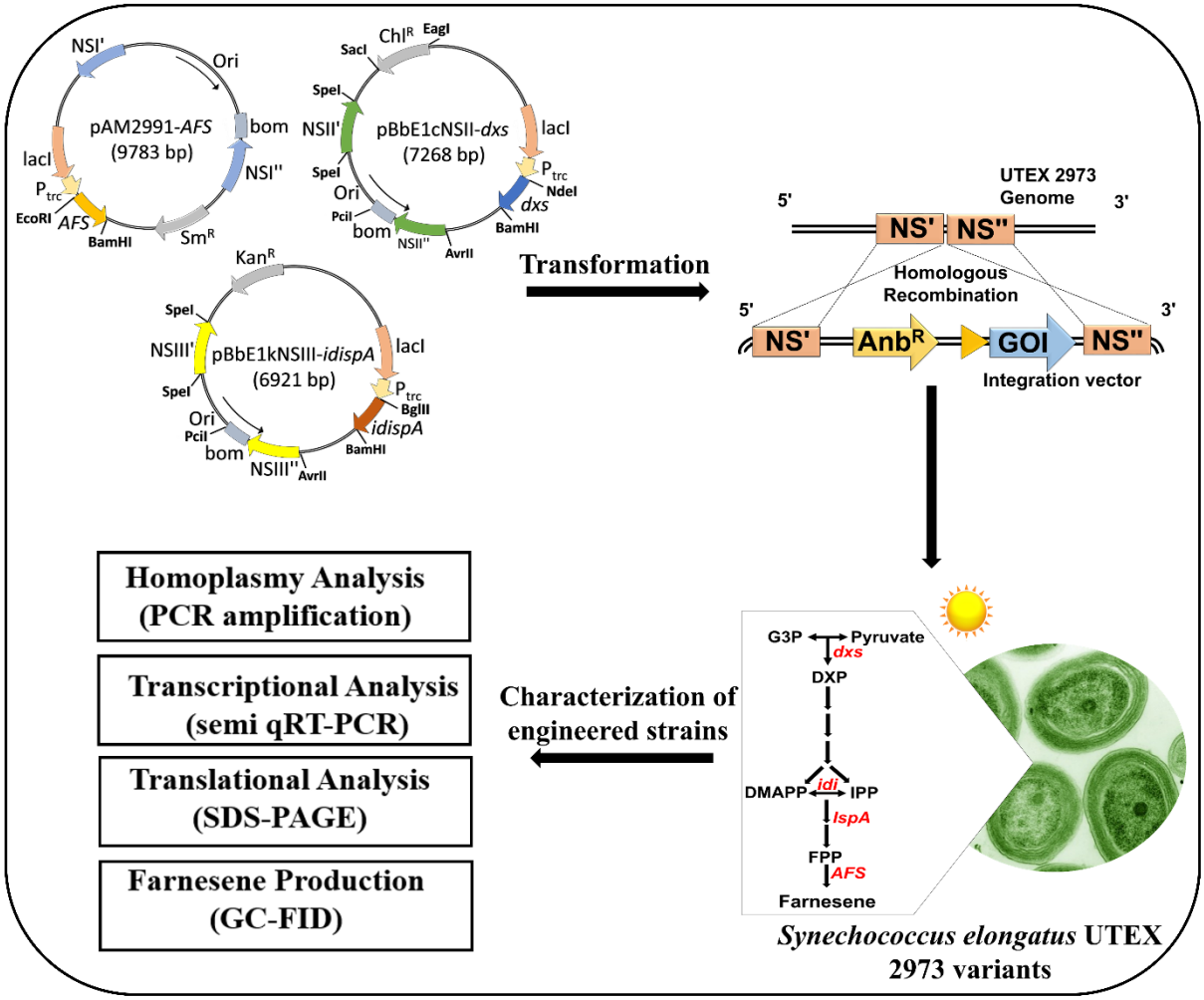
CHAPTER 4

Modification of *Synechococcus elongatus* UTEX 2973 for farnesene production*



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This chapter focuses on the transformation of integration vectors constructed in chapter 3 into the *Synechococcus elongatus* UTEX 2973 (hereafter, UTEX 2973) through triparental conjugation. The cyanobacterial strains investigated thus far for farnesene production, like *Synechococcus elongatus* PCC 7942 and PCC 7002, have a doubling time of 4.9 h and 4.1 h, respectively. UTEX 2973 demonstrates a high genomic similarity with PCC 7942, with a total of 55 single nucleotide polymorphisms and insertion deletions observed between the two genomes (Yu et al., 2015). Despite this similarity, UTEX 2973 exhibits a growth rate that is more than double that of PCC 7942. UTEX 2973 holds potential as an industrially important strain which can endure elevated temperatures (up to 41 °C) and high light intensity (500 µmol photons/m^2/s). A shorter doubling time corresponds to increased organism growth, indicating more production of the desired metabolite. To explore this prospect, UTEX 2973 was genetically engineered with a codon-optimized *M. domestica AFS* gene to develop an enhanced and robust photosynthetic production system for farnesene. Additionally, the methylerythritol phosphate (MEP) pathway was optimized by overexpressing 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) and/or fusion of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase (*idispA*) to increase the flux of isoprenoids precursor. The previous studies utilized neutral sites I and II (NSI and NSII) of the PCC 7942 strain for gene integration (Lee et al., 2021, 2017; Pattharaprachayakul et al., 2019). The present study expands upon this framework by not only utilising NSI and NSII but also neutral site III (NSIII) for gene integration. The genetically modified strains were characterized by doing homoplasmy, transcriptional and translational analysis. The farnesene productivity was checked every month for up to three months to check the genetic stability of the engineered UTEX strains. This provides the biosolar platform for the direct conversion of light and CO_2, which can be further optimized to expedite farnesene production.

4.1. Materials and methods

4.1.1. Strains and growth conditions

Escherichia coli HB101 (recA- mutant) strain was used for triparental conjugation to avoid undesirable recombination events. The *E. coli* strains were routinely maintained at 37 °C in Luria-Bertani (LB) broth and agar with the addition of appropriate antibiotics, wherever required. UTEX 2973 (provided by The Pakrasi Lab, Department of Biology, Washington University, St. Louis, USA) was used as a platform for metabolic engineering. UTEX 2973 was maintained in BG-11 broth and agar at 38 °C in constant light (100 µmol photons/m^2/s) (Yu et al., 2015). The recombinant UTEX 2973 strains were supplemented with appropriate antibiotics and grown in the same conditions as mentioned.

4.1.2. Contrivance of engineered UTEX 2973 strains

The recombinant strains generated in this study are listed in Table 4.1. The genes of interest were engineered in UTEX 2973 genome through homologous recombination. For this purpose, the constructed plasmids having gene(s) of interest flanked by homologous sequences of neutral sites were introduced in UTEX 2973 by triparental mating (Gale et al., 2019) (Fig. 4.1). Briefly, the vectors constructed were isolated from *E. coli* DH5α and transformed into *E. coli* HB101. Transformation of pAM2991-*AFS*, pBbE1cNSII-*dxs* and pBbE1kNSIII-*idispA* into *E. coli* HB101 leads to the first, second and third cargo strains generation, respectively. In addition to this, *E. coli* HB101 helper strain was required harboring pRL443 (Addgene, Plasmid #70261) and pRL623 (Addgene, Plasmid #58494). pRL443 (conjugal plasmid) and pRL623 (helper plasmid) possess resistance to ampicillin and chloramphenicol, respectively, and are transformed into competent *E. coli* HB101, generating helper strain. Cargo and helper strains were cultured overnight in LB broth, while the UTEX 2973 strain was grown in BG-11 to OD730 = 0.5-1.0. Media for the helper strain was supplemented with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol, whereas the cargo strain was supplemented with the antibiotic according to the plasmid to

Table 4.1 List of strains used and generated in the study.

Strains	Genetic constitution	Resistance to antibiotic	Reference
UTEX 2973	Wild type	–	Yu et al., 2015
UTEX *AFS*	ΔNSI::Sm^R-lacI-*AFS*	Streptomycin, spectinomycin (SS)	This study
UTEX *AFS::dxs*	ΔNSI::Sm^R-lacI-*AFS*, ΔNSII::Chl^R-lacI-*dxs*	Streptomycin, spectinomycin, and kanamycin (SSK)	This study
UTEX *AFS::idispA*	ΔNSI::Sm^R-lacI-*AFS*, ΔNSIII::Kan^R-lacI-*idispA*	Streptomycin, spectinomycin, and chloramphenicol (SSC)	This study
UTEX *AFS::dxs::idispA*	ΔNSI::Sm^R-lacI-*AFS*, ΔNSII::Chl^R-lacI-*dxs*, ΔNSIII::Kan^R-lacI-*idispA*	Streptomycin, spectinomycin, chloramphenicol, and kanamycin (SSCK)	This study

be transferred. To remove antibiotics, the bacterial and cyanobacterial cultures were pelleted down and washed with LB and BG-11 medium, respectively. Washed strains were mixed (450 µl of cargo strain + 450 µl of helper strain + 900 µl of UTEX 2973) in a 2 ml micro-centrifuge tube and left undisturbed for 2 h at room temperature in the dark. Subsequently, the mixture was spread on a cellulose ester membrane (MF-Millipore® Membrane Filter, 0.45 µm pore size) on BG-11 (5% LB) agar plates (antibiotic negative). The plates were incubated at 38 °C and 100 µmol photons/m^2/s light. Then, the membrane was transferred to the appropriate antibiotic BG-11 agar plate after 24 h and incubated at similar conditions. The transformed colonies emerged after 4-5 days of incubation, which were further re-streaked 3-4 times for proper segregation of the DNA. Foremost, UTEX 2973 was mated with the first cargo strain bearing pAM2991-*AFS* plasmid, leading to the generation of UTEX *AFS* strain. Further, the UTEX *AFS* strain, when mated with the second cargo strain (bearing pBbE1cNSII-*dxs* plasmid), leads to the development of the UTEX *AFS::dxs* strain. Similarly, the UTEX *AFS* strain, when mated with the third cargo strain carrying pBbE1kNSIII-*idispA* plasmid, generated the UTEX *AFS::idispA* strain. To

develop the strain having all genes of interest, strain UTEX *AFS::dxs* was mated with the third cargo strain, leading to strain UTEX *AFS::dxs::idispA*.

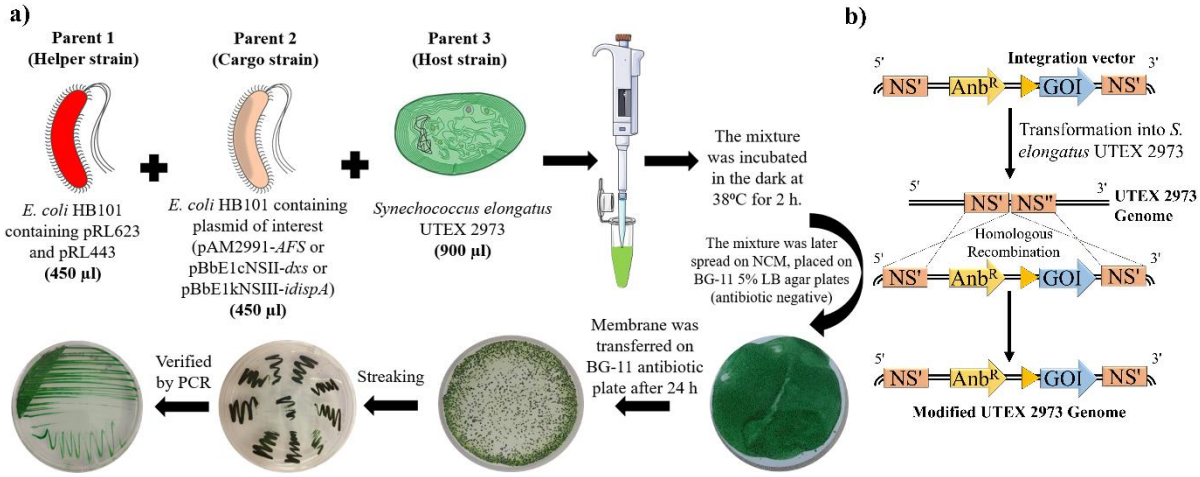

Fig. 4.1. a) Schematic representation of triparental mating. **b)** homologous recombination after transformation of the integrative vector into UTEX 2973.

4.1.3. Genomic DNA extraction and PCR analysis

Genomic DNA extraction followed a previously described method (Yadav et al., 2023a). UTEX 2973 and engineered UTEX strains were freshly grown to an OD_{730} ~ 0.75 ± 0.05 with suitable antibiotics. For genomic DNA extraction, cells were pellet down by centrifuging at 3000 × g for 10 minutes at room temperature. The resulting pellet was resuspended in 400 µl of lysis buffer (containing 4 M urea, 0.2 M Tris-HCl at pH 7.4, 20 mM NaCl and 0.2 M EDTA). To this, 50 µl of proteinase K (20 mg/ml) was added, and the mixture was incubated at 55 °C for one hour with gentle mixing every 10-15 minutes. After incubation, 1 ml of prewarmed DNA extraction buffer (composed of 3% cetyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 0.5 M Tris-HCl at pH 8.0, 1% sarkosyl and 1% β-mercaptoethanol) was introduced. The mixture was further incubated at 55 °C for an hour by gently mixing every 10 min. Two volumes of chloroform: isoamyl alcohol (24:1 v/v) were added to the mixture, and it was centrifuged at 10000 × g for 5 min. The upper aqueous phase was retained, and two volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate at pH 5.2 were added to it. The solution was then

incubated at -20 °C for an hour and centrifuged at 10000 × g for 3 min. The supernatant was discarded, and the pellet was washed with 70% ice-cold ethanol. Further, the pellet was dried and stored in 100 µl of sterile nuclease-free water. The purity and concentration of the DNA samples were quantified by NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The genomic DNA was used as a template to check the integration of *AFS*, *dxs*, and *idispA* at the respective neutral sites in the genome of UTEX 2973. A test of campbell like integration was also done using the genomic DNA as a template.

4.1.4. Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was conducted, as reported previously (Yadav et al., 2023a). The UTEX 2973 and modified strains (UTEX *AFS*, UTEX *AFS::dxs*, UTEX *AFS::idispA* and UTEX *AFS::dxs::idispA*) were grown to an OD_{730} ~ 0.75 ± 0.05 using appropriate antibiotics. Each recombinant strain underwent induction with isopropyl β- d-1-thiogalactopyranoside (IPTG) at concentrations of 0.1, 0.5, and 1 mM IPTG for 24 h. As a control, one set of cultures for the recombinant strains remained uninduced. Cells were harvested separately by centrifuging at 5000 × g for 10 minutes at 4 °C. The harvested pellets were grounded in a pre-cooled mortar and pestle using liquid nitrogen. Whole cell RNA was isolated as per the manufacturer's instructions with the RNeasy kit from Qiagen (Hilden, Germany) and was subsequently converted into cDNA using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA). DNase digestion was performed directly on the column using the RNase-free DNase set (Qiagen, Hilden, Germany) to remove DNA contamination from the RNA samples. The quality, purity and integrity of the RNA samples were assessed by NanoDrop spectrophotometer and gel electrophoresis. The total cDNA served as a template for semi-quantitative RT-PCR. *AFS*, *dxs*, and *idispA* genes were amplified from the cDNA using forward and reverse primers

AFS rt FP and AFS rt RP; dxs rt FP and dxs rt RP; idispA rt FP idispA rt RP, respectively, for 30 cycles. To serve as an internal control, housekeeping gene, RNA polymerase subunit α (*rpoA*) was amplified using primers rpoA rt FP and rpoA rt RP for the same number of cycles. Each primer was designed such that an amplicon of ~ 130 bp from the coding region of the gene was obtained (Table 4.2). The PCR products were electrophoresed on a 2% agarose gel and observed using the Gel Doc system.

4.1.5. Protein analysis

Protein extraction and analysis were performed as described by Melis's group with some modifications (Betterle and Melis, 2018). Briefly, UTEX 2973 and recombinant strains were grown to an OD_{730} ~ 0.75 ± 0.05 using appropriate antibiotics. Induction was done by adding 1 mM IPTG to the cultures. After 24 h of induction, cells were pellet down by centrifugation at 3000 × g for 10 min at 4 °C. The resulting cell pellets were resuspended in 500 µl sonication buffer (comprising of 50 mM Tris-HCl at pH 8.0, 50 mM NaCl, 10 mM $CaCl_2$, 10 mM $MgCl_2$, 1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride). The cell suspension underwent sonication with a probe sonicator set at 50% amplitude for five cycles with 30 seconds on and 30 seconds off while placed on an ice bath. After sonication, the lysate was subjected to centrifugation at 2250 × g for 3 minutes to eliminate unbroken cells. The supernatant was supplemented with an equal amount of solubilization buffer (consisting of 250 mM Tris-HCl, pH 6.8, 7% sodium dodecyl sulfate, 20% glycerol, 4 M urea and 5% β-mercaptoethanol). The mixture was left at room temperature for 2 h to facilitate the solubilization of total proteins. Following solubilization, the mixture was then centrifuged at 21000 × g for five minutes. The protein content in the supernatant having total solubilized proteins were quantified using the Bradford assay, and protein samples (10 µg per lane) were analyzed using sodium dodecyl sulfate-polyacrylamide gel

electrophoresis (SDS-PAGE) with 12% polyacrylamide gel (Yadav et al., 2023a). The gels were subjected to silver staining for protein visualization.

Table 4.2 List of primers used for semi-quantitative RT-PCR.

Primers	Sequences
rpoA rt FP	GACATCTTGCTCAACGTCC
rpoA rt RP	CTTCAACTTCAGGGCCAAAG
dxs rt FP	GTATTAAGCGTCGGGCATTC
dxs rt RP	CCATGCCTGCGGTAATCG
idispA rt FP	GCTGTTTAATGCCAAAGGAC
idispA rt RP	GATCACTGCGTCTTCGTTG
AFS rt FP	CCTGAAGGGCATGCTGGAAC
AFS rt RP	GCTATCGGGATAGCAAATGTG

4.1.6. Growth conditions for producing farnesene from engineered strains and quantification

The farnesene-producing recombinant strains derived from UTEX 2973 were cultivated in a custom-designed 250 ml bioreactor (Fig. 4.2(a)) which were placed in constant and continuous illumination of 100 µmol photons/m^2/s light in a photo-incubator. The recombinant strains were inoculated in a 250 ml Erlenmeyer flask having 100 ml BG-11 media supplemented with appropriate antibiotics and incubated at the conditions mentioned above. These acted as seed cultures and were grown till OD_{730} = 1.5-2.0. For production culture, the seed cultures were transferred to a custom-designed 250 ml bioreactor and diluted to an initial approximate OD_{730} of 0.75 to make up the final volume of 200 ml with BG-11 medium supplemented with 10 mM $NaHCO_3$ and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH-8.0). The cultures were supplemented with 25 µg/ml streptomycin, and/or 25 µg/ml spectinomycin, and/or 25 µg/ml kanamycin, and/or 25 µg/ml chloramphenicol for selection pressure. The production cultures were incubated at 38 °C and 180 rpm under constant and continuous illumination of 100 µmol

photons/m^2/s for 6 days (including one day of acclimatization). A mixture of filtered 95% (v/v) air and 5% (v/v) CO_2 was bubbled in the medium through 2 μm pore size stainless steel sparger at 0.2 vvm. The cultures were induced by 1.0 mM IPTG after 24 h of acclimatization. An organic overlay of isopropyl myristate (IM) at 2.5% (v/v) was made over the production medium to trap farnesene, and 100 μl of IM was sampled out every 24 h for farnesene quantification. The stability of the engineered strains in terms of farnesene productivity was estimated. The engineered strains were sub-cultured every week to a fresh BG-11 medium over a period of three months, and the strains were assessed for farnesene production every month.

The IM samples were subjected to gas chromatography (Nucon system 5765, New Delhi, India) coupled with a flame ionization detector using a Supelcowax capillary column (30m×0.25mm×0.25μm). Farnesene was quantified using a calibration curve made by pure farnesene, Biofene (trans-β-farnesene, CAS# 18794-84-8), provided by Amyris Inc., USA. The calibration curve was made for different farnesene concentrations in IM. The initial oven temperature was set at 60 °C for one minute, which was then ramped up to 230 °C at a rate of 15 °C/min and held for 5 minutes. Subsequently, the temperature was further increased to 250 °C at a rate of 25 °C/min and held for 15 minutes. The injector and detector temperature were maintained at 250 °C and 280 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 1.5 ml/min. To determine the isomeric form of farnesene, the IM samples were subjected to Gas chromatography-Mass spectrometry, GC-MS analysis (Agilent Technologies, USA) equipped with a DB5 column with the same conditions as mentioned above.

4.1.7. Statistical analysis

All data are presented as mean ± standard deviation from biological triplicate experiments. Statistical analysis was performed using analysis of variance and Tukey's test

to determine the statistical differences between engineered strains for farnesene productivity (mg/L/day) and yield (mg/g dry cell weight (DCW)).

4.2. Results and discussion

4.2.1. Metabolic engineering strategy for the production of farnesene

UTEX 2973 does not have *AFS* gene activity (confirmed by Protein BLAST search of UTEX 2973 genome and *AFS* gene) and, therefore, does not produce farnesene (Halfmann et al., 2014). For this purpose, the *AFS* gene was integrated into the NSI of genomic UTEX 2973 with the help of vector pAM2991-*AFS*. The vector was transformed into UTEX 2973 through triparental conjugation emanating UTEX *AFS* strain via homologous recombination (resistant to streptomycin and spectinomycin). Further, as seen in Fig. 4.2(b), the MEP pathway bottleneck genes (shown in red) were overexpressed. To increase the flux of DMAPP and IPP, the *dxs* gene was overexpressed in UTEX 2973. The vector pBbE1cNSII-*dxs* was transformed into UTEX *AFS* strain generating UTEX *AFS*::*dxs* strain (Fig. 4.2(c)) (resistant to streptomycin, spectinomycin, and chloramphenicol). Since the fusion of *idi* and *ispA* genes with the help of the linker (amino acid sequence $(GGGGS)_2$) increased the terpenoid production in PCC 7942, the genes were fused with overlap extension PCR and targeted to the NSIII of genomic UTEX 2973 (Choi et al., 2017a). The vector generated for this purpose, pBbE1kNSIII-*idispA*, was used to transform UTEX *AFS* and UTEX *AFS*::*dxs* strains, giving rise to UTEX *AFS*::*idispA* (resistant to streptomycin, spectinomycin, and kanamycin) and UTEX *AFS*::*dxs*::*idispA* (resistant to streptomycin, spectinomycin, kanamycin, and chloramphenicol), respectively. As an outcome, several strains were constructed, some only having MEP pathway bottleneck genes while others in conjunction with the *AFS* gene. Considering that the study's goal is farnesene synthesis, the strains with the *AFS* gene were only mentioned in Table 4.1.

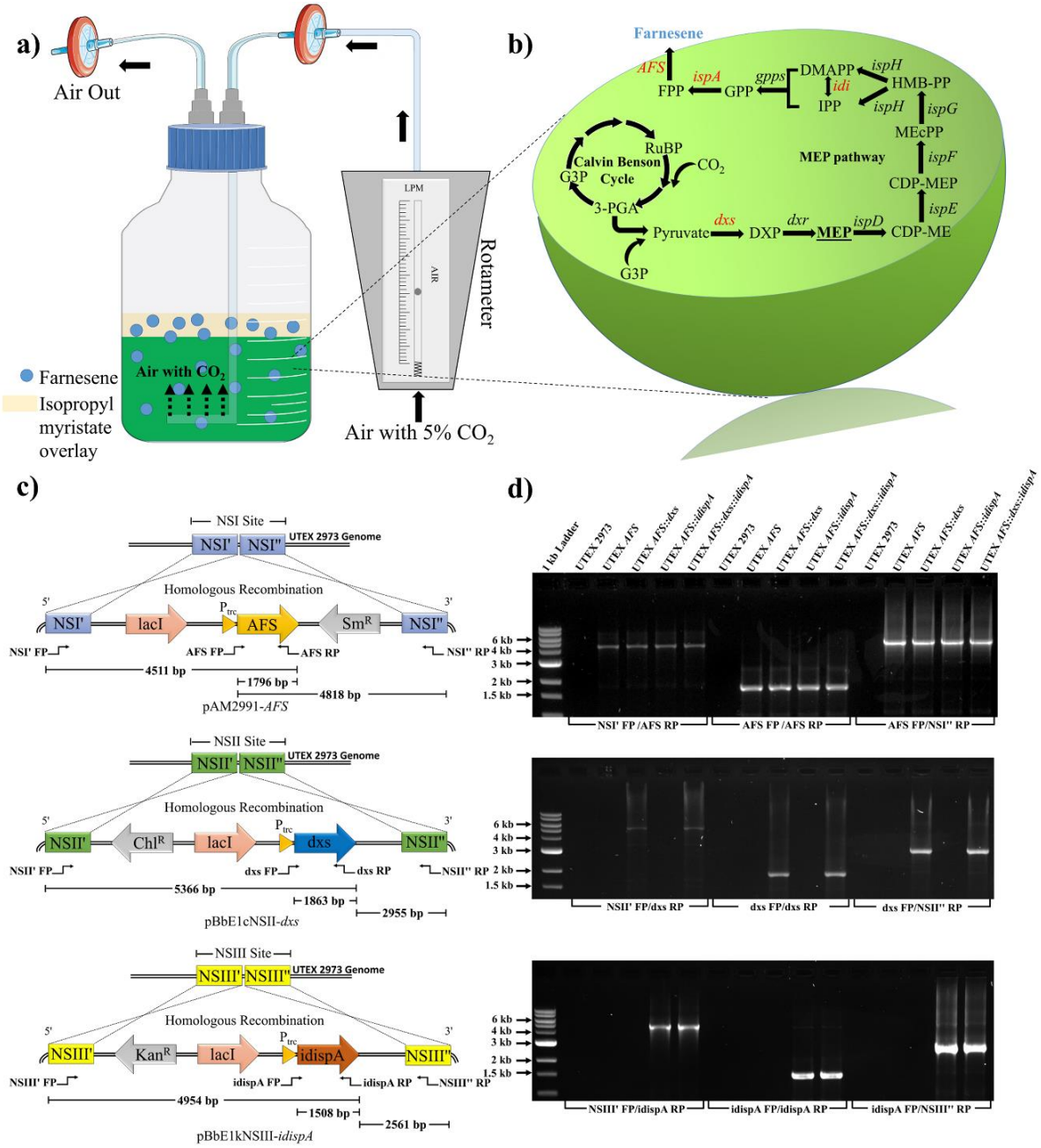

Fig. 4.2. a) Experimental setup for farnesene production through engineered strains. **b)** Schematic representation of native MEP pathway (black) and metabolic engineering strategy utilized (red) in the study for farnesene production. **c)** Schematic representation of the construction of engineered UTEX 2973 strains by integrating genes at respective sites through homologous recombination. The elbow arrow indicates primer attachment sites and the expected amplicon to be generated after PCR. **d)** Genomic DNA PCR analysis with specific primers (below each gel) to demonstrate the integration of each gene in recombinant strains (above the first gel). Abbreviation used, *RuBP*: ribulose-1,5-bisphosphate; *3-PGA*: 3-phosphoglyceric acid; *G3P*: glyceraldehyde 3-phosphate; *DXP*: 1-deoxy-D-xylulose 5-phosphate; *MEP*: methylerythritol-4-phosphate; *CDP-ME*: 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; *CDP-MEP*: 2-phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol; *MEcPP*: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; *HMB-PP*: 4-hydroxy-3-methylbut-2-enyldiphosphate; *DMAPP*: dimethylallyl diphosphate; *IPP*: isopentenyl diphosphate; *GPP*: geranyl diphosphate; *FPP*: farnesyl diphosphate; *dxs*: DXP synthase; *dxr*: DXP reductoisomerase: *IspD*: CDP-ME cytidylyltransferase; *IspE*: CDP-ME kinase; *IspF*: MEC synthase; *IspG*: HMBPP synthase; *IspH*: HMBPP reductase; *idi*: isopentenyl diphosphate isomerase; *gpps*: GPP synthase; *ispA*: FPP synthase; *AFS*: farnesene synthase.

4.2.2. Homoplasmy analysis

Genomic DNA PCR analysis was carried out to scrutinize the integration of the gene of interest at proper neutral sites. The results of the analysis are shown in Fig. 4.2(d). The marking above the first gel shows recombinant strains generated genomic DNA PCR product in the respective lanes along with wild-type strain which continues for the lower two gels. Below each gel, the marking shows the combination of primers used to amplify genomic DNA. The region of annealing of primers employed and the expected size of the amplicon can be seen in Fig. 4.2(c). UTEX 293 genomic DNA was used as a negative control, where no amplification was discerned. The first gel image uses primers that anneal to the NSI region along with the *AFS* gene, while the second and third gel use primers of NSII and *dxs*, and NSIII and *idispA*, respectively. Since the wild-type strain does not contain *AFS*, *dxs*, and *idispA* genes, no band was visualized on the gel. As the *AFS* gene was common to all the strains generated, the band can be seen in all the engineered strains in the first gel. Similar results were envisaged in the second and third gels, where strains with the *dxs* and *idispA* genes show the band, respectively. Owing to the presence of all the engineered genes in strain UTEX *AFS::dxs::idispA*, the amplification was seen with every set of primers. These results confirm the integration of genes at the respective neutral sites. To corroborate the results, an antibiotic survival test of the engineered strains was performed (Fig. 4.3) (Betterle and Melis, 2018). This was done to inspect the attainment of antibiotic-resistance genes in the characteristic strain. The UTEX 2973 strain could not grow on streptomycin, spectinomycin, chloramphenicol, and kanamycin containing BG-11 agar. As the UTEX 2973 strain transforms with configured vectors, the engineered strains acquire an antibiotic resistance gene along with the gene of interest (Table 4.1).

Plasmid transformation by triparental conjugation carries the risk of Campbell-type integration, which allows single homologous recombination (Leenhouts et al., 1988). This

type of integration duplicates the homology region on either side after the recombination (Fig. 4.4). A Campbell integration test uses primers which attach on either side in case the Campbell-type integration would have occurred. Fig. 4.4(a,b,c) shows two possibilities apart from the double homologous recombination that might occur after transformation. To confirm this genomic DNA PCR analysis was done with a neutral site upstream forward and reverse primers and downstream forward and reverse primers. As seen in Fig. 4.4(d) in all the strains, amplicon of 500 and 900 bp was only observed compared to the full amplification (7533 bp for pAM2991-*AFS*, 8321 bp for pBbE1cNSII-*dxs*, and 7515 bp for pBbE1kNSII-*idispA*). This confirms that Campbell-type integration did not occur, and the genes were integrated through homologous recombination.

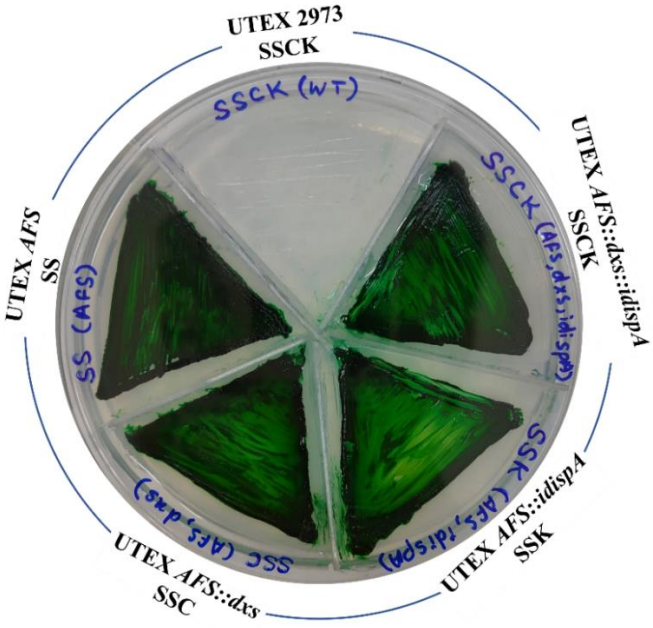

Fig. 4.3. Antibiotic survival test of UTEX 2973 as it gets transformed by the plasmid constructed and attains antibiotic resistance. UTEX 2973 was not able to survive in the presence of antibiotics. UTEX *AFS* – resistance to streptomycin and spectinomycin, UTEX *AFS::dxs* – resistant to streptomycin, spectinomycin, and chloramphenicol, UTEX *AFS::idispA* – resistant to streptomycin, spectinomycin, and kanamycin, UTEX *AFS::dxs::idispA* – resistant to streptomycin, spectinomycin, chloramphenicol, and kanamycin. S – streptomycin and spectinomycin (25 µg/ml), C – chloramphenicol (25 µg/ml), K – kanamycin (25 µg/ml).

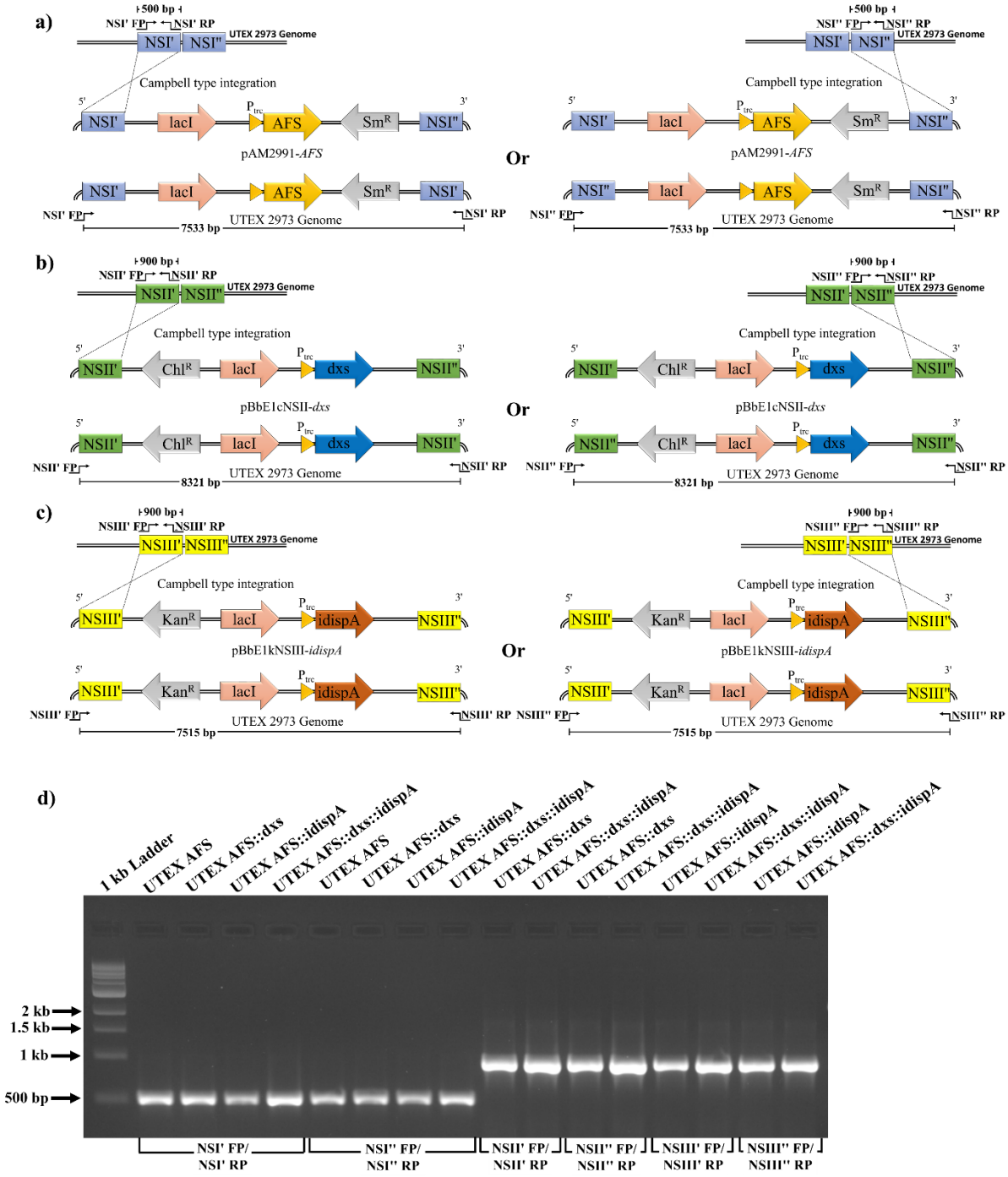

Fig. 4.4. Campbell-type integration when UTEX 2973 was transformed with **a)** pAM2991-*AFS*, **b)** pBbE1cNSII-*dxs*, **c)** pBbE1kNSII-*idispA*, **d)** Genomic DNA PCR analysis with specific primers (below each gel) to demonstrate the integration of each gene in recombinant strains (above the first gel).

4.2.3. Transcriptional and translational analysis

Prior to the production studies, the concentration of IPTG required for the effective expression of engineered genes was determined. Since the trc promoter is an IPTG

inducible promoter and all the engineered genes (*AFS*, *dxs*, *idispA*) are under its influence, semi-quantitative RT-PCR was carried out to analyze the effect of varying IPTG concentration on the mRNA levels of engineered genes (Fig. 4.5(a)). For this purpose, mRNA from induced (0.1, 0.5, and 1.0 mM IPTG) and uninduced UTEX 2973 and recombinant strains were extracted, which was further converted to cDNA and used as a template for semiquantitative RT-PCR analysis. The *rpoA* was used as a control for the experiment (Lin et al., 2020). As expected, all the strains showed increased transcriptional levels of *AFS*, *dxs*, and *idispA* genes as the IPTG concentration increased. mRNA levels of all the engineered genes were highest at 1.0 mM IPTG concentration, and therefore, all the further experiments were conducted at this concentration which was in accordance with the previous study (Yadav et al., 2023a). Some level of expression was also observed when recombinant strains were uninduced (no IPTG); this could be due to the leakiness of the trc promoter reported by many researchers (Huang and Lindblad, 2013; Markley et al., 2015). Besides the amplification of the *rpoA* gene, no amplification of *AFS*, *dxs*, and *idispA* genes was observed in the UTEX 2973, stipulating the absence of *AFS*, *dxs*, and *idispA* genes in the UTEX 2973. The relative expression level for the *AFS*, *dxs*, and *idispA* genes were assessed via densitometric analysis using ImageJ software, with the *rpoA* gene serving as an internal reference to standardize expression levels (Fig. 4.5(b)). Standardized expression levels of the uninduced recombinant strains were determined to be 0.15, 0.26, 0.25, and 0.4 for the *AFS* gene in the UTEX *AFS*, UTEX *AFS::dxs*, UTEX *AFS::idispA*, and UTEX *AFS::dxs::idispA*, respectively. As mentioned, the expression level was due to the leakiness of the trc promoter without induction. A similar pattern was observed for other genes. The expression level of *AFS* changed from 0.33 to 1.0 when the IPTG concentration was increased from 0.1 mM to 1.0 mM. The expression of *AFS* was increased in the same manner upon increasing the IPTG concentration in other recombinant strains. There was a

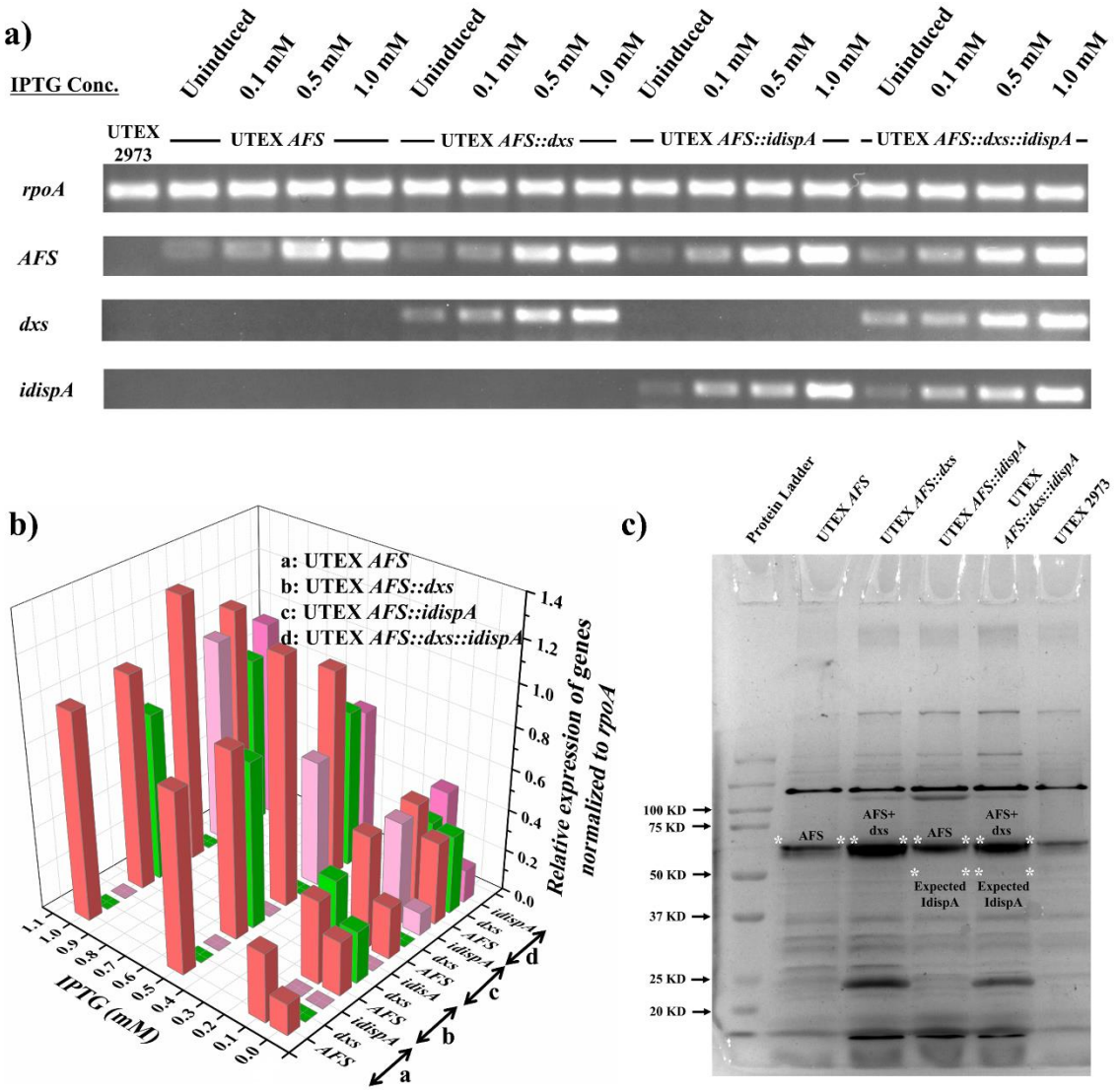

Fig. 4.5. Transcriptional and translational analysis of UTEX 2973 and engineered strains. **a)** Semi-quantitative RT-PCR analysis of the total RNA isolated from engineered strains to determine the expression level of overexpressed genes. The strains were induced overnight with varying IPTG concentrations. Strains and different IPTG concentrations are mentioned at the top, whereas the genes analyzed are shown on the left. For the genetic constitution of the strains Table 1 can be referred. **b)** Densitometric analysis of semi-quantitative RT-PCR to test the expression levels of *AFS*, *dxs*, and *idispA* genes in uninduced and induced (0.1, 0.5, and 1 mM IPTG) conditions using ImageJ 1.53t software. The *rpoA* gene was used as an internal control to normalize the expression level. **c)** Protein expression analysis. Total proteins were resolved by 12% SDS-PAGE and visualized by silver staining. Sample loading corresponds to 10 µg of protein per lane.

1.8 and 2-times increase in the expression of *dxs* in UTEX *AFS::dxs* and UTEX *AFS::dxs::idispA*, respectively, as the IPTG concentration was increased. Whereas approximately a 1.98-times increase in *idispA* was seen in UTEX *AFS::idispA* and UTEX *AFS:: dxs::idispA*.

Seeing that the highest level of expression of mRNA was obtained by inducing recombinant UTEX 2973 strains with 1 mM IPTG, the total protein fraction was obtained from strains inducing with 1 mM IPTG. Protein profiles of each strain are obtained by subjecting 10 µg of total protein samples to SDS-PAGE and stained with silver staining. The results of SDS-PAGE Silver staining are consistent with the recent findings (Yadav et al., 2023a). Upon equal protein loading, a dominant band at around 65 kDa was observed in all the recombinant strains except UTEX 2973 (Fig. 4.5(c)). AFS and dxs protein correspond to a size of 66.1 and 67.6 kDa, respectively, which can be seen as a combined one prominent band. In the strains, UTEX *AFS* and UTEX *AFS::idispA,* the intensity of a band at around 65 kDa is low in comparison to the engineered strains (UTEX *AFS::dxs* and UTEX *AFS::dxs::idispA*) due to the fact that the strains express only AFS protein. The idispA protein band is expected to be at 52 kDa (idi of 20 kDa and ispA of 32 kDa) in the strains UTEX *AFS::idispA* and UTEX *AFS::dxs::idispA*, which was not observed in the SDS-PAGE gel, suggesting instability or aggregation of the protein (Formighieri and Melis, 2016). Despite this, the strain engineered with *idispA* showed improved farnesene synthesis (discussed later), suggesting the functionality of the idispA protein. A band at 25 kDa corresponds to chloramphenicol acetyltransferase. Since UTEX *AFS::dxs* and UTEX *AFS::dxs::idispA* strains are resistant to chloramphenicol, a prominent band of chloramphenicol acetyltransferase protein at 25 kDa is seen.

4.2.4. Farnesene production by engineered UTEX 2973

The production studies were done in a setup, as shown in Fig. 4.2(a). Farnesene concentration was measured every 24 h after induction by extracting the IM layer. Terpenes like mono and sesquiterpenes have negative feedback or toxicity on cells, and therefore to prevent this, an organic solvent overlay is applied for the sequestration of terpenes (Choi et al., 2016; Davies et al., 2014). According to Davies et al. (2014), dodecane, hexadecane,

and tetradecane proved to be biocompatible organic overlays for PCC 7942. However, when dodecane was applied as an overlay on UTEX 2973, it impeded growth (Fig. 4.6). This contrasts with the findings of Lee et al., who observed no detrimental effects of dodecane on cyanobacteria during farnesene extraction (Lee et al., 2017). Besides this, hexadecane, being a volatile oil, evaporates upon continuous bubbling, serving no purpose of trapping farnesene. Consequently, a more stable organic solvent was needed that would not inhibit cyanobacterial growth while sustaining bubbling, and this need was addressed by using IM (Kato et al., 2017; Lin et al., 2021). In addition to this, IM provides better peak resolution with farnesene during gas chromatography analysis. A single-phase photobioreactor culturing system was checked for farnesene productivity. A reduced farnesene productivity was observed in a single-phase photobioreactor compared to a two-phase culturing system (having overlay of IM). This result was consistent with the previous study by Tippmann et al. (2016a) where it was presumed that product loss was there due to gas stripping.

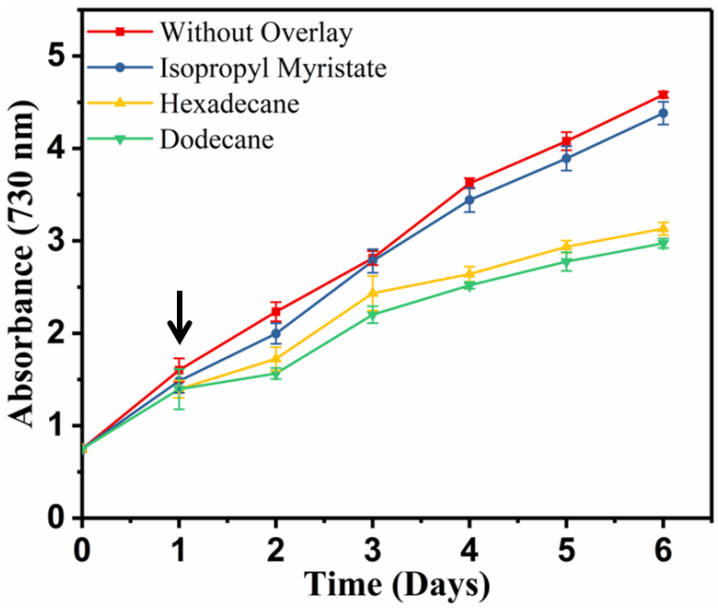

Fig. 4.6. The growth profile (absorbance at 730) of wild type *S. elongatus* UTEX 2973 with 5% (v/v) overlay of isopropyl myristate (IM), hexadecane and dodecane. The arrow indicates the addition of organic solvent overlay. Error bars represent the mean ± standard deviation (SD) from the biological triplicate experiments.

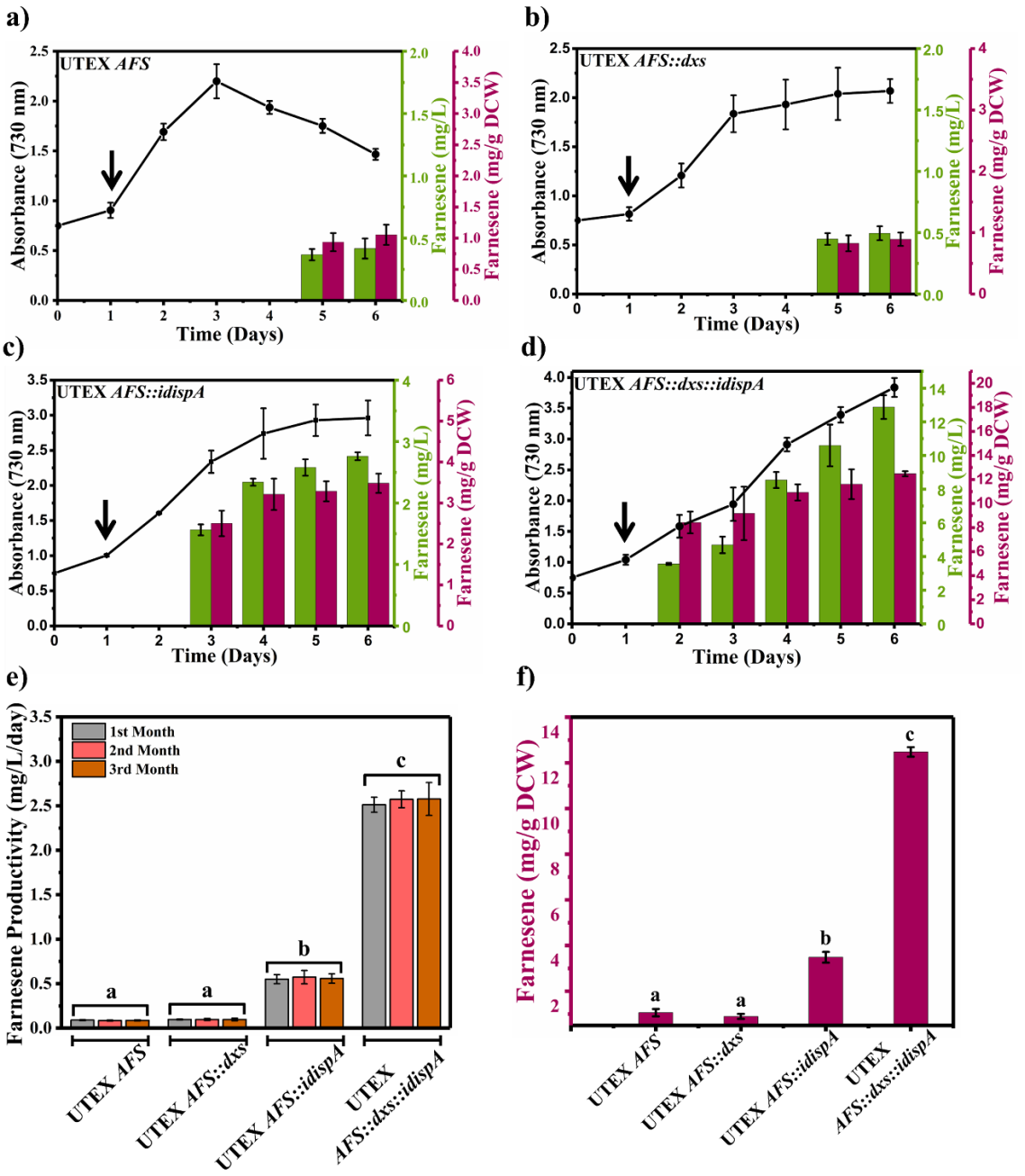

Fig. 4.7. The growth profile (absorbance at 730) and photosynthetic farnesene production (mg/L and mg/g DCW) studies for engineered UTEX 2973 strains were performed in custom-made 250 ml bioreactors bubbled with 5% CO_2 (v/v). **a)** UTEX *AFS*. **b)** UTEX *AFS::dxs*. **c)** UTEX *AFS::idispA*. **d)** UTEX *AFS::dxs::idispA*. **e)** The impact of duration (over 3 months) on farnesene productivity (mg/L/day) by engineered UTEX strains. **f)** Comparative analysis of cumulative farnesene yield (mg/g DCW) among different strains. The black arrow denotes the addition of IPTG in the cultures. Error bars represent the mean ± standard deviation (SD) from the biological triplicate experiments. Different letters indicate statistically significant differences ($p<0.05$).

Four recombinant UTEX 2973 strains with *AFS* and MEP pathway bottleneck gene(s) were checked for farnesene production, which was secreted into the IM overlay made upon the production culture. 100 µl of IM was taken for analysis which was

compensated with the addition of 100 µl of fresh IM. Strain UTEX *AFS* could not produce the quantifiable amount of farnesene for the first three days (lower than the limit of quantification <0.2 mg/L). The result was in accordant with the previous studies where lone expression of farnesene and isoprene synthase in PCC 7942 and UTEX 2973, respectively, produced no quantifiable product (Lee et al., 2017; Yadav et al., 2023a). However, on days 5 and 6, due to the accumulation of farnesene in the IM overlay, a production of 0.41 ± 0.08 mg/L was obtained (Fig. 4.7(a)). This was slightly higher than the farnesene produced by *Anabaena* sp. PCC 7120 (0.30 mg/L), which could be due to the difference in catalytic efficiency of *P. abies AFS* and *M. domestica AFS* (Halfmann et al., 2014). Kinetic parameters (K_{cat} and K_m) were only available for the *M. domestica AFS* (Pechous and Whitaker, 2004). The higher production can also be ascribed to the genetic stability of the *AFS* compared to the *P. abies AFS* since the *M. domestica AFS* is chromosomally integrated, whereas *P. abies AFS* is cloned in an expression vector. The integration strategy aids in prolonged expression of the foreign gene(s) without losing them. Additionally, when PCC 7942 strain was engineered with *P. abies AFS* and *M. domestica AFS* no farnesene was detected (Lee et al., 2017).

The farnesene production by UTEX *AFS* was subpar and exhibited growth constraints (Fig. 4.7(a)). This could potentially be attributed to the shortage of farnesene precursor, FPP. From Fig. 4.2(b), it is evident that optimizing the MEP pathway by overexpression of bottleneck gene(s) (in red) can increase the IPP/DMAPP and FPP pool and hence farnesene. In recent studies, optimization of the MEP pathway showed increased isoprene production (0.17 mg/L and 2.98 mg/L) in UTEX 2973 (Yadav et al., 2023a, 2023b). Moreover, earlier research has indicated a rise in farnesene (4.6 mg/L) and various isoprenoids derived from FPP, including amorpha-4,11-diene (19.8 mg/L), squalene (1.03 mg/L), bisabolene (9 mg/L), and following the engineering of the MEP pathway by

expressing pathway's bottleneck gene(s) (Lee et al., 2017; Choi et al., 2016; Choi et al., 2020; Rodrigues and Lindberg, 2021). For this purpose, overexpression of *dxs*, and/or *idi*, and *ispA* from *E. coli* into UTEX 2973 were inspected to ratify if they are good targets for increased farnesene production. Overexpression of *dxs* in strain UTEX 2973 *AFS* (generating UTEX *AFS::dxs*) moderately increased the farnesene production (0.49 ± 0.05 mg/L). As envisaged, *dxs* overexpression ameliorated growth constraints slightly in UTEX *AFS::dxs* compared to the UTEX *AFS* (Fig. 4.7(b)). Since there was only a 1.2-times increase in farnesene production (compared to UTEX *AFS*), it can be presumed that IPP and other intermediates were being accumulated and not being converted to farnesene. Excessive IPP buildup due to overexpression of the *dxs* gene hampers the functioning of isoprene synthase (Gao et al., 2016). This phenomenon may similarly have occurred with *AFS*.

To scrutinize the negative effect of *dxs* and to redirect the IPP pool to FPP and hence farnesene, *idi* and *ispA* without *dxs* were overexpressed into UTEX *AFS*. The *idi* and *ispA* were overexpressed together by fusing them. The *idi* converts IPP to DMAPP, which is further converted to FPP with the help of *ispA*. The *idispA* overexpression to the formation of UTEX *AFS::idispA* significantly increased the farnesene production (2.76 ± 0.06 mg/L). This was 5.6-times higher than the farnesene produced by UTEX *AFS::dxs*. A notable improvement in the growth of UTEX *AFS::idispA* was also observed (Fig. 4.7(c)), which may be due to the reduced IPP pool as it is shown to be toxic to the cyanobacteria (Gao et al., 2016). This also shows that *idispA* alone could escalate farnesene production compared to *dxs* alone, alluding that the IPP pool is naturally enough in UTEX 2973, and just redirecting the flux increases the farnesene production.

This gave a glimmering sense to engineer *idispA* into UTEX *AFS::dxs* to generate UTEX *AFS::dxs::idispA*. This hypothesis proved to be unerring as the UTEX

AFS::dxs::idispA produced the highest farnesene level of 12.87 ± 0.7 mg/L in 5 days, equivalent to 12.48 mg/g DCW. This was 26.2 and 4.6-times greater than the UTEX *AFS::dxs* and UTEX *AFS::idispA*, respectively. The strain UTEX *AFS::dxs::idispA* might have a high IPP pool due to *dxs* overexpression, and this IPP was redirected to FPP due to *idispA* overexpression leading to increased farnesene. This also suggests reduced IPP accumulation toxicity which substantially improved growth in UTEX *AFS::dxs::idispA* and hence farnesene production (Fig. 4.7(d)). With a productivity of 2.57 mg/L/day, UTEX *AFS::dxs::idispA* emerges as the superior photosynthetic farnesene producer compared to the existing literature (Pattharaprachayakul et al., 2019; Lee et al., 2021; Chenebault et al., 2023). The productivity was slightly higher (1.15-times) than the PCC 6803 (2.22 mg/L/day) when a similar engineering strategy was applied (Sun et al., 2023). When *AFS* gene integration into the neutral site was coupled with plasmid-borne expression in PCC 6803, a production of 32 mg farnesene/L was achieved in 21 days with reduced productivity (Blanc-Garin et al., 2022). Compared to PCC 7942 (8 mg farnesene/L) and PCC 7002 (2 mg farnesene/L), there was a 1.6-times and 6.4-times increase in production, respectively (Chenebault et al., 2023). In both strains, the integration and plasmid-borne expression approach was used, indicating that the production can increase several times if the same approach is utilized for UTEX 2973. The genetic stability of engineered UTEX strains for farnesene productivity is shown in Fig. 4.7(e). It was observed that there was no significant change in the farnesene productivity tested over a three-month period (~91 generations), indicating that engineered UTEX strains can stably produce farnesene. GC-MS analysis showed the isomeric form of farnesene to be α-farnesene.

Since the highest farnesene productivity was achieved by using 1 mM IPTG from the strain UTEX *AFS::dxs::idispA*, to check whether the highest IPTG concentration gives highest productivity the strain UTEX *AFS::dxs::idispA* was induced with 5 mM IPTG. As

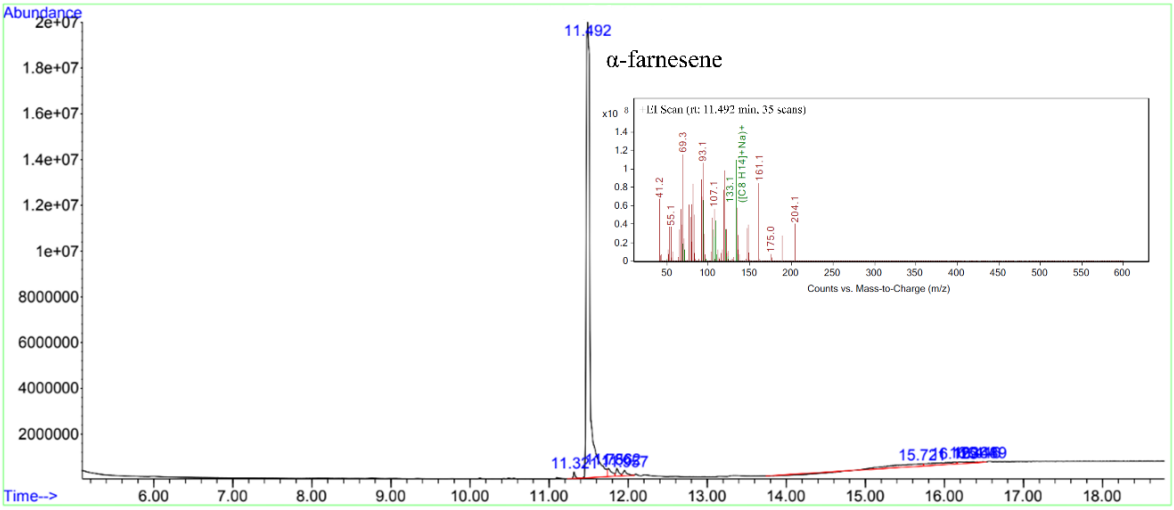

Fig. 4.8. Samples from the isopropyl myristate overlay analyzed by GC-MS. Relative abundance of the peak can be seen in the inset.

can be seen in Fig. 4.9 the strain induced with 5 mM IPTG showed reduced productivity (2.12 mg/L/day). This could be due to the fact that higher IPTG concentrations can be toxic to cells and can also lower protein expression (Einsfeldt et al., 2011, Rizkia et al., 2015).

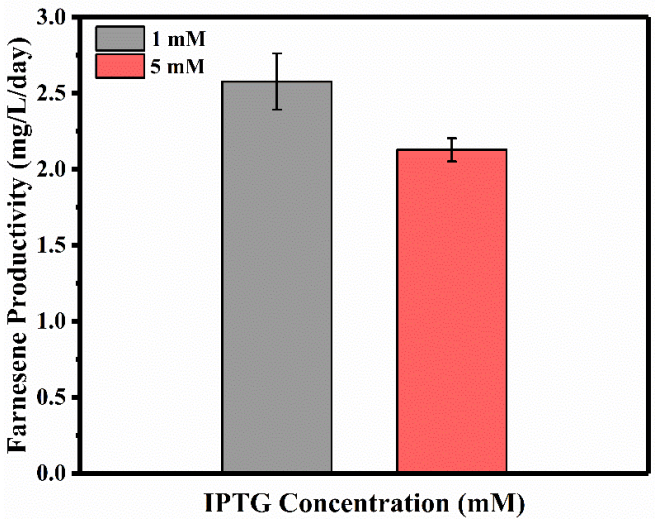

Fig. 4.9. Test of productivity at 1 mM and 5 mM IPTG concentration of the strain UTEX *AFS::dxs::idispA* strain.

All-inclusive, the strain UTEX *AFS::dxs::idispA* showed the highest productivity and yield with no detrimental effect on the growth due to the reduced cellular toxicity by co-expressing terpene synthase (here *AFS*) and MEP pathway genes. The increased productivity of strain UTEX *AFS::dxs::idispA* can be attributed to the genes integrated at

different neutral sites than the PCC 7942, PCC 6803 and PCC 7002 (Sun et al., 2023; Chenebault et al., 2023). The order of the genes and fusion of *idi* and *ispA* through linker molecules could also serve as a rationale (Lv et al., 2013). This was in concordant with the previous studies in different organisms (Kim et al., 2019; Wu et al., 2021). Increased biomass generation is correlated with high metabolite production. In this context a recently discovered robust cyanobacteria strain *Synechococcus* sp. PCC 11901 can be used as a potential platform for metabolic engineering in future studies (Włodarczyk et al., 2020). Further, the production can be improvised by adaptive evolutionary engineering, process parameters optimization and different modes of reactor operation.

4.3. Conclusion

This study demonstrates the sustainable production of farnesene through CO_2 sequestration by engineered strains of *S. elongatus* UTEX 2973. Insertion of the *dxs* showed a 1.2-times increase in farnesene production in comparison to the UTEX *AFS*. Whereas the incorporation of *idispA* resulted in a 5.6-times increase compared to the UTEX *AFS::dxs*. Furthermore, insertion of both *dxs* and *idispA* to UTEX *AFS* exhibited a 31.3-times increase in production with a productivity of 2.57 mg/L/day, the highest among existing cyanobacterial studies. This study could serve as a foundation for engineering any terpene synthase gene into UTEX 2973 by further modulating the genetic elements that control gene expression.