ENGINEERING *SACCHAROMYCES CEREVISIAE* AS A PLATFORM FOR DIVERSE COUMARIN BIOSYNTHESIS

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by
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ABSTRACT

Plant natural products are secondary metabolites synthesized in the plant with great pharmaceutical, nutritional, or agricultural uses. Specifically, coumarins are a valuable family of phenylpropanoids that are essential and widely distributed in plants. They have exhibited a vast array of pharmaceutical values, such as antioxidant, anti-cancer, anti-inflammatory, anti-depressant. In addition, coumarins have been commonly used in cosmetics, perfumes, beverages, food and tobaccos industries. Despite the extensive applications of coumarins and their derivatives, current research of them is very limited, which hinders the synthesis of coumarins. Here, we report the reconstruction of a coumarin biosynthetic pathway in *Saccharomyces cerevisiae* to enable the synthesis of diverse coumarins. Feeding caffeic acid to our engineered *S. cerevisiae* strain yielded a representative coumarin, scopoletin. This study paves the way to the biomanufacturing of more diverse coumarin compounds in engineered microbes.

BIOGRAPHICAL SKETCH

Yubo Xu was born in Zhejiang, China. Two-year high school experience at an international school in China and the fascination of American culture have encouraged her to go to American alone at the age of 17. She went to Whittier Christian High School in California and ended up graduating with the highest honor. Influenced by the medical background of her family, she chose to study biochemistry at the University of Washington, where she has accumulated solid fundamental knowledge and lab skills. From all the coursework and laboratory experience at the University of Washington, she found her true interest was not only in the medical field but more specifically, in the research part. After graduating from the University of Washington in 2018, she decided to pursue a Master of Science in Chemical Engineering at Cornell University. Influenced by her grandfather, a traditional herbalist in China, she chose to join Prof. Sijin Li's group to biosynthesize plant natural products in *S. cerevisiae.*

To mom, dad, Yikai, and 11

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I acknowledge and appreciate all the support from all my friends at Cornell University. Particularly, I am thankful to Weiyao Wang and Dr. Yinan Wu for providing help for my research, and Franklin, Chang, Jianing, and all other Li group members for their support and encouragement.

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Chapter 1. Introduction

1.1 Plant Natural Products Overview

1.1.1 History of Plant Natural Products Utilization

Human beings have relied on nature to satisfy their essential needs for millennia. From ancient times, humans have grasped the significance of natural products, especially for treating a wide range of human ailments. Particularly, plant sources have formed the foundation of the earliest traditional medicinal system, and the earliest history of plant natural products used for disease prevention and treatment could be traced back to 2600 BCE in Mesopotamia. Among more than 1000 recorded plant-derived products in Mesopotamia, oils of the *Cedrus sp.* (cedar), *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (licorice), *Commiphora sp.* (myrrh), and *Papaver somniferum* (poppy juice) are still used today for treating diseases like parasitic infections, inflammation, colds and coughs. [1] In Chinese history, the first documented medical text was *Recipes of Fifty-two Ailments,* which consisted of fifty-two prescriptions, and it was dated from approximately 1100 BCE. Other famous ancient medical texts included the *Shennong Herbal* from about 100 BCE, which documented 365 drugs, and the *Tang Herbal* from around 659 CE recording 850 drugs. [2] Long history of herbal use around the globe has revealed the significance of plant natural products in human life.

1.1.2 Pharmaceutical Applications of Plant Natural Products

Plant natural products have been continuously playing a vital role in the human medical field and contributed a lot to modern drug developments. More than 22% of all new molecular entities approved before 1950 were plant natural products. [3] In 1985, the World Health Organization reported that about 65% of the world's population depended on plant-derived medicines for their primary health care. [2] Also, 25% of drugs in the United States are plant-derived medicines. [4] According to the evaluation of the history of all FDA-approved natural products derived new molecular entities by the Yale Center for Molecular Discovery, many of them had been widely used throughout history, which was much earlier before they got officially approved. [3] In 1804, morphine, the active component in opium, was first isolated by Friedrich Serturner. This means people in the 19th century had been aware of the analgesic properties of opium. [5] Likewise, guaifenesin, a plant derivative from guaiacum resin, was approved officially in 1952 as a cough suppressant; however, it was popular in the 16th century for the treatment of syphilis. [6] Moreover, people in the 16th century had seized the medicinal properties of *Digitalis lanata*, a foxglove plant, but the digoxin, the active component in *Digitalis lanata* was first isolated in the 1930s. [7] People have gained lots of inspirations and knowledge from history. Remarkably, one of the most severe life-threatening diseases, malaria, was found to be cured by artemisinin, a plant natural product. In the 1960s, chloroquine was the primary drug used to treat malaria; however, disease rebounded and evolved with the resistance of chloroquine. To find a new effective antimalarial drug was an extremely urgent task for all human beings at that time. Youyou Tu, a Chinese pharmaceutical chemist, discovered that the extract from *A. annua L.* showing promising antimalarial

activity after reviewing lots of traditional Chinese medicine records and literature. Inspired by one of the most famous Chinese ancient texts, "*Handbook of Prescriptions for Emergencies*", she managed to extract the compounds at lower temperatures to ensure the stability of the active ingredients. In 2015, Youyou Tu was awarded the Nobel Prizes in Physiology or Medicines for the discovery of artemisinin, a novel antimalarial drug. [8]

Nature has always been generous for providing humans with live-saving therapies. After centuries, people are still getting benefits from plant natural products. Their capability to interact with more than one biological target enables them to have more than one pharmaceutical effects. Therefore, they could be used to treat diseases effectively through multiple putative physio-pathologic mechanisms. [9] There are still lots of remaining for humans to exploit plant natural products discovery based on this property.

1.1.3 Other Applications of Plant Natural Products

Despite the pharmaceutical applications of plant natural products, they also have contributed a lot in cosmetics, perfume, food, beverages, and other important industries. *C. arabica* originated from Ethiopia, and its fruits were initially used as food by nomads. Around 1000, people in Arabia used roasted beans to make a drink, "qahwah". After AD 1600, it was brought to Europe as "kahveh", and soon became popular there. [10] Nowadays, caffeine has been widely used in drinks such as coffee, sodas, and tea. It is reported that adults' average daily intake of caffeine is 280 mg, which is about two cups of filtered coffee, and three and a half cups of black tea. Also, 90% of adults in North America are consuming caffeine each day. [11] Humans have used many plants that

contain phenylpropene as condiments and herbal remedies for thousands of years. For example, people use cloves which are the unopened flower buds of *Syzygium aromaticum* as a spice, and the main active compound in cloves is the phenylpropene eugenol. [10] Another example is cinnamon, which has been a popular and pricy condiment since antiquity. It is extracted from *Cinnamomun ceylanicum* and contains trans-cinnamaldehyde as the major flavor component. [10] After centuries, people are still getting benefits from plant natural products from all aspects of life.

Papaver somniferum

Morphine

Artemisia annua

Artemisinin

Pinus

Abietic acid

Cannabis sativa

Cannabinoid

Cupressus nootkatensis

Nootkatone

Figure 1. Natural products and selected source plants. The compounds represent Morphine from *Papaver somniferum*, cannabinoid from *Cannabis sativa*, artemisinin from *Artemisia annua*, abietic acid from *Pinus*, and nootkatone from *Cupressus nootkatensis*.

1.2 Production of Plant Natural Products

1.2.1 Direct Extraction from Plants

Plant natural products have displayed a vast array of application values as medicines, cosmetics, fragrances, pigments, flavors, and other essential benefits; however, people are facing a tremendous amount of challenges while obtaining the plant natural products on a large scale. In the past, herbal drugs were usually delivered orally as crude extracts, while crude extracts might contain some compounds that were toxic and deleterious to human health. There might be not enough active components in the crude extracts to treat the specific ailments. [12] Direct extract from plants generally has low efficiency and high cost since the desired compounds are usually at low concentration in plants in nature. Yet, the demand for these compounds is continuously increasing. Also, this method is under the restraint of seasonal cycles of slow growth with low content [13], and some plants are geographically specific. Therefore, these factors make it becomes even harder to obtain plant-derived products in a sufficient quantity for commercially manufacturing. Limited supplies of these molecules often cannot meet the demand for the research and the market. Moreover, extracting compounds from plant species for commercial production might threaten the plant population and even result in species extinction. For example, not only of the Pacific yew but of all organisms within the genus were endangered because of the massive production of paclitaxel, the source of the anticancer drug Taxol®. [14] Therefore, for mass production of some plant natural products, direction extraction is not an ideal way.

1.2.2 Total Chemical Synthesis of Plant Natural Products

Chemical synthesis can avoid plant species extinction and does not rely on seasons and geography. It can be very precise and reliable. Still, it can cause environmental concerns since the chemical process usually involves organic solvent, strong bases or acids, and other toxic reagents. More importantly, some plant natural products have complex structures that contain many chiral centers or polycyclic rings; thus, it is difficult to be synthesized via chemical methods. [4] Thus, these poses numerous challenges to chemists to perform the chemical synthesis of complex plant natural products from isolated precursors in an environmentally friendly and commercially feasible way.

1.2.3 Microbial Synthesis of Plant Natural Products

In order to find an alternative feasible and practical approach that could produce plant natural products on a large scale without bringing any harm to the environment, people have put lots of effort into genetic and metabolic engineering. People found that they could use engineered *S. cerevisiae* or *E. coli* as microbial hosts to produce desired products from inexpensive and renewable precursors. There are lots of examples of plant natural products successfully produced by engineered microbes that prove the feasibility of this method, such as limonene [13], and cannabinoids [15]. Microbial synthesis uses enzymes as biocatalysts, so that it can conduct complex reactions with efficiency and selectivity under mild operating conditions. [16] Also, compared to chemical synthesis, microbial synthesis is more environmentally friendly because it avoids any uses of organic

reagents, heavy metals, and strong bases and acids. Moreover, microbial synthesis uses renewable precursors as feedstocks (e.g., glucose or starch) in a closed fermentation vessel, and this allows rapids and sustainable production of desired plant natural production without any concerns of seasonal and regional variations and the possibility of species extinction. These advantages highlight the feasibility, low cost, and effectiveness of microbial synthesis.

Although many challenges are remaining in terms of microbial synthesis, scientists and researchers have developed strategies for microbial biosynthesis of valuable plant natural products. To biosynthesize important plant natural products using engineered microbial strains, the first and key step is the reconstruction of plant biosynthetic pathways. However, in most cases, the biosynthetic pathways of plant natural products have not been completely elucidated since lots of enzymes catalyzing critical steps remain unknown. There are three general methods proposed for enzyme discovery and engineering, including identifying enzymes from the genomes and transcriptomes of native hosts, mining enzymes from non-native hosts, engineering missing enzymes via protein evolutions. [4] Each approach can be applied flexibly in different situations based on its strengths and weakness. During the reaction, intracellular microenvironments of microbial hosts are not the same as those of the native plant hosts. Consequently, many enzymes might not work as effectively in microbial hosts as in the native hosts due to incorrect folding, decreased expression levels catalytic efficiencies. One representative example is plant cytochrome P450 enzymes (P450s). [4] To improve the metabolic flux to the desired plant natural products, protein engineering can be applied to achieve the enhancement of catalytic efficiency. Protein engineering can improve the levels of

transcriptions and proteins, enhance the folding and kinetic properties, reduce substrate inhibition, and increase selectivity for target substrates. [2] Besides, there are two strategies specifically concerning the improvement of plant cytochrome P450s' catalytic efficiencies, including applying N-terminus engineering and increasing the electron transfer to P450s. [4] When biosynthesizing the complex plant natural products, the pathway usually involves multiple reactions. Due to the interaction between metabolites and enzymes, the efficiency of the pathway is very likely to be reduced. This issue can be resolved by dynamic control, subcellular compartmentalization of heterologous enzymes, and optimizing the host's metabolism. [4] There are numerous methods in terms of the production of useful plant natural products, and each method has its strengths and weakness. Based on different conditions, a different approach can be applied to achieve effective production of desired compounds.

1.3 Coumarin Overview

1.3.1 Categories of Coumarin Compounds

Coumarins, a major group of plant natural products, are a family of phenylpropanoids which exhibit a vanilla-smell and a bitter taste. They could be found in many higher plants, and function as a chemical defense to discourage the predators in nature. About 1000 coumarin derivatives of more than 800 species have been isolated. [17] The word coumarin originates from a French term for the Tonka bean, *coumarou*, seeds of *Dipteryx odorata* (*Coumarouna odorata*) (*Fabaceae*/*Leguminosae*), one of the sources from which coumarin was first isolated as a natural product in 1820. Because of

its vanilla-odor, it has been used in perfumes since 1882. [18] Coumarins consist of four categories: simple coumarins, pyrone-substituted coumarins, furanocoumarins, and pyranocoumarins. (Figure. 2) Simple coumarins contain the simplest coumarin compounds and its derivatives. Pyrone-substituted coumarins include substitution and modifications on the pyrone ring. Furanocoumarins consist of compounds that have a furan ring attached to the coumarin nucleus. Pyranocoumarins contain a pyran ring. Furanocoumarins and pyranocoumarins could be further divided into a linear or angular type according to the position of the furan ring or pyran ring, respectively. [19]

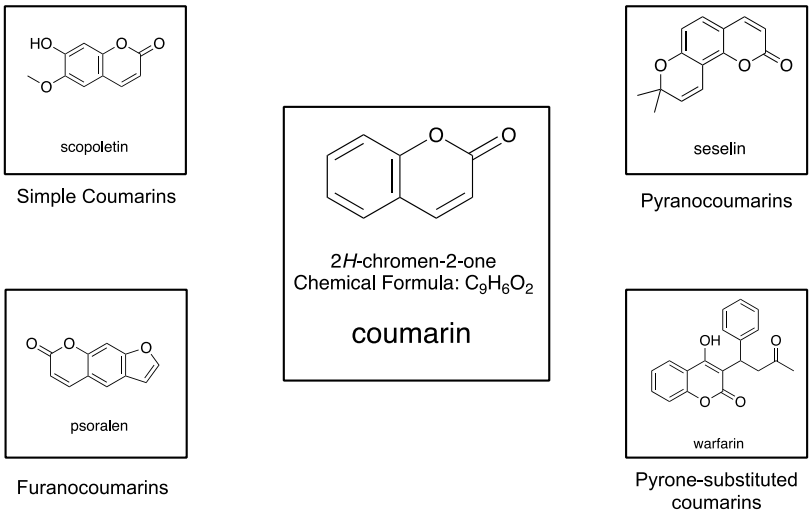


Figure 2. Categories of coumarin compounds. Coumarins are subdivided into four categories, including simple coumarins (e.g. scopoletin), furanocoumarins (e.g. psoralen), pyranocoumarins (e.g. seselin), and pyrone-substituted coumarins (e.g. warfarin).

1.3.2 Applications of Coumarins

The simplicity and flexibility of coumarin's scaffold make it a wonderful starting point for a vast range of applications, such as cosmetics, fragrances, food, and other industrial additives. For instance, like the use in perfume, some derivatives could also be added in tobaccos and certain alcoholic drinks to enhance the aroma. [18] Despite the role of coumarins as the additive, coumarins have shown various pharmaceutical activities including antioxidant [20], anti-cancer [21], anti-inflammatory [22], anti-HIV [23], anticoagulant, and other essential medicinal activities because of their strong pharmaceutical activity, low toxicity and side effects, fewer drug resistance, high bioavailability, broad-spectrum, and other properties. [18] In 1868, coumarin was successfully synthesized for the first time in the pharmaceutical industry. It was synthesized as a precursor in the synthesis of a variety of synthetic anticoagulant drugs. Some notable anticoagulant drugs derived from coumarin have been extensively used in clinics till now. [18] One of the most popular drugs is Warfarin®, a member for pyrone-substituted coumarins, which is used as an anticoagulant to treat blood clots, deep vein thrombosis, and pulmonary embolism. [24] In addition, lots of compounds that are derived from coumarin nucleus such as umbelliferon, scopoletin, marina show strong anti-inflammatory and antioxidant activities. [22] Hymecromone can be used as an antispasmodic and choleretic agent, and carbochromen benefits to treat coronary disease. [25]

1.4 Scopoletin Study

1.4.1 Introduction of Scopoletin

Scopoletin, 7-Hydroxy-6-methoxy-2*H*-1-benzopyran-2-one, is a constituent of many different medicinal plants. Scientists have put lots of effort into the investigation of this compound due to lots of reasons. First, scopoletin is a potentially biologically active compound that shows various pharmaceutical activities. For example, isolated from many species of plants and has antioxidant, hepatoprotective, anti-inflammatory and, antifungal effects. [22] It can also prevent leukemic cells proliferation by inducing apoptosis to inhibit cancer formation. [25] Then, it is an intermediate in the synthesis of other coumarins, such as fraxetin. [34] Due to the potential applications in the pharmaceutical industry, extensive efforts and research have been made to synthesize this vital compound.

1.4.2 Biosynthetic Pathway of Scopoletin in Plant and *E. coli*

The general coumarin biosynthesis scheme was hypothesized decades ago, indicating that the simple coumarin molecular skeleton biosynthesis starts with $_{L}$-phenylalanine through the intermediates *trans*-cinnamate, *trans*-2-coumarate, *trans*-2-coumarate-ß-$_{D}$-glucoside, *cis*-2-coumarate-ß-$_{D}$-glucoside, and *cis*-2-coumarate.[26] (Figure. 3) However, lots of genes encoding the enzymes have not been identified until now, and this has become an enormous challenge to complete the proposed coumarin biosynthetic pathway. In 2013, with the discovery and characterization of some key enzymes, the complete artificial pathway was assembled in *E. coli,* and de novo biosynthesis of umbelliferon and scopoletin were completed without adding any additional

precursor.[19] In their work, they connected the coumarin synthetic pathway with bacterial primary metabolism. Both umbelliferon and scopoletin biosynthetic pathway used tyrosine as the precursor since *E.coli* could produce tyrosine endogenously. [19]

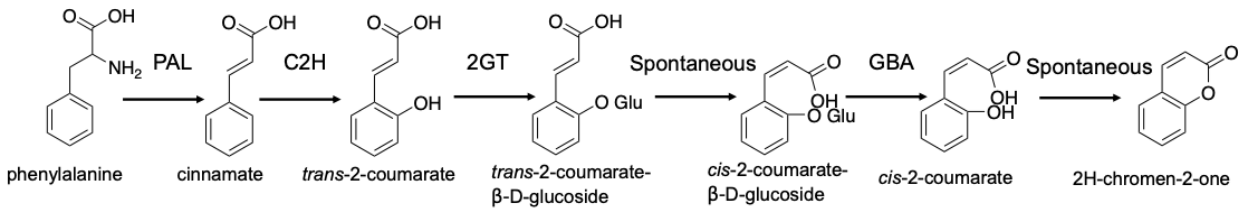


Figure 3. Proposed coumarin biosynthetic pathway in plant. PAL, phenylalanine ammonia lyase; C2H, cinnamate 2-hydroxylase; 2GT, 2-coumarate O-β-glucosyltransferase; GBA, β-glucosidase.

1.4.3 *Saccharomyces cerevisiae* as a Platform

Except for *E. coli*, *S. cerevisiae* is also a well-studied strain, with the amount of engineered tools. Lots of research groups have successfully produced desired products by using engineered *S. cerevisiae* strains, such as limonene [13] and resveratrol [30]. Furthermore, compared to *E. coli*, *S. cerevisiae* has demonstrated structure similarity to plant cells, which endowers *S. cerevisiae* a great microbial platform for the biosynthesis of plant natural products. [35] *S. cerevisiae* is capable of expressing enzymes from various species ranging from bacteria to mammals. It even can express some complex plant enzymes, such as P450 enzymes. [4] More importantly, *S. cerevisiae* has high homologous recombination efficiency, which makes it extremely convenient to assemble DNA fragments. [36] Together, all the properties make *S. cerevisiae* a suitable and efficient platform for the biosynthesis of certain plant natural products. Here, we used engineered *S.cerevisiae* strains to testify not only its ability to biosynthesize coumarins and their derivative but also its influence towards productivity.

Chapter 2. Materials and Methods

2.1. Strains, Medium, and Culture Conditions

E. coli DH5α (Zymo Research) was employed as the host for DNA manipulation and amplification. Luria-Bertani (LB) broth (Fisher Scientific) with antibiotics [50 μg/mL kanamycin (Sigma Aldrich)] was used for culture of *E. coli* strain at 37 ℃ and 250 rpm.

S. cerevisiae CEN.PK2-1D was the original yeast strain used for the scopoletin producing strain construction. YPD [1% yeast extract (VWR), 2% peptone (Fisher Scientific), and 2% dextrose (Fisher Scientific)] and synthetic complete drop-out medium (SD-His medium or SD-Leu medium) were used for culture of yeast strains at 30℃ and 400 rpm, when appropriate.

Table 1. Strains used in this study

Strains	Relevant Genotype	Reference or Source
DH5α	F⁻ φ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_K^-, m_K^+) *pho*A *sup*E44 λ⁻ *thi*-1 *gyr*A96 *rel*A1	Labstock
CEN.PK2-1D	MATalpha; ura3-52; trp1-289; leu2-3_112; his3del1; MAL2-8C; SUC2	EUROSCARF
Constructed scopoletin producing strain	CEN.PK2-1D, Locus 2::yAt4CL1-yCCoAOMT-his5-yAtF6'H	This study

2.2. Plasmid and Strain Construction

This section involves three parts: codon optimization of gene fragments, construction of gene expression cassette, and pathway integration. Different species show bias against the use of some codons for the same amino acid over others, which can significantly affect transgenic expression. Construction of gene expression cassette helps gene be able to be transcribed in *S. cerevisiae* and be expressed to protein in the further step. After that, all the DNA fragments are transferred to *S. cerevisiae,* and strain is finally constructed.

Based on the constructed coumarin biosynthetic pathway, At4CL1 (GenBank accession number AEE32699), AtCCoAOMT1 (GenBank accession number AT4G26220), and AtF6'H1 (GenBank accession number NP187970) were selected according to their activities. For the optimal expression of enzymes in *S. cerevisiae*, they were all codon optimized by GeneArt GeneOptimizer Program, Life Technologies. After codon optimization, corresponding genes, yAt4CL1, yCCoAOMT, and yAtF6'H were synthesized by Twist Bioscience.

The codon-optimized genes and their corresponding promoters and terminators were all PCR amplified using NEBuilder® Q5 High-Fidelity DNA polymerase (New England Biolabs). After amplification, they were all went through the purification step by using DNA concentrate and clean kit (Zymo Research). Each purified PCR product was confirmed by gel electrophoresis regarding its size. If necessary, gel recovery can be performed to extract DNA by using the Zymo clean Gel DNA Recovery Kit (Zymo Research). After confirmation, the codon-optimized genes and their corresponding promoters and terminators were assembled together by using Gibson Assembly with

NEBuilder® HiFi DNA Assembly Master Mix (New England Biolabs) based on New England Biolabs Protocol. This step helps to ensure plasmid stability and prevent homologous recombination. After assembly, assembled plasmids were transferred into *E. coli* DH5α competent cell and grown on LB plates with kanamycin. The plates were incubated at 37℃ overnight. After getting colonies on the plates, *E. coli* colony PCR was performed to verify the insertions using OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs). The correctly verified colony was cultured in LB liquid medium with kanamycin overnight. Then, plasmids were extracted from *E. coli* by miniprep. All the buffers, including buffer P1 (suspension buffer), buffer P2 (lysis buffer), buffer N3 (neutralizing and binding solution, and buffer PE (wash buffer), used in plasmid miniprep process were prepared by our lab. When plasmids were successfully purified, they were prepared by adding M13F/M13R primer for Sanger sequencing performed by Cornell University Institute of Biotechnology.

The protein expression cassettes, selection marker and homologous fragments (500 bp upstream and downstream to the inserted site), with 25 bp overlap sequences between each other, were amplified by PCR and transferred to the yeast cell by electroporation. *S. cerevisiae* has strong homologous recombination ability, which allows it to assemble all the cassettes together and integrate onto the locus we designed on yeast chromosome (YDR514C). The resulting strain was grown on the synthetic complete drop-out plate, and the plate was incubated at 30℃ for three days. After getting colonies on the plates, colony PCR using OneTaq® 2X Master Mix with Standard Buffer (New England Biolabs) was performed to verify each fragment. Positive results from the

verification step meant all the fragments were assembled correctly, and ready for the feeding experiment.

Table 2. Plasmids constructed in this study

Plasmids	Gene cassette reconstructed	Reference
pSL75	TPI1p-yAt4CL1-STE2t	This study
pSL74	PYK1p-yCCoAOMT-MFa1t	This study
pSL73	GPD1p-yAtF6'H-ADH1t	This study

Table 3. Primers used for pathway integration

Target gene cassette	Name of primer	Primer sequence
TPI1p-yAt4CL1-STE2t	TPI1.rev	5’-ttttagtttatgtatgtgtttttgtagttatagatttaagcaagaaaagaatac-3’
	STE2.fwd	5’-tcaaaatttacggctttgaaaaagtaatttcgtgaccttc-3’
PYK1p-yCCoAOMT-MFa1t	PYK1.rev	5’-atgttttatttgttttgattggtgtcttgtaaatagaaacaagaga-3’
	MFa1.fwd	5’-gcccgactgataacaacagtgtagatg-3’
GPD1p-yAtF6'H-ADH1t	GPD1.rev	5’-aggatccactagttctagaatccgtcg-3’
	ADH1.fwd	5’-cgcgccacttctaaataagcgaatttcttatg-3’
HIS5	HIS5. rev	5’-atgataaactcgaagagctcagaagtaactgcagttcgacaacc-3’
	HIS5. fwd	5’-gagaattcggccactagtggatctgatatcac-3’
Locus2L	Locus2L.rev	5’-actacacagattataagctattgttgaactcgtaaaagc-3’
	Locus2L.fwd	5’-gtttgtttcttcttatcttcagctgctgag-3’
Locus2R	Locus2R.rev	5’-gcaataattcttgtggcaaacaacgtgataatgataataacaatta-3’
	Locus2R.fwd	5’-ctctaccggcagatcatctagctagaagtttgtaggta-3’

Table 4. Primers used for PCR amplification of plasmid inserts

Target gene	Name of primer	Primer sequence
yAt4CL1	yAt4CL1_fwd	5’-aactacaaaaaacacatacataaact aaaatatacaatggccccacaagaacaa-3’
	yAt4CL1_rev	5’-accaagttggctaacggtttgtgatcaa aatttacggctttgaaaaagtaattt-3’
yCCoAOMT1	yCCoAOMT1_fwd	5’-aaaacaaataaaacataaaaaaatgg ctaaggatgaagctaag-3’
	yCCoAOMT1_rev	5’-atccactagtggccgaattctcttaggatt cgattcac-3’
yAtF6'H	yAtF6'H_fwd	5’-gaactgcagttacttctgagctcttcgag tttatcattatcaatac-3’
	yAtF6'H_rev	5’-aacttctagctagatgatctgccggtaga ggtgt-3’

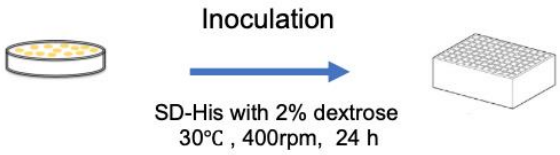
Table 5. Codon-optimized gene sequences

Name of codon-optimized gene	Codon-optimized gene sequence
yAt4CL1	ATGGCTCCACAAGAACAAGCTGTTTCTCAAGTTATGGAAAAGCAGTCGAACAACAACAACTCCGATGTTATCTTCA GATCCAAGTTGCCAGATATCTACATCCCAAACCATTTGTCCTTGCACGATTACATCTTCCAGAACATTTCTGAATTC GCTACCAAGCCATGTTTGATTAACGGTCCAACTGGTCATGTTTACACCTACTCTGATGTTCACGTTATCTCCAGACA AATTGCTGCTAACTTTCACAAGTTGGGTGTTAACCAGAACGATGTTGTCATGTTGTTATTGCCAAACTGCCCAGAAT TCGTCTTGTCTTTTTTGGCTGCTTCTTTTAGAGGTGCTACTGCTACAGCTGCTAATCCATTTTTTACTCCAGCTGAAA TTGCTAAGCAAGCTAAGGCTTCTAACACCAAGTTGATTATTACCGAAGCTAGATACGTCGACAAGATCAAGCCATT GCAAAATGATGATGGTGTTGTTATCGTCTGCATCGATGATAATGAATCCGTTCCAATTCCAGAAGGTTGTTTGAGAT TCACTGAATTGACTCAGTCTACTACTGAAGCCTCCGAAGTTATTGATTCCGTTGAAATTTCTCCAGATGATGTTGTT GCTTTGCCATACTCTTCAGGTACTACTGGTTTGCCAAAAGGTGTTATGTTGACTCACAAAGGTTTGGTTACATCCGT TGCTCAACAAGTTGATGGTGAAAATCCAAACTTGTACTTCCACTCCGATGATGTCATTTTGTGTGTTTTGCCAATGT TCCATATCTACGCCTTGAACTCTATTATGTTGTGCGGTTTGAGAGTTGGTGCTGCTATTTTGATTATGCCAAAGTTC GAGATCAACCTGTTGTTGGAATTGATCCAAAGATGCAAGGTTACTGTTGCTCCAATGGTTCCACCAATAGTTTTGG CTATTGCTAAGTCATCTGAAACCGAAAAGTACGACTTGTCCTCTATCAGAGTTGTTAAGTCAGGTGCTGCTCCATTG GGTAAAGAATTGGAAGATGCTGTTAATGCTAAGTTCCCAAACGCTAAATTAGGTCAAGGTTACGGTATGACTGAAG CTGGTCCAGTTTTAGCTATGTCTTTGGGTTTTGCTAAAGAGCCATTTCCAGTAAAATCTGGTGCTTGTGGTACTGTT GTTAGAAACGCTGAAATGAAGATCGTTGATCCAGATACTGGTGACTCCTTGTCTAGAAATCAACCAGGTGAAATCT GCATCAGAGGTCATCAAATTATGAAGGGCTACTTGAACAATCCAGCTGCTACTGCAGAAACCATTGATAAGGATGG TTGGTTGCATACAGGTGATATTGGTTTGATTGATGATGACGACGAGTTGTTCATCGTCGACAGATTGAAAGAACTG ATCAAGTACAAGGGTTTTCAAGTTGCTCCTGCTGAATTAGAAGCTTTGTTGATTGGTCATCCAGATATTACCGATGT TGCTGTTGTAGCTTGA
yCCoAOMT1	ATGGCTAAGGATGAAGCTAAGGGTTTGTTGAAATCTGAAGAGTTGTACAAGTACATCCTGGAAACATCTGTTTACC CAAGAGAACCAGAAGTCTTGAGAGAATTGAGAAACATCACTCACAATCATCCACAAGCTGGTATGGCTACTGCTCC AGATGCTGGTCAATTGATGGGTATGTTGTTGAATTTGGTTAACGCCAGAAAGACCATCGAAGTTGGTGTTTTTACT GGCTACTCTTTGTTGTTGACTGCTTTGACTTTGCCAGAAGATGGTAAGGTTATTGCCATCGATATGAACAGAGACT CCTACGAAATTGGTTTGCCAGTTATTAAGAAAGCCGGTGTTGAACACAAGATCGACTTCAAAGAATCTGAAGCTTT GCCAGCCTTGGACGAATTATTGAACAACAAGGTTAATGAAGGTGGTTTCGATTTCGCTTTTGTTGATGCTGATAAG CTGAACTACTGGAACTATCACGAAAGATTGATCAGGTTGATTAAGGTCGGTGGTATCATCGTTTACGATAATACTTT GTGGGGTGGTTCTGTTGCTGAACCAGATTCTTCTACTCCAGAATGGCGTATTGAAGTTAAGAAGGCTACTTTGGAG CTGAACAAAAAGTTGTCTGCTGATCAAAGAGTCCAAATTTCCCAAGCTGCTTTAGGTGATGGTATTACTATATGCAG AAGGCTGTACTAA
yAtF6'H	ATGGCTCCAACTTTGTTGACTACCCAATTTTCTAATCCAGCCGAAGTTACTGATTTCGTCGTTTACAAAGGTAACGG TGTTAAGGGTTTGTCTGAAACTGGTATTAAGGCTTTGCCAGAACAGTACATTCAACCATTGGAAGAGAGACTGATC AACAAGTTTGTTAACGAAACCGATGAAGCCATTCCAGTTATCGATATGTCAAACCCAGATGAAGATAGAGTTGCTG AAGCTGTTTGTGATGCTGCTGAAAAATGGGGTTTTTTCCAAGTTATCAACCACGGTGTTCCTTTGGAAGTTTTGGAT GATGTTAAGGCTGCTACCCATAAGTTTTTCAATTTGCCAGTCGAAGAGAAGAGGAAGTTTACCAAAGAAAACTCTTT GTCCACCACCGTTAGATTCGGTACTTCTTTTTCTCCATTGGCTGAACAAGCTTTGGAATGGAAAGATTACCTGTCCT TGTTCTTCGTTTCTGAAGCTGAAGCAGAACAATTTTGGCCTGATATCTGTAGAAACGAAACCTTGGAGTACATCAAC AAGAGCAAGAAAATGGTCAGAAGGTTGTTGGAATACTTGGGCAAGAATCTGAACGTCAAAGAATTGGACGAGACA AAAGAATCCTTGTTCATGGGTTCCATCAGAGTCAATTTGAACTACTACCCTATTTGCCCAAATCCAGATTTGACTGT TGGTGTTGGTAGACACTCTGATGTTTCTTCATTGACCATCTTGTTGCAAGATCAAATTGGTGGCTTGCACGTTAGAT CTTTGGCTTCAGGTAATTGGGTTCATGTTCCACCAGTTGCTGGTTCTTTCGTTATTAACATTGGTGATGCCATGCAG ATTATGTCTAACGGCTTGTACAAATCCGTTGAACATAGAGTTTTGGCCAACGGTTACAACAACAGAATCTCTGTTCC AATCTTCGTTAACCCAAAGCCAGAATCTGTTATTGGTCCATTGCCTGAAGTTATTGCTAATGGTGAAGAACCCATCT ACAGAGATGTCTTGTATTCCGATTACGTCAAGTACTTCTTCAGAAAGGCTCACGATGGTAAAAAGACTGTTGATTAC GCCAAGATCTAA

2.3. Feeding Experiment

A feeding experiment was performed to examine the production of scopoletin from caffeic acid, with two biological replicates. It includes two parts: seed culture experiment and substrate feeding experiment. (Figure. 4) For the seed culture experiment, the goal is to generate enough cell for the next step, and it started with the inoculation step. Single colony of the fully integrated strain was inoculated in a 96-well plate with 500μL SD-His medium supplemented with 2% dextrose for each well. Colony from plasmid pAG413-ccdB-1 was also inoculated in the same medium as the negative control. The plate was shaken at 30℃ and 400rpm for 24 h. A substrate feeding experiment was performed to evaluate the activity of the constructed strain. 25μL overnight culture was re-inoculated into 470μL SD-His medium supplemented with 2% dextrose. Caffeic acid was dissolved in ethanol with an initial concentration of 100mM, and 5μL of the solution was fed to each well at a concentration of 1mM. 5μL ethanol was fed to each well as the negative control. The final volume in each well was 500μL. The cultures were grown at 30℃ and 400rpm for 48 h. Samples were collected after 48 h. 400μL sample was transferred into a 1.5-mL centrifuge tube and centrifuged at 14000 rpm for 10 min. Supernatants were filtered with 0.2 μM filters and 200μL of filtered supernatants was transferred to autosampler vials (Fisher Scientific) for LC-MS analysis.

- Seed Culture Experiment



- Substrate Feeding Experiment

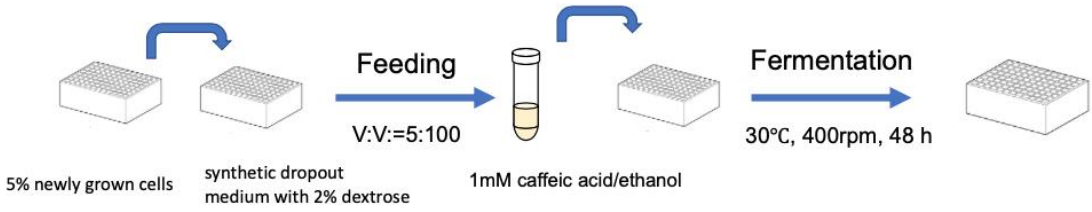


Figure 4. Workflow of feeding experiment. Feeding experiment includes seed culture experiment and substrate feeding experiment.

2.4. Analytical Procedures

Scopoletin (Tokyo Chemical Industrial Co.) and caffeic acid (Cayman Chemical) were used as standards. For each chemical, at least five standards with different concentrations were analyzed. Both the standards and samples were analyzed and quantified by LC-MS (Agilent 6545B Q-ToF) with a reverse-phase ZORBAX RRHD Eclipse Plus C18 column using positive ion mode, with 1 μL injection. The mobile phase was water (A) and acetonitrile (B), both with 0.1% (v/v) formic acid. The elution procedure was 95% A between 0 and 0.5 min; 95−50% A between 0.5 and 4.5 min; 50-5% A between 4.5 and 6.5 min; and 95% A between 6.5 and 7.5 min with a flow rate of 0.4 mL/min.

Chapter 3. Results

3.1. Reconstruction of Scopoletin Biosynthetic Pathway in Yeast

The goal of the pathway design is engineering *S.cerevisiae* as a platform for coumarin biosynthesis. In plants, the synthesis of coumarin starts from L-phenylalanine. The pathway involves two plant-specific cytochrome P450 monooxygenase, cinnamate 4-hydroxylase (C4H) and coumarate 3-hydroxylase (C3H). Since C4H and C3H are cytochrome P450 dependent enzymes, they both need their associated cytochrome P450 reductase for functional expression, which makes them hard to be purified from plants and to be expressed in microbes. To circumvent the use of cytochrome P450 dependent enzymes, there is an alternative pathway starting from L-tyrosine, which avoids the use of C4H. [27] In our study, we designed a scopoletin biosynthetic pathway starting from caffeic acid, which avoids the involving of both C3H and C4H. (Figure. 5) The full biosynthetic pathway involves four steps, in which 4CL1 (4-coumarate: CoA ligase), CCoAOMT (caffeoyl-CoA O-methyltransferase), and F6'H (Feruloyl-CoA 6'hydroxylase) were selected according to their activities. 4CL1 converts caffeic acid to caffeoyl-CoA. Then, CCoAOMT catalyzes the conversion of caffeoyl-CoA to feruloyl-CoA. F6'H demonstrated hydroxylase activity towards feruloyl-CoA forming 6'hydroxyferuloyl-CoA. Finally, scopoletin is biosynthesized from 6'hydroxyferuloyl-CoA through spontaneous reaction. Each gene was codon-optimized and assembled into a holding vector with a specific promoter and terminator as indicated in 2.2. Each gene cassette was PCR-amplified with homologous sequences to its neighbor cassettes and assembled into the chromosome

site YDR514C. After that, colony PCR was performed to verify if all the fragments were assembled successfully and correctly.

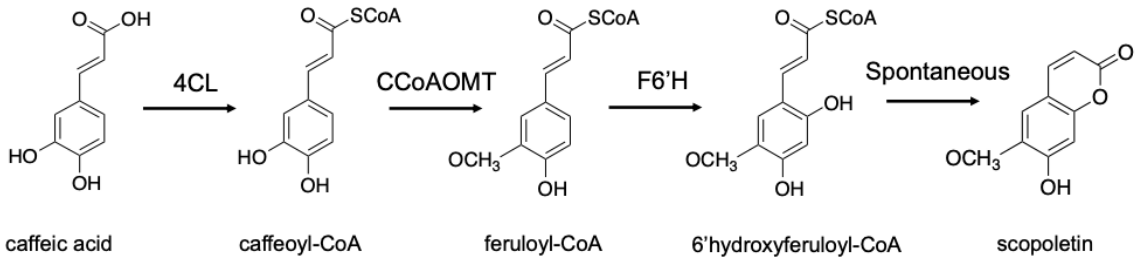


Figure 5. Reconstructed scopoletin biosynthetic pathway in *S. cerevisiae*. 4CL,4-coumarate: CoA ligase; CCoAOMT, caffeoyl-CoA O-methyltransferase; F6'H, feruloyl-CoA 6'hydroxylase.

3.2. Use of Auxotrophic Marker

Auxotrophic markers (*HIS3*, *TRP1*, *LEU2*, and *URA3*) are used to produce corresponding amino acids (e.g. histidine, tryptophan, leucine) essential for yeast growth on synthetic drop-out medium. Thus, efficient assembly of auxotrophic marker overlapping with upstream and downstream biosynthetic gene cassette detects and selects transformed cells, which indicates the successful assembly of the entire scopoletin biosynthetic pathway. [28] In our study, *LEU2* was selected as the auxotrophic marker to indicate the correct assembly of all gene cassettes into yeast chromosome. (Figure. 6a) However, the reconstructed strain showed limited cell growth and no scopoletin production with *LEU2* marker. As reported previously, auxotrophic markers are associated with varied level of cell growth inhibition. In order to lower the growth inhibition due to the usage of *LEU2* marker, the auxotrophic marker should be chosen in the order of *HIS* & *TRP* >*URA* >*LEU* in the previous study. [29] As *LEU2* showed the greatest decrease in growth rate among the auxotrophic markers. [29] We hypothesized that *LEU2* might redirect the metabolic flux toward leucine synthesis, or complete with the scopoletin precursor biosynthesis, resulting in inhibition of cell growth and scopoletin synthesis. Thus, *HIS5* was chosen to replace *LEU*2, which led to a fully functioned scopoletin producing strain. (Figure. 6b)

Figure 6a. Pathway integration with *LEU2* marker.

Figure 6b. Pathway integration with *HIS5* marker.

3.3. Bioconversion of Caffeic Acid into Scopoletin

In our study, we have designed a scopoletin biosynthetic pathway starting from caffeic acid. Caffeic acid was used as the feeding stock because feeding caffeic acid instead of amino acids is more likely to provide more upstream substrate, thus increasing the metabolic flux towards the product. After successfully constructing the coumarin integration strain, we performed feeding experiment and analyzed the samples by using LC-MS. Feeding experiments includes two parts: seed culture experiment and substrate feeding experiment. In order to generate sufficient cells for the real production, seed culture experiment was first performed. We picked the colonies and cultured them in SD-His medium that contains all the essential nutrients except histidine for the yeast to grow. 2% dextrose was also provided as the carbon source for cells growth. To maintain the best productivity and enough cell density, the cells were cultured at 30 ℃, which is the optimal temperature for yeast growth. Moreover, the plate was shaken at 400 rpm to ensure cell to have sufficient oxygen in the liquid medium. We cultured them for 24 h to guarantee enough cell density and have robust cells for future production. Then, the substrate feeding experiment was performed, we assayed scopoletin production by growing constructed strain in SD-His medium supplemented with 2% dextrose and feeding with 1mM caffeic acid. Also, a negative control was generated by feeding ethanol to the engineered strain. After fermentation at 30℃ and 400rpm for 48 h, the culture was taken and prepared for the analysis by LC-MS. The production of scopoletin was confirmed through comparison with the chemical reference standard. The exact mass of scopoletin is 193.0495. According to LC-MS results (Figure. 7a, 7b, 7c), each line represents the standard scopoletin chemical (grey line), constructed strain fed with

5μL1mM caffeic acid (yellow line), constructed strain fed with 5μL ethanol (green line), respectively. For the grey line, there was a peak at 5.4 min that represents scopoletin. The yellow line also had a peak at 5.4 min, it showed the same retention time and molecular weight as the standard scopoletin. For comparisons, we analyzed the sample of negative control, which contained the engineered strain but fed with ethanol instead of caffeic acid. Theoretically, it would not produce the product, scopoletin. At 5.4 min, there was no peak shown for the green like, like we predicted. Thus, we confirm that our constructed strain does produce the desired compound, scopoletin. Although the titer is not optimistic, further optimization experiments could be done to improve the titer of scopoletin.

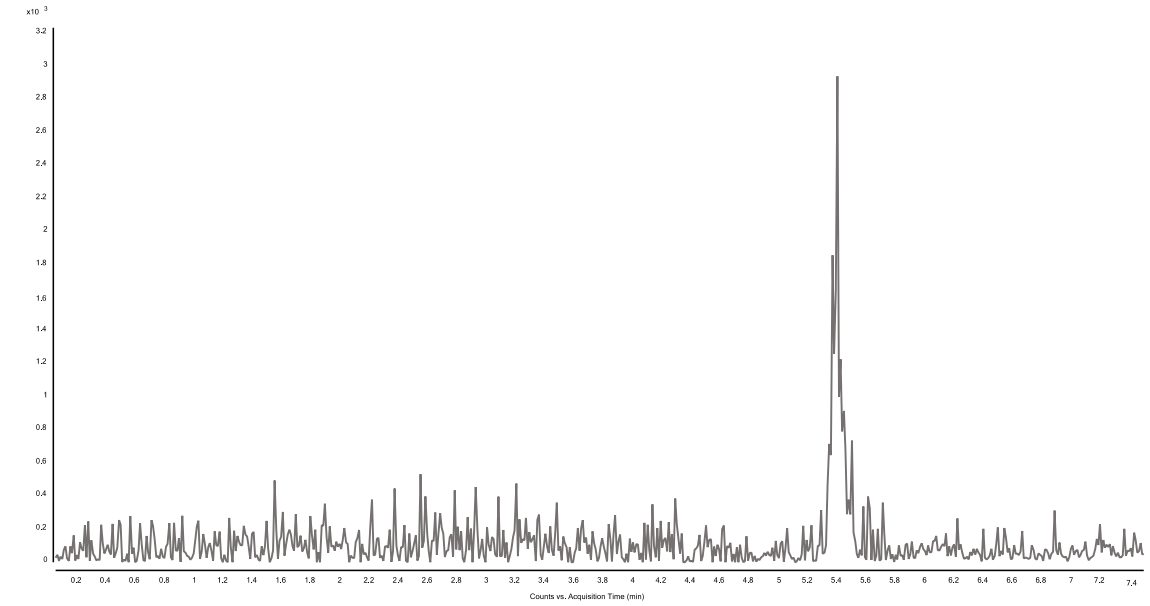


Figure 7a. LC-MS result of standard scopoletin

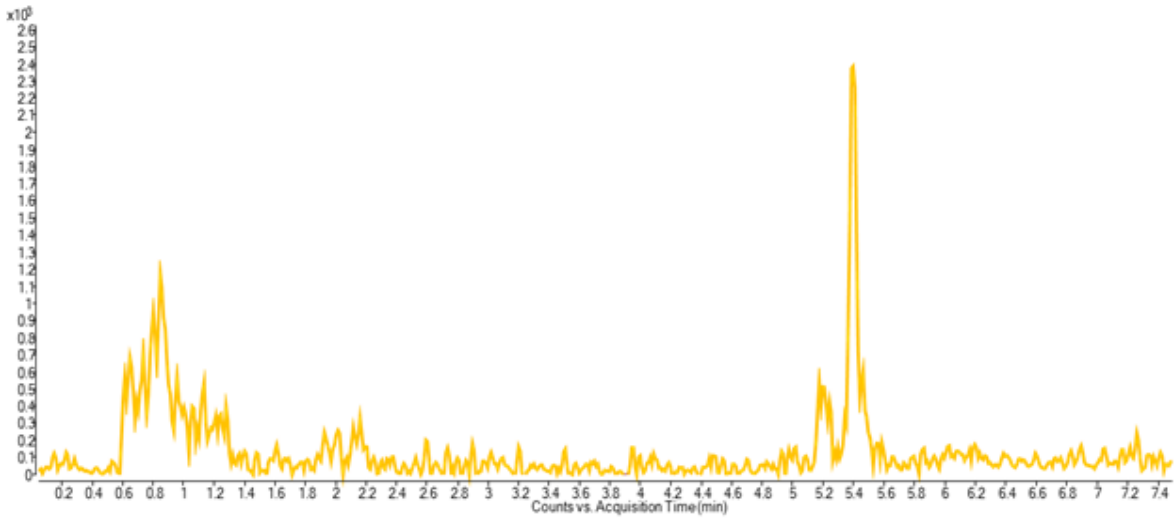


Figure 7b. LC-MS result of constructed strain fed with 5μL1mM caffeic acid

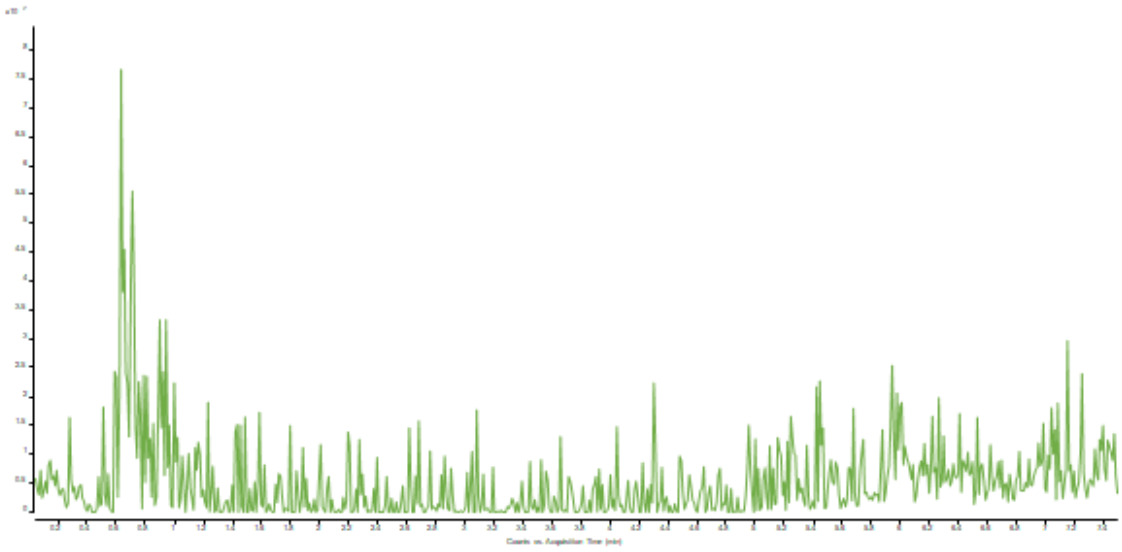


Figure 7c. LC-MS result of constructed strain fed with 5µL ethanol.

Chapter 4. Conclusion and Future Work

For millennia, human has been continuously getting benefits from plant natural products. They have played essential roles in all aspects of human life. Among them, coumarins and their derivative have been increasingly attracting many attentions due to their broad application in food, cosmetics, fragrances, and pharmaceutical industries. Scopoletin, a member of the coumarin family, shows potent antioxidant, hepatoprotective, anti-inflammatory, and antifungal activities. [22] Moreover, it is a critical intermediate in the complex coumarin biosynthesis pathway.

Most plant natural products are hard to be synthesized chemically because of their complex structures and multiple stereocenters. [4] Moreover, chemical synthesis is associated with environmental concerns in many cases. Efficient plant extraction is hampered by low levels of desired compounds in plants, and slow growth of plants. It's also hard to get high-purity compound, and not sustainable. With the knowledge of metabolic engineering and synthetic biology, heterologous biosynthesis in engineered microbes provides an alternative way to produce plant natural products using inexpensive and renewable precursors.

In this study, we managed to establish a synthetic platform in *S.cerevisiae* to achieve the production of scopoletin from caffeic acid. Although the yield of scopoletin is relatively low, and this may be due to lots of factors in the experiment. The reasons might include some enzymes' activities are low, and they may also be toxic to the pathway. A number of optimizations to improve the production of scopoletin could be attempted. First, the additive amount of caffeic acid could be optimized for scopoletin biosynthesis. In the study, we only fed the constructed strain with 1mM caffeic acid. The engineered coumarin

integration strain could be fermented with different concentrations of caffeic acid. After comparing the production of scopoletin measured and analyzed by LC-MS, the optimized concentration could be used in further study.

Second, we propose that optimize the fermentation time would further enhance scopoletin production. In the study, we collected samples after 48 h of feeding caffeic acid. The samples could be collected every 12 h in a 48-h period, or we can extend the fermentation time to several days. Then, we can analyze these samples by LC-MS to see if time could influence the production of scopoletin.

Third, we propose that optimize the concentration of the fermentation medium would also improve scopoletin yield. There are several papers published reporting that production of their desired products (e.g., resveratrol and caffeic acid) [30][27] in YPD medium containing 4% glucose was higher than in synthetic complete drop-out medium containing 4% glucose. The YPD medium containing 4% glucose could also shorten fermentation time. [27] In our study, we attempted to use YPD medium containing 4% glucose instead of SD-His medium in the seed culture step. It seemed no influence on the yield of scopoletin. It is possibly worth trying to use YPD medium containing 4% glucose in both seed culture step and substrate feeding step. Moreover, YPD medium containing 4% glucose is much cheaper than synthetic complete drop-out medium containing 4% glucose. [27] Thus, if YPD medium containing 4% glucose could improve the yield of scopoletin, it would be a better choice in the fermentation step.

Last, the activity of each enzyme should also be considered, low activity of any enzyme in the pathway could be the bottleneck limiting the high production of scopoletin. More research needs to be done about increasing the activities of the enzymes used in

the pathway. Also, more work could focus on if there are alternative enzymes that could function the same way or if there are enzymes from different hosts having a better function.

Instead of using caffeic acid as the precursor for the biosynthesis of scopoletin, in further study, we propose to establish the complete biosynthetic pathway toward scopoletin from tyrosine. Although using caffeic acid can provide more metabolic flux, the cost of tyrosine is much lower than that of caffeic acid. The biosynthetic pathway from tyrosine to caffeic acid is fully elucidated [27], and we have successfully constructed a tyrosine overproduction strain. This may not only help to improve the production of scopoletin, but also lower the cost of the experiments.

We have successfully achieved the production of scopoletin from caffeic acid. Based on this, we can reconstruct a pathway to biosynthesize fraxetin. Fraxetin belongs to a broad group of natural phenolics and demonstrated many vital activities in healthcare. It is reported that fraxetin shows the protective effects on modification on reduced endogenous glutathione, intracellular oxygen species, and apoptotic death on rotenone-mediated cytoxicity. [31] It's antihyperglycemic effect also makes it may be useful in the treatment of diabetes, although clinical studies are needed to be warranted. [32] Fraxetin can be biosynthesized from caffeic acid by a four-step pathway. (Figure. 8) Based on the biosynthetic pathway of scopoletin, it could be further converted to fraxetin with the activity of scopoletin 8-hydroxylase (S8H).

Production of umbelliferon can be achieved by reconstructing a biosynthetic pathway starting from 4-coumaric acid. Umbelliferon is a pharmacologically active compound and shows anti-inflammatory, anti-hyperglycemic, molluscicidal, and anti-tumor activities. Moreover, it is fluorescent and can be used in sunscreen. [33] The pathway

involves three steps from 4-coumaric acid to umbelliferon. (Figure. 9) 4-coumaric acid is converted to 4-coumaroyl-CoA by 4-coumarate: CoA ligase (4CL). With the activity of coumaroyl-CoA 2'-hydroxylase (C2'H), 4-coumaroyl-CoA is hydroxylated, and then umbelliferon is spontaneously synthesized.

In conclusion, we provide the first report on the biosynthesis of scopoletin from caffeic acid in *S.cerevisiae*. This study can potentially pave the way for coumarin production through further optimization and improvement. The reconstruction of the scopoletin biosynthesis pathways can be utilized to facilitate the construction of other coumarin compounds.

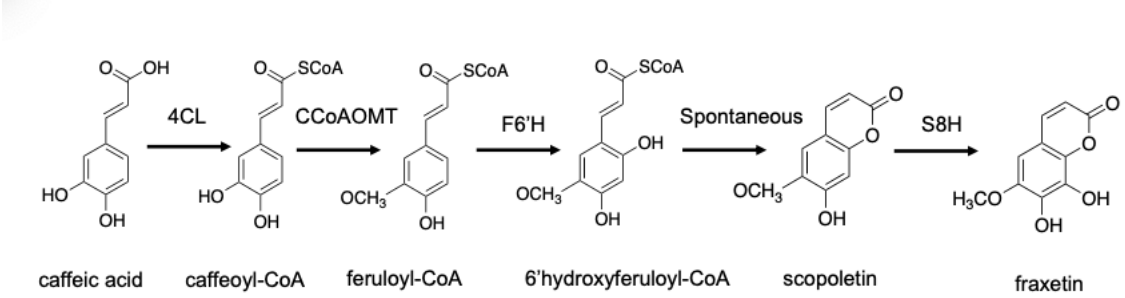


Figure 8. Proposed fraxetin biosynthetic pathway in *S. cerevisiae*. 4CL,4-coumarate: CoA ligase; CCoAOMT, caffeoyl-CoA O-methyltransferase; F6'H, feruloyl-CoA 6'hydroxylase; S8H, scopoletin 8-hydroxylase.

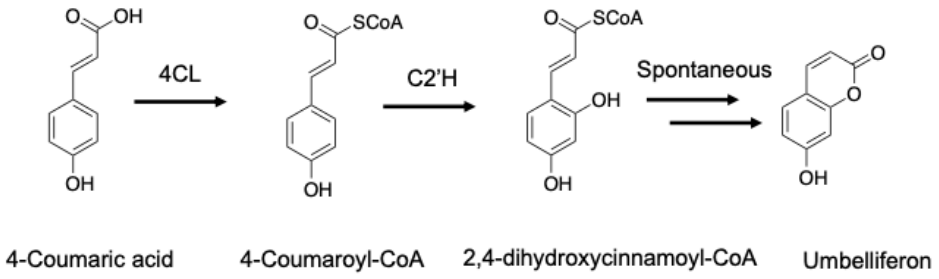


Figure 9. Proposed umbelliferon biosynthetic pathway in *S. cerevisiae*. 4CL,4-coumarate: CoA ligase; C2'H, coumaroyl-CoA 2'-hydroxylase.

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