



SYNTHETIC BIOLOGY-GUIDED REPROGRAMMING OF MICROBIAL METABOLISM FOR ENHANCED PRODUCTION OF BIOACTIVE SECONDARY METABOLITES

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Abstract

A combination of pathway engineering, host optimization, and controlled cultivation of microbial hosts is a powerful strategy for enhancing their production of high-value bioactive secondary metabolites using synthetic biology. In this work, the effects of designed pathway design, microbial chassis, cultivation media and growth-related parameters on the production of flavonoids and alkaloids were investigated. Comparative analysis revealed that screening of the designed flavonoids in an iterative fashion could significantly increase the production of pinocembrin, and the best-flavoured second round resulted in a mean concentration of 9.723 mg/L. The selection of microbial chassis also had a significant effect; as the MDS42 chassis with the design 2165 had the highest overall mean for pinocembrin production, the MG1655 chassis with the design 3382 had the best strain–design combination. Metabolite yield was strongly affected by the type of cultivation medium, with AIM providing significantly more than M9. In the case of metabolic optimization, significant and positive correlations were obtained between the production of induction biomass and pinocembrin and cinnamate concentrations, with biomass accounting for only a small proportion of the total production variation. Alkaloid pathway analysis showed that pathway products were redistributed in a pathway design-specific manner, with optimal production of tetrahydropapaveroline, reticuline and scoulerine achieved by different pathway configurations.

Keywords: Synthetic biology; Metabolic engineering; Microbial cell factories; Pinocembrin; Alkaloids

1. Introduction

Microorganisms and plants are a source of bioactive secondary metabolites that serve as a source of pharmaceuticals, nutraceuticals, pigments, antioxidants and other high-value products. Flavonoids, terpenoids and alkaloids have various biological activities like antimicrobial, antioxidant, anti-inflammatory and therapeutic. In other cases, though, their abundance may be low in nature; they may be hard to harvest from native hosts; and/or chemical synthesis may be complicated and environmentally problematic. This has led to the rise of engineered microbial systems as alternative production platforms for valuable secondary metabolites (Hemmerling & Piel, 2022). The development of synthetic biology tools has also opened the door to the reconstruction and optimization of natural-product biosynthesis pathways in genetically manipulable microbial hosts (Bureau et al., 2023).

Microbial cell factories can serve as a controllable platform for heterologous expression of biosynthetic pathways and can be systematically engineered to increase the availability of precursors, regulate enzyme expression, suppress competing reactions and optimize product formation. System metabolic engineering is a technique that uses genetic engineering, pathway analysis, synthetic biology, and process optimization to reroute cellular resources toward the production of a target substance (Ko et al., 2020). More recently, there has been a broader appreciation that microbial cell-factory capacities are not only about pathway structure, but also the physiology of the host cells, metabolic resources, and tolerance to the demands imposed by the engineered biosynthetic pathway (Kim et al., 2025). This means that the biosynthesis of these secondary metabolites is a complex process, which needs coordinated manipulation of many characteristics of the pathway and host.

The flavonoids are an important class of secondary metabolites of microbial engineering importance due to their wide biological activities and their commercial significance. The production of flavonoids using recombinant bacterial and yeast systems has been developed, which involves the introduction of enzymes from plants and the modification of central metabolic pathways (Isogai et al., 2022). However, the production of flavonoids by microorganisms is limited by various factors, including the availability of precursor compounds, imbalance of enzyme activities, toxicity of the pathways, low catalytic efficiency and competition from endogenous metabolic pathways (Li et al., 2022). Hence, metabolic engineering approaches have been directed towards optimizing precursor supply, engineering high levels of enzymes, removing alternative pathways and adjusting the growth environment to enhance flavonoid production (Sheng et al., 2020). Such strategies have shown their ability to produce structurally divergent flavonoids *de novo* which are conventionally extracted from plants or synthesized through multiple steps in chemical synthesis (Tariq et al., 2023).

Another important parameter for biosynthetic performance is the selection of an appropriate microbial chassis. There are significant differences in the availability of precursors, cofactor balance, capacity for protein expression, stress resistance, and metabolic burden between different hosts and strain backgrounds. Therefore, the cellular environment can be tailored to suit heterologous production through chassis engineering by creating the cellular environment more compatible with complex biosynthetic pathways (Liu et al., 2022). Furthermore, some metabolic pathways may need dynamic control rather than constitutive control since over-activity of the pathway can have negative consequences for cell growth and overall production. Dynamic regulation allows the cells of the microorganisms to allocate resources to biomass production and product formation in a more efficient manner (Ni et al., 2021). These days, scientists are employing modular systems of regulation, biosensors, and systems for controlling pathways to achieve this balance.

The Design-Build-Test-Learn approach has become increasingly important in contemporary synthetic biology due to its ability to facilitate the iterative improvement of designed biological systems. This approach allows building candidate pathway designs, experimental testing, and then improvement of the designs based on the results of the tests (Gurdo et al., 2022). The use of this iterative loop has grown even more common in recent years, and it has been combined with high throughput experiments and computational learning to discover better configurations of the pathways (Foldi et al., 2024). Also, computational recommendation systems have been shown to inform subsequent engineering iterations by learning relationships between various biological design parameters and experimentally measured outcomes (Radivojević et al., 2020). These developments are especially relevant for complex pathways leading to the production of natural products in which many interacting genetic and environmental factors are involved in the final production.

In addition, synthetic biology is beginning to transcend the narrow scope of traditional pathway reconstruction, aiming for more complex control of the metabolism of precursors and the production of aromatic compounds. With modern tools, it is possible to regulate the key pathways leading to the production of aromatic amino acids, which are key precursors of many flavonoids, alkaloids and other secondary metabolites (Tang et al., 2024). Likewise, engineered microorganisms are now being utilized as environmentally friendly manufacturing platforms, cutting back on the need to rely on the agricultural extraction of resources or traditional chemical synthesis (Jones et al., 2024). Although much progress has been made, the correlation between engineered pathway configuration, microbial chassis, cultivation environment, microbial growth and final production of the secondary metabolites is highly context-dependent (Lou et al., 2021).

For this reason, the present work deals with the investigation of the effects of synthetic biology-supported reprogramming on the microbial production of secondary metabolites, specifically flavonoids and alkaloids.

Objectives:

- To test the impact of various synthetic biology "engineered" setups on the synthesis of bioactive secondary metabolites.
- To evaluate the effects of chassis and growth conditions on secondary-metabolite biosynthetic productivity.
- To investigate the correlations between microbial growth, pathway-related metabolites and product generation and detect trends that correlate with better metabolic reprogramming.

2. Materials and Methods

2.1 Study Design

To assess the impact of synthetic biology-inspired engineering of microorganisms for the production of bioactive secondary metabolites, this study was designed using a quantitative secondary data analysis. The analysis utilized experimentally constructed data from the SYNBIOCHEM Design-Build-Test-Learn pipeline, including engineered microbial constructs, strain-level comparison, cultivation conditions, microbial growth indicators, and measurements of metabolite production. The overall analytical process adopted was Design-Build-Test-Learn with alternative engineered biological configurations being compared, and those with improved biosynthetic performance being identified.

2.2 Data Source and Dataset Structure

The study employed the SYNBIOCHEM Design-Build-Test-Learn pipeline dataset which consists of 6 CSV files with data on the production of flavonoids and alkaloids in engineered microbial systems. The flavonoid datasets consisted of first-round and second round design screening, microbial chassis comparison, culture medium screening and pathway optimization experiments. Multiple engineered designs were tested for the measurement of the alkaloids tetrahydropapaveroline (THP), reticuline and scoulerine in the alkaloid dataset. The principal outcome of flavonoids was pinocembrin and measurements of cinnamate were utilized to support the interpretation of precursor-product relationships (Carbonell et al., 2018).

2.3 Variable Selection and Classification

Engineered design, microbial strain and cultivation medium were the main independent variables. The engineered design was an alternative synthetic-biological configuration, and the strain variable were different *Escherichia coli* chassis backgrounds. The variables measured were related to growth, such as induction and harvest OD. The main dependent variables were the concentrations of bioactive secondary metabolites such as pinocembrin, THP, reticuline and scoulerine. In the optimization experiments of flavonoids, cinnamate was also measured as a precursor in the pathway.

2.4 Data Preparation and Quality Assessment

Before analysis, all data sets were reviewed for missing data, duplicate records, inconsistencies in variable types and non-numeric data. Treatment, strain, medium and sample identifiers were treated as categorical while optical-density and metabolite-concentration measurements were treated as continuous. To estimate within-group variability, experimental replicates were kept. Extreme metabolite values were examined within the experimental groups for which they were found and not automatically excluded as being due to genuine high producing engineered designs.

2.5 Analysis of Synthetic-Biology Design Performance

The production of metabolites was compared in engineered designs to find out which designs were related to higher biosynthetic performance. The mean, SD, median, minimum, and maximum flavopigments levels of pinocembrin were reported separately for the first and second design rounds in the experiments for screening flavopigments. Production levels were ranked based on design and percentage improvements, and fold changes were computed against appropriate reference designs. Originally the two screening rounds were analyzed separately to maintain the experimental structure of the two rounds in order to make a wider comparison of production ranges and designs with high performance before that. Before, the two screening rounds were analyzed separately so that the experimental structure of the two screening rounds was maintained and a wider comparison of production ranges and designs that showed good production was made.

2.6 Microbial Chassis and Culture-Medium Analysis

The secondary-metabolite production in *E. coli* was analyzed by comparing the amount of pinocembrin produced by various *E. coli* strains in the microbial chassis. Average production was measured to assess if chassis selection influenced the performance of engineered pathways. Likewise, the culture-medium dataset was analyzed for variation of metabolites produced under different nutrient conditions. In some cases, the synergy of engineered design and the cultivation medium was studied to assess the effect of genetic design, environmental conditions or both on production enhancement.

2.7 Metabolic Pathway Optimization Analysis

A relationship between induction optical density, accumulation of cinnamate and production of pinocembrin was evaluated using the flavonoids optimization dataset. Correlation and regression analysis were performed to see if there was any relationship between the metabolite yield and the microbial biomass at induction. The accumulation of cinnamate and the lack of production of pinocembrin were compared to see if there was a precursor-product relationship; cinnamate levels were higher and pinocembrin levels were low, which is interpreted with prudence as a possible constraint in the pathway. Pathway effects were inferred from the production profiles observed, without the measurements of metabolic flux.

2.8 Alkaloid Production Profiling

The alkaloid data set was subsequently analysed separately to compare the production of THP, reticuline and scoulerine for the engineered designs. The mean concentrations and variability were computed for each metabolite and design. Correlation analysis was used to check for relationships between the three compounds, and to determine designs that would show a preference for upstream intermediates or downstream products. With this analysis the engineered configurations with comparatively better alkaloid biosynthetic performance were identified.

2.9 Statistical Analysis

Continuous variables were summarised using descriptive statistics: mean, standard deviation, median, range and coefficient of variation. One-way analysis of variance (ANOVA) was used to compare differences in metabolite production between several engineered designs, strains, or culture conditions provided that the parametric assumptions were met. When variances were not homogeneous, Welch's ANOVA was applied, and for distributions that were significantly not normally distributed, the Kruskal-Wallis test was applied. Correlation analysis (Pearson or Spearman) was used to determine the relationship of the variables, and linear regression was used, when relevant, to establish the numerical correlation between growth indicators and metabolite production. Level of statistical significance was established as $p < 0.05$ and effect sizes and confidence intervals were analyzed along with the level of significance.

2.10 Ethical Considerations

No humans were involved directly in the study, nor was personal data collected; no new biological samples were collected, nor were animal experiments conducted. Hence, no further institutional ethical approval and informed consent were needed for this analysis. Data was analysed and used only for academic research, and its original structure and experimental context were maintained. All of the interpretations were limited to variables available in the dataset, and no unvalidated statements were made about genome-wide regulation, the intracellular metabolic flux, or unmeasured molecular mechanisms.

3. Results

3.1 Dataset Characteristics and Experimental Coverage

The SYNBIOCHEM Design-Build-Test-Learn pipeline consisted of six experimental datasets from different phases of pathway engineering of flavonoids and alkaloids. The first, and second flavonoid screening rounds had 51 and 132 observations of 17 and 32 engineered configurations, respectively as shown in Table 1. The 81 observations in the microbial chassis experiment covered 9 *Escherichia coli* strains, while the culture-medium screening experiment comprised 112 observations spanning 7 different culture media. The numbers of observations in the flavonoid optimization data set were 238, and for the alkaloid data set were 42, which were the numbers of engineered designs.

Table 1. Experimental structure and coverage of the SYNBIOCHEM datasets

Experimental dataset	Observations	Experimental groups/designs	Principal outcome
Flavonoid screening—Round 1	51	17 designs	Pinocembrin
Flavonoid screening—Round 2	132	32 designs	Pinocembrin
Microbial chassis screening	81	9 strains; 3 designs	Pinocembrin
Culture-medium screening	112	7 media; 2 designs	Pinocembrin
Flavonoid optimization	238	2 metabolite targets	Pinocembrin and cinnamate
Alkaloid pathway screening	42	14 designs	THP, reticuline and scoulerine

The mean concentration of pinocembrin during the two screening rounds was 0.137 ± 0.572 mg/L for Round 1 and 1.012 ± 1.899 mg/L for Round 2, which is about 7.36-fold higher for the overall mean production. A similar increase was noted in the maximum concentration as it rose from 2.556 mg/L (Round 1) to 10.438 mg/L (Round 2). This was statistically significant ($U = 899.0$, $p < 0.001$) between the two rounds, meaning that the new second-round design space was found to represent a significant number of higher-yielding designs.

3.2 Synthetic-Biology Design Performance

There were significant differences in the production of pinocembrin across engineered configurations. The pQLINKN configuration had the highest mean production in Round 1 (2.396 ± 0.139 mg/L) compared to the other configurations in the round. There was significant heterogeneity between first-round designs ($F = 171.76$, $p < 0.001$) as confirmed by Welch's ANOVA.

The second round of screens showed a much wider range of production. As presented in Table 2, design 3382 produced the highest mean pinocembrin concentration (9.723 ± 0.712 mg/L), followed by designs 3391 (4.307 ± 1.142 mg/L) and 3353 (4.304 ± 0.709 mg/L). Design 3382, in turn, achieved about 2.26 times the average concentration of the next highest Design.

Table 2. Highest-performing engineered designs based on pinocembrin production

Screening round	Design	n	Mean (mg/L)	SD	Median (mg/L)	Maximum (mg/L)
Round 1	pQLINKN	3	2.396	0.139	2.320	2.556
Round 1	1049	3	0.134	0.062	0.156	0.183
Round 1	1043	3	0.025	0.011	0.028	0.034
Round 1	1040	3	0.010	0.008	0.012	0.017
Round 1	1041	3	0.009	0.005	0.011	0.013
Round 2	3382	3	9.723	0.712	9.718	10.438
Round 2	3391	3	4.307	1.142	3.902	5.597
Round 2	3353	6	4.304	0.709	4.316	5.433
Round 2	3381	5	2.962	0.271	2.911	3.384
Round 2	3373	5	2.448	2.191	3.322	4.467

Round 2	3364	6	1.909	0.170	1.887	2.114
Round 2	3372	6	1.671	0.881	1.467	3.402
Round 2	3375	3	1.377	0.378	1.188	1.812
Round 2	3360	3	1.193	0.033	1.205	1.217
Round 2	3388	3	0.978	0.056	0.966	1.039

The differences were statistically significant in second round using both Welch's ANOVA ($F = 169.49$, $p < 0.001$) and Kruskal–Wallis testing ($H = 124.68$, $p < 0.001$). The relative performance of the top 10 producing designs can be seen in Figure 1, which clearly shows the superiority of design 3382 over the other engineered designs.

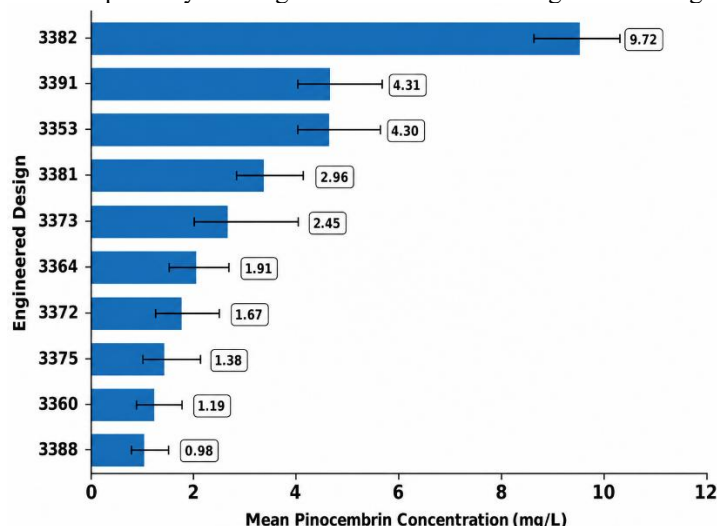


Figure 1. Mean pinocembrin concentration among the ten highest-performing synthetic-biology designs during the second flavonoid screening round

3.3 Effect of Microbial Chassis on Pinocembrin Production

Secondary-metabolite productivity was significantly affected by the choice of microbial chassis. As summarized in Table 3, MDS42 exhibited the highest overall mean pinocembrin concentration (30.043 ± 3.705 mg/L), followed by MG1655 (24.677 ± 14.611 mg/L) and W3110 (21.737 ± 8.308 mg/L). On an average basis, however, MDS42 produced 16.45 more times than BW25113 producing the lowest mean production of 1.827 ± 2.312 mg/L.

Table 3. Pinocembrin production across different Escherichia coli chassis

E. coli strain	n	Mean (mg/L)	SD	Median (mg/L)
MDS42	9	30.043	3.705	30.925
MG1655	9	24.677	14.611	27.542
W3110	9	21.737	8.308	20.650
DH5a	9	14.179	3.485	14.003
DH1	9	7.884	3.302	9.715
Mach1	9	7.768	4.436	6.576
DH10b	9	5.971	3.249	4.826
BL21	9	3.570	1.881	3.498
BW25113	9	1.827	2.312	0.573

Two-way ANOVA demonstrated a significant main effect of strain ($F = 33.76$, $p < 0.001$) and design ($F = 9.18$, $p < 0.001$). Additionally, there was a significant interaction between the pathway and the design ($F = 2.03$, $p = 0.028$), indicating that pathway performance was partly dependent on the genetic background of the microorganisms. In the strain design level, the highest mean concentration (35.099 mg/L) was obtained for strain MG1655 with the design 3382, followed by strain MDS42 with design 3382 (33.283 mg/L) and MDS42 with design 3353 (31.289 mg/L). The results in Figure 2 confirm the clear differences in the production of pinocembrin, with the strains of MG1655–3382 and MDS42 and their combinations producing the most pinocembrin.

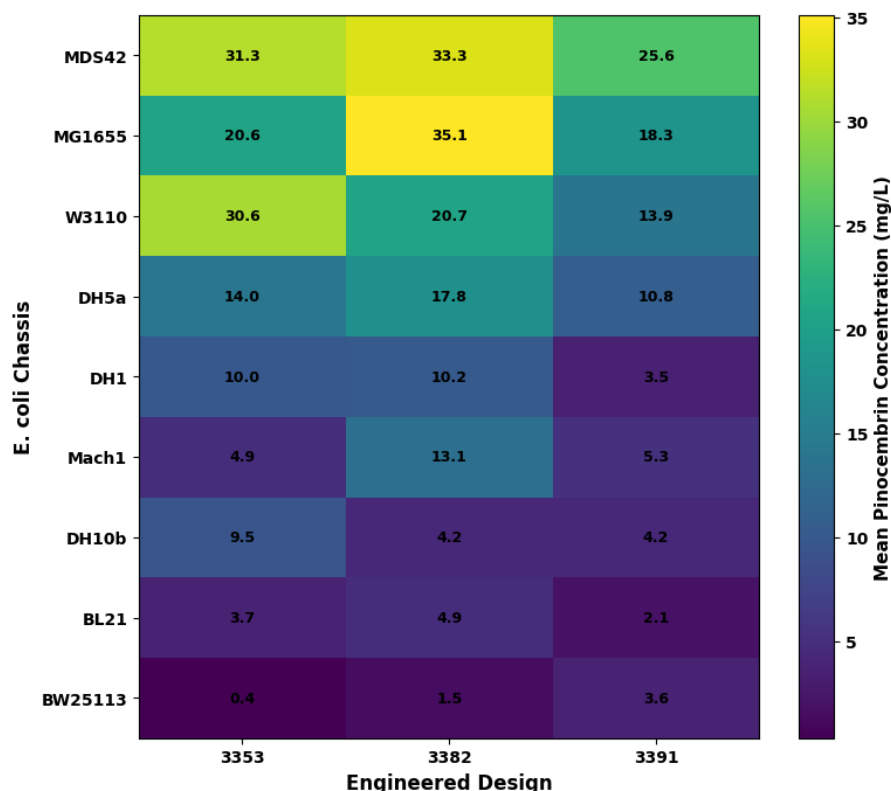


Figure 2. Heatmap of mean pinocembrin production across Escherichia coli chassis and engineered synthetic-biology designs

3.4 Effect of Cultivation Medium on Metabolite Production

Pinocembrin production was also highly affected by the cultivation environment. As shown in Table 4, AIM medium produced the highest overall mean concentration (147.004 ± 146.341 mg/L), closely followed by TBsb (142.161 ± 115.785 mg/L) and TB (141.675 ± 125.283 mg/L). A production of 124.462 ± 106.499 mg/L was obtained in EZ medium, which was relatively high. However, M9 had the lowest mean concentration (31.542 ± 36.420 mg/L).

Table 4. Pinocembrin production under different culture-medium conditions

Medium	n	Mean (mg/L)	SD	Median (mg/L)
AIM	16	147.004	146.341	62.646
TBsb	16	142.161	115.785	138.916
TB	16	141.675	125.283	130.033
EZ	16	124.462	106.499	112.132
SOB	16	61.809	55.686	50.157
MOPS	16	42.807	69.137	7.029
M9	16	31.542	36.420	17.962

The average production in AIM was about 4.66 times greater than that of M9. The overall design effect was not significant ($F = 0.032$, $p = 0.859$), but there was a statistically significant effect of culture medium ($F = 3.96$, $p = 0.001$) based on factorial ANOVA. Design $\times$ medium was also not significant ($F = 0.443$, $p = 0.848$), indicating that the major source of variation in this experiment was the environment.

Figure 3 presents the concentration of pinocembrin in all seven cultivation media. In AIM, TBsb, TB, and EZ, higher and more variable production was found, but in M9 and MOPS, generally lower production of metabolites was observed.

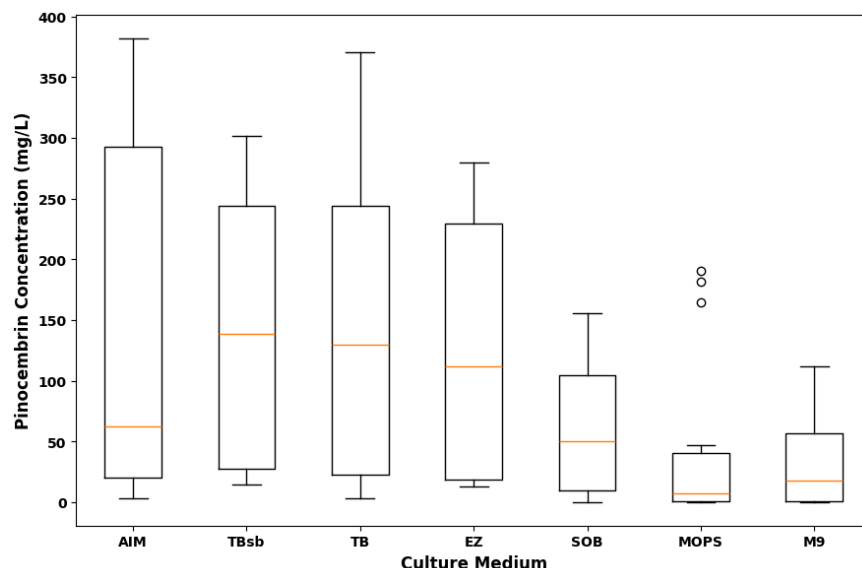


Figure 3. Distribution of pinocembrin concentrations across seven microbial cultivation media used during synthetic-biology pathway screening

3.5 Metabolic Optimization and Association with Induction Biomass

There was a wide range of pinocembrin and cinnamate content in the flavonoid optimization data set. The concentrations of cinnamate were significantly higher, though, with a mean of 163.444 ± 136.852 mg/L and a maximum of 591.053 mg/L. Microbial biomass at induction and the concentration of metabolites are summarized in Table 5. Both the concentration of pinocembrin ($r = 0.293$, $p = 0.004$) and cinnamate ($r = 0.288$, $p = 0.004$) were significantly correlated with the induction OD600.

Table 5. Relationship between induction OD600 and flavonoid-pathway metabolite production

Metabolite	Paired n	Pearson r	p-value	Regression slope	R ²
Pinocembrin	96	0.293	0.004	2.952 mg/L per OD unit	0.086
Cinnamate	96	0.288	0.004	20.618 mg/L per OD unit	0.083

Each unit increase in OD600 of induction was estimated to yield a 20.618 mg/L increase in cinnamate and a 2.952 mg/L increase in pinocembrin by linear regression, respectively. However, the low R² values suggested the induction biomass accounted for a small fraction of the variability in metabolite production.

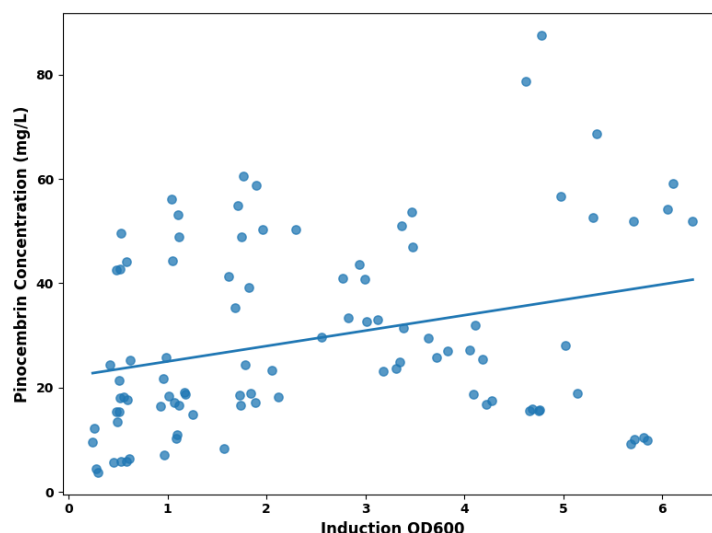


Figure 4. Relationship between microbial induction optical density (OD600) and pinocembrin concentration during flavonoid pathway optimization

The concentration of pinocembrin remained constant from optimization point 1 (18.591 mg/L) to optimization point 2 (21.539 mg/L), then increased at optimization point 3 (26.664 mg/L), optimization point 4 (30.072 mg/L) and optimization point 5 (30.639 mg/L), and then slightly decreased at optimization point 6 (29.597 mg/L). A statistically significant positive monotonic relation was seen between the optimization point with the concentration of pinocembrin (Spearman's $r_s = 0.224$, $p = 0.020$).

The production trend of induction OD600 and pinocembrin concentration is shown in Figure 4, which exhibits a positive

but scattered trend of production.

3.6 Alkaloid Pathway Reprogramming

It is also noted that the alkaloids production differed significantly among the different designs. The mean concentration of THP was 29.266 ± 21.550 mg/L for all the experimental observations, the mean concentration of reticuline was 19.885 ± 16.399 mg/L and the mean concentration of scoulerine was 1.044 ± 2.417 mg/L.

Table 6 shows that the best configurations varied significantly depending on the target metabolite. The highest THP concentration (76.899 ± 14.379 mg/L) was obtained in design 203 while the highest concentration of reticuline (49.946 ± 4.829 mg/L) was achieved in design 208. Design 209, on the other hand, strongly favored the production of scoulerine downstream and yielded a mean concentration of 9.406 ± 1.491 mg/L.

Table 6. Highest-performing synthetic-biology designs for alkaloid metabolite production

Metabolite	Highest-performing design	Mean (mg/L)	SD	Second-highest design	Mean (mg/L)
THP	203	76.899	14.379	213	43.528
Reticuline	208	49.946	4.829	216	47.937
Scoulerine	209	9.406	1.491	214	1.179

The effect of engineered design was statistically significant for THP ($F = 58.88$, $p < 0.001$), reticuline ($F = 72.32$, $p < 0.001$), and scoulerine ($F = 84.20$, $p < 0.001$). There was a significant inverse correlation between the concentrations of THP and reticuline (Pearson $r = -0.759$). A significant inverse correlation was observed between the concentration of THP and reticuline (Pearson $r = -0.759$). In contrast, an upstream increase in scoulerine was positively correlated with an increase in reticuline (Spearman's $r_s = 0.628$), indicating a coordinated increase in production of these two alkaloids in a number of the engineered designs. The metabolic production profiles for each of the 14 synthetic-biology configurations are shown in Figure 5. Design 203 showed a significant amount of THP accumulation, designs 208 and 216 showed a preference for the formation of reticuline and design 209 showed the most distinct increase in the production of scoulerine.

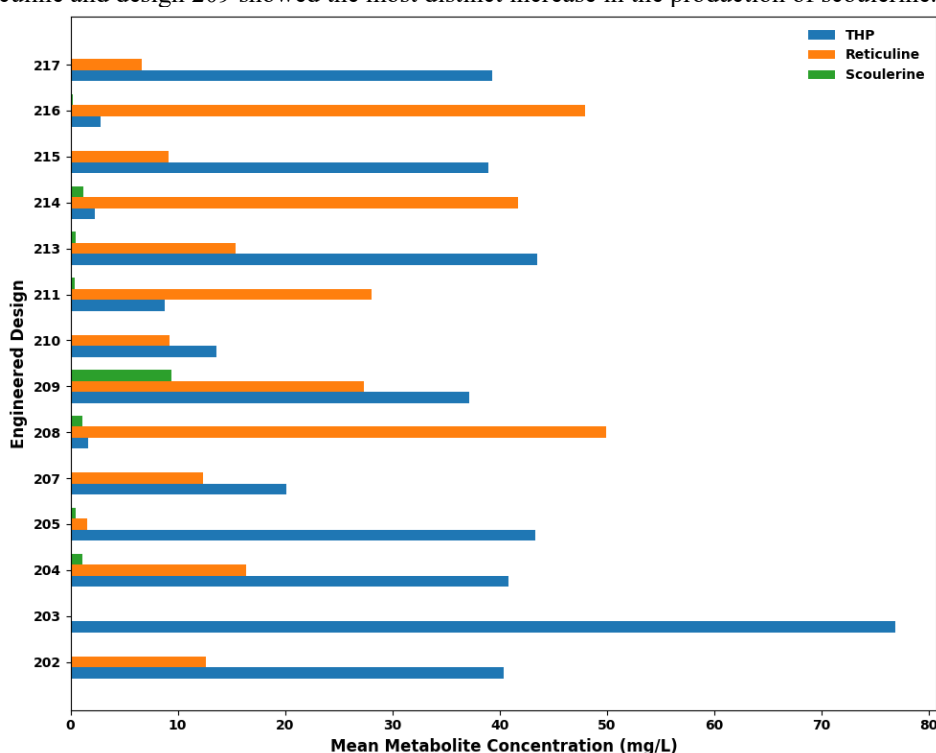


Figure 5. Design-specific mean production profiles of tetrahydropapaveroline (THP), reticuline, and scoulerine across engineered alkaloid biosynthetic configurations

3.7 Summary of Results

Overall, the engineering approach using synthetic biology has significantly increased the yield of the production of secondary metabolites. The production of pinocembrin was higher in all the rounds of screening of flavonoids, with the design 3382 having a higher yield of 9.723 mg/l in the 2nd round. The highest production was obtained in microbial chassis MDS42 (30.043 mg/L) and the lowest production was obtained in microbial chassis M9 (4.66 mg/L). There was a slight positive correlation between induction biomass and the production of pinocembrin and cinnamate. Production of the alkaloids was also strongly design-dependent with different designs producing maximum THP, reticuline, and scoulerine. Overall, the results demonstrate that pathway design, microbial chassis and environmental conditions play an important role in the improvement of secondary-metabolite production.

4. Discussion

The present study shows that the synthetic biology toolbox can make a significant impact on the production of microbial secondary metabolites by pathway engineering, host background, culture conditions and metabolic state. The greatest improvement was noted during the screening of flavonoids, with the mean production of pinocembrin growing around 7.36 times from the first to second design round. This pattern suggests that iterative pathway refinement can yield a lot more productive pathway configuration. Incorporating gene additions and alterations at the strain level and pathway level to enhance pinocembrin-derived product formation, as demonstrated for the production of flavonoids from *E. coli*, (Hanko et al., 2024), has yielded similar results. Rational pathway restructuring, therefore, has been shown to be a very powerful tool to increase the allocation of carbon to the desired product of flavonoids and can be done in a rational manner with the good performance of design 3382.

It is also noteworthy that there are significant differences between engineered designs, thus signaling the importance of controlling pathway flux, not simply boosting pathway expression. In this study, only a few of the configurations resulted in high levels of pinocembrin, indicating a high requirement for pathway balance to facilitate efficient pinocembrin production. Precursor depletion and metabolic imbalance during production can be prevented by using dynamic control strategies. For instance, the Autonomous Cascaded Artificial Dynamic regulation system has demonstrated the coordinated regulatory control can enhance the performance of the pathway by regulating the metabolic activity based on the state of the cells (Jiang et al., 2022). Similarly, flux rerouting towards flavonoids has been demonstrated to increase naringenin yield in engineered *E. coli* cells (Kim et al., 2022).

Another key factor in production was the selection of microbial chassis. The highest overall mean concentration of pinocembrin was observed from the MDS42 with the best strain-design combination being obtained from the MG1655 harbouring the design 3382. The distinct difference in biosynthetic performance seen between MDS42 and BW25113, of 16.45, shows how much an identical biosynthetic goal can vary as a function of different host physiologies. Chassis-dependent effects could be related to variations in precursor pools, cofactor balance, stress resistance, protein-expression potential and endogenous metabolic competition. Other examples are the case of heterologous secondary-metabolite production in engineered microbial hosts where chassis selection was found to have a significant impact on biosynthetic efficiency (Lee et al., 2024). A second example of the usefulness of host optimization can be found in engineered *Yarrowia lipolytica*, where a chassis capable of production of naringenin was successfully used to diversify flavonoids as downstream products (Marsan et al., 2024).

The culture media also significantly affected the culture production of pinocembrin, with approximately 4.66-fold higher mean production in culture medium AIM than that in M9. This result highlights the crucial importance of nutritional and physiological conditions—where engineered pathways are able to reach their full production potential or not—in addition to genetic optimization. A richer or better-balanced environment can allow for more availability of precursors, energy production, and cofactor regeneration while a nutrient limited environment can limit biosynthesis. Concurrently, overactivity of the pathways can put a metabolic strain on the cells, which decrease their robustness and productivity. Reducing such burden using appropriate strategies has been proven to enhance microbial fitness and bioproduction performance (Mao et al., 2024).

Optimization analysis also revealed positive correlation between induction biomass and pinocembrin and cinnamate concentrations with relatively low R² values indicating low production variability associated with biomass. This indicates that as cell density increases, metabolites can be formed but the nature of the pathway configuration, the conversion of precursors, enzyme activity and the allocation of cellular resources will be more significant factors in determining eventual yield. It might also be possible to further optimise pathway efficiency by spatially confining enzymes in intracellular compartments to promote intermediate transfer and minimise competing reactions, as is done in synthetic biology approaches (Wang et al., 2023).

The alkaloid results add to the evidence of pathway-specific metabolic redistribution. The THP, reticuline and scoulerine were optimized in different engineered designs, indicating that optimizing one part of a pathway does not necessarily increase the production of downstream products. The artificial biosynthetic construction of (S)-reticuline in engineered *E. coli* has further demonstrated that enzyme and pathway selection for a specific biosynthetic pathway has a strong impact on the formation of benzyloquinoline alkaloids (Guo et al., 2021). More recent studies have also shown that alternative precursor supplies can enhance benzyloquinoline alkaloid titre, selectivity and yield in an engineered yeast system (Narcross et al., 2026).

In summary, the results demonstrate that coordinated optimization of pathway architecture, microbial chassis, cultivation conditions, and metabolic regulation is critical for the successful production of bioactive secondary metabolites by the microorganisms. Unlike a single engineering intervention, the results suggest a systems-level approach to optimize genetic design and host physiology to achieve higher titres, selectivity, and pathway efficiency.

5. Conclusion

In this study, we show how the integration of pathway design, host selection and cultivation optimization driven by synthetic biology can significantly enhance the production of bioactive secondary metabolites using microbes. The results revealed that pinocembrin production was significantly raised by iterative flavonoid engineering, with certain engineered combinations showing a significantly higher yield compared to others. The selection of microbial chassis was also found to play an important role as there were significant differences among the different *Escherichia coli* backgrounds in their biosynthetic capacity, which suggests that host physiology is a major factor that influences pathway performance. Environmental conditions were also important to optimise in addition to genetic modification, as culture-medium composition was also seen to have an effect on metabolite yield. The positive correlations of the induction biomass with

both pinocembrin and cinnamate production levels indicate that microbial growth enhances biosynthetic productivity, but the low level of explanation suggest that the efficiency of the pathways also relies on the interplay of multiple factors. The analysis of the alkaloid distribution also showed a high degree of pathway-specific redistribution of the metabolites indicating THP, reticuline and scoulerine, thereby demonstrating a pathway-specific effect of the pathway engineering that favours specific metabolites or end products. In summary, the findings point to the possibility of designing synthetic metabolic pathways in the context of the chassis, cultivating the engineered strain under the optimal conditions, and optimizing these three components in parallel in microbial metabolic engineering.

References

1. Bureau, J. A., Oliva, M. E., Dong, Y., & Ignea, C. (2023). Engineering yeast for the production of plant terpenoids using synthetic biology approaches. *Natural product reports*, 40(12), 1822-1848.
2. Carbonell, P., Jervis, A. J., Robinson, C. J., Yan, C., Dunstan, M., Swainston, N., Vinaixa, M., Hollywood, K. A., Currin, A., Rattray, N. J. W., Taylor, S., Spiess, R., Sung, R., Williams, A. R., Fellows, D., Stanford, N. J., Mulherin, P., Le Feuvre, R., Barran, P., ... Scrutton, N. S. (2018). SYNBIOCHEM Design-Build-Test-Learn pipeline (Version 1) [Data set]. The University of Manchester. <https://doi.org/10.17632/8g4wfwtd43.1>
3. Foldi, J., Connolly, J. A., Takano, E., & Breitling, R. (2024). Synthetic biology of natural products engineering: Recent advances across the discover–design–build–test–learn cycle. *ACS synthetic biology*, 13(9), 2684.
4. Guo, D., Kong, S., Sun, Y., Li, X., & Pan, H. (2021). Development of an artificial biosynthetic pathway for biosynthesis of (S)-reticuline based on HpaBC in engineered *Escherichia coli*. *Biotechnology and Bioengineering*, 118(12), 4635-4642.
5. Gurdo, N., Volke, D. C., & Nikel, P. I. (2022). Merging automation and fundamental discovery into the design–build–test–learn cycle of nontraditional microbes. *Trends in Biotechnology*, 40(10), 1148-1159.
6. Hanko, E. K., Robinson, C. J., Bhanot, S., Jervis, A. J., & Scrutton, N. S. (2024). Engineering an *Escherichia coli* strain for enhanced production of flavonoids derived from pinocembrin. *Microbial Cell Factories*, 23(1), 312.
7. Hemmerling, F., & Piel, J. (2022). Strategies to access biosynthetic novelty in bacterial genomes for drug discovery. *Nature Reviews Drug Discovery*, 21(5), 359-378.
8. Isogai, S., Tominaga, M., Kondo, A., & Ishii, J. (2022). Plant flavonoid production in bacteria and yeasts. *Frontiers in Chemical Engineering*, 4, 880694.
9. Jiang, T., Li, C., Zou, Y., Zhang, J., Gan, Q., & Yan, Y. (2022). Establishing an Autonomous Cascaded Artificial Dynamic (AutoCAD) regulation system for improved pathway performance. *Metabolic engineering*, 74, 1-10.
10. Jones, E. M., Marken, J. P., & Silver, P. A. (2024). Synthetic microbiology in sustainability applications. *Nature Reviews Microbiology*, 22(6), 345-359.
11. Kim, D. H., Hwang, H. G., & Jung, G. Y. (2022). Optimum flux rerouting for efficient production of naringenin from acetate in engineered *Escherichia coli*. *Biotechnology for biofuels and bioproducts*, 15(1), 90.
12. Kim, G. B., Kim, H. R., & Lee, S. Y. (2025). Comprehensive evaluation of the capacities of microbial cell factories. *Nature Communications*, 16(1), 2869.
13. Ko, Y. S., Kim, J. W., Lee, J. A., Han, T., Kim, G. B., Park, J. E., & Lee, S. Y. (2020). Tools and strategies of systems metabolic engineering for the development of microbial cell factories for chemical production. *Chemical Society Reviews*, 49(14), 4615-4636.
14. Lee, S. Q. E., Ma, G. L., Candra, H., Khandelwal, S., Pang, L. M., Low, Z. J., ... & Liang, Z. X. (2024). *Streptomyces sungeiensis* SD3 as a microbial chassis for the heterologous production of secondary metabolites. *ACS Synthetic Biology*, 13(4), 1259-1272.
15. Li, H., Lyv, Y., Zhou, S., Yu, S., & Zhou, J. (2022). Microbial cell factories for the production of flavonoids—barriers and opportunities. *Bioresource technology*, 360, 127538.
16. Liu, J., Wang, X., Dai, G., Zhang, Y., & Bian, X. (2022). Microbial chassis engineering drives heterologous production of complex secondary metabolites. *Biotechnology advances*, 59, 107966.
17. Lou, H., Hu, L., Lu, H., Wei, T., & Chen, Q. (2021). Metabolic engineering of microbial cell factories for biosynthesis of flavonoids: a review. *Molecules*, 26(15), 4522.
18. Mao, J., Zhang, H., Chen, Y., Wei, L., Liu, J., Nielsen, J., ... & Xu, N. (2024). Relieving metabolic burden to improve robustness and bioproduction by industrial microorganisms. *Biotechnology advances*, 74, 108401.
19. Marsan, C. B., Lee, S. G., Nguyen, A., Sierra, A. R. G., Coleman, S. M., Brooks, S. M., & Alper, H. S. (2024). Leveraging a *Y. lipolytica* naringenin chassis for biosynthesis of apigenin and associated glucoside. *Metabolic Engineering*, 83, 1-11.
20. Narcross, L., Pyne, M. E., Kevvai, K., Siu, K. H., Dueber, J. E., & Martin, V. J. (2026). Benzyloquinoline Alkaloid Production in Yeast via Norlaudanosoline Improves Titer, Selectivity, and Yield. *ACS Synthetic Biology*, 15(3), 1187.
21. Ni, C., Dinh, C. V., & Prather, K. L. (2021). Dynamic control of metabolism. *Annual Review of Chemical and Biomolecular Engineering*, 12, 519-541.
22. Radivojević, T., Costello, Z., Workman, K., & Garcia Martin, H. (2020). A machine learning Automated Recommendation Tool for synthetic biology. *Nature communications*, 11(1), 4879.
23. Sheng, H., Sun, X., Yan, Y., Yuan, Q., Wang, J., & Shen, X. (2020). Metabolic engineering of microorganisms for the production of flavonoids. *Frontiers in bioengineering and biotechnology*, 8, 589069.
24. Tang, M., You, J., Yang, T., Sun, Q., Jiang, S., Xu, M., ... & Rao, Z. (2024). Application of modern synthetic biology technology in aromatic amino acids and derived compounds biosynthesis. *Bioresource Technology*, 406, 131050.
25. Tariq, H., Asif, S., Andleeb, A., Hano, C., & Abbasi, B. H. (2023). Flavonoid production: current trends in plant

- metabolic engineering and de novo microbial production. *Metabolites*, 13(1), 124.
26. Wang, R., Liu, X., Lv, B., Sun, W., & Li, C. (2023). Designing intracellular compartments for efficient engineered microbial cell factories. *ACS Synthetic Biology*, 12(5), 1378-1395.