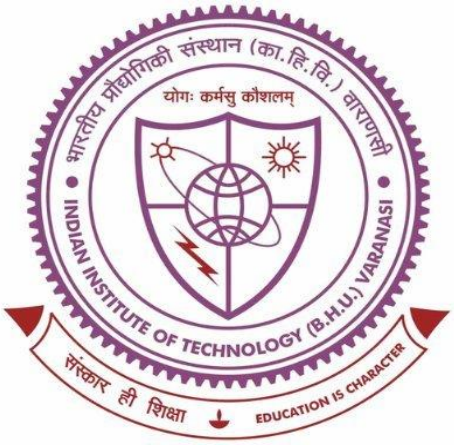
Production of Farnesene by Engineered Cyanobacteria



Thesis submitted in partial fulfilment for the Award of Degree

DOCTOR OF PHILOSOPHY

By

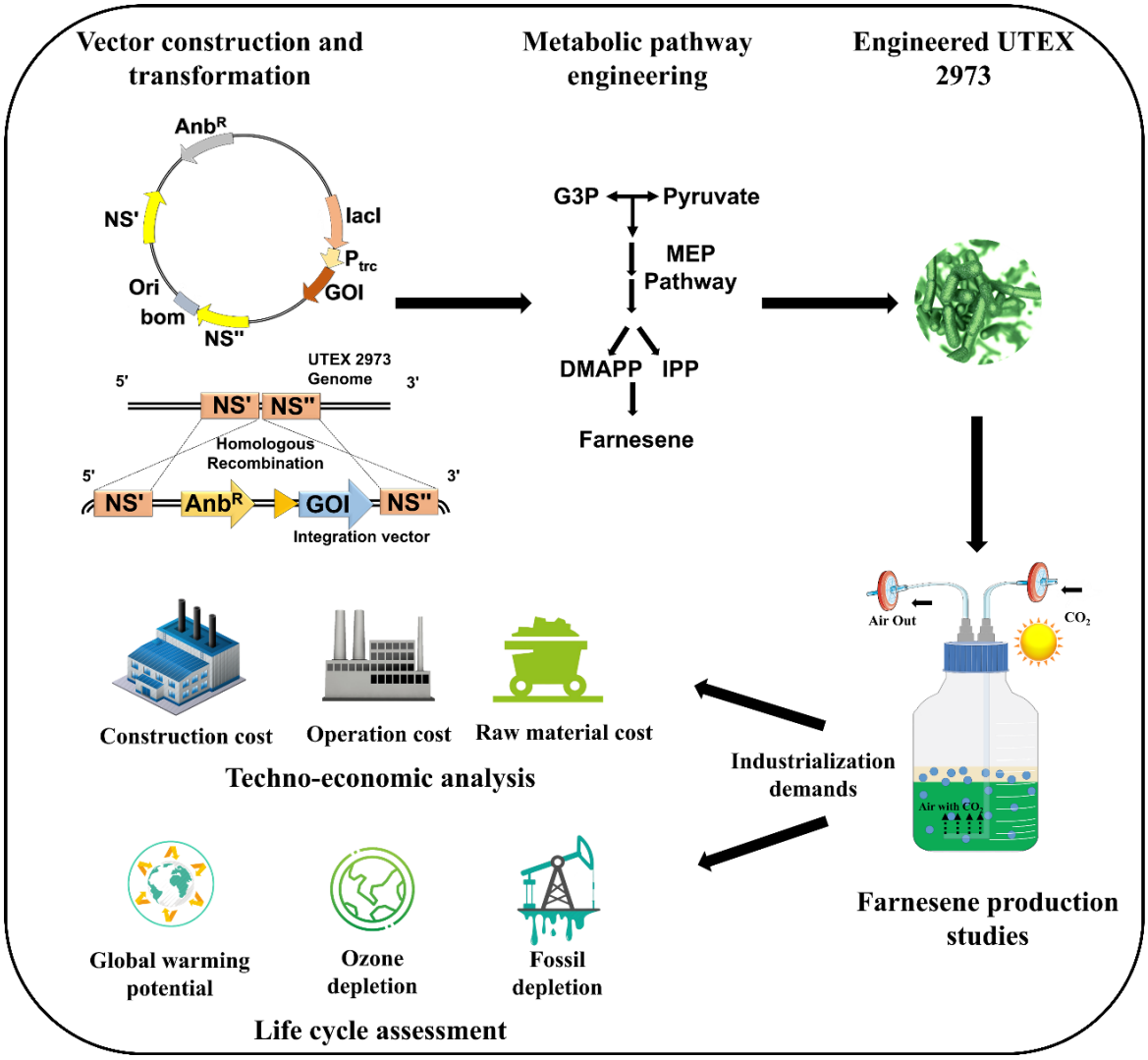
AKHIL RAUTELA

School of Biochemical Engineering
Indian Institute of Technology
(Banaras Hindu University)
Varanasi – 221005
India

17011501 **2024**

CHAPTER 6

Conclusion and future perspectives



Cyanobacteria are the prospective microbial cell factories for the production of value-added products. In the present study *Synechococcus elongatus* UTEX 2973 was genetically modified for sustainable production of farnesene. For this purpose, foremost integrative vectors were constructed. The integrative vector strategy represents a significant advancement in the field of genetic engineering as it enables the stable integration of genes into the genome of host organism without compromising the expression of target genes. The MEP pathway which generates precursor molecules for farnesene synthesis was targeted. Three integration vectors to target MEP pathway bottleneck gene(s) were made namely pAM2991-*AFS*, pBbE1cNSII-*dxs*, and pBbE1kNSIII-*idispA*. Since cyanobacteria lacks *AFS* gene, pAM2991-*AFS* targets codon-optimized *M. domestica AFS* gene to the NSI of UTEX 2973. The pBbE1cNSII-*dxs* target 1-deoxy-D-xylulose-5-phosphate synthase (*dxs*) to the NSII of UTEX 2973. While the pBbE1kNSIII-*idispA* target fusion of isopentenyl diphosphate isomerase and farnesyl diphosphate synthase (*idispA*) to NSIII of UTEX 2973. The vectors constructed were verified at each step with colony PCR and restriction digestion followed by sequencing to confirm the addition of gene/fragment.

The metabolic pathway engineering of UTEX 2973 by the integration vectors constructed showed the highest farnesene productivity. Integration vectors were transformed into UTEX 2973 through triparental conjugation generating number of strains namely, UTEX *AFS*, UTEX *AFS::dxs*, UTEX *AFS*::*idispA*, and UTEX *AFS*::*dxs*::*idispA*. The strains generated were characterized and antibiotic survival test, homoplasmy analysis, transcriptional, and translational analysis were done. A Campbell integration test reveals that Campbell-like integration has not taken place, suggesting that the gene(s) were integrated into the UTEX 2973 genome by homologous recombination. After characterization of the strains and confirmation that the genomic integration of *AFS*, *dxs*, and *idispA* at NSI, NSII, and NSIII, respectively, is successful, farnesene production and

growth studies of the strains were done. Strain UTEX *AFS* produced 0.41 ± 0.08 mg/L farnesene and showed growth inhibition. The production was underwhelming, which was presumed to be due to the shortage of farnesene precursor, FPP. Insertion of the *dxs* gene only showed a 1.2-times increase (0.49 ± 0.05 mg/L by UTEX *AFS::dxs*) in farnesene production in comparison to the UTEX *AFS*. The fusion of *idi* and *ispA*, which converts IPP to DMAPP and further to FPP, respectively, escalates farnesene production in UTEX *AFS::idispA*. The UTEX *AFS::idispA* showed a 5.6-times increase (2.76 ± 0.06 mg/L) in farnesene and improved growth compared to the UTEX *AFS::dxs*. This shows the negative effect of *dxs* in UTEX *AFS::dxs* due to the excessive buildup of IPP and the inability to convert it to FPP and farnesene, which was resolved by engineering the *idispA* gene into UTEX *AFS::idispA*. Furthermore, insertion of both *dxs* and *idispA* genes to UTEX *AFS* exhibited a 31.3-times increase (12.87 ± 0.7 mg/L by UTEX *AFS::dxs::idispA*) in production with a productivity of 2.57 mg/L/day, the highest among existing cyanobacterial studies. The *dxs* overproduction might have increased the IPP pool, and *idispA* overexpression has led this IPP pool to farnesene production.

Testing the productivity in two-phase and single-phase photobioreactors suggests that product loss occurs in the single-phase culture due to gas stripping, making two-phase culturing the preferred choice. Moreover, the increased IPTG concentration (5 mM) does not increase farnesene productivity due to the toxicity of higher concentrations of IPTG and lower protein expressions. 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. The results obtained from this study demonstrate the effectiveness of the integrative vector strategy in enhancing farnesene production in engineered cyanobacterial strains. The incremental improvements achieved through the integration of genes encoding key enzymes involved in farnesene biosynthesis underscore the potential

for further enhancement through targeted genetic modifications. Notably, the synergistic effect observed upon integrating multiple genes highlights the importance of comprehensive metabolic engineering strategies in maximizing product yields.

To check the economic and environmental feasibility of large-scale farnesene production by engineered UTEX 2973, the techno-economic analysis and life cycle assessment were conducted. The analysis serves as a conceptual framework for scaling up farnesene production with 100 tonnes annual capacity. The study highlights key factors influencing the minimum farnesene selling price (MFSP) and potential avenues for increasing productivity and reducing the cost of the process. The estimated CapEx for the plant amounts to $28.16 MM, encompassing equipment, installation and other costs. Taking into account both fixed and variable operating costs, the total annual OpEx is projected to be $30.75 MM. The key cost drivers of the MFSP were determined by single-point sensitivity analysis. The study identified the farnesene productivity and cost of organic solvent and inducer, majorly influencing the MFSP. An MFSP of $5.57/kg was observed with a 70-fold increase in productivity, which was further reduced following the reduction in the cost of organic solvent. Moreover, a life cycle assessment of the conceptual process is assessed, indicating that the process is carbon neutral. The study provides future insight into the commercialization of sustainable farnesene production by cyanobacteria.

The advancements made in genetically engineering *Synechococcus elongatus* UTEX 2973 for farnesene production provide a solid foundation for future work in microbial cell factory development. For instance, the study's focus on the MEP pathway has laid the groundwork for future enhancements through fine-tuning enzyme expression, improving precursor supply, and eliminating metabolic bottlenecks. Future research could investigate novel synthetic biology approaches, such as CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa), to regulate gene expression dynamically. The upstream

and downstream regions of the neutral sites can be modified according to the different neutral sites or the host organism. The *AFS* and other MEP pathway bottleneck gene(s) expression can be increased by modulating the genetic elements such as strong promoter and/or ribosome binding site engineering, which can further increase the farnesene production. Additionally, the genes of interest in the integrative vectors are cloned at the unique restriction site in the integrative vectors constructed and can be used to integrate any terpene synthase gene(s) or any other gene(s) into the genome of UTEX 2973 leading to simultaneous production of multiple value-added compounds, such as other terpenes or biofuels. By engineering additional metabolic pathways, these cyanobacteria could serve as versatile platforms for producing a range of chemicals, turning them into the cornerstone of multi-product biorefineries.

While this study outlines a promising path for farnesene production on a small scale, transitioning to industrial-scale operations will be crucial. Continuous efforts to optimize fermentation conditions, reactor designs, and nutrient supplies will help increase yield efficiency while reducing the costs associated with organic solvents and inducers. Moreover, investigating alternative culturing systems, such as continuous photobioreactors or engineered light-harvesting complexes, could maximize photosynthetic efficiency and growth rates. Dairy waste can be used as an alternative to freshwater for the cultivation of cyanobacteria. Moreover, the lactose present in dairy waste can act as an alternative to IPTG for inducing trc promoter. In addition to the MEP pathway, the introduction of the MVA pathway into cyanobacteria can further increase the yield of farnesene. Improvement in farnesene yield, alternative sources to IPTG and use of waste water for cyanobacteria cultivation can lead to low farnesene production cost and making the process feasible.

As the study emphasizes, the economic feasibility of large-scale production is a critical consideration. Future research should focus on improving the cost-effectiveness of

farnesene production through innovations in downstream processing, such as solvent recovery and recycling. Additionally, optimizing the supply chain for biomass feedstocks and photobioreactor materials could further reduce CapEx and OpEx. Expanding life cycle assessments to include real-world data will help refine projections of carbon neutrality, allowing for clearer market positioning in the growing field of sustainable bioproducts.

The carbon-neutral outcome of the process is a vital selling point. Future work could aim to reduce energy inputs by integrating renewable energy sources into the production process or by engineering strains that require less light or nutrients to grow. Research into more efficient light distribution in photobioreactors could also help lower energy consumption. Combining these optimizations with a life cycle assessment will provide a clearer picture of how large-scale microbial production can fit into global sustainability efforts.