Introduction

The global beauty and cosmetics industry has witnessed exponential growth, driving a significant increase in demand for natural resources. Among these, sandalwood oil stands out as one of the most coveted ingredients, valued for its distinctive woody fragrance and therapeutic benefits. Indian sandalwood (Santalum album), known for its high concentrations of α-santalol and β-santalol, has held a special place in global markets for centuries, representing both cultural heritage and economic value. However, unsustainable harvesting practices and overexploitation have led to its alarming decline, earning it a "Vulnerable" classification on the IUCN Red List of Threatened Species.

In recent years, the scarcity of Indian sandalwood has reached critical levels. Major manufacturers, including Karnataka Soaps and Detergents Limited (KSDL), now rely heavily on imported sandalwood oil, with up to 90% of their supply sourced from Australia to produce iconic products like Mysore Sandal Soap. This shift underscores the urgent need for sustainable alternatives to reduce dependence on imports and conserve India’s natural resources. Traditional sandalwood cultivation is resource-intensive, requiring 15 to 20 years for trees to mature. Furthermore, the cultivation process demands significant space and host plants, adding to the challenges of restoring natural populations and meeting rising global demands. Simultaneously, the world grapples with another pressing environmental challenge: plastic pollution. PET (polyethylene terephthalate), a widely used plastic, contributes millions of tons to global waste annually. Its persistence in the environment has severe ecological consequences, including contamination of soil, waterways, and marine ecosystems. Addressing this issue requires innovative solutions that not only manage waste but also convert it into valuable resources.

Our project seeks to tackle these interconnected challenges through an innovative approach inspired by Dr. Joanna Sadler's groundbreaking research on upcycling PET into vanillin. We propose a novel solution to synthesize sandalwood oil using PET plastic waste as the primary feedstock. By employing genetically engineered microorganisms, we aim to transform PET-derived compounds into high-value sesquiterpenes like α-santalol and β-santalol. This process not only alleviates pressure on natural sandalwood populations but also offers a sustainable method for mitigating plastic waste. This pioneering approach combines synthetic biology and sustainability to address critical environmental and industrial challenges. By creating a circular economy model, where waste materials are repurposed into high-value products, we aim to contribute to a more sustainable future for the beauty and cosmetics industry while preserving one of India’s most treasured resources.

Project design

We aim to engineer *Pseudomonas putida* KT2440 for the biosynthesis of sandalwood oil components using PET monomers as a carbon source. Our project integrates metabolic engineering, synthetic biology, and computational modelling to create an innovative solution for plastic upcycling and high-value compound production. This antedisciplinary approach allows us to design around the challenges of metabolic engineering more efficiently than traditional experimental methods alone. By combining in silico predictions with wet lab techniques, we can explore possibilities and overcome obstacles that would be time-consuming or impractical to tackle solely through experiments, especially within the constraints of an iGEM project timeline. The experimental aspects of our project include:

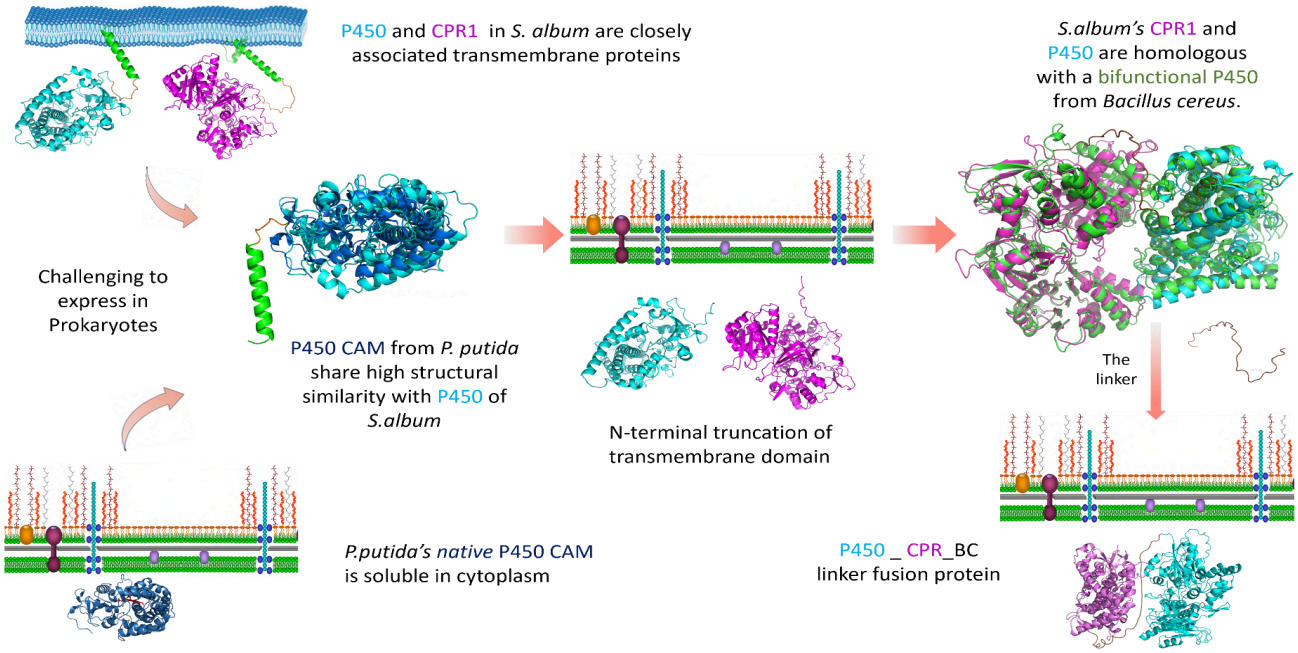
1. Chassis Selection: We chose *P. putida* KT2440 as our chassis organism due to its metabolic versatility, robustness, and ability to grow on various substrates. Specifically, we utilize the TA7-EG strain, engineered to metabolise both terephthalic acid (TPA) and ethylene glycol (EG) at neutral pH.
2. Pathway Engineering: Our strategy involves redirecting the carbon flux from PET monomers towards the native MEP pathway for terpenoid synthesis and introducing the santalol biosynthetic pathway. This consists of expressing three key enzymes from the *Santalum album:*Farnesyl pyrophosphate synthase (FPPS) ,Santalene synthase (SaSSy),Cytochrome P450 monooxygenase (CYP736A167) and its reductase partner (CPR1)

All these composite enzyme parts were designed to achieve modularity by using replaceable promoters and RBS sites, acting as a repository for future teams and learners to test further. This allows for fine-tuning the expression levels and testing the impact of different regulatory elements. While the experimental approach, as seen above, is straightforward, our computational approach answers the working and optimization of our system as a whole.

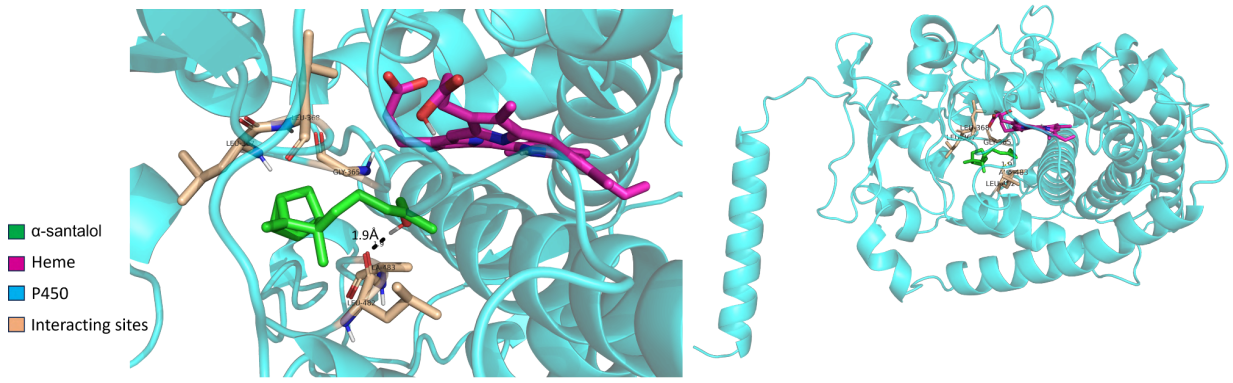
Metabolic and structural modelling:

1. Protein Engineering: A significant challenge in our project is the expression of the eukaryotic membrane-bound cytochrome P450 enzyme in our prokaryotic host. We have designed and ideated an innovative protein engineering strategy to overcome this. Our P450 engineering approach could address the challenges of expressing plant enzymes in bacteria. This strategy could apply to other systems involving eukaryotic P450s, broadening the impact of our project beyond sandalwood oil production.
    1. Transmembrane Domain Truncation: We identified and removed the hydrophobic N-terminal transmembrane domain of both CYP736A167 and CPR1. This modification aims to improve the solubility and expression of these enzymes in the bacterial cytosol.
    2. Fusion Protein Design: Inspired by self-sufficient bacterial P450s and previous studies, we designed a fusion protein combining the truncated CYP736A167 and CPR1, using a 36-amino acid linker (BC_linker) derived from a self-sufficient

P450 from *Bacillus cereus* to join these two enzymes, this could enable the functional co-expression of P450-CPR.Following image describes fusion protein design



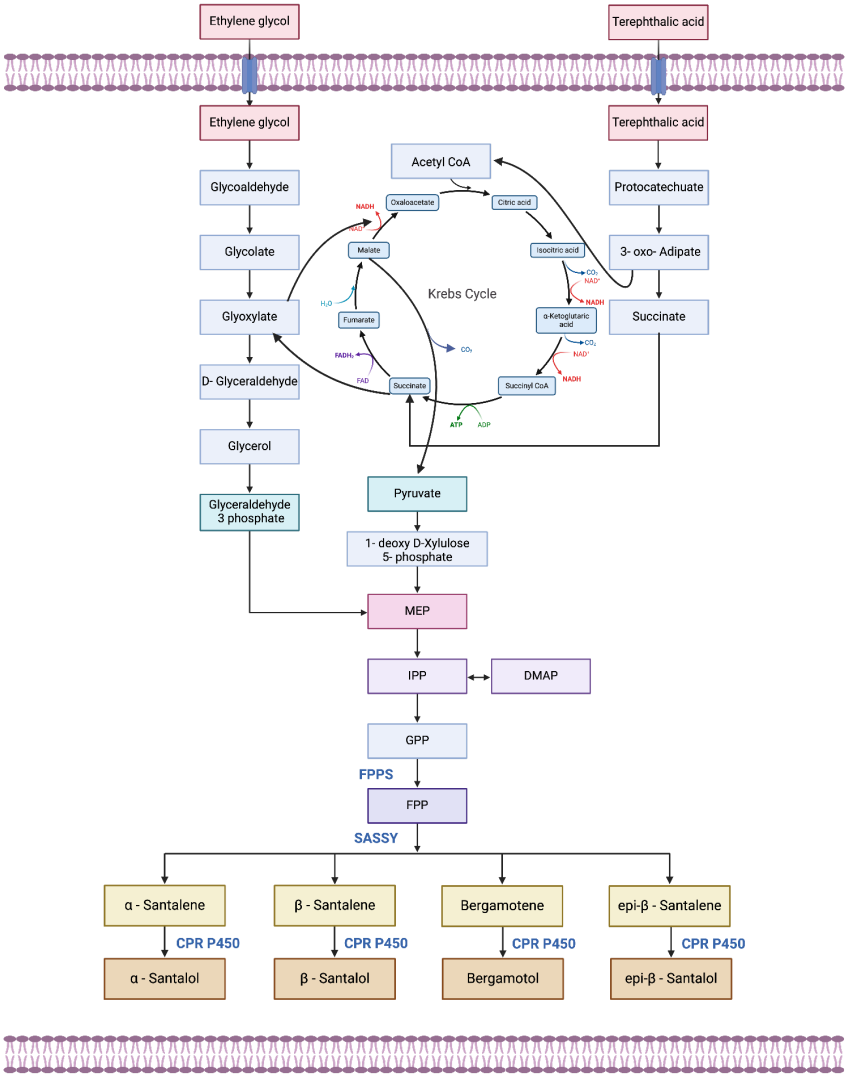
2. <u>Structural Modeling:</u> We used AlphaFold2 to predict the structures of our engineered P450-CPR pair. With the predicted structures, we also looked at their binding energies with santalene to understand if they can effectively bind and catalyse santalene to santalol.The conversion of α-santalene to α-santalol takes the least amount of energy; thus, as per our assumption, alpha santalol is going to be produced more comparatively to other isomers. Following image shows docking of α santalol molecule with P450-Heme which gives us predicted catalytic site.



3. Metabolic Modeling: We utilise Flux Balance Analysis (FBA) to optimise growth conditions, analyse metabolic fluxes, and identify key regulatory points in the engineered pathway. This computational approach guides our experimental design and helps predict potential bottlenecks in the production process.

   By integrating these approaches, we aim to create a microbial cell factory capable of upcycling PET waste into valuable sandalwood oil components. This project serves as a proof-of-concept for sustainable production of plant secondary metabolites from plastic waste. It could be extended to other high-value compounds with therapeutic or fragrant properties.

Following is the engineered pathway of uptake of ethylene glycol and terepthalic acid(PET monomers) into *P. putida* to produce *Santalol*



Experimental design and engineering cycles

From the use and characterisation of modified strains that can take up TPA (Terephthalic Acid) and EG (Ethylene Glycol) to the Assembly and Expression of Gene Constructs associated with the Sandalwood Oil Pathway, our experimental core is firmly embedded within the Design, Build, Test, Learn Framework. Through an extensive literature review, we identified *P. putida* TA7-EG as our optimal chassis, leveraging its proven ability to metabolise PET monomers.

In pursuing sandalwood oil production, we first explored the established pCDFDuet system but encountered limited santalene yields. This led us to engineer constructs for SaSSy-FPPS and Cytochrome P450-CPR, which we successfully integrated into pSEVA631 and pSEVA241 plasmids through Gibson Assembly and traditional ligation methods. To overcome the inherent challenges of bacterial P450-CPR expression, we developed solutions, including truncated variants and fusion proteins, with all designs thoroughly documented. Our engineering refinement included analytical techniques with GC-MS for precise quantification of target compounds - santalene and santalol combined with computational modelling through FBA (Flux Balance Analysis) via detailed in silico models of the strain's metabolic pathways.

Characterising the strain

Our first engineering cycle began with a crucial decision: develop new strains or adapt existing ones. Literature review revealed that *Pseudomonas putida* KT2440 could naturally utilise ethylene glycol through laboratory evolution and possessed the β-ketoadipate pathway with protocatechuate (PCA) as an aromatic acid catabolite. However, the strain lacked natural TPA-to-PCA conversion capability. This led us to focus on incorporating the *tphII* operon from *Comamonas sp.*, which encodes TphAII and TphBII enzymes that convert TPA to PCA.
We obtained the wild-type strain from Dr Phale from IIT Bombay to start with our approach. To obtain more insight into this idea, we discussed engineering the strain with Dr Oliver Brandenburg to enable terephthalic acid and ethylene glycol uptake. He reviewed our proposed timeline and concluded that engineering from scratch alongside developing the santalol pathway was too ambitious within the given timeframe. Instead, he recommended leveraging his existing engineered strains of *Pseudomonas putida* KT2440, which could take up both TPA and EG monomers. Incorporating this adapted design into our project, we sought to characterise the growth of TA7-EG strain on terephthalic acid (TPA), ethylene glycol (EG), and a combination of both substrates in minimal M9 media. However, during our initial experiments, we encountered several challenges related to the irregular growth of the strain. One of the primary difficulties we faced was the growing strain on the M9 media caused by the precipitation of CaCl2 and MgSO4 in the media. This issue was further exacerbated by the pH changes resulting from adding TPA and EG to the media.To address this issue, we fine-tuned the concentrations of the components of the media and monitored the pH at all times to balance TPA dissolution and ensure no precipitate formation. We also made sure to add the components just before further experiments to ensure the whole media was prepared each time and to avoid pH and precipitate issues. With all these challenges faced, we were able to successfully grow the *P. putida* TA7-EG strain on the different substrates, and one such is shown below.

The second challenge we encountered during the characterisation of the *P. putida* TA7-EG strain was its slow growth rate on PET monomers. Upon receiving the strain from Dr Oliver, we observed that the bacteria required 24-36 hours to achieve substantial growth on terephthalic acid (TPA) and ethylene glycol (EG), which was not optimal for real-world applications. We implemented an adaptive evolution strategy using decreasing glucose concentrations alongside PET monomers, starting at 5% glucose and gradually reducing to media containing only TPA and/or EG. Growth curve analysis revealed our adapted strain reached log phase faster than the original TA7-EG strain, with strongest growth in TPA+EG media, while EG-only media showed slower growth rates.

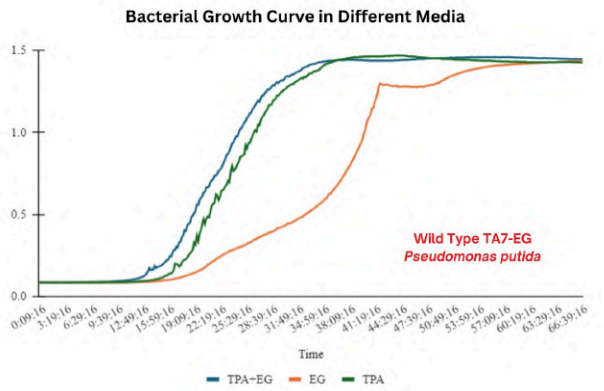


Fig.2.: Growth Curve of TA7-EG in M9 minimal media, with 20mM of each TPA and EG

This result enabled us to learn that the adaptive evolution approach successfully improved the growth characteristics of the *P.putida* TA7-EG strain on PET monomers, bringing us one step closer to developing an efficient bacterial platform for PET bioconversion.

SaSSy-FPPS Expression Testing

Our second engineering cycle focused on testing the expression of SaSSy-FPPS enzymes, which catalyse the conversion of IPP/DMAPP into santalene, the first component of our sandalwood oil. Dr Bohlmann previously studied the expression of these enzymes in *E. coli* using the pCDFDuet expression vector. In our discussions with him, he mentioned that while his exact construct might not be optimally expressed in our *P.putida* TA7-EG strain and may require further modifications, he kindly agreed to provide us with the pCDFDuet containing SaSSy-FPPS for testing in our chassis.

To test the initial pCDFDuet construct's expression in our chassis. We transformed the pCDFDuet with SaSSy-FPPS into our TA7-EG strain via electroporation. After successful transformation, we induced the expression of SaSSy and FPPS with 1 mM IPTG and assessed the expression through Coomassie staining of purified protein and chemical analysis using GC-MS. The SaSSy enzyme, which already had a His-tag upstream from the original construct [3], allowed us to purify the protein using Ni-NTA columns with His-trap resin. Despite some

purification issues, the Coomassie-stained gel revealed a thick band at 65 kDa, corresponding to the expected size of the SaSSy protein

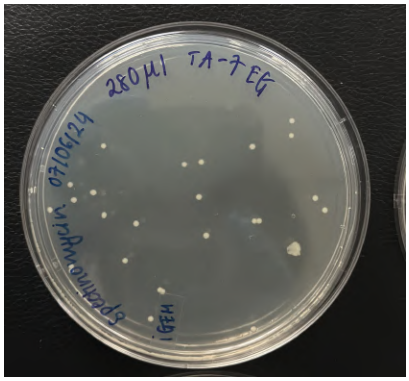


Fig.3.: Electroporation of TA7-EG.

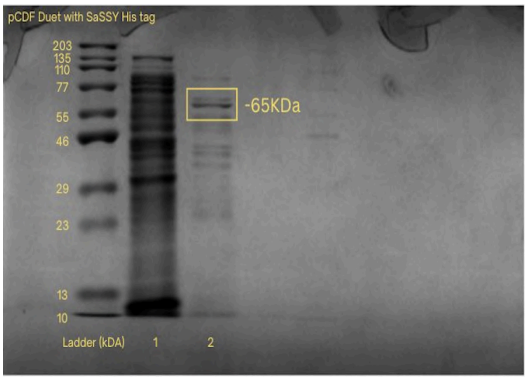


Fig.4.: Coomassie gel for pCDFDuet

GC-MS analysis was performed using our institute's Agilent 8890gc system 597 7 GC/MSD with a suitable column. We observed a peak at an m/z ratio of 204, which corresponds to the known ratio of santalene as reported in previous literature. However, the abundance of this compound within the sample was relatively low, confirming Dr Bohlmann's suggestion that the construct might not work optimally in our chassis.
To address this issue, we devised several construct designs that could improve the expression of SaSSy-FPPS in our chassis. These designs were based on principles such as bicistronic expression and dual expression systems, aiming to optimise the expression of SaSSy-FPPS in different settings.

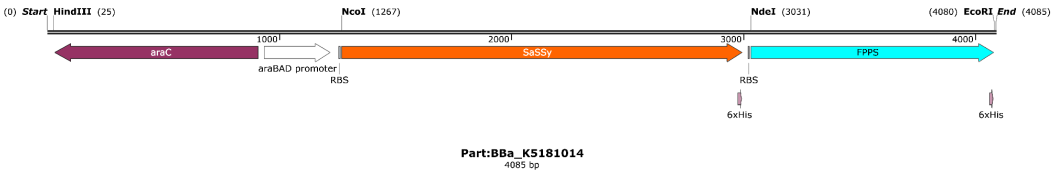


Fig.6.: The araBAD_SaSSy_FPPS insert.

Due to time and logistics constraints, we initially worked with our bicistronic SaSSy-FPPS construct under the control of an arabinose promoter, BBa_K5181014. The complete part was received as three separate fragments built using the NEBuilder HiFi DNA Assembly kit. Following assembly, we PCR-amplified the entire construct to obtain a high yield and to verify successful assembly and PCR
Following digestion, we ligated the gene insert with the vector and transformed it into TA7-EG via electroporation, selecting transformants on gentamicin plates. Plasmid isolation and subsequent EcoRI/HindIII restriction analysis yielded expected 3 and 4 kb bands, confirming successful plasmid integration in our TA7-EG strain.

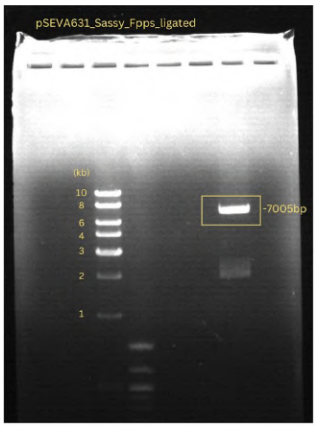


Fig.8.: Single digested SaSSy_FPPS.

Fig.9: Double Digested SaSSy_FPPS

We also tested the expression of this construct by inducing various concentrations of arabinose (0.2%, 0.5%, 0.7%, 1%, 1.5% and 2%). We found the best results from the 0.7% and 1.5% inductions. The proteins were purified using the His Trap NI NTA column, and the expression of these proteins was confirmed by the bands observed in the following Coomassie-stained gel. To analyse their functionality, we tested the production of santalene using our designed construct through GCMS.

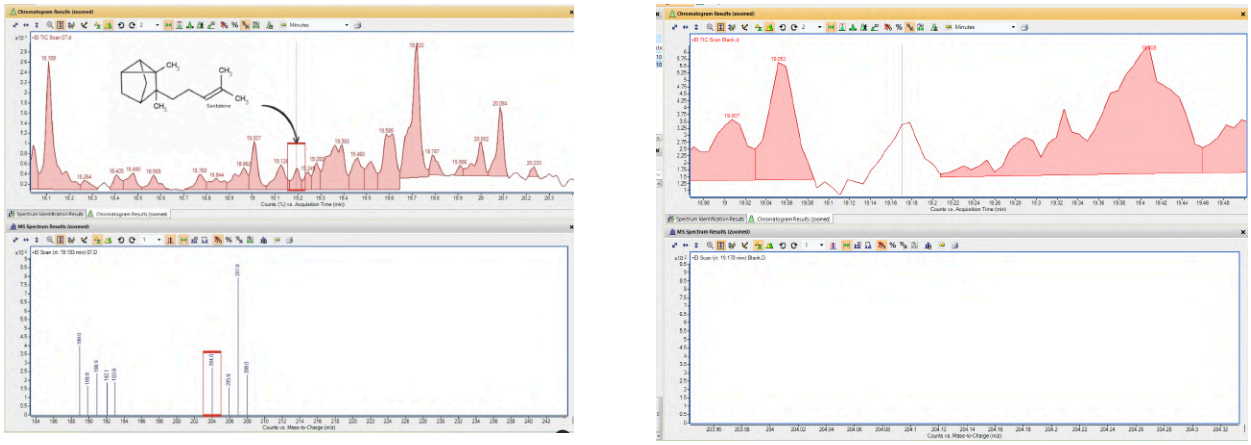


Fig.11.: The Left shows the presence of the santalene with a sharp peak at 204 m/z resembling the molecular weight; on the right side of the image, there is an untransformed sample showing no peak at the same retention time for 0.7% induction

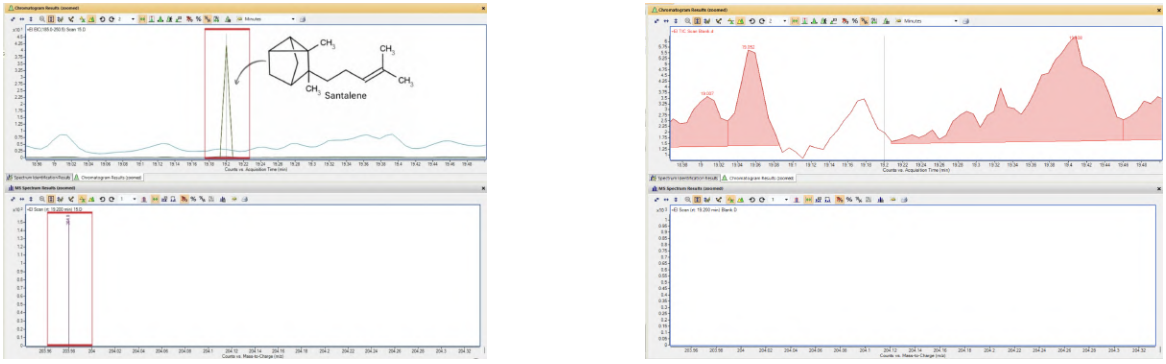


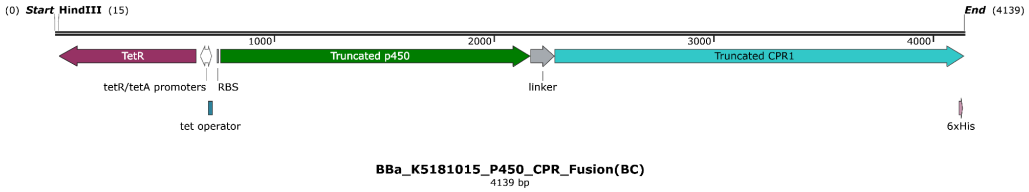
Fig.12.: A peak of 204 m/z presence strongly suggests the santalene expression in 1.5% arabinose induction in the left one; on the right side, the control shows no peak at the same retention time.

Through these processes, we learned that our designed SaSSy-FPPS construct (BBa_K5181014) is functionally expressed in our TA7-EG strain when TPA and EG are the sole carbon sources. The functionality of the enzymes was confirmed by GC-MS analysis. To our knowledge, this is the first demonstration of santalene production in *P.putida* using PET monomers as the carbon source. While this is a promising result for us undergrad students, it presents new directions for further testing. Future work includes investigating part modularity through promoter and RBS optimisation. Our alternative design, BBa_K5181018, expressing SaSSy and FPPS under different promoters in pRGPDuo, offers potential for independently tuned expression.

Assembly and Expression of P450-CPR

For our third engineering cycle, our goal was to design constructs for the expression of our P450-CPR enzymes. The P450-CPR enzymes catalyse the conversion of santalene to santalol, the final major component of sandalwood oil. The expression of these enzymes in prokaryotic systems remains a challenge that is still being tackled by many worldwide. Challenges faced include expression of the eukaryotic membrane-bound cytochrome P450 enzyme in prokaryotic systems like *P.putida*, differences in membrane composition, incorrect anchoring of the enzymes, and lack of optimal expression conditions in bacteria. To overcome these challenges, we adopted transmembrane domain truncation and fusion with membrane-anchoring proteins to enhance the expression and solubility of membrane-bound P450s in our bacterial system.
To test our design, we built a composite part BBa_K5181015 with tet promoter and truncated fusion P450-CPR, an optimized fusion protein sequence for expression in *P. putida.* This part is also modular with respect to the promoters and RBS sites, as shown below.
We attempted Gibson assembly of three fragments using the NEBuilder HiFi kit, followed by PCR confirmation. Despite multiple approaches using both conventional ligation and Gibson assembly with various pSEVA vectors (241, 631, and 424), transformations either failed or yielded colonies with problematic plasmids showing only smears on gels. Sequencing of the first fragment showed perfect alignment at 130 bp,though primer binding sites couldn't be confirmed. The sequencing of the third fragment revealed numerous overlapping peaks throughout the sequenced region, suggesting potential issues related to the sequence, primer binding, or the

presence of a homopolymer region.
Our analysis suggests successful PCR amplification but potential ligation issues due to sequence or structural complications. We propose investigating ligation efficiency and testing our alternative design BBa_K5181017, which uses truncated P450 and CPR genes under tac promoters. We also conducted molecular docking studies to analyze binding energies between our designed P450-CPR and santalene derivatives, providing insights into their theoretical



functionality. The binding energies between P450-CPR constructs and key substrates. α-santalene showed a favourable binding energy of -7.37 kcal/mol, suggesting efficient potential conversion to α-santalol. These findings support the theoretical functionality of our designed P450-CPR genes despite experimental challenges.

Biosafety

For our project, we selected our strains based on their potential to synthesise our required metabolites and the risks they pose to humans and the environment. As per the Department of Biotechnology of India, microorganisms are classified into four categories based on their ability to cause diseases in humans/animals/plants.

Pseudomonas putida KT2440

The chassis organism that we will be using in our project is Pseudomonas putida KT2440 and two of its derivatives, Pseudomonas putida TA7 and Pseudomonas putida TA7-EG which were engineered by Dr Oliver Brandenberg, by incorporating the genes phA1II, tphA2II, tphA3II and tphBII under constitutive promoter PEM7 [1]. As per the FDA guidelines, the strain, P. putida KT2440, is HV1 certified, indicating it is safe to use in a P1 or ML1 environment [2]. It is a non-virulent strain, and hence, its potential to cause harm to humans is minimal.

Before starting our project, we were given Biosafety level-II training by our laboratory technician, Mrs. Nithya Rani, wherein she provided extensive courses on safe work in the laboratory. We were given prior training on the proper and safe use of autoclave sterilisers, bio-safety cabinets and the usage of ethidium bromide and UV for visualising electrophoresis results. All chemicals and equipment were handled with necessary precautions.