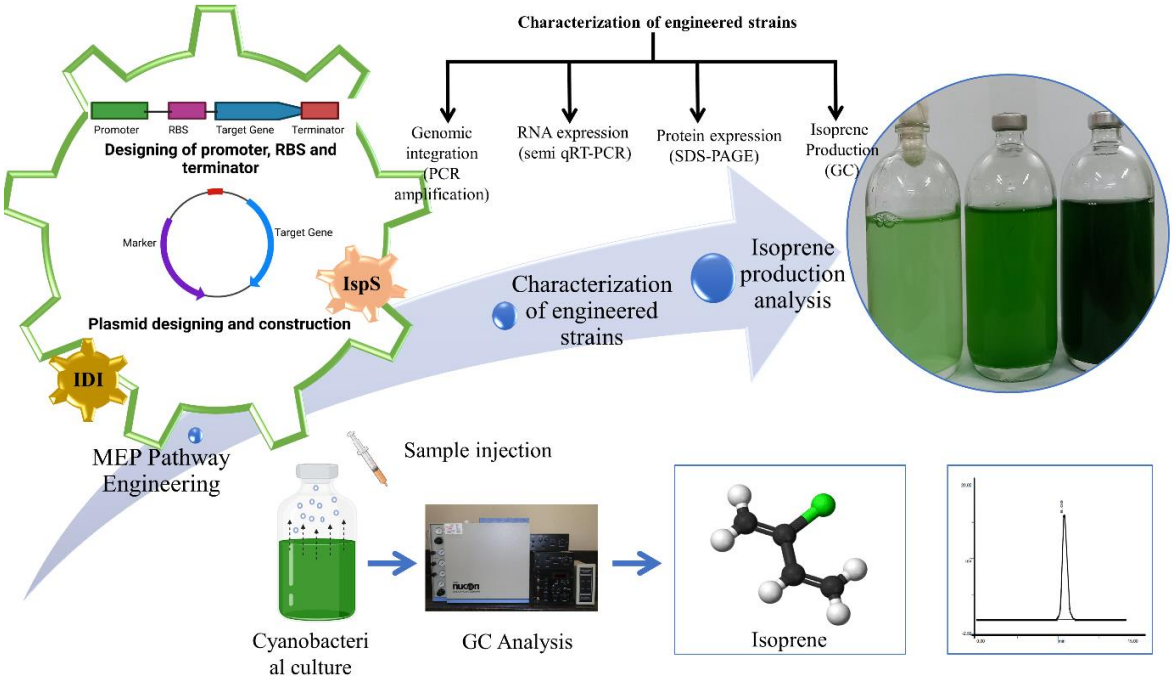
Chapter – 3

Engineering of MEP pathway of *S. elongatus* UTEX 2973 for isoprene production



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3.1 Background

This chapter deals with the engineering of a fast-growing cyanobacterial strain, *S. elongatus* UTEX 2973 for the isoprene production by modifying the MEP pathway. To accomplish the objective, the codon optimized *IspS* gene of plant *Pueraria montana* was cloned in pAM2991 vector under P_{trc} promoter targeted for the integration of *IspS* gene at NSI in the genome of *S. elongatus* UTEX 2973. The pAM 2991 plasmid also contains the upstream and downstream NSI genomic DNA segments of *S. elongatus* PCC 7942 which are 100% similar to the NSI sequence of *S. elongatus* UTEX 2973. Similarly, another plasmid construct pBbe1k-IDI-NSIII was prepared for the integration of *IDI* gene at NSIII in the *S. elongatus* UTEX 2973 genome through the homologous recombination process. The triparental conjugation process was used for the transfer of plasmid constructs into *S. elongatus* UTEX 2973 wild type cells. The recombinant cyanobacterial cells were selected and characterized growth, gene expression, protein expression and isoprene production.

3.2 Materials and methods

3.2.1 Strains and culture conditions

E. coli DH5α cells were routinely used for cloning and propagating plasmids. *E. coli* HB101 cells were used for triparental conjugation. All of the *E. coli* strains were cultivated and maintained in a Luria–Bertani (LB) broth medium for liquid cultures and LB agar for solid-state plate cultures at 37 °C in the presence of suitable antibiotics, where necessary. A fast-growing cyanobacteria strain, *S. elongatus* UTEX 2973, was provided by Professor Pakrasi, Washington University (St. Louis, MI, USA) (Yu et al., 2015). The *S. elongatus* UTEX 2973 cells were grown and maintained in a liquid BG-11 medium or on solid BG-11 agar plates at 38 °C in daylight fluorescent tubes that emit 100 μmol photon/m^2/s PAR (photosynthetically active radiation). Recombinant strains of *S. elongatus* UTEX 2973

produced by genetic modification were grown and maintained under the same conditions as mentioned above in the presence of suitable antibiotics.

3.2.2 Preparation of plasmid constructs

To obtain the isoprene synthase gene (*IspS*), plasmid pBA2SkIKmA2 (Addgene plasmid #39214) was purchased, which contains the codon-optimized *IspS* gene of the *Pueraria montana* plant (Lindberg et al., 2010). Plasmid pAM2991 (Addgene plasmid #40248) was also purchased, which contains upstream and downstream DNA segments of the NSI region of the *S. elongatus* PCC 7942 genome. An advantage of using the pAM2991 plasmid was that it also contains a basis of mobility (BOM sequence, oriT), which is essential for triparental conjugation. Another plasmid, pBbE1k-RFP (Addgene plasmid #35333), was purchased, which was genetically modified further for insertion into the NSIII site of the *S. elongatus* UTEX 2973 genome (Lee et al., 2011). In addition, the conjugal plasmid pRL443 (Addgene plasmid #70261) and the helper plasmid pRL623 (Addgene plasmid #58494), to be used in triparental conjugation, were also purchased (Elhai et al., 1997). A first vector was constructed, containing the *IspS* gene under the P_{trc} promoter as well as upstream and downstream segments of the NSI region targeted for integration at the NSI in the genome of *S. elongatus* UTEX 2973. For this, the *IspS* gene was amplified using high fidelity Phusion Plus DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA) from a pBA2SkIKmA2 plasmid with gene-specific primers, having EcoRI and BamHI flanking recognition sequences at the 5′ and 3′ ends, respectively. PCR was conducted through the use of a Veriti Thermal Cycler and PCR conditions have been mentioned in Tables 3.1 and 3.2 (Applied Biosystems, Waltham, MA, USA). The amplified PCR product (*IspS* gene) was purified by a QIAquick gel extraction kit (Qiagen, Hilden, Germany) and cloned into pAM2991 between the restriction sites mentioned above. After ligation, the constructed resulting vector was named pAM2991-*IspS* (targeting integration at NSI) and transformed

into *E. coli* DH5α cells. Transformed cells were selected in the presence of 50 µg/mL of spectinomycin and streptomycin, and insert verification was carried out by colony PCR as well as double restriction digestion using EcoRI and BamHI. The pAM2991-*IspS* plasmid was isolated from *E. coli* DH5α cells, sequenced for confirmation of the *IspS* gene, and further transformed into competent *E. coli* HB101 cells (first Cargo HB101 strain) for conjugation. Another vector was constructed that contains the NSIII upstream region (NSIII'), the NSIII downstream region (NSIII") fused with a BOM sequence, and *IDI* gene under the P_{trc} promoter in pBbE1k-RFP targeted for integration at the NSIII site in the genome of *S. elongatus* UTEX 2973. For this, sequential addition and the screening of 912 bp NSIII', 549 bp IDI gene, and 900 bp NSIII" fused with a 141 bp BOM sequence in a pBbE1k-RFP plasmid were performed. The PCR amplifications of the NSIII' segment, IDI gene, and NSIII" segment were carried out using gene-specific primers and PCR conditions mentioned in Tables 3.1 and 3.2. The DNA sequences NSIII' (SpeI restriction site at both ends) and NSIII" (AvrII and PciI restriction sites at the 5′ and 3′ ends) were amplified by PCR from the genomic DNA of *S. elongatus* UTEX 2973 using Phusion Plus DNA Polymerase. Target DNA products were gel purified. The purified NSIII′ segment and the pBbE1k-RFP plasmid were digested with the SpeI restriction enzyme, gel purified, and ligated. The ligated product, pBbE1k-RFP-NSIII′, was transformed into competent *E. coli* DH5α cells. Transformed cells were selected with kanamycin (50 µg/mL). The *IDI* gene was amplified from the *E. coli* genome with gene-specific primers with an NdeI cut site at the 5′ end and a BamHI cut site at the 3′ end (Tables 3.1 and 3.2). The pBbE1k-RFP-NSIII′ plasmid and the *IDI* gene were digested with the same restriction enzymes, gel purified, ligated, and transformed into competent *E. coli* DH5α cells. The NSIII" sequence and the BOM site were fused by overlap extension PCR. The pBbE1k-IDINSIII' plasmid and fused NSIII" BOM sequence were digested with restriction enzymes PciI and AvrII. The digested

plasmid and insert were gel purified and ligated. The ligated product (pBbE1k-IDI-NSIII) was transformed in competent *E. coli* DH5α cells, and transformants were selected in the presence of kanamycin (50 µg/mL). The pBbE1k-IDI-NSIII plasmid was isolated from *E. coli* DH5α, sequenced for confirmation of the *IDI* gene as well as NSIII site, and transformed into competent *E. coli* HB101 cells (second Cargo HB101 strain) for conjugation. All of the cloned DNA segments were confirmed by colony PCR as well as by digestion of recombinant plasmids with referred restriction sites.

Table 3.1 List of primers used cloning and semiquantitative PCR.

Name	**Sequence (5´- 3´)**	**Tm (°C)**
IspS FP	CGGAATTCATGCCCTGGCGTGTAATCTGTG	64.4
IspS RP	CGGGATCCCCTCTAGATTACACGTACATTAATTG	63.2
NSI´ FP	GACTAGTCAGCTTAGTCCTGCGCAATCT	51.8
NSI´ RP	GACTAGTCGAAATGTTCTGGACTTGCAGC	52.4
NSI´´ FP	CCTAGGTGAAACAAACCACGGGCA	48.0
NSI´´ RP	CCTAGGGACACCAAAATCACCACG	48.0
NSIII´ FP	GACTAGTCTCGAGATCAGCCAGCTC	57.0
NSIII´ RP	GACTAGTCGACCGACCGATCAACCA	58.0
NSIII´´ FP	ATA CCTAGG GACAAGCCGGGGCAG	62.0
NSIII´´ RP	ATTCTGTGGATAACCGTATTACCGCCTTTGACAGTCGGCGTCACGG	51.1
bom FP	CAAAGGCGGTAATACGGTTATCCACAGAATGCGTCGGTACTGGGTC	51.1
bom RP	AGAACATGTGGACTACGCCATAAAAGAGG	58.0
IDI FP	GCATGACAT ATGCAAACGGAACACGTC	59.7
IDI RP	CGGGATCCTTATTTAAGCTGGGTAAATGCAG	61.7
IspS rt FP	TCGGTGGTGGACTGAAATG	51.1
IspS rt RP	GTCACTGCCTTACGACACT	51.1
IDI rt FP	GCTGTTTAATGCCAAAGGAC	49.7
IDI rt RP	GATCACTGCGTCTTCGTTG	51.1
rpoA FP	GACATCTTGCTCAACGTCC	51.1
rpoA RP	CTTCAACTTCAGGGCCAAAG	51.8

Table 3.2 Amplification of gene/DNA segment by PCR.

Gene/DNA	Amplification conditions
IspS	Initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, **annealing at 57 °C for 20 sec, extension at 72 °C for 2 min** and final elongation at 72 °C for 10 min
IDI	Initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, **annealing at 55 °C for 20 sec, extension at 72 °C for 1 min** and final elongation at 72 °C for 10 min
NSIII´; NSIII´´; NSIII´´.bom	Initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, **annealing at 55 °C for 20 sec, extension at 72 °C for 1.5 min** and final elongation at 72 °C for 10 min
Bom	Initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, **annealing at 55 °C for 20 sec, extension at 72 °C for 30 sec** and final elongation at 72 °C for 10 min
Semiquantitative PCR of IspS/IDI/rpoA	Initial denaturation of 95 °C for 5 min, **followed by 35 cycles of denaturation at 94 °C for 1 min, annealing 20 sec at gene-specific temperatures (Supplementary Table S1), extension at 72 °C for 30 sec** with a final elongation at 72 °C for 10 min
Verification of genomic DNA integration	Initial denaturation of 95 °C for 5 min, **followed by 30 cycles of denaturation at 94 °C for 1 min, annealing 30 sec at gene-specific temperatures (Supplementary Table S1), extension at 72 °C (time according to amplicon size at rate 1 kb/min)** with a final elongation at 72 °C for 10 min

3.2.3 Conjugal transfer of plasmid into *S. elongatus* UTEX 2973

The triparental conjugation technique was performed to produce two recombinant strains of *S. elongatus* UTEX 2973 that carried the heterologous gene *IspS* (*S. elongatus* UTEX IspS) and *IspS* along with *IDI* (*S. elongatus* UTEX IspS.IDI), using a previously described method (Gale et al., 2019). For this, a helper HB101 strain was prepared through transformation with the pRL443 and pRL623 plasmids, and the two Cargo HB101 strains were prepared through transformation with a vector pAM2991-IspS/pBbE1k-IDI-NSIII, as mentioned earlier. To produce the first recombinant strain, *S. elongatus* UTEX IspS, wild-

type UTEX 2973 cells and the first Cargo HB101 strain carrying the vector (pAM2991-IspS) were used; however, to produce the second recombinant strain, *S. elongatus* UTEX IspS.IDI, the first recombinant strain, *S. elongatus* UTEX IspS, and the second Cargo HB101 strain carrying the vector, pBbE1k-IDI-NSIII, were used. A fresh culture of *S. elongatus* UTEX 2973 was grown to OD730 ~0.75 ± 0.05 in 50 mL of BG 11 medium. Cultures grown overnight of helper and cargo strains were centrifuged at 3000 g for 10 min at room temperature using a centrifuge (Thermo Fisher Scientific, Waltham, MA, USA). Pellets were washed twice with an LB broth to remove residual antibiotics and then resuspended in half the volume of the LB medium of the initial culture volume. The helper and cargo HB101 strains were mixed in a 1:1 ratio (450 µL of the helper strain and 450 µL of the cargo strain) and kept at room temperature. One milliliter of the *S. elongatus* UTEX 2973 culture was taken for each conjugation reaction and centrifuged at 1500 g for 10 min at room temperature. The supernatant was discarded, and the pellet was washed twice with BG11 medium and resuspended in the same. Washed *S. elongatus* UTEX 2973 cells, 900 µL, were mixed with previously combined helper and cargo strains in a microfuge tube and incubated in the dark at room temperature for 2 h. Mixed cells were centrifuged at 1500 g for 10 min at room temperature; 1.6 mL of supernatant was discarded and the pellet was resuspended in the remaining supernatant. Resuspended cells were spread onto a MF Millipore 0.22 µm pore size membrane filter (Sigma-Aldrich, St. Louis, MI, USA) placed on BG11 plus 5% LB (v/v) agar plates without antibiotics and incubated at 38 °C for 24 h in light fluorescent tubes emitting 100 µmol photon/m^2/s PAR light intensity. After incubation, the membrane filter was transferred to fresh BG-11 agar plates containing 50 µg/mL of spectinomycin and streptomycin and incubated at 38 °C for 4–5 days in light (100 µmol photon/m^2/s). Transformed *S. elongatus* UTEX 2973 colonies were streaked on fresh BG-11 agar plates containing the abovementioned antibiotics. Transformed colonies were

subsequently re-streaked several times (4 to 5 times) for the proper segregation of DNA. The transformed colonies of the recombinant cyanobacterial strain were named *S. elongatus* UTEX IspS. A similar procedure was used to develop the recombinant strain *S. elongatus* UTEX IspS.IDI, using *S. elongatus* UTEX IspS instead of *S. elongatus* UTEX 2973, and positive transformants were selected in the presence of kanamycin, spectinomycin, and streptomycin (50 µg/mL).

3.2.4 Genomic DNA isolation and PCR analysis of recombinant strains of *S. elongatus* UTEX 2973

Genomic DNA isolation was performed using the protocol described previously (Singh et al., 2011). Genomic DNA was isolated from *S. elongatus* UTEX 2973 and recombinant strains of *S. elongatus* UTEX 2973 (*S. elongatus* UTEX IspS and *S. elongatus* UTEX IspS.IDI) using freshly grown cultures (50 mL; OD730 ~0.75 ± 0.05) by centrifuging at 3000 g for 10 min at room temperature. Cell pellets were resuspended in 400 µL of lysis buffer (Urea 4M, Tris-HCl 0.2 M (pH of 7.4), NaCl 20 mM, EDTA 0.2 M, 50 µL proteinase K (20 mg/mL)) and incubated for 1 h at 55 °C. Furthermore, 1 mL of a prewarmed DNA extraction buffer (CTAB 3%, NaCl 1.4 M, EDTA 20 mM, Tris-HCL 0.5 M (pH of 8.0), sarkosyl 1%, and β-mercaptoethanol 1%) was added to the lysed solution. The mixture was divided into two parts, and equal volumes of chloroform and isoamyl alcohol (24:1) were mixed and centrifuged at 10,000 g for 5 min. The upper aqueous phase was taken in a new tube, and 2 volumes of absolute ethanol in addition to 0.1 volume of 3M sodium acetate (pH of 5.2) were added; the mixture was incubated at –80 °C for 1 h. After incubation, the mixture was centrifuged at 10,000 g for 10 min at 4 °C; the supernatant was discarded and the pellet was washed with 70% ethanol. The pellet was air-dried and resuspended in an elution buffer. Thus, isolated DNA samples were quantified by a NanoDrop One UV–Vis spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and used as templates

to perform PCR for the integration of *IspS* and *IDI* genes within the genomic DNA of *S. elongatus* UTEX 2973 using appropriate primers and PCR conditions.

3.2.5 Growth profile and dry cell weight determination

Growth profile studies on wild-type and recombinant strains of *S. elongatus* UTEX 2973 were performed in cotton-plugged serum bottles under ambient CO_2 conditions, a temperature of 38 °C, continuous white light (100 μmol photon/m^2/s), and shaking at 180 rpm in a Orbitek shaker photo incubator (Scigenics Private Limited, Chennai, TN, India). Samples (1 mL) were taken every 24 h, and the absorbance was measured by using a Cary 60 UV–Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) at 730 nm. The DCW of recombinant strains and the wild-type strain of *S. elongatus* UTEX 2973 was determined by the method described previously (Bentley and Melis, 2012).

3.2.6 Expression analysis of *IspS* and the *IDI* genes in recombinant *S. elongatus* UTEX 2973 strains through semi-quantitative RT-PCR

Semi-quantitative RT-PCR was performed with the method described earlier with slight modification (González et al., 2014). For the expression analysis, *S. elongatus* UTEX 2973 and the recombinant strains (*S. elongatus* UTEX IspS and *S. elongatus* UTEX IspS.IDI) were grown to OD730 ~0.75 ± 0.05 in 50 mL of BG-11 medium by using suitable antibiotics (50 μg/mL) under the same growth conditions as described earlier. Subsequently, cultures of the recombinant strains were induced, with a final concentration of 1 mM IPTG for 24 h. Another set of cultures of recombinant strains without any induction was also cultivated under similar growth conditions. For the total RNA extraction, 50 mL of *S. elongatus* UTEX 2973 and recombinant strain cultures with and without induction were harvested by centrifuging at 5000 g for 10 min at 4 °C after 24 h of induction; pellets were powdered in a precooled mortar and pestle in the presence of liquid nitrogen. Total RNA was isolated from the resulting cell pellet biomass by using RNeasy

Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The complete removal of genomic DNA traces was performed using on-column DNase I digestion. RNA quality and purity were measured using a NanoDrop spectrophotometer, and RNA integrity was checked by electrophoresis. cDNA was synthesized through the reverse transcription of 1 µg of total RNA using a GoScriptTM cDNA synthesis kit (Promega, Madison, WI, USA) and random primers in a 20 µL reaction volume according to the manufacturer's instructions. The cDNAs were diluted with autoclaved distilled water and stored at – 20 °C until further analysis. Gene-specific primers for *IspS* and *IDI* genes were designed with the NCBI primer design tool, targeting the coding region with an amplicon size of approximately 130 bp, and are listed in Table 3.1. The cDNA (50 ng) was used as a template for PCR amplification carried out with gene-specific primers and PCR conditions described in Tables 3.1 and 3.2. A housekeeping gene, the DNA-dependent RNA polymerase subunit alpha (rpoA), was used as an internal control. The PCR products were run on 2% agarose gel, visualized under the Gel Doc system, and the level of expression of genes was measured using ImageJ software.

3.2.7 Analysis of protein by SDS-PAGE

Protein was extracted from samples of wild-type and recombinant *S. elongatus* UTEX 2973 strains and run on SDS-PAGE, as in a previously described protocol (Formighieri and Melis, 2015). Fresh cultures of wildtype and recombinant *S. elongatus* UTEX 2973 strains were grown in 50 mL of BG11 with a suitable antibiotic up to OD730 ~0.75 ± 0.05 and induced with 1 mM IPTG. After 24 h of induction, cells were centrifuged at 3000 g for 10 min at 4 °C. Cell pellets were resuspended in 300 µL of Tris-HCl buffer (50 mM, pH of 8.0) supplemented with the protease inhibitor 1 mM phenylmethylsulphonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MI, USA), 5% glycerol, and 1% Triton X-100. The resuspended samples were lysed by sonication at 50% intensity for 10 min (10 s on and 15

s off). The lysed cell samples were centrifuged at 16,000 g for 30 min. Supernatants were taken and mixed with 62 mM Tris-HCl (pH of 6.8), 1% SDS, 5% β-mercaptoethanol, and 10% glycerol in a 1:1 ratio. The protein contents of the diluted supernatant samples were determined by a Bradford assay (Kruger, 1994). Protein samples (10 µg) of each strain were run on 12% SDS-PAGE resolving gel and stained using the silver staining method.

3.2.8 Isoprene production conditions and quantification

Isoprene production experiments were executed in a batch culture mode in sealed wheaten serum bottles (Sigma-Aldrich, St. Louis,MI, USA). Wild type *S. elongatus* UTEX 2973 and Recombinant strains (*S. elongatus* UTEX IspS and *S. elongatus* UTEX IspS.IDI) were inoculated with an active culture and cultivated in cotton-plugged serum bottles in BG-11 media for 4 days till OD_{730} reaches ~0.75. Cultures were then supplemented with sodium bicarbonate (50 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM), IPTG (1mM), and/or the sodium salt of an alendronate inhibitor (25 µg/mL); cultures were sealed using aluminum crimp and teflon septa by a crimper (Sigma-Aldrich, St. Louis, MI, USA). The sealed serum bottles contain 120 mL of recombinant cyanobacterial cultures and 40 mL of gas headspace. Gas samples of 1 mL were taken from the headspace of serum bottles by using a gas-tight syringe at 24 h intervals as well as analyzed by a Nucon gas chromatography system 5765 (Nucon Engineers Private Limited, New Delhi, India) through the use of a Porapack Q packed column (80/100) (Thermo Fisher Scientific, Waltham, MA, USA) and flame ionization detector (FID). Isoprene was quantified by using a standard curve prepared from the known concentrations of vaporized pure isoprene (Sigma-Aldrich, St. Louis, MI, USA). Nitrogen was used as a carrier gas with a flow rate of 40 mL/min. The initial oven temperature was kept at 50 °C for 2 min, with an increase of 15 °C/min until 120 °C, followed by 1 min of hold time. Furthermore, the oven temperature was raised to 200 °C, with a ramp temperature of 10 °C/min. The injector

temperature was kept at 150 °C and the detector temperature was set at 280 °C (Bentley and Melis, 2012).

3.3 Results and discussions

3.3.1 Construction of plasmids and recombinant *S. elongatus* UTEX 2973 Strains

The *IspS* gene of the *Pueraria montana* plant was selected because it had shown efficient isoprene synthase activity in previously reported studies when expressed under a light-regulated psbA2 promoter in *Synechocystis sp.* PCC 6803 model cyanobacteria to produce isoprene (Bentley and Melis, 2012; Lindberg et al., 2010). We used the pAM2991 plasmid, previously made for the integration at the NSI site in the genome of *S. elongatus* PCC 7942 by adding an NSI′ upstream region and NSI′′ downstream region to accomplish homologous recombination (Ivleva et al., 2005). *S. elongatus* UTEX 2973 is a close relative of *S. elongatus* PCC 7942 and shares 99.8% of genome sequences (Yu et al., 2015). The NSI upstream and downstream sequences are 100% identical, as checked by the NCBI nucleotide BLAST. The codon-optimized *IspS* gene, without the chloroplast transit peptide, was cloned between the EcoRI and BamHI restriction sites under the P_{trc} promoter in the pAM2991 plasmid, forming pAM2991-IspS (Table 3.3; Figure 3.1A). Next, the pBbE1k-IDI-NSIII plasmid construct was prepared, in which the *IDI* gene from *E. coli* DH5α was cloned under the P_{trc} promoter (Table 3.3; Figure 3.1B). The NSIII′ and NSIII′′ sequences from the *S. elongatus* UTEX 2973 genome were cloned at the upstream and downstream regions of the IDI gene, respectively.

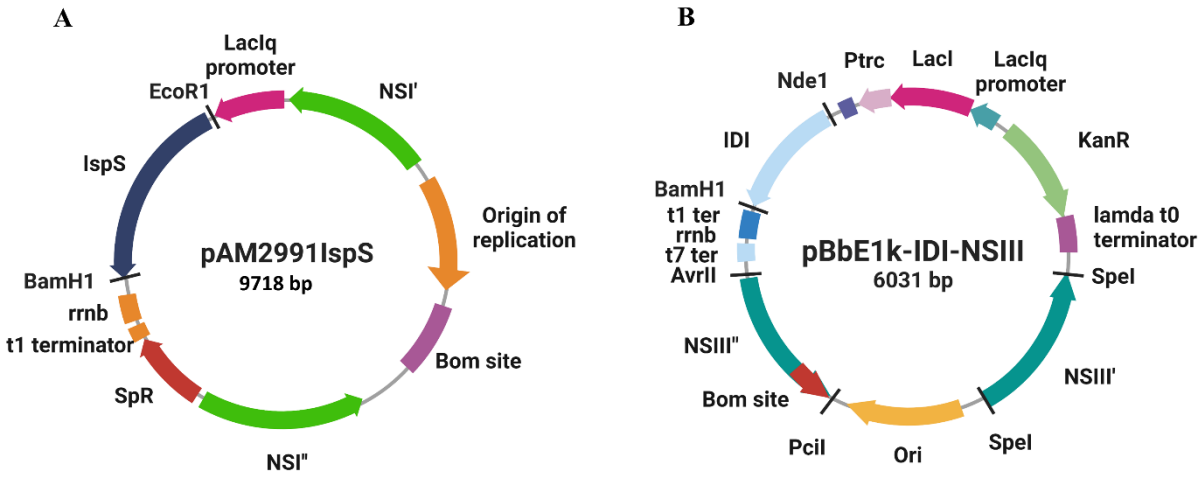

Figure 3.1 Schematic plasmid maps showing the major DNA inserts in constructed integrative vectors (A) pAM2991IspS and (B) pBbE1k-IDI-NSIII.

The *S. elongatus* UTEX IspS recombinant strain was developed by transforming (triparental conjugation) the pAM2991-IspS plasmid into *S. elongatus* UTEX 2973. The *IspS* gene, together with a spectinomycin resistance gene, was integrated at the NSI site of the UTEX 2973 genome by double homologous recombination, forming the recombinant strain *S. elongatus* UTEX IspS (Figure 3.2A). Transformant cells were grown for several generations in the presence of a kanamycin antibiotic for the proper segregation of *S. elongatus* UTEX IspS DNA (DNA homoplasmy), resulting in the replacement of the DNA segment NSI with the *IspS* transgene. Similarly, the *S. elongatus* UTEX IspS.IDI strain was developed by transforming the pBbE1k-IDI-NSIII plasmid into the *S. elongatus* UTEX IspS strain, resulting in the integration of the *IDI* gene at NSIII into the *S. elongatus* UTEX IspS genome. A scheme of genomic DNA integrations of the *IspS* and *IDI* genes in the *S. elongatus* UTEX Isps.IDI strain is represented in Figure 3.2B. The genomic integrations of the *IspS* and *IDI* genes were confirmed by a PCR analysis using genomic DNA of recombinant strains (Figure 3.2C). The cultivation scheme of recombinant strains of *S. elongatus* UTEX 2973 for growth and isoprene production is given in Figure 3.2D.

Table 3.3 List of plasmids and strains used in this study.

Plasmids and strains	Description	Antibiotic resistance	Reference
Plasmids			
pBA2SkIKmA2	Contains codon-optimized *IspS* gene for cyanobacteria	Kanamycin	Lindberg et al. (2010)
pAM2991	One-Step cloning vector for overexpression between EcoRI and BamHI restriction sites	Spectinomycin	Ivleva et al. (2005)
pRL443	Conjugal plasmid suitable for the mobilization of cargo plasmids in cyanobacteria	Ampicillin	Elhai et al. (1997)
pRL623	Helper plasmid for bacterial conjugal DNA transfer	Chloramphenicol	Elhai et al. (1997)
pBbE1k-*RFP*	Contains *RFP* gene between NdeI and BamHI	Kanamycin	Lee et al. (2011)
pAM2991-*IspS*	Derivative of pAM2991, *IspS* gene cloned between EcoRI and BamHI site under P_{trc} promoter	Spectinomycin	This work
pBbE1k-*IDI*-NSIII	Made from pBbE1k-*RFP*, *IDI* gene cloned between NdeI and BamHI sites under P_{trc} promoter	Kanamycin	This work
Strains			
UTEX 2973	Wild-type	None	Yu et al. (2015)
UTEX 2973 *IspS*	The *IspS* gene integrated at NSI site of the UTEX 2973 genome	Spectinomycin	This work
UTEX 2973 *IspS.IDI*	*IspS* and *IDI* genes integrated at the NSI and NSIII sites of UTEX 2973 genome respectively	Spectinomycin and kanamycin	This work

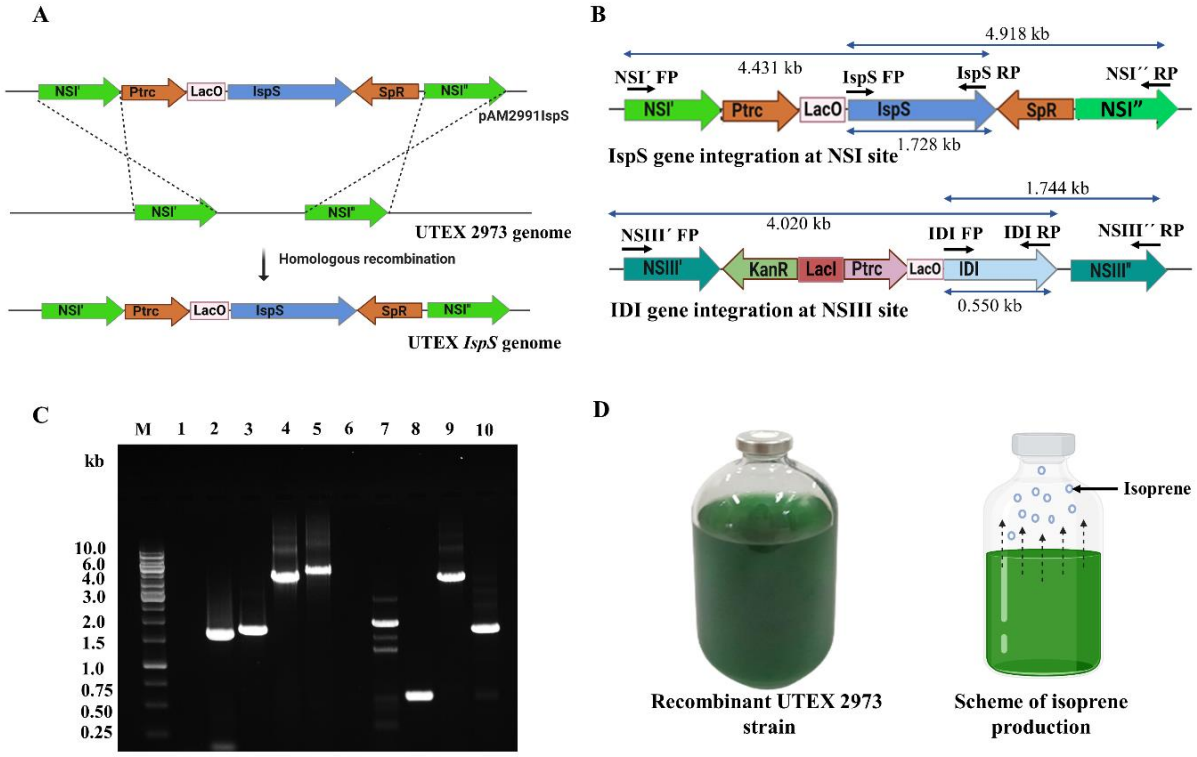

Figure 3.2 The homologous recombination process is used for the genomic DNA integration of the *IspS* and *IDI* genes at neutral site I (NSI) and neutral site III (NSIII), respectively. (A) Scheme of homologous recombination for *IspS* gene integration at the NSI site in the UTEX 2973 genome. (B) Schematic representation of the IspS and IDI genes integrated into the UTEX 2973 genome at the NSI and NSIII sites, respectively. Ptrc—trc promoter; LacO—lac operator; SpR—spectinomycin resistance; KanR—kanamycin resistance; and LacI—lac repressor. (C) Gel image shows the genomic DNA integration of the *IspS* and *IDI* genes by PCR. The genomic DNA template of cyanobacterial strains, the primer pair used, and the amplified product size are as follows: M: DNA ladder; 1: primers—IspS FP and IspS RP, template—the UTEX 2973 genome, and no product was amplified; 2: primers—NSI′ FP and NSI′′ RP, template—the UTEX 2973 genome, and 1.6 kb of amplified product; 3: primers—IspS FP and IspS RP, template—the UTEX IspS.IDI genome, and 1.728 kb of amplified product; 4: primers—NSI′ FP and IspS RP, template—the UTEX IspS.IDI genome, and 4.431 kb of amplified product; 5: primers—IspS FP and NSI" RP, template—the UTEX IspS.IDI genome, and 4.918 kb of amplified product; 6: primers—IDI FP and IDI RP, template—the UTEX 2973 genome, and no product was amplified; 7: primers—NSIII′ FP and NSIII" RP, template—the UTEX 2973 genome, and 1.8 kb of amplified product; 8: primers—IDI FP and IDI RP, template—the UTEX IspS.IDI genome, and 0.550 kb of amplified product; 9: primers—NSIII' FP and IDI RP, template—the UTEX IspS.IDI genome, and 4.020 kb of amplified product; and 10: primers—IDI FP and NSIII" RP, template—the UTEX IspS.IDI genome, and 1.744 kb of amplified product. (D) Scheme of isoprene production by recombinant UTEX 2973 strains using serum bottle cultivation. Dashed arrows denote the shifting of isoprene molecules from the aqueous phase to gas phase in the sealed culture bottle.

3.3.2 Expression of *IspS* and *IDI* transgenes and the growth profile of recombinant *S. elongatus* UTEX 2973 Strains

The expression of heterologous genes, *IspS* and *IDI*, in recombinant strains, *S. elongatus* UTEX IspS and *S. elongatus* UTEX IspS.IDI, at the transcription level was determined by mRNA detection by using semi-quantitative RT-PCR (Figure 3.3A). Maximum levels of *IspS* and *IDI* transcripts have been reported in genetically engineered cyanobacteria at a 1 mM concentration of IPTG in a previous study (Gao et al., 2016). To perform transcriptional analyses, *S. elongatus* UTEX 2973 and recombinant *S. elongatus* UTEX 2973 cultures (in the absence and presence of IPTG) were used for mRNA extraction and semiquantitative RT-PCR analysis. The results showed the presence of intense DNA bands in cultures supplemented with IPTG for the *IspS* gene in strains *S. elongatus* UTEX IspS and *S. elongatus* UTEX IspS.IDI. Similarily, an intense band of *IDI* was seen in *S. elongatus* UTEX IspS.IDI strains (Figure 3.3A). However, faint bands of *IspS* in strains *S. elongatus* UTEX IspS and *S. elongatus* UTEX IspS.IDI strains, as well as *IDI* in *S. elongatus* UTEX IspS.IDI, were observed in cultures grown without IPTG due to the leaky nature of the P_{trc} promoter (Camsund et al., 2014). Since the *S. elongatus* UTEX 2973 strain does not have *IspS* and *IDI* (from *E. coli*) genes, no amplification was observed when using gene-specific semi-quantitative RT-PCR primers. Constitutively expressing housekeeping gene rpoA was used as an internal control, as suggested previously (Pinto et al., 2012). An amplified product of the rpoA gene was observed in strains *S. elongatus* UTEX 2973, *S. elongatus* UTEX IspS, and *S. elongatus* UTEX IspS.IDI (Figure 3.3A). Relative expression levels of the *IspS* and *IDI* genes were determined by using the *rpoA* gene as an internal control to normalize expression levels via densitometric analyses using ImageJ software. Normalized expression levels of the *IspS* gene were ascertained to be 0.20 (uninduced) and 0.9 (induced) in the *S. elongatus* UTEX IspS strain and 0.22 (uninduced) and 0.94 (induced) in

the *S. elongatus* UTEX IspS.IDI strain; however, *IDI* gene expression levels were 0.15 (uninduced) and 0.93 (induced) in the *S. elongatus* UTEX IspS.IDI strain. These findings suggest that the expression levels of the *IspS* and *IDI* genes are nearly equivalent to the internal control rpoA gene (Lin et al., 2021; Pinto et al., 2012). Equal amounts of protein samples (10 µg) from the soluble fraction obtained from wild-type and recombinants of *S. elongatus* UTEX 2973 were run on SDS-PAGE and visualized under Gel Doc for protein analysis. A prominent band of IspS of 65.85 kDa was observed in the *S. elongatus* UTEX IspS and *S. elongatus* UTEX IspS.IDI strains. Similarly, a 20.5 kDa protein band of IDI was observed in the *S. elongatus* UTEX IspS.IDI strain. No IspS and IDI protein bands were seen in *S. elongatus* UTEX 2973 (Figure 3.3B). A growth study on *S. elongatus* UTEX 2973, *S. elongatus* UTEX IspS, and *S. elongatus* UTEX IspS.IDI was performed to see the effect of transgene expression on the growth characteristics of recombinant strains. Changes observed in the growth profiles of the recombinant strains were negligible when compared to the wild-type *S. elongatus* UTEX 2973 in the present study (Figure 3.3C,D). These findings are supported by a previous study in which the *IspS* and *IDI* genes were transformed into *S. elongatus* PCC 7942 without hampering the growth properties (Gao et al., 2016).

3.3.3 Isoprene production by recombinant *S. elongatus* UTEX 2973 Strains

All of the production studies were performed in closed serum bottles. Culture bottles were incubated in an inverted position to avoid the evaporation of produced isoprene, as it is highly volatile. Isoprene concentration was measured in the gaseous phase of bottle headspace every 24 h after inducer and/or inhibitor addition. Previous studies have shown that isoprene and oxygen accumulation in a closed vessel inhibit cell growth and isoprene productivity (Gao et al., 2016; Rana et al., 2022). Therefore, the headspaces of culture

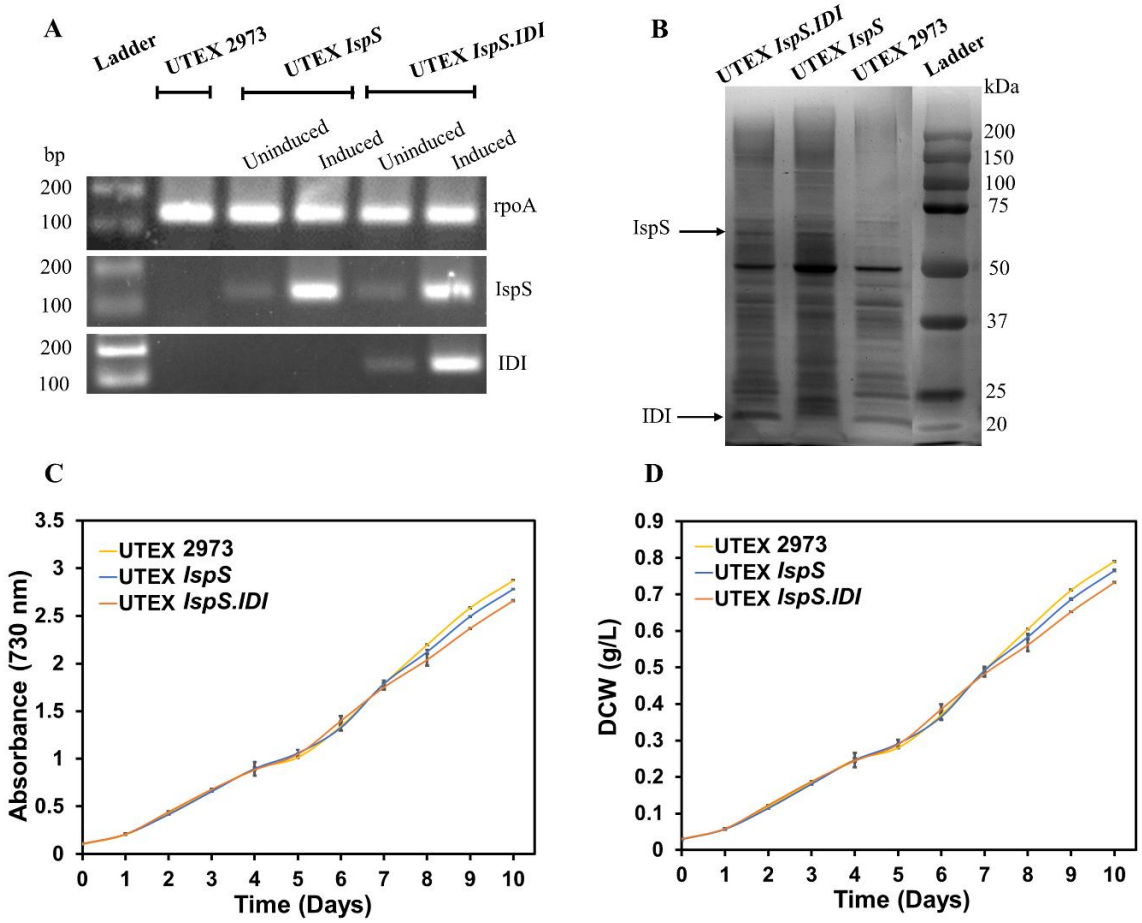

Figure 3.3 Gene expression analysis and growth profiles of S. elongatus UTEX 2973, S. elongatus UTEX IspS, and S. elongatus UTEX IspS.IDI strains. (A) Semiquantitative RT-PCR: Constitutively expressing rpoA gene was used as a positive control, and gene-specific primers were used for the amplification of 130 bp DNA segments from *IspS*, *IDI*, and *rpoA* coding regions. (B) Protein analysis by SDS-PAGE: Prominent band of IspS (65.85 kDa) in UTEX IspS and UTEX IspS.IDI strains and protein band of IDI (20.5 kDa) was observed in UTEX IspS.IDI strain. (C) and (D) Growth profiles of UTEX 2973, UTEX IspS, and UTEX IspS.IDI strains in cotton plugged serum bottle cultures (100 µmol photons/m^2/s, 38 °C, ambient CO_2, and 180 rpm) expressed in absorbance (730 nm) and DCW (g/L).

bottles were vented every 24 h to remove the accumulated isoprene and oxygen during the production phase. The isoprene level in recombinant strain *S. elongatus* UTEX IspS could not be quantified in the presence of IPTG, since the isoprene concentration was detected as being below the limit of quantification (LOQ) of the GC system (Figure 3.4A). The IspS enzyme utilizes DMAPP as a substrate and converts it into isoprene, thus decreasing the intracellular concentration of DMAPP. Since IDI enzyme catalyzes the interconversion of

IPP into DMAPP, we overexpressed the *IDI* gene in the *S. elongatus* UTEX IspS.IDI strain to increase the intracellular DMAPP flux towards isoprene production. We achieved a maximum isoprene value of 34.34 ± 1.87 µg/L of culture, equivalent to 79.97 ± 2.54 µg/g DCW of culture, in a single day, and a cumulative isoprene titer value of 0.16 ± 0.0064 mg/L of culture and 0.41 ± 0.0063 mg/g DCW in 6 days of cultivation in the *S. elongatus* UTEX IspS.IDI strain (*IspS* and *IDI* genes harboring) in the presence of inducer (Figure 3.4B). The average isoprene productivity was calculated as being 1.11 µg/L/h, equivalent to 2.8 µg/g DCW/h, in 6 days with an inducer.

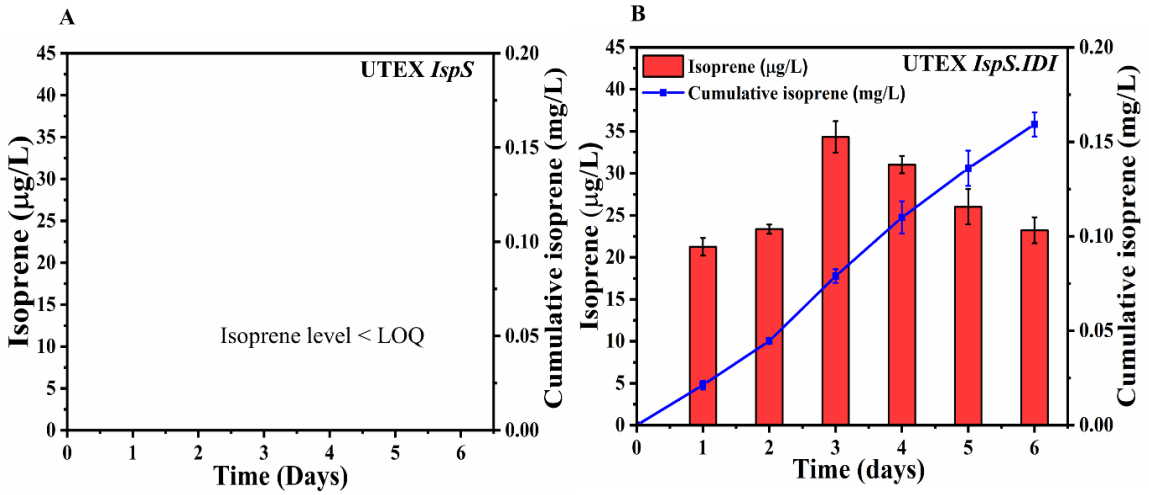

Figure 3.4 Isoprene production profiles of recombinant *S. elongatus* UTEX 2973 strains. (A) *S. elongatus* UTEX IspS (B) *S. elongatus* UTEX IspS.IDI. Cotton-plugged cultures were grown (100 µmol photons/m^2/s, 38 °C, ambient CO_2, and 180 rpm) upto OD_{730} 0.75 ± 0.05. Furthermore, cultures were supplemented with 50 mM $NaHCO_3$, 10 mM HEPES buffer, and 1 mM inducer and sealed to entrap the produced isoprene in the bottle headspace. All data are expressed as the average values of triplicate experiments with standard deviation. The bar diagrams show the isoprene production, and the line plot shows the cumulative isoprene production. Limit of detection (LOD), 0.5 µg isoprene/L of gas phase; limit of quantification (LOQ), 2.0 µg isoprene/L of the gas phase.

Studies reporting on the isoprene production from CO_2 when using recombinant cyanobacteria in closed cultivation and open continuous culture systems have been previously performed. Pioneering work reported 50 µg/g DCW isoprene production per day

by integrating a codon-optimized IspS gene under a psbA2 promoter in *Synechocystis sp.* PCC 6803 from the *Pueraria montana* plant (Lindberg et al., 2010). Further several studies were performed by researchers utilizing different engineering strategies and culture conditions to enhance isoprene yields. We observed a cumulative isoprene yield of 0.41 ± 0.0063 mg/g DCW in presence of IPTG in engineered *S. elongatus* UTEX IspS.IDI strain. The isoprene production level could be further enhanced by other bottleneck gene modification of the MEP pathway. Furthermore, culture conditions and nutrients could be optimized for increasing the isoprene production in engineered cyanobacterial strain.

3.4 Conclusion

Isoprene, having the properties of advanced fuels, could be a potential alternative to fossil-based fuels. The sustainable production of isoprene using recombinant photosynthetic cyanobacteria has been our approach in this study. We successfully modified an MEP pathway by integrating the *IspS* gene from the *Pueraria montana* plant and the *IDI* gene from *E. Coli* DH5α under the control of a strong inducible promoter, P_{trc}, in the genome of *S. elongatus* UTEX 2973 at the NSI and NSIII sites, respectively. Recombinant *S. elongatus* UTEX 2973 strains were cultured in serum bottles containing BG11 media supplemented with 1 mM IPTG and 50 mM $NaHCO_3$ for isoprene production studies. The *S. elongatus* UTEX IspS.IDI strain produced a cumulative isoprene amount of 0.41 mg/g DCW in 6 days in the presence of IPTG. The production level of isoprene could be significantly enhanced by making additional modifications to bottleneck genes within the MEP pathway. Additionally, it is possible to optimize the culture conditions and nutrient supply to further maximize isoprene production in the genetically engineered cyanobacterial strain.