ENGINEERING *Y. LIPOLYTICA* FOR THE BIOSYNTHESIS OF TERPENOIDS IN THE STRICTOSIDINE PATHWAY

Ayushi Agrawal

Advisor: Mark A. Blenner

Committee Members: Eleftherios Papoutsakis, Aditya Kunjapur, Jesus Beltran

April 14, 2025, Monday, 11:00 AM (EDT)

AP Bio-526, https://udel.zoom.us/j/99411294541

Natural products derived from plants, animals and microorganisms like bacteria, yeast, and fungi are valuable sources of bioactive compounds with diverse pharmaceutical applications. However, their native production occurs in minute quantities and as complex mixtures of compounds, making extraction labor-intensive, expensive and inefficient. This challenge underscores the need for alternative hosts to facilitate natural product synthesis to meet the increasing market demands.

Microbial cell factories offer a promising solution and have been used extensively for this purpose. Traditionally, hosts like *E. coli* and *S. cerevisiae* are preferred due to their well-characterized genomes, widely available genetic engineering tools, and ease of manipulation. However, *E. coli*, as a bacterium, lacks essential organelles such as vacuole, mitochondria, peroxisomes, endoplasmic reticulum etc. which are crucial for expressing certain plant-based enzymes in heterologous hosts. While *S. cerevisiae* possesses these organelles and serves as an industrial workhorse for numerous compounds, its natural acetyl-CoA flux, the central intermediate for terpenoid production, is relatively low.

In contrast, *Yarrowia lipolytica*, not only contains all the organelles found in *S. cerevisiae* but also offers additional advantages, including a high acetyl-CoA flux for terpenoid biosynthesis, high heterologous protein expression, and high oil production capacity. Therefore, this dissertation focuses on leveraging *Yarrowia lipolytica* as a host of terpenoid biosynthesis.

In this work, we focus on the biosynthesis of two key intermediates, geraniol and 8-hydroxy geraniol, in the strictosidine pathway. Strictosidine serves as a precursor to over 3,000 monoterpene indole alkaloids with significant therapeutic potential. Notably, anti-cancer derivatives of strictosidine such as vinblastine and vincristine, are included in the WHO List of Essential Medicines.

First, we engineered a *Yarrowia lipolytica* strain for geraniol production through random genome integration. Additionally, we enhanced geraniol yield by overexpressing multiple mevalonate pathway genes, optimized the gene copy numbers, and optimizing the fermentation medium. As a result, we achieved a titer of 1 g/L in shake-flask fermentation, the highest reported to date.

Moreover, we metabolically engineered a geraniol producing strain through directed integration to ensure stable gene expression and enable further pathway optimization. In addition to this, we examined the enzymes involved in the degradation of geraniol and investigated the role of the P450 enzyme, geraniol 8-hydroxylase as a potential bottleneck. To this end, we tested cytochrome P450 reductase and cytochrome b5 enzymes from two plant variants *Arabidopsis thaliana* and *Catharanthus roseus* and observed that the activity of the enzyme activity nearly doubled upon incorporating reductase partners.

Finally, we performed computational modeling using AutoDock Vina of the G8H enzyme using the predicted structure from AlphaFold and discovered several key site residues that were critical for protein function, along with additional residues influencing enzyme activity. Based on these findings, we constructed a site-saturation mutagenesis library, which will be tested in the future to identify mutants with enhanced activity. Overall, we leveraged metabolic engineering and synthetic biology to investigate the potential of *Yarrowia lipolytica* as a heterologous host for natural product synthesis, establishing a foundation for future research on terpenoid production.