De Novo Biosynthesis of Sakuranetin from Glucose by Engineered Saccharomyces cerevisiae

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Abstract

Sakuranetin is a plant-natural product, which has increasingly been utilized in cosmetic and pharmaceutical industries for its extensive anti-inflammatory, anti-tumor, and immunomodulatory effects. Sakuranetin was mostly produced via extraction technology from plants, which is limited to natural conditions and biomass supply. In this study, a novel strategy to produce sakuranetin via *de novo* synthesis from glucose by engineering *S. cerevisiae* was introduced . After a series of heterogenous genes integration, a biosynthetic pathway of sakuranetin from glucose was successfully constructed in *S. cerevisiae* which sakuranetin yield reached only 4.28 mg/L. Then, a multi-module metabolic engineering strategy was applied for improving sakuranetin yield in *S. cerevisiae*: (1) adjusting the copy number of sakuranetin synthesis genes; (2) removing the rate-limiting factor of aromatic amino pathway and optimizing the synthetic pathway of aromatic amino acids to enhance the supply of carbon flux for sakuranetin; (3) introducing acetyl-CoA carboxylase mutants $ACC1^{S659A,\ S1157A,}$ and knocking-out *YPL062W* to strengthen the supply of malonyl-CoA which is another synthetic precursor of sakuranetin. The resultant mutant *S. cerevisiae* exhibited a more than 10-fold increase of sakuranetin titer (50.62 mg/L) in shaking flasks. Furthermore, the sakuranetin titer increased to 158.65 mg/L in a 1-L bioreactor, which is the highest sakuranetin titer among all publications reported yield of the engineered microbial cell.

De Novo Biosynthesis ofSakuranetin from Glucose by Engineered *Saccharomyces cerevisiae*

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Abstract: Sakuranetin is a plant-natural product, which has increasingly been utilized in cosmetic and pharmaceutical industries for its extensive anti-inflammatory, anti-tumor, and immunomodulatory effects. Sakuranetin was mostly produced via extraction technology from plants, which is limited to natural conditions and biomass supply. In this study, a novel strategy to produce sakuranetin via *de novo*synthesis from glucose by engineering *S. cerevisiae* was introduced. After a series of heterogenous genes integration, a

biosynthetic pathway of sakuranetin from glucose was successfully constructed in *S. cerevisiae* which sakuranetin yield reached only 4.28 mg/L. Then, a multi-module metabolic engineering strategy was applied for improving sakuranetin yield in *S. cerevisiae* : (1) adjusting the copy number of sakuranetin synthesis genes; (2) removing the rate-limiting factor of aromatic amino pathway and optimizing the synthetic pathway of aromatic amino acids to enhance the supply of carbon flux for sakuranetin; (3) introducing acetyl-CoA carboxylase mutants $ACC1^{S659A,\ S1157A,}$ and knocking-out *YPL062W* to strengthen the supply of malonyl-CoA which is another synthetic precursor of sakuranetin. The resultant mutant *S. cerevisiae* exhibited a more than 10-fold increase of sakuranetin titer (50.62 mg/L) in shaking flasks. Furthermore, the sakuranetin titer increased to 158.65 mg/L in a 1-L bioreactor, which is the highest sakuranetin titer among all publications reported yield of the engineered microbial cell.

Keywords: Sakuranetin; *Saccharomyces cerevisiae* ; De novo biosynthesis; Pathway balancing; Metabolic engineering

Introduction

Flavonoids, which are well-known plant secondary metabolites, have anti-oxidant, anti-cancer, anti-aging, and antimicrobial effects (Benkherouf et al., 2019; Berim & Gang, 2016; Li et al., 2018; Stompor et al., 2019; Stompor & Zarowska, 2016). The majority of flavonoids are produced via extraction technology from plants, which is limited to natural conditions and biomass supply and is not stable and uniform for useful flavonoid production (Newman & Cragg, 2007). Therefore, it is necessary to develop effective microbial systems for the synthesis of flavonoids via metabolic engineering (Pirie et al., 2013).

Sakuranetin (chemical name: 4 ' ,5-dihydroxy-7-methoxyflavanone) is a dihydroflavonoid compound originally separated from the bark of the cherry tree (Asahina, 1908). Current studies have found that sakuranetin has anti-inflammatory activity (Kim & Kang, 2016), anti-tumor (Chen et al., 2016), and antimicrobial effects (Grecco et al., 2014), especially has a therapeutic effect on asthma (Sakoda et al., 2016; Santana et al., 2019), exhibiting a wide range of medicinal application potential. Meanwhile, it can effectively resist melanin deposition and improve the dullness of the skin, and play a role in whitening and rejuvenating the skin due to its high antioxidant activity (Stompor, 2020).

So far, there have been few reports on the synthesis of sakuranetin in microorganisms. However, the synthesis of its precursor naringenin has been widely studied in *S. cerevisiae* and *Escherichia coli* . The biosynthetic pathway of naringenin begins with aromatic amino acids and is primarily mediated by four enzymes: tyrosine ammonia lyase (TAL) or phenylalanine ammonia-lyase (PAL), 4-coumaric acid-CoA ligase (4CL), chalcone synthase (CHS), and chalcone isomerase (CHI) (Winkel-Shirley, 2001). A complete synthetic pathway of naringenin by introducing the above four genes (*PAL* , *4CL* , *CHS*, and *CHI*) and cinnamic acid monooxygenase (C4H) was constructed in *S. cerevisiae* for naringenin production via the phenylalanine pathway, the yield of naringenin increased to 54.4 mg/L in shaking flask cultures (Koopman et al., 2012). Four genes (*TAL* , *4CL* , *CHS* , and *CHI*) were integrated into *S. cerevisiae* for naringenin production via the tyrosine pathway, and the yield of naringenin reached 648.63 mg/L via fed-batch fermentation by the addition of*p* -coumaric acid (Gao, Lyu, et al., 2020). The naringenin yield reached 100.64 mg/L starting with glucose in *E. coli* via a multi-module metabolic engineering strategy including (1) increasing the number of gene copies of the naringenin synthesis pathway, and (2) regulating the expression intensity of the promoter (Wu et al., 2014). The yield of naringenin reached 90 mg/L via metabolic engineering of precursor supply and promoter control in *S. cerevisiae* (Lyu et al., 2017). Furthermore, a fatty acid catabolic pathway was systematically designed for the synthesis of naringenin in *S. cerevisiae* , and the naringenin yield reached 1.13 g/L in a 5-L bioreactor (Zhang et al., 2021). After efficient pathway optimization via promoter engineering based on the promoter library in *S. cerevisiae* , a high naringenin production of 1.21 g/L from*p* -coumaric acid was achieved in a 5-L bioreactor (Gao, Zhou, et al., 2020).

After the key enzyme from naringenin to sakuranetin in rice, naringenin-7-O-methyltransferase (NOMT), was identified (Shimizu et al., 2012). Several genes including *NOMT* were introduced for sakuranetin synthesis in *E. coli* , and the sakuranetin production in the shaking flask reached 40.1 mg/L (Kim et al., 2013). A

two-module co-culture strategy of p -coumaric acid and sakuranetin was designed in *E. coli* , and the yield of sakuranetin reached 79 mg/L in a 2.5-L bioreactor (Wang et al., 2020). However, there are few reports on sakuranetin synthesis in*S. cerevisiae.* Given its higher ability to produce SAM than that of prokaryotes, *S. cerevisiae* has a natural advantage as a chassis cell to produce sakuranetin.

In this study, we proposed to construct a pathway for *de novo*production of sakuranetin from glucose in *S. cerevisiae* (Fig. 1) and strengthen the biosynthesis of sakuranetin via a multi-modules metabolic engineering, including (1) enhancing sakuranetin biosynthesis by adjusting the genes copy number; (2) removing the rate-limiting factor of aromatic amino pathway and optimizing the synthetic pathway of aromatic amino acids (L-Phe and L-Tyr) to enhance the supply of carbon flux of sakuranetin; and (3) introducing acetyl-CoA carboxylase mutants$ACC1^{S659A,\ S1157A}$ and knocking-out*YPL062W* to strengthen the supply of malonyl-CoA, another synthetic precursor of sakuranetin. The resultant mutant *S. cerevisiae* exhibited a more than 10-fold increase of sakuranetin at a titer of 50.62 mg/L in shake-flask cultures and 158.65 mg/L in a 1-L bioreactor, respectively.

2. Material and methods

2.1 Gene amplification and plasmid construction

Escherichia coli DH5α was employed for the replication and construction of plasmids. The information on all yeast strains and plasmids utilized in this research are presented in Table 1 and Table S1. All genes utilized in this research presented in Table S2, including*HaTAL* (from *Hemiphyllodactylus aurantiacus*), *MtPDH1*(from *Medicago truncatula*), *OsNOMT* (from *Oryza sativa*), *PhCHS* (from *Petunia hybrida*), and *MsCHI*(from *Medicago sativa*). All primers used in this study have been listed in Table S3. The plasmids of pH1, pH2, pH3, pH4, pH5, and pH6 (Lian & Zhao, 2015) were utilized as empty vectors for PCR amplification of all native promoters, genes, and terminators in*S. cerevisiae* CEN.PK2-1C. Codon-optimized heterologous genes were amplified by PCR using synthetic fragments or available plasmids. Genes of *AtPAL2* , *AtC4H, AtATR2,* and *At4CL1* were amplified from plasmids of pH1-AtPAL2, pH2-AtC4H, pCfB2767, and pCfB2584, respectively.*EcaroL* and *HaTAL* were amplified from *E. coli*genomic DNA and plasmid of pTAL. The mutant genes of$ARO4^{K229L}$, $ARO7^{G141S}$, and $ACC1^{\ S659A,\ S1157A}$ were obtained by overlap extension PCR.

These candidate genes, promoters, or terminators were then cloned into template plasmids (pH1, pH2, pH3, pH4, pH5, pH6) (Lian & Zhao, 2015), using restricted ligation or Gibson assembly to produce gene cassette plasmids. In addition, these gene cassettes are amplified and assembled by using DNA assembly methods into multi-gene pathways. Then they were integrated into genomic locis where heterologous genes could be efficiently and stably expressed (Apel et al., 2017). Using plasmid pKan10-ADE2.1 (Lian et al., 2018) as a template, all guide RNA (gRNA) plasmids were constructed via Gibson assembly with the corresponding primers. Guide RNAs and integration sites were designed on E-CRISP website (*http://www.e-crisp.org*) (Heigwer et al., 2014) and listed in Table S4.

2.3 Strain construction and cultivation conditions

All strains used or constructed were listed in Table 1, gene knockout and insertion of DNA fragments in *Saccharomyces cerevisiae* were operated by CRISPR/Cas9 system (Stovicek et al., 2015). YT00 (CEN.PK2-1C, *IX1* :: *TEFp-SpCas9-ADH2t*) (Xiao et al., 2022) was adopted as the host strain for the integration of sakuranetin synthesis pathway genes. LiAc/ssDNA/PEG method was used to co-transform an equal amount of purified linearized fragments (50-100 ng/kb) with the corresponding gRNA plasmids (˜300-500 ng) into *S. cerevisiae* , and YPD agar plate containing 200 μg/mL G418 was used for the selection of the resulting strains. The clones were selected to verify whether knockout or integrate into the corresponding position of genome by PCR. And then, the right module-integrated clones were cultured in YPD for an entire night before being streaked onto plates without antibiotics to loop out of gRNA vectors.

E. coli was cultivated at 37 in Luria-Bertani medium containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 100 μg/mL of ampicillin or kanamycin if necessary. *S. cerevisiae* was cultivated in YPD

medium containing 20 g/L peptones, 10 g/L yeast extract, 20 g/L glucose, and 200 μg/mL of G418 for the selection of engineered strains. For shaking-flasks fermentation, the engineered yeast strains were selected to cultivate in 5 mL YPD tubes under 30°C and 220 rpm. The broth was then inoculated into 50 mL of basic medium with an initial OD_{660} of 0.05, and incubated under 30°C and 220 rpm.

For bioreactor cultivation, the selected yeast strain was incubated into a 5 mL YPD tube at 30, 220 rpm for 24h, 4% of seed solution was then transferred to 250 mL flasks, which contains 50 mL of YPD medium, and cultivated for 22 h. The obtained culture was transferred to a 1-L bioreactor containing 500 mL of YPD medium (2%) with an initial OD_{660} of 0.68. The fermentation was carried out in a bioreactor at 30 C, 400 rpm, with the pH maintained at 5.5, and the airflow rate was 1 L/min by automatic addition of control. The concentration of sakuranetin and OD_{660} was determined by regular sampling during fermentation.

2.4 Analysis of the metabolites

To determine the concentration of *p* -coumaric acid, naringenin, and sakuranetin, 800 μL ethyl acetate was added to 800 μL *S. cerevisiae* culture. After mixing by vortex and centrifugation at 12,000 rpm for 10 min, the top layer of 400 μL (ethyl acetate organic phase) was transferred to a 2 mL centrifuge tube. Then 400 μL of ethyl acetate was added to the remaining *S. cerevisiae* culture for the second extraction. A total of 800 μL ethyl acetate organic phase extracts were mixed and concentrated in a vacuum centrifuge at 50 °C for 40 min. Then the dry residue was dissolved in 800 μl methanol. Before HPLC analysis, the samples were filtered via a 0.22 μm organic filter membrane.

HPLC analysis was performed using Agilent 1260 HPLC system and a C18 column (250×4.6 mm, 5 μm). The mobile phases consisted of solvent A (0.2% acetic acid in water) and B (0.2% acetic acid in methanol). The gradient program was performed as follows: 0.00-10.00 min:75-25% B;10.00-20.00 min:25% B; 20.00-23.00 min:25%-75% B;23.00-25.00 min: 75% B. The flow rate was 1mL/min and the injection volume was 10 μL with stable column temperature at 30, *p* -coumaric acid, naringenin, and sakuranetin were detected at 8.2 min (308 nm), 11.5 min (288 nm), and 13.9 min (288 nm), respectively. Thermo Ultimate 3000 UPLC equipped with a mass spectrometer (LC-MS) was used to analyze the mass values of sakuranetin. The mass spectra data were processed and analyzed by using the software UniDec (Marty et al., 2015) to produce meaningful mass distributions. The optical density at 660 nm was measured by an ultraviolet spectrophotometer to monitor cell concentration. A biosensor analyzer SBA-40D (Shandong, China) was utilized to analyze the concentration of glucose in the supernatant.

3. Results

3.1 Construction of *de novo* sakuranetin biosynthetic pathway in

S. cerevisiae

In *S. cerevisiae* , a novel sakuranetin biosynthetic pathway was designed and constructed to synthesize sakuranetin from glucose, which contained two parallel branching pathways (Fig. 1). One is the formation of *p* -coumaric acid from the L-tyrosine branch catalyzing by tyrosine ammonia lyase, codon-optimized *HaTAL* from *Hemiphyllodactylus aurantiacus* . Another is from the L-phenylalanine branch, which is catalyzed by phenylalanine ammonia-lyase from *Arabidopsis thaliana* (AtPAL2) to generate cinnamic acid, and then cinnamic acid is catalyzed by cinnamate-4-hydroxylase from *Arabidopsis thaliana* (AtC4H), P450 reductase (AtATR2), and yeast native cytochrome b5 (CYB5), to synthesize *p* -coumaric acid.

Then, for sakuranetin synthesis starting from *p* -coumaric acid, 4-coumarate-CoA ligase from *A. thaliana* (At4CL1), chalcone synthase from *Petunia hybrida* (PhCHS), chalcone isomerase from *Medicago sativa* (MsCHI) and naringenin-7-O-methyltransferase from *Oryza sativa* (OsNOMT) were chosen as the target genes to integrate into *S. cerevisiae* . The gene cluster of the abovementioned four enzymes under four constitutive promoters (Fig. 2A), was introduced into strain YT02 (Xiao et al., 2022) (Table 1). The resulting strain YHS01 (YT02, *X3* ::*PGK1p-At4CL1-HXT7t-TPI1p-PhCHS-TPI1t-ENO2p-MsCHI-PGK1t-TEF1p-OsNOMT-TEF1t*) produced 4.28 mg/L sakuranetin (Fig. 2D), indicating that the synthesis pathway of sakuranetin was successfully constructed in *S. cerevisiae.* To improve the expression of target genes responsible for

the transformation process from *p* -coumaric acid to sakuranetin and the yield of sakuranetin, the effect of galactose promoters instead of constitutive promoters was evaluated. However, it is necessary to eliminate the dependence of galactose promoter on expensive inducer galactose, while maintaining a high expression of target genes under a *GAL*regulation system (Xie et al., 2015). Thus, *GAL80* was deleted in strain YT02. Then, a series of expression cassettes were constructed and introduced into the YT02 mutant strain which *GAL80* was deleted for the expression of the above four genes (*At4CL1, PhCHS, MsCHI, OsNOMT*) under the control of GAL promoters (Fig. 2B). The resultant strain YHS02 (YT02,*GAL80* ::*ADH1t-At4CL1-GAL10p-GAL7p-PhCHS-TPS1t-PGK1t-MsCHI-GAL2p-GAL1p-OsNOMT-CYC1t*) exhibited increased productivity of sakuranetin (Fig. 2C, D, E), which titer (9.19 mg/L) was twice higher than that of strain YHS01. These results suggested that the yield of sakuranetin modified by *GAL*regulation system is higher than that by constitutive regulation system.

Although Δ*GAL80* does not require galactose for induction, these strains still require the utilization of galactose, Δ*GAL1 /7 /10* can eliminate the utilization of galactose (Westfall et al., 2012). Here, the resulting strain YHS05 (YHS04, Δ*GAL1/7/10*) produced 13.59 mg/L sakuranetin (Fig. 3B) without improvement.

3.2 Enhanced sakuranetin biosynthesis by adjusting the number of gene copies

Although the constructed strain YHS02 can *de novo* synthesize sakuranetin from glucose, the yield is low. To improve the yield of sakuranetin, increasing the number of gene copies of heterologous genes is a feasible strategy for pathway optimization (Lyu et al., 2017). Firstly, one more gene copy of phenylalanine ammonia-lyase (AtPAL2) was introduced into strain YHS02. The resultant strain was named strain YHS03 (YHS02, *HO-1* ::*GPM1p-AtPAL2-ADH1t*), in which sakuranetin titer was 8.24 mg/L. YHS03 exhibited no significant improvement in sakuranetin production. Meanwhile, more carbon flux can flow to *p* -coumaric acid. However, when one more copy of the tyrosine ammonia lyase (HaTAL) was integrated into strain YHS03, the resultant strain YHS04 (YHS03, *XVI1* :: *GAL7p-HaTAL-TPS1t*) produced 17.97 mg/L of sakuranetin (Fig. 3A), which increased by 95.5% compared with YHS02.

After one more copy of chalcone synthase (PhCHS) and naringenin-7-O-methyltransferase (OsNOMT) was introduced into YHS04, the resultant strain YHS07 (YHS04, *X5* ::*TPI1p-PhCHS-TPI1t-TEF1p-OsNOMT-TEF1t*) produced 25.37 mg/L sakuranetin, 41.2% higher than strain YHS04(17.97 mg/L)(Fig. 3A), which indicated increasing copy number of *PhCHS* and *OsNOMT*can improve the yield of sakuranetin efficiently.

3.3 Optimizing the synthetic pathway of aromatic amino acids(L-Phe/L-Tyr)

Phenylalanine and tyrosine, are the direct precursor of sakuranetin biosynthesis. Therefore, relieving feedback inhibition of tyrosine synthesis may release more carbon flux into the sakuranetin pathway.*ARO4* and *ARO7* are feedback inhibition genes in the tyrosine synthesis pathway, it has been reported that feedback-insensitive DAHP synthase ($ARO4^{K229L}$) and chorismate mutase ($ARO7^{G141S}$) can alleviate the feedback inhibition regulation (Liu et al., 2019). Unexpectedly, the resulting strain YHS09 (YHS07, *TRP1* ::*TEF1p-*$ARO4^{K229L}$*-TEF1t-PGK1p-*$ARO7^{G141S}$*-HXT7t*) produced 20.51 mg/L sakuranetin by enhancing the expression level of$ARO4^{K229L}$ and$ARO7^{G141S}$, decreased by 19.1% compared with YHS07 (25.37 mg/L) (Fig. 3B), we speculated that there may be a bottleneck in the synthesis of sakuranetin due to the weak metabolic flux in the downstream pathway. Meanwhile, knocking out of the bypass metabolic flux genes of phenylpyruvate decarboxylase (ARO10) and pyruvate decarboxylase (PDC5), can increase carbon flux into aromatic amino acids (Koopman et al., 2012; Rodriguez et al., 2015). Then,*ARO10* and *PDC5* were deleted respectively in YHS09 and YHS10. The resultant strains, YHS10 (YHS09, ^{ARO10})$and YHS11(YHS10,^{PDC5})produced 24.91mg/L and 26.65mg/L of sakuranetin after 72h culture(Fig.3B), whichindicat$

Endogenous genes of*ARO2/ARO1/PHA2* , MtPDH1 (tyrosine prephenate dehydrogenase from*Medicago truncatula*), and EcaroL (shikimate kinase from *E. coli)* have been demonstrated beneficial to release more carbon flux into aromatic amino acid pathway (Liu et al., 2019). These genes were introduced into strain YHS11 one by one via a series of the expression cassette, the sakuranetin production was improved to 33.05 mg/L, 25.96 mg/L, 26.23 mg/L, 23.89 mg/L, and 43.82 mg/L in the resultant strains YHS12 (YHS11, *XII5* :: *GPDp-ARO2-CYC1t*), YHS13 (YHS11,*ARO1p* :: *GPDp-ARO2-CYC1t-ENO2p*), YHS14 (YHS13,*III1*

:: *GPM1p-PHA2-ADH1t*), YHS15 (YHS14, *X2* ::*FBA1p-MtPDH1-CYC1t*), and YHS16 (YHS15, *HO-2* ::*PDC1p-EcaroL-ADH3t*), respectively (Fig. 3B). The strain YHS16 produced 43.82 mg/L sakuranetin, 64.4% higher than that of YHS11 (26.65 mg/L), which suggested that introducing more carbon flux into the downstream pathway of sakuranetin synthesis could significantly improve the production of sakuranetin.

3.4 Enhancing malonyl-CoA biosynthetic pathway

In a previous publication, it was reported that the supply of malonyl-CoA is of great importance for flavonoid production in *S. cerevisiae* (Leonard et al., 2007; Zhang et al., 2021). Thus, the effect of malonyl-CoA supply on sakuranetin synthesis was evaluated by two methods. One method is to delete *YPL062W* in *S. cerevisiae* , which can decrease the transcription of downstream gene*ALD6* and increase acetyl-CoA accumulation (Chen et al., 2019; Leonard et al., 2007). However, when *YPL062W* was deleted, the resultant strain YHS17 (YHS16, ^{YPL062W})$produced 35.38 mg/L sakuranetin, which decreased by 19.2\% compared to YHS16 (Fig. 4). This results suggested that the$
$CoA and the weak transformation to downstream malonyl-CoA might occur. Another method is to convert more acetate-$
$CoA to malonyl-CoA. Acetyl-CoA carboxylase (ACC1) can catalyze acetyl-CoA to malonyl-$
$CoA in S. cerevisiae, expression of a double-point mutant at Ser659 and Ser1157 (ACC1^{S659A,\ S1157A}) has been verified to increas$
$CoA production (Ferreira et al., 2018; Sun et al., 2019). Thus, one copy of ACC1^{S659A,\ S1157A} was further introduced into the biosy$
$CoA to malonyl-CoA, YHS18 (YHS16, ^{YPL062W} :: PGK1t-ACC1^{S659A,\ S1157A}\text{-}GAL2p) produced 50.62 mg/L of sakuranetin, i$
$CoA was beneficial for sakuranetin production.$

3.5 Production of sakuranetin in a 1-L bioreactor fermentation

To scale up sakuranetin production, the engineered strain YHS16 was selected for fermentation in a 1-L bioreactor with 500 mL YPD medium. As shown in Fig. 5, the cell densities increased gradually with the prolongation, and the broth OD_{660} reached a maximum of 25.7 after 46 h fermentation, while sakuranetin titer increased continuously and finally reached 158.65 mg/L after 70 h. To our knowledge, it is the highest titer of sakuranetin via microbial cell factories method among all in the past publications.

4. Discussion

The heterologous biosynthesis of flavonoids has attracted increasing researchers and significant progress has been achieved (Shah et al., 2019). However, sakuranetin is one natural flavonoid, and few reports about its microbial synthesis were found in the previous publication. Only *Escherichia coli* was reported as the chassis cell for sakuranetin synthesis with low yield. Few reports were found on the synthesis of sakuranetin in *S. cerevisiae* . Here, the biosynthetic pathway of sakuranetin from glucose was constructed in *S. cerevisiae* and the yield of sakuranetin was improved through a multi-modules strategy.

We first constructed a complete sakuranetin synthetic pathway from glucose (*AtPAL2, AtC4H* , *AtATR2* , *At4CL1* ,*PhCHS* , *MsCHI* , and *OsNOMT*) in *S. cerevisiae* . The resultant strain of YHS01 successfully achieved de novo biosynthesis of sakuranetin. Furthermore, we compared the ability of the constitutive regulation system and modified *GAL* regulation system to synthesize sakuranetin. The result showed that the yield of sakuranetin in *GAL* regulation system YHS02 (9.19 mg/L) was two times higher than that of constitutive regulation system YHS01 (4.28 mg/L), which showed the potential of the *GAL* regulation system in sakuranetin production.

In module 1, the pathway genes of sakuranetin synthesis (*AtPAL2* ,*AtC4H* , *HaTAL* , *PhCHS* , and *OsNOMT*) were enhanced to further strengthen the metabolic flow. The sakuranetin yield of the engineered strain YHS07 is 25.37 mg/L, which is 2.76 times compared with YHS02. It was confirmed that increasing the copy number of key enzyme genes makes a significant improvement in sakuranetin production.

Optimizing the synthetic pathway of aromatic amino acids can introduce more carbon flux into the downstream pathway (Liu et al., 2019). In module 2, we knocked out the bypass metabolic flux genes of *ARO10*and *PDC5* and further removed the rate-limiting factors to enhance the supply of the precursor *p* -coumaric acid, including enhancing the expression of endogenous genes $ARO4^{K229L}$,$ARO7^{G141S}$, *ARO1* , *ARO2* ,*PHA2,* exogenous genes *MtPDH1* and *EcaroL* . The accumulation of sakuranetin reached 43.82 mg/L in strain

YHS16, which increased by 72.7% compared with YHS07 (25.37 mg/L). This result confirmed that introducing more carbon flux into the downstream pathway could improve the yield of sakuranetin, but the improvement effect is limited. This may result from the weakness of the downstream metabolic flow of the sakuranetin synthesis pathway.

Another crucial precursor of flavonoids is malonyl-CoA, which is of great significance for flavonoid production (Zhang et al., 2021). In module 3, we aimed to enhance the supply of precursor malonyl-CoA by deleting *YPL062W* and introducing$ACC1^{S659A,\ S1157A}$. The yield of sakuranetin in the resultant strain YHS18 increased to 50.62 mg/L, suggesting the supply of malonyl-CoA was beneficial to sakuranetin production. However, the production of p -coumaric acid and naringenin accumulated significantly, resulting in the inhibition of conversion to sakuranetin effectively.

The sakuranetin pathway genes were transformed into *Escherichia coli* via plasmids and the resultant recombinant strains produced 40.1 mg/L (Kim et al., 2013) and 79 mg/L (Wang et al., 2020) sakuranetin in shaking flask and a 2.5-L bioreactor, respectively. Whereas, these could result in issues like plasmid instability, excessive metabolic pressure, the requirement of selective burden, and the synthesis of sakuranetin in*S. cerevisiae* has not been reported so far. In comparison to prior publications on engineering *Escherichia coli* for the systhesis of sakuranetin, we realized de novo synthesis of sakuranetin with the pathway genes inserted into the genome of *S. cerevisiae*, which was stable without selective pressure. Using the strategy of metabolic engineering of multiple modules, the yield of sakuranetin increased by 10.8-fold. The best-performing mutant strain exhibited enhanced sakuranetin production at a titer of 50.62 mg/L in shaking flask cultures and 158.65 mg/L in a 1-L bioreactor, which is the highest reported sakuranetin production in microbial cell factories. This study also established the foundation for the biosynthesis of sakuranetin and its derived metabolites.

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Conflict of interests

The authors declare no conflicts of interest.

Author contributions

Shuai Tu was responsible for drafting the manuscript, performing experiments, data analysis, and revision. Feng Xiao was responsible for the acquisition of metabolomics data, study conception, and design. Chengyu Mei and Shuang Li reviewed and revised the manuscript. Pei Qiao guided data processing and manuscript revision. Ziyan Huang and Zhixing Gong performed experiments. Weihong Zhong was responsible for funding acquisition, project administration, supervision, and revision.

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