Methods of Enhancing Triterpenoid Production in Yeast

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ABSTRACT

Microbial cell factories, particularly those using eukaryotic yeasts, are ideal platforms for producing plant secondary metabolites, including flavonoids, alkaloids, and terpenoids. Accordingly, this study aimed to increase triterpenoid production in the *Saccharomyces cerevisiae* strain BY4743 through CRISPR/Cas9 and cultivation engineering. The ROX1, DGK1, and PAH1 genes were targeted for knockout experiments. Sanger sequencing showed all three targets were successfully mutated; however, only the DGK1 knockout strain had a significant change in triterpenoid production at 130% compared to the wild-type. Various cultivation strategies were also explored, but none increased triterpenoid production significantly. Additionally, to illustrate the potential applications of engineered yeast, five uncharacterized oxidosqualene cyclases (OSCs) from *Erysimum cheiranthioides* were tested in the ROX1 knockout strain, revealing one responsible for producing the steroid core of medicinal cardenolides. In summary, this thesis provides engineered yeast strains with improved MVA pathway derivative production potential and comprehensive CRISPR/Cas9 methodologies for *S. cerevisiae*.

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CHAPTER 1. LITERATURE REVIEW

1.1 Plant Natural Products and Triterpenoid Production

Plant natural products are widely used in industry and medicine, with more than 80% of drug substances being natural products or inspired by a natural compound (Harvey, 2008). Plant natural products are usually in the form of secondary metabolites, including flavonoids, alkaloids, and terpenoids. These metabolites are major bioactive constituents, which has allowed them to be widely used in the cosmetic and pharmaceutical industries (Li et al., 2021). Of these secondary metabolites, terpenoids (also known as isoprenoids) are the largest and most diverse group of naturally occurring compounds primarily found in plants (Cox-Georgian et al., 2019).

Terpenoids are frequently used in the flavor and fragrance industry (for example, menthol), as well as in the pharmaceutical industry (for example, the antimalarial and anticancer drugs artemisinin and taxol; Moses et al., 2013 and Zhang et al., 2017). Within the compound class of terpenoids, some subclasses of compounds have been under-explored within the research field, one of these being triterpenoids. Triterpenoids can be used for various industrial and pharmaceutical applications (Moses et al., 2013). The industrial application of triterpenoids includes vaccine adjuvants, anti-cancer drugs, food supplements, and agronomic agents (Miettinen et al., 2018). Triterpenoids' pharmaceutical applications are based on their medicinal properties, including anti-

inflammatory, hepatoprotective, analgesic, antimicrobial, antimycotic, virostatic, immunomodulatory, and tonic effects (Dzubak et al., 2006). Despite their extensive potential applications and medicinal properties, the production of triterpenoids, like most natural products, relies on specific industry standards that present their own set of challenges.

The current industry standard for producing most natural products, including triterpenoids, is either through chemical synthesis or phytochemical extraction from plants. Chemical synthesis uses several simple organic compounds in a reaction to generate other more complex chemicals. This process can require large amounts of starting materials due to the complexity of the synthesis, which drives up costs (Carsanba et al., 2021). Accordingly, phytochemical extraction is the primary source of plant natural products which involves extracting chemicals from the plant material using various solvents. This method also has downsides due to the slow nature of plant growth, poor extraction yields, and the amount of plant material required to extract one gram of a compound is considered to be endangering some species, for example, the ancient tree species *Ginkgo biloba* (Isah, 2015). The complexity, low yields, and environmental impacts of chemical synthesis and phytochemical extraction have caused a shift towards finding new methods to obtain plant chemicals.

1.2 Cell Factories and Saccharomyces Yeast

Microbial cell factories are one of the most popular methods to replace traditional plant natural product production processes. Cell factories are engineered microbial strains and the environment in which they grow. These can be used to produce a wide range of chemicals, including biofuels, bulk and fine chemicals, polymers, amino acids, natural products, and drugs (Yoo-Sung et al., 2020). Over the last century, commercial chemicals have been mainly derived from fossil resources; however, this has been found to have serious environmental effects. Cell factories have addressed this issue because they can produce chemicals using various carbon sources, including renewable, non-edible biomass and carbon dioxide (Sung Cho et al., 2022). Industrial cell factories seek to reduce the need to produce carbon dioxide through the combustion of fossil fuels and sequester carbon dioxide from the atmosphere using microorganisms (Bo-Liang et al., 2020).

The most widely used microorganisms for cell factories are *Escherichia coli* and various types of yeast (*Saccharomyces* species and *Yarrowia* species). Both of these options have their inherent benefits. Still, yeast stands out because it is a eukaryote and thus has plant-like subcellular compartments (such as the endoplasmic reticulum), facilitating engineering efforts (Guo et al., 2020). Metabolic engineering of yeast has had many successes over the last few decades in producing important industrial and pharmaceutical compounds such as insulin proteins and advanced biofuels in high

amounts, or ‘titers’ (Nandy and Srivastava, 2018). Titer is the term used to indicate the concentration or amount of a compound generated in a cell factory culture. Due to *S.cerevisiae’s* robustness in low pH conditions, genetic accessibility (it has a small and relatively simple genome), efficient conversion of raw materials like sugars, and tolerance to product toxicity, it is one of the most common types of yeast used to produce plant natural products and many other industrial chemicals, (Borodina and Nielsen, 2014; Nielsen et al., 2013). In addition, yeast's structural similarity to plant cells allows for the efficient expression of plant genes while alleviating toxic effects by storing products in subcellular compartments (Cao et al., 2020). These attributes enable yeast to (i) be a highly efficient mode of production for complex chemicals and (ii) a less time-dependent alternative to harvesting the chemicals from plant material.

The engineering efforts to increase titer yields in yeast cell factories are (i) metabolic engineering and (ii) cultivation engineering methods. The metabolic engineering strategies actively explored are engineering precursor, regulatory, and membrane composition genes to be overexpressed, underexpressed, or turned off through gene knockouts. Overexpression of the genes encoding the precursor pathway bottlenecks and other genes in the metabolic pathways have significantly increased most product titers (Guo et al., 2020). Over or under-expression of other genes, including regulatory and membrane composition genes, has also considerably increased product titers (Broker et al., 2018; Zhang et al., 2020). Overexpression and underexpression are controlled by the efficiency of promoters and terminators that are linked to that gene, as well as the

relocalization of a gene to a different location within the genome (Zhang et al., 2015; Patrow et al., 2010; Ahmed et al., 2019; Reider Apel et al., 2016). The ability to apply various metabolic engineering strategies has been improved in recent years by developing more efficient metabolic engineering strategies, particularly Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) proteins genetic engineering methods that can be used to add exogenous DNA or modify genomic DNA (Degreif et al., 2018). Compared to other genomic engineering methods, CRISPR/Cas9 is a fast, marker-free, versatile, and highly targeted genome-editing technique (Jakociunas et al., 2016). The application of CRISPR protocols has been optimized in the model organism *Saccharomyces cerevisiae* (baker's yeast) throughout many studies over the past few years, creating an efficient platform for metabolic engineering (Reider Apel et al., 2016; Degreif et al., 2018). This platform allows for various genetic modifications, including (i) gene elimination via knock-out and (ii) over or under-expression via knock-in, which are further described in Chapter 2.1.1.

In concordance with metabolic engineering, cultivation methods have also been an important research focus in developing efficient cell factories. Cultivation methods that positively impact chemical production include different carbon sources, resource limitations, cofactor supply, fermentation strategies, and continuous growth in highly controlled reactor environments (Nieto-Taype et al., 2020). Particularly for yeast, the modulation of metabolism based on carbon sources is attainable due to its ability to grow on many different carbon sources. This trait allows for a metabolic shift of the cell

factory from a growth state to an enzyme or chemical production state, which allows for elevated levels of target product production. Similar effects can be seen when employing resource limitation strategies which generally include either carbon, nitrogen, or phosphorus limitation (Czarnotta et al., 2017). Lastly, highly controlled fermentation and bioreactor environments allow for the optimization of growth and chemical balance throughout the fermentation process.

The combination of the metabolic and cultivation engineering methods has produced cell factories for various chemicals that have achieved an industrially efficient productivity rate, typically around 2–5g l^{-1} h^{-1} (Gustavsson and Lee, 2016). Although these efficient cell factories have been developed for particular plant natural products, there is still a significant need to develop these strategies to expand the production abilities for more structurally diverse phytochemicals.

1.3 Triterpenoid Production in Yeast

Structurally complex chemicals such as plant natural products are some of the most pursued chemicals to be produced in yeast cell factories. Of all the plant natural product chemical classes, terpenoids are popular candidates for production in cell factories because of their known biosynthetic pathways with relatively few steps. Although triterpenoids are considered the least researched class of terpenoids, research has shown they are involved in plant communication, defense, and sensory regulation (Chen et al., 2021; Lucini et al., 2018; Sun et al., 2021). However, the functions of

pentacyclic triterpenoids include more than just plant biology. For example, they have also been positively linked to human health based on the amount present in the diet (Li et al., 2022). Recent studies have shown that yeast cell factories can produce these economically important triterpenoids at an industrially efficient chemical production rate (Wang et al., 2019; Zhao and Li, 2018). These high yields were enabled by metabolically engineering individual parts of the triterpenoid precursor pathways in yeast. Triterpenoids are mainly synthesized via the mevalonate (MVA) and the methylerythritol phosphate (MEP) pathways. The main recognized pathway engineered to produce triterpenoids is the MVA pathway, which produces 2,3-oxidosqualene under the catalysis of squalene epoxidase. 2,3-oxidosqualene is the direct precursor to triterpenoids, and this reaction is catalyzed by an oxidosqualene cyclase (OSC). OSCs can generate hundreds of triterpenoids, but these are separated into two classes: tetracyclic and pentacyclic. Within the pentacyclic class of triterpenoids, there are four main skeletons that 2,3-oxidosqualene can be cyclized into, which include lupine-type, oleane-type, ursane-type, and friedelane-type (Figure 1). These triterpenoid scaffolds can be further modified by Cytochrome P450-mediated oxygenation of their R groups, shown in the inset box in Figure 1. In addition to oxygenation by CYP450s, UDP-glycosyltransferase (UGT) can generate triterpenoid saponins through glycosylation (inset box, Figure 1; Moses et al., 2013).

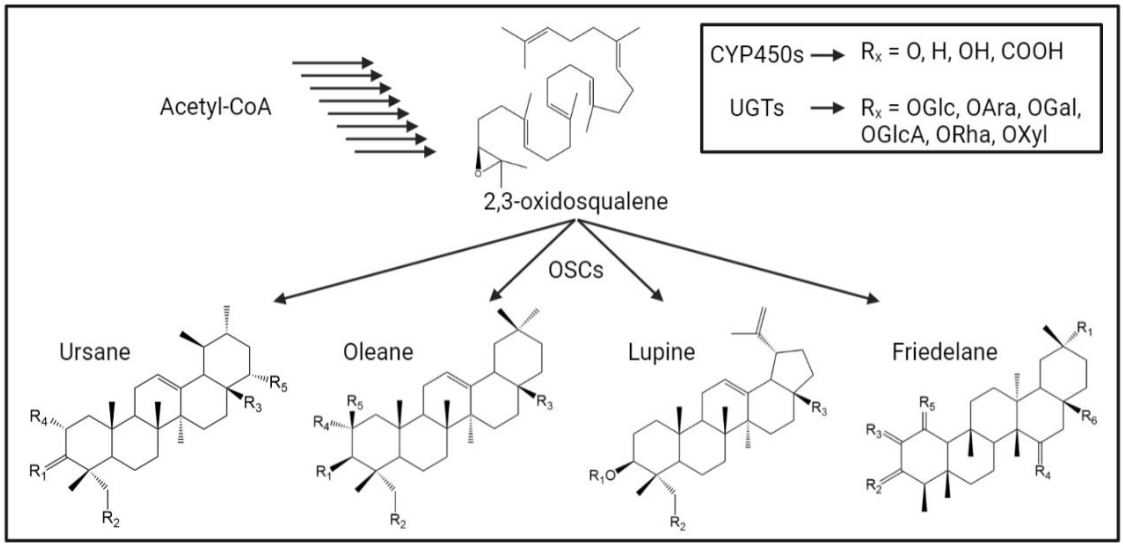


Figure 1. *The biosynthetic pathway to the backbone structures of the four pentacyclic triterpenoid classes including, Ursane, Oleane, Lupine, and Friedelane-type triterpenoids.*

A triterpenoid's exact structure is one factor in achieving high production of these products in yeast cell factories. Pentacyclic triterpenoids have received less focus than tetracyclic triterpenoids in creating highly optimized model yeast cell factories to produce such compounds. The research gap regarding pentacyclic triterpenoids is partially due to their low content in plant tissues and the long growth cycle of plants, making them more difficult to study (Li et al., 2023). Tetracyclic triterpenoids, such as the dammarane-type triterpenoids, have reached up to 11 grams per liter but pentacyclic lupine-type, oleane-type, ursane-type, and friedelane-type triterpenoid titers are comparably low at ~2.6 grams per liter (Wang et al., 2019; Guo et al., 2020; Du et al., 2022). Certain types of triterpenoids having lower titers are thought to result from higher levels of metabolic

imbalance, which increases the burden of cells, reduces metabolic efficiency, and can lead to unexpected negative regulation (Du et al., 2022). By advancing engineering efforts, challenges like metabolic imbalance can be resolved to further increase the production of pentacyclic triterpenoids in yeast.

Specifically for triterpenoids, current engineering efforts have focused on overexpressing, under-expressing, or turning off precursor, regulatory, and membrane composition genes. The primary methods are newly developed CRISPR/Cas9 genetic engineering protocols. CRISPR/Cas9 can be used to re-localize genes from the precursor pathway to different locations within the genome with a particular expression level based on whether the targeted gene is over or under-expressed. These protocols have been used in the overexpression of the genes encoding the precursor pathway bottlenecks along with all the other genes in the triterpenoid pathway, which has been shown to increase most triterpenoid titers (Guo et al., 2020). They have also been used to engineer key membrane composition genes involved in lipid and fatty acid production, increasing the yeast cells' capacity to store triterpenoids. Through increased storage, titers of triterpenoids were shown to be increased due to distorting the fatty acid content in the cellular membranes (Zhang et al., 2020; Yu et al., 2020). While the successful implementation of CRISPR/Cas9 protocols and modification of key genes has yielded notable advancements in triterpenoid production, the frontier of this field is yet to be fully explored.

One mostly unexplored way to increase triterpenoid titers is the engineering of transporters to reduce the intracellular abundance of toxic products, promoting metabolic balance (Bu et al., 2020). Although specific modes of extracellular transport of triterpenoids in yeast are yet to be determined, many hypotheses proposed include diffusion through amphiphilic molecular stabilization, protein-mediated transport, and vesicle transport (Fang and Xiao, 2021). Also, relocalizing genes to specific intracellular locations using genetic tags can further increase the yeast cell's ability to store many triterpenoid pathway intermediates and products. The intracellular sites that were shown to increase the titers of multiple different types of triterpenoids are lipid bodies and peroxisomes. Specifically, the peroxisomes were shown to help improve the storage and production of much of the pathway intermediates. In contrast, the lipid bodies helped store and produce the final products of the triterpenoid pathway (Shi et al., 2021; Du et al., 2022). Further manipulations to localize the triterpenoid pathway will need to be researched to identify any additional benefits to the production of triterpenoids. The engineering strategies discussed in Chapter 1 are further described and specified in Chapters 2 and 3 of this document.

Along with triterpenoid pathway and membrane composition genes, cultivation strategies contribute immensely to the growth and metabolism of yeast to produce triterpenoids and elevate their production (Czarnotta et al., 2017). The first of these strategies is feeding ethanol to yeast as a carbon source at a constant rate and in pulses. Czarnotta and colleagues found that feeding ethanol as a carbon source is the preferred

carbon source for the production of cyclic triterpenoids. This characteristic may be due to increased production of the precursor acetyl-CoA or the redox cofactor NADPH, which this study addressed as a shared observation within the literature. This study also found that resource limitations, such as nitrogen-limited fermentation, can reduce the competition of carbon, redox, and energy cofactors with triterpenoid synthesis. Furthermore, they identified an optimal fermentation strategy for triterpenoid production: ethanol pulse feed coupled with nitrogen limitation. There have also been conclusions about the increase in triterpenoids resulting from a change in gene expression based on carbon sources and resource limitations (Slavov and Botstein, 2011). Another strategy identified as a bottleneck for the production of triterpenoid precursor squalene is Coenzyme A concentration in the initial growth media. Some studies addressed this bottleneck by adding an excess of pantothenate in the media, the precursor to Coenzyme A (Ebert et al., 2018). Overall, the genetic and fermentation engineering strategies mentioned are examples of progress toward generating efficient yeast cell factories for the production of triterpenoids.

1.4 Summary and Statement of the Problem

Genetic engineering and cultivation techniques in yeast cell factories have enabled us to produce simple hydrophobic compounds, such as oils, in the hundreds of grams per liter (Darvishi Harzevili, 2014). Still, we have yet to be able to accomplish this for certain complex hydrophobic compounds derived from plants. In the case of

triterpenoids, the current highest reported oleanane-type triterpenoid titers were reported at 2.6 g/L (Du et al., 2022). Further method discovery and novel combinations of existing methods are required to produce high yields of various types of pentacyclic triterpenoids. To do this, we must systematically explore methods of engineering model classes of phytochemicals since developed knowledge in that area could likely be translated to producing a wide variety of structurally diverse phytochemicals. Triterpenoids are a promising model for complex hydrophobic phytochemicals due to their known biosynthetic pathways with relatively few steps. They are also easy to purify and detect with analytical instruments (Moses et al., 2013).

The project's aims include (i) combining previously reported methods to create model yeast strains for triterpenoid production and (ii) exploring the applications of these engineered yeast strains. The genetic engineering approaches explored to accomplish these aims include modifying key precursor pathway genes, regulatory genes, fatty acid synthesis genes, and membrane permeability genes. The effects of cultivation methods on product titers are also explored. The applications explored for this yeast strain include enzyme screening for plant natural product biosynthesis in the pharmaceutical industry and large-scale natural product production for the food and flavoring industries. Chapter 2 of this thesis details the methods and results of optimizing a yeast strain to produce triterpenoids and other Mevalonate pathway-derived natural products. Chapter 3 discusses the developed engineered yeast strains' potential applications and potential implications for the more prominent pharmaceutical and agricultural industries. The

results and methods reported here will be utilized and leveraged for other research projects within the Busta Lab at the University of Minnesota Duluth, not only for the applications discussed in Chapter 3.

CHAPTER 2. METHODS OF ENHANCING TRITERPENOID PRODUCTION IN YEAST

2.1 Introduction

Chapter Two of this thesis focuses on the intricate and technical ways triterpenoid production can be enhanced in yeast, specifically by exploring genetic and cultivation methodologies. This area of study is at the front of the synthetic biology field, where genetic engineering and nuanced fermentation protocols promise great strides in optimizing product yield. These tactics offer a clear path to unlocking the potential of yeast, an organism with a rich reservoir of untapped biotechnological possibilities.

The introduction of this chapter is divided into two critical sections. In Section 2.1.1, "Genetic Engineering Strategies," I first inspect the techniques employed to modify and optimize the yeast genome for chemical production. These techniques include strategies such as heterologous pathway engineering and optimizing gene expression using CRISPR/Cas9, which are leveraged to push yeast beyond its natural limitations and enhance triterpenoid production. Additionally, I discuss the genetic targets of this research to increase precursor availability and reduce competition for triterpenoid production. Next, in Section 2.1.2, "Cultivation Engineering Strategies," I pivot to the growth and cultivation factors that influence yeast's triterpenoid production capabilities. I examine how techniques like nutrient management, environmental stress, and bioprocess control are harnessed to cultivate yeast to maximize triterpenoid yield. Together, these

sections offer a comprehensive overview of the cutting-edge methods used to enhance triterpenoid production in yeast, setting the stage for our subsequent exploration of this innovative research's broader implications and potential applications.

2.1.1 Genetic Engineering Strategies

The CRISPR/Cas9 engineering strategy has emerged as a powerful tool for precise and efficient manipulation of DNA sequences, offering significant potential for enhancing the production of plant natural products in yeast. In this section, I explore the molecular mechanism of CRISPR/Cas9 and how I utilize this technology to engineer the genome of our yeast strains. Specifically, I discuss the targets and modifications I make to the yeast genome using CRISPR/Cas9 to optimize the triterpenoid biosynthetic pathway, ultimately leading to improved triterpenoid production in our yeast strains.

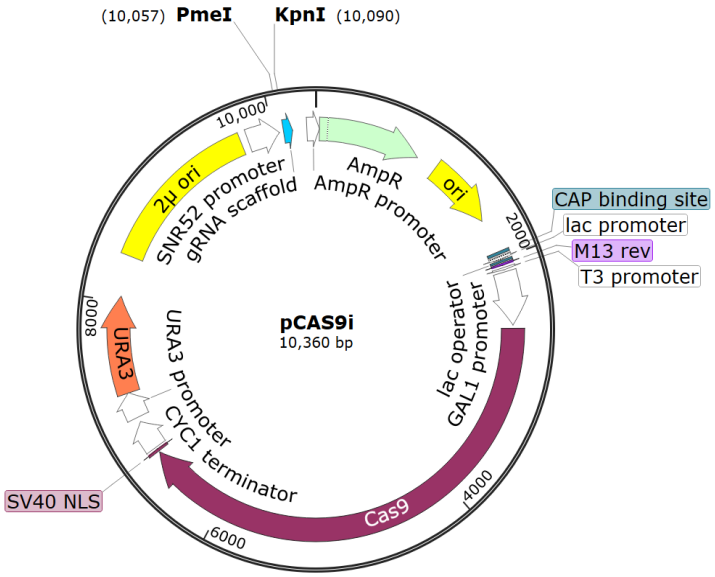


Figure 2. *Vector Map of plasmid pCAS9i. Used for the transformation of Cas9 into yeast. Cut sites PmeI and KpnI are where the sgRNA has homologous ends and where the sgRNA is inserted during recombination in vivo.*

Using restriction enzymes, a pCAS9i plasmid that codes for the Cas9 enzyme can be cut at its restriction sites of PmeI and KpnI to linearize the plasmid (Figure 2). Once linearized, the restriction digest is analyzed and purified using gel electrophoresis. The linearized plasmid is then transformed into yeast with two primers that code for the sgRNA (small guide RNA) and are homologous to the restriction sites, allowing the reformation of the circular plasmid. This recombinant plasmid contains the DNA coding sequence for the Cas9 gene and a sgRNA which will guide Cas9 to a target region in the yeast's genome. The sgRNA is a single-stranded piece of RNA designed to contain two regions, shown in Figure 3. The first region is a 17-24 base pair targeting sequence complementary to the genomic DNA sequence adjacent to the PAM (Protospacer Adjacent Motif) sequence "NGG". The second region is the complementary binding motif to the CAS9 enzyme, which allows the sgRNA to bind to the CAS9 enzyme.

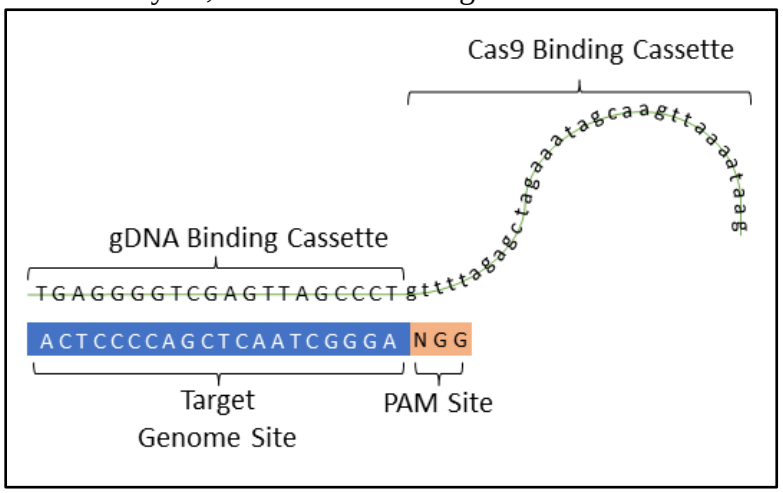


Figure 3. *Design of a DNA primer equivalent to the sgRNA and how it interacts with genomic DNA (gDNA). The green line is the sgRNA, containing a gDNA binding cassette and a Cas9 binding cassette. The blue region is the site in the genome the gDNA binding cassette targets, which is adjacent to the orange region containing the PAM sequence.*

After confirming transformation efficiency with colony PCR (Polymerase Chain Reaction), the cells are grown in raffinose to ensure they metabolize all internal glucose from growing on YPD-amino acid plates. Growing the yeast cells in raffinose prevents glucose from inhibiting the GAL (galactose-inducible) promoter. Once the yeast cells have finished growing in raffinose, Cas9 expression is induced by washing the cells and resuspending them in galactose growth media. For a gene knockout, after 24 hours, the cells are transferred to galactose-containing plates for two days to continue Cas9 expression. The galactose controls the expression of Cas9 through the inducible GAL promoter, which turns on expression when in the presence of galactose. The resulting cells having undergone Cas9 induction can be screened using colony PCR and Sanger sequencing, further described in Section 2.2.2 and summarized in Figure 5. Since the sgRNA codes for the location of the cut site, the only change to the plasmid that needs to be made for different cut sites is different sgRNA primers. For gene knockouts, if the cut site corresponds to a region of the gene you are trying to knock out, then that gene is turned off. This “knockout” results from faulty non-homologous end-joining (NHEJ) repair, which is an error-prone repair system that usually inserts or deletes small fragments of native DNA at the cut site (Guirouilh-Barbat et al., 2004; Moore and Haber, 1996) (Figure 4). Overall, gene knockouts are a potentially short (~3 weeks) and cost-effective (~$100) process for the metabolic engineering of yeast.

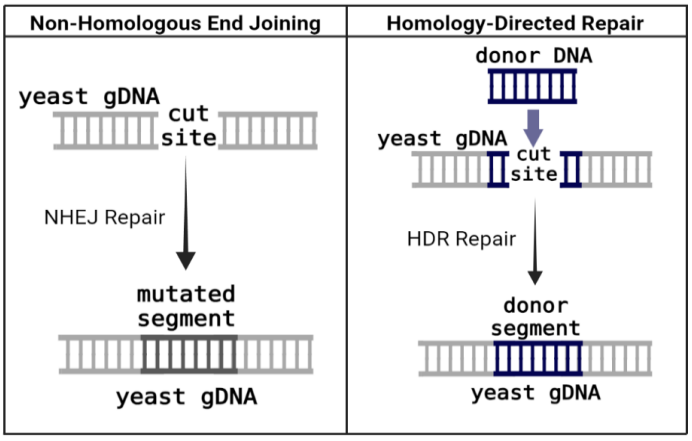


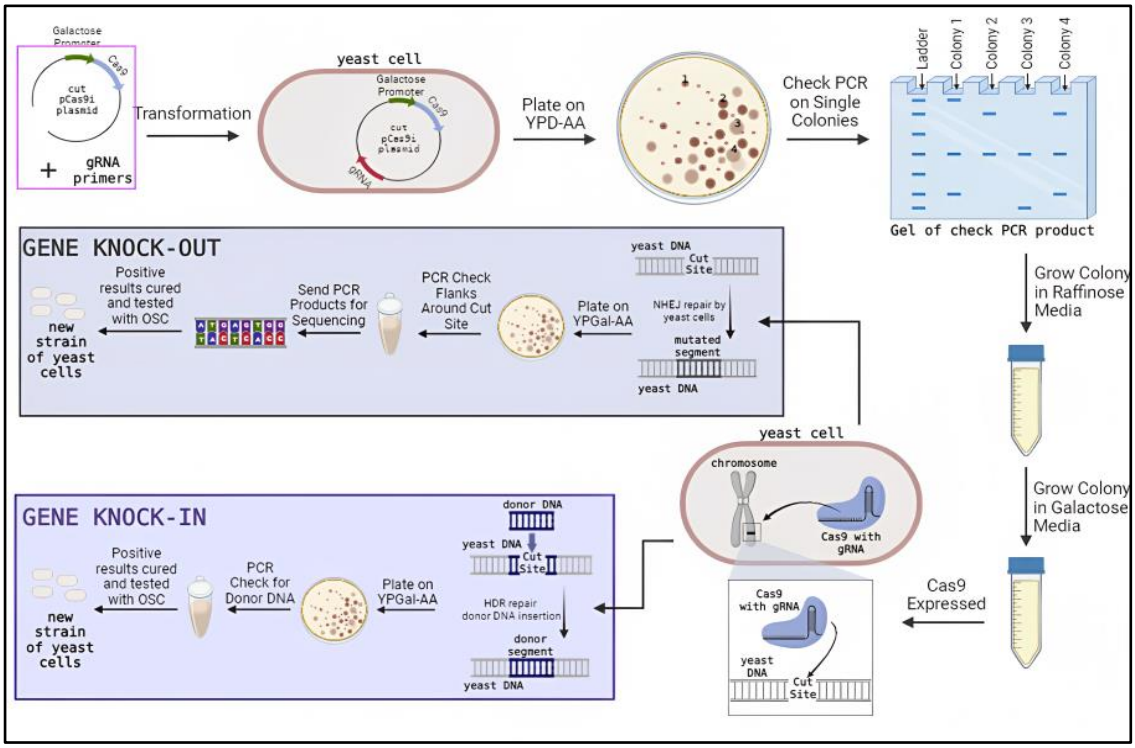
Figure 4. *Molecular mechanisms of the two native yeast repair systems, non-homologous end joining (NHEJ) and homology-directed repair (HDR).*

When knocking-in a gene into the cut site, the yeast culture is incubated in liquid galactose media for 2 hours before being used in an additional transformation. The transformation introduces many linear pieces of donor DNA with flanking regions homologous to the Cas9 cut site into the yeast cells. The donor DNA is then present around the Cas9 cutting sites and inserted into that location via homology-directed repair (HDR) (Degrief et al., 2018). HDR allows error-free DNA repair, which can introduce an exogenous donor DNA template into the cut site (Paques and Haber, 1999). This results in cells that have donor DNA inserted into a specific region determined by the sgRNA (Figure 4).

Although there are extra steps, the time of conducting a gene knockout or a gene addition is roughly the same. The introduction of the donor DNA does come with a significant cost difference compared to gene knockouts. The price for an over or underexpression using donor DNA costs about $400, depending on the length of the donor DNA, as it is purchased from a gene synthesis company. After the expression of

Cas9 on galactose media, single colonies are chosen to confirm the presence of a genetic alteration for both the gene knockouts and gene knock-ins. Confirmation is done by first amplifying the surrounding region of the genetic alteration using PCR. The amplification increases the amount of the DNA surrounding the alteration, which yields the required amount of DNA needed for sequencing. The corresponding PCR products are then sequenced using Sanger sequencing, and the results are aligned to the wild-type genome to confirm either the gene knockout or an addition of a gene. At this point, a new strain has been created and can be used to make competent cells for further genetic modifications (Figure 5).

Figure 5. *CRISPR/Cas9 genetic engineering flow chart. Summarizes creating a mutant yeast strain starting from the plasmid and primers and ending with a cured new yeast strain. Figure developed in collaboration with Nicole Babineau.*



The design of the donor DNA used for over or under-expressing a gene involves changing promoter strength, terminator strength, and genomic location. Promoter and terminator strength can either increase or decrease the relative expression of a specific gene. For overexpression, the strongest promoter and terminator are linked to each gene that is inserted into the genome. TDH3 is currently regarded as yeast's strongest and most stable promoter (Zhang et al., 2015; Partow et al., 2010). The strongest synthetic terminator in yeast currently known is SINter10, a short synthetic terminator that has surpassed the efficiency of most native terminators in yeast (Ahmed et al., 2019). The synthetic terminators' size also drastically reduces the cost of purchasing donor DNA fragments. TDH3 and SINter10 can be used to overexpress any genes targeted for insertion into the genome. Under-expression utilizes weak promoters and terminators. These consist of the MET3 promoter and the gene's native terminator, which can be employed for under-expression as they have been shown to downregulate genes in yeast effectively (Broker et al., 2018). Furthermore, designing the sgRNA to target the insertion of donor DNA into highly expressed chromosomal locations has been shown to increase the expression efficiency of specific genes (Reider Apel 2016). These donor DNA and sgRNA design strategies can improve triterpenoid production in yeast by modifying and relocating various genes involved in the mevalonate pathway, lipid metabolism, and precursor availability.

Utilizing the previously described CRISPR/Cas9 and gene expression strategies, *Saccharomyces cerevisiae* can be genetically engineered for the overproduction of triterpenoids and, more generally, Mevalonate pathway derivatives. Candidate genes and their effect on triterpenoid production were identified as potential genetic engineering targets for triterpenoid overproduction. The candidate genes relevant to this research are categorized into two sections: (i) the three genes targeted in the current study and (ii) potential gene targets for future investigations. The current study prioritized three gene targets, ROX1, DGK1, and PAH1, to validate the gene knockout methods before venturing into gene knock-ins, considered the more challenging engineering technique. The knockout of these three genes has been demonstrated to increase triterpenoid production in yeast. For instance, ROX1 encodes a transcription factor that regulates the mevalonate pathway. By knocking out this gene, the precursor supply for triterpenoid biosynthesis can be enhanced (Broker et al., 2018). For instance, knocking out ROX1, which encodes a transcription factor that regulates the mevalonate pathway, can increase the precursor supply for triterpenoid biosynthesis (Broker et al., 2018). Additionally, knocking out DGK1, which encodes a diacylglycerol kinase, could lead to an accumulation of diacylglycerol, which modifies lipid metabolism to increase the ability to store triterpenoids and its precursors (Zhang et al., 2020). Similarly, knocking out PAH1, encoding phosphatidate phosphatase, could also lead to diacylglycerol accumulation, which would further positively affect the storage of essential molecules (Zhang et al., 2020; Zhang et al., 2021).

The genes described in the following paragraphs will be explored in future research. They were chosen due to their tested impacts on triterpenoid production using similar genetic engineering methods in the literature. Many of these genes are either (i) competing with or are (ii) directly involved in the biosynthetic pathway of triterpenoids (Figure 6). The primary competing pathway shown in Figure 6 is sterol biosynthesis catalyzed by ERG7. ERG7, encoding lanosterol synthase, is the first step in sterol biosynthesis, and underexpression would reduce this pathway that competes for triterpenoid precursors. When controlled by the CTR3 promoter and the presence of 150 um CuSO4, ERG7 underexpression increased triterpenoid production by 660% (Broker et al., 2018). Although other minor competing pathways have been identified, underexpression of sterol biosynthesis is the most effective in triterpenoid overproduction (Guo et al., 2020).

The genes directly involved in the triterpenoid biosynthetic pathway start with encoding the conversion of acetyl-CoA to HMG-CoA (Figure 6). This conversion is catalyzed by ERG10 and ERG13, encoding acetyl-CoA C-acetyltransferase and 3-hydroxy-3-methylglutaryl-CoA synthase, respectively, are essential for the mevalonate pathway, and overexpression can increase the production of isoprenoids, including triterpenoids. When overexpressed, each of these genes helped to increase triterpenoid output by 30 percent (Li et al., 2019). tHMGR, encoding truncated 3-hydroxy-3-methylglutaryl-CoA reductase, is a rate-limiting enzyme in the mevalonate pathway that many studies have prioritized. Its overexpression has been shown to increase triterpenoid

production by 260% (Li et al., 2019). ERG8 and ERG12, encoding phosphomevalonate and mevalonate kinase, respectively, are involved in the mevalonate pathway. Overexpression of these genes can improve the flux through the mevalonate pathway leading to a 50 and 110% increase in triterpenoid production, respectively (Li et al., 2019). ERG19, encoding squalene synthase, is vital for producing squalene, a precursor for triterpenoid biosynthesis. IDI, encoding isopentenyl diphosphate isomerase, is involved in the mevalonate pathway, and overexpression can improve the flux through the pathway leading to a 55% increase in triterpenoid production (Sun et al., 2019). ERG20, encoding farnesyl pyrophosphate synthase, is also involved in the mevalonate pathway, and overexpression can increase the production of farnesyl pyrophosphate, a precursor for triterpenoid biosynthesis. The elevated levels of farnesyl pyrophosphate have aided in the production of triterpenoids in yeast, increasing output by 100% (Li et al., 2019). Finally, ERG9 and ERG1, encoding squalene epoxidase and squalene monooxygenase, respectively, are involved in the biosynthetic steps of converting farnesyl pyrophosphate to squalene and squalene to 2,3-oxidosqualene. Overexpression of ERG9 results in a 130% increase in triterpenoid production (Li et al., 2019). Additionally, the overexpression of *C. albicans*-derived ERG1 increased triterpenoid production by 210% (Zhang et al., 2015). Overexpression of these genes can improve the availability of precursors required for triterpenoid and sterol biosynthesis.

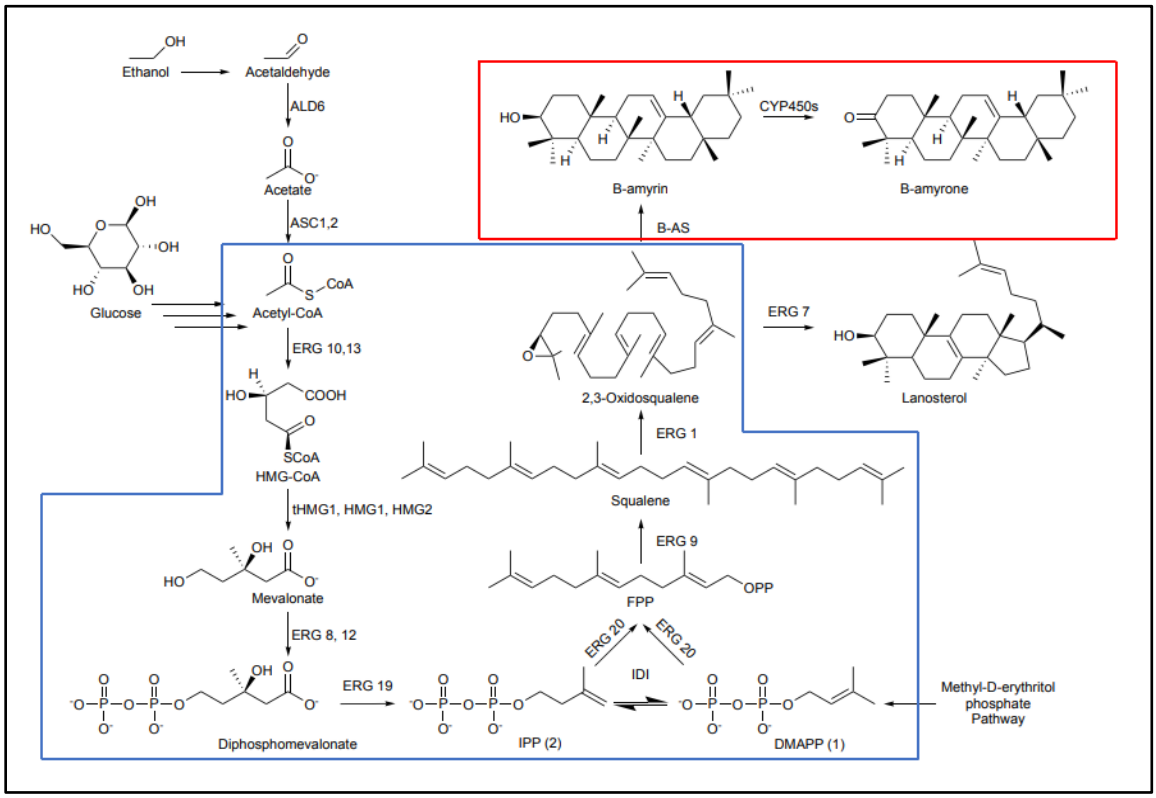


Figure 6. *Overview of the metabolic pathway that leads to β-amyrone. Oxidosqualene cyclases (OSCs) that produce β-amyrin are generally written as BAs (β-Amyrin synthase), which is shown. The colored lines correspond to different pathways; blue outlines the mevalonate pathway, and red outlines the heterologous triterpenoid pathway. All the pathway steps shown here are native to Saccharomyces cerevisiae except those outlined in red.*

There are also gene targets for overexpression involved in lipid metabolism, including DGA1 and OLE1. The overexpression of DGA1, encoding diacylglycerol acyltransferase, can increase lipid accumulation and be used as a storage module for both precursors and triterpenoids. OLE1, encoding delta-9 desaturase, can further increase the

storage capabilities of both precursors and triterpenoids by increasing the unsaturated fatty acid content in the cell membrane (Zhang et al., 2020). Overall, many gene candidates for engineering are directly involved in triterpenoid biosynthesis and indirectly in triterpenoid storage and transport. Engineering these genes for the overproduction of triterpenoids and their precursors provides a yeast strain capable of characterizing plant natural product synthases and constructing biosynthetic pathways to economically important molecules such as pharmaceuticals. Table 1 provides an overview of all the gene editing targets for developing a yeast strain that can aid in the overproduction of triterpenoids. This table includes the gene edits accomplished in this study and all genes identified for future research studies. Through this research, I refined our ability to conduct gene knockouts efficiently, and continued research will be aimed at developing the ability to perform gene knock-ins of various gene targets efficiently. The priority scores of these gene knock-ins are indicated in parenthesis in Table 1.

Table 1. *Genes for overexpression, underexpression, and gene knockouts that are in consideration for this study. Abbreviations in the table include OE (Overexpression), UE (Underexpression), KO (gene knockout), DAG (diacylglycerol), FA (fatty acid), MVA (Mevalonate), IP (in progress), ✓ (completed), and UG (undergraduate). The color coordination for difficulty: Easiest ( ), ( ), ( ), ( ) Hardest.*

Gene	Expression	Pathway	Genome Location	Cellular Location	Difficulty (Priority)
ROX1	KO	MVA deregulation	Endogenous	NONE	1 (✓)
DGK1	KO	Diacylglycerol metabolism	Endogenous	NONE	1 (✓)
PAH1	KO	Phosphatidic acid Phosphatase	Endogenous	NONE	1 (✓, UG)
DGA1 & OLE1	OE	DAG/FA metabolism	308a	Endogenous	2 (IP)
ERG7	UE	Sterol metabolism	Endogenous	Endogenous	2 (1)
ERG19 & tHMG1	OE	MVA	106a	Peroxisome	3 (1)
ERG12 & ERG8	OE	MVA	1622b	Peroxisome	3 (1)
ERG13 & ERG10	OE	MVA	1309a	Peroxisome	3 (1)
ERG1	OE	Squalene	416d	Lipid Body	3 (3)
ERG9 & ERG20 & IDI	OE	Squalene	208a	Peroxisome	4 (2)

The development of these CRISPR/Cas9 methods has given us the ability to genetically engineer yeast with the previously discussed genetic alterations for producing Mevalonate pathway derivatives, primarily triterpenoids. However, confirming the genetic alterations in the edited strains is essential to ensure that the desired changes have been made. Genomic sequencing methods such as PCR amplification and Sanger sequencing can confirm genetic alterations but may not ensure a chemical phenotypic difference from wild-type yeast strain (BY4743). Therefore, it is also necessary to utilize

chemical analysis methods such as gas chromatography-mass spectrometry (GC-MS) to confirm the presence of the desired product(s). By using a combination of these methods, it can be ensured that the genetic alterations have been made and that the yeast strain is producing the desired natural product(s) at differential quantities when compared to the wild-type.

2.1.2 Cultivation Engineering Strategies

Several cultivation engineering strategies can be implemented to further increase the production of triterpenoids in yeast, including ethanol pulse feed, nitrogen limitation fermentation, media supplementation, and biphasic cultures. Ethanol pulse feed has been identified as one of the most effective strategies for enhancing triterpenoid production in yeast (Carsanba et al., 2021). By providing the yeast cells with a readily available carbon source through ethanol feeding, the metabolic activity of the cells can be enhanced, and the biosynthesis of triterpenoids stimulated. Ethanol pulse feeding is also thought to invoke several stress responses in yeast. These stress responses are because ethanol is a toxic compound that can disrupt cell membranes and cause protein denaturation, leading to cellular damage. When yeast is exposed to ethanol, it can activate various stress response pathways, such as the unfolded protein response, oxidative stress response, and heat shock response (Ding et al., 2009). These pathways can lead to changes in gene expression, protein synthesis, and cellular metabolism, ultimately leading to an adaptation to the stressor. In the case of triterpenoid production, the ethanol pulse feeding

may be increasing the production of triterpenoids as a stress response, as triterpenoids are known to play a role in membrane composition and defense mechanisms in plants (González-Coloma et al., 2011).

Comparably, in nitrogen-limited conditions, a stress response is induced in the yeast cells, promoting triterpenoid production. When yeast is grown in nitrogen-limited conditions, it undergoes a metabolic shift known as the "nitrogen catabolite repression" response. This response is a regulatory mechanism that allows the cell to prioritize the utilization of nitrogen-containing compounds for essential cellular functions (Hofman-Bang, 1999). As a result, the cells redirect their metabolic pathways toward producing amino acids and other nitrogen-containing compounds, essential building blocks for biomass synthesis. This shift leads to an increase in acetyl-CoA production, a key precursor molecule for triterpenoid biosynthesis. Additionally, nitrogen limitation can induce stress responses in yeast cells, such as the unfolded protein response and the general stress response, which can redirect metabolic fluxes toward the production of triterpenoids. Furthermore, nitrogen limitation can also affect the expression of genes involved in triterpenoid biosynthesis and regulation, leading to an upregulation of the relevant pathways. All these factors contribute to increased triterpenoid production observed under nitrogen-limited conditions.

The most effective feeding strategy for increasing triterpenoid production was nitrogen limitation in combination with ethanol pulse feed, which increased triterpenoid

production by 470% (Czarnotta et al., 2017). In addition to these feeding strategies, the media can be supplemented with pantothenate, a precursor of coenzyme A (Co-A). As a bottleneck for producing squalene, coenzyme-A overproduction can increase the presence of necessary precursors for triterpenoid production in yeast (Ebert et al., 2018). Combining cultivation engineering strategies such as nitrogen limitation, ethanol pulse feed, and media cofactor optimization can increase the production of triterpenoids in yeast and facilitate the development of other engineered yeast strains for plant natural product production.

Biphasic cultures are another cultivation strategy to increase yields and extract natural products from yeast cultures (Jiao et al., 2022). This method uses two immiscible phases, an aqueous phase, and an organic solvent phase, to extract the desired products. While a biphasic culture can be an effective method for natural product extraction, it can also present challenges for downstream analysis using gas chromatography-mass spectrometry (GC-MS). These challenges arise from the organic layers used, such as dibutyl phthalate, which can introduce impurities that interfere with GC-MS analysis, making it challenging to identify and quantify the extracted natural products accurately. Therefore, in this study, I have chosen not to use biphasic culture to extract natural products from the yeast cultures to ensure the accuracy and reliability of our GC-MS analysis. The discussed CRISPR/Cas9 engineering strategy, its molecular mechanism, and the plans to use it to optimize the triterpenoid biosynthetic pathway in yeast have been outlined. The sequencing and chemical phenotypic analysis of the accomplished

gene knockouts made using CRISPR/Cas9 are described in Section 2.3. The results of various cultivation strategies, including ethanol pulse feed and nitrogen limitation, are also described in Section 2.3. Chapter 2 presents the genetic engineering and cultivation strategies employed to increase heterologous triterpenoid production in the wild-type BY4743 *Saccharomyces cerevisiae* is presented.

2.2 Methods

2.2.1 Plasmids

In each experiment before cultivation, a plasmid containing a β-amyrin synthase from *Glycyrrhiza glabra* (GgBAs) was transformed into each strain to produce β-amyrin. The pCAS9i plasmid was obtained from AddGene and was used to make genetic alterations. The sequence of the β-amyrin synthase (BAS) (*Glycyrrhiza glabra)* used was synthesized from (Site) after codon optimization using the JCAT software (http://www.jcat.de) to optimize gene expression in *S. cerevisiae* strain BY4743. The β-amyrin synthase gene was expressed under the strong inducible promoter (GAL) in an expression cassette (PGAL-BAs-Tcyc1) and placed into the pESC-HIS plasmid. Transformants were grown on YPD-HIS agar for 48 hr at 30C, and positive colonies were selected and plated on YPD-HIS master plates. Master plates were also grown on YPD-HIS agar for 48 hr at 30C. The resulting master plates were then used for B-amryin production using GC mass spectrometry.

2.2.2 Strain construction

The *S. cerevisiae* strain BY4743 was used to carry out all genetic alterations in this study. The CRISPR/Cas9 method described in (Degrief et al., 2018) was used for DNA transformation into the *S. cerevisiae* genome. Before DNA transformation, plasmid pCAS9i was linearized with a restriction digest at sites Pme1 and Kpn1, and efficiency was checked with gel electrophoresis. The linearized pCAS9i plasmid and sgRNA primers are then transformed into *S. cerevisiae* using the Zymogen Frozen-EZ Yeast Transformation II kit. The pCAS9i plasmid and sgRNA recombine in vivo to form a circular plasmid. The transformants were plated on YPD-URA and grown for 48 hr at 30C. Transformation and recombination efficiency of the plasmid in the single colonies is checked using colony PCR and gel electrophoresis. In this PCR, one primer binds on the sgRNA coding sequence, and the other binds on the plasmid ~200 bp upstream of the sgRNA on the pCAS9i plasmid. A 200 bp PCR product indicates a positive transformant, and the respective single colony was used in the next steps of the gene knockout.

Positive transformants are selected and grown in YPRaf-URA for 24 hr at 30 C to ensure they metabolize all internal glucose. Cells are harvested using centrifugation at 600g for 4 minutes. Cas9 expression is induced by washing the cells and resuspending them in YPGal-URA at OD 0.2-0.3 and then growing them for either 1 or 24 hours at 30 C depending on if an expression cassette is being inserted or a gene is being knocked out, respectively. When inserting an expression cassette, the cells from the 1-hour culture are

harvested using centrifugation and transformed with the expression cassette. Transformants are plated on YPGal-URA for 48 hr at 30C. In the case of a gene knockout, the 24-hour culture cells were harvested by centrifugation and plated on YPGal-URA for 48 hr at 30C. In either case, master plates were made by plating positive transformants on YPD-URA for 48 hr at 30C. Single colonies of the positive transformants are streaked and replated two times to ensure homogeneity within the master place colonies. Final master plate colonies are screened by conducting a colony PCR on the respective single colonies and amplifying a 200-500 base pair region around the target mutation site. These PCR products are then purified and sent to get Sanger sequenced by a sequencing company. The results are then aligned and compared against the wild-type yeast strain to identify mutations. A yeast colony found to have the mutated sequence is then cured to remove any plasmid DNA and made into competent cells, resulting in a new yeast strain. The primers used for the PCRs and sequencing mentioned throughout the methods section are listed in Table 2.

Table 2. *List of the primers used in this study.*

PRIMER NAME	**PRIMER SEQUENCE**
ROX1_gRNA_F	TGAGGGGTCGAGTTAGCCCTgttttagagctagaaatagcaagttaaaataag
ROX1_gRNA_R	AGGGCTAACTCGACCCCTCAgatcatttatctttcactgcggag
ROX1_cut_flank_F	CACCATCTTCCTCGGTGTCAAGCTCGA
ROX1_cut_flank_R	GAGGAATGTGATGATGCGTAGGGGTAG
pCAS9i_gRNA_check_R	GCGGCGACCGAGTTGCTC
ADE2_cut_flank_F	GTTGCATGGCTACGAACCGGG
ADE2_cut_flank_R	CCACAGGAACACTTTGGGTAACTGC

PAH1_cut_flank1_F	GGCCATCGTGGGGGCTT
PAH1_cut_flank1_R	CGTCAGGGACATCAGTG
pCAS9i_gRNA_check_F	GCAACACGAGCTGCGCACATAC
ADE2_gRNA_F_TVA	AGTTACCCAAAGTGTCCCTGGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG
ADE2_gRNA_R_TVA	CAGGGACACTTTGGGTAACTGATCATTTATCTTTCACTGCGGAG
ADE2_gRNA_check_F_TVA	AGTTACCCAAAGTGTCCCTG
PAH1_gRNA_F	TTGGGTCTGTGTCTAAAACAgttttagagctagaaatagcaagttaaaataag
PAH1_gRNA_R	TGTTTTAGACACAGACCCAAgatcatttatctttcactgcggag
PAH1_gRNA_check1_F	TTGGGTCTGTGTCTAAAACA
DGK1_gRNA_F_v2	CGTGCTATTTGGAAGGGCAAgttttagagctagaaatagcaagttaaaataag
DGK1_gRNA_R_v2	TTGCCCTTCCAAATAGCACGgatcatttatctttcactgcggag
DGK1_gRNA_check_R_v2	CGTGCTATTTGGAAGGGCAA
DGK1_cut_flank_F_3	CCTGAGCGGTGTTGTCG
DGK1_cut_flank_R_3	CTGTCACTGGAACGTGTGC
ADE2_cut_flank_F_TVA	GTTGCAGGGCTACGAACCGGG
ADE2_cut_flank_R_TVA	CCACACCAAATATACCACAACCGGG
TEF_HYG_TEF_F	GTTATGTCACGCTCTAGAGCGGCC
TEF_HYG_TEF_R	GCCACCGGGAACAAAAGCTGGAGCTCC
TEF_HYG_TEF_check	GCGCGGCCGTCTGGAC

2.2.3 Cultivation, Media, and Metabolite Extraction

To produce β-amyrin, the pESC-HIS plasmid containing expression cassette (PGAL-GgBAs-Tcyc1) was transformed into *S. cerevisiae*. Transformants were plated on YPD-HIS for 48 hr at 30 C. Master plates were then made by plating positive transformants on YPD-HIis for 48 hr at 30C. Master plate colonies were then grown in YPRaf-HIS for 24 hr at 30 C to ensure they metabolized all internal glucose. The cells

were harvested by centrifugation and induced in YPGal-HIS at OD 0.2-0.3 for 96 hr at 30C. The ethanol pulse feed trials were conducted with additions of 25 g/L (243 µL of 95% ethanol) approximately every 24 hours. The nitrogen limitation trials were done by growing cells until they reached the stationary phase, about 60 hours, in YPGal-His. Then the cells are transferred to a fresh 50 mL falcon tube and resuspended in 8 mL fresh YP-HIS medium without any nitrogen and carbon source (Czarnotta et al., 2017). All engineered strains were grown under aerobic conditions in 50mL falcon tubes and stored on an angled tube holder in a shaking incubator at 150rpm. At the end of cultivation, optical density at 600 nm was measured for all cultures. An ANOVA test indicated that the cultivation treatments and strain difference statistically significantly affected cell density with a p-value of 5.93e-7. Data used in statistical analysis are provided in supplementary tables 1 and 2. Although significant, the change in cell density was not negative for any of the cultivation treatments. Studies show that triterpenoid titers can be reported as mg/L (culture performance) or adjusted with an optical density as mg/g cell density weight (Guo et al., 2022). Since whole culture performance is of interest in this study rather than individual cell performance, triterpenoid titers were not adjusted based on cell density.

The cells and media of the resulting culture were separated to have their chemical contents extracted independently. For the extracellular fraction, the media was separated from the cells by centrifugation and then transferred to 8 mL glass tubes. Hexane and 10 µL of a 0.05 mg/mL tetracosane standard were added to the tubes. The tubes were

vortexed, and the chloroform fraction was removed and evaporated, yielding the media extract that was further analyzed to prepare it for the GC-MS. The intracellular fraction is the dry cell pellet from the growth culture. To break open the cells, glass beads and 200 µL of methanol were added to the cells in an 8 mL glass tube. Then the tube was vortexed and sonicated to ensure the intracellular components had been released into the methanol. Water, hexane, and 1 µL of a 0.1 mg/mL octadecanol standard were added to the tubes to separate the hydrophobic and hydrophilic compounds. After being vortexed, the hexane fractions were removed and added to the evaporated chloroform extract. The remaining hexane was evaporated, and what was left was the total extract from the culture. A saponification method is then applied to the sample, removing fatty acids that can interfere with GC-MS data. The saponification method is conducted as follows: To the glass tubes, 2 mL of ethanol was added and incubated at 90^{o}C for 5 minutes. Then 166 µL of 80% w/v potassium hydroxide was added, vortexed, incubated at 90^{o}C for 5 minutes, vortexed, and incubated again at 90^{o}C for five more minutes. Immediately following the incubation, the tubes were placed on ice, and approximately 1 mL of ice-cold water was added, vortexed, and put back on ice. 1 mL of hexane was added to the tubes, vortexed, and then the hexane fraction was removed and evaporated until 200 µL was left. The remaining 200 µL fraction was transferred to a GC vial insert and completely evaporated. Lastly, 15 µL of a 1:1 BSTFA/pyridine mixture was added to the insert and incubated at 70^{o}C for 60 minutes.

Prepared samples were analyzed on a GC-MS with an autosampler attachment to inject samples. 3uL of the samples were injected with a split/splitless injector in splitless mode at a temp of 300^{o}C. At the time of injection, the oven is at 50^{o}C; following injection, it is held at that temperature for 2 min. Then the temperature is ramped at 40 C/min to 200^{o}C and held there for 2 min. The last temperature ramping is done at 3^{o}C/min to 320^{o}C and then held for 10 min. The total run time of this GC-MS method is 58 minutes. This method separates analytes using an HP-5MS column with a helium mobile phase and a 1 mL/min flow rate. After gas chromatography separation, analytes were detected with a 5977B mass spectrometer. Analytes were ionized with an electron ionization source running in positive mode with an ionization energy of 70 eV. The mass spectrometer is programmed to scan *m/z* 40 to *m/z* 800 at two scans per second. After GC-MS analysis of the yeast culture samples, a correction factor was applied based on the ionization efficiency of the internal standard octadecanol and a structurally similar triterpenoid betulin. Mass spectral data is analyzed manually using the Agilent data analysis software, and figures are generated through R studio software. Statistical analysis was done using the ANOVA and Tukey test to determine the significance of triterpenoid titers. Data collected are provided in Supplementary Tables 1 and 2.

2.3 Results

In this section, I present the results of the experiments conducted to enhance triterpenoid production in yeast via genetic and cultivation engineering methods. These results offer valuable insights into the strategies' efficacy and potential implications. To streamline the comprehension of the findings and provide a clearer structure, I have categorically separated the results into three main divisions. This approach allows a more focused exploration of the outcomes and enables the reader to comprehend each strategy's influences and impacts readily. The first subsection is dedicated to the sequencing results derived from my genetic engineering strategies. Herein, I discuss the impact of our targeted CRISPR/Cas9-mediated genetic modifications, assessed through rigorous sequencing analysis. The subsequent subdivision delves into the chemical phenotyping of the yeast strains produced by knocking out genes using CRISPR/Cas9 genetic engineering methods. This section discusses the impact of genetic modification on the production of triterpenoids in novel yeast strains. Lastly, the third section discusses the impact of testing various cultivation engineering strategies on one of the engineered yeast strains developed in this study. These cultivation strategies encompass the effects of the yeast's environment and cultivation conditions, effectively correlating them with observed shifts in triterpenoid yield. This clear delineation of the results based on sequencing, chemical phenotyping, and cultivation facilitates better comprehension and provides a robust platform for subsequent discussion and interpretation in later sections of this thesis.

2.3.1 Sequencing Results

Once Cas9 expression is induced and the sgRNA leads Cas9 to the target region, single colonies can be screened for genetic mutations caused by the native NHEJ repair systems of yeast. The following shows the sequencing results and interpretation for the three genes that were knockout targets in our genetic engineering strategy. These genes are a mevalonate pathway regulator (ROX1), diacylglycerol kinase (DGK1), and phosphatidate phosphatase (PAH1). Although chemical phenotyping was done before the succeeding genetic mutation of each knockout strain, the sequencing and chemical phenotyping results are presented separately for the reader's convenience. Additionally, DGK1 and PAH1 knockouts were performed in parallel as PAH1 was part of an undergraduate research project and could not be performed after the DGK1 knockout.

This study's first genetic engineering target was a ROX1 knockout in the wild-type BY4743 yeast strain. ROX1 is a negative regulator of the MVA pathway, so deletion of it is hypothesized to increase amyrin titers by increasing the precursor pool. The ROX1 deletion, combined with a tHMGR and ERG13 overexpression, increased triterpenoid production by 1500% (Broker et al., 2018). The Sanger sequencing results, in Figure 7, of one of the screened single colonies generated through the CRISPR/Cas9 methods showed a single base pair deletion in the genomic DNA coding sequence of ROX1. The sequencing results also indicated that the single base pair deletion was homozygous due to the single peak signal within the sequencing data. When the mutated

genomic DNA sequence is translated into a protein coding sequence, a premature stop occurs at amino acid 247. This mutation truncated the last 33% of the protein and was hypothesized to render the resulting enzyme incapable of regulating the MVA pathway. Therefore, the yeast colony that showed the ROX1 mutation was cured to remove any plasmid DNA and then made into competent cells, which resulted in the SS1 strain.

Figure 7. *Shown is the ROX1 DNA coding sequence subsection that the ROX1 sgRNA targets. The SS1 mutant shows a deviation from the BY4743 wild-type due to a deletion of a singular base pair in the DNA coding sequence. The sequencing results showed that this mutation was homozygous, indicated in the parenthesis. The highlighted area shows the changed base pairs compared to the reference yeast strain 288C. Dashes ("-") indicate a missing base.*

This study's second genetic engineering target was a DGK1 knockout in the previously engineered SS1 yeast strain. DGK1 encodes a diacylglycerol kinase, so a deletion of it is hypothesized to cause an accumulation of diacylglycerol. This deletion would subsequently alter lipid metabolism and increase the ability to store triterpenoids and their precursors. A DGK1 knockout increased triterpenoid production by 230% when combined with a PAH1 knockout (Zhang et al., 2020). The altered lipid metabolism through a DGK1 knockout and the deregulation of the MVA pathway through the ROX1 knockout would likely further increase triterpenoid production. Multiple colonies'

showed a multiple peak signature in the Sanger sequencing results, and it was determined that the mutations were likely heterozygous, only affecting one of the chromosomes in the diploid yeast genome. Additionally, the genomic DNA of the wild-type yeast strain was Sanger sequenced and showed that the wild-type could also be heterozygous at the target location in the DGK1 sequence (Figure 8). Tools beyond Sanger sequencing would need to be utilized to determine the heterozygosity of the wild-type accurately. In an alternative study, the heterozygosity could be accurately determined using next-generation sequencing. Although these results were inconclusive due to the heterozygous nature of this genomic location, they did not entirely rule out the possibility of a successful knockout. Therefore, the yeast colony that showed the greatest likelihood of a DGK1 mutation was cured to remove any plasmid DNA and then made into competent cells, which resulted in the SS2 strain. If the DGK1 knockout worked as designed, the DNA sequence would contain a mutation in the coding sequence where there are several missing base pairs in both the wild-type sequence (Figure 8). This mutation could potentially truncate the protein-coding sequence, rendering the resulting enzyme incapable of acting as a diacylglycerol kinase.

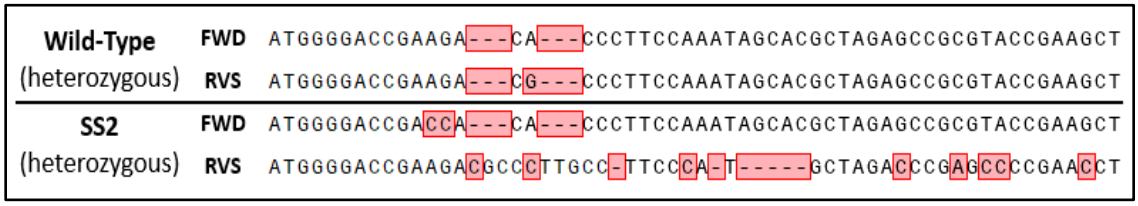


Figure 8. *Shown is the DGK1 DNA coding sequence that the DGK1 sgRNA targets. The wild type BY4743 shows differentiation in its coding sequence. Similarly, the SS2 mutant shows differentiation, but there is no distinguishable difference from the wild-type BY4743 DNA coding sequence. The sequencing data for both strains inferred that this mutation was heterozygous, indicated in the parenthesis under the strain names. The highlighted area shows the changed base pairs compared to a reference yeast strain 288C. Dashes ("-") indicate a missing base.*

This study's third genetic engineering target was a PAH1 deletion in the previously engineered SS1 yeast strain. PAH1 encodes a phosphatidate phosphatase, and when knocked out, it leads to an accumulation of diacylglycerol. This knockout would positively affect the storage of products and precursors, similar to the DGK1 knockout. A PAH1 knockout combined with a DGK1 knockout increased triterpenoid production by 330% (Zhang et al., 2020). It was hypothesized that the altered lipid metabolism through a PAH1 knockout, in combination with the deregulation of the MVA pathway through the ROX1 knockout, would likely further increase triterpenoid production. This gene knockout was done in the SS1 strain and not in the SS2 strain due to it being a part of an undergraduate research project conducted by Nicole Babineau. A large part of the PAH1 gene knockout in SS1 was done by Nicole and is included here as it is an extension of the

work and methods developed throughout this thesis project. It allows this document to directly compare the three different mutant yeast strains developed in this study.

All four PAH1 knockout colonies' sanger sequencing results showed homozygous 12-18 base pair deletions in the genomic DNA coding sequence of PAH1. Although the results showed successful mutations in the PAH1 coding sequence, all four colonies showed mutations that were multiples of three starting at the beginning of an amino acid sequence (Figure 9). This mutation means that whole amino acids were removed, resulting in no frameshift mutations that would otherwise cause the presence of early stop codons within the PAH1 coding sequence. Additionally, due to heterozygosity events with DGK1, the BY4743 PAH1 coding sequence was Sanger sequenced to determine if it deviated from the reference strain 288C. It was found that the BY4743 sequence for PAH1 did not deviate from the PAH1 sequence in 288C; therefore, the wild-type forward and reverse reads are not included in Figure 9.

Figure 9. *Shown is the PAH1 DNA coding sequence that the PAH1 sgRNA targets. The SS3 mutant shows deviation from the BY4743 wild-type due to an 18-base pair deletion in the DNA coding sequence. The sequencing data inferred that this mutation was homozygous, indicated in the parenthesis under SS2. The highlighted area shows the changed base pairs compared to the reference yeast strain 288C. Dashes ("-") indicate a missing base.*

In further analysis of the PAH1 mutation, Nicole and I decided to compare the crystal structure of the translated PAH1 sequence from a colonies with an 18-base pair deletion to the crystal structure of the wild-type PAH1 protein. In comparing these two crystal structures, it was hypothesized that the loss of the six amino acids would have little to no effect on protein function. This hypothesis was based on the fact that the lost amino acids were (i) not in critical areas in the structure (i.e., active site or alpha folds) and (ii) were not present in the resulting crystal structure due to them being close to the beginning of the protein sequence. Even though the computational results indicated that the mutation was unlikely to affect the resulting protein structure, it was still decided that chemically phenotyping the mutant would provide meaningful information. Therefore the yeast colony with the 18 base pair PAH1 mutation was cured to remove any plasmid DNA and made into competent cells, which resulted in a strain named SS3.

2.3.2 Chemical Phenotyping Results

To test whether or not the knockout strains showed elevated triterpenoid production, a chemical phenotype test needed to be performed. This test included integrating a plant synthase into yeast that produced triterpenoids, an MVA pathway derivative. This study used a highly efficient β-amyrin synthase from *Glycyrrhiza glabra* named “GgBAs” as the model production enzyme. It has been widely tested, which showed it to be the most efficient β-amyrin synthase. A plasmid containing GgBAs (pESC-GgBAS) was transformed into yeast strains, and positive transformants were

selected based on the histidine auxotrophic marker located on the pESC-GgBAs plasmid (Figure 10). Also shown on the vector map of pESC-GgBAs in Figure 10 is that GgBAs is linked to the galactose promoter; therefore, its expression can be controlled through the presence of galactose. Positive transformant cells from the pESC-GgBAs transformation were then grown in 2 mL of raffinose YPD-HIS media for 24 hours to grow the cell biomass needed for expressing GgBAs. The cells from the raffinose cultures were then transferred to 8 mL galactose cultures and adjusted to a 0.2 - 0.3 optical density at 600 nm. Triplicates of the cultures were made to account for deviations in culture performance. After growth at 30C for four days, 4 mL of each culture was harvested for analysis with GC-MS.

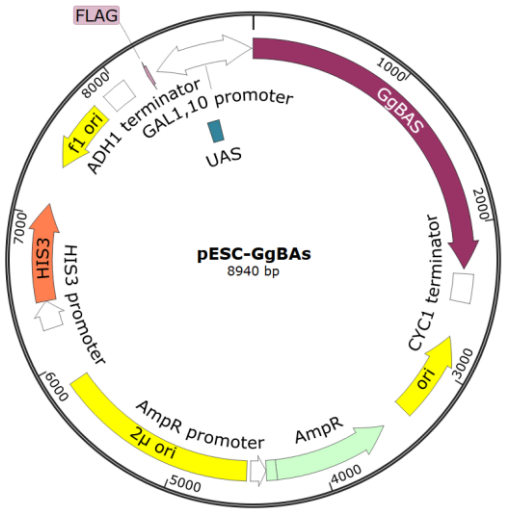


Figure 10. *Vector Map of plasmid pESC-GgBAs. Based on plasmid pESCC*-HIS *with an insertion of a β-amyrin synthase from Glycyrrhiza glabra (GgBAs). The expression of GgBAs is controlled by a galactose inducible promoter (GAL1, 10 promoter).*

The GC-MS analysis results indicated that the ROX1 deletion increased β-amyrin titers in the SS1 strain, with an average titer of 123 µg/L β-amyrin. This result represented a 40% increase in amyrin titers compared to the control strain of BY4743

with no genetic modifications (Figure 11). These results initially showed a chemical phenotypic difference between BY4743 and SS1, further indicating that ROX1 was successfully truncated in the SS1 strain. This process also determined that subsequent gene knockouts would take around four weeks to complete genetic and chemical phenotype testing. The successful genetic modification of the ROX1 gene using CRISPR/Cas9 was the first step in demonstrating the potential of using this approach for engineering yeast strains to produce high-value compounds. In the DGK1 deletion strain, SS2, GC-MS analysis showed that β-amyrin titers in the SS2 strain increased significantly, with an average 211 μg/L titer. This finding represented a 130 and 70% increase in amyrin titers compared to the control strain BY4743 and the ROX1 mutant SS1, respectively (Figure 11). These results show a significant chemical phenotypic difference between SS1 and SS2, indicating that DGK1 was successfully truncated in the SS2 strain, even considering its indistinguishable sequencing results. The successful genetic modification of DGK1 using CRISPR/Cas9 further demonstrates the accuracy and reproducibility of gene knockouts using our developed CRISPR/Cas9 methods. Furthermore, it highlights the importance of doing chemical phenotyping in the case of indistinguishable sequencing results.

The PAH1 deletion strain SS3's GC-MS analysis results did not increase amyrin titers in the SS3 strain compared to its parent strain SS1. The titers of SS3 were within the margin of error of SS1, with an average titer of 121 μg/L β-amyrin (Figure 11). These results did not show a chemical phenotypic difference between SS1 and SS3, indicating

that PAH1 was not successfully truncated in the SS3 strain. The unsuccessful knockout of PAH1 solidified our hypothesis that the missing 18 base pairs from the beginning of the DNA coding sequence would not impact the protein structure. Furthermore, these results indicate that targeting a more prominent location of the DNA coding sequence would be advantageous, such as a region encoding an alpha fold or the active site. Statistical analysis was done using the ANOVA and Tukey test to determine the significance of amyrin titers between the engineered yeast strains. Using the ANOVA test, there was a statistically significant difference in amyrin titers between the different engineered strains and the wild-type with a p-value of 0.000764. This prompted the use of a Tukey test to determine which strains were driving the significant difference, and it was found that only SS2 was statistically different from the other strains. Furthermore, it showed that the increase in average amyrin titers in strains SS1 and SS3 was not statistically different from the wild-type.

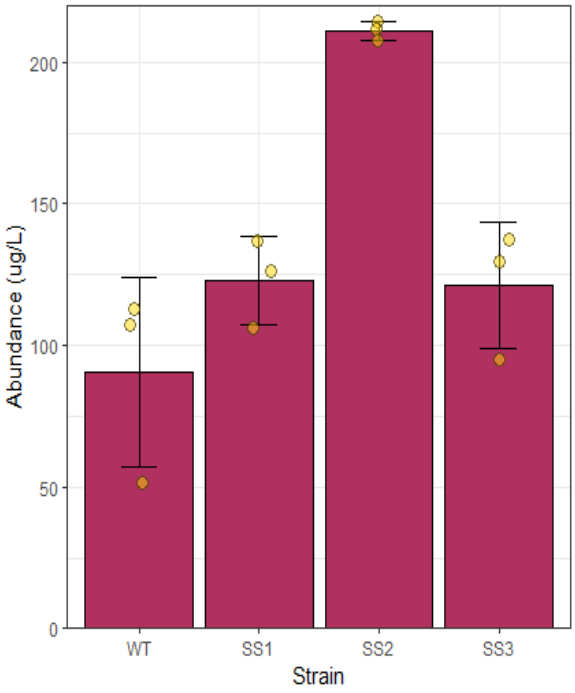


Figure 11. *Titers of control strain BY4743, ROX1-knockout SS1, DGK1-knockout SS2, and PAH1-knockout SS3, expressed with pESC-GgBAs. Error bars are indicative of triplicate measurements for each strain. The yellow data points show each strain's relative abundance of individual samples. ANOVA and Tukey tests showed a significant difference in triterpenoid titers only between strain SS2 and the other strains. This figure was generated using R studio.*

2.3.3 Cultivation Strategies

To increase the production of triterpenoids in the previously engineered yeast strains, various cultivation strategies were employed. The cultivation strategies tested in this study consisted of ethanol pulse feeding, nitrogen limitation, and a combination of the two methods. These methods have been previously shown to increase triterpenoid production in yeast and are the most appropriate methods for small-scale fermentations (Czarnotta 2017); therefore, it was hypothesized that similar results would be seen in this study.

Using ethanol pulse feeding at the 8 mL culture volume resulted in a 30% decrease in the average amyrin production compared to the SS1 control culture with normal cultivation conditions (Figure 12). This result indicates that ethanol pulse feeding did not enhance amyrin production using the SS1 yeast strain at this culture volume. The nitrogen-limited fermentation strategy resulted in a 40% decrease in average amyrin production compared to the SS1 control culture without ethanol pulse feeding (Figure 12). The results indicate that nitrogen limitation at this culture volume did not enhance amyrin production in the engineered yeast strain. Combining the two methods showed that ethanol pulse feeding and nitrogen limitation had a 40% decrease in average amyrin production compared to the SS1 control culture with normal cultivation conditions (Figure 12). Statistical analysis was done using the ANOVA test to determine the statistical significance of amyrin titers between the different cultivation techniques. It was found that with a p-value of 0.595, there was no significant difference between the amyrin titers.

Overall, cultivation strategies such as ethanol pulse feeding and nitrogen limitation did not enhance triterpenoid production at the 8 mL culture volume using the SS1 yeast strain. These results do not refute increasing triterpenoid production using ethanol pulse feeding and nitrogen limitation but probably reflect the lack of application at lower culture volumes. (see discussion below). The optical density of all the cultures reached a critical point, suggesting that all cultivation treatments had no significant adverse effects on culture performance. Further investigation may look into the difference

in culture performance between cultivation strategies.

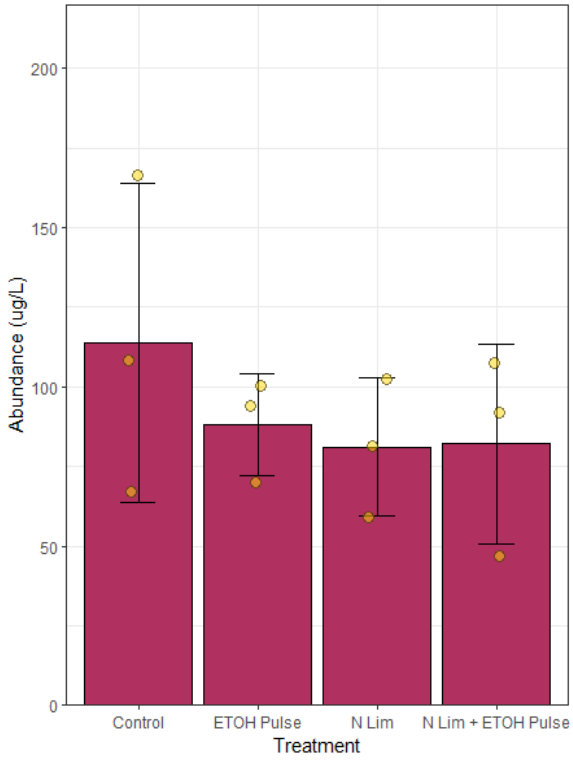


Figure 12. *Ethanol pulse feeding (ETOH Pulse), nitrogen limitation (N lim), and the combination of ethanol pulse feed and nitrogen limitation treatment (N Lim + ETOH Pulse) results compared to the control cultivation. All culture treatments harbored the SS1 strain containing plasmid pESC-GgBAs. Error bars indicate triplicate measurements for each strain. The yellow data points show the relative abundance of individual samples for each strain. The ANOVA test showed no significant difference between the triterpenoid titers across all cultivation techniques. This figure was generated using R studio.*

2.4 Discussion

The results of this study demonstrated multiple successful gene knockouts using CRISPR/Cas9 technology. The first of these knockouts was the ROX1 gene in the wild-type BY4743 yeast strain resulting in a 40% increase, on average, in β-amyrin titers. This result is similar to that observed in the literature, which found a 140-290% increase in

triterpenoid production (Gao et al., 2022). Although the chemical phenotype tests showed an increase in average β-amyrin titer compared to the wild-type, an ANOVA test identified that this result was non-significant. Additionally, the data showed that this difference in average β-amyrin titers was likely due to a single point in the wild-type data. Sanger sequencing results of ROX1 showed a clear and definitive single base pair deletion, which caused the required frameshift mutation in the ROX1 knockout. The successful knockout of ROX1, as confirmed by sequencing results, also indicated the potential of using this approach to engineer yeast strains to produce high-value compounds.

This study's second genetic engineering target was a DGK1 knockout in the previously engineered SS1 yeast strain. The hypothesis was that the deletion of DGK1 would cause an accumulation of diacylglycerol, subsequently altering lipid metabolism and increasing the ability to store triterpenoids and their precursors. The altered lipid metabolism through a DGK1 knockout and the deregulation of the MVA pathway through the ROX1 knockout was expected to increase triterpenoid production further. The results from the DGK1 knockout showed a 130% increase in β-amyrin production. The significance of this result was confirmed by ANOVA and Tukey test. This increase in triterpenoid production was less than that observed in the literature, which found that a DGK1 KO had a 330% increase in triterpenoid production (Zhang et al., 2020). Although this study’s results were less than reported in the literature, it is important to note that the cited studies DGK1 KO were done in combination with a successful PAH1. This

additional PAH1 KO is likely the cause of the large discrepancy in the percent increase in triterpenoid production. Sanger sequencing results of DGK1 showed that the target locus was heterozygous, which caused the results to be challenging to interpret. In subsequent gene knockout investigations, it will be necessary to preliminarily sequence the wild type's target genomic region to ensure homozygosity.

PAH1 was expected to have similar effects on diacylglycerol metabolism as DGK1. Chemical phenotype results from the PAH1 gene knockout strain SS3 showed no change in β-amyrin production compared to the SS1 strain. This result was confirmed by an ANOVA test which showed no statistically significant difference in β-amyrin titers. If conducted successfully, the literature reports that the PAH1 KO, in combination with a DGK1 KO, would increase triterpenoid production by 330% (Zhang et al., 2020). This lack of impact is likely due to the Sanger sequencing results showing that six whole amino acids were removed from the genomic sequence. Mutations of whole three base pair codons do not cause a frameshift mutation, which is needed to introduce premature stop codons that truncate the coding sequence. Furthermore, the missing amino acids in the PAH1 coding sequence did not negatively impact the protein's function, likely due to them being at the beginning of the coding sequence. In further studies, it may be possible that this mutation would have disrupted the protein function if it were located in a critical structural region such as the active site. Overall, the genetic engineering strategies deployed in this study resulted in three unique situations, including (i) a single base pair deletion causing a frameshift mutation and successful knockout, (ii) an undetectable

successful mutation due to heterozygosity, and (iii) an 18 base pair deletion that did not affect the translated protein's function. Understanding these potential outcomes using CRISPR/Cas9 engineering strategies will help design and analyze future genetic engineering experiments.

The cultivation strategies employed in this study, including ethanol pulse feeding and nitrogen limitation, were shown in previous studies to increase triterpenoid production in yeast (Czarnotta et al., 2017). Ethanol pulse feeding is thought to increase β-amyrin titers by activating various stress response pathways, ultimately leading to an adaptation to the stressor. Comparably, nitrogen limitation is believed to increase β-amyrin titers by inducing a metabolic shift that increases acetyl-CoA production, a key precursor molecule for triterpenoid biosynthesis. Additionally, nitrogen limitation also induces stress responses in yeast cells, which can redirect metabolic fluxes toward the production of triterpenoids. These literature results and hypotheses were not congruent with the results from this study. In this study, all cultivation methods had no significant effect on β-amyrin titers, as confirmed by ANOVA. Compared to the control fermentation, ethanol pulse feed and nitrogen limitation showed a non-significant 30 and 40% decrease in average β-amyrin titers, respectively. Ethanol pulse feeding and nitrogen limitation were also tested in combination, resulting in a non-significant 40% reduction in average β-amyrin titers compared to the control fermentation. The poor performance of applying these strategies seems to be the discrepancy in the control of and the size of cultures in literature versus our study. Many of the literature results are derived from

highly controlled experiments done in bioreactors at the 0.5 L to 1 L scale. These conditions likely impact the culture performance and overall success of using ethanol pulse feed and nitrogen limitation cultivation strategies, seen in the literature as an increase in triterpenoid production by 470% (Czarnotta et al., 2017). A higher culture volume in a bioreactor also provides the conditions necessary for yeast to achieve higher cell densities than would usually be seen in conditions such as those in this study. These higher cell densities increase the production capacity and efficiency within the cell factory, directly correlating to higher triterpenoid yields. Collectively, the results of this study showed that at the 8 mL culture volume in falcon tubes, the application of ethanol pulse feeding and nitrogen limitation did not result in increased triterpenoid production.

Overall, this study analyzed the use of CRISPR/Cas9 technology and cultivation strategies to engineer yeast strains for increased production of triterpenoids. The genetic engineering strategies utilized have great potential for increasing the production of various natural products of interest, such as other Mevalonate pathway derivatives. Additionally, these strategies could be applied to produce other plant compounds of interest in yeast, such as fatty acid natural products. This study provides a foundation for future research on optimizing yeast strains for increased production of high-value natural products.

2.5 Conclusions

In summary, the significant findings from this chapter compromise three major points: (i) DGK1 and ROX1 knockout strains were confirmed by sequencing and chemical phenotyping, respectively, whereas the PAH1 strain sequencing showed a mutation but had no effect on the chemical phenotype, (ii) the framework and methods that were developed for completing gene knockouts are efficient and reproducible, and (iii) cultivation strategies such as ethanol pulse feeding and nitrogen limitation were not effective methods of increasing triterpenoid production at the 8 mL culture scale. In the first significant finding, I achieved successful gene knockout in two engineered strains. These knockouts resulted in strains SS1 and SS2, but only strain SS2 showed a significant increase significance increase of β-amyrin titers to 211 µg/L, which was confirmed by ANOVA and Tukey tests. A PAH1 knockout in strain SS2 must be conducted at a homozygous region of the PAH1 coding sequence to further build the first significant finding. Combining all three gene knockouts will be beneficial in creating a strain even more capable of producing triterpenoids at elevated levels. In the second point, a reproducible and efficient CRISPR/Cas9 methodology was built through troubleshooting and subsequent development of the methods throughout the three gene knockouts. In developing these methodologies, I also identified challenges in determining potential NHEJ mutations due to wild-type and mutation heterozygosity.

The next step in developing the CRISPR/Cas9 methods is to conduct a gene knock-in utilizing the native yeast repair system of homology-directed repair (HDR). This gene knock-in will be conducted in one of the previously developed engineered strains, as the goal is to create a genetically engineered yeast strain that can produce triterpenoids at an increasing rate. The last finding in Chapter 2 was that the application of ethanol pulse feeding and nitrogen limitation did not significantly increase triterpenoid production in the SS1 yeast strain. Although these strategies failed to increase β-amyrin titers at the 8 mL scale, it would be beneficial to recreate this study at a larger culture scale and in a more controlled environment to confirm or refute the results found in the literature.

Chapter 3 delves into the practical applications of engineered yeast strains using the various strains and methods developed in Chapter 2. The two prominent industry examples discussed in the introduction to Chapter 2 are the pharmaceutical and agricultural industries. In each of these industries, I discuss the broad application of engineered yeast strains and give primary examples of how the current study has sought to address some challenges they encounter. The enormous potential of engineered yeast strains as a platform for industrial biotechnology is demonstrated through the primary examples. Overall, the engineering of yeast strains represents a significant advance in biotechnology, enabling the production of valuable compounds more sustainably and efficiently. As these technologies develop and refine, their impact on numerous industries and our environment is extremely promising.

CHAPTER 3. ENGINEERED YEAST APPLICATIONS

3.1 Introduction

Engineered yeast has shown great promise for applications beyond ethanol biofuel production (Nandy and Srivastava, 2018). With the ability to efficiently produce complex natural molecules and proteins, yeast has become a valuable tool for various industries, including pharmaceuticals and agriculture. By harnessing the power of genetic engineering and synthetic biology, yeast can be tailored to produce specific products of interest, providing a sustainable and cost-effective alternative to traditional production methods (Yang et al., 2022). These traditional production methods mainly include organic chemical synthesis and chemical extraction from plant material. In this chapter, I explore some significant applications of engineered yeast, focusing on its potential for use in the pharmaceutical and agricultural industries.

In the pharmaceutical industry, engineered yeast has shown promise in producing natural products such as artemisinic acid and taxol, which are used as antimalarial and anticancer drugs (Ro et al., 2006; Engels et al., 2008). Using yeast as a host for drug production offers advantages over traditional methods, which face many challenges, such as cost-effectiveness and scalability. The pharmaceutical industry faces many challenges, including long drug development timelines, high costs, and difficulty producing certain high-value small molecules. Engineered yeast has the potential to address some of these challenges by offering a faster and more cost-effective means of producing high-value

small molecules, including therapeutics and vaccine compounds. Yeast-based production systems can be optimized for the efficient and high-yield production of specific molecules, and genetically modified yeast strains can further enhance the productivity of these systems. Additionally, yeast can be engineered to produce complex macromolecules, such as glycosylated proteins, which are often challenging to produce using traditional techniques (Fidan and Zhan, 2015). By using engineered yeast to produce high-value small molecules, the pharmaceutical industry can potentially reduce drug development timelines and costs, ultimately improving patient access to these essential treatments. However, nearly all plant-derived pharmaceutical biosynthetic pathways are yet to be identified or fully characterized. Yeast is an exceptional model organism for studying and characterizing these plant-derived pharmaceuticals' biosynthetic pathways that have yet to be fully elucidated. By introducing the relevant plant genes into the yeast genome or on an inducible plasmid, researchers can recreate the plant biosynthetic pathways in yeast cells, allowing for the production and analysis of target molecules. This approach allows for the rapid and efficient characterization of biosynthetic pathways, as yeast cells can be grown quickly and are amenable to genetic manipulation. Furthermore, by using yeast as a platform, researchers can identify and engineer key biosynthetic pathway enzymes to improve the yield and purity of the target compound, ultimately improving the overall efficiency of the production process. Utilizing these microbial engineering techniques and yeast as a model organism, further innovation and discovery within the pharmaceutical industry are inevitable.

Similarly, engineered yeast has been proposed as an alternative to producing small molecules in the agricultural industry as crop by-products. Producing plant natural products, such as flavors and fragrances, in yeast can reduce the need for agricultural production of these high-value molecules. Traditional agricultural practices for producing these molecules face several challenges, including production efficiency, environmental impacts, and the potential to endanger some wild plant species (Liu et al., 2022). These challenges have led to exploring alternative methods for producing food and other agricultural products, such as using engineered microorganisms like yeast. This approach could reduce reliance on traditional agricultural practices while providing a sustainable source of high-value products. One way to reduce the reliance on traditional agricultural practices is by utilizing alternative methods like microbial engineering. For example, vanillin, the primary flavor molecule in vanilla beans, is often extracted from the beans or synthesized chemically. However, the process of extracting vanillin from vanilla beans is time-consuming, expensive, and environmentally unsustainable due to the high demand for vanilla products (Qiu et al., 2022). On the other hand, engineered yeast can be used to produce vanillin from simple, sustainable sources such as glucose. The same is true for different flavor and fragrance molecules, such as safranal, the primary aroma molecule found in saffron, and methyl jasmonate, the major component of the jasmine scent (Sun, L. et al., 2021; Naziz et al., 2019). Producing these molecules using engineered yeast could provide a more sustainable and cost-effective alternative to the traditional production of flavor and fragrance molecules.

Additionally, yeast can be engineered to produce and study prospective natural pesticides and herbicides that could replace less biodegradable or more toxic synthetic chemicals in agriculture, creating environmentally safe alternatives. For instance, researchers have engineered yeast to produce pyrethrins, a class of natural insecticides found in Chrysanthemums. Pyrethrins are effective against a wide range of insects, including mosquitoes, flies, and fleas, and they have low toxicity to mammals (Mohamed et al., 2016). Additionally, scientists have used yeast to produce essential oils from plants with insecticidal and herbicidal properties, such as thyme, peppermint, and rosemary. These essential oils can be natural alternatives to synthetic pesticides and herbicides (Wang et al., 2022). By producing these natural molecules using engineered yeast, it is possible to reduce the reliance on traditional agricultural practices that involve synthetic chemicals, which can negatively affect the environment and human health (Rani et al., 2021). The widespread application of engineered yeast in these industries has the potential to revolutionize the way we approach drug and crop production.

In summary, the engineered yeast strains I reported in Chapter 2 contribute to the development of both the pharmaceutical and agricultural industries. In the pharmaceutical industry, engineered yeast can significantly reduce costs and increase the accessibility of plant-derived therapeutics and medicines. Moreover, the engineered yeast can help characterize the biosynthetic pathways of these molecules. In agriculture, our strain can be used for the sustainable production of flavor and fragrance molecules, natural pesticides, and herbicides, reducing the environmental impact of traditional agricultural

practices. Overall, engineering yeast can lead to more sustainable and cost-effective production of high-value molecules for various industries. In the following sections of this chapter, I describe two specific examples of how my engineered yeast and/or the protocols I have developed can be utilized in the pharmaceutical and agricultural industries. First, I discuss the characterization and elucidation of biosynthetic pathways to cardenolides in Wallflower, a class of molecules known for their medicinal properties (Mosleh et al., 2018). Next, I describe a project I have designed to engineer beer yeast to produce hop-derived monoterpenes, molecules essential for beer flavor and aroma. These examples highlight the versatility and potential of engineered yeast in various industries and how it can be harnessed for an alternative production method.

3.2 Methods

3.2.1 Wallflower OSC Strain Development

In each experiment before cultivation, a copy of the pESC-HIS plasmid containing the respective oxidosqualene cyclase (OSC) from *Erysimum cheiranthioides* (Wallflower) was transformed into the ROX1 mutant strain SS1 using the Zymogen Frozen-EZ Yeast Transformation II kit. Dr. Holland from Williams College in Massachusetts identified the candidate wallflowers OSCs using a BLAST search to query the *Erysimum cheiranthioides* genome (https://www.erysimum.org). OSCs from Arabidopsis thaliana were used as queries. Using RNA-seq datasets from Dr. Georg Jander at the Boyce Thompson Institute (unpublished), Dr. Holland confirmed that the

candidate Wallflower OSCs were expressed in planta. The OSCs were expressed under the strong inducible promoter (GAL) in an expression cassette (PGAL-OSC-Tcyc1) and synthesized into the pESC-HIS plasmid. Transformants were grown on YPD-HIS plates for 48 hr at 30C, and positive colonies were selected and plated on YPD-HIS master plates. Master plates were also grown on YPD-HIS plates for 48 hr at 30C.

3.2.2. Wallflower OSC Strain Cultivation, Media, and Metabolite Extraction

The first step in cultivating the SS1 strains with the pESC-HIS plasmid containing an OSC expression cassette (PGAL-OSC-Tcyc1) is growing the master plate colonies in YPRaf-HIS for 24 hr at 30 C to ensure they metabolized all internal glucose. The cells were harvested by centrifugation and induced in YPGal-HIS at OD 0.2-0.3 for 96 hr at 30C. All cultivations were grown under aerobic conditions in 50 mL falcon tubes and stored on an angled tube holder in a shaking incubator at 150rpm.

The cells and media of the resulting culture were separated to have their chemical contents extracted independently. For the extracellular fraction, the media was separated from the cells by centrifugation and then transferred to 8 mL glass tubes. 2 mL of hexane and 10 µL of a 0.05 mg/mL tetracosane standard were added to the tubes. The tubes were vortexed, and then the hexane fraction was removed and evaporated, yielding the media extract that was analyzed in a further step to prepare it for the GC-MS. The intracellular fraction was the cell pellet generated after centrifugation. To break open the cells, glass beads and 200 µL of methanol were added to the cells in an 8 mL glass tube. Then the

tube was vortexed and sonicated to ensure the intracellular components had been released into the methanol. Water, hexane, and 10 µL of a 0.05 mg/mL tetracosane standard were then added to the tubes to separate the hydrophobic and hydrophilic compounds. After being vortexed, the hexane fractions were removed and added to the evaporated chloroform extract. The remaining hexane was evaporated, and what was left was the total extract from the culture. A saponification method is then applied to the sample, removing FAs that can interfere with GC-MS data. The saponification method is conducted as follows: To the glass tubes, 2 mL of ethanol was added and incubated at 90^{o}C for 5 minutes. Then 166 µL of 80% w/v potassium hydroxide was added, vortexed, incubated at 90^{o}C for 5 minutes, vortexed, and incubated again at 90^{o}C for five more minutes. Immediately following the incubation, the tubes were placed on ice, and approximately 1 mL of ice-cold water was added, vortexed, and put back on ice. 1 mL of hexane was added to the tubes, vortexed, and then the hexane fraction was removed and evaporated until 200 µL was left. The remaining 200 µL fraction was transferred to a GC vial insert and completely evaporated. Lastly, 15 µL of a 1:1 BSTFA/pyridine mixture was added to the insert and incubated at 70^{o}C for 60 minutes.

Prepared samples were analyzed on a GC-MS with an autosampler attachment to inject samples. Samples were injected with a split/splitless injector in split mode at 4ul at 300^{o}C. At the time of injection, the oven is at 50^{o}C; following injection, it is held at that temperature for 2 minutes. Then the temperature is ramped at 40 C/min to 200^{o}C and held there for 2 min. The last temperature ramping is done at 3^{o}C/min to 320^{o}C and then held

for 10 min. The total run time of this GC-MS method is 58 minutes. This method separates analytes using an HP-5MS column with a helium mobile phase and a 1 mL/min flow rate. After gas chromatography separation, analytes were detected with a 5977B mass spectrometer. Analytes were ionized with an electron ionization source running in positive mode with an ionization energy of 70 eV. The mass spectrometer is programmed to scan m/z 40 to m/z 800 at two scans per second. Mass spectral data is analyzed manually using the Agilent data analysis software, and figures are generated through R studio software.

3.3. Results

In this section, I present the results of the experiments conducted to test the application of the CRISPR/Cas9 methods and engineered yeast strains. I also offer valuable insights into the applications of these findings and their effect on the pharmaceutical and agricultural industries. This section has been divided into two parts. The first subsection utilizes the engineered yeast strain SS1, described in Chapter 2, to explore cardenolide biosynthesis in Wallflower. The findings about cardenolide biosynthesis preambles discussion about further applications of engineered yeast for cardenolides and the discovery and production of pharmaceutical compounds derived from plants. The subsequent subsection delves into applying the CRISPR/Cas9 genetic engineering methods I developed in Chapter 2 for the heterologous production of important agricultural compounds. The target compounds in this study are hop-derived

monoterpenes, essential flavor and fragrance components in beer. Separating the application of the engineered yeast strains and engineering methods developed in this study facilitates better comprehension. Later in this thesis, it also provides an avenue for investigating the applications in the pharmaceutical and agricultural industries.

3.3.1 Wallflower Cardenolides

Cardenolides are a class of natural products with potent biological activities, including anticancer and antiarrhythmic properties (Wen et al., 2016). They are commonly found in plants of the Apocynaceae family, such as *Erysimum cheiranthioides*, widely known as the Wallflower. The biosynthesis of cardenolides involves a complex network of enzymatic reactions that ultimately leads to the production of structurally diverse compounds. Understanding the biosynthetic pathway of cardenolides is crucial for various reasons. Firstly, it provides insights into the evolutionary origins and diversification of this class of natural products. Secondly, it enables identifying and characterizing key enzymes and genes involved in their biosynthesis, which can be helpful in metabolic engineering and synthetic biology applications. Finally, elucidating the cardenolide biosynthetic pathway contributes to our knowledge of plant secondary metabolism and opens avenues for exploring these compounds' potential medicinal properties.

The Wallflower has long been recognized as a rich source of cardenolides (Perez-Alonso et al., 2016). However, despite their importance, this plant's biosynthetic pathway

responsible for cardenolide production remained largely unknown until recent advancements in molecular biology and metabolomics techniques. Previous studies have hinted at the involvement of several enzyme families, including cytochrome P450 monooxygenases and glycosyltransferases, in cardenolide biosynthesis (Kreis and Muller-Uri, 2012). However, the precise sequence of enzymatic reactions, the specific genes responsible, and the regulatory mechanisms controlling the pathway have yet to be discovered. Unraveling the cardenolide biosynthetic pathway in Wallflower will shed light on these intriguing compounds' biosynthesis and provide a foundation for understanding their ecological roles and potential medical applications. This knowledge paves the way for further investigations into manipulating and optimizing cardenolide production, offering opportunities for developing novel therapeutic agents and studying the biological functions of these compounds in their natural context.

One of the critical enzymes hypothesized to catalyze the initial step in the cardenolide biosynthetic pathway in Wallflower is the 2,3-oxidosqualene cyclase (OSC). OSCs are responsible for cyclizing 2,3-oxidosqualene, a common precursor in sterol and triterpenoid biosynthesis, into various cyclic triterpenoids. Dr. Holland and previous research studies have suggested that OSCs are crucial in initiating the cardenolide pathway by converting oxidosqualene into cycloartenol, a structurally important intermediate. Cycloartenol is a key compound in sterol biosynthesis and has been proposed as a precursor to cardenolide production due to its structural similarity to other known cardenolides. However, the direct involvement of OSCs in the cardenolide

biosynthetic pathway and the formation of cycloartenol as an intermediate is still subject to further investigation. Figure 13 highlights the structural similarity of 2,3-oxidosqualene, cycloartenol, and cardenolides, as well as the hypothesized biosynthetic steps involved in cardenolide synthesis, including an OSC catalyzing the formation of cycloartenol. Understanding the enzymatic activity and specificity of OSCs in the context of cardenolide biosynthesis is essential for unraveling the complete pathway and identifying potential targets for metabolic engineering.

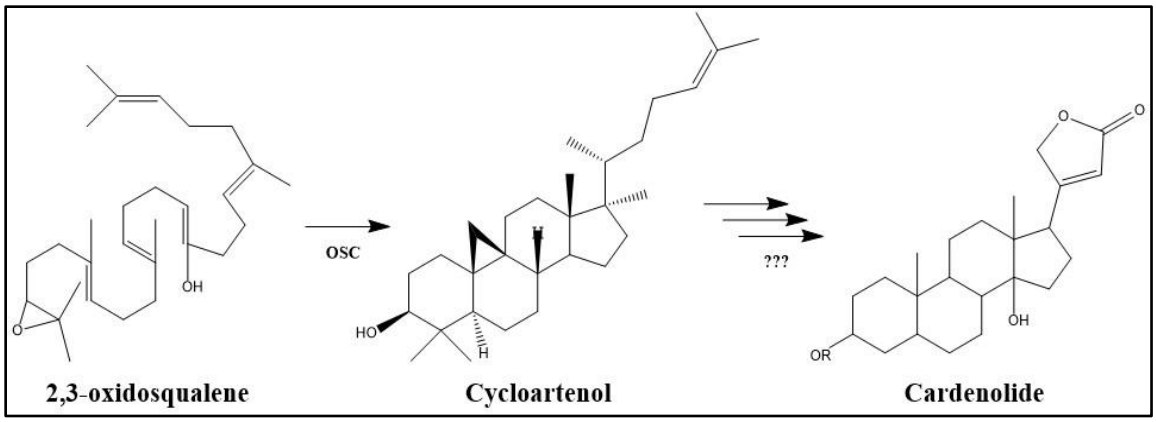


Figure 13. *Summarized biosynthetic steps from 2,3-oxidosqualene to cardenolides. Cycloartenol was identified as the likely result of the first biosynthetic step. The arrows and question marks following cycloartenol represent the unknown genes and the number of steps required to convert cycloartenol into a cardenolide.*

Our study aimed to express and characterize five OSCs identified from Wallflower to investigate the role of OSCs in the cardenolide biosynthetic pathway. These OSCs identified by Dr. Holland are potential candidates for catalyzing the first step in cardenolide production. To facilitate their expression and analysis, I utilized the SS1 *S.*

cerevisiae strain that contains a non-functional version of the ROX1 gene, which regulates genes involved in squalene synthesis. Using the SS1 yeast strain offers several advantages for this study. Firstly, relative to the wild-type yeast strain, SS1 contains elevated levels of squalene, a precursor in the cardenolide biosynthetic pathway, providing a conducive environment for expressing and detecting OSC activity. Secondly, the absence of functional ROX1 allows for exploring OSCs' role in regulating cardenolide production, as ROX1 is known to control genes participating in squalene synthesis. By expressing the identified Wallflower OSCs in this yeast strain, I aim to evaluate their enzymatic activity and assess their ability to catalyze the formation of cycloartenol or other potential intermediates in the cardenolide biosynthesis pathway. This approach was anticipated to gain insights into the enzymatic function and specificity of the Wallflower OSCs, elucidating their involvement in the initial steps of cardenolide biosynthesis. This research contributes to a deeper understanding of the cardenolide pathway and provides a foundation for future studies aiming to engineer microbial hosts for producing valuable cardenolide compounds.

To produce the Wallflower OSCs in the SS1 strain, I first had to transform the 5 OSCs, each on their own pESC-HIS vector, into the SS1 strain. These vectors had the same structure as the GgBAs vector described in Section 2.3.2. The Wallflower OSC-containing yeast strains' transformation, cultivation, and GC-MS analysis were completed using the methods described in Section 3.2. Figure 14 shows the chromatograms from GC-MS analysis of the 5 OSC expression cultures. In this figure, the 5 OSC names are

shown, which are OSC1, OSC2, OSC3, OSC4, and OSC5. Not shown in Figure 14 is that we compared the chromatographic data to a sample of an SS1 culture containing pESC-HIS with no expression cassette. The control sample allowed us to overlay the total chromatograms of the control and each of the individually expressed OSC samples to identify differences. Through doing this, I identified that OSC1 makes a new product, likely some derivative of squalene. The spectrum is similar to squalene's mass spectrum but differs in some regard. OSC2 appeared to make beta-amyrin, which we could confirm through its mass spectrum. OSC3 and OSC4 did not appear to make any new products, at least not those with a high enough abundance to detect. OSC5 made multiple molecules, two unknown and one identified as cycloartenol. These molecules were characterized by comparing their mass spectrum in Figure 15 to a mass spectrum database. The two unknown compounds had yet to be recorded within this database or had their mass spectrums identified. The molecule identified as cycloartenol in the Ec5g034600 cultures had been previously hypothesized to be the precursor for the cardenolide biosynthesis pathway, which prompted us to conduct further investigation into the function of Ec5g034600.

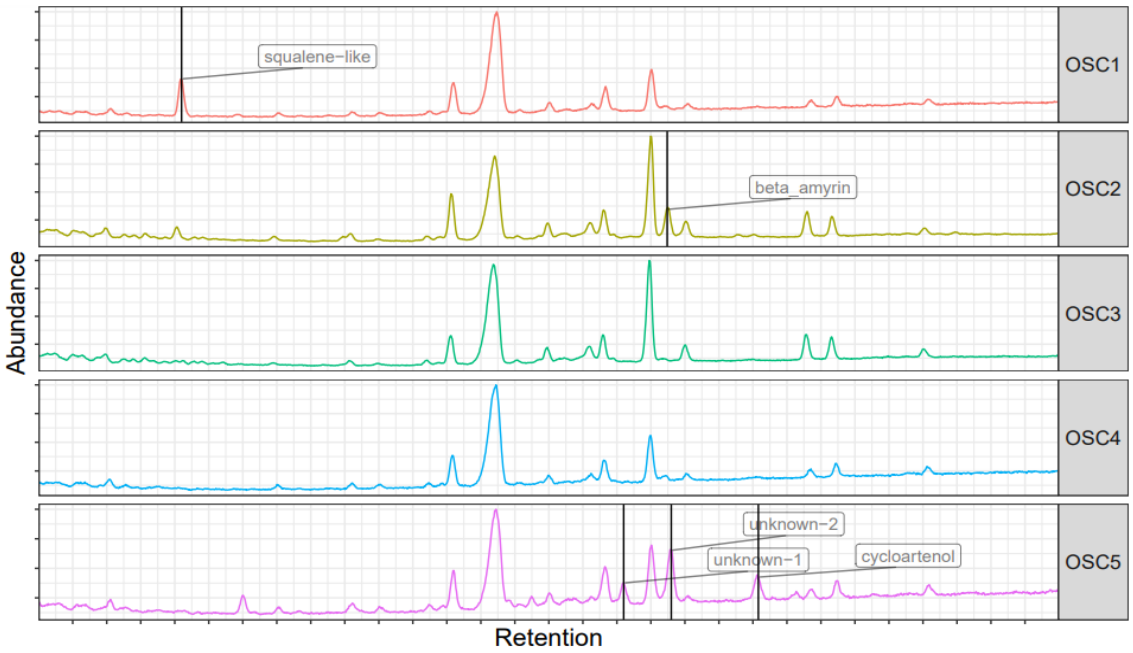


Figure 14. *Chromatograms of the 5 Wallflower OSCs expressed independently in yeast strain SS1. All non-labeled peaks were present in a control culture harboring the SS1 strain containing an empty pESC-*HIS *vector.*

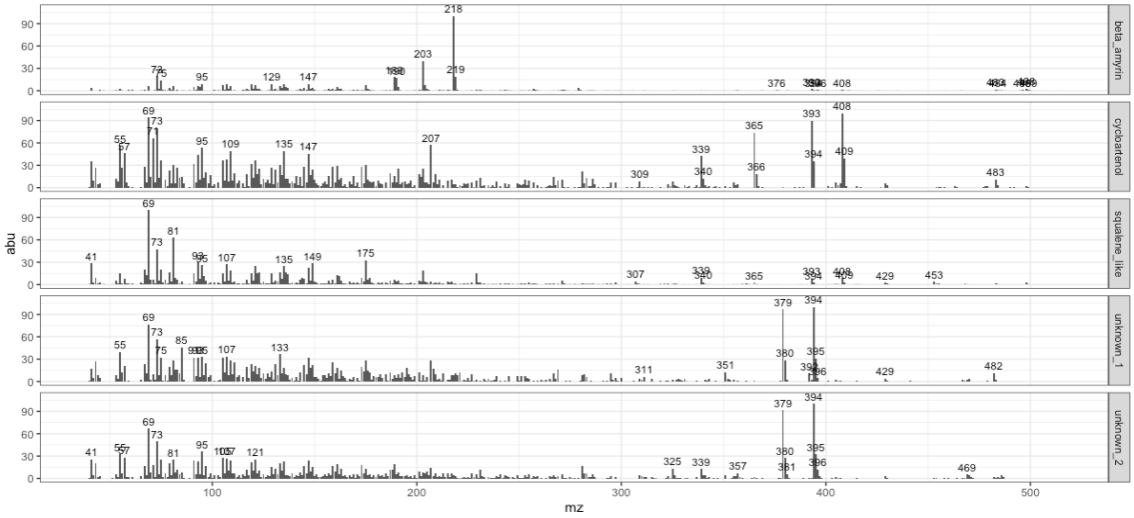


Figure 15. *The mass spectra of the five compounds identified in Figure 13.*

Further research into OSC5 started with reaffirming the presence of cycloartenol in the SS1 strain expressing the OSC. To do this, I compared the expression of OSC5 in SS1 to the expression of a known cycloartenol synthase from sorghum (Sobic. 137200), identified by Busta and Colleagues (Busta et al., 2021). This cycloartenol synthase was identified by. In the growth of these cultures before GC-MS analysis, it was determined that growing them in shake flasks could produce more of the target molecule. Increased analyte concentrations would allow for more reliable and defined GC-MS results. Additionally, these cultures were performed in triplicates, and product concentrations were determined by adding an internal standard in the GC-MS sample preparation. Figure 16 shows the GC-MS chromatograms of OSC5, Sobic.137200, and a control culture of SS1 harboring the empty pESC-HIS vector. In this experiment, I identified five definitive peaks in OSC5 and only 4 in Sobic. 137200. This analysis showed that there were two additional peaks that the shake flask cultures produced in high enough abundance that the previous 8 mL cultures did not. Peaks two and three are isomers of the same compound, four is cycloartenol, and 1 is another sterol-like compound. as previously described. Peak 5 is unique to the wallflower OSC but was quite difficult to analyze as it was in a relatively low abundance to extract detailed mass spectra. The characterization of these compounds was made through a comparison of their mass spectra to a mass spectra database. These findings were communicated to our collaborator Dr. Holland at Williams College in Massachusetts, and it was concluded that OSC5 was likely responsible for the first catalytic step in the biosynthetic pathway to cardenolides in Wallflower. Additionally, this means that cycloartenol must be modified further in the next steps of

the biosynthesis of cardenolides. Further research investigation will explore the next steps of cardenolide biosynthesis using the methods developed in this study.

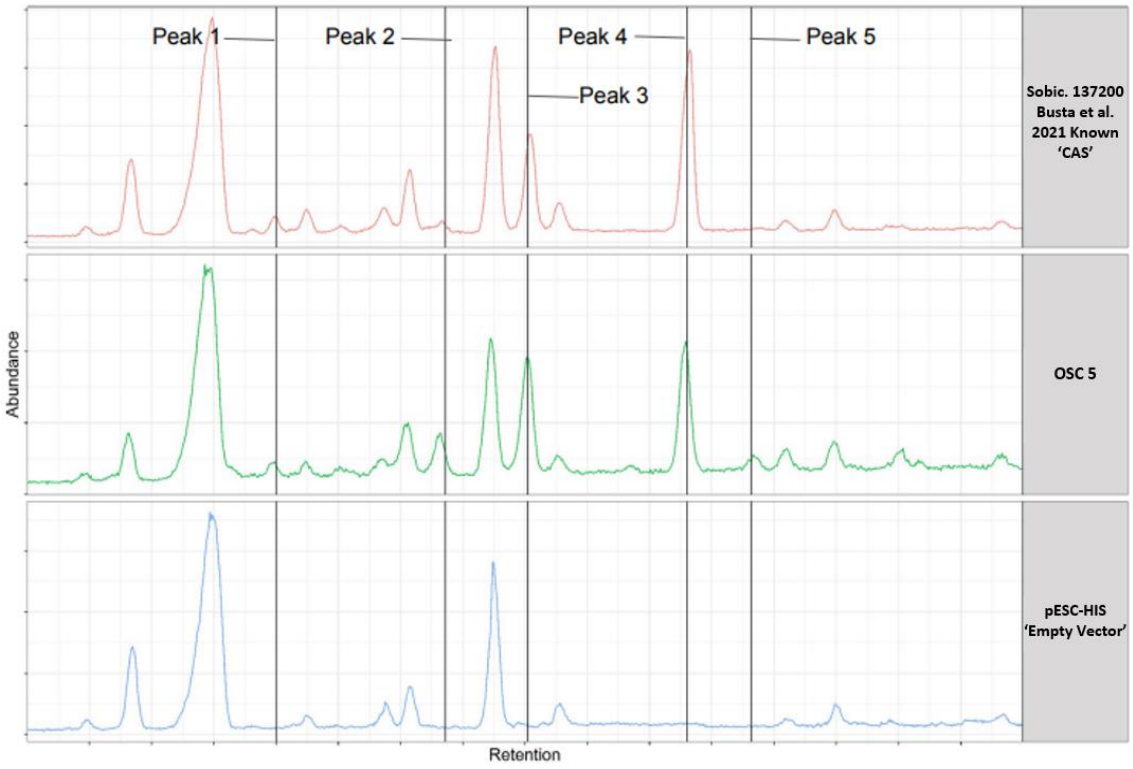


Figure 16. *GC-MS analysis product comparison of wallflower OSC 5 to a known cycloartenol synthase from sorghum (Sobic. 137200) and an empty pESC-HIS vector. All non-labeled peaks were present in the control culture harboring the SS1 strain containing an empty pESC-*HIS *vector.*

In conclusion, the investigation of oxidosqualene cyclases (OSCs) in the cardenolide biosynthetic pathway of Wallflower holds significant promise for unraveling the enzymatic steps involved in cardenolide production. While previous studies have hypothesized the involvement of OSCs in catalyzing the initial step and the formation of cycloartenol as an intermediate, further research is needed to validate these hypotheses.

By utilizing a ROX1 knockout yeast strain to express and study the identified Wallflower OSCs, I aimed to shed light on their enzymatic activity and role in cardenolide biosynthesis. This research contributes to our understanding of the intricate pathways involved in plant natural product biosynthesis. It also paves the way for future efforts in metabolic engineering and producing valuable cardenolide compounds. Ultimately, a comprehensive understanding of the cardenolide biosynthetic pathway will facilitate the development of sustainable strategies for accessing these bioactive molecules and their derivatives with potential applications in the pharmaceutical industry.

3.3.2 Beer Yeast and Hops Monoterpenes

Yeast has emerged as a versatile microbial platform for producing flavor and fragrance molecules. Its ability to efficiently convert sugars into desired compounds, combined with the advancements in genetic engineering tools such as CRISPR/Cas9, has unlocked new possibilities for harnessing yeast as a manufacturing system (Rainha et al., 2020). By introducing specific genes into the yeast genome, metabolic pathways can be directed toward synthesizing desired flavor and fragrance molecules. This approach has produced many molecules, including monoterpenes, sesquiterpenes, esters, and alcohols. These molecules contribute to the complex aromas and tastes of foods, beverages, and personal care products. Using yeast as a production platform offers advantages such as scalability, cost-effectiveness, and sustainability compared to traditional extraction methods from natural sources (Carsanba et al., 2021). Furthermore, the ability to fine-

tune the yeast's genetic makeup allows for the tailored production of specific compounds, creating unique and novel flavor and fragrance profiles.

Engineering yeast for desired flavor and fragrance molecules offers an exceptional opportunity to revolutionize the production of yeast-fermented beverages, with a particular focus on beer. Traditionally, beer's unique taste and aroma are achieved by adding plant-derived ingredients like hops. However, by harnessing the power of yeast engineering, the reliance on external sources is bypassed and instead creates yeast strains capable of producing the same flavor and fragrance compounds. Additionally, hops are expensive and contribute significantly to beer production's environmental footprint (Bahl et al., 2020). This research aims to develop genetically engineered brewer's yeast capable of producing the same flavor chemicals in hops. By eliminating the need for hops in the brewing process, this innovation could decrease production costs, reduce the environmental impact of beer-making, and offer brewers access to a broader range of flavor profiles that are not easily achievable with hops alone. To achieve these research aims, our research group has collaborated with Castle Danger Brewery, one of Minnesota's largest breweries. With access to their beer yeast strain and the ability to grow it in our lab, the hop chemical synthesis genes can be integrated into the yeast's genome, and production of the desired flavor chemicals can be evaluated. The successful implementation of this project will not only have significant implications for the brewing industry, particularly large-scale breweries, and align to foster industry-university connections as part of MPact2025, the University of Minnesota’s commitment to

research, teaching, and service. Furthermore, the potential applications of this research extend beyond beer production, encompassing other fermented beverages and foods, thereby expanding its market reach and impact.

My contribution to this project was to create a roadmap for going forward using experimental design and method development. Others will follow this roadmap to generate the transgenic yeast strain described. The first step in the experimental design was identifying what compounds in hops are responsible for their flavor profile in a beer. Previous studies identified three key chemicals—linalool, geraniol, and myrcene—primarily responsible for beer's distinct hop flavor (Figure 17; Lafontaine et al., 2019). These chemicals are produced through the activity of a linalool synthase from *Mentha citrata, a* geraniol synthase from *Ocimum basilicum*, and a myrcene synthase from *Antirrhinum majus.* These genes will have their promoters and terminators optimized and inserted into the beer yeast genome obtained from the local Castle Danger Brewery.

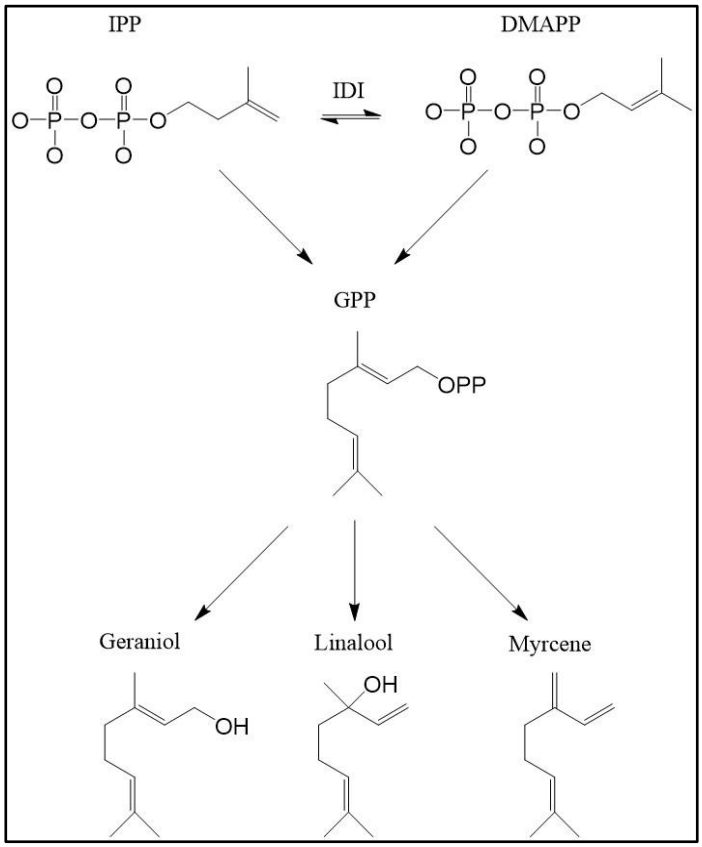


Figure 17. *Summarized biosynthetic pathway to the hop terpenes geraniol, linalool, and myrcene. IPP and DMAPP are produced through the MVA (mevalonate) and MEP (methylerythritol phosphate) pathways and converted to GPP before further downstream synthesis into terpenes.*

The method development portion of my contribution included leveraging my CRISPR/Cas9 engineering techniques discussed in Section 2.1.1. These techniques were further optimized for this project and will be utilized to insert genes into the beer yeast. To engineer the beer yeast strain, I first had to sequence its genome to design primers and insertion fragments accurately. The sequencing of the beer yeast was accomplished by extracting the genomic DNA, bead cleaning the gDNA, and using next-generation Illumina sequencing through the University of Minnesota Genomics Center. Once the genomic sequence of the yeast strain was received, I could design the monoterpene insertion fragments and sgRNA primers needed to engineer the beer yeast. Additionally,

since the beer yeast strain isn’t a pre-engineered lab strain compatible with auxotrophic markers, a method of screening yeast transformations needed to be developed. In this case, I decided that inserting an antibiotic-resistant marker on the pCAS9i plasmid would allow for the accurate screening of transformations. The antibiotic resistance marker chosen was hygromycin, which is cytotoxic to most eukaryotic cells, including plant, mammalian, and fungi (Kaster et al., 1984). The hygromycin resistance marker will be inserted into the pCAS9i plasmid using a restriction digest and Gibson Assembly. The resulting plasmid will be screened using gel electrophoresis, and the hygromycin resistance will be tested by transforming the plasmid into the beer yeast strain and plating it on YPD plates with 200 µg/mL hygromycin. The previous paragraphs describe this project's current stage, and the following sections include my additional experimental design for the rest of the project.

Once hygromycin resistance is confirmed, the sgRNA primers are designed to conduct the ADE2 knockout. Knocking out the ADE2 gene in yeast results in a distinct phenotype characterized by losing the ability to produce a pigment called adenine. The mutant yeast cells with a non-functional ADE2 gene exhibit a red color due to the accumulation of a red intermediate compound. In contrast, the wild-type yeast cells do not accumulate this intermediate and therefore appear white or pale. This phenotype serves as a useful selectable marker in genetic experiments. By observing the color of yeast colonies or individual cells, researchers can quickly identify and select strains that have undergone successful knockout of the ADE2 gene. Moreover, the ADE2 mutant

strain provides a genetic background that can be further engineered or manipulated for various purposes, such as the insertion of desired genes or metabolic pathways, as in the case of monoterpene insertion described previously. Knocking out the ADE2 gene in yeast can be achieved using the CRISPR/Cas9 gene editing methods previously described in Section 2.1.1. The first step is to design a small guide RNA (sgRNA) that explicitly targets the ADE2 gene using the sequenced genome of the beer yeast strain. This sgRNA will guide the Cas9 enzyme to the ADE2 sequence location in the yeast genome. The Cas9 enzyme then introduces double-strand breaks at the targeted site of the ADE2 gene, leading to gene disruption or knockout. After generating the ADE2 knockout strain, and selecting colonies exhibiting the accumulation of red pigment, the cells will be cured to remove the pCAS9i plasmid containing the ADE2 gRNA.

The next step will be to insert a monoterpene insertion fragment into the ADE2 site of the knockout strain. This insertion fragment was designed to contain all three monoterpene genes and regulatory elements, including promoters and terminators. Additionally, it includes the wild-type ADE2 gene that will renew the ADE2 function (Figure 18). sgRNA primers were designed for inserting the fragment into a different location within the ADE2 gDNA coding sequence. Once the sgRNA primers are transformed into the pCAS9i plasmid, the cells will be screened on YPD plates containing hygromycin. Positive transformants will be master plated and then grown in raffinose media to grow the required cell mass for transformation. Once achieved, the cells are induced in galactose for two hours before transformation with the Donor DNA

fragment containing the three monoterpene and ADE2 gene. Transformants will be screened on YPD plates containing hygromycin, and those with a reinstated white phenotype will be selected for further testing. The white phenotype indicates that the ADE2 gene on the monoterpene insertion fragment restored the function of the ADE2 gene in the yeast strain. The ADE2 functionality allows for a simple colorimetric screening of the yeast colonies, which can be further analyzed with Sanger sequencing. The engineered yeast would likely now produce the desired monoterpene compounds while maintaining the essential function provided by ADE2.

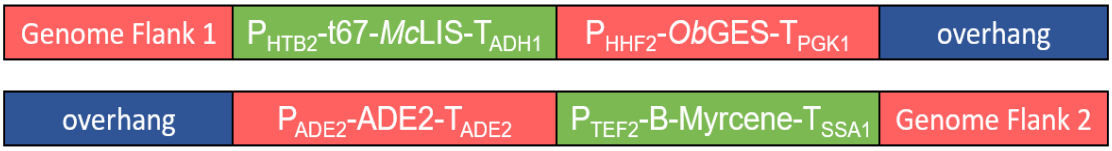


Figure 18. *Insertion Fragments designed for the overexpression of the monoterpene genes. Promoters and terminators were chosen based on their relative effect on monoterpene production, as described in Denby 2018. Genome flanks are designed based on the ADE2 cut site within the beer yeast genome. The overhangs are complementary to one another for applying Gibson assembly in creating a single linear piece of donor DNA.*

To test for the production of monoterpenes, multiple colonies that colorimetrically show insertion events will first be cured of the pCAS9i plasmid containing the ADE2 sgRNA. These colonies will then be put under a simulated brewing fermentation method described in Denby 2018. A GC-MS analysis will be conducted on the beer yeast cultures

to test for the presence of linalool, geraniol, and myrcene. This process involves extracting the monoterpenes from the cultures using minimal amounts of ethyl acetate and adding an internal standard. The organic extract will be used directly for GC-MS analysis. Further sample preparation, such as saponification, cannot be performed due to the volatile nature of the monoterpene compounds.

In summary, the experimental design and methods developed allow for further investigation into this research project. Over time a genetically engineered brewer's yeast capable of producing the same flavor chemicals found in hops will be developed. By accomplishing the outlined re search, we can potentially decrease production costs, reduce the environmental impact of beer-making, and offer a creative and sustainable future for the brewing industry. The following discussion section highlights the importance of the previously described research and the potential for innovation in both the pharmaceutical and agricultural industries.

3.4 Discussion

Yeast-based production systems are invaluable for pharmaceutical research and producing agriculturally derived chemicals. The yeast-based system engineered in this study allows for identifying and characterizing the activity of known and unknown plant enzymes, which provides valuable insights for engineering pharmaceutical pathways derived from plants. Furthermore, the yeast strain employed in this study holds great potential for further testing and identifying phytochemicals that share the same precursor

pathway and exhibit similar storage mechanisms as triterpenoids. Additionally, the utilization of yeast engineering methods developed serves as a platform for producing agriculturally derived chemicals, such as hop monoterpenes. This chemical production strategy offers several advantages over traditional approaches, including environmental benefits, scalability, and the ability to produce complex plant natural products. The following discussions highlight (i) the benefit of the developed yeast-based production systems in comparison to traditional production methods, (ii) further efforts to develop these yeast-based production systems, and (iii) their potential contributions to the fields of pharmaceuticals and agriculture.

3.4.1 Pharmaceutical Applications

Investigating oxidosqualene cyclases (OSCs) in the cardenolide biosynthetic pathway of Wallflower using a ROX1 knockout yeast strain illustrates the potential of yeast-based production systems for pharmaceutical production. This approach offers advantages over traditional methods, providing precise control over metabolic pathways, scalability, and enhanced production efficiency (Bapat et al., 2022). Additionally, these findings highlight the ability of yeast to perform as a model platform for elucidating biosynthetic pathways of plant-derived pharmaceuticals. Integrating genetic and metabolic engineering in yeast-based systems holds significant potential for developing innovative and sustainable drug discovery and production approaches. Going forward, the Busta research lab intends to apply advanced genetic engineering strategies, such as

CRISPR/Cas9 and vector-based gene expression, to our yeast-based systems to further dissect the metabolic pathway of cardenolide compounds in Wallflower. Building on our success with the ROX1 knockout strain SS1, we have already identified that OSC5 likely plays a pivotal role in the first step of cardenolide biosynthesis in Wallflower. We envision employing a combinatorial approach of CRISPR-engineered yeast strains and vector-based gene expression for overexpressing desired enzymes. This strategy provides a highly controlled environment for dissecting and understanding the complex biosynthetic pathway of cardenolides.

The next step is to focus on the formation of the lactone ring present in the cardenolide structure. By leveraging the power of our engineered yeast strains and vector-based gene expression, we aim to investigate the activity of 14 candidate wallflower cytochrome P450s in conjunction with Wallflower OSC5. Dr. Cynthia Holland has identified these enzymes through analysis of the Wallflower genome, and they will be subjected to integration into a vector-based expression model. Each P450 will be selectively expressed in our engineered yeast, and their effect on potential lactone ring formation will be carefully studied. This research will not only shed additional light on the cardenolide biosynthesis pathway but can reaffirm our previous results that identified cycloartenol as the candidate precursor. This knowledge is crucial for understanding the biosynthetic pathway and facilitating the production of cardenolides within microbial systems. Application of the CRISPR-engineered strains and vector-based gene expression can aid in further investigations into the cardenolide pathway and

most plant-based pharmaceuticals with unidentified biosynthetic pathways. Furthermore, identifying these enzymes can contribute to the broader field of metabolic engineering, enabling the development of strategies for plant natural product production in the pharmaceutical industry.

3.4.2 Agricultural Applications

Traditional agricultural production methods for plant natural products typically involve cultivating specific plants, such as hops, for use directly in industrial purposes or by extracting the desired compounds from their tissues. The cultivation of hop is subject to various environmental factors, including climate conditions and seasonal variations, which can impact the yield and consistency of the desired flavor and fragrance compounds (Romero-Suarez et al., 2022). In comparison, yeast-based production systems offer several advantages over traditional agricultural methods. Yeast-based production provides greater control over the production process, optimizing the target compounds' yields, purity, and consistency.

Our yeast-based production system for producing hop monoterpenes directly in the brewing process aligns with the themes discussed in the previous sections. Firstly, it presents an alternative approach to traditional agricultural production methods for obtaining hop flavor compounds. Instead of relying on the cultivation of hops or extracting their chemical components, genetically engineered brewer's yeast can be utilized to produce the desired monoterpenes. This alternative technique circumvents the

need for extensive land areas dedicated to hop cultivation, reduces water usage, and eliminates pesticide applications typically associated with traditional hop farming (Diwan and Gupta, 2020). In terms of comparison, our yeast-based production system offers distinct advantages over conventional methods. By integrating the production of hop monoterpenes directly into the brewing process, a streamlined production workflow can be developed, saving time and costs associated with the sourcing, processing, and transportation of hops (Bahl et al., 2020). Moreover, the economic impact of our yeast-based system on the traditional hop farming industry needs to be considered. While our approach could disrupt traditional agricultural sectors reliant on hop production, it also opens up new opportunities for sustainability, diversification, and value-added products. Farmers and agricultural stakeholders may explore alternative crops or embrace the production of other high-value plant products (McLaughlin, 1985). Collaborations and partnerships between the brewing industry and traditional hop farmers could also be established to explore new business models that benefit both parties.

Although the development of our hops monoterpene-producing brewer's yeast strain is still in its infancy, preliminary research and industry insights provide a well-laid foundation for its success. After the development of the hop monoterpene yeast strain, there is room for further enhancements, such as refining precursor engineering techniques and integrating additional sought-after flavor compounds into the yeast's genome. Our yeast-based production methods allow for this extension beyond hop monoterpenes and hold great potential for producing other sought-after flavor compounds by brewers. By

leveraging the previously developed CRISPR/Cas9 engineering methods, brewer's yeast can be manipulated to synthesize various flavor molecules contributing to different beer styles' unique taste and aroma profiles. This opportunity opens up exciting possibilities for brewers to explore new flavors, experiment with novel combinations, and create distinctive brews that push the boundaries of traditional beer production. With our yeast engineering platform, brewers can access a diverse array of flavor compounds typically found in various plant sources. For instance, specific enzymes or pathways involved in the biosynthesis of compounds such as esters, phenols, terpenes, or other flavor-active molecules can be targeted (Kutyna and Borneman, 2018). One example is the heterologous production of raspberry ketones in a wine yeast strain that can produce raspberry chardonnay (Lee et al., 2016). Introducing these genetic modifications into the yeast enables the production of these compounds directly during fermentation, eliminating the need for external additives or costly extraction procedures. Engineering yeast also offers a versatile and sustainable solution that empowers brewers to explore new flavors, innovate in beer production, and craft unique sensory experiences for beer enthusiasts. This technology has the potential to expand the brewing industry by providing a more efficient, scalable, and customizable approach to flavor production, opening up a world of possibilities for brewers to elevate their craft.

Although yeast-based natural product production offers exciting opportunities, it also has technical challenges that must be addressed for successful scaling up. One major challenge is the complexity of genetic engineering required to introduce and optimize the

biosynthetic pathways in yeast. Simultaneously manipulating multiple genes and regulatory elements can be demanding and time-consuming, but it has been made more achievable using systems like CRISPR/Cas9. Additionally, achieving high yields of the desired natural products can be challenging due to metabolic limitations and competition for cellular resources (Kastberg et al., 2022). Another limitation of yeast-based production is the potential toxicity of the desired natural products, which can reduce the growth and productivity of heterologous systems (Gottardi et al., 2017). Balancing the expression of biosynthetic enzymes and cellular tolerance to these compounds becomes crucial for achieving optimal yields.

Looking ahead, continuous advancements in genetic engineering tools, high-throughput screening methods, and process optimization techniques offer promising avenues for overcoming the current limitations of yeast-based production. Future improvements involve the development of more efficient and robust yeast strains, the discovery of novel biosynthetic enzymes, and the integration of advanced bioprocess technologies. In conclusion, yeast-based production of natural products presents both opportunities and challenges. While technical challenges such as complex genetic engineering, low yields, and downstream processing limitations exist, ongoing research and development efforts are addressing these issues. With the application of metabolic engineering, synthetic biology, and process optimization strategies, yeast-based production technology has the potential to significantly improve, paving the way for sustainable and scalable production of valuable natural products in the future.

3.5 Conclusion

Chapter 3 has presented a broad exploration of the application of our developed CRISPR/Cas9 methods and engineered yeast strains to produce plant pharmaceutical compounds and agricultural compounds. Throughout this chapter, I have discussed this research's advantages, challenges, and potential impacts. By leveraging the power of genetic engineering and yeast as a host organism, I have demonstrated the potential to revolutionize the production of plant natural products and offer sustainable alternatives to traditional agricultural methods. One of the key achievements highlighted in this chapter is the successful utilization of our engineered yeast strains to explore and characterize the enzymes involved in the biosynthesis of plant pharmaceutical compounds. By engineering a yeast strain that produces high amounts of MVA pathway derivatives, valuable insights into the enzymatic steps involved in the biosynthesis of cardenolides in *Erysimum cheiranthioides* (Wallflower) have been gained. This approach has deepened our understanding of intricate biosynthetic pathways and opened new avenues for further research and development into plant-derived pharmaceuticals. Moreover, the success of our CRISPR/Cas9 engineering methods has enabled us to investigate the production of agricultural compounds, such as hops, to reduce the need for traditional agricultural production.

In addition to the scientific advancements achieved in this chapter, I have also addressed this research's environmental and economic impacts. Many industries can

significantly reduce their environmental footprint associated with traditional agricultural practices by employing yeast-based production systems. These systems require less land, water, and pesticide usage, minimizing the negative impact on ecosystems and promoting sustainability. Furthermore, the economic implications of our research are substantial. By offering more efficient and controlled production methods, yeast-based systems can disrupt traditional agricultural sectors, leading to increased productivity, reduced costs, and improved market competitiveness.

The potential applications of our developed CRISPR/Cas9 methods and engineered yeast strains extend beyond the scope of this chapter. Our research has laid the foundation for further investigations into the production of various plant pharmaceutical and agricultural compounds. By refining and expanding our genetic engineering techniques, the full potential of yeast as a versatile production platform can be unlocked. Additionally, our engineered yeast strains can be used to screen plant enzymes, elucidating previously uncharacterized biosynthetic pathways in plants. Furthermore, the insights gained from this research can contribute to developing sustainable strategies for accessing valuable compounds, thereby benefiting the pharmaceutical industry, agricultural sector, and society.

In conclusion, Chapter 3 has demonstrated the significant advancements and potential of our CRISPR/Cas9 methods and engineered yeast strains for producing plant pharmaceutical compounds and agricultural compounds. By combining cutting-edge

genetic engineering techniques with the scalability and versatility of yeast, I have set the stage for future research and innovation that builds off this study. This research's environmental and economic impacts cannot be understated, as yeast-based production systems offer sustainable and efficient alternatives to traditional agricultural practices. With continued dedication and exploration, the power of yeast can be harnessed to drive positive change in the production of valuable natural products and contribute to a more sustainable future.

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APPENDIX

Supplemental Table 1. Strain comparison data. A correction factor of 6.4:1 was applied to the amyrin titers based on the ionization efficiency of a structurally similar triterpenoid betulin and internal standard octadecanol, respectively. PA (Peak Area), OD (optical density at 600 nm).

Sample	Replicate	PA B-amyrin	PA octadecanol	Corr. Fac PA B-amyrin	ug octadecanol	ug/L amyrin	OD
BY4743 + GgBAs	1	25134719	187653622	160862201.6	0.5	107.2	1.299
BY4743 + GgBAs	2	22914836	162611742	146654950.4	0.5	112.7	1.3
BY4743 + GgBAs	3	11733218	180126949	75092595.2	0.5	52.11	1.314
SS1 (ROX1)+ GgBAs	1	33788789	197871254	216248249.6	0.5	136.6	1.314
SS1 (ROX1)+ GgBAs	2	26590121	168818683	170176774.4	0.5	126.0	1.308
SS1 (ROX1)+ GgBAs	3	27152550	205388244	173776320	0.5	105.8	1.292
SS3 (PAH1)+ GgBAs	1	22669818	189799240	145086835.2	0.5	95.55	1.332
SS3 (PAH1)+ GgBAs	2	38725194	224921394	247841241.6	0.5	137.7	1.277
SS3 (PAH1)+ GgBAs	3	28121818	173224284	179979635.2	0.5	129.9	1.299
SS2 (DGK1)+ GgBAs	1	37490955	139868797	239942112	0.5	214.4	1.271
SS2 (DGK1)+ GgBAs	2	50579304	191639888	323707545.6	0.5	211.1	1.29
SS2 (DGK1)+ GgBAs	3	45980521	177434246	294275334.4	0.5	207.3	1.266

Supplemental Table 2. Cultivation comparison data. A correction factor of 6.4:1 was applied to the amyrin titers based on the ionization efficiency of a structurally similar triterpenoid betulin and internal standard octadecanol, respectively. PA (Peak Area), OD (optical density at 600 nm).

Sample	Replicate	PA B-amyrin	Corr Fact. PA amyrin	PA octadecanol	ug octadecanol	ug/L amyrin	OD
ETOH	1	18372953	117586899.2	156456395	0.5	93.95	1.24
ETOH	2	12547320	80302848	143130904	0.5	70.13	1.208
ETOH	3	20695648	132452147.2	164548703	0.5	100.6	1.245
N limitation	1	14996011	95974470.4	147618266	0.5	81.27	1.375
N limitation	2	11045910	70693824	149044380	0.5	59.29	1.325
N limitation	3	19453968	124505395.2	151317908	0.5	102.9	1.419
N limitation+ ETOH	1	18738427	119925932.8	139435409	0.5	107.5	1.426
N limitation+ ETOH	2	16128186	103220390.4	140688544	0.5	91.71	1.508
N limitation+ ETOH	3	5932282	37966604.8	101151274	0.5	46.92	1.525
SS1 Control	1	29546079	189094905.6	141967950	0.5	166.5	1.315
SS1 Control	2	20603810	131864384	152367729	0.5	108.2	1.233
SS1 Control	3	12943171	82836294.4	154877218	0.5	66.86	1.288