data. The pAWG plasmid which was used is a larger broad-host plasmid with a RSF1010 replicon.[56] The

replicon has been known to work for *Synechocystis*, however the stability over a longer time span could have resulted in the fluctuating results.[35]

The specific activity of the ene-reductase was only calculated for the g_{CDW} but the amount of active protein inside the cells was not considered. To validate the two systems on the level of protein expression, the amount of mRNA of *YqjM* could be investigated with Northern Blot. Additionally, protein extractions could be done to also validate the protein concentration. This would provide a thorough understanding of the two expression systems and their respective capabilities. One method which is currently under development in our laboratory is the establishment of an in vitro method for the measurement of the active protein. For this method a cell lysate is prepared and the NADPH absorbance is measured once the co-factor and substrate is added. Furthermore, for a direct measurement of the protein concentration a mass spectrometer could be used.

To further improve the ability of pAWG to act as a self-replicative plasmid for *Synechocystis* some adjustments could be made. As some promoters like the T7 promoter are known to be strong promoters in *E. coli*, they can act weak for *Synechocystis*.[57] To elucidate wether the self-replicative pAWG plasmid system is advantageous for the production of proteins for biotechnological applications, an exchange to the native cpc promoter is valuable. This would ensure the comparability of the self-replicative system with the genomic integration system with independency in regards to the promoter.

Additionally, to further tune and enhance the expression of a heterologous gene RBS and terminators can be optimized. A recent study was conducted exploring a novel library of terminators and RBS for *Synechocystis*.[37]

6.2 Influence of varying the cell concentration for the biotransformation of 2-methylmaleimid

To determine the effects of the cell concentration on the biotransformation of 2-methylmaleimid, four concentrations (OD_{750}: 5, 10, 15 and 20) were chosen and the specific activity was calculated through the detection of the product with GC-FID. This experiment was set up slightly differently than the biotransformation mentioned previously (see 4.12.2). The harvesting OD_{750} was set lower, as well as the total reaction volume. The experiment showed that for all expression systems the specific activity increased with decreasing cell density. For the OD_{750}: 10, 15 and 20 the genomic integration system with the native *cpc* and *psbA2* showed close specific

activity. For OD_{750}: 5 the genomic *cpc* expression system showed the highest specific activity. This discrepancy between *cpc* and *psbA2* could be attributable to the different natures of the native promoters. Whereas the gene downstream of the *cpc* promoter encodes for the C-phycocyanin beta chain, which is part of the light adsorption complex, the *psbA2* promoter is downstream of a protein that encodes for the D1 subunit of PS 2.[11,15] The regulation mechanisms in terms of light intensity could vary for each system and therefore result in the divergence of the specific activity at lower cell densities.

The self-replicative pAWG_T5 system showed the lowest activity for all tested cell concentrations (Fig 9). With an increasing cell density the light availability is decreased, which also decreases the photosynthetic efficiency. The observed decrease of activity (Fig 9) with an increase of cell density can be explained as self-shading effect. This effect has also been described as problematic by Böhmer *et al*. in their study, in which they expressed CHMO as a recombinant enzyme in *Synechocystis*.[5] Jahn *et al*. have elucidated that the *Synechocystis* proteome is not optimized for the growth under low light conditions. The authors suggested that the proteome of Synechocystis is not adapted to prolonged depletion of light.[58] Therefore, a biotechnological setup with a high cell concentration is rather problematic. Duan and Shi describe the challenge of photo-limited zones in the centre, as well as photoinhibition at the peripheral zones as one of the main issues in the construction of photopbioreactors. Moreover the overheating via the light exposure is another main challenge. Photobioreactors have been designed to make algae and other phototrophic organisms such as cyanobacteria available for biotechnological applications. These include for example tubular, plastic bag and flat panel photobioreactors.[59]

6.3 **Promoter exchange for the self-replicative pAWG plasmid system**

To fully elucidate the effect of the self-replicative plasmid system vs. the genomic integrative system, the same expression cassette with the same promoter should be used to allow for comparability. This would ensure that the recombinant enzyme activity, which is achieved, is due to the system with independency to the promoter choice. To achieve this, the *T5* promoter was replaced with the native *cpc* promoter, which was used for the genomic integration (Fig 10). Unfortunately, the electroporation of the newly created plasmid was not successful; despite varying the

used parameters (see 4.11.1). The difficulties of the electroporation could stem from the size of the plasmid (roughly 9.7 kb). With its larger size, the plasmid was found to be difficult to work with, not only in terms of electroporation but also for sequencing and cloning. Additionally, the pAWG plasmid is not specifically designed for the expression in *Synechocystis* which could also contribute to the difficulties which were faced. For the integration of the pAWG_cpc_YqjM, conjugation with *E. coli* as a shuttle is another possibility. This method was also used by Nowaczyk *et al*. (unpublished) in order to transform wild type *Synechocystis* with pAWG_T5_YqjM and is therefore a promising alternative to the electroporation.

6.4 **Double integration of YqjM & CHMO into *Synechocystis***

In order to explore the capacity of *Synechocystis* to perform cascade reactions, the enzymes YqjM and CHMO were integrated into the genome via a double integration. The double integration should be performed at the neutral site *slr0168* for YqjM and *N15* for CHMO. The *slr0168* locus has been known as a neutral site and has been used for years as an integration site for heterologous genes.[3,32,33] *YqjM* was integrated into this locus under the control of either the *cpc* or *psbA2* promoter previously by Hanna Büchsenschütz. The *N15* locus was discovered by Pinto *et al*. and due to its low impact on the hosts growth was chosen as the neutral site for the integration of CHMO.[34]

To achieve this, the antibiotic resistance had to be exchanged on one plasmid, to ensure the selection pressure. For this, the Kan resistance of the plasmid pSN15K was exchanged to Cm (Fig 11). To further assess and improve the expression of the enzymes, CHMO was cloned under the control of either the native *cpc* or *psbA2* promoter (Fig 12). By transforming *Synechocystis* psbA2_YqjM or cpc_YqjM with the newly cloned plasmids, a double integration at the neutral sites *slr0168* and N*15* was tried to be achieved.[3,33,34] The transformation of the *Synechocystis* strains did not result in the formation of colonies albeit changing parameters of the transformation protocol (see 4.11.2). Two approaches were tested: the regeneration on either Kan/Cm (50/10 µg/mL) BG11 agar plates or just Cm plates (10 µg/mL). The second approach was done, as no colonies were formed on the double antibiotic plate. As the *Synechocystis* strains harbouring the YqjM are already fully segregated and stable, the focus of the selection pressure was put on the newly integrated CHMO and therefore the Cm resistance. Despite changing this parameter, no colonies were formed.

The natural transformation could be repeated with freshly thawed cells from a glycerol stock. In addition other transformation methods, such as electroporation or conjugation could be explored to ensure the double integration.

Once the transformation is successful, biotransformations with 2-methylcyclohexenone should be conducted to determine the efficiency of the double integrative expression system for the cascade reaction.

David *et al*. have also expressed three homologous enzymes in *Synechocystis* by the double homologous recombination into the neutral site *slr0168*.[60] This could also be an option which could be explored for the expression of YqjM and CHMO.

6.5 *S*-selective YqjM

In an attempt to change the stereoselectivity of YqjM towards the *S*-enantiomer of 2-methylcyclohexanone, two consecutive QuikChange™ reactions were performed. The *S*-selective YqjM mutant would be useful for the cascade reaction described in Fig 5 to favour the conversion of 2-methylcyclohexanone to its corresponding lactone. The mutations were based on a study conducted by Bougioukou *et al*., who did an iterative saturation mutagenesis on YqjM and 3-methylcyclohexenone as substrate.[51] After the successful exchange of the amino acids (Fig 14), no activity towards 2-methylcyclohexenone could be detected when performing whole cell biotransformations with *Synechocystis*. As the iterative saturation mutagenesis was conducted with 3-methylcyclohexenone this was not too surprising. In order to however rule out the possibility that the enzyme is not expressed, a control experiment was set up with the achiral cyclohexenone as the substrate. This biotransformation showed activity, indicating that the enzyme is expressed and active. As the cascade reaction in this study involved a different substrate than from the mutagenesis study performed by Bougioukou *et al*. The changes performed at position 26 and 60 seem to have influenced the ability of YqjM to convert 2-methylcyclohexenone. The amino acids which are usually found at these positions (Gly and Ala respectively) are thought to be involved in the tuning of the redox potential of the FMN cofactor, as well as redox sensing.[45,46] To change the stereoselectivity, the scaffolding sampling described by Nett *et al*. could also be implored. This strategy is based on the transferability of known engineered residues to proteins of the same family.[52] With this strategy active site mutations could be done to ensure conversion and stereoselectivity for 2-methylcyclohexenone.

7 Conclusion

In this study two expression systems (genomic integration vs. self-replicative plasmid) for *Synechocystis* were investigated to improve the reaction rate for the homologous ene-reductase YqjM. Due to the premise of a faster NADPH decay (Fig 15) the self-replicative pAWG_T5_YqjM plasmid was expected to show a higher specific activity. This hypothesis was however not proven (Fig 8). The genomic integration cpc_YqjM construct at *slr0168* showed the fastest conversion for 2-methylmaleimid (76.8 U/g_{CDW}). Köninger *et al*. were able to achieve an activity of 123 U/g_{CDW} for the exact same biotransformation with *Synechocystis* cpc_YqjM. [4] The reason for this discrepancy could not be elucidated.

Moreover, this study determined the effects of cell concentration on the biotransformation of 2-methylmaleimid. The results showed that, a smaller cell concentration lead to an increase of specific enzyme activity for all tested expression systems. For all tested cell concentrations the self-replicative pAWG_T5 system was the lowest in terms of activity. Both genomic integrative systems (*cpc* and *psbA2*) showed similar activities for OD_{750}: 10, 15 and 20. For OD_{750}: 5 the cpc_YqjM showed a higher activity compared to the psbA2_YqjM system (Fig 9).

For a better comparison of the two expression systems the T5 promoter was exchanged for the native *cpc* promoter for the pAWG self-replicative system (Fig 10). For the transformation of wt *Synechocystis* with the newly created pAWG_cpc_YqjM plasmid, an electroporation protocol (adapted from Hoschek *et al*.) was tried to be established.[6] The implementation of this protocol could not be achieved, which could be due to the size of the plasmid (~ 10 kb). Therefore, other transformation methods should be considered.

Furthermore plasmids were cloned to allow for the implementation of a cascade reaction (Fig 11 & Fig 12) via genomic double integration at the gene loci *slr0168* & *N15*. This reaction consists of the reduction of 2-methylcyclohexenone to 2-methylcyclohexanone via the previously mentioned YqjM, followed by a BVO via CHMO to ε-caprolactone (Fig 5). The transformation of *Synechocystis* cpc_YqjM and psbA2_YqjM via natural transformation was not successful. Other transformation methods could therefore be explored to attain the double integrative strains.

Lastly two QuikChange™ reactions were performed to obtain an *S*-selective YqjM mutant, which would favour the cascade reaction (Fig 5). The changes were based on a study on iterative saturation mutagenesis of YqjM conducted on 3-

methylcyclohexenone.[51] The mutations were verified via sequencing (Fig 14), however no activity was could be determined. In a control experiment with cyclohexenone the C26G/A60V YqjM mutant showed conversion of the substrate. This suggests that the performed changes might have led to a change of the active sit so that 2-methylcyclohexenone is no longer accepted. To allow for the conversion of this substrate, modelling could be applied to ensure for the desired stereoselectivity.

Overall, the potential of *Synechocystis* for biotechnological applications could be shown. The implementation of the herein mentioned self-replicative pAWG plasmid did not lead to an increase in the activity of the heterologous expressed YqjM. This indicates for the creation of suitable and stable vectors, which are suitable for *Synechocystis*.

8 References

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9 Appendix

9.1 Chemicals

Table 10: Chemicals: all chemicals used in this study are listed below, including name, company and CAS No.

Name	**Company**	**CAS No.**
10x Pfu Buffer	Promega, USA	-
2-Methylmaleimid	synthetised from Chiracon GmbH, Germany	
(R) 2-Methylsuccinimid	synthetised from Chiracon GmbH, Germany	
5x HF Phusion Buffer	Thermo Scientific, USA	-
Agar	Roth, Germany	9002-18-0
Agarose	Roth, Germany	9012-36-6
Ammonium ferric citrate	Roth, Germany	1185-57-5
$CaCl_2$ x 2 H_2O	Roth, Germany	10035-04-8
Chloramphenicol	Roth, Germany	56-75-7
Citric acid	Roth, Germany	77-92-9
$CuSO_4$ x 5 H_2O	Roth, Germany	7758-99-8
Decanol	Roth, Germany	112-30-1
DMSO	Sigma Aldrich, USA	67-68-5
dNTPs (100 mM each)	Thermo Scientific, USA	-
dpn1 (10 U/µL)	Thermo Scientific, USA	-
EDTA	Sigma Aldrich, USA	60-00-4
Ethylacetate	Honeywell, USA	141-78-6
$FeCl_3$ x 6 H_2O	Roth, Germany	10025-77-1
GelGreen (LabQ Green)	Lab Consulting, Austria	CAT: IDMG04
Glucose	Roth, Germany	50-99-7
Glycerol	Roth, Germany	56-81-5
H_3BO_3	Roth, Germany	10043-35-3
HEPES	Roth, Germany	7365-45-9

HS-Taq Mix	Biozym, Germany	CAT: 331126
KCl	Roth, Germany	7447-40-7
K_2HPO_4	Roth, Germany	7758-11-4
Kanamycin Monosulphate	Roth, Germany	25381-94-0
$MgCl_2$	Roth, Germany	7786-30-0
$MgSO_4$	Roth, Germany	7487-88-9
MnCl x 4 H_2O	Sigma-Aldrich, USA	13446-34-9
NaCl	Roth, Germany	7647-14-5
Na_2CO_3	Roth, Germany	7487-88-9
Na_2MoO_4 x 2 H_2O	Roth, Germany	10102-40-6
$NaNO_3$	Roth, Germany	7631-99-4
NaOH plateletts	Roth, Germany	1310-73-2
Pfu Polymerase	Promega	
Phusion Polymerase (2 U/µL)	New England Biolabs, USA	
Sodium thiosulfate	Roth, Germany	7772-98-7
TRIS	Roth, Germany	1185-53-1
Trypton	Roth, Germany	91079-40-2
Yeast extract	Roth, Germany	8013-01-2
$ZnSO_4$ x 7 H_2O	Roth, Germany	7447-20-0

9.2 Plasmid Maps

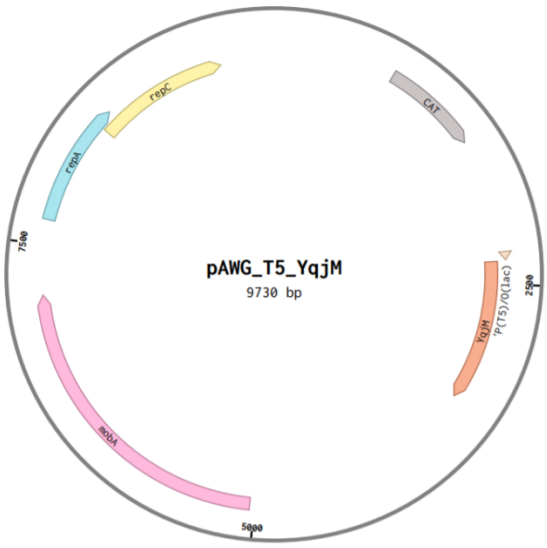

Fig 16: pAWG_T5_YqjM plasmid map

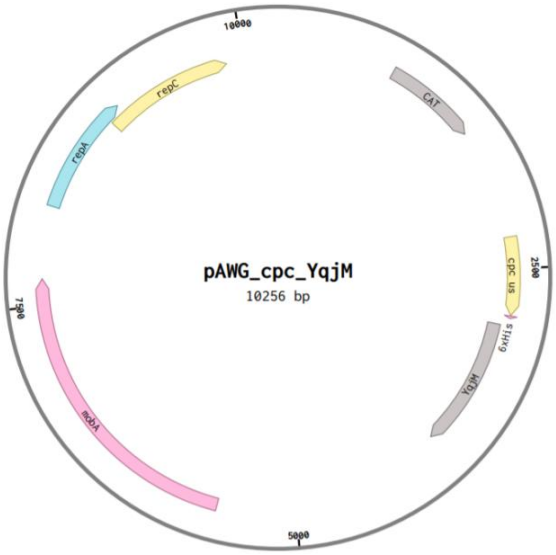

Fig 17: pAWG_cpc_YqjM plasmid map

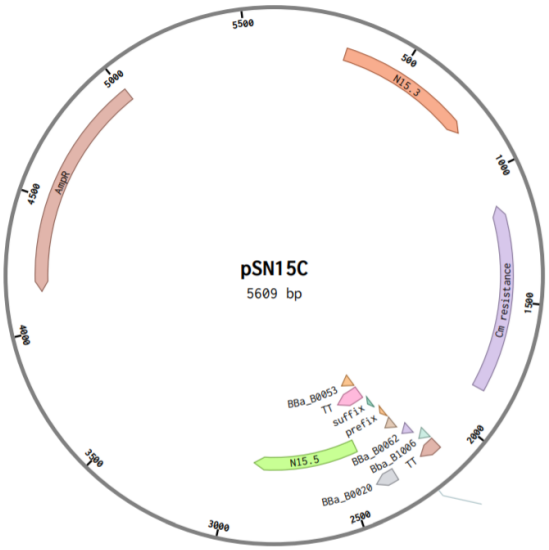

Fig 18: pSN15C plasmid map

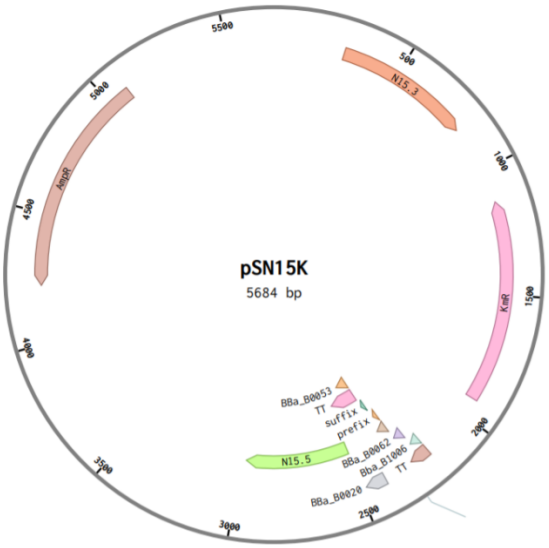

Fig 19: pSN15K plasmid map

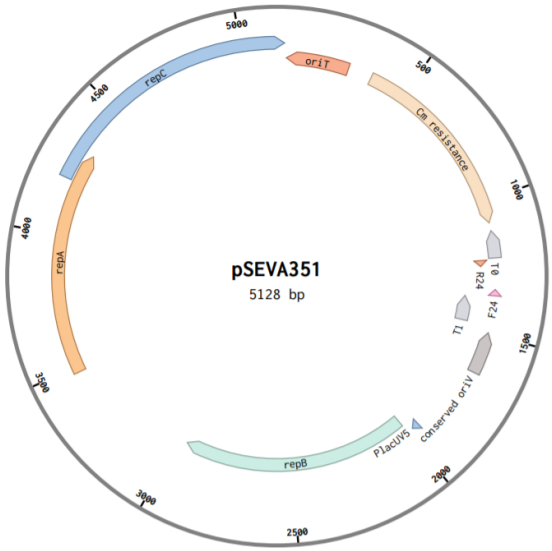

Fig 20: pSEVA351 plasmid map

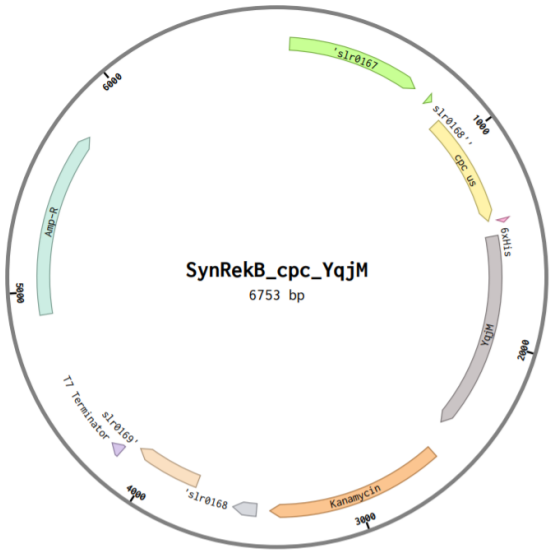

Fig 21: SynRekB_cpc_YqjM plasmid map

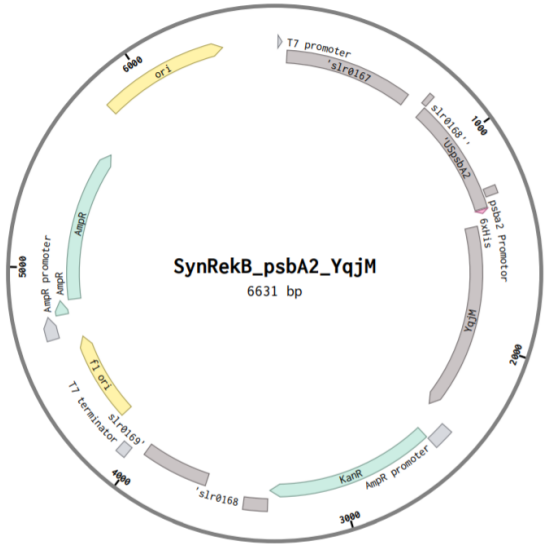

Fig 22: SynRekB_psbA2_YqjM plasmid map

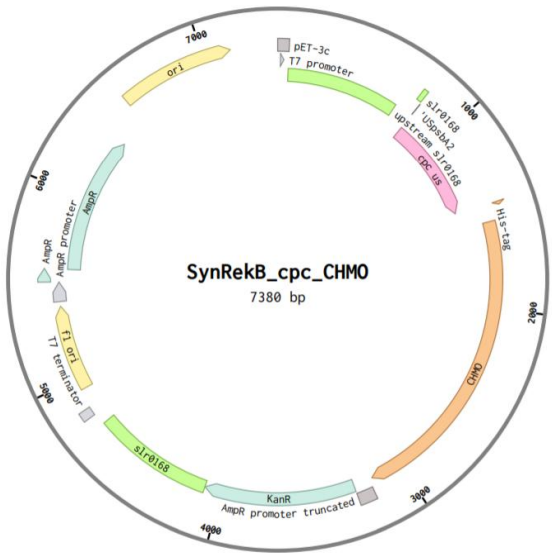

Fig 23: SynRekB_cpc_CHMO plasmid map

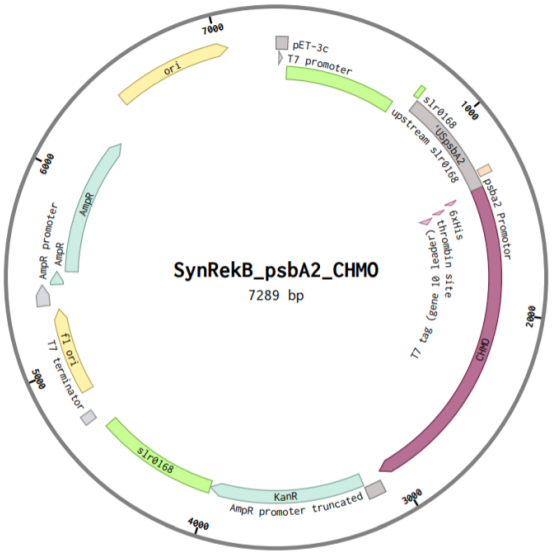

Fig 24: SynRekB_psbA2_CHMO plasmid map

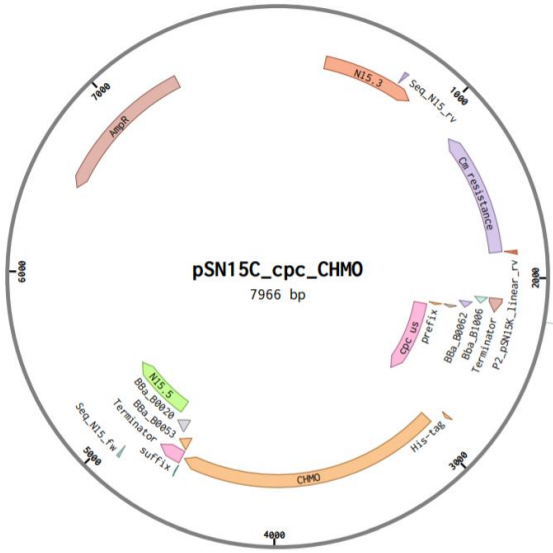

Fig 27: pSN15C_cpc_CHMO plasmid map

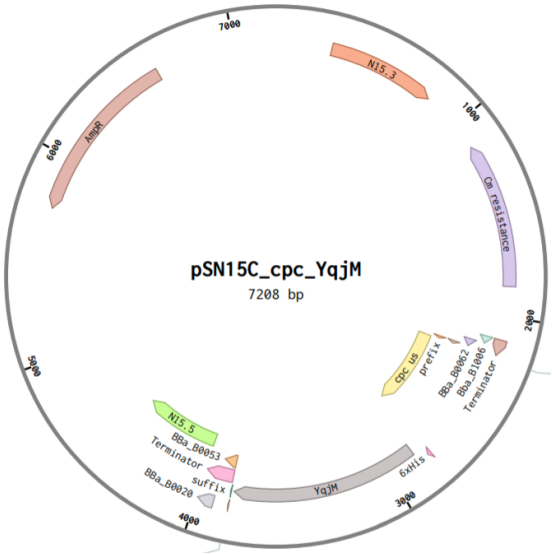

Fig 25: pSN15C_cpc_YqjM plasmid map

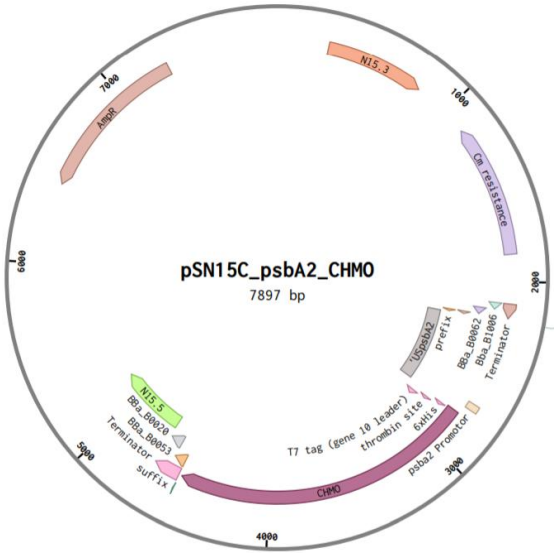

Fig 28: pSN15C_psbA2_CHMO plasmid map

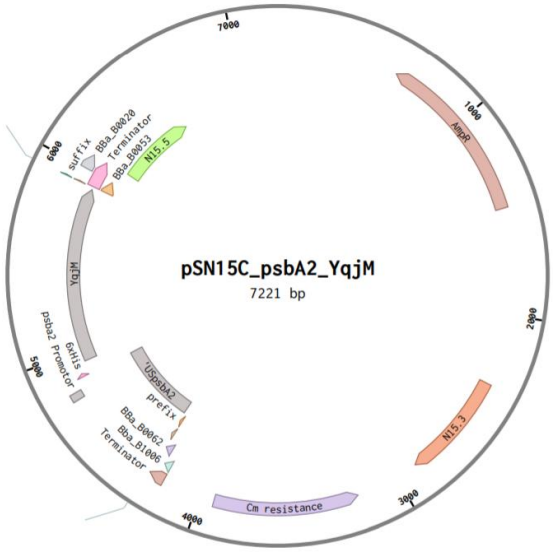

Fig 26: pSN15C_psbA2_YqjM plasmid map