Improve isoprene production in *Syechocystis* by downregulating native competitive pathways via RNA interference

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Abstract

The alarming numbers of dependency of fossil fuels are a big concern that affect the all society, increasing the carbon dioxide level in atmosphere and the greenhouse effect. The sustainability of population due international conflicts involving energy sources such as petrol and natural gas, push leaders around the world to invest in renewable energy sources. To counter these problematics, the production of biofuels by photosynthetic microorganisms is an interesting solution, that for one hand use the CO_2 present in the atmosphere as carbon source for growth, and for the other hand, produces biofuel and renewable chemicals decreasing the need of fossil fuels. The hemiterpene isoprene can be an excellent candidate as precursor for biofuel production or valuable chemicals due it structures and the possibility to be produced by photosynthetic bacteria. The model strains *Synechocystis* sp. PCC 6803 had been engineered to produce isoprene from CO_2 and light as carbon source and energy source, respectively. To improve the isoprene production in this cyanobacterium model, RNA interference tools were tested in the engineered strain ΔNS1::2MEP_IspS2 (Rana, Gomes et al. 2022). Through the Hfq-MicC complex, the *crtE* gene was targeted downregulating more than 20% of transcription and enhanced the isoprene production, the maximum yield obtained with this strain was 0.951 mg L^{-1} OD_{750} $^{-1}$ after 24 hours of inoculation.

Keywords: RNA interference, Cyanobacteria, Synechocystis, Isoprene

Resumo

A dependência dos combustíveis fósseis é uma problemática que afeta toda a sociedade, levando ao aumento dos níveis de dióxido de carbono bem como do efeito estufa. A sustentabilidade das populações, devido aos conflitos internacionais envolvendo fontes de energia como o petróleo e o gás natural, conduziu os líderes de todo mundo a investir em fontes de energias renováveis. Para inverter essa situação, a produção de biocombustível por microrganismos sintéticos é uma solução viável, que por um lado utiliza o CO_2 presente na atmosfera como fonte de carbono para o crescimento desses microrganismos, e por outro lado, produz biocombustível e químicos renováveis diminuindo a necessidade de recorrer à combustíveis fósseis. O hemiterpeno isopreno pode ser um excelente candidato como precursor para a produção de biocombustíveis e químicos de valor elevado, devido à sua estrutura e a possibilidade de ser produzido por bactérias fotossintéticas. A estirpe modelo, *Synechocystis* sp. PCC 6803 foi metabolicamente manipulada para produzir isopreno a partir de CO_2 e luz, como fontes de carbono e energia, respetivamente. De modo a elevar a produção de isopreno neste modelo biológico, ferramentas de interferência de RNA foram testadas na estirpe ΔNS1::2MEP_IspS2 (Rana, Gomes et al. 2022). Através do complexo Hfq-MicC, a tradução do gene *crtE* teve uma diminuição de 20% que levou a um aumento na produção de isopreno, o rendimento máximo obtido por esta estirpe atingiu os 0,951 mg L^{-1} OD_{750}^{-1} após 24 horas de incubação.

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List abbreviations

EU	European Union
Synechocystis	*Synechocystis* sp. PCC 6803
E. coli	*Escherichia coli*
DMAPP	Dimethylallyl pyrophosphate
IPP	Isopentenyl-pyrophosphate
MVA	Mevalonic acid pathway
MEP	2-deoxyxylulose 5-phosphate/2-methylerythritol 4-phosphate pathway
CO_2	Carbon dioxide
N_2	Nitrogen
RNAi	Interference RNA
asRNA	Antisense RNA
mRNA	Messenger RNA
dsRNA	Dual strand RNA
RBS	Ribosomal binding site
PTRNA	Paired termini antisense RNA
PCR	Polymerase chain reaction
FWD	Forward
REV	Reverse
crtE	Geranylgeranyl diphosphate synthase
IspS	Isoprene synthase
α*crtE*	Antisense *crtE*
α*IspS*	Antisense *IspS*
O/N	Over night
WT	Wild type
EVC	Empty vector contained
OD	Optical density
TAE	Tris-acetate-EDTA

Introduction

The dependency of fossil fuels, natural gas and crude oil presents a risk for society in several levels such as, climate changes, political conflicts, crisis, and wars. The greenhouse effect and global warming are a big problematic that are increasing over the years, the burning of fossil fuels was the major contributor for increase the carbon dioxide (CO_2) concentrations in the past (Wuebbles and Jain 2001). According to Martins et al. most of European countries consume, at least, 60% of fossil fuel in the total energy consumption and affirm that is a long way ahead to decrease the fossil fuel usage and shift to low carbon energy systems, but the renewable energy is the key to achieve it (Martins, Felgueiras et al. 2019).

The recent conflict between Russia and Ukraine warned the EU (European Union) and the world for the importance to bet in more renewable energy sources and to become more sustainable and independent. About 40% of Europe's gas, and 25% of EU crude oil import are supplied by Russia, and after the invasion European leaders conclude that now is a good opportunity to accelerate the transition to renewable energy (Hosseini 2022).

Occupying more than 60% of total demand for oil are the transport sector, 16% for the industrial sector, 5% for energy industry, 3% for services, 7% for residential and 4% for other purposes (Econometrics and Garden 2016). Fortunately, with the advances in battery technology, the development of electric vehicles and electrical energy devices have been accelerated. But for long transportations and aircraft, the solution remains in the development of biofuels (Liu, Cruz-Morales et al. 2021). However, the most popular biofuels production derived from food-crops and posteriorly fermentation by microorganism. This method come with limitations such as extract and separate target intermediates from bioenergy crops, high cost of enzymes used to produce fermentable sugars, and farmland limitations. Engineered microorganisms that can directly produce advanced biofuels from carbon dioxide and light emerge as solution for biofuels production (Keasling, Garcia Martin et al. 2021).

Terpenoids

It is known that terpenoids, also called isoprenoids, are one of the largest groups of natural compounds (Lin and Pakrasi 2019). The dimethylallyl-pyrophosphate (DMAPP)

and isopentenyl-pyrophosphate (IPP) are the 5-carbon building blocks that produces all natural terpenoids from two different pathways (Pattanaik and Lindberg 2015). These hydrocarbon molecules can be rearrangement into several structures from hemiterpenes (C5), monoterpenes (C1 0), sesquiterpenes (C15), diterpenes (20), triterpenes (C30), tetraterpenes (C40) to polyterpenes (>C40) (Lin and Pakrasi 2019). Isoprenoids are essential for all living organisms, in plants and photosynthetic organisms are vital, interfering with the photosynthetic reaction centers (Pattanaik and Lindberg 2015). They are also requisite in electron transport chain and in the synthesis and stability of membranes and cell wall (Englund 2016). At the industrial level, they can be attractive to the food, cosmetic, pharmaceutical and chemical industries (Rodrigues and Lindberg 2021). The table 1 summarize and demonstrate the range of terpenoids and their applications.

Table 1: Examples of terpenoids and their applications

Compound	Application	Industry	Reference
Artemisinin	Malaria treatment	Pharmaceutical	(Van Agtmael, Eggelte et al. 1999)
Paclitaxel	Anti-cancer agent	Pharmaceutical	(Slichenmyer and Von Hoff 1991)
Betulinic acid	Anti-HIV-1 activity	Pharmaceutical	(Fujioka, Kashiwada et al. 1994)
Valencene	Flavour and Fragrance (citrus)	Food	(Schempp, Drummond et al. 2017)
Stevio glycosides	Sweet	Food	(Schempp, Drummond et al. 2017)
Squalene	Cosmetic oil	Cosmetic	(Leavell, McPhee et al. 2016)
β-Farnesene	Surfactants, lubricants, and fuels	Cosmetic, Fine chemistry	(Leavell, McPhee et al. 2016)
Isoprene	Biofuel, feedstock	Chemistry	(Lindberg, Park et al. 2010)

Isoprene

One of the simplest terpenoids, isoprene is a volatile hydrocarbon (C_5H_8) produced by plants under some environmental conditions, for example to oppose heat stress (Lindberg, Park et al. 2010). Isoprene is becoming a desired compound for the production of biofuel due to its structure, diffusion, and the possibility of being produced in biotechnological ways (Rana, Gomes et al. 2022). Either in plants or microorganisms isoprene is made from the DMAPP by the isoprene synthase in two different pathways, the mevalonic acid (MVA) pathway and the 2-deoxyxylulose 5-phosphate/2-methylerythritol 4-phosphate (MEP) pathway (Sharkey and Yeh 2001). The production of isoprenoids in bacteria in more often through the MEP pathway with a higher efficiency in the carbon utilization and loss (Gruchattka, Hädicke et al. 2013). The scheme in figure 1 is a representation of the biosynthetic pathway for the production of isoprene and others terpenoids adapted from Cyanobacteria Biotechnology (Rodrigues and Lindberg 2021).

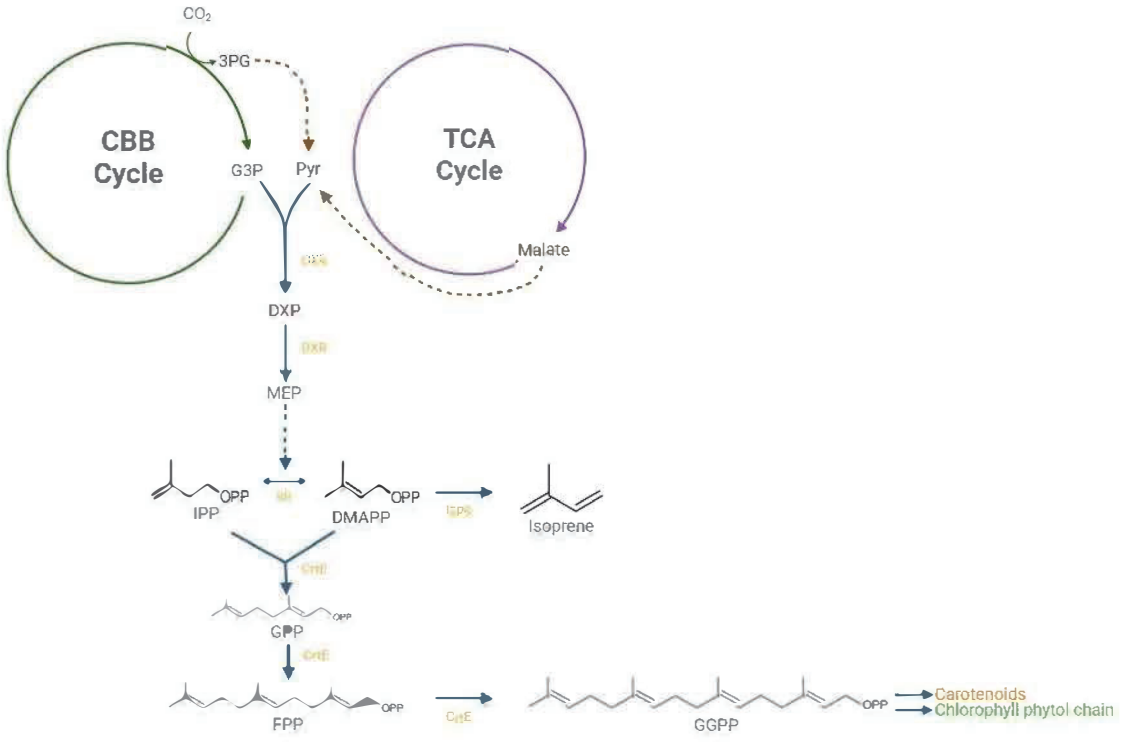

Figure 1: Biosynthetic pathway for production of isoprene and other terpenoids such as pigments, adapted from Cyanobacteria Biotechnology 2021

In 2010, Lindberg developed the production of isoprene in photosynthetic cyanobacterium *Synechocystis* by the heterologous expression of the *Pueria montana*

isoprene synthase gene (*IspS*) (Lindberg, Park et al. 2010). In a very similar work, Gao in 2016 engineered the cyanobacterium *Synechococcus elongatus* to produce isoprene by the redirection of the carbon flux from photosynthesis (Gao, Gao et al. 2016).

Cyanobacteria

Cyanobacteria are the most ancient prokaryotes in Earth's history and the only microorganism that has the capability to produce oxygenic photosynthesis (Knoot, Ungerer et al. 2018) (Hamilton, Bryant et al. 2016). They transformed the earth's atmosphere 2.3 billion years ago and evolved into what is now the chloroplasts in plants and algae (Tamagnini, Leitão et al. 2007). With the capability to inhabit most ecological niches, cyanobacteria are a versatile group that exhibits morphological differences among the unicellular, filamentous and colonial strains (Heidorn, Camsund et al. 2011). Cyanobacteria play a key role in ecosystem as primary producers (up to 25% of all primary productivity), using CO_2 as carbon source, sunlight and water as energy source and electron donor, respectively (Knoot, Ungerer et al. 2018). However, filamentous strains of cyanobacteria have the ability to fix not only CO_2 but also nitrogen ($N_{2)}$ under specific conditions, contributing to their relevance in biotechnology (Tamagnini, Leitão et al. 2007). These characteristics made them extremely attractive as hosts for the biotechnological industry.

The ability to convert CO_2 in desired chemicals by direct or indirect pathways (synthetic pathways), cyanobacteria provide advantages when compared to plants and algae. Cyanobacteria grows faster than higher plants and shows a higher efficiency in the conversion of solar energy into organic matter, 9% in cyanobacteria against 0.5-3% in plants (Hamilton, Bryant et al. 2016). The fact of cyanobacteria does not need to waste energy growing roots or stems like plants, they can direct more carbon to production of commodity compounds. When compared to algae, cyanobacteria can be more easily manipulated and genetically accessible than the eukaryotic algae (Englund 2016).

The unicellular bacterium *Synechocystis* sp. PCC 6803 is one of the most studied cyanobacteria and it is widely used as a potential chassis in production and as a model organism in research. This strain was isolated in 1968 from freshwater and had their entire genome sequenced in 1996 by Kaneko and co-workers (Kaneko, Sato et al. 1996). This event opened a novel range of molecular and genetic tools to elucidate some

functional genes present in the photosynthesis process and to manipulate and engineer them as cell factories for biofuel or feedstocks production (Yu, You et al. 2013).

The fact that many strains of cyanobacteria are amenable to natural transformation make the incorporation of exogenous DNA quite easy. Strains like *Synechocystis* can receive DNA from natural transformation, conjugation, and electroporation (Vavitsas, Kugler et al. 2021). Although many techniques developed for heterotrophic chassis such as *Escherichia coli* can be adapted to cyanobacteria, engineering those strains are not always straightforward and can become unstable (Al-Haj, Lui et al. 2016).

Metabolic engineering

The aim of any metabolic engineering is to enhance the production of a native compound that is residual or to redirect the cell metabolism to create new products by introducing synthetic enzymes and/or synthetic pathways. The introduction of these new pathways requires a certain level of regulation to track the production specially if the compound is toxic for the cell. Among the most commonly used promoters in cyanobacteria we have the P*psbA2*, P*nrsB* ,and P*cpcG560* that on one hand respond to external signals that the cell is used to, but on the other hand, are hard to predict and regulate (Ferreira, Pacheco et al. 2018). In a recent study that compared different promoters for biotechnological applications in *Synechocystis*, the author showed that the P*nrsB* was a safe choice regarding strength, versatility and regulation (Englund, Liang et al. 2016).

RNA interference

In other cases, metabolic engineering techniques also require the inactivation or downregulation of competitive genes for higher productivity. One way to archive these regulations without interfering with the cell genome (knocking out) is through the interference RNA (RNAi) mechanism (absent in bacteria). The expression of antisense RNAs (asRNA) seems appropriate and versatile for bacteria (Nakashima, Tamura et al. 2006).

The most developed technologies using asRNA, the regulation is mediated by the interaction of the asRNA to the target mRNA creating a double strand RNA (dsRNA). By blocking the ribosome binding site (RBS) and preventing the ligation to the ribosome, the dsRNA is degraded by RNases (Villa, Su et al. 2018).

In 2018, Sun et al. tested two different small RNA regulatory tools described before for *E. coli* in *Synechocystis*. They archived the downregulation in both techniques and re-direct the carbon flux to malonyl-CoA. The first mechanism was inspired by the paired termini antisense RNA (PTRNA), where the asRNA was inserted between the 38bp PT sequences and the second tool was based on the Hfq-MicC complex (Sun, Li et al. 2018) (Na, Yoo et al. 2013).

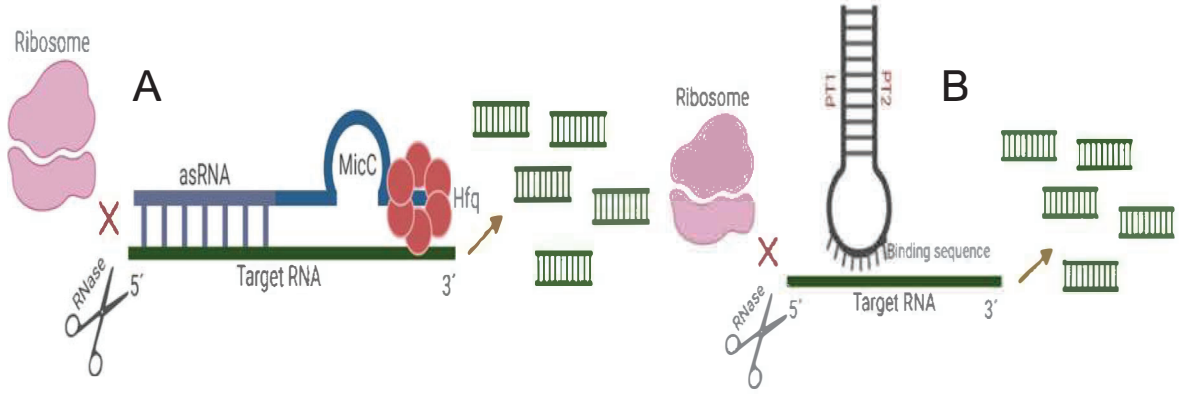

*Figure 2: Representation of RNA interference tools. **A** Schematic of the Hfq-MicC complex tool. An 24bp sequence binding to the target gene is design together with the 79bp scaffold MicC that recognize by the chaperone Hfq blocking the ribosome binding and promoting the degradation via RNase. B Schematic of the PTRNA tool. The 38bp PT1 and PT2 were added after and before an 100bp binding sequence contained the asRNA. After transcription the two paired terminus bind each other forming a hairpin, blocking the ribosome, and promoting the degradation via RNase.*

Aim

The aim of this thesis is to improve the production of isoprene in the cyanobacteria strain ΔNS1:: 2MEP_*IspS*2 (Rana, Gomes et al. 2022) by downregulating native competitive pathways via RNA interference. Using techniques described in Sun et al. 2018, vectors were created containing asRNA in two different mechanisms. The asRNA was designed to target the geranylgeranyl diphosphate synthase (*crtE*) gene in the MEP pathway and the isoprene synthase (*IspS*) gene as control. For one hand, targeting the *crtE* gene is expected to increase the isoprene production with more dimethylallyl-pyrophosphate available. For the other hand, targeting the *IspS* gene is expected the opposite effect in the production.

Methods and materials

Construction of plasmids for asRNAs expression in *Synechocystis* sp. PCC 6803

PCR Amplification

The construction of the plasmids started with the amplifications of the asRNAs. For the PTRNA constructs against the *IspS* gene, a 100bp asRNA was amplified using as template the pEERM3_2MEP_*IspS*2 vector. The forward (Fwd5) and reverse (Rev5) primers contained the PT1 and PT2 sequence, respectively. The same mechanism was used for the asRNAs against the *crtE* gene. It was used two different sequences as asRNA also with 100bp, α*crtE*1 (Fwd1 and Rev1) and α*crtE*2 (Fwd2 and Rev2), both amplified from gDNA. The P*trc20* (VF2 and Rev3) promoter and the P*nrsB* (Fwd4 and Rev4) promotor was amplified with the PT1 sequence in the beginning of the reverse primer. The P*trc20* was amplified from the pEEC+ (pEEC1 plasmid plus a toxin/anti-toxin system) plasmid and the P*nrsB* from the pEERM3+ plasmid.

Table 2: PCR reaction and conditions to amplify the PTRNA constructs.

PCR reaction	Final concentrations	PCR conditions (24 cycles)	Time
dH2O	Up to 50 µL	Initial denaturation (98°C)	30''
5X Phusion HF Buffer	10 µL	Denaturation	10''
10mM dNTPs	1 µL (0.2mM)	Annealing (54-64°C)	30''
Primer forward 10mM	2,5 µL	Extension (72°C)	30'' / Kbp
Primer reverse 10mM	2,5 µL	Final extension (72°C)	5'
Template DNA	5-6 ng (pDNA) / ~200 ng (gDNA)	Hold 12°C	∞
Phusion High Fidelity DNA Polymerase	0,02 U/µL (0,5 µL)		

All the amplifications were confirmed by electrophoresis (1% TAE agarose gel + 0.1% 1 0mM thiazole orange dye, 120V for 15'-20') and the PCR products were purified using the GeneJET PCR Purification Kit (Thermo Scientific).

To finish the construct of the PTRNAs, an overlap extension PCR was used to fuse the fragments contained the promoter + PT1 and the fragments contained the PT1 + asRNA

+ PT2. The protocol for overlap extension PCR consists in two steps. In the first step, both fragments are fused without primers occurring homologous recombination (6 cycles). In the second step, the primers (VF2 and Rev6) were added and the entire construct was amplified (30 cycles).

Table 3: Overlap extension PCR reaction and conditions.

Step I			**Step II**		
PCR reaction	Final concentration	PCR conditions (6 cycles)	PCR reaction	Final concentration	PCR conditions (25 cycles)
d H_2O	Up to 50 µL	Initial denaturation (98°C) for 30''	d H_2O	Up to 50 µL	Initial denaturation (98°C) for 30''
5X Phusion HF Buffer	10 µL	Denaturation (98°C) for 8''	5X Phusion HF Buffer	10 µL	Denaturation (98°C) for 8''
10mM dNTPs	1 µL (0.2mM)	Annealing 15''	10mM dNTPs	1 µL (0.2mM)	Annealing 15''
Template DNA 1 (larger fragment)	150 ng	Extension (72°C) 30''/Kbp	Primer forward 10mM	2,5 µL	Extension (72°C) 1'
Template DNA 2 (smaller fragment)	X ng (1:1 molar ratio)	Final extension (72°C) for5'	Primer reverse 10mM	2,5 µL	Final extension (72°C) for5'
Phusion HF DNA Polymerase	0,02 U/µL (0,5 µLl)	Hold 12°C (∞)	Template DNA (3 µL from step I)	3 µL from step I	Hold 12°C (∞)
			Phusion HF DNA Polymerase	0,02 U/µL (0,5 µLl)	

For the construction of the Hfq-MicC-asRNA, the constructs contained the P*trc* core promoter plus the small asRNA with 24bp against both genes, and the MicC sequence were ordered as gBlocks sequences.

Table 4: gBlocks sequences for the Hfq-MicC tool.

gBlock	Sequence
P*trc*_core_small_asRNA_crtE	ATCGAATTCGAGCTGTTGACAATTGTGAGCGCTCACAATATAATGTGTGGAAACTAGAAACCATACTGTCACTGGATACTAATTTCTGTTGGGCCATTGCATTGCCACTGATTTTCCAACATATAAAAAGACAAGCCCGAACAGTCGTCCGGGCTTTTTTTACTAGTTGC
P*trc*_core_small_asRNA_IspS	ATCGAATTCGAGCTGTTGACAATTGTGAGCGCTCACAATATAATGTGTGGAAACTAGAGAAGGAGGAAAAAAATGGACTACATTTCTGTTGGGCCATTGCATTGCCACTGATTTTCCAACATATAAAAAGACAAGCCCGAACAGTCGTCCGGGCTTTTTTTACTAGTTAC

The gBlocks sequencies were furthermore cloned into a plasmid contained the chaperone (Hfq) sequence with the P*nrsB* promoter.

All the PCRs were cleaned using the GeneJET PCR Purification Kit from Thermo Fisher and DNA concentrations was measured in the Nanodrop spectrophotometer.

Digestion with restriction enzymes

Digestions steps with restriction enzymes were used to obtain the desired plasmid. All digestions were performed using enzymes from the New England Biolabs. It was used the Fast AP to dephosphorylate the plasmids. The enzymes used are listed below:

Table 5: Restriction enzymes used in this thesis.

Enzyme	Temperature (°C)	Time (min)
EcoRI	37	30
XbaI	37	30
PstI	37	30
BcuI (SpeI)	37	30
Fast AP	37	30

The digestion reaction was performed in a total of 30µL reaction contained:

- 3µL of 10x Fast Digestion Buffer (New England Biolabs)
- Restriction enzymes and Fast AP in a total of 3µL
- The tDNA (~400ng for PCR fragments and ~1µg for plasmids)
- dH_2O up to 30µL

The mixture was incubated at 37°C for 30min in a water bath then purified using the DNA Clean & Concentrator^{TM} – 5 (Zymo Research) Kit according to the fabricant instructions followed by the measurement in the nanodrop.

Ligation

The last step for the cloning process was the ligation of the insert into the plasmid. The reaction was prepared in ice and incubated at room temperature for 15min and then used to transform *E.coli* cells. The molar ratio was 1:3 (vector:insert).

Table 6: Final volumes in the ligation mix.

Ligation reaction	Volume (µL)
dH_2O	Up to 10µL
5x Quick Ligase Buffer	5µL
Vector	~1µL
Insert	~0,5µL
Quick ligase	0,5µL

Transformation

For the transformation, the *E. coli* DH5α Z1 were used as competent cells (pre-made and stored at -80°C). The cells were removed from the freezer and rested on ice for 20 min. After that, the ligation mixture was added on the Eppendorf tube with the competent cells and mixed gently and incubated on ice for 30 min followed by a heat shock at 42°C for 40 sec and added 950µL of LB (Luria-Bertani) media and placed in the incubator at 37°C shaker for 1 hour. After incubation, a shot spin was done and removed 950µL of the supernatant and the cells were resuspended in the left 100µL and plated in the LB agar plates with Cm^{35} 1000x diluted (35µg/µL) and incubated O/N at 37°C.

Screening/Colony PCR

Colonies from the previous day transformation were picked up and resuspended in 20µL of dH_2O, one colony for each PCR tube. This resuspension was used as template for the screening PCR. The reaction and conditions are described below:

Table 7: Reaction mix and PCR conditions for a colony PCR.

PCR reaction	Final concentrations (20µL)	PCR conditions (29cycles)	Time
d H2O	13,5µL	Initial denaturation (95°C)	1'
10x DreamTaq™ Green Buffer	2µL	Denaturation (95°C)	30''
10mM dNTPs	0,4µL (0.2mM)	Annealing (58-64°C)	30''
Primer forward 10mM	1µL	Extension (72°C)	1' / Kbp
Primer reverse 10mM	1µL	Final extension (72°C)	5'
Template DNA	2µL	Hold (12°C)	∞
DreamTaq™ DNA polymerase	5U/µL (0,1µL)		

The colony PCR was confirmed through electrophoresis. The colonies who showed bands on the expecting size were inoculated in 10mL LB Cm^{35} and incubated O/N at 37°C. From this liquid culture, 800µL was added into a 2mL tube with 200µL of glycerol 85% for cryo-stock. The remainder culture was used for plasmid extraction using the GeneJET Plasmid Miniprep Kit (Thermo Fisher) and then, sent for sequencing (15µL of plasmid ((>50-100)ng/µL) and 2µL of primer) by Eurofins.

Conjugation

Successful sequencing from the plasmids led to the next step in this work, *Synechocystis*'s transformation. Through conjugation, *Synechocystis* was transformed using the *E. coli* cargo cells (DH5α Z1 expression cells) and the *E. coli* helper cells (HB101 pRL443-Amp^R).

First, O/N fresh cultures were inoculated for both E. coli strains from the cryo-stocks. The strains grew in LB media with the respective antibiotics at 37°C in a shaker incubator. The base strains of *Synechocystis* were grew till a $O.D_{750nm}$ = 0.5. It was used four base strains: WT, ΔNS1, ΔNS1::2MEP and ΔNS1::2MEP_*IspS*2.

For each conjugation plate, 1mL of the cargo strain and 1mL of the helper strain were centrifuged, separately, at 13000 x *g* for 60''. The supernatant was removed and added 1mL of fresh LB without antibiotics in each tube and resuspended. This step was repeated. After the second centrifugation, the pellet was resuspended in 100µL of LB and both strains were mixed. A similar procedure was done for the *Synechocystis* strains, 1mL of liquid culture, then centrifuge at 4000 x *g* for 10'. The supernatant was discarded and resuspended in same volume of fresh BG11 without antibiotics (2x). In the end, the

pellet was resuspended in 200µL of LB. Then, around 20µL of the cyanobacteria culture was added in the tube containing the *E. coli* strains.

The conjugation mix was incubated for 2 hours in a shaking incubator at 30°C and then, the cells were spread on nitrocellulose filter on BG11 agar plates (1,5%) with no antibiotics. In the next day (24h later), the filters were transferred to new BG11 plates with antibiotics (Km^R and/or Cm^R). The plates were incubated at 30°C until was possible to observe single colonies. After that, colonies were picked up and scratched into new BG11 plates with the same antibiotics until have enough biomass for colony PCR. The positives colonies were incubated in 6 well plates in 3mL of BG11 with antibiotics then after couple days, inoculated again in 20mL BG11 in 100 mL Erlenmeyer-flasks. When the cultures achieve $O.D_{750}$ of 1, cryo-stocks were done. For the cryo-stock, 5mL of culture were pelleted at 4000 x *g* for 10 minutes, resuspended on 1mL of media. Then, 950µL of cells were added in a 2mL screw cap tube with 50µL of filtered DMSO and stored at -80°C.

Isoprene assay

The first isoprene assay, cells from the cryo-stock grew in 6-well plates into BG11 with 25µg mL^{-1} kanamycin plus 10µg mL^{-1} chloramphenicol until the culture has an light green colour, then 1mL of the cultures were inoculated in 20mL of BG11 plus antibiotics on 100mL E-flasks. When the $O.D_{750}$ was around 1, pre-cultures for the experiment were made. The cells were inoculated in a new BG11 supplemented with 50mM TES, 50mM $NaHCO_3$, 25µg mL^{-1} kanamycin plus 10µg mL^{-1} chloramphenicol and with/without 2.5µM mL^{-1} $NiCl_2$ with a final $O.D_{750}$ = 0.5. The pre-culture was divided for 8mL screw tubes each one with 5mL of culture, 3 biological replicates for 3 time points, the tubes were incubated at 30°C. For the isoprene measurements, 150µL of the gas in the head space was injected in Clarus 580 Perkin Elmer FID gas chromatograph (GC) with a packed column 353 (1.8 m x 2mm i.d., Cat No. N9305013-ZW5531, Perkin Elmer). The carrier gas was N_2 at 20 mL min^{-1}, then the sample was injected through a packed injector and the oven temperature was 200°C for 2.5 min. To measure the isoprene concentration, a standard curve was made using an isoprene standard that converted the peak area to µg/L, then compared with the isoprene peak at 1.74 min. The measurements were done after 8h, 24h and 48h after the incubation. After the measurements, the $O.D_{750}$ was also measured and 2mL of culture was seeded and then resuspended in 1mL of PBS, seeded again and the pellet was frozen at -80°C for crude cell extraction.

A second isoprene assay very similar to the first one was done, but with slight differences. The pre-cultures were started with a OD_{750} around 0.5, in a supplemented BG11 and this time, with 4 different concentrations of the inducer for the P*nrsB* promoter ([0; 2.5; 5 and 7.5]µM mL^{-1} $NiCl_2$). The pre-cultures grew for 24h with induction and then diluted again to a $O.D_{750}$ = 0.5, divided into the 8 mL screw tubes and the isoprene peak was measured after 3h, 8h and 24h after incubation in the same way as for the first assay. After the isoprene measurements, the O.D750 was checked, 4 mL of the culture was seeded and frozen for the RNA extraction and 1 mL for the crude cell extraction.

Chlorophyll *a* and carotenoids assay

Cells from the cryo-stock grew in 6-well plates into BG11 with 25µg mL^{-1} Km plus 10µg mL^{-1} Cm until the culture have a light green colour, then 1mL of the cultures were inoculated in 20mL of BG11 plus antibiotics on 100mL E-flasks. When the $O.D_{750}$ was around 1, pre-cultures for the experiment were made. The cells were re-inoculated in a new BG11 supplemented with [0; 2,5; 5; 7,5]µM $NiCl_2$ plus the appropriate antibiotics in a final $O.D_{750}$ = 0.2 with three biological replicates. After 1, 2, 4 and 6 days from the beginning of the experiment, 20% of the culture was collected for measure the $O.D_{750}$, pigments extraction and RNA extraction and replaced for fresh media. From this samples, 1mL of culture was centrifuged in 2mL Eppendorf tubes at 18000 x *g* for 10min at room temperature, the supernatant was discarded and the pellet frozen at -80°C. To determine the pigments concentration, the pellets were defrosted and resuspended in 1mL of precooled (4°C) methanol 100% and homogenized by pipetting up and down or by briefly vortexing (2000rpm). The samples were placed into a paper box and covered with aluminium foil and incubated at 4°C for 1 hour. After the incubation, the samples were centrifuged at 18000 x *g* at 4°C for 7 min. The supernatant was transferred to a new tube and used for measuring the absorbance in the spectrophotometer (Varian Cary 50 BIO) from 720nm to 450nm, the methanol was used as blank and as baseline correction. ~

The concentration of Chlorophyll *a* and Carotenoids was calculated using the following equations:

1. [Chl a] = 12,9447 (A_{665}-A_{720}) (Ritchie 2006)
2. [Car] = (1000 (A_{470}-A_{720}) – 2,86 [Chl a]) / 221 (Wellburn 1994)

Cell crude extraction

The cell crude extraction was performed according describe in (Ivleva and Golden 2007). The frozen pellets were defrosted, kept in ice, and resuspended in 200µL of PBS + PA (100x diluted), 1 PCR tube of glass beads acid washed (Sigma) was added. The cells were broken at the Precellys 24 bead beater (Bertin Technologies) in 3 cycles of 30s at 5800rpm and rested on ice for 60s between the cycles. A short spin was performed for concentrate the beads and the cell debris on bottom and 200µL of the supernatant was transferred to a new 1,5mL tube. The protein concentration was calculated using the DC protein assay from BIO-RAD according to the manufacturing instructions, and the absorbance was measured at the plate reader at 750nm and compared with the standard curve made for BSA (albumin from bovine serum – Sigma).

Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and Western immunoblotting

The isoprene was separated from the other proteins by SDS-PAGE from the soluble fraction of the crude cell extraction. The SDS-PAGE mixture contained 7-9ng of protein, pre-mixed protein buffer 4x and dH_2O up to 80% of the total volume of the well. A hot temperature treatment was performed incubating the SDS-PAGE mixture at 95°C for 5min and then rested in the fridge at 4°C for 1min and centrifuged at 18000 x *g* for 1min as well. The mixture was loaded in the Mini-PROTEAN TGX™ gels (BIO-RAD) and left run at 200V for 45min. The gel was transferred to PVDF membrane using the Trans-Blot Turbo Transfer Pack from BIO-RAD in the following order (bottom to top): blotting paper – membrane – gel – blotting paper. The transference was done with the protocol for Standard SD for 30min at 25V constant in the Trans-Blot Turbo (BIO-RAD).

The membrane was incubated in blocking buffer TBST (TBS + and 0.05% Tween 20) with 5% of milk (1g in 20mL) for 2 hours at room temperature in the shaker (100rpm). After the blocking step, the membrane was washed with TBST for 15min three times (same procedure for all wash steps). Then the primer antibody anti-FLAG in mouse (5000x diluted) was added in the membrane and incubated for more 2 hours and washed, this antibody was recycled. The final incubation step was done with the second antibody Rabbit anti-mouse (5000x diluted) for another 2 hours and washed in the end.

In the transilluminator the membrane was checked using the Clarity™ Western ECL Substrate kit from BIO-RAD.

RNA extraction

The RNA extraction was performed using the Zymo Research RNA extraction Kit with some variations. The frozen pellets from the samples were mixed with glass beads and 500µL of Trizol, then the cells were broken by vortexing for 30sec at 5500 x rpm and chilled on ice for 2min, this step was repeat 3 times. The samples were centrifuged for 10min at 4°C max speed and the supernatant was used for RNA extraction with the kit. The DNAse treatment from the kit was not performed and bypassed directly to the pre-wash buffer (the DNAse treatment was performed with different enzyme after the RNA elution). After all wash steps, the RNA was eluted in 25-30µL nuclease free water. The eluent was passed a second time in the same column for a higher RNA concentration, and then measured in the nanodrop.

The DNAse treatment was carried on using 1µg of RNA in a 20µL reaction with 1µL of Thermo Fisher Scientific DNase. The reaction was incubated for 1h at 37°C, after incubation 2µL of 10x EDTA (Thermo Fisher) was added and the incubated for 10min at 65°C for enzyme inactivation. A new digestion was performed, using half of the volume from the previous digestion plus 2µL of DNase in a 20µL total volume. This digestion was incubated again at 37°C for 1h and 2µL of 10x EDTA was added and incubated for 10min at 65°C. In the end the concentration of RNA was measured in the nanodrop and their respective absorbance ratio (A260/A280). The RNA concentrations, the absorbance and the reaction are described in the table below.

Table 8: Samples used for RNA extraction, reaction mix and RNA concentration.

	Before digestion		1st digestion					2nd digestion			After digestion	
Sample	[RNA] ng.µL^{-1}	A260/A280	Volume 1µg of RNA (µL)	Volume of DNase (µL)	Volume of Buffer (µL)	Nuclease free water	Volume of previous digestion (µL)	Volume of DNase (µL)	Volume of Buffer (µL)	Nuclease free water	[RNA] ng.µL^{-1}	A260/A280
EVC - $NiCl_2$	180.1	1.98	5.6	1	2	11.4	10	2	2	6	16.9	1.79
EVC + 7,5µM $NiCl_2$	166.3	1.91	6.0	1	2	11.0	10	2	2	6	18.9	1.69
***αcrtE* - $NiCl_2$**	201.1	1.99	5.0	1	2	12	10	2	2	6	18.7	1.73
***αcrtE* + 7,5µM $NiCl_2$**	170.6	1.62	5.9	1	2	11.1	10	2	2	6	16.0	1.57
***αIspS* - $NiCl_2$**	165.5	1.97	6.0	1	2	11.0	10	2	2	6	16.6	1.74
***αIspS* + 7,5µM $NiCl_2$**	136.5	1.90	7.3	1	2	9.7	10	2	2	6	19.9	1.63

To confirm that the RNA was clean without gDNA contamination, a PCR was done at the end of digestion. Primers for housekeeping genes like the qPCR_rnpB forward and reverse were used with an amplicon of 100bp length. The protocol for the PCR was similar that the one used for previous PCR using the Phusion HF DNA polymerase, with 2µL of RNA template, and for the positive control 200ng of genomic DNA in 20µL reactions and negative control with dH_2O. The cycle conditions were: 30sec for initial denaturation (98°C), 8sec for denaturation (98°C), 15sec for annealing (56°C), 30sec extension (72°C) and final extension also at 72°C for 5min, 40 cycles. Unfortunately, the fragment was too short and did not amplify. A second amplification was performed but this time with primers that amplifies a 400bp fragment from another gene in the genome (NS2). The final concentration of reagents in the mix and the conditions of the cycles were the same as the first PCR except that the annealing temperature was 56°C.

cDNA synthesis

Once the gel from the PCR was without any amplification in the lines with RNA samples, the work continued with the cDNA synthesis using the iScript™ cDNA Synthesis Kit (BIO-

RAD). The process followed the instructions of the kit, 240ng of RNA was used as template in a 20µL reaction. Using the same pair of primers that before, a new PCR was made to confirm the cDNA synthesis, using 1/10 of the cDNA reaction. The conditions of the cycle were the same that before but with less cycles (30).

Real time-quantitative Polymerase Chain Reaction

The RT-qPCR was executed using the iTaq Universal SYBR® green kit (BIO-RAD) following the supplier protocol and, melting curve according to BIO-RAD. The table x is a demonstration of the reaction and conditions of the procedure. Three pairs of primers were used: the qPCR_rnpB (forward/reserve) was used as control, the qPCR_*IspS*_forward and qPCR_*IspS*_reverse for the isoprene synthase gene, and the q_PCR_*crtE*_forward and q_PCR_*crtE*_reverse for the geranylgeranyl diphosphate synthase gene. After running the RT-qPCR the C_t values were taken and then the difference between the C_t "target" and the C_t "rnpB" was calculated achieving the dC_t value. It was also calculated the ddC_t, it consists in the difference between the dC_t "target" and the dC_t "EVC".

Table 9: PCR reaction and conditions for RT-qPCR.

PCR reaction	Final concentrations (10µL)	PCR conditions (40cycles)	Time
Nuclease-free H2O	3.2µL	Polymerase activation (95°C)	5'
Primer forward 400nM	0.4µL	Denaturation (95°C)	30''
Primer reverse 400nM	0.4µL	Annealing (56°C)	30''
Template cDNA	1µL	Extension (60°C)	30''
iTaq Universal SYBR® green 2x	5µL	Melting curve	BIO-RAD

Table 10: Plate reader in the qPCR machine. The blue samples were amplified with the qPCR_IspS primer pair, the green samples were amplified with the qPCR_crtE primer pair, and the black samples were amplified with the qPCR_rnpB primer pair. NC indicates the negative control where the template was substituted for dH_2O.

	1	2	3	4	5	6	7	8	9	10	11	12
A	EVC-	EVC-	EVC-	EVC+	EVC+	EVC+	EVC-	EVC-	EVC-	-	-	-
B	*IspS*-	*IspS*-	*IspS*-	*IspS*+	*IspS*+	*IspS*+	*IspS*-	*IspS*-	*IspS*-	-	-	-

C	NC	NC	NC	NC	NC	NC	*crtE-*	*crtE-*	*crtE-*	-	-	-
D	EVC-	EVC-	EVC-	EVC+	EVC+	EVC+	EVC+	EVC+	EVC+	-	-	-
E	*crtE-*	*crtE-*	*crtE-*	*crtE+*	*crtE+*	*crtE+*	*IspS+*	*IspS+*	*IspS+*	-	-	-
F	NC	NC	NC	NC	NC	NC	*crtE+*	*crtE+*	*crtE+*	-	-	-
G	-	-	-	-	-	-	NC	NC	NC	-	-	-
H	-	-	-	-	-	-	-	-	-	-	-	-

Results and discussion

This study has as aim improve the isoprene production in Synechocystis by downregulating the native competitive pathways via RNA interference. As mentioned, the study leans on two different small RNA mechanisms, and target two differents genes. To improve the isoprene production, an asRNA targeting the *crtE* gene downregulates this gene increasing the amount of DMAPP for isoprene production by *IspS*. This gene was tested in two different strains, the ΔNS1::2MEP_*IspS*2 (Rana, Gomes et al. 2022) that was engineered with the heterologous enzyme (*Eucalyptus globulus IspS*) and two more enzymes upstream in the MEP pathway DXS (1-deoxy-D-xylulose-5-phosphate synthase) and IDI (isopentenyl-diphosphate isomerase), tested the isoprene yield. The ΔNS1 strain used has control to validate the efficiency of the regulation on *crtE* comparing the concentration of pigments with the EVC. Known that the isoprene production can be toxic for the cell, would be interesting to have a system that could switch between asRNAs, downregulating the *IspS* in the exponential phase of *Synechocystis* growth, and have more DMAPP for pigments production and others terpenoids, and then, in the stationary phase, downregulate the *crtE* and produce higher titres of isoprene. For this reason, strains contained asRNA for *IspS* were also tested, even if they do not increase the isoprene production.

asRNA with the PTRNA system

The engineered strain ΔNS1::2MEP_*IspS*2 (Rana, Gomes et al. 2022) was conjugated with self-replicating plasmid carrying the asRNA mechanism and the chloramphenicol resistance cassette, under control of the synthetic promoters P*trc*, or the inducible promoter P*nrsB*. The first strains tested were those that contained the PTRNA system, in this experiment the strains were inoculated with BG11, 50mM TES, 50mM $NaHCO_3$, 25μg mL^{-1} kanamycin plus 10μg mL^{-1} chloramphenicol, and 2.5μM mL^{-1} $NiCl_2$ in the ΔNS1::2MEP_*IspS*2-pEEC_PT1_PnrsB_α*IspS*_PT2 and

ΔNS1::2MEP_*IspS*2_pEEC_EVC strains, these strains were also tested with no inductor. The ΔNS1::2MEP_*IspS*2_pEEC_Ptrc_ α*IspS* was tested in the same experiment. Once the target was the *IspS*, we expected to have a lower isoprene production when compared to the empty vector control in the ΔNS1 ::2MEP _*IspS*2_pEEC_PT1_Ptrc_α*IspS*_PT2 and in the ΔNS1 ::2MEP _*IspS*2-pEEC_PT1_PnrsB_α*IspS*_PT2 supplemented with 2.5μM mL^{-1} $NiCl_2$. The yield in the EVC should not change with the addition of $NiCl_2$ (fig.4). The isoprene peak was measured at GC after 8h, 24h and 48h. The peak area of isoprene curve was converted to mass value (isoprene [mg] / L liquid culture) using an isoprene standard curve. Protein and RNA samples were taken, and the OD was measured at 750nm (fig.3).

To study the downregulation in the pigment's concentration, the engineered plasmids containing the asRNA targeting the *crtE*, Cm^R followed by the P*trc* promoter were tested in the *Synechocystis* ΔNS1 strain. Initially, we first tested two different targets in the *crtE* gene and compared with the EVC. The cultures were inoculated in 20mL of BG11 supplemented with 25μg mL^{-1} Km^R and 10μg mL^{-1} CmR in 100mL e-flasks, strains under the inducible promoter P*nrsB* were not tested. The optical density, the concentrations (mg L^{-1} culture) of chlorophyll a and carotenoids are represented (fig.5, 6 and 7).

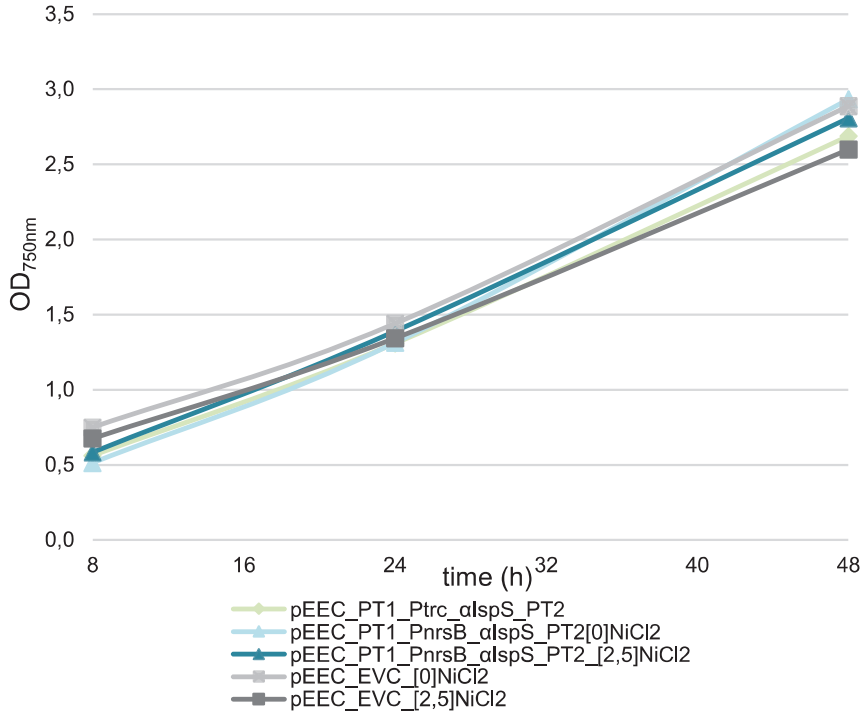

Figure 3: Optical density at 750nm of all strains transformed with the PTRNA tool after 8h, 24h and 48h the isoprene assay.

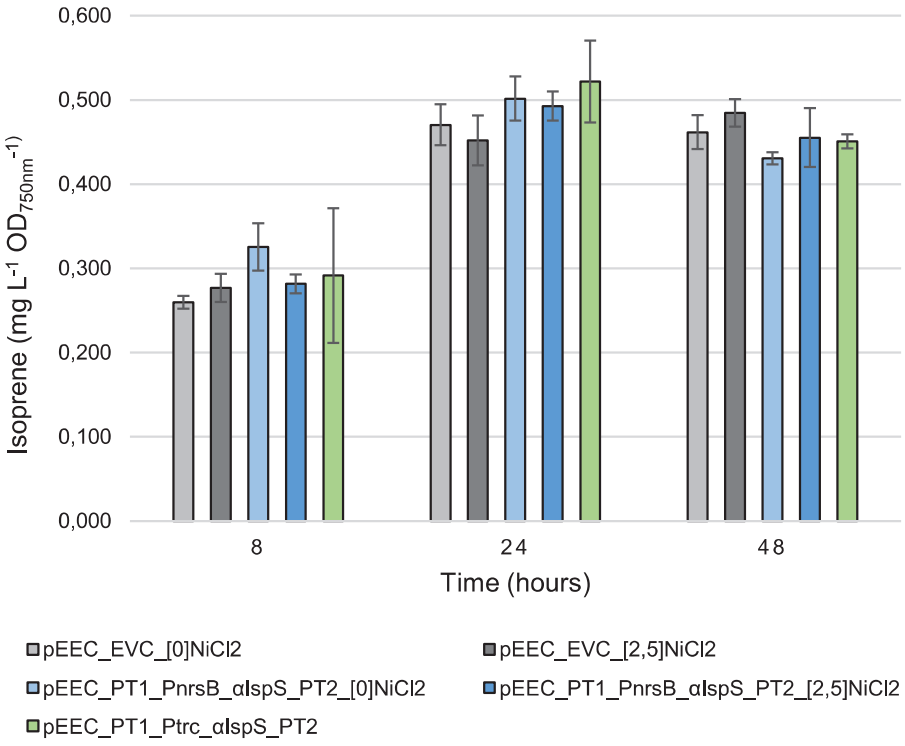

Figure 4: Isoprene production titres measured the isoprene concentration in the headspace in GC and converted to mg L^{-1} culture OD_{750nm}^{-1} in all ΔNS1::2MEP_IspS2 transformed strains contained the PTRNA systems and EVC.

According to the graphic, we can observe that the expression of engineered plasmid contained the PTRNA system does not change the growth curve when compared with the control strain (EVC). At the isoprene production level, all strains containing the PTRNA regardless the promoter or the induction, have similar production or even more than the empty vector. Considering that the base strain in Rana et al. 2022 achieved an isoprene titre of 1.60 mg L^{-1} culture after four days (~0.400 mg L^{-1} $24h^{-1}$), the EVC at 24 hours produced between 0.452 mg L^{-1} to 0.471 mg L^{-1}, with 2.5μM of Ni^{2+} and without inducer, respectively, the results are consistent (Rana, Gomes et al. 2022). The standards deviations in isoprene production overlap each other's, implying that the mechanism did not work, and small variations is due variations of initial ODs. These results can be the outcome of the failure of the cloning step due the lack of base pairs in the PT2 region. Unfortunately, this same pattern was observed in the pigments experiment, the strains with the PTRNA did not have any detectable regulation when compared with the EVC. The growth curve between the ΔNS1_pEEC_PT1_Ptrc_α*crtE*2_PT2 and the ΔNS1_pEEC_EVC was very similar, for the ΔNS1_pEEC_PT1_Ptrc_α*crtE*1_P2 the growth curve has a slight difference on the fourth day compared with the others, but the final OD is very close to the other two strains. Looking at the pigment content in the three strains, it is evident that has no variations at the concentration of chlorophyll a, the only strain that seem to have lightly less chlorophyll has a considerable standard deviation that overlap the EVC. The profile repeats at the carotenoids content. After this experiment, I tried several times to have a clean cloning with all base pairs, once I was not successful regardless the order of cloning or technique, I decided to focus only in the Hfq-MicC mechanism.

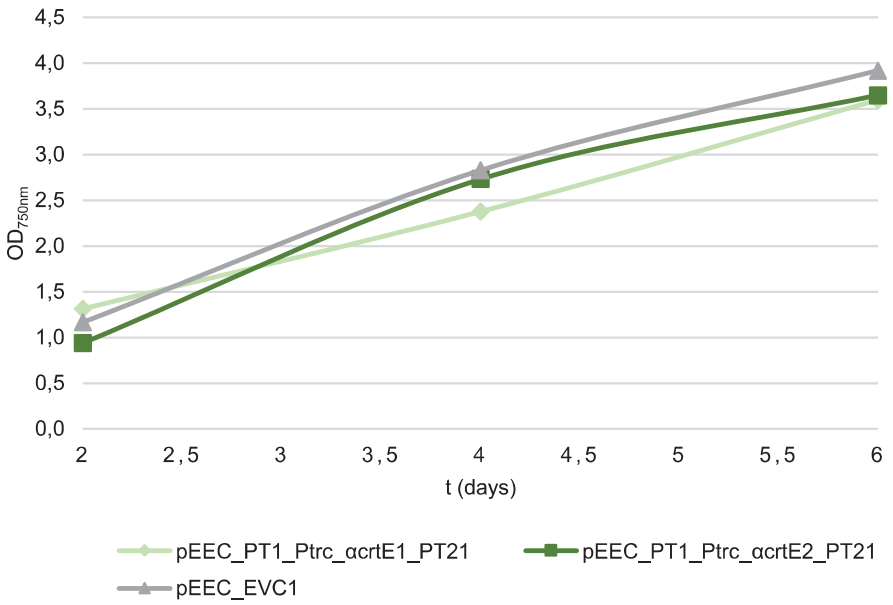

Figure 5: Growth curve of all ΔNS1 transformed strains with the PTRNA system after 6 days of cultivation.

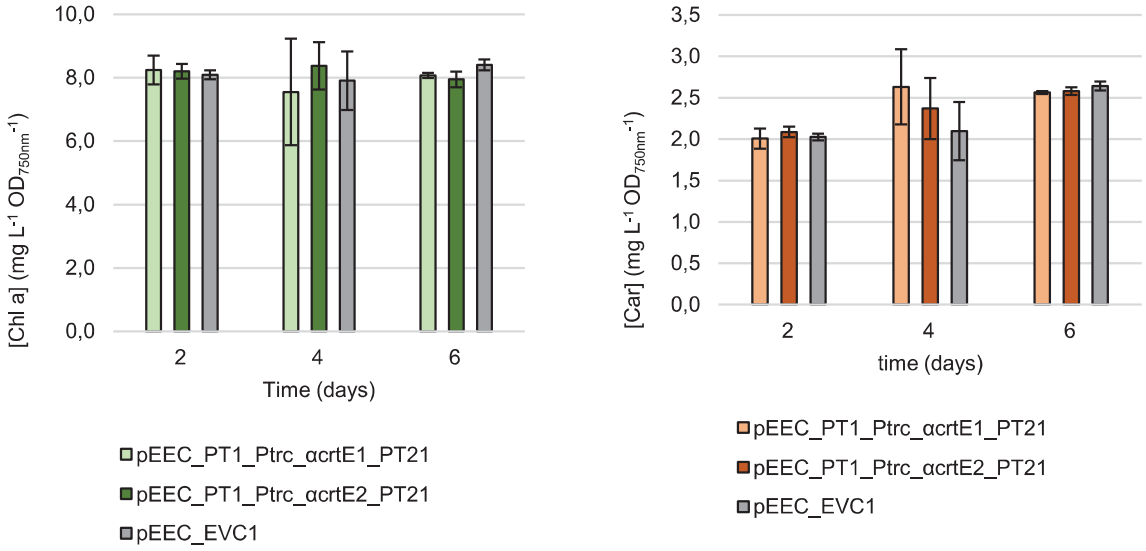

Figure 6: Pigment contents of all ΔNS1 transformed strains with the PTRNA and EVC after 2, 4 and 6 days of growth, represented in mg L^{-1} culture OD_{750nm} $^{-1}$. Left: Chlorophyll a concentration. Right: Carotenoids concentration.

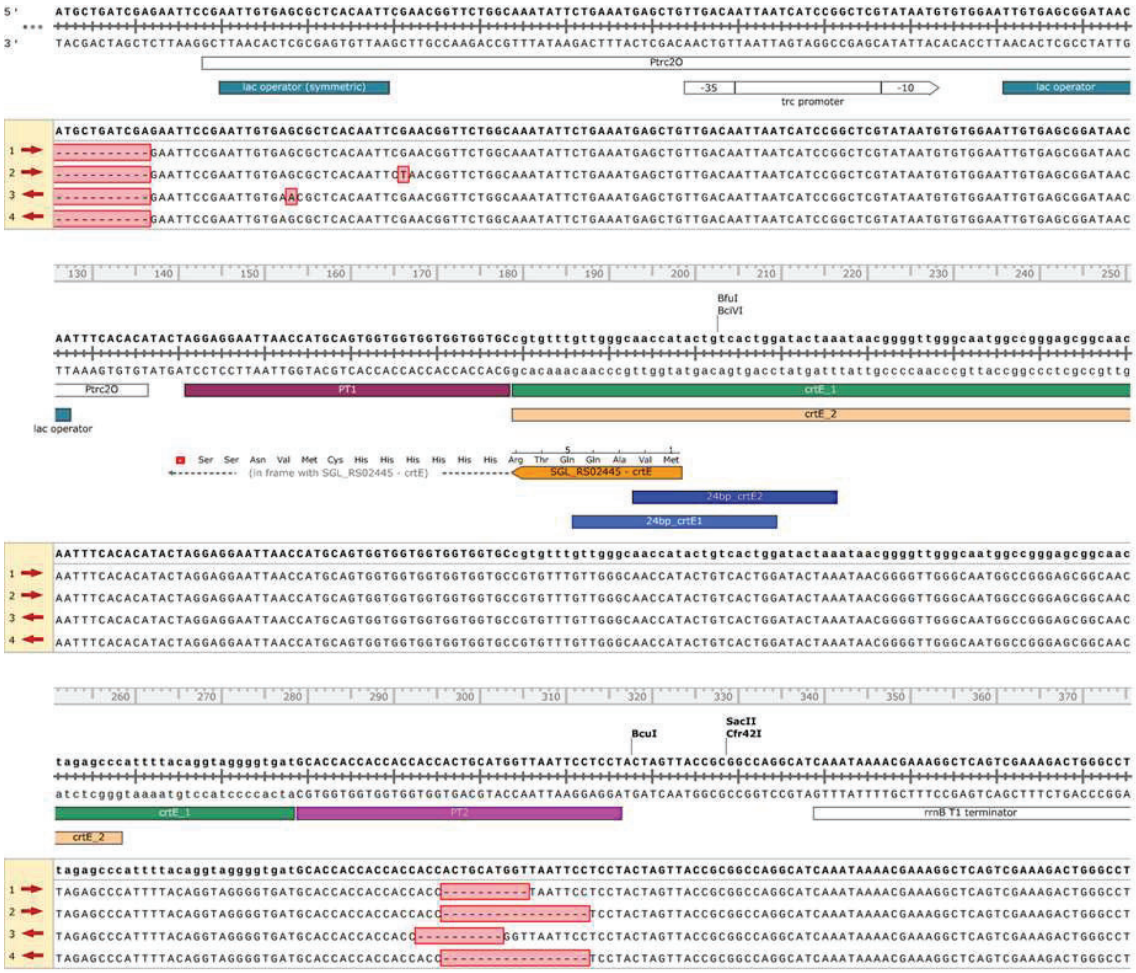

Figure 7: pEEC_PT1_Ptrc_αcrtE1_P2 sequencing. Example of the missing base pairs in the PTRNA.

asRNA with the Hfq-MicC complex

Following the aim of this thesis and previous studies, the next phase to improve the yield of isoprene in *Synechocystis* using RNA interference, the Hfq-MicC complex was tested. The ΔNS1::2MEP_*IspS*2 strain was engineered with the following plasmids: pEEC_EVC (EVC), pEEC_Ptrc_α*crtE*+MicC_PnrsB_Hfq (α*crtE*) and pEEC_Ptrc_α*IspS*+MicC_PnrsB_Hfq (α*IspS*). The chaperone was inserted under the inducible promoter P*nrsB* in order to control the complex activity, turning on by the addition of $NiCl_2$ (inducer) and to investigate if the asRNA was functional regardless the chaperone protein, or if the MicC sequence inserted interact with *Synechocystis* native chaperones. The cells were maintained in liquid culture with an optical density around 1, then the pre-culture for isoprene measurements were made at OD 0.5. Four concentrations of inducer were used, although previous studies showed that 2.5μM Ni^{2+}

leads to high induction of P*nrsB* and present low toxicity for the cells (Englund, Liang et al. 2016), we also tested 5μM, 7.5μM and no induction. The pre-cultures grew for 24 hours with induction and then the O.D. was normalized to 0.4 (due an error in one culture I had to normalize to O.D=0.4 instead 0.5) and distributed to closed vials to measure the isoprene accumulated in the headspace. The isoprene peak area was measured after 3h, 7h, and 24h after the incubation, three replicates for each strain and for each time point.

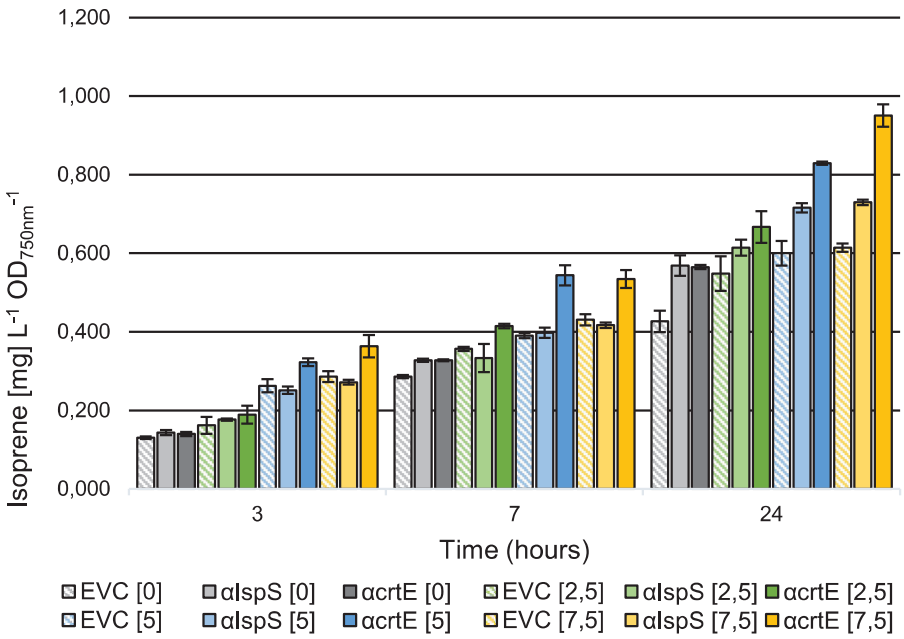

Figure 8: Isoprene production titres measured the isoprene concentration in the headspace in GC and converted to mg L^{-1} culture OD_{750nm}^{-1} in all ΔNS1::2MEP_IspS2 transformed strains contained the Hfq-MicC complex and EVC. The grey bars indicate the strains treated with no inducer, the green bars the strains treated with 2.5 μM of Ni^{2+}, the blue bars the strains treated with 5 μM of Ni^{2+} and the yellow bars the strains treated with 7.5 μM of Ni^{2+}. The striped bar represented the EVC.

In 2016, Englund et al. demonstrated that higher concentrations of inducer lead to more transcription at the cell level, translated to higher production levels (Englund, Liang et al. 2016). In fig.8 it's clear that the production of isoprene increases along with the increase in induction, passing from the same as the EVC to an increase of 20% - 40% (the ratio between the α*crtE* and EVC values) with 5μM and 7.5 μM in the α*crtE*. The maximum yield of isoprene produced by pEEC_Ptrc_α*crtE*+MicC_PnrsB_Hfq was 0.951 mg L^{-1} OD_{750}^{-1} after 24 hours. Comparing the production with previous studies, 2.8 ng / g dry

cell weight or 1.0 mg L^{-1} at lest after 24 hours (Englund, Shabestary et al. 2018), 102.24 mg L^{-1} after 24h (1.26 g L-1 or 4.26 mg L^{-1} h^{-1} (Gao, Gao et al. 2016) and 1.104 mg L^{-1} after 24h (46 µg L^{-1} h^{-1} or 12.3 mg / g dry cell weight) (Chaves and Melis 2018), the results obtained in this experiment are promising. For the other hand, the strain α*IspS* did not decrease the isoprene production as expected, in fact in some points the production was higher than the EVC control (5 µM and 7.5 µM at 24h). This behaviour does not make sense and is hard to explain. To better understanding this results, the RT-qPCR will clarify what happens at the transcriptional level.

To validate the results obtained in the isoprene assay, strains contained the α*crtE* (ΔNS1_Ptrc_α*crtE*_PnrsB_Hfq) were tested. Evaluating the chlorophyll *a* and carotenoids concentrations between the EVC and the α*crtE*, it is possible to validate that the difference in isoprene production is due the downregulation in the *crtE* gene providing more DMAPP to isoprene synthase. The assay consists in cultivate the strains in BG11 supplemented only with the antibiotics for selection of transformant cyanobacterium and different concentrations of inducer for the *PnrsB* promoter. The cultures started with OD 0.2 and 20% of the culture was taken for samples and replaced for fresh media on the first, second, fourth and sixth days. From the samples, was analysed the growth curve, the concentration of chlorophyll *a*, carotenoids, and frozen pellets were frozen for posterior RNA extraction and RT-qPCR. The cells culture treated with no $NiCl_2$ looked healthy with no big difference between the EVC and α*crtE*, but with the increase of $NiCl_2$ concentration cells show lower growth in both strains. In fig. 9 is a representation of the cultures on the sixth day of experiment, fig. 10 and fig.11 the ODs.

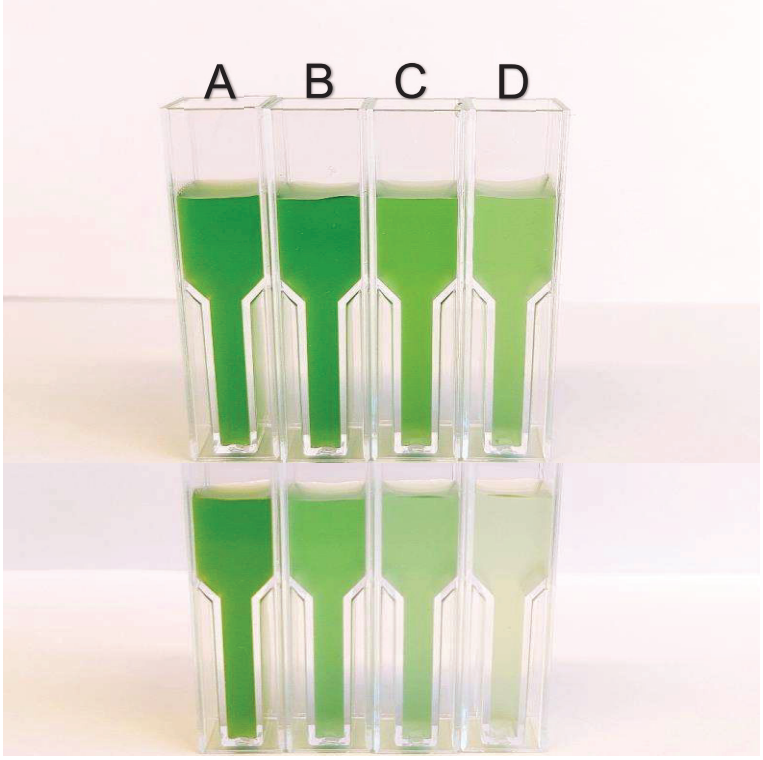

*Figure 9: Liquid cultures ΔNS1 strains after 6 days of the pigments assay. **A** strains treated with no inducer, **B** strains treated with 2.5 μM of Ni^{2+}, **C** strains treated with 5 μM of Ni^{2+}, **D** strains treated with 7.5 μM of Ni^{2+}. Top: strains with the EVC. Bottom: strains with the αcrtE.*

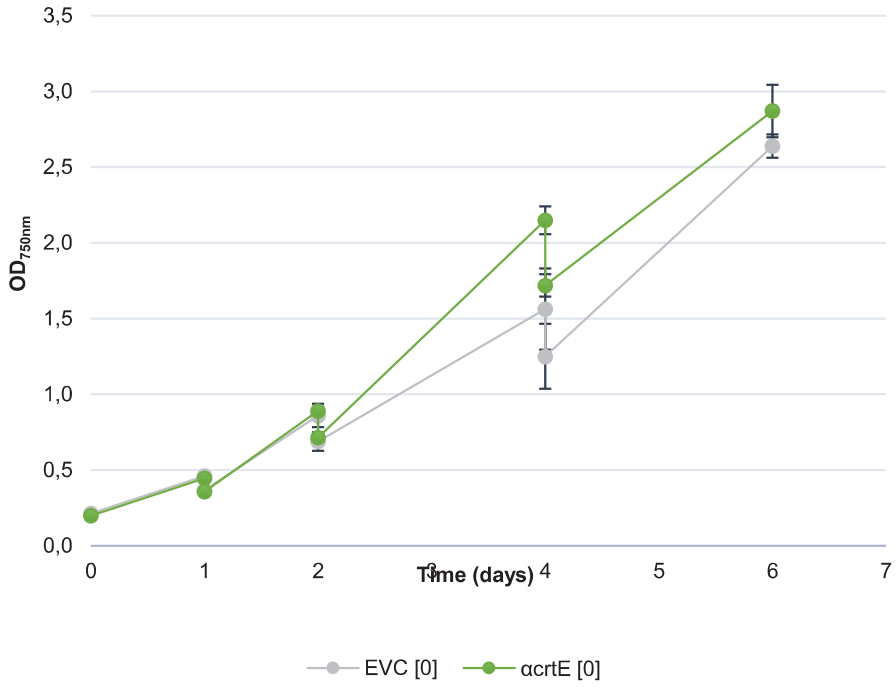

Figure 10: Growth curve of ΔNS1 strains with EVC and with the αcrtE during the pigments assay with no inducer.

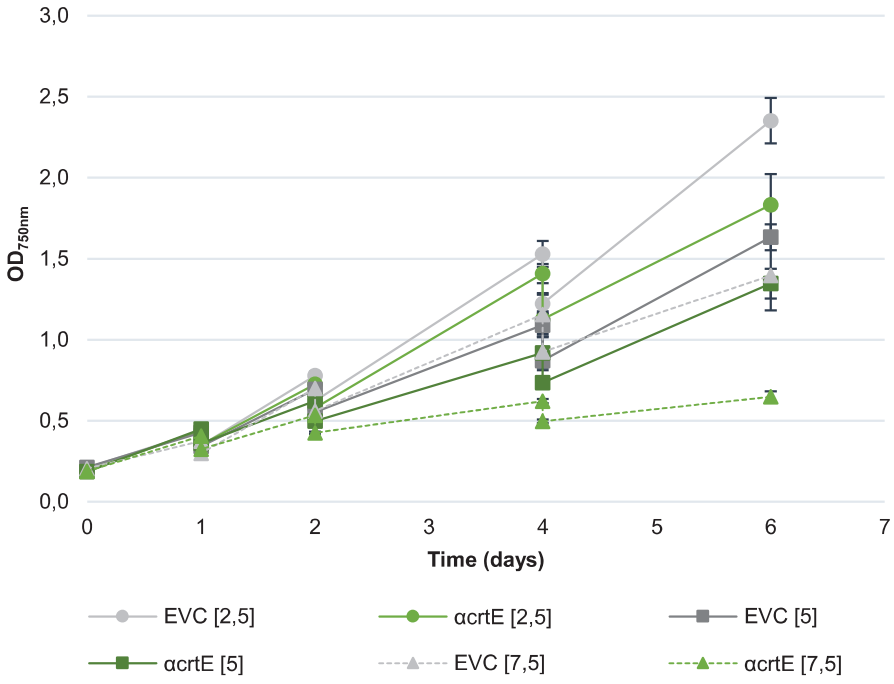

Figure 11: Growth curve of ΔNS1 strains with the EVC and the αcrtE during pigments assay treated with [2.5; 5; 7.5] μM of Ni^{2+}.

During the experiment the cultures were diluted when the samples were taken, for that reason the OD needed to be calculated after the dilution to see the real growth of the cultures. In this way, after the samples were taken, the OD was calculated using the equation: $OD_{real} = OD_{measured} \times 0.8$. Looking at the growth pattern between the EVC and α*crtE* with no induction of the Hfq protein, the asRNA system is not activated and does not compromise the growth. In fact, the growth is very identical until the fourth day. When the inducer was added, the cells grow lower in both strains, but the effect in more significant in the α*crtE* since the downregulation system is activated and target the *crtE* gene, the production of pigments drop. The pigments are essential for cell growth. To prove that were less production of pigments, the pigments in the culture were extracted and measured the concentration, the results are in fig. 12.

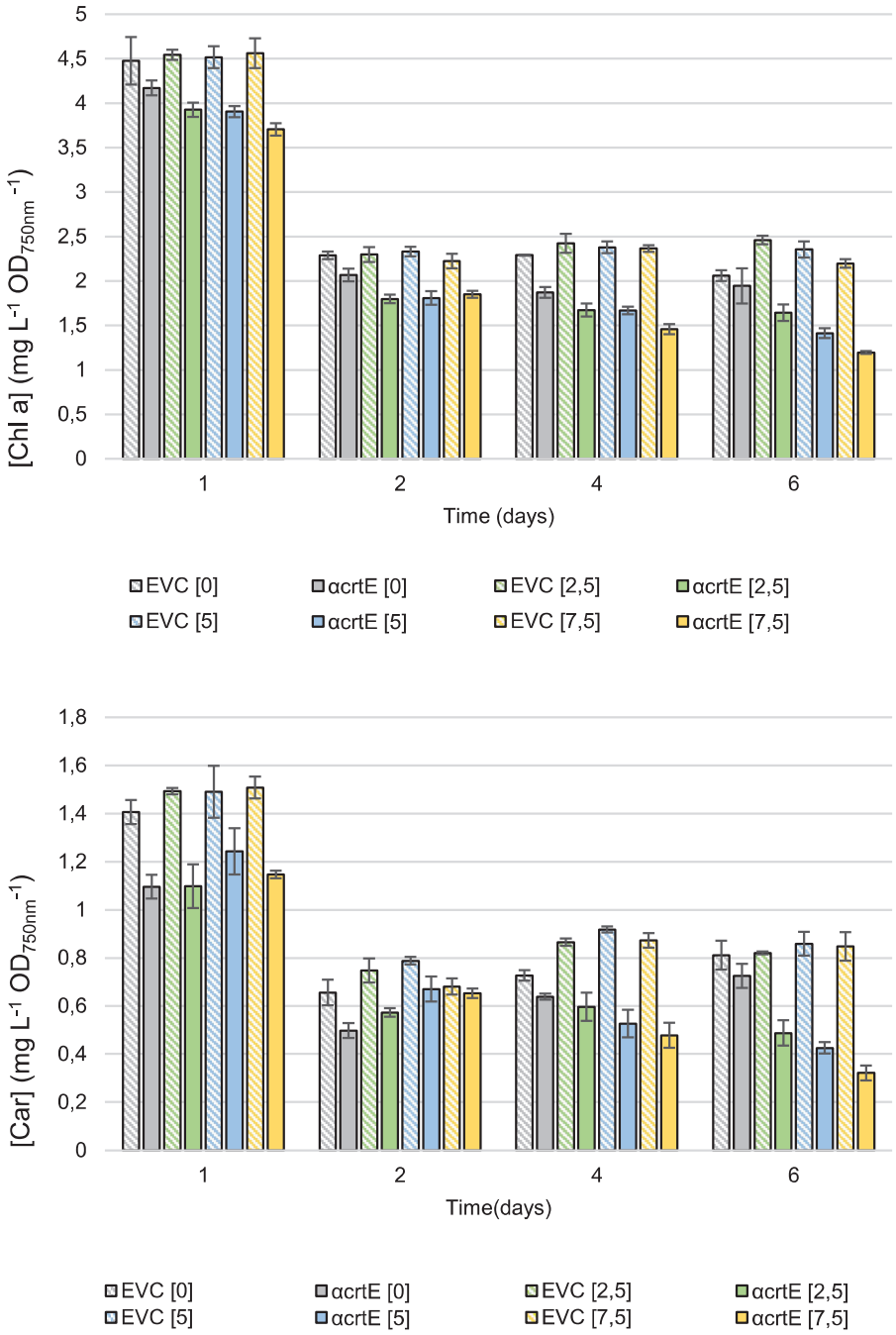

Figure 12: Pigment contents of all ΔNS1 transformed strains with the Hfq-MicC complex and EVC after 1, 2, 4 and 6 days of growth, represented in mg L^{-1} culture OD_{750nm} $^{-1}$. The grey bars indicate the strains treated with no inducer, the green bars the strains treated with 2.5 μM of Ni^{2+}, the blue bars the strains treated with 5 μM of Ni^{2+} and the yellow bars the strains treated with 7.5 μM of Ni^{2+}. The striped bar represented the EVC. Top: Chlorophyll a concentration. Bottom: Carotenoids concentration.

From the graphics, the concentrations of pigments in the strains contained the α*crtE* (full bars) are significantly lower than the EVC (striped bars) in all time points and

concentrations except the [Car] at second day with 7.5 μM $NiCl_2$. Comparing the strains treated with no inducer, the α*crtE* also present less amounts of pigments than the EVC, but in the last day of experiment this difference is more subtle. Other observation is that after the first day both Chl a and Car concentrations decrease nonetheless maintain similar in the 4th and 6th day in the EVC strain. The decrease in the pigment's concentrations is more intense in the α*crtE* with the increase of induction. The difference between the first day and the following days, might be due the different growth stages that the cells are passing over (Rodrigues and Lindberg 2021).

RNA extraction and cDNA synthesis

Frozen pellets preserved from the isoprene assay were further used to extract the RNA and synthesize new cDNA for posterior RT-qPCR analysis. As representative samples we chose: ΔNS1::2MEP_*IspS*2_ pEEC_Ptrc_α*crtE*+MicC_PnrsB_Hfq ([0; 7.5] μM $NiCl_2$), ΔNS1::2MEP_*IspS*2_ pEEC_Ptrc_α*IspS*+MicC_PnrsB_Hfq ([0; 7.5] μM $NiCl_2$) and ΔNS1::2MEP_*IspS*2_ pEEC_EVC ([0; 7.5] μM $NiCl_2$), all from the 7h time point. After RNA extraction, an PCR was performed in order to confirm that was no gDNA contamination, the primers pair NS2 (250-500bp) were used, 200 ng of gDNA was used as positive control and dH_2O as negative control. After cDNA synthesis a new PCR was

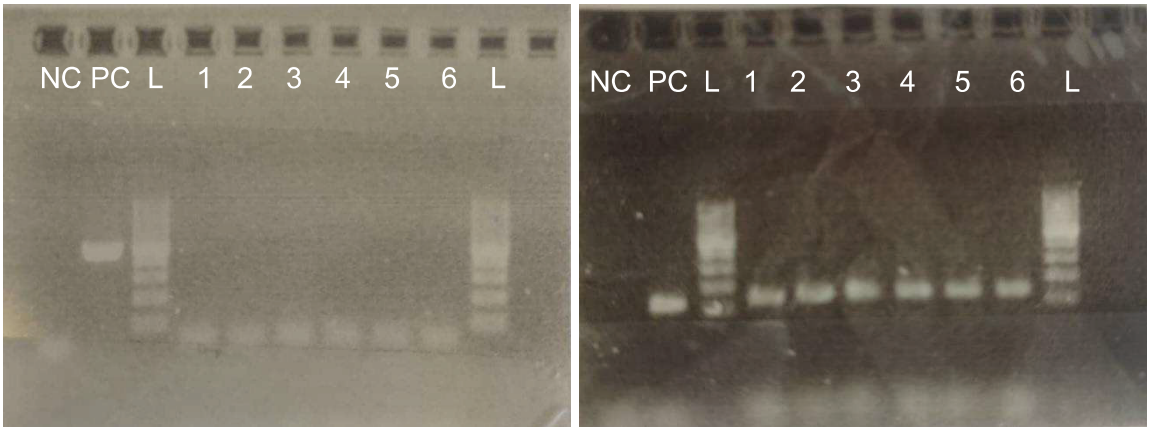

Figure 13: Agarose gel after PCR. NC: negative control; PC: positive control; L: GeneRuler 1kb; 1: EVC – Ni^{2+}; 2: IspS - Ni^{2+}; 3: crtE – Ni^{2+}; 4: EVC + 7.5 μM Ni^{2+}; 5: IspS + 7.5 μM Ni^{2+}; 6: crtE + 7.5 μM Ni^{2+}. Left: after RNA extraction and amplified with NS2 primers pair. Right: after cDNA synthesis amplified with qPCR_rnpB primer pair.

Figure 14: Agarose gel after PCR. NC: negative control; PC: positive control; L: GeneRuler 1kb; 1: EVC – Ni^{2+}; 2: IspS - Ni^{2+}; 3: crtE – Ni^{2+}; 4: EVC + 7.5 μM Ni^{2+}; 5: IspS + 7.5 μM Ni^{2+}; 6: crtE + 7.5 μM Ni^{2+}. Left: after RNA extraction and amplified with NS2 primers pair. Right: after cDNA synthesis amplified with qPCR_rnpB primer pair.

made with the qPCR_rnpB primers pair (100bp) using 1/10 of the cDNA reaction as template, 200 ng of gDNA as positive control and dH_2O as negative control.

As it is possible to see there was no amplification in all RNA samples, and after the cDNA synthesis all samples showed amplification in the desired size. With this two PCR we prove that all differences obtained in the RT-qPCR is due differences in the transcriptional level (RNA) and do not come from DNA contaminations.

During this master thesis, I tried to change the promoters in the Hfq-MicC complex, even though the cloning was successful, I did not get colonies after the conjugation, what make we believe that maybe the system was much stronger, downregulating the essential *crtE* gene and interfering with the pigments limiting the growth of recent transformed strains.

RT-qPCR

The qPCR was performed using the SYBR® green (BIO-RAD) kit, the C_t values was measured using the qPCR_rnpB (Fwd/Rev) for housekeeping genes, the qPCR_*IspS* (Fwd/Rev) for the *IspS* gene and que qPCR_*crtE* (Fwd/Rev) for the *crtE* gene. The EVC samples were amplified with all primers for posterior comparison between them and the tested strains. The α*IspS* samples were amplified with the qPCR_*IspS* primers, the α*crtE* with the qPCR_*crtE* primers, and the both were also amplified with the qPCR_rnpB primers.

Table 11: C_t values after qPCR

Samples	*IspS* PCR	*crtE* PCR	rnpB PCR
EVC – NiCl2	19.21	25.62	17.05
EVC + 7.5 µM NiCl2	19.16	25.29	16.97
α*IspS* – NiCl2	19.25	-	16.69
α*IspS* + 7.5 µM NiCl2	18.98	-	16.93
α*crtE* – NiCl2	-	25.36	16.91
α*crtE* + 7.5 µM NiCl2	-	26.51	17.89

To normalize the results, the ΔC_t was calculated between the C_t of target gene and C_t of rnpB (housekeeping gene), ΔC_t = C_t "target" – C_t "rnpB".

Table 12: ΔCt values

Samples *IspS* = target	ΔC_t	Standard deviation	Samples *crtE* = target	ΔC_t	Standard deviation

EVC – NiCl2	2.16	0.08	EVC – NiCl2	8.57	0.05
EVC + 7.5 µM NiCl2	2.19	0.08	EVC + 7.5 µM NiCl2	8.32	0.07
α*lspS* – NiCl2	2.56	0.09	α*crtE* – NiCl2	8.45	0.15
α*lspS* + 7.5 µM NiCl2	2.05	0.21	α*crtE* + 7.5 µM NiCl2	8.63	0.16

The $\Delta\Delta C_t$ was calculated by deducting the ΔC_t "EVC" from the ΔC_t "target" in order to normalize the results with the control strain. The differences observed in the transcription of the target genes are due the asRNA.

Table 13: ΔΔCt values and standard deviation

Samples *lspS* = target	**$\Delta\Delta C_t$**	**Standard deviation**	**Samples *crtE* = target**	**$\Delta\Delta C_t$**	**Standard deviation**
α*lspS* – NiCl2	0.40	0.12	α*crtE* – NiCl2	-0.12	0.16
α*lspS* + 7.5 µM NiCl2	-0.13	0.22	α*crtE* + 7.5 µM NiCl2	0.31	0.18

To evaluate the fold change in gene transcription the $2^{-\Delta\Delta Ct}$ equation was applied. The results are in table 14.

Table 14: Fold change in gene transcription

Strain	**$2^{-\Delta\Delta Ct}$**	**Standard deviation**
α*lspS* – NiCl2	0.76	0.06
α*lspS* + 7.5 µM NiCl2	1.10	0.2
α*crtE* – NiCl2	1.08	0.12
α*crtE* + 7.5 µM NiCl2	0.81	0.10

Comparing the results from the qPCR with the results obtained in the isoprene assay, the α*lspS* do not correspond with what was expected. The isoprene production was barely affected, and the $2^{-\Delta\Delta Ct}$ values do not make much sense. The α*lspS* when not induced has a fold-change of 0.8, the transcriptional level is lower than the EVC but when induced, the transcription level is equal to the EVC. On the other hand, the results for α*crtE* are very interesting. Comparing the $2^{-\Delta\Delta Ct}$ values, the α*crtE* has lower transcript levels when induced and remain unchanged when the inducer is not present. The

induced α*crtE* present a decrease in almost 20% in the transcriptional level, this percentage is very similar to the percentage of increasement in the isoprene production when the α*crtE* was induced.

Conclusion

In this study, we aimed at downregulating the native competitive pathway to enhance the isoprene production in Synechocystis via RNA interference. The Hfq-MicC complex seems to work very well, improving the isoprene titers by 20%, this result was confirmed by the qPCR where the amount of transcription was measured in the strains transformed with the α*crtE* and compared with the EVC. The downregulation obtained in this study was not as strong as the observed in Sun et al. 2018, where they reached 90% of downregulation of *glgC* gene using the same mechanism. However, Sun et al. used different promoters, the Hfq protein was under a constitutive promoter (P*cpc560*) and the micC scaffold with the asRNA under a light inducible promoter (P*psbA2M*) while in this study, the Hfq protein was under the inducible promoter (P*nrsB*) and the asRNA plus the micC sequence under the constitutive promoter (P*trc*). Since the system was only activated when the Hfq protein is present, we assume that the regulation can be stronger when the Hfq is under a constitutive promoter since it belongs at the protein level and not in the mRNA level.

According to the results of this thesis, the asRNA targeting the *IspS* gene did not seemed to work, or the regulation was too weak to interfere with the isoprene production. Also, the results acquired from the qPCR, were confusing and did not make sense, why the αIspS when not induced decrease the transcription and when induced the transcriptional level as similar to the EVC. Maybe the fact that *IspS* is a heterologous proteins expressed in *Synechocystis* is more difficult to regulate compared to a native protein like the *crtE*. In order to understand beter the mechanism and take better advantage of it, it should target different parts of the gene to downregulate the transcription.

Use the PTRNA as an RNA interference tool was another aim of this study, although the usage of this mechanism was not successful. The difficulties found along the cloning process made it impossible to study, even when we performed the assays no difference in production was achieved.

To conclude, in this study we reached a downregulation between 20% to 40% in the *crtE* gene, enhancing the production of isoprene in *Synechocystis* achieving a total of 0.951 mg L^{-1} OD_{750} $^{-1}$. The RNAi using the Hfq-MicC complex is a valuable tool for downregulating gene expression and using it in *Synechocystis* for production of biofuel

can be very interesting for enhance titers and make the scale up for industrial production possible. More improvements need to be done, in particular to target different parts of the gene and different genes for higher improvement. For the PTRNA, a better technique for cloning needed to be performed in order to achieve the full cloning without missing base pairs.

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