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# (E)-α-bisabolene production in *Synechocystis* sp. PCC 6803

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# Abstract

## (E)-α-bisabolene production in *Synechocystis* sp. PCC 6803

*Sara Nilsson*

The world is currently facing many environmental challenges related to fossil fuels and meanwhile the global energy demand is increasing rapidly. The terpenoid (E)-α-bisabolene is a precursor of the potential diesel fuel replacement bisabolane, and has been proven possible to produce in the cyanobacterial strain *Synechocystis* sp. PCC 6803. The aim of this project was to increase the bisabolene production in said strain, as well as introducing the same engineered system into S*ynechococcus elongatus* PCC 7942 for comparison. For this purpose, several constructs were constructed which all contained the first enzyme of the terpenoid biosynthesis pathway; 1-Deoxy-D-xylulose 5-phosphate synthase (DXS). These constructs were then conjugated into the cyanobacterial strains after which the bisabolene production of positive colonies was evaluated. One of the constructs proved to increase the production of bisabolene in *Synechocystis* sp. PCC 6803, however the introduced DXS gene could not be detected neither on a genetic nor a translational level. Furthermore, the conjugation of *Synechococcus elongatus* PCC 7942 proved unsuccessful and did not give any colonies.

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# Sammanfattning

Världen står just nu inför många miljömässiga utmaningar orsakade av fossila bränslen, så som luft- och vattenföroreningar samt global uppvärmning. Samtidigt ökar den globala energiförbrukningen i en alarmerande takt. Tillsammans medför detta ett brådskande behov av hållbara energikällor som varken släpper ut giftiga ämnen eller ökar mängden växthusgaser i atmosfären. En sådan energikälla, som redan idag används till att producera el och värme, är solen. Större delen av världens energikonsumtion sker dock i form av bränsle, vilket ännu inte tillverkas kommersiellt från solenergi. Just detta är dock en vision för många forskare och forskning pågår för att göra detta till verklighet, exempelvis genom att utnyttja naturens egna soldrivna bränsleproduktion; fotosyntesen. Fotosyntes är en komplicerad reaktion som används av bland annat växter för att producera bränsle till sina egna celler med hjälp av solenergi. I praktiken används solenergin för att omvandla vatten och koldioxid till syre och kolväten. Kolvätena lagras som ett slags bränsle i cellerna och används som energikälla vid behov, till exempel för att växa.

Genom att manipulera arvsmassan av en fotosyntetiserande organism kan energin från fotosyntesen omdirigeras för produktion av önskade ämnen så som läkemedel, parfymer och till och med kommersiellt användbara bränslen. I detta avseende är fotosyntetiserande bakterier, så kallade cyanobakterier, på många sätt fördelaktiga i jämförelse med växter såväl som alger. Eftersom varken cyanobakterier eller alger är bundna till marken så kan de odlas på mark som inte är brukbar för exempelvis jordbruk. Dessutom har de högre fotosyntetisk effektivitet eftersom de inte spenderar energi på att odla delar så som stammar och rötter. Cyanobakterier har dock fördelen jämfört med alger att de är just bakterier och därmed relativt snabbväxande och lätta att genetiskt manipulera.

(E)-α-bisabolen (bisabolen) är ett kolväte som genom en enda kemisk reaktion kan omvandlas till en potentiell ersättare för dieselbränsle och som tidigare har bevisats kunna produceras i cyanobakterien *Synechocystis* sp. PCC 6803. Målet med detta projekt var att öka produktionen av bisabolen i denna bakterie samt att introducera samma designade system i *Synechococcus elongatus* PCC 7942 i hopp om att öka produktionen ytterligare. I detta avseende tillverkades flera genetiska konstrukt i syfte att öka cellkoncentrationen av ett visst enzym; 1-Deoxy-D-xylulos 5-fosfat syntas (DXS). Detta enzym katalyserar den första i en lång rad av kemiska reaktioner som resulterar i produktionen av bisabolen. Konstrukten introducerades sedan i både *Synechocystis* sp. PCC 6803 och *Synechococcus elongatus* PCC 7942, varpå bisabolenproduktionen av positiva kolonier evaluerades. Ett av konstrukten visade sig öka produktionen i *Synechocystis* sp. PCC 6803 trots att den introducerade DXS genen inte kunde detekteras varken på en genetisk nivå eller som protein. Vad gäller *Synechococcus elongatus* PCC 7942 erhölls inga positiva kolonier efter introduktionen av konstrukten.

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# Abbreviations

| | |
|---|---|
| DMAPP | Dimethylallyl diphosphate |
| DXP | 1-Deoxy-D-xylulose 5-phosphate |
| DXS | 1-Deoxy-D-xylulose 5-phosphate synthase |
| Bisabolene | (E)-α-bisabolene |
| *E. coli* | *Escherichia coli* DH5α |
| FPP | farnesyl pyrophosphate |
| GC | Gas chromatography |
| GC-MS | Gas chromatography mass spectrometry |
| Idi | Isopentenyl diphosphate isomerase |
| IPP | Isopentenyl diphosphate |
| PAGE | Polyacrylamide gel electrophoresis |
| PBS | Phosphate-buffered saline |
| SDS | Sodium dodecyl sulfate |
| *Synechococcus* | *Synechococcus elongatus* PCC 7942 |
| *Synechocystis* | *Synechocystis* sp. PCC 6803 |
| TBS | Tris-buffered saline |

# 1. Introduction

The world is facing many urgent environmental issues related to fossil fuels, such as global warming and air and water pollution. At the same time, the energy demand of the world is growing rapidly, hence the need of sustainable energy sources with a net zero addition to greenhouse gas levels is pressing. One such sustainable solution could be the utilization of the complicated endergonic reaction oxygenic photosynthesis, where water and carbon dioxide are converted to carbohydrates and oxygen using light energy (Johnson 2016). With the help of genetic engineering the energy flow of this reaction can be directed towards production of valuable compounds such as fragrances, pharmaceuticals or even fuels. To achieve this, one need to alternate the metabolic flux of a photosynthesizing organism at the expense of non-vital secondary metabolites. For this purpose, the oxygenically photosynthesizing cyanobacteria are advantageous in many ways compared to plants, and even algae. Since neither cyanobacteria nor algae are earth bound they can be grown on non-agricultural land and they also have a higher photosynthetic efficiency since they do not spend energy on growing parts such as stems and roots (Dismukes *et al*. 2008). However, given that cyanobacteria, as opposed to algae, are prokaryotes they have the advantageous features of being relatively fast growing and easy to engineer.

Cyanobacteria were the first oxygenically photosynthesizing organisms and are the progenitors of today's chloroplasts (Herrero and Flores 2008). They comprise of various kinds of unicellular, filamentous and colonial strains, the most studied being the model unicellular strain *Synechocystis* sp. PCC 6803 (herein referred to as *Synchocystis*) (Trautmann *et al*. 2012). This project has aimed to genetically engineer said strain to become a biological factory producing a precursor of a potential diesel fuel replacement; (E)-α-bisabolene. Currently commercial bisabolene is extracted from plant material and is thereby only available in small amounts and is generally impure (Peralta-Yahya *et al*. 2011). Furthermore, the aim was to introduce the same engineered system in another unicellular strain, *Synechococcus elongatus* PCC 7942 (herein referred to as *Synechococcus*), for comparison.

## 1.1 Project outline

Bisabolene is a so called sesquiterpenoid, which is a terpenoid consisting of 15 carbons. Terpenoids are a group of various organic compounds that are all synthesized from a combination of the same two precursor compounds; isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). In most bacteria, including cyanobacteria and *E. coli*, IPP and DMAPP are in turn produced through the so called MEP pathway, initializing with the 1-Deoxy-D-xylulose 5-phosphate synthase (DXS) driven synthesis of 1-Deoxy-D-xylulose 5-phosphate (DXP) (figure 1) (Pattanaik and Lindberg 2015).

Even though the terpenoid biosynthetic pathway is native in cyanobacteria they do not produce the particular terpenoid bisabolene. However, it has been shown that by introducing a plant gene from the tree *Abies grandis* encoding a bisabolene synthase (AgBIS, figure 1) it is possible to produce small amounts of bisabolene in *Synechococcus* sp. PCC 7002 (Davies *et al*. 2014). In addition, in preliminary, unpublished work by Pia Lindberg's research group the same synthase has been shown to produce bisabolene also in *Synechocystis* sp. PCC 6803. Furthermore said research group have confirmed the farnesyl pyrophosphate (FPP) synthase

to be a bottleneck in the bisabolene production (see figure 1), as hypothesised in Davies *et al*. 2014. The hypothesis was addressed by introducing two different non-native FPP synthases respectively into *Synechocystis* sp. PCC 6803 together with the bisabolene synthase and confirming the increased production by Gas chromatography mass spectrometry (GC-MS). The synthases introduced were CrFPPS from *Chlamydomonas reinhardtii* and IspA from *E. coli* (figure 1).

To further upregulate the bisabolene production this project aimed to introduce two more non-native enzymes further upstream in the pathway that have been shown to be potential bottlenecks in terpenoid production (Englund, E., unpublished). The first of the two enzymes was the DXP synthase, overexpression of which hopefully would increase the metabolic flux into the MEP pathway. The other is the isopentenyl diphosphate isomerase (Idi), which maintain a necessary balance between the two building blocks of all terpenoids; IPP and DMAPP (figure 1).

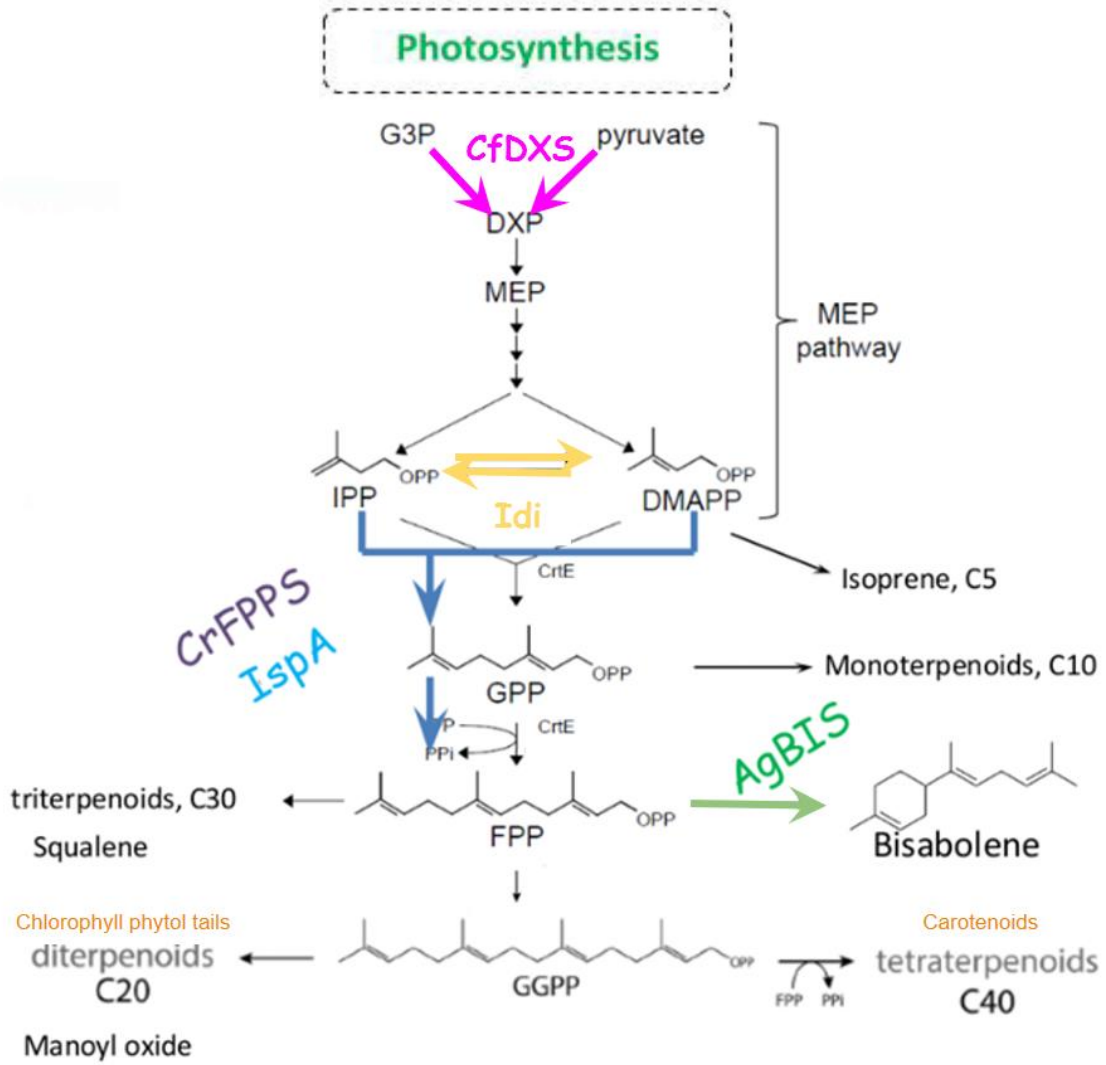


**Figure 1.** Terpenoid biosynthesis through the MEP pathway with introduced non-native enzymes marked with different colors.

## 1.2 Constructs

The constructs previously created by Pia Lindberg's research group have all been placed on the chloramphenicol resistant, pPMQAK1-based pEEC1 vector described in a paper by Englund and Lindberg from 2016. Both pEEC1 and pPMQAK1 are self-replicating plasmids with the broad-range-replicon RSF1010 (Englund, E., unpublished; Huang *et al*. 2010). The genes are regulated by the strong, constitutive trc20 promoter (ptrc20) and tagged for protein detection by western blot (see figure 2a and b). The promoter was chosen based on the initial hypothesis that production rate is positively related to high expression levels.

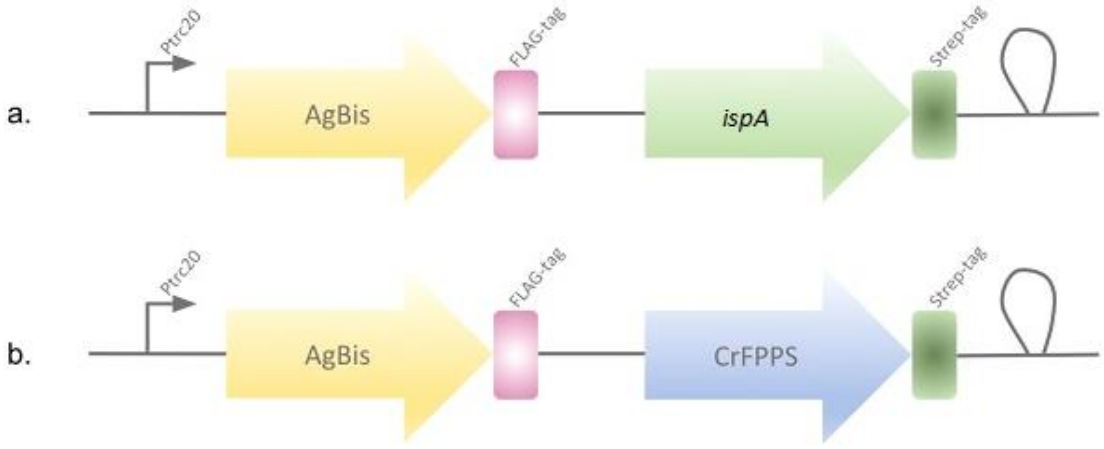


**Figure 2.** Constructs previously created by Pia Lindberg's research group consisting of a chloramphenicol resistant pEEC1 plasmid with a trc20 promoter, the FLAG-tagged bisabolene synthase gene AgBIS, a strep-tagged FPP synthase gene and a terminator. The two constructs contain **(a)** the *E. coli* FPP synthase gene *ispA* and **(b)** the *C. reinhardtii* FPP synthase gene CrFPPS respectively.

For the purpose of this project four new constructs were created (see figure 3a-d). Two of these constructs were self-replicating plasmids containing the constructs shown in figure 2b and c with an additional trc20 promotor and a tagged *Coleus forskohlii* DXS gene (figure 3a and b). Initially the new operon was to be cloned into the already constructed plasmids in figure 2. However, due to cloning difficulties, a three-fragment cloning with the chloramphenicol resistant, pPMQAK1-based pPMQAC1 vector was performed instead (see section 4.3 for details). pPMQAC1 is very similar to pEEC1 and share its broad-range-replicon RSF1010 (Englund, E., unpublished).

The two other constructs each consists of only one operon. Both are placed on a kanamycin resistant so called pEERM3 vector and contain the tagged CfDXS gene, however one also contains the *E. coli idi* gene and an additional strep-tag (figure 3c and d). The pEERM3 vectors are designed to be integrated into the *Synechocystis* genome by homologous recombination (Englund *et al*. 2015), but are unable to self-replicate in its host. The pMB1 origin allows it to replicate in *E. coli*, which is useful for cloning purposes, but not in *Synechocystis*. For both constructs a neutral integration site was chosen and therefore disrupting the site does not affect any known biological function. The genetic sequence is however specific for *Synechocystis*, meaning that the constructs cannot be introduced into *Synechococcus*.

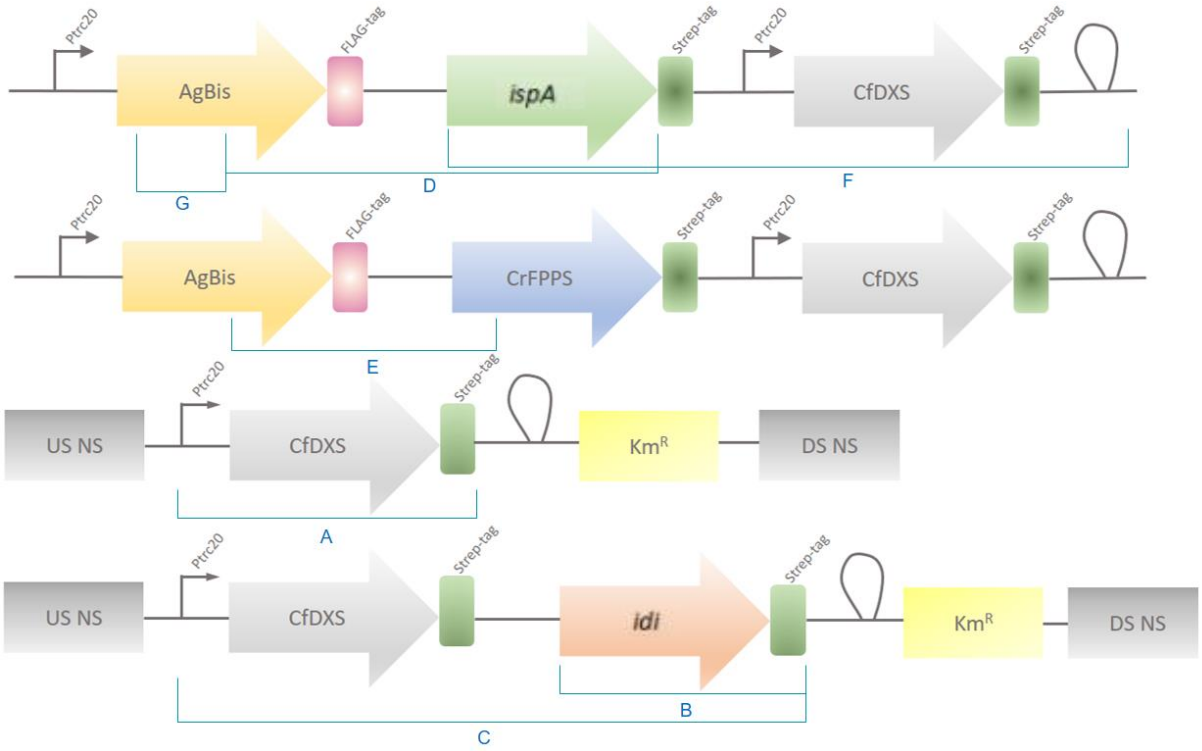


**Figure 3.** Constructs created during this project with primer pairs indicated by blue lines. **(a and b)** pPMQAC1 plasmids with two operons. First operon contains a trc20 promoter, the FLAG-tagged bisabolene synthase gene AgBIS and a strep-tagged (a) *E. coli* FPP synthase gene and (b) *Chlamydomonas reinhardtii* FPP synthase gene respectively. The second operon consists of the strep-tagged *Coleus Forskohlii* DXS gene regulated by the trc20 promoter and is followed by a terminator. **(c and d)** pEERM3 plasmids, with upstream neutral site (US NS) and downstream neutral site (DS NS) for integration into the *Synechocystis* genome, containing one operon each. Both contain a trc20 promoter regulating the strep-tagged *Coleus Forskohlii* DXS gene followed by (c) a terminator and (b) a strep-tagged *idi* gene and a terminator.

# 2. Results

In preliminary, unpublished work Pia Lindberg's research group has produced bisabolene in *Synechocystis* sp. PCC 6803 by introducing a bisabolene synthase. Said research group has also shown that addition of a non-native FPP synthase lead to an increase in production levels. The aim of this project has been to increase the production in cyanobacteria even further by introducing two more non-native genes of the MEP-pathway (figure 1). For this purpose, four constructs were to be created using basic cloning with restriction enzymes and then conjugated into *Synechocystis* and *Synechococcus*. The mutants were then to be grown and experiments to be conducted in order to evaluate potential differences in bisabolene production levels. However, due to many unforeseen complications the experimental outline was altered during the progression of the project.

The DNA ladder used for analysis of PCR products is shown in figure 4. Table 1 show all primer pairs used with their respective expected product length, which are also illustrated in figure 3. All wells of the DNA gels are marked with template DNA followed by primer pair used. See section 4.2 for a description of each template strain.

**Table 1.** PCR primer pairs with expected product length.

| Primer pair | Primers | Expected product length |
|---|---|---|
| A | speI_EcoRI_Ptrc20_F<br>CfDXS_streptag_PstI_R | ~2,2 kb |
| B | XbaI_Idi_F<br>Idi_streptag_SpeI_PstI_R | 623 bp |
| C | speI_EcoRI_Ptrc20_F<br>Idi_streptag_SpeI_PstI_R | ~2,9 kb |
| D | AgB_F2<br>IspA_BgI_F | ~1,7 kb |
| E | AgB_F2<br>CrF_R1 | 882 bp |
| F | IspA_Nde_R<br>VR | ~1,3 kb alt. ~3,5 kb |
| G | AgB_F1<br>AgB_R2 | 930 bp |

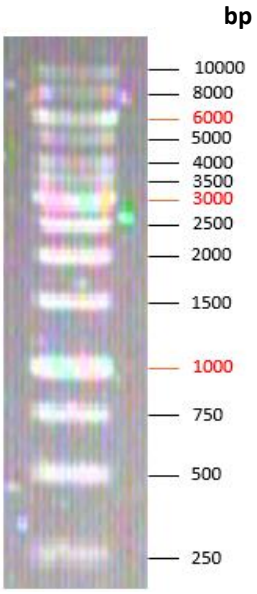


**Figure 4.** DNA ladder used for analysis of PCR products.

## 2.1 Cloning

For construction of the constructs in figure 3 overhang PCR and classic restriction-based cloning were used. See section 4.3 for details. The colony PCR showed that one colony each of the pPMQAC1 constructs had the DXS gene (figure 5) and were thereby kept for further analyzes. As for the pEERM3 constructs (see figure 3 c and d) there were many positive colonies of E_p3DXS and at least two of E_DXS_Idi (figure 6 resp. 7). The later had three colonies, of which two gave products of the expected size for all three primer pairs, although primer pair C also gave some unspecific binding (figure 7).

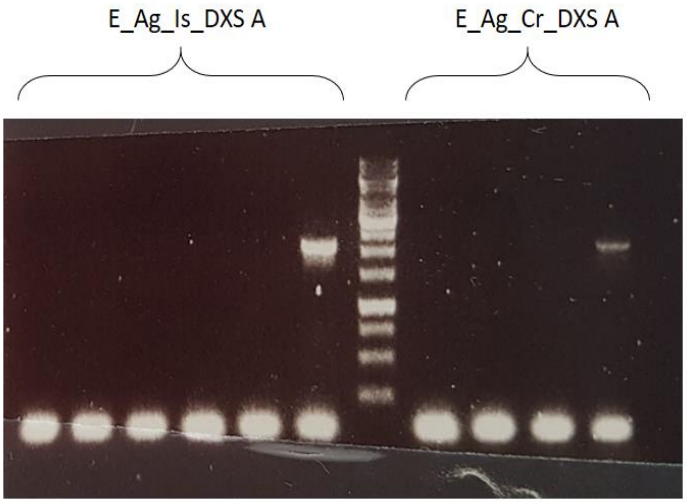


**Figure 5.** Colony PCR of potential E_Ag_Is_DXS and E_Ag_Cr_DXS strains. The letter A after the template name indicate the primer pair used. Templates are described in section 4.2.

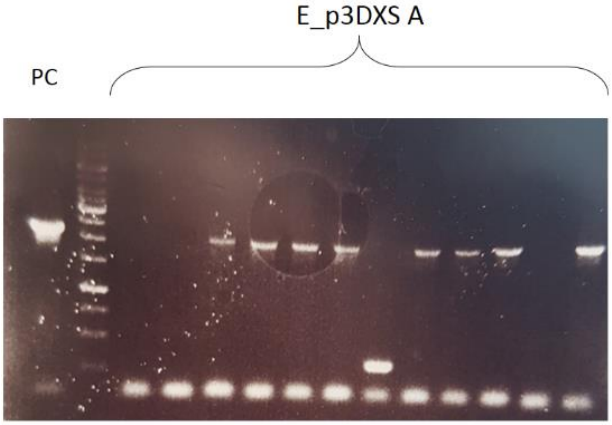


**Figure 6.** Colony PCR of potential E_p3DXS strains. E_p3DXS indicate the template and A the primer pair. Templates are described in section 4.2.

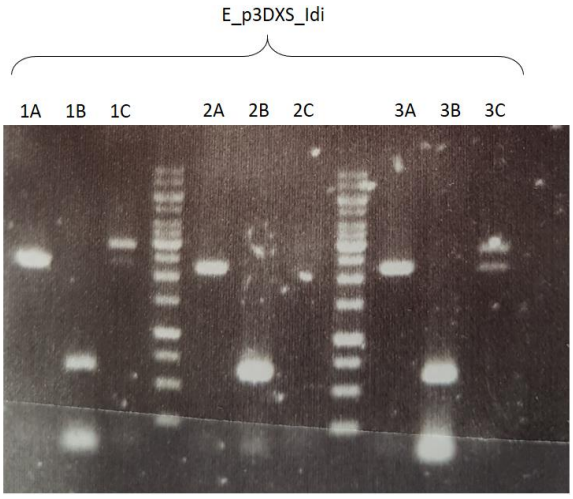


**Figure 7.** Colony PCR of potential E_p3DXS_Idi strains. The number indicate colony and the letter indicate primer pair. Templates are described in section 4.2.

In order to verify the whole sequence of each constructs they were sent for sequencing multiple times (data not shown). However, sequencing proved problematic and therefore not all constructs were fully sequenced. In fact, only the pEERM3 constructs were completely verified, whereas the pPMQAC1 constructs were more troublesome. The sequence of the construct containing the *ispA* gene indicated that it might lack the last nucleotide in the stop codon following the DXS strep-tag. Furthermore, the other pPMQAC1 construct could not be verified to contain neither the bisabolene synthase nor the CrFPPS gene. Only the primers targeting the CfDXS gene generated a sequence.

## 2.2 Conjugation of constructs

After cloning, the constructs where transferred into *Synechocystis* and *Synechococcus* using conjugation. For plasmid conjugation into cyanobacteria a cargo, a conjugal, and in some cases a helper, *E. coli* strain is needed (Heidorn *et al*. 2011). The cargo strain contains the plasmid one wish to introduce to the cyanobacterial cells, also called the cargo plasmid, whereas the conjugal strain contains a plasmid which is able to conjugate into other bacteria, a so called conjugal plasmid. These two strains are mixed, leading to the transfer of the conjugal plasmid into the cargo strain. Cyanobacteria are then added to the *E. coli* mixture, after which the conjugal plasmid will help transfer the cargo plasmid into the cyanobacterial cells. (Heidorn *et al*. 2011) The protocol in section 4.14 has been developed based on this theory and the experiences of members of Pia Lindberg's research group.

The pPMQAC1 constructs were successfully conjugated into *Synechocystis* at the first attempt, whereas the pEERM3 constructs proved unsuccessful. The attempt was repeated several times with small alterations of the protocol in section 4.14, such as lowering of kanamycin concentration to 10 µg/ml, however no colonies were obtained. Conjugation into both wild type *Synechocystis* as well as into S_Ag_Is and S_Ag_Cr was attempted. As for the conjugation into *Synechococcus*, it was also unsuccessful. Both of the pPMQAC1 constructs gave colonies, however when restreaked or grown in liquid culture the cells died. The pEERM3 vectors were never intended for conjugation into *Synechococcus* since they are specifically designed for homologous recombination into the *Synechocystis* genome.

Five colonies of each strain were grown in liquid culture, after which DNA was extracted and analyzed by PCR. According to the results in figure 8, there were no positive S_Ag_Cr_DXS colonies and only one colony of S_Ag_Is_DXS (colony 1) contained the bisabolene synthase and FPP synthase, but lacked the CfDXS gene. The control strain, S_Ag_Is, did however not give a product for primer pair D, indicating that it might have lost its construct. Furthermore, it seemed as if the E_Ag_Cr_DXS strain lacked either the bisabolene synthase, the FPP synthase or both (see figure 8c, well 8), meaning that it was something wrong with the construct itself.

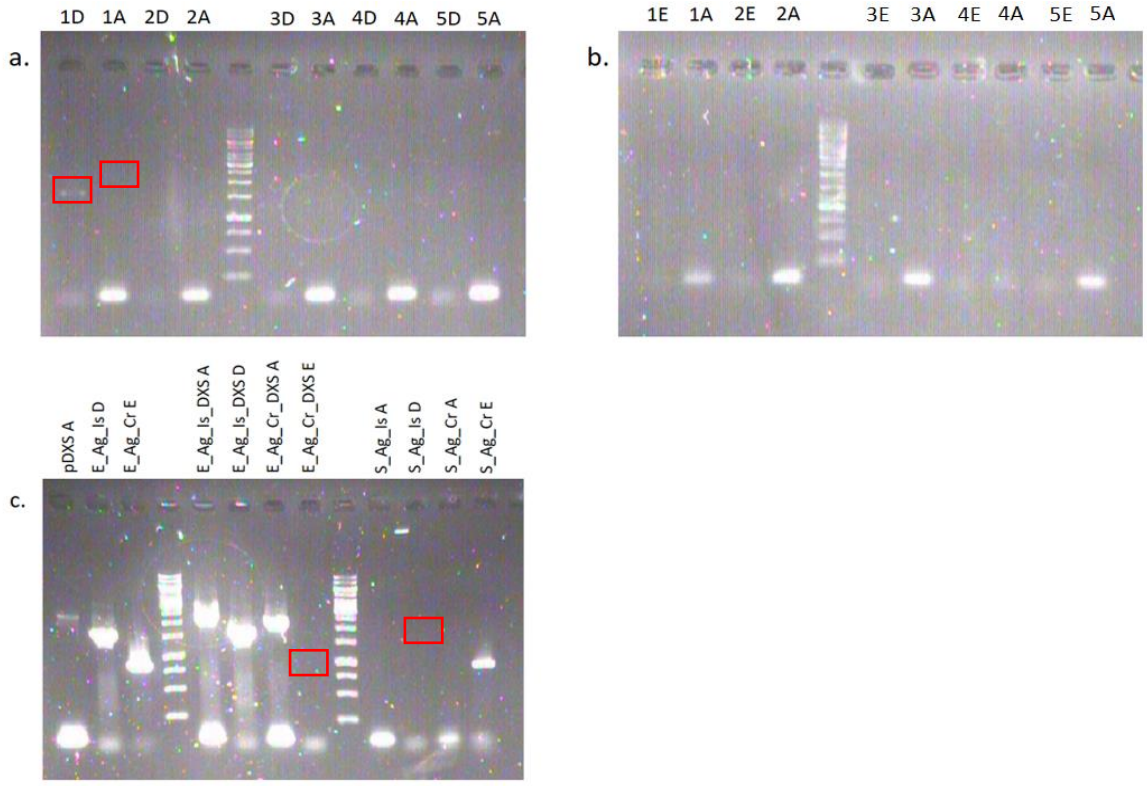


**Figure 8.** Colony PCR of potential **(a)** S_Ag_Is_DXS and **(b)** S_Ag_Cr_DXS strains. **(c)** Control reactions. S_Ag_Is A and S_Ag_Cr A are negative controls and the rest are positive controls. The letters after the templates indicates the primer pairs. Templates are described in section 4.2.

To further analyze colony 1 of S_Ag_Is_DXS, and the S_Ag_Is, strain another PCR was performed with more template. Though the band is week, the results indicate that S_Ag_Is do contain both the AgBIS and the *ispA* gene, and thereby has not lost its construct after all (see figure 9a, well 2). These genes are also in the S_Ag_Is_DXS strain, however it appears to lack CfDXS (see figure 9a, well 7). When attempting to verify whether it contains DXS or not by amplification using primer pair F there is no product (see figure 9b). This might simply be due to incompatible primers.

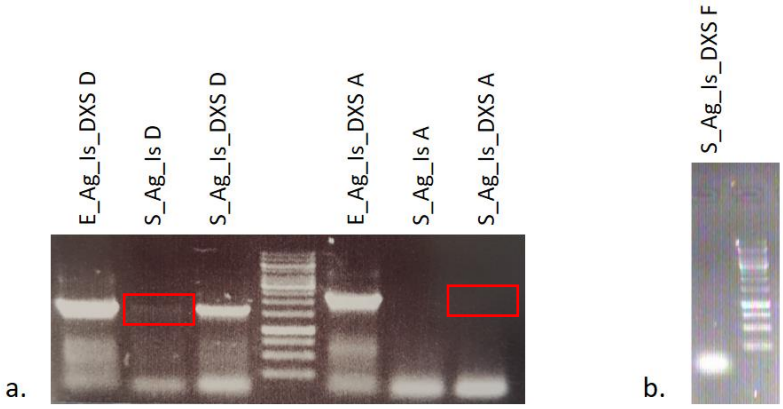


**Figure 9.** Further analysis of S_Ag_Is_DXS colony 1, and the S_Ag_Is strain, using more template. Templates are described in section 4.2. **(a)** Analysis to determine whether S_Ag_Is_DXS and S_Ag_Is contains all inserted genes. **(b)** Additional analysis to determine whether S_Ag_Is_DXS contain DXS.

Given that conjugation of the pEERM3 constructs was unsuccessful, their effect of bisabolene production could not be analyzed in *Synechocystis*. Furthermore, since they are self-replicating in *E. coli* and does not integrate into the genome, a production analyzes in *E. coli* would not be representative to their effect in *Synechocystis*. Nevertheless, a relative comparison between the constructs containing respectively lacking the *idi* gene might give an indication of the genes importance in the bisabolene biosynthesis pathway. In order to measure the pEERM3 vectors effect on the production, however, strains containing both a bisabolene synthase and respective constructs needed to be created. For this purpose, several double transformations were performed into *E. coli*. The transformations where one of the constructs was the pEERM3 vector lacking *idi* yielded no colonies and due to limited time the attempt was not repeated. The other transformations, however, yielded many colonies which unfortunately were all negative. They all contained only one of the plasmids; the pPMQAC1-based (see figure 10a). Furthermore, the control in figure 10b once again indicate that E_Ag_Cr_DXS lack either the bisabolene synthase, the FPP synthase or both.

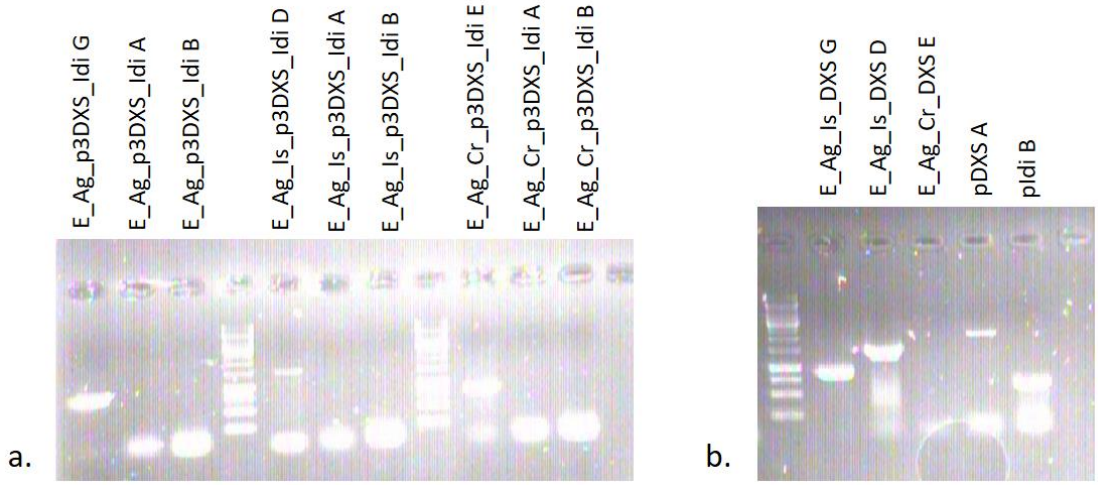


**Figure 10.** Colony PCR of potential E_Ag_p3DXS_Idi, E_Ag_Is_p3DXS_Idi and E_Ag_Cr_p3DXS_Idi strains (a), and positive controls (b). Templates are described in section 4.2.

## 2.3 Western blot and SDS-PAGE

Other than by PCR, the strains were evaluated based on their protein expression using SDS-PAGE and Western blot (see ladder in figure 11). Most of the SDS-PAGE gels did not show any difference between the controls and the constructs, however figure 12 indicate a quite high overexpression of a protein of approximately 30 kDa in E_p3DXS_Idi. Since the size of the protein does not match that of neither CfDXS nor Idi, it is unclear which protein it is. However, the corresponding strep-tag Western blot gave no bands (data not shown), indicating that it might be a truncated version of CfDXS lacking the tag.

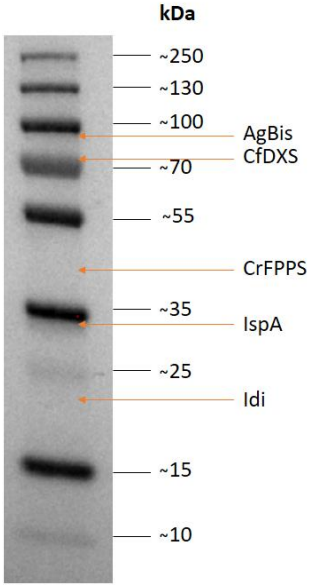


**Figure 11.** Protein ladder used for analysis by SDS-PAGE and Western blot. The expected protein sizes are 95 kDa for AgBIS, 75 kDa for CfDXS, 42 kDa for CrFPPS, 34 kDa for IspA and 21 kDa for Idi.

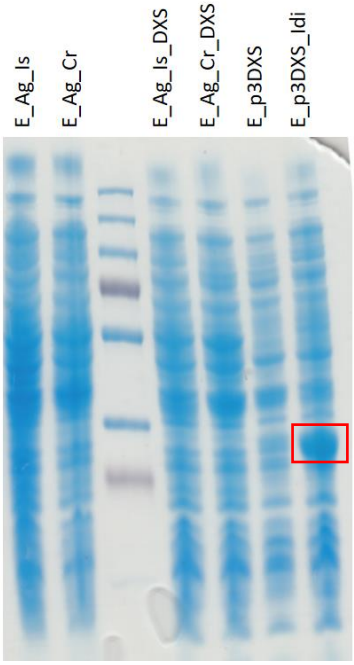


**Figure 12.** Loading control SDS-PAGE gel for western blot. Strains are described in section 4.2.

As for the western blots, they all had the lack of CfDXS signal in common. The strep-tag antibody blot in figure 13 show bands for the control E_Ag_Is and E_Ag_Cr strains, but not for the E_p3DXS strain. The band from E_Ag_Is appears to be too big to be IspA, however based on the bent ladder this is likely because the gel did not run straight. Furthermore, the E_Ag_Cr strain give several bands, the thicker of which seems to be the FPP synthase and the others are probably unspecific binding.

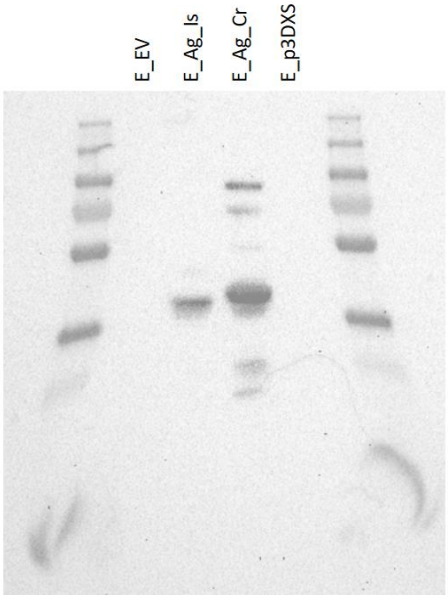


**Figure 13.** Western blot of *E. coli* strains with strep-tag antibodies. Strains are described in section 4.2.

Western blots of *E. coli* strains containing all constructs, including controls, are presented in figure 14. Since the bisabolene synthase is the only protein that is FLAG-tagged, only the controls and the strains with the pPMQAC1 constructs were expected to give a signal for the FLAG-tag antibodies. However, though it was expected that the pEERM3 strains would lack AgBIS, it seems as if E_Ag_Cr_DXS lacks it as well (figure 14a). Furthermore, the corresponding strep-tag blot gives unspecifric binding at just below 25 kDa for the controls and pPMQAC1 based strains, but no signal for CfDXS from any of the constructs. Strain E_Ag_Cr_DXS has a band of approximately the size of CfDXS, however it is probably an unspecific band since the protein pattern is just like the pattern of control strain E_Ag_Cr in figure 13. As for the E_p3DXS_Idi strain there was no detection of Idi, and IspA was not detected in neither E_Ag_Is_DXS nor the control strain E_Ag_Is.

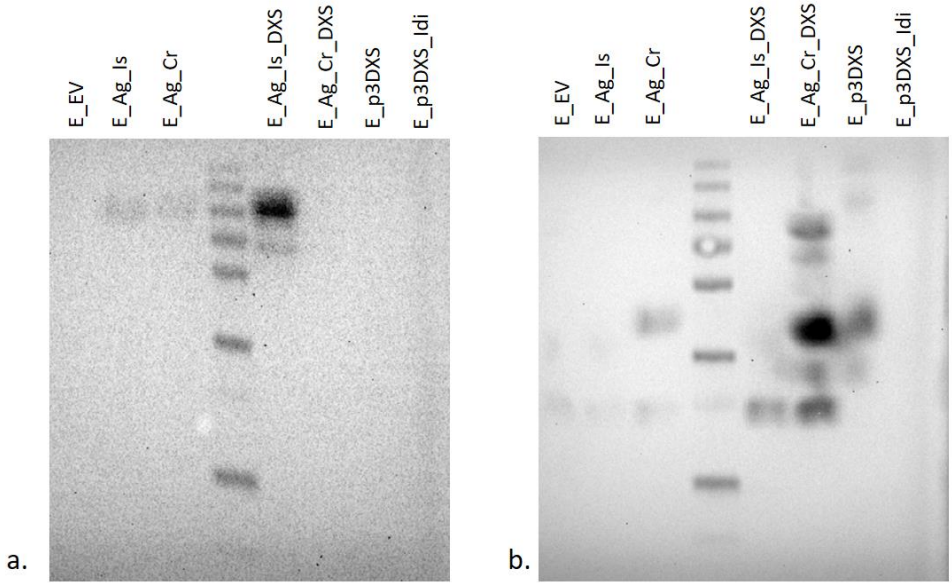


**Figure 14.** Western blots of *E. coli* strains with **(a)** FLAG-tag and **(b)** strep-tag antibodies. Strains are described in section 4.2.

The five colonies of each engineered *Synechocystis* strain were also analyzed with western blot (figure 15). However, no signal was detected except for the unspecific binding at just below 25 kDa that was also detected in the *E. coli* blot (see figure 14b). Not even the FPP synthases could be detected in any of the engineered strains or the controls. The bisabolene synthase was not detected either by the FLAG-tag antibodies (data not shown).

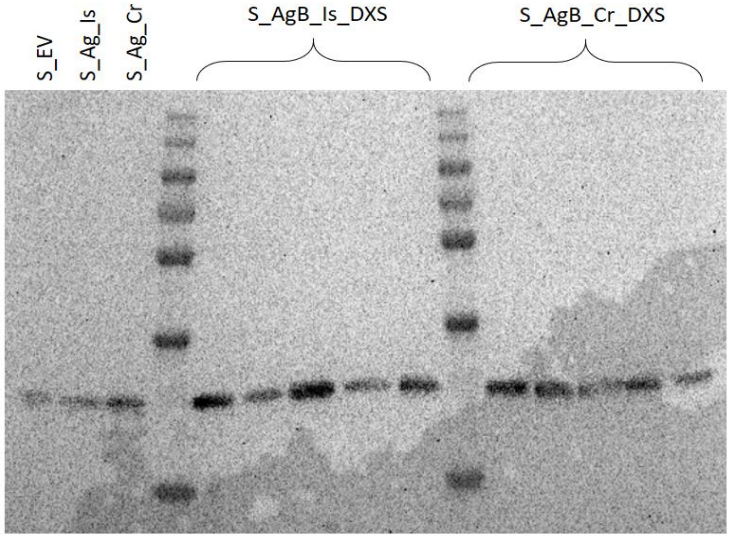


**Figure 15.** Western blot of *Synechocystis* colonies with strep-tag antibodies. Strains are described in section 4.2.

## 2.4 Production experiments

The bisabolene production was measured for each colony of the S_Ag_Is_DXS and S_Ag_Cr_DXS strains, as well as for all *E. coli* strains containing the pPMQAC1 constructs. The measurements were performed using the gas chromatography (GC) method described in section 4.21, resulting in a (E)-α-bisabolene peak at approximately 10.8 minutes (see figure 16). The purpose of the first measurement of the *Synechocystis* colonies was not to measure the level of production, but to determine whether they produced bisabolene at all. The only colony producing bisabolene was S_Ag_Is_DXS colony 1 (data not shown) and therefore it was also the only colony kept for further analysis.

The second measurement was a relative comparison between the production levels of S_Ag_Is_DXS and the previously engineered strains S_Ag and S_Ag_Is. The experiment was performed in biological triplicates and all GC and OD measurements were performed in technical triplicates. Figure 17 indicate a significant increase in production level for the S_Ag_Is_DXS strain compared to the other two strains.

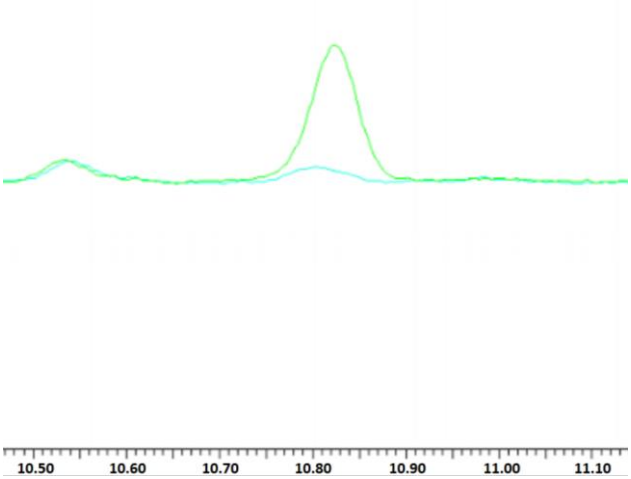


**Figure 16.** GC analysis of S_Ag_Is (green) and pure dodecane solvent (turquoise). The S_Ag_Is strain give a bisabolene peak at just over 10.8 minutes.

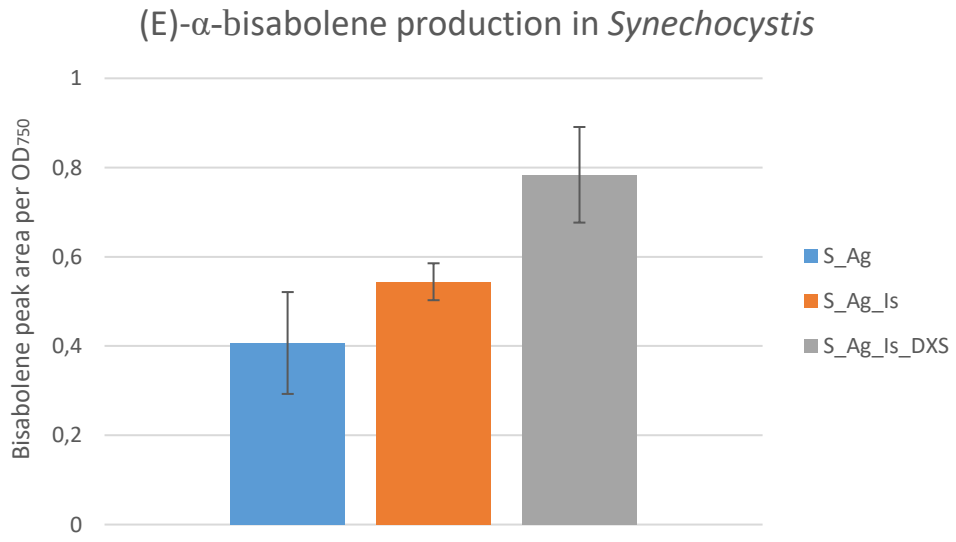


**Figure 17.** (E)-α-bisabolene production in bisabolene producing *Synechocystis* strains measured by gas chromatography. The production is measured in bisabolene peak area per $OD_{750}$, were the peak area is normalized to the peak area of the internal standard β-caryophyllene. The value for each strain is the mean value of the three biological triplicates, and the error bars represent the standard deviation. The strains are described in section 4.2.

Furthermore, since the CfDXS gene could not be detected by PCR in the *Synechocystis* strain S_Ag_Is_DXS, but was detected in the corresponding *E. coli* strain E_Ag_Is_DXS the bisabolene production was also evaluated in *E. coli*. In addition, the E_Ag_Cr_DXS strain was included in order to confirm the loss of the bisabolene synthase, which had been indicated by both PCR and western blot. The previously created constructs, and an empty pPMQAC1 vector, were also included for comparison. The experiment was performed in biological triplicates for all strains except S_Ag_Cr, for which too little internal standard was accidentally added to one of the triplicates. In addition, all GC and OD measurements were performed in technical triplicates. The results are presented in figure 18. See section 4.19 and 4.20 for a more extensive description of the culturing conditions.

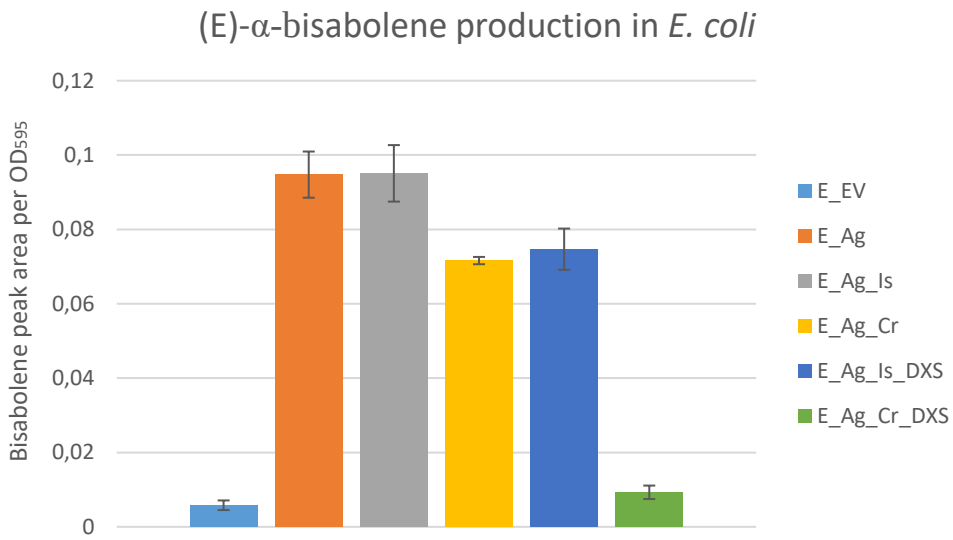


**Figure 18.** (E)-α-bisabolene production in *E. coli* strains containing the pEEC1 and pPMQAC1 constructs measured by gas chromatography. The production is measured in bisabolene peak area per $OD_{595}$, were the peak area is normalized to the peak area of the internal standard β-caryophyllene. The value for each strain is the mean value of the three biological triplicates, and the error bars represent the standard deviation. The strains are described in section 4.2.

# 3. Discussion

The results of this project are somewhat contradictory and leave a lot of questions unanswered. Already at conjugation and cultivation things became problematic when the *Synechococcus* strains would not grow in liquid culture and the *Synechocystis* strains with the pEERM3 constructs did not grow at all. The reason for why these cells would not grow is unclear, however since conjugation of other constructs did work it does not seem to be anything wrong with the conjugation method itself. Therefore, it is most likely that the pEERM3 vectors were in fact conjugated into the cells, but for some reason failed to introduce the construct into the genome by homologous recombination. Furthermore, protein analysis of the E_p3DXS_Idi strain show an overexpression of an unknown protein, which might be a truncated version of CfDXS. As for why the *Synechococcus* strains did not grow in liquid culture my best, and only, guess is that the concentration of antibiotics was too high. In previous publications, a working concentration of 7.5-10 µg/ml chloramphenicol has been used for *Synechococcus* (Heidorn *et al*. 2011), whereas I used the same concentration as for *Synechocystis*; 25 µg/ml. However, even though the fact that cells are more sensitive to antibiotics in liquid culture could explain why they survived on the first plate but not in the media, it does not explain why the cells did not grow when they were restreaked on new plates.

The conjugations of the pPMQAC1 constructs were successful, however other problems were accounted instead. As indicated by PCR and western blot (figure 8c resp. 14a), and confirmed by bisabolene measurements (figure 18), the construct containing the CrFPPS lacks the bisabolene synthase. This was not discovered after cloning since, in theory, the cloning method is designed so that the plasmid fragments can only ligate together in one way, and therefore the colony PCR only screened for the DXS gene (figure 5). How the construct was assembled without the bisabolene synthase is a mystery, especially considering that the western blot in figure 14b indicate expression of CrFPPS, which is located after AgBIS in the same operon. The western blot of the *Synechocystis* colonies however, did not give any signal for neither the FLAG-tag nor the strep-tag antibodies (figure 15). Although, considering that the positive controls did not give a signal either, no conclusions can be drawn from that blot.

As for the pPMQAC1 construct with the *ispA* gene, the results are quite contradictive. The gel picture in figure 9 indicate that the construct itself has been cloned successfully, however neither the IspA protein nor the CfDXS protein was detected by the western blot (figure 14b). In addition, sequencing indicated that the last nucleotide of the stop codon following the strep-tagged CfDXS gene might be missing. On the other hand, the IspA protein was not detected in the positive control either, and the CfDXS protein was not detected in any construct even though the PEERM3 vectors were completely confirmed by sequencing. Furthermore, a frame shift at the stop codon would give a longer protein but leave an intact strep-tag, meaning that it would still be detectable by western blot. Why the CfDXS protein was not detected in any of the strains is unclear, however it seems unlikely that it was due to lack of expression from all constructs.

After conjugation into *Synechocystis* however, CfDXS could no longer be detected even on a genetic level (figure 9) and yet, it gave an increased bisabolene production compared to both

the S_Ag and the S_Ag_Is strain. The increase of production level from S_Ag_Is to S_Ag_Is_DXS was 44%, making it evident that something related to terpenoid production differ between the strains. Apart from expression of the CfDXS protein I have no ideas regarding what that difference might be. On the other hand, I see no possible explanation for the PCR amplification of CfDXS not working other than one of the primers of primer pair A not annealing. If the CfDXS is indeed expressed, the promotor must be intact, however the protein might be functional without the last amino acids of the C-terminal. This hypothesis would mean that the truncated CfDXS did not have a step-tag and thereby would not be detectable by western blot. It is however important to remember that it does not explain why the protein was not detected by the blot of the *E. coli* strain E_Ag_Is_DXS.

Apart from the bisabolene production measurements of the *Synechocystis* strain S_Ag_Is_DXS, all results indicate that CfDXS is not expressed from any of the constructs. The protein could not be detected by western blot and the E_Ag_Is_DXS strain did not give an increased production level. However, the control constructs with the FPP synthases, which have proven to give an increased bisabolene production in *Synechocystis*, did not increase the production level in *E. coli* either. In fact, one of the control strains, E_Ag_Cr_DXS, somewhat decreased the production, which leads me to believe that the bottlenecks of the terpenoid biosynthesis pathway may not be the same for *E. coli* as for *Synechocystis*. Nevertheless, DXS has previously been shown to increase the production of another terpenoid, isoprene, in *E. coli* (Englund, E. unpublished). Provided that CfDXS is expressed in E_Ag_Is_DXS, this means that the bottleneck of bisabolene production is downstream of the MEP pathway (see figure 1). In addition, according to figure 18 the FPP synthase can be ruled out, leaving the bisabolene synthase itself as the only possible bottleneck candidate.

In conclusion, the effect of the DXS enzyme on the bisabolene production can not be determined based on the results of this project. One of the engineered *Synechocystis* strains give a significant increase in production, however CfDXS can not be detected on neither a genetic or a translational level. In addition, the corresponding *E. coli* strain, E_Ag_Is_DXS, show no increase in bisabolene production compared to the control strains even though the CfDXS was detected by PCR. Because of the somewhat contradictive results, it is unclear whether the strains actually express CfDXS and therefore no conclusions can be drawn regarding its effects on the bisabolene production. It seems however as if the bottlenecks of the bisabolene biosynthesis pathway may be different for *E. coli* compared to *Synechocystis*.

# 4. Material and methods

## 4.1 Instruments

Spectrophotometer

Varian cary 50 bio UV-visible spectrometer

Thermo scientific nano-drop 2000 UV-visible spectrometer

Thermocycler

BioRad MJ mini gradient thermal cycler

Plate reader

Hidex Chameleon 4.47

Cell homogenizer

Betin technologies - precellys 24 homogenizer

Blotting transfer machine

BioRad Trans-Blot Turbo System

## 4.2 Bacterial strains

*Escherichia coli* DH5α

*Synechocystis* sp. PCC 6803

*Synechococcus elongatus* PCC 7942

**Table 2.** Successfully and unsuccessfully engineered strains.

| Strain name | Description | Successful / Unsuccessful |
|---|---|---|
| E_EV | *E. coli* containing an empty pPMQAC1 vector | Successful |
| E_Ag | *E. coli* containing a pEEC1 vector with the AgBIS gene | Successful |
| E_Ag_Is | *E. coli* containing a pEEC1 vector with the AgBIS and *ispA* genes | Successful |
| E_Ag_Cr | *E. coli* containing a pEEC1 vector with the AgBIS and CrFPPS genes | Successful |
| E_Ag_Is_DXS | *E. coli* containing a pPMQAC1 vector with the AgBIS, *ispA* and DXS genes | Successful |
| E_Ag_Cr_DXS | *E. coli* containing a pPMQAC1 vector with the AgBIS, CrFPPS and DXS genes | Unsuccessful |
| E_p3DXS | *E. coli* containing a pEERM3 vector with the DXS gene | Successful |

| | | |
|---|---|---|
| E_p3DXS_Idi | *E. coli* containing a pEERM3 vector with the DXS and *idi* gene | Successful |
| E_Ag_p3DXS | *E. coli* containing an pEEC1 vector with the AgBIS gene and a pEERM3 vector with the DXS gene | Unsuccessful |
| E_Ag_p3DXS_Idi | *E. coli* containing an pEEC1 vector with the AgBIS gene and a pEERM3 vector with the DXS and *idi* genes | Unsuccessful |
| E_Ag_Is_p3DXS | *E. coli* containing an pEEC1 vector with the AgBIS and *ispA* genes and a pEERM3 vector with the DXS gene | Unsuccessful |
| E_Ag_Is_p3DXS_Idi | *E. coli* containing an pEEC1 vector with the AgBIS and *ispA* genes and a pEERM3 vector with the DXS and *idi* genes | Unsuccessful |
| E_Ag_Cr_p3DXS | *E. coli* containing an pEEC1 vector with the AgBIS and CrFPPS genes and a pEERM3 vector with the DXS gene | Unsuccessful |
| E_Ag_Cr_p3DXS_Idi | *E. coli* containing an pEEC1 vector with the AgBIS and CrFPPS genes and a pEERM3 vector with the DXS and *idi* gene | Unsuccessful |
| S_EV | *Synechocystis* containing an empty pEEC1 vector | Successful |
| S_Ag | *Synechocystis* containing a pEEC1 vector with the AgBIS gene | Successful |
| S_Ag_Is | *Synechocystis* containing a pEEC1 vector with the AgBIS and *ispA* genes | Successful |
| S_Ag_Is_DXS | *Synechocystis* containing a pPMQAC1 vector with the AgBIS, *ispA* and DXS genes | ? |
| S_Ag_Cr_DXS | *Synechocystis* containing a pPMQAC1 vector with the AgBIS, CrFPPS and DXS genes | Unsuccessful |
| S_Ag_Is_p3DXS | *Synechocystis* containing an pEEC1 vector with the AgBIS and *ispA* genes and a pEERM3 vector with the DXS gene | Unsuccessful |
| S_Ag_Cr_p3DXS | *Synechocystis* containing an pEEC1 vector with the AgBIS and CrFPPS genes and a pEERM3 vector with the DXS gene | Unsuccessful |
| S_Ag_Is_p3DXS_Idi | *Synechocystis* containing an pEEC1 vector with the AgBIS and *ispA* genes and a pEERM3 vector with the DXS and *idi* genes | Unsuccessful |

## 4.3 Cloning

The two constructs containing the bisabolene synthase, FPP synthase and CfDXS gene (figure 3a and b) were created using a three-fragment cloning. This was done by ligating two PCR fragments and the pPMQAC1 vector together. The primers VF2 and VR (see appendix A, table 3) were used to amplify the constructs previously constructed by Pia Lindberg's research group, whereas the DXS gene, including promoter, was amplified from another plasmid using the speI_EcoRI_Ptrc20_F and CfDXS_streptag_PstI_R primers (see appendix A, table 3). These fragments where then digested with EcoRI and SpeI, and SpeI and PstI restriction enzymes respectively and cloned into the EcoRI and PstI-digested pPMQAC1 vector, resulting in the two constructs in figure 3 a and b.

The EcoRI restriction enzyme was used also for the cloning of the pEERM3-vector constructs (figure 3c and d). However, since the *C. forskohlii* DXS gene contain an EcoRI site it first had to be removed by site-directed mutagenesis using the DXS_SDmut_F and DXS_SDmut_R primers (see appendix A). The DXS gene, including the promotor was then amplified using the speI_EcoRI_Ptrc20_F and CfDXS_streptag_SpeI_R primers (see appendix A), digested with EcoRI and SpeI, and ligated into the EcoRI and SpeI digested pEERM3 vector (figure 3c). Finally, the resulting plasmid was used to create the last construct containing the idi gene (figure 3d). It was digested using the SpeI and PstI restriction enzymes and ligated together with an XbaI and PstI-digested PCR fragment amplified from another plasmid using the XbaI_Idi_F and Idi_streptag_SpeI_PstI_R primers (see appendix A).

## 4.4 Cyanobacterial cultivation

Cyanobacteria was cultivated either in liquid BG11 media (see appendix B for recipe) or on BG11 plates with 1.5% agar. Mutants were selected and cultivated by addition of appropriate antibiotics. If not otherwise stated chloramphenicol and kanamycin were added to a final concentration of 20 µg/ml and 25 µg/ml respectively.

## 4.5 *Escherichia coli* cultivation

*E. coli* was cultivated either in liquid LB media or on LB agar plates of 1.5% agar. Mutants were selected and cultivated by addition of appropriate antibiotics. If not otherwise stated chloramphenicol and kanamycin were added to a final concentration of 35 µg/ml and 50 µg/ml respectively.

## 4.6 Plasmid preparation

Plasmid preparations were performed on 20 ml *E. coli* overnight cultures using Thermo Scientific GeneJET Plasmid Miniprep Kit. The plasmids were eluted in deionized water and then stored at -20°C.

## 4.7 Restriction enzyme digestion

Digestion was performed using Thermo Scientific Fast Digest enzymes according to manual from the manufacturer with the exception of a longer digestion time of 30 minutes.

## 4.8 Purification of restriction digest

Purification of restriction digest was performed using Zymo Research DNA Clean and Concentrator kit according to manual from the manufacturer.

## 4.9 Ligation of constructs

Ligation of constructs was performed using Biolabs QuickLigase according to manual from the manufacturer.

## 4.10 Transformation of *Escherichia coli*

Transformation of *E. coli* was performed using the following protocol:

1. Thaw component cells on ice for 15 minutes.
2. Add 5 µl ligation mixture or approximately 10 ng whole plasmid to 50 µl of competent cells.
3. Incubate for 5 minutes on ice.
4. Heat shock for 45s at 42°C.
5. Incubate for 5 minutes on ice.
6. Add 950 µl of LB media (pre-heated to 37°C).
7. Incubate for 1-1.5h at 37°C.
8. Spread mixture on an LB agar plate with the appropriate antibiotics.

The amount of cells plated varied, 50 µl being the most common. In some cases, the remainder of the mixture was spun down after which the pellet was resuspended and spread.

## 4.11 Polymerase Chain Reaction (PCR)

PCR was performed using two different enzymes; phusion HS polymerase and DreamTaq polymerase.

### 4.11.1 Phusion HS polymerase

Here follows a recipe for a 20 µl reaction and a thermocycler program using phusion HS polymerase. The reaction can be scaled up or down as desired.

| | |
|---|---|
| $dH_2O$ | 12.4 µl |
| 5x buffer | 4 µl |
| 10 mM dNTPs | 0.4 µl |
| Primer 1 (10 µM) | 1 µl |
| Primer 1 (10 µM) | 1 µl |
| Template | 1 µl |
| Polymerase | 1 µl |

Program:

| | | |
|---|---|---|
| | 98°C | 30 s |
| x 35 | 98°C | 10 s |
| | $T_A$ | 30 s |
| | 72°C | 30 s/kb |
| | 72°C | 5 min |

### 4.11.2 DreamTaq polymerase

Here follows a recipe for a 20 µl reaction and a thermocycler program using DreamTaq polymerase. The reaction can be scaled up or down as desired.

| | |
|---|---|
| $dH_2O$ | 12.5 µl |
| 10x buffer | 2 µl |
| 10 mM dNTPs | 0.4 µl |
| Primer 1 (10 µM) | 2 µl |
| Primer 1 (10 µM) | 2 µl |
| Template | 1 µl |
| Polymerase | 0.1 µl |

Program:

| | | |
|---|---|---|
| | 95°C | 3 min |
| | 95°C | 30 s |
| x 35 | $T_A$ | 30 s |
| | 72°C | 2 min/kb |
| | 72°C | 15 min |

## 4.12 Purification of PCR products

Purification of PCR products was performed using Thermo Scientific GeneJET PCR Purification Kit according to manual from the manufacturer.

## 4.13 Sequencing

Samples were mixed with appropriate primers according to instructions and sent to Eurofins Genomics for Sanger sequencing.

## 4.14 Conjugation into cyanobacteria

Conjugation into cyanobacteria was performed using pRL443 as conjugal plasmid according to the following protocol:

1. Grow *E. coli* cultures containing plasmids to be conjugated and pRL443 respectively over night.
2. Centrifuge 1 ml of each overnight culture at maximum speed for 1 minute.
3. Remove supernatant.
4. Wash pellet twice with fresh LB without antibiotics.
5. Resuspend pellet in 50 µl LB without antibiotics.
6. Mix the two cultures.
7. Depending on the cyanobacterial culture density either
    a. add 20 µl cyanobacterial culture to the *E. coli* mixture.
    b. concentrate the cyanobacterial culture before adding 20 µl to the *E. coli* mixture.
8. Place cellulose filters on BG11 agar plates without antibiotics.
9. Spread the culture mixture on the cellulose filters.
10. Place the plates in 30°C and low light for 24-48 hours.
11. Move filters to BG11 agar plates containing the appropriate antibiotics.
12. Place in 30°C and high light. After approximately 2 weeks single colonies will appear given that the conjugation was successful.

## 4.15 DNA extraction from *Synechocystis*

DNA extraction from *Synechocystis* was performed using the following protocol:

1. Centrifuge 30-50 ml of culture at maximum speed for 10 minutes.
2. Discard supernatant.
3. Resuspend pellet in 500 µl of 50 mM Tris-HCl, pH 8.0, with 10 mM EDTA.
4. Add 0.6-mm-glass beads, 12.5 ml 20% SDS and 500 µl of phenol:chloroform (1:1 [v/v]).
5. Disrupt the cells using a cell homogenizer. 3 cycles of 5800 rpm for 30 seconds with 2 minutes on ice between the runs.
6. Centrifuge at 4°C, maximum speed for 15 minutes.
7. Move the supernatant into a new Eppendorf tube, taking care not to disrupt the interface between the phases.
8. Add an equal volume of chloroform and mix.
9. Repeat steps 6-8.
10. Repeat steps 6 and 7.
11. Add 3/2 volume of 2 M sodium acetate (pH 5.2) and mix.
12. Add 2.5 volumes of 100% ethanol.
13. Incubate in -20°C for at least 30 minutes.
14. Centrifuge at 4°C, maximum speed for 20 minutes.
15. Wash pellet with ice cold 70% ethanol.
16. Air dry the pellet and resuspend in $dH_2O$.

## 4.16 Crude cell extract preparation from *Synechocystis*

Crude cell extract preparation from *Synechocystis* was performed using the following protocol:

1. Centrifuge 30-50 ml culture at maximum speed for 10 minutes.
2. Resuspend pellet in 2 ml PBS buffer.
3. Centrifuge at maximum speed for 5 minutes. From here on keep the samples on ice.
4. Add 200 µl PBS, 3µl protease inhibitors and glass beads.
5. Disrupt the cells using a cell homogenizer. 4 cycles of 5800 rpm for 30 seconds with 2 minutes on ice between the runs.
6. Add 100 µl PBS.
7. Centrifuge at 4°C, maximum speed for 1 minute.
8. Collect supernatant.
9. Repeat step 7 and 8.

## 4.17 SDS-PAGE

Preparation of protein samples were different for *E. coli* and *Synechocystis*. For *E. coli* 1 ml of overnight culture was centrifuged for 1 minute at maximum speed. The pellet was then resuspended in 35 µl Tris (50 mM) - EDTA (10 mM) to a total volume of approximately 40 µl and then mixed with 20 µl 3x sample buffer with 3% 2β-mercaptoethanol. For *Synechocystis* on the other hand 40 µl of the crude cell extract (see 5.15) was directly mixed with 20 µl of the sample buffer.

The remaining procedure was performed according to the following protocol:

1. Denature protein samples in 95°C for 10 minutes.

2. Load the gel and run at 200 V for approximately 30 minutes.
3. Stain gel with Coomassie Brilliant Blue for at least 1 hour.
4. Destain gel with $dH_2O$ for at least 3 hours.

## 4.18 Western blot

For protein analysis of the mutant strains western blot was used, a technique in which proteins are detected using antibodies. This technique is carried out in three steps. First the proteins are separated according to size using a gel based method such as SDS-PAGE. The proteins are then transferred to a solid support membrane, after which they are detected using target specific primary and secondary antibodies (Mahmood and Yang 2012). In this case, the target of the primary antibodies were the tags of the introduced genes; the FLAG- and strep-tag respectively. As a loading control a separate SDS-PAGE gel was always run and stained with Coomassie Brilliant Blue.

Western blot was performed according to the following protocol:

1. Run an SDS-PAGE until step 2 of the protocol.
2. Transfer proteins from gel to membrane using the BioRad Trans-Blot Turbo System according to instructions.
3. Shake membrane in 0.05% tween-TBS with 5% milk powder slowly for at least 1 hour.
4. Shake membrane in 0.05% tween-TBS for 5 minutes.
5. Repeat step 4 twice.
6. Add primary antibody diluted in appropriate amount of 0.05% tween-TBS.
7. Shake for at least 1 hour.
8. Wash ones with 0.05% tween-TBS.
9. Shake membrane in 0.05% tween-TBS for 5 minutes.
10. Repeat step 9 twice.
11. Add secondary antibody diluted in appropriate amount of 0.05% tween-TBS.
12. Shake for at least 1 hour.
13. Wash ones with 0.05% tween-TBS.
14. Shake membrane in 0.05% tween-TBS for 5 minutes.
15. Repeat step 9 twice.
16. Add substrates for horseradish peroxidase (HRP) according to BioRad.
17. View membrane in the BioRad ChemiDoc XRS+ system.

## 4.19 Bisabolene experiment in *Synechocystis*

Biological triplicates of each strain were inoculated in 100 ml E-flasks at an $OD_{750}$ of 0.05 and placed in a light intensity of 50 µE (µmol photons $s^{-1}$ $m^{-2}$). They were cultured in 20 ml BG11 media containing 20 µg/ml chloramphenicol and a 2 ml dodecane overlay, in which the bisabolene accumulates (Davies *et al*. 2014). When sampling, the $OD_{750}$ was measured and a sample of the dodecane layer was stored for analysis. The bisabolene measurements were performed using gas chromatography (GC), however since no pure bisabolene standard was available the production was only evaluated relatively to already existing strains. All GC and OD measurements were performed in technical triplicates.

## 4.20 Bisabolene experiment in *Escherichia coli*

Biological triplicates of each strain were inoculated in 100 ml E-flasks using 20 ml LB media containing 35 µg/ml chloramphenicol, 1 ml overnight culture and a 2 ml dodecane overlay. When sampling, the $OD_{595}$ was measured and a sample of the dodecane layer was stored for analysis. The bisabolene measurements were performed using gas chromatography (GC), however since no pure bisabolene standard was available the production was only evaluated relatively to already existing strains. All GC and OD measurements were performed in technical triplicates.

## 4.21 Gas chromatography

To all samples 1,6% β-caryophyllene was added as internal standard. However, the β-caryophyllene was impure and therefore gave several peaks, one of which was chosen as a reference. The peak chosen was eluted at approximately 6,1 minutes and was well-separated from the bisabolene peak at approximately 10.8 minutes, as well as any peaks of the dodecane solvent. The GC run was a Perkin Elmer GC 580 with a FID detector and am Elite-WAX Polyethylene Glycol Series Capillary, 30m x 0.25 mm x 0.25 µm, column. The oven was set to the following conditions: 100 ° C for 1 min, ramp at 5 °C $min^{-1}$ to 160 °C, hold 2 min, ramp at 10 °C $min^{-1}$ to 240 °C.

# 5. References


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# 7. Appendix A. Primer sequences

**Table 3.** Primer names, sequences and annealing temperatures used.

| **Primer** | **Sequence** | **$T_A$ [°C]** |
|---|---|---|
| DXS_SDmut_F | CAAACAATAAAGGAATCCCATTAGAGGTTG | 55 |
| DXS_SDmut_R | CAACCTCTAATGGGATTCCTTTATTGTTTG | 55 |
| speI_EcoRI_Ptrc20_F | GAGCACTAGTCTCATATCATGAATTCCGAATTGTGAG | 55 |
| CfDXS_streptag_SpeI_R | GAGCTGACTAGTTTACTTCTCGAACTGAGGATGACTCCAA CTTCCGCTACCCATGTTGATCAAATGAAGA | 55 |
| CfDXS_streptag_PstI_R | GAGCTGCTGCAGTTACTTCTCGAACTGAGGATGACTCCA ACTTCCGCTACCCATGTTGATCAAATGAAGA | 55 |
| XbaI_Idi_F | GAGATCTAGATAGTGGAGGTCATTGAATGC | 60 |
| Idi_streptag_SpeI_PstI_R | GATACTGCAGGCTGACTAGTTTACTTCTCGAACTGAGGAT GACTCCAACTTCCGCTACCTTTAAGCTGGGTAAATGCAG | 60 |
| VF2 | TCACGAGGCAGAATTTCAGA | 55 |
| VR | GCTCACTCAAAGGCGGTAAT | 55 |
| MevS2F_NS | TGGACAGTCAGGAATGGCAT | 55 |
| AgB_F1 | TTGATAATATCGTCCGGTTG | 55 |
| AgB_F2 | TGATATTGTGCACGAAGTTG | 55 |
| AgB_R2 | CAACTTCGTGCACAATATCA | 55 |
| IspA_BgI_F | GCGCGCAGATCTTTTATTACGCTGGATGAT | 55 |
| IspA_Nde_R | GCGCGCCATATGGACTTTCCGCAGCAACTC | 55 |
| CrF_R1 | CTTCTTTAAACCACTCTTGCG | 55 |

# 8. Appendix B. BG11 recipe

| | |
|---|---|
| $NaNO_3$ | 1.5 g |
| $K_2HPO_4$ | 0.04 g |
| $MgSO_4 \cdot 7H_2O$ | 0.075 g |
| $CaCl_2 \cdot 2H_2O$ | 0.036 g |
| Citric acid | 0.006 g |
| Ferric ammonium citrate | 0.006 g |
| EDTA (Disodium salt) | 0.001 g |
| $Na_2CO_3$ | 0.02 g |
| Trace metal mix A5 (see below) | 1.0 ml |
| Distilled water | 1.0 L |
| **Trace metal mix A5:** | |
| $H_3BO_3$ | 2.86 g |
| $MnCl_2 \cdot 4H_2O$ | 1.81 g |
| $ZnSO_4$ | 0.222 g |
| $Na_2MoO_4 \cdot 2H_2O$ | 0.39 g |
| $CuSO_4 \cdot 5H_2O$ | 0.079 g |
| $Co(NO_3)_2 \cdot 6H_2O$ | 49.4 mg |
| Distilled water | 1.0 L |