# Production of Farnesene by Engineered Cyanobacteria

**Thesis submitted in partial fulfilment for the Award of Degree**

## DOCTOR OF PHILOSOPHY

**By**

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It is certified that the work contained in the thesis titled "**Production of Farnesene by Engineered Cyanobacteria**" by **Akhil Rautela** has been carried out under my supervision and that this work has not been submitted elsewhere for a degree.

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***Dedicated to my loving***

***parents and brother***

# Acknowledgement

*Knowledge without humility is worthless.*

– **Mahamana Pt. Madan Mohan Malviya**
(Creator of the pious seat of learning, Banaras Hindu University)

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Akhil Rautela

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## List of Abbreviations

| | |
|---|---|
| 1G | First generation |
| 2G | Second generation |
| 3G | Third generation |
| 4G | Fourth generation |
| AFS/FS | Farnesene synthase |
| ASTM | American Society of Testing and Materials |
| AtoB | Acetoacetyl-CoA thiolase |
| bom | Basis of mobility |
| CapEx | Capital expenditure |
| CBB | Coffee berry borer |
| $Chl^R$ | Chloramphenicol resistance gene |
| CpcB | β-subunit of phycocyanin |
| CRISPR | Clustered regularly interspaced short palindromic repeats |
| DCW | Dry cell weight |
| DMAPP | Dimethylallyl diphosphate |
| DPOAEs | Distortion product otoacoustic emissions |
| DPPH | 2,2-diphenyl-1-picryl-hydrazyl |
| DXP | 1-deoxy-D-Xylulose-5-phosphate |
| DXR | DXP reductoisomerase |
| dxs | 1-deoxy-D-Xylulose-5-phosphate synthase |
| EBF | (E)-β-farnesene |
| ENDS | Electronic nicotine delivery systems |
| FID | Flame ionization detector |
| FOC | Fixed operating cost |
| FP | Forward primer |
| FPP | Farnesyl diphosphate |
| FPPS/ispA | Farnesyl diphosphate synthase |
| FSLFPPS | Fusion of FS and FPPS genes by linker |
| GAP | Glyceraldehyde 3-phosphate |
| GC | Gas chromatography |
| GGPP | Geranyl geranyl diphosphate |
| GHG | Greenhouse gas |
| GPP | Geranyl diphosphate |
| GPPS | GPP synthase |
| GLS | Geranyllinalool synthase |
| HMBPP | 4-hydroxy-3-methylbut-2-enyldiphosphate |
| HMG-CoA | 4-hydroxy-3-methyl-glutaryl-CoA |
| HMGS | HMG-CoA synthase |
| HMGR | HMG-CoA reductase |
| $IC_{50}$ | Half-maximal inhibitory concentration |
| idi | Isopentenyl diphosphate isomerase |

| | |
|---|---|
| idispA | Fusion of *idi* and *ispA* genes by linker |
| IM | Isopropyl myristate |
| IspG | HMBPP synthase |
| IspH | HMBPP reductase |
| IPP | Isopentenyl diphosphate |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| IspS | Isoprene synthase |
| $kan^R$ | Kanamycin resistance |
| $K_{cat}$ | Turnover number |
| kDa | Kilo Dalton |
| $K_m$ | Michaelis constant |
| LB | Luria-Bertani broth |
| LCA | Life cycle assessment |
| LDH | Lactate dehydrogenase |
| MEP | Methyl-D-erythritol-4-phosphate |
| MeSa | Methyl salicylate |
| MFSP | Minimum farnesene selling price |
| MK | Mevalonate kinase |
| MVA | Mevalonate |
| NADH | Nicotinamide adenine dinucleotide |
| NASA | National Aeronautics and Space Administration |
| $NO_X$ | Nitrogen oxides |
| NREL | National Renewable Energy Laboratory |
| NSI | Neutral site I |
| NSI′ | Upstream NSI region |
| NSI″ | Downstream NSI region |
| NSII | Neutral Site II |
| NSII′ | Upstream NSII region |
| NSII″ | Downstream NSII region |
| NSIII | Neutral site III |
| NSIII′ | Upstream NSIII region |
| NSIII″ | Downstream NSIII region |
| OBPs | Odorant binding proteins |
| OpEx | Operating expenditures |
| ORs | Odorant receptors |
| OUFP | Orientation-specific upstream forward primer |
| PBR | Photobioreactor |
| PCR | Polymerase chain reaction |
| PCX | Paclitaxel |
| PHLS | β-phellandrene synthase |
| PT | Prenyl transferase |
| PMD | Mevalonate 5-pyrophosphate decarboxylase |
| PMK | Mevalonate 5-phosphate kinase |

| | |
|---|---|
| ppm | Parts per million |
| RBS | Ribosome binding site |
| RDRP | Reversible deactivation radical polymerization |
| RFP | Red fluorescent protein |
| RP | Reverse primer |
| SARS | Severe acute respiratory syndrome |
| SCN | Soybean cyst nematode |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SIP | Iso paraffin |
| $Sm^R$ | Streptomycin and spectinomycin resistance |
| TEA | Techno-economic analysis |
| TPEs | Thermoplastic elastomers |
| tHMGR | Truncated HMGR |
| $T_g$ | Glass transition temperature |
| TPS | Terpene synthase |
| VOC | Variable operating cost |

# Notations

| | |
|---|---|
| α | Alpha |
| β | Beta |
| γ | Gamma |
| °C | Degree celsius |
| °C/min | Degree celsius per minute |
| h | Hour |
| Gt | 1 billion tonnes |
| % | Percentage |
| $ | Dollar |
| $/kg | Dollar per kilogram |
| mg | Milligram |
| mg/L | Milligram per liter |
| mg/g DCW | Milligram per gram dry cell weight |
| mg/L/day | Milligram per liter per day |
| g/L | Gram per liter |
| $g/m^3/day$ | Gram per meter cube per day |
| $\mu mol\ photon/m^2/s$ | Micromole photons per meter square per second |
| µl | Microliter |
| µg | Microgram |
| µg/L | Microgram per liter |
| µg/L/day | Microgram per liter per day |
| M | Molar |
| mM | Millimolar |
| rpm | Revolution per minute |
| v/v | Volume by volume |

# Preface

In the current energy paradigms, most of the requirements, be it fuel for transportation, electricity generation, or thermoplastic materials production, are fulfilled by utilizing fossil-based fuels such as coal, petroleum, and natural gases. This leads to the generation of substantial amounts of greenhouse gases (GHG) into the atmosphere causing global warming. Carbon dioxide is the highest and accounts for 76% of total GHG emissions. Apart from fossil fuel combustion, deforestation and industrial processes also add up to the atmospheric $CO_2$. This represents a significant global challenge, impacting climate, ecosystem, and human health by climate fluctuations, irregular drought and flood patterns, and decreased crop productivity. Considering these repercussions, substantial research efforts and advancements have been dedicated over the past decade to support the objectives outlined in the "Agenda 2030" for sustainable development. The aim is to address 17 key goals encompassing social, economic, and environmental dimensions on a global scale. The primary target include limiting the average temperature increase to below 2 °C and halving greenhouse gas emissions by 2030. There are several strategies to sequester $CO_2$, including physical, chemical and biological methods. Out of these methods biological fixation has emerged to be the most feasible one to achieve carbon neutrality. Cyanobacteria transform atmospheric $CO_2$ into oxygen and can generate a variety of valuable products. Cyanobacteria are known for their biofixation efficiency which is 10-15 times higher than the terrestrial plants. Following the biofixation of $CO_2$ the cyanobacteria can produce carbohydrates, lipids, proteins which can be utilized in food and feed additives and also can be used as biofuel directly or indirectly.

The thesis reports the sustainable production of farnesene through $CO_2$ sequestration by cyanobacteria. The study is designed in three different sections. Wherein the first section integration vectors were constructed. Three integration vectors were constructed to genetically modify fast growing cyanobacteria *Synechococcus elongatus* UTEX 2973. Conventional cloning, which includes restriction digestion and ligation, was used for the vector construction. The vectors namely were constructed such that the gene(s) of interest is inserted at the neutral site of the genome. The vectors were checked at each step to confirm the correct gene/fragment ligation, which was followed by the sequencing.

In the next section, the integration vectors constructed were transformed into *Synechococcus elongatus* UTEX 2973, emanating four genetically modified strains, UTEX *AFS*, UTEX *AFS::dxs*, UTEX *AFS::idispA*, and UTEX *AFS::dxs::idispA*. 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. With a productivity of 2.57 mg/L/day, UTEX *AFS::dxs::idispA* emerges as the superior photosynthetic farnesene producer compared to the existing cyanobacterial literature for farnesene production.

Further, a conceptual farnesene production plant is designed using engineered cyanobacteria which can utilize $CO_2$ from the flue gas. Techno-economic analysis and life cycle assessment of this conceptual plant are done. The capital expenses (CapEx), operating expenses (OpEx) and minimum farnesene selling price (MFSP) was calculated. The estimated CapEx for the plant amounts to $74.36 MM. The total annual OpEx is projected to be $19.42 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 $5.77/kg was observed with a 40-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.

Thus, the present work successfully provides the framework for sustainable production of farnesene from cyanobacteria. The platform can also be used to produce other value-added products by varying genes of interest in the vectors constructed and, hence, the modified cyanobacteria strains. The modified UTEX strain was able to produce farnesene with the highest productivity reported to date in cyanobacteria, which can be further improvised by adaptive evolutionary engineering, process parameters optimization and different modes of reactor operation. The TEA and LCA reveal that the process can be successfully upscaled and can give competition to the existing technologies marking a significant shift to a carbon-neutral economy.