De novo biosynthesis of complex natural product sakuranetin using modular co-culture engineering

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Abstract

Flavonoids are a large family of plant and fungal natural products, among which many have been found to possess outstanding biological activities. Utilization of engineered microbes as surrogate hosts for heterologous biosynthesis of flavonoids has been investigated extensively. However, current microbial biosynthesis strategies mostly rely on using one microbial strain to accommodate the long and complicated flavonoid pathways, which presents a major challenge for production optimization. Here, we adapt the emerging modular co-culture engineering approach to rationally design, establish and optimize a microbial *E. coli*-*E. coli* co-culture for *de novo* biosynthesis of flavonoid sakuranetin from simple carbon substrate glucose. Specifically, two *E. coli* strains were employed to accommodate the sakuranetin biosynthesis pathway. The upstream strain was engineered for pathway intermediate *p*-coumaric acid production, whereas the downstream strain converted *p*-coumaric acid to sakuranetin. Through step-wise optimization of the co-culture system, we were able to produce 29.7 mg/L sakuranetin from 5 g/L glucose within 48 h, which is significantly higher than the production by the conventional monoculture-based approach. The co-culture biosynthesis was successfully scaled up in a fed-batch bioreactor, resulting in the production of 79.0 mg/L sakuranetin. To our knowledge, this is the highest bioproduction concentration reported so far for *de novo* sakuranetin biosynthesis using the heterologous host *E. coli*. The findings of this work expand the applicability of modular co-culture engineering for addressing the challenges associated with heterologous biosynthesis of complex natural products.

Keywords: complex natural product, sakuranetin, *E. coli*, modular co-culture engineering, *de novo* biosynthesis

Key points

- *De novo* biosynthesis of sakuranetin was achieved using *E. coli-E. coli* co-cultures.
- Sakuranetin production by co-cultures was significantly higher than the mono-culture controls.
- The co-culture system was optimized by multiple metabolic engineering strategies.
- The co-culture biosynthesis was scaled up in fed-batch bioreactor.

Introduction

Engineering rationally designed microbial co-cultures has recently emerged as an effective method for biosynthesis of various biochemicals (Jawed et al. 2019; Jones and Wang 2018; Roell et al. 2019). In particular, modularization of the biosynthesis pathway in the context of co-cultures, referred to as modular co-culture engineering, has been widely used to biosynthesize products ranging from simple commodity chemicals to complex nutraceuticals (Chen et al. 2019; Zhang and Wang 2016). Compared with the conventional mono-culture approach, modular co-culture engineering harnesses the power of two or more microbial strains for reconstitution of a target biosynthesis pathway. As such, it largely diminished the biosynthesis labor on each strain and reduced the associated metabolic burden (Wu et al. 2016). Also, the balancing between the pathway modules for biosynthesis optimization can be achieved by conveniently changing the ratio of the co-culture subpopulations. These advantages are more outstanding for biosynthesis of complex natural products which involves long and complicated pathways and requires extensive and yet delicate engineering efforts. To this end, considerable progress has been made in recent years to utilize modular co-culture engineering approaches for overcoming the challenges of complex natural products biosynthesis. For example, Jones *et al*. developed an *E. coli*- *E. coli* co-culture to produce flavan-3-ols with biosynthesis performance 970-fold higher than the mono-culture approach (Jones et al. 2016). Li *et al*. adapted a co-culture system with three *E. coli* strains and increased the rosmarinic acid biosynthesis by 38-fold (Li et al. 2019). Jones *et al*. constructed an *E. coli* poly-culture consisting of four strains and achieved *de novo* production of anthocyanins from glucose (Jones et al. 2017). Similar strategies had been used for biosynthesis of naringenin, resveratrol, resveratrol glucosides and apigetrin (Camacho-Zaragoza et al. 2016; Ganesan et al. 2017; Thuan et al. 2018a; Thuan et al. 2018b). Efforts had also been made for using cross-species

co-cultures to combine the biosynthetic powers of the recruited microbes to serve the need of biosynthesis. For instance, Zhou *et al*. developed *E. coli*-*S. cerevisiase* co-culture systems that showed much stronger capabilities for biosynthesis of oxygenated taxanes, tanshinone precursors and functionalized sesquiterpenes (Zhou et al. 2015). In another study, Sgobba *et al*. utilized *C. glutamicum*-*E. coli* co-cultures to achieve biosynthesis of cadaverine and pipecolic acid from starch (Sgobba et al. 2018).

These previous achievements provide strong incentive to leverage modular co-culture engineering approach to facilitate biosynthesis of other challenging natural products. In the present study, we designed, constructed and optimized an *E. coli*-*E. coli* co-culture for high efficiency biosynthesis of sakuranetin using glucose as the carbon substrate. Sakuranetin is a complex natural product belonging to the flavonoid family. This molecule is naturally produced by some plants, such as some shrubs and rice, as a phytoalexin for pathogen infection. Sakuranetin had been found to possess various biological activities, including antimicrobial, anti-inflammatory, antimutagenic, anti-Helicobacter pylori, antileishmanial and antitrypanosomal activities (Grecco et al. 2012; Miyazawa et al. 2003; Zhang et al. 2008; Zhang et al. 2006). Due to its potential values for nutraceutical and pharmaceutical markets, it is of great research interest to develop robust methods for high efficiency biosynthesis of sakuranetin. In particular, to circumvent the issue of low growth rate and product titer using the native sakuranetin producers, utilization of genetically tractable microorganisms as heterologous hosts for *de novo* sakuranetin production holds strong research and application significance.

The sakuranetin biosynthetic pathway is shown in Fig. 1. Carbon substrate, such as glucose, is first utilized to make amino acid tyrosine through the tyrosine pathway. Subsequently, tyrosine is converted to *p*-coumaric acid by enzyme tyrosine ammonia lyase (TAL). *p*-coumaric acid is then

used as the precursor to produce naringenin through a series of enzymatic steps involving 4-coumarate-CoA ligase (4CL), malonate synthetase (MatB), malonate carrier protein (MatC), chalcone synthase (CHS) and chalcone isomerase (CHI). Finally, naringenin is o-methylated by naringenin 7-O-methyltransferase (NOMT) to generate the product sakuranetin. Overall, there are seven heterologous enzymes required for the sakuranetin biosynthesis in *E. coli*. In a previous study, Kim *et al*. engineered bacterium *E. coli* to reconstitute the sakuranetin biosynthesis pathway using the conventional mono-culture approach and achieved 40.1 mg/L sakuranetin production (Kim et al. 2013). In this work, we adapted a co-culture composed of two *E. coli* strains to further improve the sakuranetin biosynthesis. Several metabolic engineering approaches were employed to modify the co-culture system for biosynthesis optimization. Bioprocess engineering techniques were also used to scale up the bioproduction in a 2.5-L bioreactor to demonstrate the scalability of the developed co-culture.

Materials and Methods

Plasmids and strains construction

Strains and plasmids used in this work are listed in Table 1. *E. coli* DH5α (New England Biolabs, USA) was adopted for DNA cloning. Sequences of the primers used in this study are listed in Supplementary Information (Table S1). The NOMT gene from *Oryza sativa* (Shimadzu et al. 2013) was codon-optimized for *E. coli* expression and synthesized by Bio basic Inc., New York. The At4CL gene from *Arabidopsis thaliana* (Lim et al. 2011) was synthesized by Bio basic Inc., New York. The NOMT gene and At4CL gene were deposited into GenBank with the accession number MT127797 and MT134263. Nucleotide sequences of NOMT gene and At4CL gene are provided in the Supplementary Information (Table S2). DNA purification and plasmids isolation used kits

from Zymo Research (Irvine, CA, USA). All restriction enzymes, DNA ligase, Q5 Master-mix enzymes and Gibson assembly builder were purchased from New England Biolabs. All cloning procedures were performed according to the manufacturer's protocols. Detail information for plasmid construction is described as follows.

To construct pRS1, a DNA fragment containing the codon-optimized NOMT gene (Shimizu et al. 2012) was synthesized (Bio Basic Inc. New York), PCR amplified with primers NOMT-F and NOMT-R and digested by *Nco*I and *Bam*HI, following by ligation with pRSFDuet-1 treated with the same restriction enzymes. To construct pRS2, a previously constructed plasmid pRP5 (Li et al. 2019) was digested by *Nde*I and *Xho*I to isolate the DNA fragment containing the Pc4CL gene, which was then ligated with pRS1 treated with the same enzymes. To construct pRS3, a DNA fragment containing the At4CL gene (Lim et al. 2011) was synthesized (Bio Basic Inc. New York), digested by *Nde*I and *Xho*I, and ligated with the plasmid pRS1 treated with the same restriction enzymes. To construct pCS1, the P*proD* promoter was PCR amplified from pBD (Guo et al. 2019) using primer PROD-F and PROD-R, digested with *Hin*dIII/*Nde*I, and then ligated with plasmid pETDuet-1 treated with the same enzymes. To construct pCS2, the P*pdc* promoter was obtained by digestion of plasmid pTY5 (Wang et al. 2019) using *Eco*NI/*Nco*I, and then ligated with plasmid pCS1 treated with the same enzymes. To construct pCS3, the CHI gene was PCR amplified from pOM-PhCHS-MsCHI (Santos et al. 2011) using primer CHI-F and CHI-R, digested with *Nco*I/*Eco*RI, and then ligated with plasmid PCS2 treated with the same enzymes. To construct pCS4, the CHS gene was PCR amplified from pOM-PhCHS-MsCHI (Santos et al. 2011) using primer CHS-F and CHS-R, digested with *Nde*I/*Eco*RV, and then ligated with plasmid PCS3 treated with the same enzymes. To construct pTB1, a DNA fragment containing the *aroB* and *aroD* genes

were obtained by digestion of pMM2 (unpublished data) with *Sal*I/*Xho*I, and then ligated with plasmid pCA3 (Zhang and Stephanopoulos 2013) digested with *Sal*I.

Medium and cultivation conditions

Luria-Bertani (LB) medium was used for cell propagation. MY1 medium was used for *p*-coumaric acid and sakuranetin production by *E. coli* (Zhang et al. 2015). One liter of MY1 medium contained 2.0 g of NH_4Cl, 5.0 g of $(NH_4)_2SO_4$, 3.0 g of KH_2PO_4, 7.3 g of K_2HPO_4, 8.4 g of 3-(N-morpholino)-propanesulfonic acid, 0.5 g of NaCl, 0.24 g of $MgSO_4$, 0.5 g of yeast extract, 40 mg of tyrosine, 40 mg of phenylalanine, 40 mg of tryptophan, 10 mg of 4-hydroxybenzoic acid, trace elements and desired amounts of glucose. The working concentrations of trace elements were 0.4 mg/L Na_2EDTA, 0.03 mg/L H_3BO_3, 1 mg/L thiamine, 0.94 mg/L $ZnCl_2$, 0.5 mg/L $CoCl_2$, 0.38 mg/L $CuCl_2$, 1.6 mg/L $MnCl_2$, 3.77 mg/L $CaCl_2$, and 3.6 mg/L $FeCl_2$. Isopropyl beta-D-thiogalactoside (IPTG) was added into the medium at the beginning of cultivation with a final concentration of 0.5 mM. The working concentrations of antibiotics were 50 mg/L for kanamycin, 34 mg/L for chloramphenicol, 100 mg/L for ampicillin and 50 mg/L for streptomycin. 2 g/L malonate was added when necessary.

Both *E. coli* mono-cultures and co-cultures were cultivated in 2 mL MY1 medium in test tubes at 250 rpm. For mono-culture cultivation, 10 % (v/v) overnight LB culture of the desired *E. coli* strains was inoculated into the MY1 medium with proper antibiotics and incubated at 37 ^{o}C for 10 h. The cells were then harvested by centrifugation and re-suspended in the fresh MY1 medium with an initial OD_{600} of 0.5. After 48 h cultivation at desired temperature, the culture samples were taken for HPLC analysis. For *E. coli–E. coli* co-cultures, 10 % (v/v) overnight LB cultures of the desired *E. coli* strains were cultivated in the MY1 medium as seed cultures, respectively. After 10 h growth at 37 ^{o}C, the upstream and downstream cell seed cultures were measured for cell density

(OD_{600}), harvested by centrifugation, and inoculated into the fresh MY1 medium containing appropriate antibiotics at desired ratios. The needed volume for individual seed cultures was calculated based on the specific inoculation ratio. The initial OD_{600} of the co-culture after inoculation was controlled at 0.5, followed by 48 h cultivation at desired temperature.

For fed-batch bioreactor cultivation, the UPBC:DGS3 co-culture was cultivated in a 2.5-L bioreactor (Eppendorf Bioflo 120) containing 1.5 L MY1 medium and 10 g/L glucose. The dissolved oxygen (DO) probe and pH probe were calibrated according to the manufacturer's protocol. As the seed culture, the overnight MY1 culture of the UPBC and DGS3 strains were added to the medium at the ratio of 1:9. The initial OD_{600} of the co-culture after inoculation was 0.5. Fermentation was carried out at 30 °C with an air flow of 1.0 L/min. The pH was maintained at 7.0 by automatic addition of 5 M sodium hydroxide. Foam formation was suppressed by adding Antifoam B (Silicone Emulsion). 400 mL of 50 g/L glucose solution was fed to the bioreactor at a rate of 0.28 mL/min from 12 h to 36 h.

Quantification of metabolite and glucose

To quantify tyrosine, *E. coli* culture samples were centrifuged at 10,000 rpm for 5 min, and the supernatants were filtered through 0.45 mm PTFE membrane syringe filters. 10 μL filtered sample was injected into an Acclaim™ AmG C18 HPLC column connected to a Shimadzu HPLC system with a diode array detector set to a wavelength of 280 nm. The samples were eluted using solvent A (0.5 % acetic acid in water) and solvent B (99.9 % acetonitrile) run at a flow rate of 0.6 mL/min. The following gradient was used for elution: 0 min, 100 % solvent A; 4 min, 80 % solvent A and 20 % solvent B; 7 min, 100 % solvent A; 12 min, 100 % solvent A.

To quantify *p*-coumaric acid, naringenin and sakuranetin, 1 mL ethyl acetate was added to l mL *E. coli* culture. After mixing by vortex and centrifugation at 10,000 rpm for 2 min, the top layer was transferred to a microcentrifuge tube for evaporation to dryness, followed by dissolving with 1 mL methanol. Such prepared samples were analyzed using a Shimadzu HPLC system with an Acclaim™ AmG C18 HPLC column. An isocratic method of 65% solvent A (0.5 % acetic acid in water) and 35% solvent B (99.9 % acetonitrile) running at a flow rate of 0.4 mL/min was used for elution. The UV absorption at 280 nm and 290 nm was measured.

Glucose concentration in the culture was determinate by 3,5-dinitrosalicylic acid (DNS) assay (Miller 1959). Specifically, the *E. coli* culture samples were centrifuged at 10,000 rpm for 5 min. 30 μL of the supernatants were mixed with 60 μL DNS reagent in microcentrifuge tubes and centrifuged at 2000 rpm for 1 min at room temperature. Subsequently, the samples were incubated at 95 C° for 5 minutes and then cooled to 10 C° for 10 min. After centrifugation at 2000 rpm for 1 min, 36 μL of the sample solution was mixed with 160 μL distilled water in a 96-well microplate, followed by another round of centrifugation. Light absorbance at 540 nm was used for the resulting samples.

Determination of strain-to-strain ratio of co-culture

The upstream strain-to-downstream strain ratio of co-culture was determined by the blue-white colony counting method (Zhang et al. 2015). 7 μL of the co-culture sample was diluted 10^6-fold with sterile water and then spread on an LB agar plate containing IPTG and X-Gal. After 12-24 h of incubation at 37 °C, the upstream strain UPBC carrying the disrupted *lacZ* generated white colonies, while the downstream strain DGS3 containing the intact *lacZ* generated blue colonies.

The numbers of the blue and white colonies on the plates were counted for strain-to-strain ratio analysis.

Results

E. coli-E. coli co-culture design and construction

The co-culture design used in this work is shown in Fig. 1. The sakuranetin pathway is divided into two modules for co-culture-based biosynthesis: the upstream module for *p*-coumaric acid provision, and the downstream module for *p*-coumaric acid-to-sakuranetin conversion. Two *E. coli* strains were engineered to accommodate the upstream and downstream pathway modules, respectively. The upstream strain contained an engineered tyrosine pathway as well as the tyrosine ammonia lyase (TAL) for biosynthesis of *p*-coumaric acid from glucose. The downstream strain was engineered to express the heterologous enzymes 4CL, CHS, CHI, MatB, MatC and NOMT for conversion of *p*-coumaric acid to sakuranetin. Notably, functional expression of these enzymes in *E. coli* had been extensively reported before (Jones et al. 2016; Kim et al. 2013; Leonard et al. 2007; Santos et al. 2012), paving the way for the co-culture biosynthesis of this work. The rationale for such division of the pathway is that *p*-coumaric acid is a relatively small molecule and can travel across cell membrane for connecting the separate modules in the context of the co-culture. Once it is CoA-activated to generate coumaroyl-CoA or further converted to naringenin chalcone, the cross-membrane relocation can become an issue for co-culture biosynthesis. Also, using more downstream metabolites as the division node may result in uneven biosynthesis labor between the two modules.

To construct the designed co-culture system, we first attempted to identify suitable strains to accommodate the upstream and downstream pathway modules, respectively. In fact, it had been found that microbial strains of the same species but distinct genetic backgrounds could have vastly different impacts on co-culture biosynthesis performance (Zhang and Stephanopoulos 2016). Therefore, we compared the biosynthetic capabilities of six strains for supporting the upstream pathway module. For the first three strains, *E. coli* P2H is a tyrosine overproducer developed by a previous study (Santos 2010), K12(DE3) is a long-used strain with only minimal genetic modifications, and BL21(DE3) is popularly used for protein expression. The other three strains are derivatives of the first three strains and were engineered to over-express the tyrosine pathway enzymes AroB and AroD. The TAL enzyme from *R. glutinis* was introduced to these 6 strains, respectively, and the resulting strains were cultivated in the MY1 medium containing 5 g/L glucose at 30 °C for *p*-coumaric acid biosynthesis comparison. As shown in Fig. 2A, the final *p*-coumaric acid concentration is considerably different among these strains. Strain UPB1 derived from *E. coli* P2H produced the highest amount of *p*-coumaric acid (265.2 mg/L), which is 4.1-fold higher than the lowest producer UP3. Strain UPB1 was thus selected as the baseline upstream strain for all the following experiments.

Similarly, we compared two strains, BL21(DE3) and BL21-Gold(DE3), for their capabilities to convert *p*-coumaric acid to sakuranetin. These two strains are both commonly used hosts engineered for protein expression, but they differ in that BL21-Gold(DE3) carries a Hte genetic modification that had been found to be helpful for co-culture biosynthesis by potentially facilitating metabolite cross-membrane transportation (Zhang and Stephanopoulos 2016). All involved downstream pathway enzymes were introduced into BL21(DE3) and BL21-Gold (DE3) via plasmid vectors for expression. For enzyme 4CL, two variants derived from *Petroselinum*

crispum and *Arabidopsis thaliana*, respectively, were used to carry out the first step of the downstream pathway module. The four strains resulted from the combinations of two background strains and two 4CL variants were fed with exogenous *p*-coumaric acid and cultivated at 30 °C for sakuranetin biosynthesis comparison. As shown in Fig. 2B, strain DG2 (BL21-Gold(DE3) expressing *Arabidopsis thaliana* 4CL and other downstream pathway enzymes) showed the highest production of 28.1 mg/L sakuranetin from 200 mg/L exogenous *p*-coumaric acid. This strain was thus chosen as the baseline downstream strain for the co-culture biosynthesis of sakuranetin.

After determination of the upstream and downstream strains, the effect of cultivation temperature on these strains' biosynthesis performance was also evaluated, respectively. In fact, although 37 °C is preferred for *E. coli* growth, a lower temperature often reduces the amount of folding stress by reducing the rates of transcription, translation, cell division and protein aggregation, which is often helpful for heterologous enzyme folding and activity (Gasser et al. 2008). We first compared the production of *p*-coumaric acid by the selected upstream strain UPB1 under 25 °C, 30 °C and 37 °C. As shown in Fig. 2C, UPB1 showed the highest *p*-coumaric acid production at 30 °C. Similarly, the conversion efficiency by the downstream strain DG2 under 25 °C, 30 °C and 37 °C was investigated by feeding the strain with 200 mg/L exogenous *p*-coumaric acid. Fig. 2D shows that the optimal temperature for DG2 to produce sakuranetin from *p*-coumaric acid was also 30 °C. Based on these results, 30 °C was selected for the following co-culture biosynthesis experiments.

Optimization of the co-culture strain inoculation ratio for sakuranetin biosynthesis

Next, the selected upstream and downstream strains were engineered to possess the same antibiotic resistance for compatible growth in one culture and the resulting strains UPBC and

DGS1 were co-cultivated together to establish the sakuranetin *de novo* biosynthesis system. In order to coordinate the biosynthetic strengths of these two strains for pathway balancing, UPBC and DGS1 were inoculated at varying ratios ranging from 4:1 to 1:19. As shown in Fig. 3A, the inoculation ratio to a large extent affected the pathway intermediate accumulation and the final product sakuranetin biosynthesis. At the inoculation ratio of 4:1, the biosynthetic strength of the upstream strain was excessively strong, and there was accordingly around 105 mg/L *p*-coumaric acid accumulation. Correspondingly, sakuranetin production was relatively weak (3.1 mg/L), as the downstream strain's population was not sufficient for *p*-coumaric acid-to-sakuranetin conversion. Increase of the downstream strain's inoculum resulted in the better balance between the upstream and downstream pathway modules, leading to reduced *p*-coumaric acid accumulation and improved sakuranetin biosynthesis. At the optimal ratio of 1:9, 11.3 mg/L sakuranetin was produced after 48 h co-culture cultivation. For comparison, the mono-culture control strains SKM1 and SKM5 were constructed by introducing the entire sakuranetin pathway into the upstream and downstream baseline strains, respectively. Under the same cultivation conditions, SKM1 and SKM5 only produced 3.0 mg/L (Fig. 3A) and 5.7 mg/L (Fig. S1) sakuranetin, respectively. The difference between the mono-culture and co-culture biosynthesis performance clearly showed that co-culture engineering is a robust approach for sakuranetin heterologous biosynthesis.

We also investigated the use of another upstream strain UPB2 derived from *E. coli* K12(DE3) for co-culture biosynthesis. As shown in Fig. S1, the sakuranetin production by the UPB2:DGS1 co-culture was also dependent on the inoculation ratio. The highest concentration of sakuranetin (9.2 mg/L at 1:9 ratio) is higher than the mono-culture controls SKM4 and SKM5, but is still lower than the co-culture using UPBC as the upstream strain. This result confirms that *E. coli* UPBC is indeed the best choice to be used as the upstream strain for co-culture biosynthesis.

Improving the malonyl-CoA availability to further increase sakuranetin bioproduction

As reported in previous studies, low level of intracellular malonyl-CoA is a bottleneck step for the flavonoid bioproduction (Leonard et al. 2007; Miyahisa et al. 2005). Therefore, we attempted to engineer the malonyl-CoA availability to further improve the sakuranetin biosynthesis. Specifically, 2 g/L exogenous malonate, the precursor of malonyl-CoA, was supplemented to the co-culture medium. In addition, two heterologous enzymes involved in malonyl-CoA formation, malonate synthetase (MatB) and malonate carrier protein (MatC) from *Rhizobium trifolii*, were over-expressed in *E. coli* using pACYC-MatBC plasmid (Leonard et al. 2008). Based on this strategy, a new co-culture UPBC:DGS2 was developed for sakuranetin biosynthesis. As shown in Fig. 3B, the pathway intermediate naringenin's concentration in the co-culture was increased at all inoculation ratios, clearly indicating that naringenin biosynthesis was strengthened by improved malonyl-CoA provision. Accordingly, the sakuranetin concentration was also increased. The highest production of 23.4 mg/L sakuranetin was achieved at the ratio of 1:9, 109% higher than the production without engineering the malonyl-CoA pathway. In the meantime, the sakuranetin production by the corresponding mono-culture control SKM2 engineered by the same strategy also increased to 9.7 mg/L. These results indicate that the availability of malonyl-CoA indeed plays an important role in sakuranetin biosynthesis. On the other hand, the highest titer of sakuranetin by the UPBC:DGS2 co-culture was still 1.6-fold higher than the SKM2 mono-culture, confirming the advantages of modular co-culture engineering in heterologous biosynthesis of complex natural products.

Strengthening the CHS and CHI expression to enhance sakuranetin bioproduction

After improving the malonyl-CoA availability, we next sought to strengthen the CHS and CHI expression to increase the efficiency of incorporating malonyl-CoA with 4-coumaroyl-CoA for

naringenin formation, which is considered another limiting step for sakuranetin biosynthesis. To this end, instead of putting the CHI and CHS genes in one operon under the control of the *E. coli* P_{GAP} promoter, the CHS and CHI genes were placed under two strong constitutive promoters, the *Z. mobilis* pyruvate decarboxylase promoter P*pdc* and the synthetic P*proD* promoter (Davis et al. 2010), respectively. The resulting new downstream strain DGS3 was then co-cultivated with UPBC for sakuranetin biosynthesis.

As shown in Fig. 3C, the new co-culture system UPBC:DGS3 accumulated considerably lower levels of *p*-coumaric acid at all tested inoculation ratios. In addition, the naringenin accumulation was improved, compared with the UPBC:DGS2 co-culture. It was therefore indicated that the strategy of strengthening the CHS and CHI gene expression indeed enhanced naringenin biosynthesis. More importantly, the new co-culture UPBC:DGS3 produced more sakuranetin product under the same cultivation conditions. The highest production of 29.7 mg/L sakuranetin was achieved at the ratio of 1:9, which was 27% higher than before the CHI and CHS expression was strengthened. The results hereby show that the CHS and CHI expression level was critical for co-culture-based biosynthesis of sakuranetin. Notably, the application of the same strategy in the mono-culture control SKM3 did not generate considerable production improvement (9.7 mg/L to 10.7 mg/L), which may result from the mono-culture strain's insufficient capacity for adequately expressing all heterologous pathway genes and the overwhelming metabolic burden associated with expressing the entire pathway in one strain. Nonetheless, based on the engineering efforts above, the optimized co-culture system was able to produce sakuranetin with significantly higher efficiency than the baseline mono-culture control (29.7 vs 5.7 mg/L).

Re-configuration of co-culture design for sakuranetin production

E. coli BL21-Gold(DE3) is a robust host for expression of heterologous enzymes. In our abovementioned efforts, enzyme TAL was expressed in the upstream strain, whereas the other heterologous enzymes were expressed in the BL21-Gold(DE3)-derived downstream strain. This situation inspired us to investigate whether re-configuration of the co-culture design could further improve the biosynthesis efficiency. In the new system shown in Fig 4A, all heterologous pathway enzymes, including TAL, 4CL, CHI, CHS, MatB, MatC, and NOMT, were expressed in the downstream strain DGS4 that was derived from BL21-Gold(DE3). The upstream strain UPBT with the *E. coli* K12(DE3) background was then dedicated to express only the tyrosine pathway which is native to *E. coli*.

Next, UPBT and DGS4 were inoculated at different ratios for biosynthesis optimization. As shown in Fig. 4B, the highest production of sakuranetin (21.8 mg/L) was achieved when the co-culture strains were inoculated at the ratio of 1:19; this titer is lower than that of UPBC:DGS3 inoculated at 1:9. Moreover, the accumulation of *p*-coumaric acid was similar to that of the UPBC:DGS3 co-culture. These findings indicated that (1) moving TAL to the downstream strain did not significantly increase the *p*-coumaric acid provision, and (2) overall sakuranetin biosynthesis was reduced, which can be due to the negative impacts of burdening the downstream strain with expressing all the heterologous enzymes.

On the other hand, previous studies had demonstrated that coumaroyl-CoA could inhibit the activity of TAL (Santos et al. 2011). As such, putting TAL and coumaroyl-CoA in the same downstream strain can generate undesired crosstalk which accounts for the suboptimal biosynthetic performance of the new co-culture design. Thus, spatial segregation of TAL and coumaroyl-CoA in the co-culture UPBC:DGS3 is beneficial for sakuranetin biosynthesis.

Scale up sakuranetin bioproduction using a fed-batch bioreactor

352 In order to investigate the scalability of the co-culture biosynthesis system, the best performing
353 co-culture UPBC:DGS3 was cultivated in a 2.5-L fed-batch bioreactor. Specifically, the
354 UPBC:DGS3 co-culture was grown in the MY1 medium containing 10 g/L glucose, and 400 mL
355 of 50 g/L glucose was fed to the bioreactor at a rate of 0.28 mL/min starting at 12 h. The dynamic
356 change of the cell density, strain-to-strain ratio and pathway metabolite concentrations were
357 monitored throughout the bioproduction process. As shown in Fig. 5A, glucose in the bioreactor
358 was quickly depleted after 12 h. Even with the exogenous feed, the glucose concentration was still
359 kept at a low level (<0.5 g/L) for the rest of the cultivation time.

360 On the other hand, the co-culture cell density increased exponentially in the first 24 h and then
361 plateaued at OD_{600}=6, despite that there was still residual glucose in the culture. Meanwhile, the
362 percentage of the upstream strain's subpopulation showed steady increase from 10% at 0 h to 38%
363 at 16 h. For the rest of the cultivation period, it fluctuated between 30% and 38%. This growth
364 trend indicates that the upstream co-culture strain grew relatively faster than the downstream strain
365 at the beginning of the cultivation, but the growth advantage disappeared after 16 h. Moreover,
366 although there was dynamic change of glucose accumulation in the culture, the cell density and
367 strain-to-strain ratio remained stable after 28 h. It is therefore indicated that the co-culture
368 population growth is not sensitive to glucose concentration change under the adapted cultivation
369 conditions and that the engineered co-culture had desired population stability after scale-up.

370 The concentration change of the pathway metabolites is shown in Fig. 5B. The sakuranetin
371 concentration was found to increase rapidly in the first 36 h, which overlapped mostly with the co-
372 culture's exponential growth phase. This indicates that the sakuranetin biosynthesis is strongly
373 coupled with the co-culture growth. The highest sakuranetin concentration of 79.0 mg/L was
374 achieved at 72 h. To our knowledge, this is the highest product concentration for *de novo*

biosynthesis of sakuranetin using *E. coli* as a heterologous host. Meanwhile, the accumulation of the pathway intermediates *p*-coumaric acid and naringenin showed different patterns. *p*-coumaric acid concentration was maintained at around 40 mg/L between 8 and 28 h, and then gradually decreased to zero toward the end of bioreactor cultivation. Interestingly, naringenin concentration was kept below 11 mg/L throughout the cultivation. The naringenin accumulation was lower than that of the test tube scale experiment (Fig. 3C), suggesting that the naringenin provision and consumption dynamics in the fed-batch bioreactor was quite different from the test tube scale. Overall, the fed-batch bioreactor cultivation results show that the developed *E. coli*-*E. coli* co-culture can be scaled up for sakuranetin biosynthesis enhancement.

Discussion

Heterologous biosynthesis of flavonoids has received increasing research interest and made significant progress in recent years (Pandey et al. 2016; Shah et al. 2019). Yet, most of the previous efforts were based on utilization of conventional mono-culture engineering strategy and often resulted in suboptimal bioproduction performance due to the complex nature of the flavonoid pathway. In contrast, modular co-culture engineering employs an innovative approach to implement pathway modularization for overcoming the existing challenges, which adds a new dimension for high efficiency flavonoid biosynthesis. For example, the application of the co-culture engineering strategy does not involve modification of any particular pathway enzyme's activity by sophisticated means such as protein engineering. It also does not need coordinated adjustment of gene expression strengths of different pathway modules, which often requires optimization of gene promoters, ribosomal binding sites, or plasmid vector copy number, for pathway balancing. Rather, modular co-culture engineering uses strain-to-strain ratio manipulation to balance the biosynthesis capabilities of different pathway modules and achieve

rational allocation of metabolic resources between them for bioproduction optimization. The sakuranetin biosynthesis improvement shown in this study demonstrates that this co-culture-based approach is straightforward to implement and is indeed effective for complex natural bioproduction.

On the other hand, one inherent challenge for co-culture engineering is maintenance of compatible co-existence of the constituent co-culture strains. Often times, due to difference in genetic background and assigned biosynthetic labor, the constituent co-culture members have uneven growth rates and biosynthesis capabilities. Such imbalance can be compensated through changing the inoculation ratio between the co-culture members at the beginning of the cultivation. For this study, the downstream strain was over-burdened to express six heterologous enzymes. As a result, it needed to be inoculated at a high percentage of 90% of the total inoculum to maintain the biosynthesis capability balance with the upstream strain. From this perspective, it is speculated that further dividing the downstream pathway module and utilizing an additional strain to share the associated metabolic burden could be an effective strategy for biosynthesis enhancement. In fact, previous studies had already shown that recruitment of three strains or four strain poly-culture is a robust approach for complex natural product biosynthesis (Jones et al. 2017; Li et al. 2019). For future efforts in sakuranetin biosynthesis, development and optimization of *E. coli* poly-cultures is one promising direction. Notably, such efforts will also provide a flexible plug-and-play platform for heterologous bioproduction of sakuranetin analogues and derivatives. For example, the last pathway enzyme naringenin 7-O-methyltransferase can be swapped with flavonoid 4'-O-methyltransferase for biosynthesis of isosakuranetin. Within the context of the co-culture system, this can be achieved by replacing the last co-culture strain with a new one over-expressing the desired flavonoid 4'-O-methyltransferase. Importantly, the other co-culture strains

421 do not need to be metabolically re-engineered to adjust the gene expression level for pathway
422 balancing; only inoculation ratio optimization is needed for establishing the new effective platform
423 for isosakuranetin biosynthesis.

424 For cultivation in test tubes, a series of engineering strategies were adapted to modify the co-
425 culture system. Specifically, identification of suitable strains for expression of the upstream and
426 downstream pathway modules, determination of optimal cultivation temperature, variation of the
427 inoculation ratio, enhancement of malonyl-CoA provision, strengthening of naringenin
428 biosynthesis genes' expression, and manipulation of pathway modularization pattern were
429 investigated in a step-wise manner for sakuranetin biosynthesis optimization. As a result, the
430 product concentration was increased from lower than 10 mg/L to 29.7 mg/L.

431 Co-culture biosynthesis using the fed-batch bioreactor further shows interesting dynamics of the
432 co-culture growth and biosynthesis performance. For example, the sakuranetin concentration
433 increased at a steady rate over the first 36 h. During the same period of time, the upstream strain's
434 subpopulation percentage changed from 10% to 35%. This demonstrated that there is not a fixed
435 optimal ratio between the co-culture strains to support the dynamic biosynthesis pathway
436 balancing during the co-culture cultivation. In this regard, the optimized inoculation ratio only
437 defined the initial condition of the relative growth and biosynthesis capacities between the co-
438 culture members; yet, such definition largely affects the subsequent development of the co-culture
439 population composition change and the corresponding behavior of sakuranetin biosynthesis over
440 the entire cultivation period. Therefore, inoculation ratio between the co-culture strains is a critical
441 and probably the most important factor to decide the complex natural product biosynthesis by co-
442 culture engineering. Fortunately, the inoculation ratio is relatively straightforward to manipulate,

443 which just requires mixing and adding of desired amount of inoculum of the co-culture strains to
444 the co-culture medium at the beginning of cultivation.

445 Also, the sakuranetin production in the fed-batch bioreactor plateaued after the pathway
446 intermediate *p*-coumaric acid and naringenin concentration fell to a low level after 36 h. This
447 finding suggests that further improvement of these pathway intermediates' provision can lead to
448 higher sakuranetin biosynthesis. To that end, although this study adapts the strategies of
449 supplementing exogenous malonate as well as strengthening the CHI and CHS genes expression,
450 there are other methods for boosting the naringenin provision to an even higher level for
451 maximizing the co-culture's capability of producing sakuranetin. For example, protein chaperone
452 can be adapted to improve the folding of the heterologous enzymes MatB, MatC, CHS and CHI in
453 the downstream strain and thus increase the naringenin availability and sakuranetin bioproduction.
454 On the other hand, increasing the co-culture's cell density by adding more carbon substrate can
455 also improve the final product concentration, although longer cultivation is needed and the overall
456 production yield may be reduced.

457 Overall, this study establishes a robust co-culture biosynthesis system and achieves *de novo*
458 sakuranetin biosynthesis with the highest concentration reported so far in the literature. Moreover,
459 in all cases, the biosynthesis by the engineered co-culture is consistently higher than the mono-
460 culture control engineered using the same strategies and cultivated under the same conditions. The
461 findings of this work clearly demonstrate the power of modular co-culture engineering and open
462 up new opportunities for further advances in complex natural product biosynthesis.

463

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human participants or animals.

Author Contribution Statement

XW and HZ conceived and designed research. XW, ZL and LP conducted experiments. MK conceived research and contributed experiment resources. XW and HZ analyzed data. ZL and XW wrote the manuscript. All authors read and approved the manuscript.

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598 # Tables

599 Table 1 Plasmids and strains used in this study.

Plasmids	Description	Source
pRSFDuet-1	double T7 promoters, Kan^R	Novagen
pETDuet-1	double T7 promoters, Amp^R	Novagen
pCDFDuet-1	double T7 promoters, Sp^R	Novagen
pACYCDuet-1	double T7 promoters, Cm^R	Novagen
pET28a	T7 promoter, Kan^R	Novagen
pET21c	T7 promoter, Amp^R	Novagen
pCA1	pTrcHis2B carrying codon optimized *R. glutinis* TAL	(Santos et al. 2011)
pBS2	pET28a carrying the *E. coli aroE*, *aroL*, *aroA*, *aroC, tyrA*^{fbr} and *aroG*^{fbr} genes with a proD promoter (P*proD*)	(Li et al. 2019)
pBD	pACYCDuet-1 carrying the *E. coli aroB and aorD* genes with a proD promoter (P*proD*)	(Guo et al. 2019)
pRS1	pRSFDuet-1 carrying codon optimized *O. sativa* NOMT with a T7 promoter	This study
pRS2	pRSFDuet-1 carrying codon optimized *P. crispum* 4CL and *O. sativa* NOMT with double T7 promoters	This study
pRS3	pRSFDuet-1 carrying *A. thaliana* 4CL and *O. sativa* NOMT with double T7 promoters	This study
pOM-*Ph*CHS-*Ms*CHI	pOM carrying *P. hybrida* CHS and *M. sativa* CHI with a P_{GAP} (constitutive) promoter	(Santos et al. 2011)
pACYC-MatBC	pACYCDuet-1 carrying *R. trifolii* MatB and MatC	(Leonard et al. 2008)
pCS1	pETDuet-1 with a T7 promoter and a constitutive a *proD* promoter (P*proD*) promoter	This study

pCS2	pETDuet-1 with a *proD* promoter (P*proD*) and a constitutive Zymomonas mobilis pyruvate decarboxylase promoter (P*pdc*) promoter	This study
pCS3	pETDuet-1 carrying *M. sativa* CHI with a constitutive Zymomonas mobilis pyruvate decarboxylase promoter (P*pdc*) promoter	This study
pCS4	pETDuet-1 carrying *M. sativa* CHI with a constitutive Zymomonas mobilis pyruvate decarboxylase promoter (P*pdc*) promoter and *P. hybrida* CHS with a *proD* promoter (P*proD*)	This study
pCA3	pCDFDuet-1 carrying the codon-optimized *R. glutinis* TAL with a trc promoter	(Zhang and Stephanopoulos 2013)
pTB1	pCDFDuet-1 carrying the codon-optimized *R.glutinis* TAL, *E. coli aroB* and *aroD* genes with a trc promoter	This study
pBS10	pET28a carrying the *E. coli aroE*, *aroL*, *aroA*, *aroC*, $tyrA^{fbr}$ and $aroG^{fbr}$ genes with a proD promoter (P*proD*), Cm^R replacing Kan^R	(Guo et al. 2019)

Strains	**Description**	**Source**
K12 (DE3)	*E. coli* F⁻ lambda- *ilvG- rfb*-50 *rph*-1(DE3)	(Santos et al. 2011)
K12(DE3)Δ*lacZ*	*E. coli* K12(DE3) *ΔlacZ*	This study
P2H	*E. coli* K12(DE3) *ΔpheA ΔtyrR lacZ::*$P_{LtetO-1}$-$tyrA^{fbr}aroG^{fbr}$ *tyrR*::$P_{LtetO-1}$-$tyrA^{fbr}aroG^{fbr}$ hisH(L82R)(DE3)	(Santos 2010)
BL21(DE3)	*E. coli* B F⁻ *ompT hsdS*($r_B^-m_B^-$) dcm^+ *gal* λ(DE3)	Invitrogen
BL21-Gold (DE3)	*E. coli* B F⁻ *ompT hsdS*($r_B^-m_B^-$) dcm^+ Tet^r *gal* λ(DE3) *endA* Hte	Agilent
UP1	P2H harboring pCA1	This study
UP2	K12(DE3)Δ*lacZ* harboring pBS2 and pCA1	This study
UP3	BL21(DE3) harboring pBS2 and pCA1	This study
UPB1	P2H harboring pCA1 and pBD	This study

UPB2	K12(DE3)Δ*lacZ* harboring pBS2, pBD and pCA1	This study
UPB3	BL21(DE3) harboring pBS2, pBD and pCA1	This study
DG1	BL21-Gold(DE3) harboring pRS2 and pOM-*Ph*CHS-*Ms*CHI	This study
DG2	BL21-Gold(DE3) harboring pRS3 and pOM-*Ph*CHS-*Ms*CHI	This study
DS1	BL21(DE3) harboring pRS2 and pOM-*Ph*CHS-*Ms*CHI	This study
DS2	BL21(DE3) harboring pRS3 and pOM-*Ph*CHS-*Ms*CHI	This study
UPBC	P2H harboring pET28a, pCA1 and pBD	This study
UPBT	P2H harboring pBD, pET28a, pET21c and pCDFDuet-1	This study
DGS1	BL21-Gold(DE3) harboring pRS3, pOM-*Ph*CHS-*Ms*CHI and pACYCDuet-1	This study
DGS2	BL21-Gold(DE3) harboring pRS3, pOM-*Ph*CHS-*Ms*CHI and pACYC-MatBC	This study
DGS3	BL21-Gold(DE3) harboring pRS3, pCS4 and pACYC-MatBC	This study
DGS4	BL21-Gold(DE3) harboring pRS3, pCS4, pACYC-MatBC and pCA3	This study
SKM1	P2H harboring pRS3, pOM-*Ph*CHS-*Ms*CHI, pBD and pCA3	This study
SKM2	P2H harboring pRS3, pOM-*Ph*CHS-*Ms*CHI, pACYC-MatBC and pTB1	This study
SKM3	P2H harboring pRS3, pCS4, pACYC-MatBC and pTB1	This study
SKM4	K12(DE3)Δ*lacZ* harboring pRS3, pOM-*Ph*CHS-*Ms*CHI, pBS10 and pTB1	This study
SKM5	BL21-Gold(DE3) harboring pRS3, pOM-*Ph*CHS-*Ms*CHI, pBS10 and pTB1	This study

Figure captions

Fig. 1 Design of the *E. coli-E. coli* co-culture system for sakuranetin biosynthesis. DAHP: 3-deoxy-D-arabino-heptulosonate-7-phosphate; DHQ: 3-dehydroquinate; DHS: 3-dehydroshikimate; SHK: shikimate; S3P: shikimate 3-phosphate; EPSP: 5-enolpyruvylshikimate 3-phosphate; CHR: chorismate; TYR: tyrosine. $AroG^{fbr}$: the feedback-resistant (fbr) derivative of the 3-deoxy-7-phosphoheptulonate synthase; AroB: 3-dehydroquinate synthase; AroD: 3-dehydroquinate dehydratase; AroE: shikimate dehydrogenase; AroL: shikimate kinase 2; AroA: 3-phosphoshikimate 1-carboxyvinyltransferase; AroC: chorismate synthase; TAL: tyrosine ammonia lyase, 4CL: 4-coumarate: CoA ligase, MatB: malonate synthetase, MatC: malonate carrier protein, CHS: chalcone synthase, CHI: chalcone isomerase, NOMT: naringenin 7-O-methyltransferase.

Fig. 2 Identification of the suitable upstream and downstream co-culture strains and optimum cultivation temperature for sakuranetin biosynthesis. (a) Comparison of *p*-coumaric acid biosynthesis by six *E. coli* strains to identify the candidate that can be used as the upstream co-culture strain. (b) Comparison of *p*-coumaric acid to sakuranetin conversion by four *E. coli* strains to identify the candidate that can be used as the downstream co-culture strain. 200 mg/L *p*-coumaric acid was fed to the culture for biosynthesis analysis. (c) The effect of cultivation temperature on the *p*-coumaric acid biosynthesis by strain UPB1. (d) The effect of cultivation temperature on the sakuranetin biosynthesis by strain DG2. 200 mg/L *p*-coumaric acid was fed to the culture for sakuranetin production comparison. The error bars represent standard deviation of the experimental measurements for at least three independent experiments.

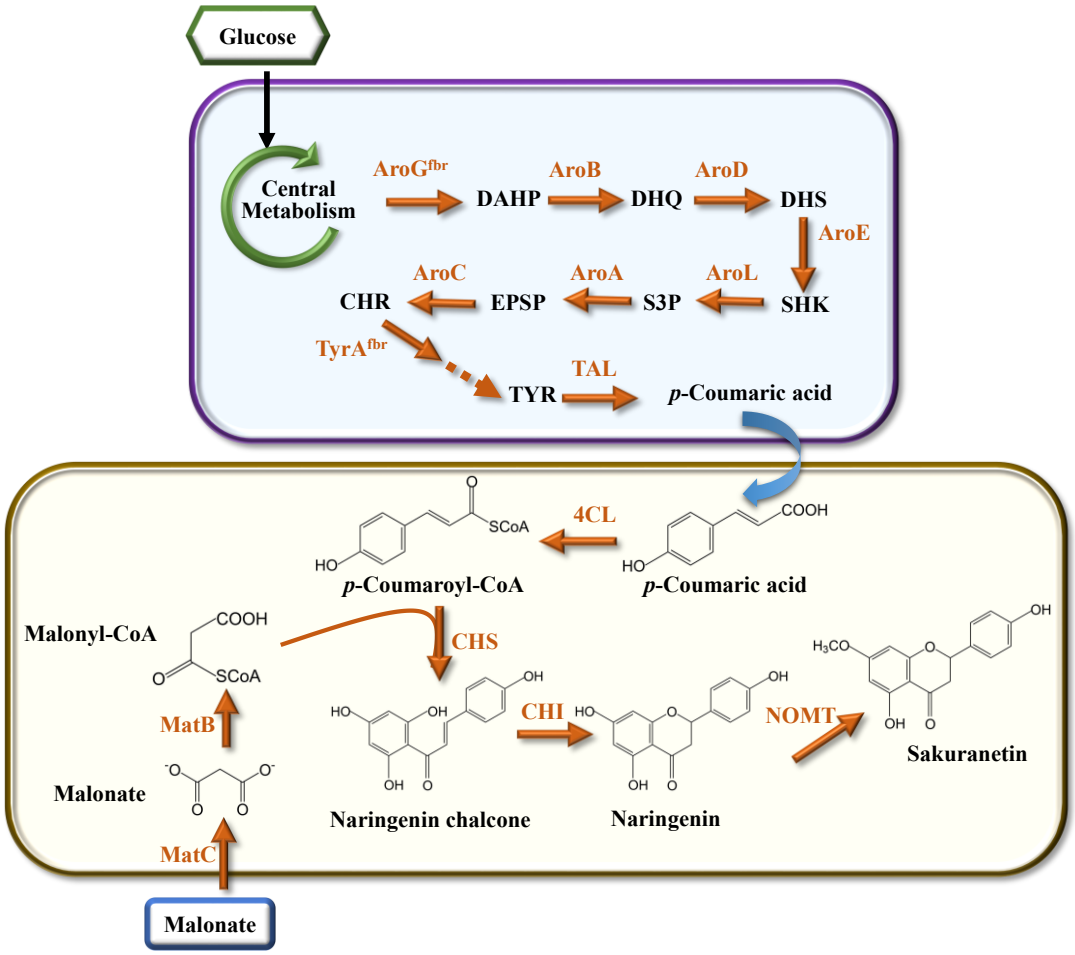
Fig. 3 Sakuranetin biosynthesis performance by the developed co-cultures and corresponding monoculture controls. The inoculation ratio between the upstream and downstream strains were varied for biosynthesis optimization. Strains harboring the whole pathway were utilized as the mono-culture controls. (a) Sakuranetin bioproduction by the UPBC:DGS1 co-culture. (b) Sakuranetin bioproduction by the UPBC:DGS2 co-culture with enhanced the malonyl-CoA availability. 2 g/L exogenous malonate was added to the culture. MatB and MatC were over-expressed in the downstream strain to facilitate the conversion of malonate to malonyl-CoA. (c) Sakuranetin bioproduction by the UPBC:DGS3 co-culture. The expression of the pathway enzymes CHS and CHI was strengthened by placing the two genes under two strong constitutive promoters (P*proD* and P*pdc*), respectively. The error bars represent standard deviation of the experimental measurements for at least three independent experiments.

Fig. 4 Re-configuration of the *E. coli-E. coli* co-culture system for sakuranetin biosynthesis. (a) The schematic of the co-culture design. The upstream strain is a tyrosine over-producer and the downstream strain is specialized in converting tyrosine to sakuranetin. (b) Sakuranetin biosynthesis by UPBT:DGS4 co-culture inoculated at different ratios. The error bars represent standard deviation of the experimental measurements for at least three independent experiments.

Fig. 5 Sakuranetin bioproduction by the UPBC:DGS3 co-culture using a fed-batch bioreactor. Initial glucose concentration was 10 g/L. From 12 h to 36 h, 400 mL of 50 g/L glucose was added to the medium at a flow rate of 0.28 mL/min. (a) The time profiles of co-culture growth and glucose

concentration. (b) The time profile of concentrations of pathway metabolites, including intermediate *p*-coumaric acid, naringenin and product sakuranetin. The error bars represent standard deviation of the experimental measurements for at least two independent experiments.

Figure 1


Glucose
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AroB
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DAHP
DHQ
DHS
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AroC
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AroL
CHR
EPSP
S3P
SHK
TyrA^fbr
TAL
TYR
p-Coumaric acid
4CL
p-Coumaroyl-CoA
p-Coumaric acid
Malonyl-CoA
CHS
MatB
Malonate
CHI
NOMT
Naringenin chalcone
Naringenin
Sakuranetin
MatC
Malonate

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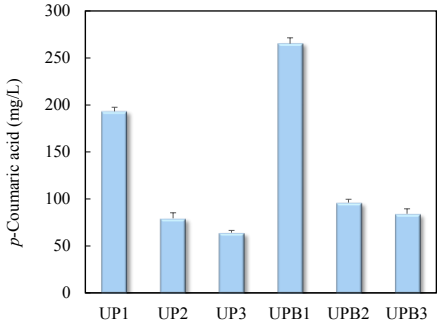
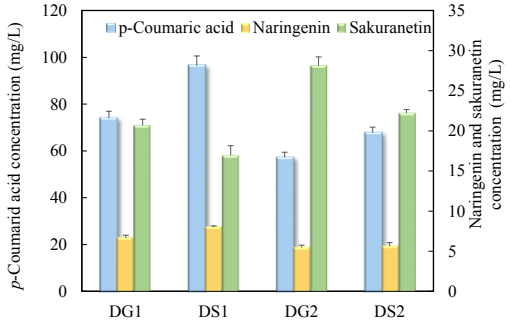
Figure 2

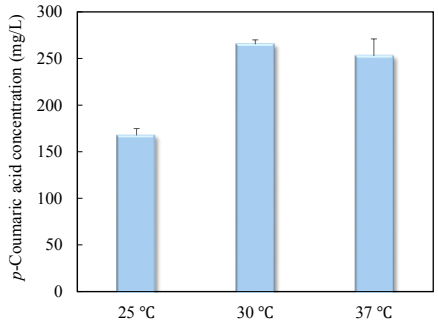
(A)



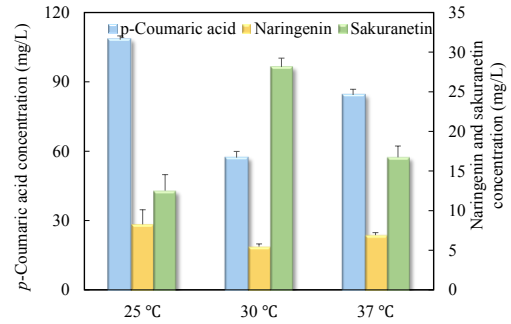
(B)



(C)

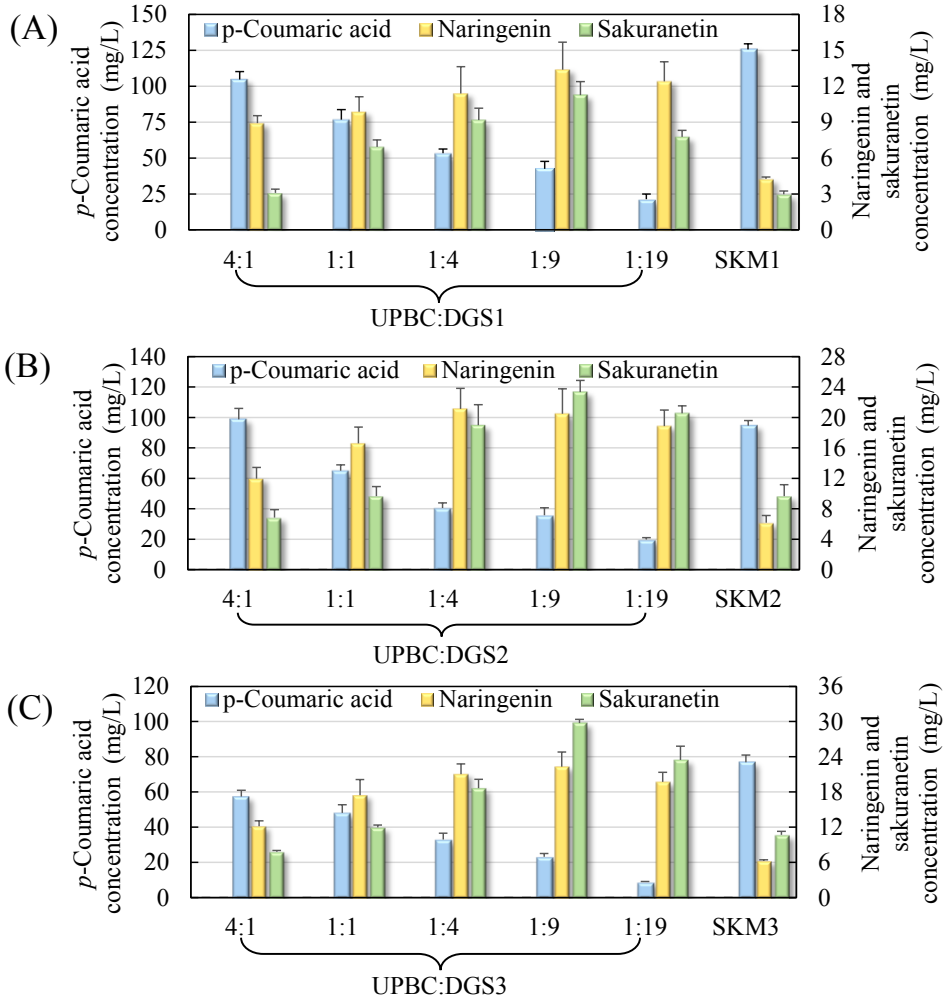


(D)



660

Figure 3



661

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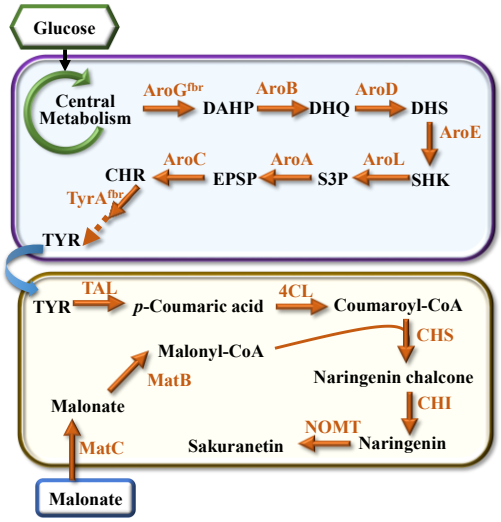
Figure 4

(A)


Glucose
Central Metabolism
AroG^fbr
DAHP
AroB
DHQ
AroD
DHS
AroE
SHK
AroL
S3P
AroA
EPSP
AroC
CHR
TyrA^fbr
TYR
TAL
p-Coumaric acid
4CL
Coumaroyl-CoA
CHS
Naringenin chalcone
CHI
Naringenin
NOMT
Sakuranetin
Malonyl-CoA
MatB
Malonate
MatC
Malonate

(B)

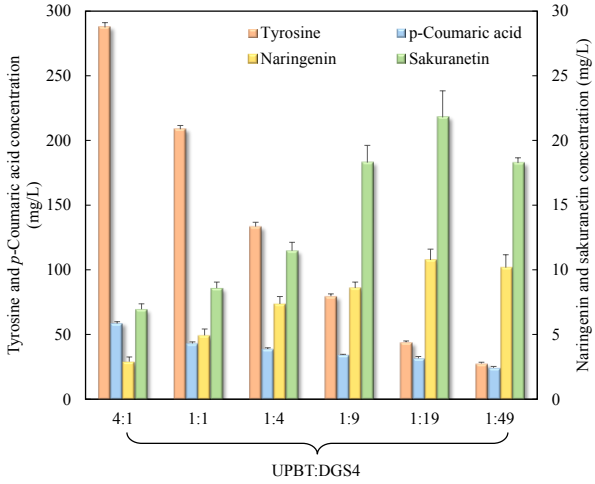
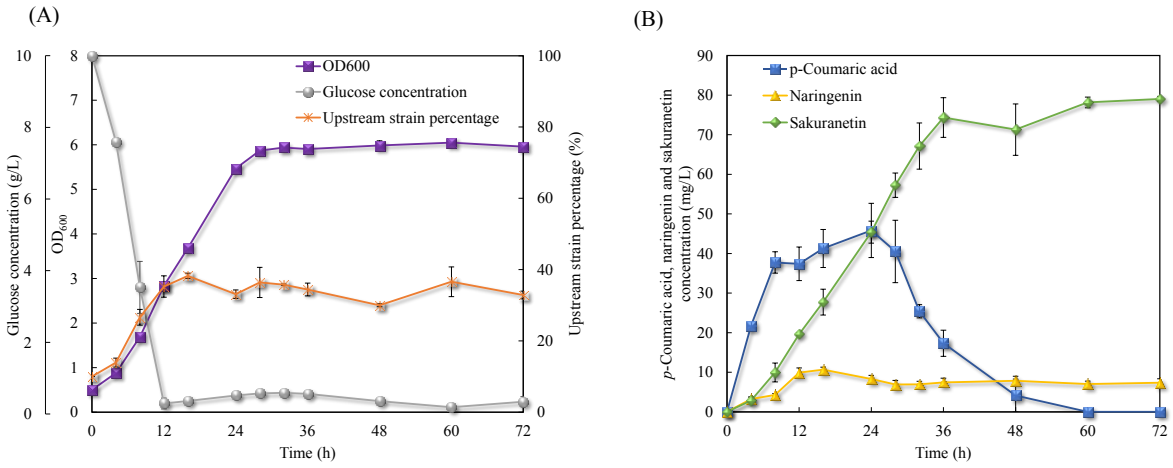

Tyrosine
p-Coumaric acid
Naringenin
Sakuranetin
Tyrosine and p-Coumaric acid concentration (mg/L)
Naringenin and sakuranetin concentration (mg/L)
300
250
200
150
100
50
0
30
25
20
15
10
5
0
4:1
1:1
1:4
1:9
1:19
1:49
UPBT:DGS4

Figure 5


(A)
OD600
Glucose concentration
Upstream strain percentage
Glucose concentration (g/L)
OD600
Upstream strain percentage (%)
Time (h)
(B)
p-Coumaric acid
Naringenin
Sakuranetin
p-Coumaric acid, naringenin and sakuranetin concentration (mg/L)
Time (h)

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