Journal of Industrial Microbiology and Biotechnology, 2023, 50, kuad030

https://doi.org/10.1093/jimb/kuad030

Advance access publication date: 20 September 2023

Metabolic Engineering and Synthetic Biology – Original Paper

Metabolic engineering and optimization of *Escherichia coli* co-culture for the *de novo* synthesis of genkwanin

Nguyen Huy Thuan [1], Vinay Bharadwaj Tatipamula[1], Nguyen Thanh Trung[1], Nguyen Van Giang[2]

[1]Center for Pharmaceutical Biotechnology, Duy Tan University, Da Nang 550000, Vietnam

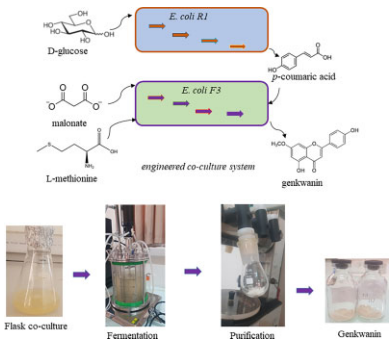
[2]Faculty of Biotechnology, Vietnam National University of Agriculture, Trau Quy, Hanoi 100000, Vietnam

Correspondence should be addressed to: Nguyen Huy Thuan at nguyenhuythuan@dtu.edu.vn

Abstract: Genkwanin has various significant roles in nutrition, biomedicine, and pharmaceutical biology. Previously, this compound was chiefly produced by plant-originated extraction or chemical synthesis. However, due to increasing concern and demand for safe food and environmental issues, the biotechnological production of genkwanin and other bioactive compounds based on safe, cheap, and renewable substrates has gained much interest. This paper described recombinant *Escherichia coli*-based co-culture engineering that was reconstructed for the *de novo* production of genkwanin from D-glucose. The artificial genkwanin biosynthetic chain was divided into 2 modules in which the upstream strain contained the genes for synthesizing *p*-coumaric acid from D-glucose, and the downstream module contained a gene cluster that produced the precursor apigenin and the final product, genkwanin. The Box–Behnken design, a response surface methodology, was used to empirically model the production of genkwanin and optimize its productivity. As a result, the application of the designed co-culture improved the genkwanin production by 48.8 ± 1.3 mg/L or 1.7-fold compared to the monoculture. In addition, the scale-up of genkwanin bioproduction by a bioreactor resulted in 68.5 ± 1.9 mg/L at a 48 hr time point. The combination of metabolic engineering and fermentation technology was therefore a very efficient and applicable approach to enhance the production of other bioactive compounds.

Keywords: Genkwanin, Co-culture, *De novo* production, *Escherichia coli*, Metabolic engineering

Graphical abstract



Engineering *E. coli* co-culture for production of genkwanin.

Highlights

- The artificial biosynthetic *Escherichia coli* consortia for the synthesis of genkwanin from D-glucose were reconstructed.
- Production of genkwanin was evaluated using the monoculture and co-culture.
- Optimization of genistein production by using response surface methodology and Box–Behnken design resulted in 48.8 ± 1.3 mg/L.
- Scale-up of the production by a high-density fed-batch bioreactor resulted in 68.5 ± 1.9 mg/L at a 48 hr time point.

Introduction

Genkwanin [systematic name: 5-hydroxy-2-(4-hydroxyphenyl)-7-methoxychromen-4-one, $C_{16}H_{12}O_5$] is one of the methylated flavonoids commonly found in some medicinal plants such as *Callicarpa americana*, *Alnus glutinosa*, and *Phaleria nisidai* (Kumarasamy et al., 2006; Porras et al., 2019; Horvath et al., 2022). Genkwanin has many remarkable anti-inflammatory, antibacterial, and anticancer activities. Specifically, its anti-inflammatory activity was investigated in lipopolysaccharide (LPS)-activated macrophages, RAW264.7, through the control of the miR-101/MKP-1/MAPK

Received: August 4, 2023. **Accepted:** September 18, 2023.

pathways (Gao et al., 2014). The compound has also been reported to have Gram-positive and Gram-negative antibacterial and antioxidant activity (Kumarasamy et al., 2006). In addition, genkwanin nanosuspensions displayed more stability, effectiveness, and stronger *in vitro* cytotoxicity against 4T1, MCF-7, MDA-MB-453, HeLa, HepG2, BT474, and A549 cells than free genkwanin (Li et al., 2017). Genkwanin derivatives such as genkwanin glycosides in *P. nisidai* extract had antidiabetic effects by inducing a non-insulin-dependent increase in glucose absorption (Horvath et al., 2022).

Biochemically, genkwanin is the methylated form of apigenin at the 7-OH position via the phenylpropanoid pathway. Plant cells use carbon sources such as dextrose (6C) to produce the amino acid tyrosine via the L-tyrosine pathway. Then, it is bioconverted to *p*-coumaric acid (3C). Subsequently, *p*-coumaric acid is used as a co-substrate to synthesize naringenin (15C) in the presence of *4CL*, *CHS*, and *CHI*. Naringenin is next converted to apigenin (15C) by the catalyst of *FNSI*. Finally, apigenin is 7-*O*-methylated (*OMT7*) to generate the product genkwanin (16C). Recently, there has been an increasing demand for genkwanin, but there are some restrictions on the direct extraction from plants, such as low yield, time consumption, and tedious isolation techniques. Therefore, microbial production of genkwanin is a useful alternative approach. Due to well-characterized biochemistry and physiology, the availability of versatile genetic manipulation tools, and favorable growth conditions, *Escherichia coli* has become an ideal platform host for the development of industrially viable productions (Pontrelli et al., 2018; Yang et al., 2021). For example, strains of *E. coli* carrying the genes *TAL*, *4CL*, *CHS*, *CHI*, and *FNS* were used to synthesize apigenin from L-tyrosine. Next, *POMT7* was added to synthesize genkwanin. The engineered *E. coli* strain synthesized approximately 41 mg/L of genkwanin (Jeon et al., 2009; Lee et al., 2015).

Lately, co-culture engineering for the production of bioactive compounds has had several advantages compared to monoculture such as simplification in the design of artificial biosynthetic pathways, restriction of the metabolic burden of the recombinant host, etc. These are causing an increased titer of desired compounds. For example, co-culture has been successfully applied to synthesize curcumin, sakuranetin, acacetin, apigetrin, resveratrol, etc. (Fang et al., 2018; Thuan et al., 2018a, 2018b; Wang et al., 2020a, 2020b; Camacho-Zaragoza et al., 2016). Particularly, co-cultures of *E. coli* and *Streptomyces* were used to synthesize about 27 mg/L of genkwanin.

In this paper, we describe the *de novo* biosynthetic co-culture approach in which genkwanin was synthesized from the simple substrate, D-glucose. Specifically, the *E. coli* R1 strain was genetically engineered to synthesize *p*-coumaric acid from D-glucose, and the *E. coli* F3 carried the gene for synthesizing genkwanin from *p*-coumaric acid. Each *E. coli* strain was also reengineered to enhance the precursor production. Furthermore, the response surface methodology, Box–Behnken, was used to empirically model the production of genkwanin and its productivity using four variables (strain-to-strain ratio, isopropyl β-D-1-thiogalactopyranoside [IPTG] concentration, induction time, and temperature). This synthesis model has provided a new perspective on the synthesis of active complex structures in *E. coli*.

Materials and Methods

Cultural Media and Chemicals

Luria-Bertani (LB) medium (LB: 10 g Bacto Tryptone, 5 g yeast extract, and 10 g NaCl per liter) was used for cloning, plasmid propagation, and screening the recombinant plasmids at 37°C. Bioconversion strains were grown in the minimal M9 medium (M9: Na_2HPO_4-$7H_2O$ 12.8 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, NH_4Cl 1 g/L, $MgSO_4$ 249 mg/L, $CaCl_2$ 11.1 mg/L, and thiamine 10 μg/L) or MY1 (MY1: 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)-propane sulfonic 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, and trace elements). The following antibiotics were added when needed: 100 µg/mL ampicillin (*Amp*), 30 µg/mL chloramphenicol (*Cm*), 100 µg/mL streptomycin (*Str*), and 30 µg/mL kanamycin (*Km*). The formula minimal M9 medium supplemented with 20 g/L glucose, 2 g/L malonate, and 2 g/L L-methionine was the so-called M9 Plus. Similarly, the formula MY1 supplemented with 10 g/L glucose, 2 g/L malonate + 2 g/L L-methionine, and 10 g/L glucose + 2 g/L malonate + 2 g/L L-methionine was the so-called MY1P1, MY1P2, and MY1P3, respectively.

Ethyl acetate, methanol, and other chemicals were purchased from Merck (Germany) or Sigma (USA). Nanodrop 2000 UV-Vis Spectrophotometer (Thermo, USA) and High Performance Liquid Chromatography (HPLC)-PDA (Agilent, USA) were used for chromatographic analysis.

Plasmid and Strain Construction and Verification

pETD-ppsA-tktA plasmid contained *ppsA* and *tktA* genes (chloramphenicol-resistant gene) (Thuan et al., 2022a). The *Salmonella typhimurium*-derived tyrosine aminotransferase (*tyrB*) gene (accession number Z68874.1) was codon-optimized and inserted into the multiple cloning site 1 (MCS1) (*BamHI*/*HindIII*) of pCBJ228 plasmid that contained *Flavobacterium johnsoniae* tyrosine ammonia-lyase (*FjTAL*) to generate plasmid *pAC-tyrB-FjTAL* (Supplementary Fig. 1) (Jendresen et al., 2015).

On the other hand, *F1OMT* was codon-optimized and then inserted into pCDFDuet-1 to generate *pCDFD-F1OMT* (*Sm*) (Supplementary Fig. 2). Subsequently, *pCDFD-F1OMT* (*Sm*) was transferred with *metK* to generate *pCDFD-F1OMT-metK* (*Sm*) (Koirala et al., 2015). Next, the *E. coli* API was transferred with the plasmid *pCDFD-F1OMT* (*Sm*), *pCDFD-F1OMT* plus *pCDF-matB-matC* (*Amp*), and *pCDFD-F1OMT-metK* (*Sm*) plus *pCDF-matB-matC* (*Amp*) to generate *E. coli* F1, F2, and F3, respectively. Various plasmids, including *pETD-ppsA-tktA*, *pCDFD-tyrB-FjTAL*, *pET28-4CL-FNSI*, *pAC-CHS-CHI*, *pCDF-matB-matC*, and *pCDFD-F1OMT-metK*, were introduced into *E. coli* BL21(DE3)/Δ*pheA* (Thuan et al., 2022a) to reconstruct the *E. coli* R1E strain.

All strains and DNA plasmids are listed in Table 1. DNA manipulation, such as DNA plasmid extraction, purification, digestion, and ligation, followed standard protocols (Sambrook & Green, 2012).

All the recombinant plasmids were digested by a pair of restriction enzymes as described, and then their molecular sizes were electrophoretically separated on 5%–7% agarose gel. Those fragment genes were independently collected and purified by using GeneJET PCR Purification Kit (Thermo Scientific, USA). Finally, they were sent to DNA sequencing by Sanger's method to check for any errors (Supplementary Table 1).

Small-Scale Bioconversion Experiments

For the monoculture experiment, a single colony of *E. coli* R1E from a fresh LB plate of the indicated strain was individually inoculated into 3 mL of the minimal M9 and MY1 medium with the appropriate antibiotics and grown at 37°C and 200 rpm overnight. For checking genkwanin production by monoculture, the R1E strain was diluted to obtain the initial net cell density of

Table 1. DNA Plasmids and Strains in This Study

Strains/plasmids	Description	Sources/references
Plasmids		
pET28a	T7 promoter, Kan^R	Novagen, USA
pETDuet-1	Double T7 promoters, Amp^R	Novagen, USA
pCDFDuet-1	Double T7 promoters, Sp^R	Novagen, USA
pACYCDuet-1	Double T7 promoters, Cm^R	Novagen, USA
pETD-ppsA-tktA (*Amp*)	Expression vector pETDuet-1, behind the T7 promoter, ampicillin resistance; ppsA and tktA	Thuan et al. (2022a)
pCDFD-tyrB-FjTAL (*Sm*)	Expression vector pCDFDuet-1, T7 promoter, spiramycin resistance; *FjTAL*: tyrosine amnonia lyase	This study
pET28-4CL-FNSI (*Km*)	Expression vector pET28a, T7 promoter, kanamycin resistance	This study
pCBJ228 (*Sm*)	pCDFDuet-1 vector, T7 promoter, *FjTAL*, spiramycin resistance	Jendresen et al. (2015)
pAC-CHS-CHI (*Cm*)	Expression vector pACYCDuet-1, behind the T7 promoter, streptomycin resistance	This study
pCDF-matB-matC (*Amp*)	Expression vector pCDF-Duet 1 containing *matB* and *matC*	Thuan et al. (2022a)
pCDFD-F1OMT-metK (*Sm*)	Expression vector pRSF-Duet, lac promoter, spiramycin resistance. *F1OMT: O-methyltransferase*, *metK: S-adenosyl-methionine*	This study
Escherichia coli strains		
E. coli XL1Blue	General cloning host	Promega, USA
E. coli BL21(DE3)	*E. coli* B F^- *ompT hsdS*(rB^- mB^-) dcm^+ *gal* λ(DE3)	Invitrogen, USA
E. coli BL21(DE3)/Δ*pheA*	*pheA* gene was knocked out	Thuan et al. (2022a)
E. coli MCA	*E. coli BL21(DE3)*/Δ*pheA* containing *pETD-ppsA-tktA* (*Amp*) and *pET28-TAL* (*Km*)	Thuan et al. (2022a)
E. coli API	*E. coli* BL21(DE3) containing *pAC-4CL-FNSI* (*Amp*), and *pAC-CHS_CHI* (*Cm*)	Thuan et al. (2018a)
E. coli RE	*E. coli BL21(DE3)*/Δ*pheA*/*pETD-ppsA-tktA/tyrB*	This study
E. coli R1E	*E. coli* BL21(DE3)/Δ*pheA* containing *pETD-ppsA-tktA* (Amp), *pAC-tyrB-FjTAL* (*Cm*), *pET28-4CL-FNSI* (*Km*), *pCDFD-F1OMT-metK* (*Sm*), *pCDF-matB-matC* (*Amp*), and *pAC-CHS-CHI* (*Cm*)	This study
E. coli R1	*E. coli* BL21(DE3)/Δ*pheA* containing *pETD-ppsA-tktA* (*Amp*) and *pAC-tyrB-FjTAL* (*Cm*)	This study
E. coli F1	*E. coli* API containing *pCDFD-F1OMT* (*Sm*)	This study
E. coli F2	*E. coli* API containing *pCDF-matB-matC* (*Amp*) and *pCDFD-F1OMT* (*Sm*)	This study
E. coli F3	*E. coli* API containing *pCDFD-F1OMT-metK* (*Sm*) and *pCDF-matB-matC* (*Amp*)	This study

3.5×10^6 cells/mL of culture into 10 mL of fresh M9 and MY1 media, respectively, and grown at 34°C and 200 rpm till the optical density at 600 nm (OD_{600}) nm of 0.6. Each system was induced with 1 mM of IPTG for protein expression, and the induction time was about 2 hr. Those broths were continuously maintained at 34°C and 200 rpm until 60 hr. Similarly, *E. coli* MCA and R1 strains were cultured in the MY1P1 to evaluate the production of *p*-coumaric acid from D-glucose. In the same way, the recombinant *E. coli* F1, F2, and F3 were grown in the MY1P3 to check the production of genkwanin. *p*-coumaric acid was supplemented gradually into the induced *E. coli* culture broth to avoid its growth inhibition by excessive substrate, that is, 10, 20, and 30 µM. Bioconversion of substrate was checked at 12 hr of interval time.

For co-culture experiments, the overnight culture was grown for each of the constituent strains, separately. Their culture broths were then centrifuged and suspended in MY1 medium plus 20 g/L of D-glucose and 2 g/L of malonate. Next, the ratio of upstream (R1) and downstream (F3) strains was adjusted as 90:10, 80:20, 60:40, 50:50, 30:70, and 10:90 (v/v) with an initial cell density of each strain of 3.5×10^6 cell/mL to generate a co-culture system. Each system was then induced by 1 mM IPTG for protein expression. In addition, the monoculture *E. coli* R1E strain was used as a control. The converted *p*-coumaric acid, apigenin, and genkwanin concentrations were checked after an interval of 12 hr until 60 hr of culture.

Experimental Design, Statistical Analysis, and Multiresponse Optimization

In order to optimize the genkwanin production by co-culture, the Box–Behnken design (BBD) was used to determine the design points for applying the response surface methodology (RSM). Four independent variables (factors), including inoculum ratios, IPTG concentration, induction time, and temperature, respectively, were designed as factors A, B, C, and D at three different levels [high (+), middle (0), and low (−)] (Supplementary Table 2). All experiments were carried out in triplicate, and the final cell density (value of OD_{600}) and the yield of genkwanin were taken as the response (Y).

The experimental response data were then supplied to the Design-Expert v13 software (Stat-Ease Inc., USA) and fitted with a second-order polynomial equation as follows:

$$Y = b_0 + b_1A + b_2B + b_3C + b_4D + b_{11}A^2 + b_{22}B^2 + b_{33}C^2 + b_{44}D^2 + b_{12}AB + b_{13}AC + b_{14}AD + b_{23}BC + b_{24}BD + b_{34}CD,$$

where Y is the predicted response (OD_{600} and genkwanin production) in this study, b_0 is the model constant, and b_i, b_{ii}, and b_{ij} are the linear coefficients, the squared coefficients, and the interaction coefficients, respectively (Ferreira et al., 2007; Xu et al., 2019; Li et al., 2019).

Scale Up Genkwanin Production by Bioreactor

The metabolically engineered bioproduction of genkwanin was finally scaled up in a 2.0 L bioreactor to demonstrate the scalability of the developed co-culture. For fed-batch cultivation, the ratio of R1:F3 with an initial net cell density of 4.5×10^6 cells/mL of each culture was cultivated in a 5.0 L bioreactor (Biotron, Korea) containing 2.0 L MY1 medium. Glucose, malonate, and methionine were gradually supplied at 6, 12, and 18 hr to reach final concentrations of 20, 4, and 4 g/L, respectively. Fermentation was carried out at a shaking speed, culture temperature, and airflow of

220 rpm and 1.0 L/min, respectively. pH and dissolved oxygen (DO) were measured using the manufacturer's protocol. pH was maintained at 7.2 by the automatic addition of 4 M sodium hydroxide (NaOH). Parameters such as growth kinetics, precursor formation, and formation of apigenin and genkwanin were evaluated continuously every 12 hr during the 60 hr culture.

Extraction and Analysis of *p*-Coumaric Acid, Apigenin, and Genkwanin by HPLC and LC–ESI/MS Methods

For quantifying *p*-coumaric acid and polyphenol, a 1000 µL aliquot of complete culture was mixed with an equal volume of ethyl acetate. The mixture was mixed thoroughly for 60 s and centrifuged, then 800 µL of the organic layer was separated in a clean microfuge tube and evaporated till dry in an Eppendorf concentrator. After that, samples were re-solubilized in 800 µL of methanol. Finally, the samples were filtered through a Whatman filter of 0.2 µm before injecting into an HPLC system.

Mobile phases consisted of acetonitrile (100%) (solvent B) and deionized distilled water containing 0.1% trifluoroacetic acid (TFA) (solvent A). The HPLC program was performed following solvent B: 10% (0–5 min), 10%–30% (5–15 min), 30%–80% (15–30 min), and 80%–100% (30–41 min) finally. The flow rate and wavelength were set at 1.0 mL/min and 280 nm, respectively. The Mightysil RP-C18 column (4.6 × 250 mm) (Kanto Chemical Co. Inc., Japan) was used to separate intermediates and GE. Under these conditions, the retention time for *p*-coumaric acid, apigenin, and genkwanin was 6.2, 10.5, and 12.8 min, respectively. For quantification of flavonoids, a calibration curve of authentic *p*-coumaric acid, apigenin, and genkwanin was individually drawn using 10-, 20-, 30-, 40-, and 50-µg/mL concentrations, respectively. Furthermore, the exact molecular weight of the concerned intermediates or targeted products was measured by Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (LC–ESI/MS) equipped with the Phenomenex Synergi Polar-RP column (150 × 4.6 mm, 4 μm).

Statistical Analysis

The Student's *t*-test was performed on the biological replicates to determine the statistical significance of the difference between control and experiment samples at each time point. Differences with a p value < 0.05 were considered statistically significant.

Design-Expert v13 software was used for the regression analysis of the experimental data obtained, and statistical parameters were examined with analysis of variance (ANOVA). The fit quality of the polynomial model equation was evaluated by the determination of coefficient, and the significance of the model, an optimum value of the parameter, was assessed with the determination coefficient and correlation coefficient. The statistical testing of the model was by *F*-value and *p*-value.

Results

Bioproduction of Genkwanin by Monoculture *E. Coli* R1E Strain

In order to monitor the *de novo* production of genkwanin from D-glucose by monoculture, the R1E strain was genetically engineered. As a result, the growth rate (OD_{600}) of R1E in the complex MY1 was higher than in the minimal M9 medium. Indeed, the strain was exponentially increased in the first 24 hr in both media and reached the maximal OD_{600} values of 7.2 and 5.8, respectively (Fig. 1a). In this condition, we also found a maximum genkwanin productivity of 0.86 ± 0.01 and 0.65 ± 0.03 mg/L/hr in MY1 and minimal M9 medium, respectively (Table 2). The titer production of genkwanin in MY1 and minimal M9 medium was progressively increased and achieved the maximal values of 28.1 ± 1.8 and 19.1 ± 2.6 mg/L at 48 hr timepoint, respectively (Fig. 1b). LC–ESI analysis in negative mode resulted in the mass-to-charge ratio

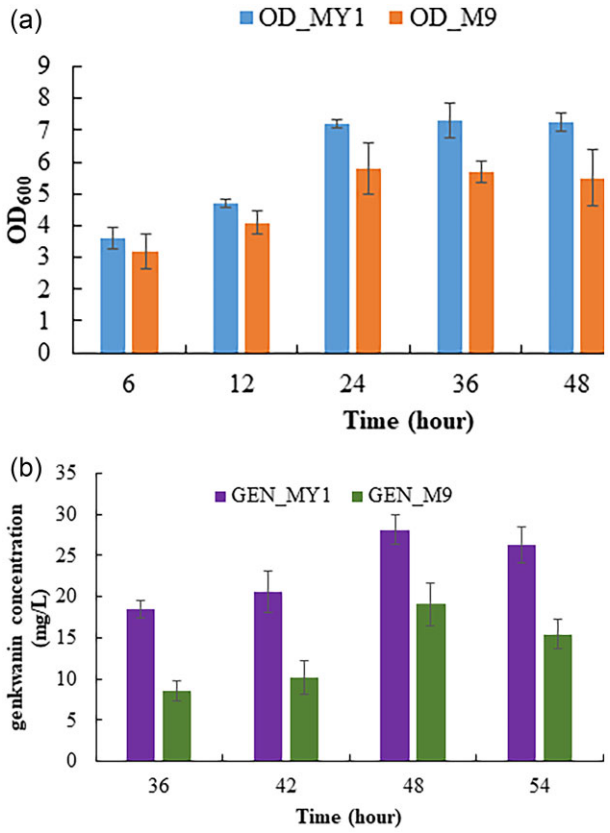


Fig. 1 *De novo* genkwanin production using the monoculture *Escherichia coli* R1E in the minimal M9 and complex MY1 media. The minimal M9 medium was exogenously fed with 20 g/L glucose, 2 g/L malonate, and 2 g/L methionine. The complex MY1 medium was exogenously supplemented with 10 g/L glucose, 2 g/L malonate, and 2 g/L methionine. (a) Comparison of growth profile in the MY1 plus (OD_MY1) and in the M9 plus (OD_M9) by indicating OD_{600} values. (b). Comparison of genkwanin production in the MY1 plus (GEN_MY1) and in the minimal M9 plus (GEN_M9). The experiments were conducted with an initial net cell density of 3.5×10^6 cells per mL of culture. The error bars represent the standard deviation of the experimental measurements for at least three independent experiments. Two-tailed Student's *t*-tests were performed to determine the statistical significance of two group comparisons, $\alpha = 0.05$.

Table 2. Genkwanin Productivity of *Escherichia coli* R1C in the Minimal M9 and MY1

Fermentation time (hr)	Productivity (mg/L/hr)	
	Minimal M9	MY1
12	0.11 ± 0.01	0.25 ± 0.04
24	0.25 ± 0.04	0.41 ± 0.03
36	0.57 ± 0.02	0.72 ± 0.11
48	0.65 ± 0.03	0.86 ± 0.01
60	0.44 ± 0.01	0.58 ± 0.05

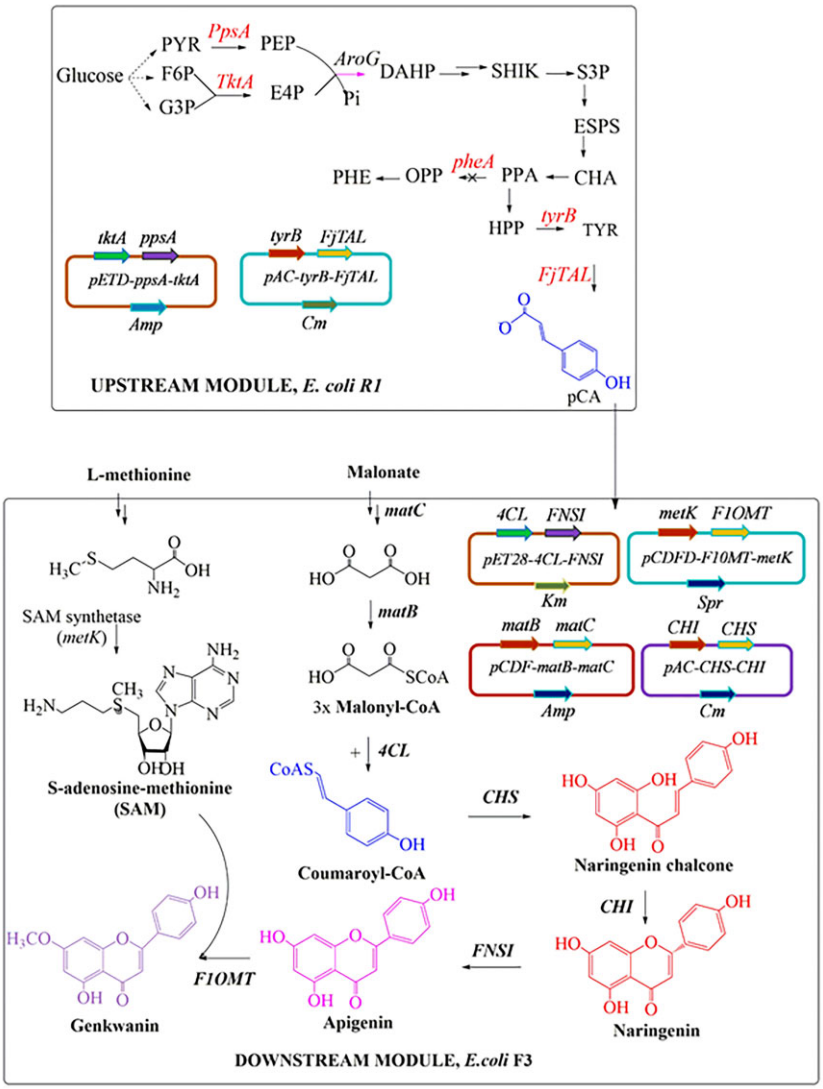


Fig. 2 Schematic representation of *Escherichia coli* modular coculture system for production of genkwanin starting from D-Glucose. The upstream strain was a *p*-coumaric acid over-producer, and the downstream strain was specialized in converting *p*-coumaric acid to genkwanin. *G6-P*, glucose-6-phosphate; *PYR*, pyruvate; *F6P*, fructose-6-phosphate; *G3P*, glucose 3-phosphate; *Pps*A, phosphoenolpyruvate synthase; *Tkt*A, transketolase A; *PEP*, 3-deoxy-D-arabino-heptulosonate-7-phosphate; *E4P*, erythrose 4-phosphate; *AroG*, DAHP synthase; *DAHP*, 3-deoxy-D-arabino-heptulosonate-7-phosphate; *SHIK*, shikimate 5-phosphate; *S3P*, 3-enolpyruvylshimate-5-phosphate; *PPA*, phenylpyruvate; *PPY*, phenylpyruvate, *L-Phe*, L-phenylalanine; *FjTAL*, tyrosine ammonia-lyase; *tyrB*, tyrosine aminotransferase ammonia-lyase; *4CL*, 4-coumarate-CoA ligase; *CHS*, chalcone synthase; *CHI*, chalcone isomerase; *matC*, malonate transporter protein; *matB*, malonyl-CoA synthetase; *pCA*, p-coumaric acid; *FNSI*, flavone synthase I; and *F1OMT*, flavonoid 7-O-methyltransferase.

(m/z) of $[M–H]^-$ of 283.3 (Supplementary Fig. 3). As a result, MY1 medium was selected for the following experiments.

Reconstruction of the Synthetic Co-Culture System

pheA gene, encoding for chorismate mutase/prephenate dehydratase, was knocked out to block L-tyrosine converting to L-phenylalanine (Patnaik et al., 2008; Fordjour et al., 2019). Hence, the *pheA*-deleted gene *E. coli* BL21(DE3) was metabolically engineered for enhancing the production of L-tyrosine (*E. coli* BL21(DE3)/Δ*pheA*). Then, *ppsA* and *tktA* genes, respectively, encoding for phosphoenolpyruvate synthase (PpsA) and transketolase A (TktA), account for the bioconversion of phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) from different carbon sources. Those genes were overexpressed using the expression vector pETDuet-1. This work aimed to enhance the intracellular accumulation of PEP and E4P, important precursors for the shikimate pathway (Koma et al., 2020). Next, the *tyrB* gene, *S. typhimurium*-derived tyrosine aminotransferase (accession number Z68874.1), was co-transferred into this *E. coli* BL21(DE3)/Δ*pheA*/*pETDuet-ppsA-tktA*/*tyrB* (so-called *E. coli* RE). This work aimed to increase gene dose (co-overexpression) to enhance the production of 4-hydroxyphenylpyruvate (HPP) as a precursor for the synthesis of L-tyrosine (Nakai et al., 1996). Furthermore, *F. johnsoniae*-originated tyrosine ammonia-lyase gene (*FjTAL*) was highly efficiently proved to convert L-tyrosine to *p*-coumaric acid, and was introduced into *E. coli* RE (Jendresen et al., 2015). Finally, the obtained *E. coli* RE/*FjTAL* was named *E. coli* R1 (upstream module).

Previously, the *E. coli* API strain encompassed *4CL*, *CHS*, *CHI*, and *FNSI* for the conversion of *p*-coumaric acid to apigenin (Katsuyama et al., 2007; Leonard et al., 2007; Thuan et al., 2018a).

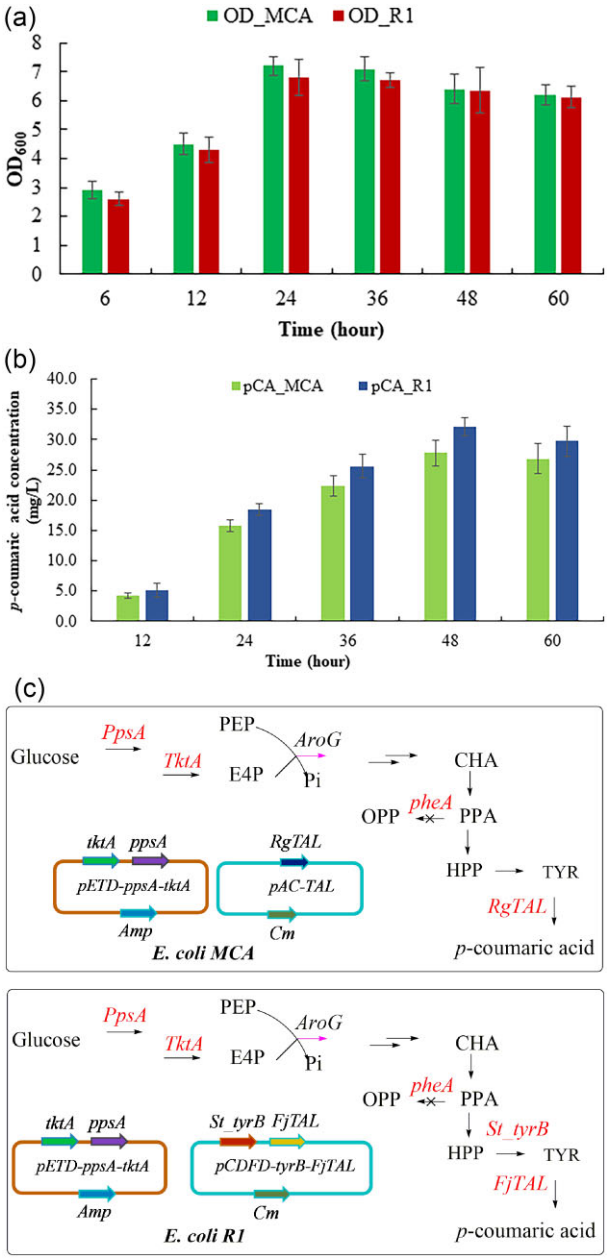


Fig. 3 Investigating the biosynthetic performance of *p*-coumaric acid by the monoculture *Escherichia coli* MCA and R1 in the MY1 medium plus 20 g/L D-glucose. (a) Growth profile of *E. coli* MCA (OD_MCA) and R1 strain (OD_R1). (b) Comparison of the *p*-coumaric acid production by MCA (pCA_MCA) and R1 (pCA_R1). The R1 was reconstructed based on MCA. The experiments were conducted with an initial net cell density of 3.5×10^6 cells per mL of culture. (c) The difference between metabolically engineered *E. coli* MCA and *E. coli* R1. The error bars represent the standard deviation of the experimental measurements for at least three independent experiments.

In addition, *pCDF-matB-matC* plasmid containing malonate synthetase (MatB) and a malonate carrier protein (MatC) increased the pool of intracellular malonyl-CoA and was introduced into API strain to improve the production of intracellular malonyl-CoA as an important precursor for the biosynthesis of naringenin (Fang et al., 2018). Furthermore, the S-Adenosyl methionine synthetase gene (*metK*; GenBank accession number K02129) was cloned from *E. coli* K12 (Koirala et al., 2015) and was introduced to enhance the production of S-Adenosyl-L-methionine (SAM) as a precursor for the methylation of apigenin. Finally, the *F1OMT* gene encoded an apigenin 7-O-methyltransferase (accession number X77467) that was codon optimized and concurrently transferred into the *E. coli* API strain to correspond to the methylation of apigenin to genkwanin. This reconstructed *E. coli* was named the F3 strain (downstream module) (Fig. 2).

Table 3. *p*-Coumaric Acid Productivity of *Escherichia coli* R1 in the MY1

Fermentation time (hr)	Productivity (mg/L/hr)
12	0.23 ± 0.06
24	0.42 ± 0.02
36	0.82 ± 0.12
48	0.96 ± 0.01
60	0.56 ± 0.07

Comparison of *p*-Coumaric Acid Production by *E. Coli* R1 and *E. Coli* MCA

Two *E. coli* strains, MCA and R1, were cultured in the complex MY1P1 medium. Consequently, OD_{600} of MCA and R1 was 7.2 and 6.8, respectively (Fig. 3a). Furthermore, *p*-coumaric acid productivity showed the highest value of 0.96 ± 0.01 mg/L/hr at a 48 hr time point in MY1P1 (Table 3). And the highest production of *p*-coumaric acid by *E. coli* MCA and R1 was 27.8 ± 2.1 and 32.2 ± 1.5 mg/L, respectively (Fig. 3b). In comparison, these results indicated that codon-optimized *tyrB* and *FjTAL* in R1 strain showed highly efficient activity than MCA strain containing *Rhodotorula glutinis*-originated *TAL*. In addition, this result was fitted with the use of complex media in co-culture for the production of sakuranetin and resveratrol (Wang et al., 2020a; Yuan et al., 2020). As a result, the R1 strain was further used in the following experiments.

Biosynthesis of Genkwanin From *p*-Coumaric Acid by *E. Coli* F1, F2, and F3 Strains

Escherichia coli F1, F2, and F3 were reconstructed based on the *E. coli* API for genkwanin synthesis, but F2 and F1 strains lacked *metK* and *matB-matC*, respectively (Table 1). All those strains were cultured in the MY1P2 medium. The concentration change of the pathway metabolites is shown in Fig. 4a. In a similar manner, OD_{600} of those strains reached the highest values of 7.3, 6.9, and 6.5 after 24 hr (Fig. 4a). The maximal yields of genkwanin produced by *E. coli* F1, F2, and F3 were achieved at 23.7 ± 0.63, 27.3 ± 1.42, and 32.7 ± 0.85 mg/L at 48 hr time point, respectively (Fig. 4b). This result could be explained by the introduction of supporting genes (*matB*, *matC*, and *metK*) to enhance the production of precursors, malonate and SAM, and this was also proven by the measurement of their productivity of F1, F2, and F3 as 0.46 ± 0.09, 0.67 ± 0.08, and 0.94 ± 0.2 mg/L/hr, respectively (Table 4). Based on this strategy, *E. coli* R1 and *E. coli* F3 were selected as constituents to construct the co-culture system for *de novo* genkwanin biosynthesis.

The Overall Strategy of *De Novo* Co-Culture and Investigation of Initial Conditions for the Production of Genkwanin

The set-up of the initial parameters for the co-cultures was based on culture conditions supporting the monoculture of *E. coli* R1E to produce genkwanin. One of the most important factors was strain-to-strain ratio due to reconstructed upstream and

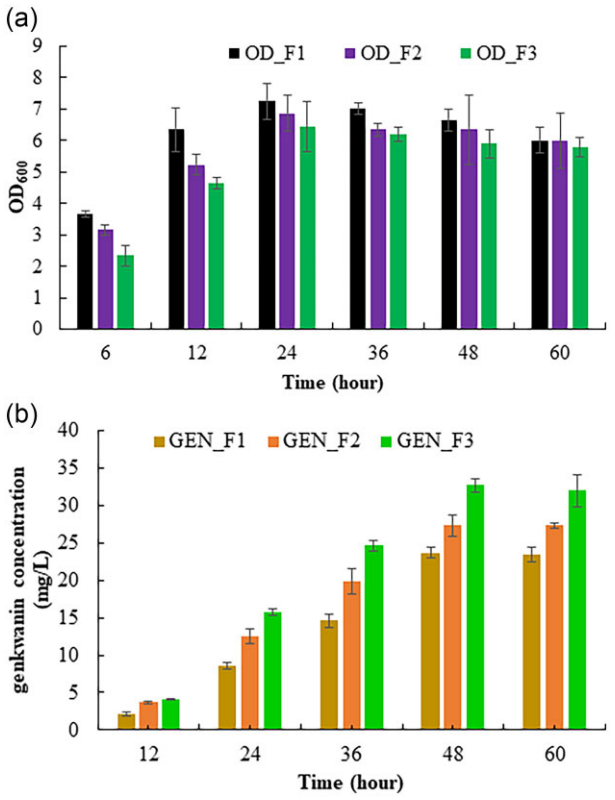


Fig. 4 Production of genkwanin by the monoculture *Escherichia coli* F1, F2, and F3 strains in MY1 medium plus 10 g/L glucose, 2 g/L malonate, and 2 g/L methionine via the whole-cell bioconversion using the substrate *p*-coumaric acid. (a) Growth profile of F1, F2 and F3 strains (OD_F1, OD_F2, and OD_F3). (b) Comparison of genkwanin production by those strains (GEN_F1, GEN_F2, and GEN_F3). F1, F2, and F3 were reconstructed based on API strain to improve genkwanin production. The experiments were conducted with an initial net cell density of 3.5 × 10^6 cells per mL of culture. The error bars represent the standard deviation of the experimental measurements for at least three independent experiments.

downstream strains forming a continuous biosynthetic pathway of genkwanin. Therefore, *E. coli* R1 and *E. coli* F3 were inoculated at varying ratios ranging from 90:10, 80:20, 60:40, 50:50, 30:70, and 10:90 (% vol. R1:F3), resulting in six co-culture groups, respectively. In comparison, the monoculture strain *E. coli* R1E containing all genes in the biosynthetic routes of genkwanin was used as the control. Temperature, IPTG concentration, and induction time were maintained at 34°C, 1 mM, and 2 hr, respectively. As shown in Supplementary Fig. 4, the concentration of *p*-coumaric acid and genkwanin was dependent on strain-to-strain ratio, a highly sensitive parameter, at the 48 hr time point. In the range of inoculum ratios (9:1, 8:2, 7:3, 6:4, and 5:5), the genkwanin concentration gradually increased and reached the highest value of 34.5 ± 2.7 mg/L at a ratio of 5:5. There is an intact relationship between upstream and downstream strains in the co-culture system to produce apigetrin, acacetin, and curcumin glycosides (Fang et al., 2018; Thuan et al., 2018a; Wang et al., 2020b). In this case, when the percentage of upstream was adjusted higher than the downstream strain, a higher concentration of *p*-coumaric acid was produced, as expected, than the production of genkwanin. In contrast, the higher production of genkwanin was observed when the percentage of downstream strain was higher in the range of inoculum ratio (4:6, 2:8, 1:9). Comparatively, this result was significantly higher efficiency than the baseline monoculture control *E. coli* R1E about 1.2-fold (34.5 ± 2.7 mg/L > 28.1 ± 1.8 mg/L). However, this result was not only dependent on the inoculum ratio but also on other factors such as IPTG concentration, induction time, temperature, etc. (Jones et al., 2016; Xu et al., 2019; Bracalente et al., 2022).

Optimization of the Co-Culture Genkwanin Productivity by RSM–BBD

Previous publications showed the various factors affecting the performance of co-culture systems, including strain-to-strain ratio, IPTG concentration, induction time, and temperature (Jones et al., 2016; Zhang et al., 2017; Bracalente et al., 2022). The impact of each of the four factors (variables) resulted in individual significance. Next, RSM–BBD, a cost-efficient multivariate statistical technique, was applied to evaluate the significance of combined factors. RSM–BBD has several advantages: (i) to allow simultaneous evaluation of factors such as strain-to-strain ratio and IPTG concentration or induction time and temperature, etc., that could not be done by the optimization of one variable at a time (OVAT), and (ii) RSM–BBD provided an empirical model to obtain optimized results by conducting as few experiments as possible.

Based on strain-to-strain ratio results and previous publications (Thuan et al., 2018a, 2022a, Wang et al., 2020a, 2020b), independent variables included inoculum ratio R1 (40%–90%) (A), IPTG concentration (0.3–1 mM) (B), induction time (1–3.5 hr) (C), and temperature (22–38°C) (D). Those four independent variables were prescribed into three levels selected for each of the experiments (Supplementary Table 1). The *de novo* biosynthetic co-culture performance and production of genkwanin were evaluated by two response functions: OD values measured at 600 nm (OD_{600}) and yield of genkwanin (mg/L).

The experiments and their responses are presented in Supplementary Table 3. Next, Supplementary Table 4A and B summarized different types of models and suggested the quadratic mathematical model is the most suitable to simulate the experimental procedure.

Based on ANOVA, the final model of OD_{600} values and four critical control factors (variables) were obtained (Table 5), which in terms of actual variables are as follows:

$$\begin{aligned} Y(OD_{600}) = {} & -72.645 + 0.338 * A + 25.131 * B + 20.629 * C \\ & + 2.869 * D + 0.067 * A * B - 0.001 * A * C \\ & + 0.007 * A * D - 0.306 * B * C - 0.201 * B * D - 0.189 * C * D \\ & - 0.005 * A^2 - 18.860 * B^2 - 2.888 * C^2 - 0.045 * D^2, \end{aligned} \quad (1)$$

where inoculum ratio (% R1 strain) = A, IPTG concentration = B, induction time = C, and temperature = D.

The statistical significance of Equation (1) was checked by *F*-test, and the results of ANOVA are shown in Supplementary Table 5A. The model *F*-value of 73.62 implies the model is more significant (<0.0001). There is only a 0.01% chance that a 'Model *F*-value' this large could occur due to noise. Values of 'Prob > *F*' less than 0.0500 indicate model terms are significant. The coefficient determination (R^2) value was found to be 0.9866, which implies that the variation of 98.66% for the $OD_{600\ nm}$ was attributed to the independent variables and only 1.34% of the total variation could not be explained by the model. The R^2 value found in this study was closer to 1, showing that the developed model could effectively increase the cell density (13.89). The 'lack-of-fit *F*-value' of 2.69 implies the lack-of-fit is

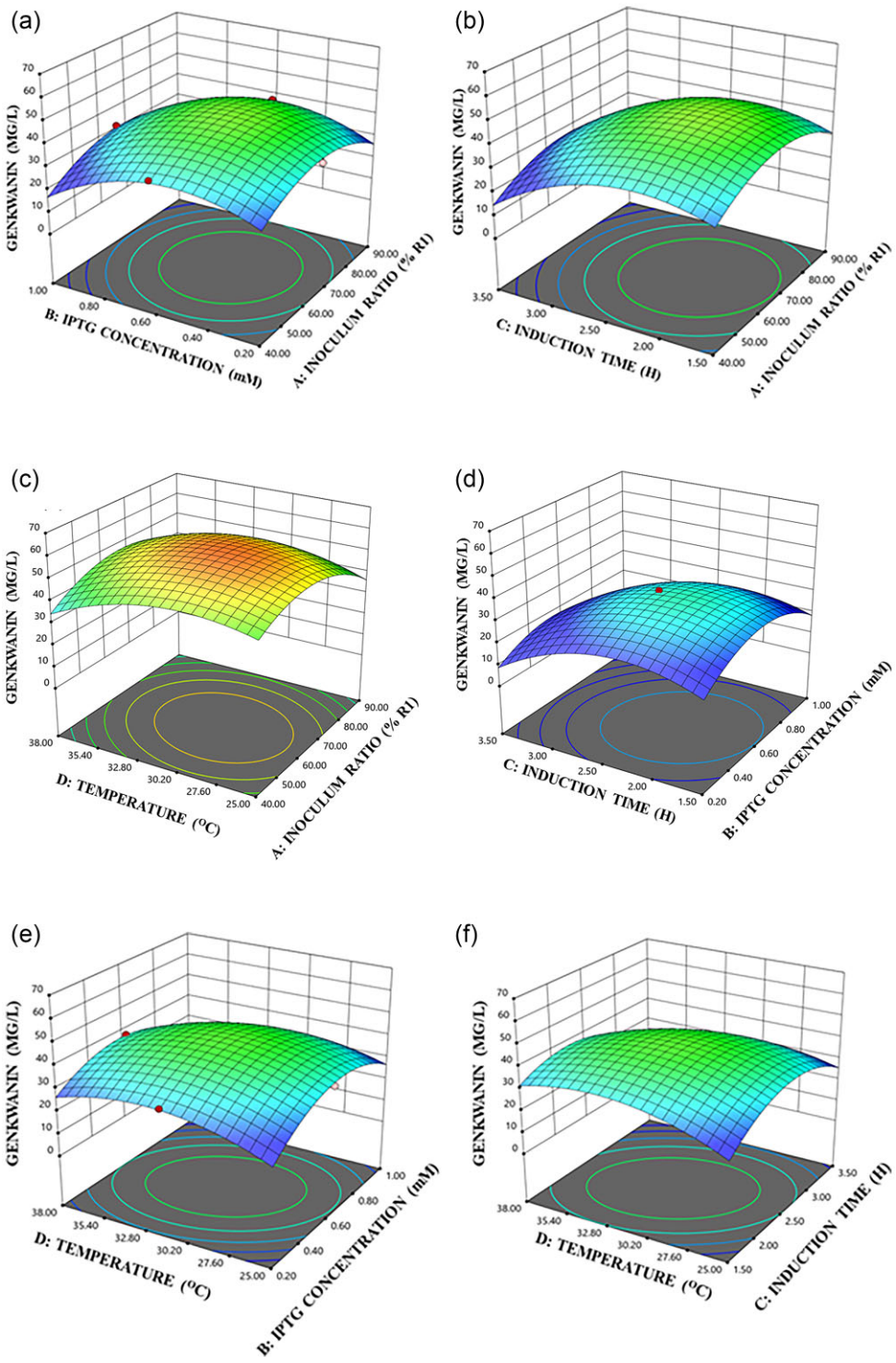


Fig. 5 Optimization of the genkwanin production by the co-culture using RSM-BBD. (a–f) Response surface plots for genkwanin production as a function of the four variables under study: inoculation ratio (% R1), inducer (IPTG) concentration (mM), induction time (H), and temperature (°C). These plots were obtained for a given pair of variables while maintaining the other one fixed at its zero value.

not significant relative to the pure error. There is a 17.63% chance that a 'lack-of-fitF-value' this large could occur due to noise. A non-significant lack-of-fit is good.

In *a* similar manner, Y (genkwanin (mg/L)) $= -239.892 + 1.137 * A + 95.089 * B + 61.415 * C + 9.671 * D + 0.112 * A * B + 0.010 * A * C + 0.014 * A * D - 2.838 * B * C - 0.808 * B * D - 0.538 * C * D - 0.013 * A^2 - 59.559 * B^2 - 9.143 * C^2 - 0.144 * D^2$. (2)

ANOVA for the response surface quadratic model of genkwanin production was shown in Supplementary Table 5.

Table 4. Genkwanin Productivity by *Escherichia coli* F1, F2, and F3 Strains

	Productivity (mg/L/hr)		
Fermentation time (hr)	*E. coli* F1	*E. coli* F2	*E. coli* F3
12	0.13 ± 0.06	0.25 ± 0.05	0.42 ± 0.05
24	0.25 ± 0.05	0.39 ± 0.03	0.62 ± 0.01
36	0.35 ± 0.04	0.51 ± 0.02	0.85 ± 0.01
48	0.46 ± 0.09	0.67 ± 0.08	0.94 ± 0.02
60	0.19 ± 0.06	0.41 ± 0.04	0.52 ± 0.06

The model *F*-value of 55.01 implies the model is significant. There is only a 0.01% chance that a 'Model *F*-value' this large could occur due to noise. The 'lack-of-fit *F*-value' of 5.02 implies the lack-of-fit is not significant relative to the pure error. There is a 6.70% chance that a 'lack-of-fit *F*-value' this large could occur due to noise. The coefficient determination (R^2) value was found to be 0.9821, which implies that the variation of 98.21% for the $OD_{600\ nm}$ was attributed to the independent variables and only 1.79% of the total variation could not be explained by the model. The R^2 value found in this study was closer to 1, showing that the developed model could effectively increase the genkwanin production (45.1 mg/L) (Fig. 5a–f).

Equations (1) and (2) showed the significance of strong interactions between the four variables in the co-culture. The quadratic interactions of four variables, as shown in the temperature and IPTG concentration, temperature and induction time, etc. (Supplementary Fig. 5A–F), indicated that when the variables were adjacent to their zero levels, the responses (OD_{600} and the yield of genkwanin) were maximized.

Predicted Versus Actual OD_{600} Values and Genkwanin Production and Model Validation

The experimental data for OD_{600} and genkwanin production were compared to the values predicted by the model (Supplementary Fig. 6A and B). Both responses showed a good correlation between the actual and predicted values ($R^2 = 0.98$ and 0.98 for OD_{600} and genkwanin production, respectively). According to the model, the highest OD_{600} value (13.9) and yield of genkwanin (45.1 mg/L) would be obtained under conditions of R1 strain (%), IPTG concentration, induction time, and temperature of 62.4%, 0.6 mM, 2.5 hr, and 31.5°C, respectively. Those values were different compared to previous data described in Supplementary Fig. 1. Indeed, RSM–BBD allowed to construct the optimum model based on the interaction of each pair of parameters simultaneously. And the integral data were obtained statistically with high confidence (Wang et al., 2017; Xu et al., 2019).

Table 6. OD_{600} Value and Yield of Genkwanin of the Validation Experiments

	OD_{600}	Genkwanin production
Predicted	13.64	45.14
Experimental average	12.9 ± 1.1	48.8 ± 1.3

Next, we performed three independent runs at predicted optimal conditions for four dependent variables to validate the model generated by the RSM–BBD design. The obtained results were very close to those predicted by the model. Those resulted in OD_{600} value and genkwanin titer of 12.9 ± 1.1 and 48.8 ± 1.3, respectively (Table 6).

The Co-Culture-Based Bioproduction of Genkwanin by Bioreactor

We carried out the culture in a 2.0 L bioreactor with the optimal parameters as described above. As described in the Materials and Methods section, the glucose, malonate, and methionine contents were gradually supplemented to obtain the final concentration

Table 5. Analysis of Variance (ANOVA) for Response Surface Quadratic Model of OD_{600}

ANOVA for quadratic model						
Response 1: OD_{600} values						
Source	Sum of squares	df	Mean square	*F*-value	*p*-value	
Model	151.80	14	10.84	73.62	<0.0001	Significant
A-inoculum ratio (% R1 strain)	1.76	1	1.76	11.97	0.0038	
B-IPTG concentration	0.1452	1	0.1452	0.9859	0.3376	
C-induction time	3.56	1	3.56	24.20	0.0002	
D-temperature	8.77	1	8.77	59.56	<0.0001	
AB	1.77	1	1.77	12.01	0.0038	
AC	0.2070	1	0.2070	1.41	0.2555	
AD	5.04	1	5.04	34.22	<0.0001	
BC	0.0600	1	0.0600	0.4076	0.5335	
BD	1.09	1	1.09	7.41	0.0165	
CD	6.05	1	6.05	41.09	<0.0001	
A^2	51.77	1	51.77	351.53	<0.0001	
B^2	59.07	1	59.07	401.07	<0.0001	
C^2	54.09	1	54.09	367.26	<0.0001	
D^2	23.79	1	23.79	161.54	<0.0001	
Residual	2.06	14	0.1473			
Lack-of-fit	1.79	10	0.1795	2.69	0.1763	Not significant
Pure error	0.2669	4	0.0667			
Cor total	153.86	28				

Note. Factor coding is coded.
Sum of squares is Type III—partial.

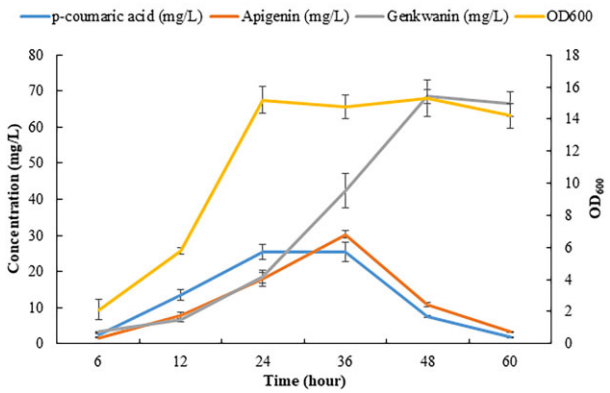


Fig. 6 Genkwanin bioproduction by the *Escherichia coli* R1:F3 co-culture using a fed-batch bioreactor. Initial glucose, malonate, and methionine concentrations were 5, 1, and 1 g/L, respectively. Those nutrients were then gradually supplied at 6, 12, and 18 h to reach final concentrations of 20, 4, and 4 g/L, respectively. Each constituent strain (R1 and F3) was used with the initial net cell density of 4.5×10^6 cells per mL of each culture. The error bars represent the standard deviation of the experimental measurements for at least three independent experiments.

Table 7. Productivity of *p*-Coumaric Acid, Apigenin, and Genkwanin in Bioproduction by Fed-Batch Fermentor

Fermentation time (hr)	Productivity (mg/L/hr)		
	p-coumaric acid	Apigenin	Genkwanin
12	0.45 ± 0.01	0.41 ± 0.03	0.34 ± 0.03
24	0.72 ± 0.01	0.71 ± 0.01	0.68 ± 0.01
36	1.16 ± 0.04	1.11 ± 0.01	0.95 ± 0.03
48	1.32 ± 0.02	1.18 ± 0.04	1.11 ± 0.02
60	0.61 ± 0.01	0.62 ± 0.02	0.56 ± 0.01

of 20, 4, and 4 g/L, respectively, compared to culture in the test tube, since we assumed that the process cultured in a bioreactor, vigorous shaking induces stronger growth of *E. coli* host. The dynamic change in the cell density was monitored throughout the bioproduction process. Specifically, the OD_{600} value was increased exponentially in the first 24 hr (15.2) and then kept in stationary phase until 48 hr. The concentration change of the pathway metabolites is shown in Fig. 6. The *p*-coumaric acid concentration was also increased fast in the first 24 hr (25.5 ± 2.1 mg/L) and then maintained around this level until 36 hr before decreasing to 1.7 ± 0.2 mg/L at 60 hr time point. The bioconversion of *p*-coumaric acid to intermediate (apigenin) and final product (genkwanin) has occurred continuously, but almost between 24 and 48 hr. The productivity of *p*-coumaric acid, apigenin, and genkwanin was increased and reached the maximal values of 1.32 ± 0.02, 1.18 ± 0.04, and 1.11 ± 0.02 mg/L/hr at 48 hr, respectively (Table 7). This indicated that the genkwanin biosynthetic performance in the co-culture system depends on all optimum parameters (strain-to-strain ratio, IPTG concentration, induction time, and temperature) in the fed-batch bioreactor. As a result, the maximal titer of genkwanin was achieved at 68.5 ± 1.9 mg/L at the same time point. In general, the genetically engineered co-culture system was used in a fed-batch bioreactor under optimum conditions, allowing successful scale-up of the production of genkwanin.

Discussion

Microbial monoculture has been traditionally used to synthesize numerous natural products such as polyketides, alkaloids, terpenoids, steroids, etc. (Malla et al., 2012; Takemura et al., 2017; Du et al., 2018; Adams et al., 2019; Liu et al., 2020). Particularly, advanced technologies, such as DNA mutagenesis, codon optimization, fusion gen, flux balance, etc., have been applied to optimize the expression of proteins and improve hosts. However, the yield of the product was still low due to the metabolic burden caused by the imbalance of metabolic fluxes, low expression of heterologous genes, growth inhibition, etc. (Wu et al., 2016; Wang et al., 2020c; Thuan et al., 2022b).

Until now, genkwanin has been synthesized using different approaches, such as direct extraction from plants and/or biological methods using biotransformation (*E. coli*, *Saccharomyces cerevisiae*, etc.) and *de novo* biosynthesis. Particularly, biotransformation of genkwanin was carried out using different substrates such as apigenin, L-tyrosine, and naringenin (Christensen et al., 1998; Jeon et al., 2009; Lee et al., 2015). In this study, our aims were: (i) to design a biological system that can synthesize a complicated compound (as genkwanin) from the simple, ready, and cheap substrate (as D-glucose); (ii) different optimum strategies (strain development, culture conditions) can be applied to improve the performance of the system to reduce metabolic burdens; and (iii) it finally could work as "plug and play" by using simple conditions (substrates, fermentation conditions, etc).

Firstly, the *E. coli* R1E strain, bearing all genes in the artificially biosynthetic pathway of genkwanin, was checked to produce this compound from D-glucose in both minimal M9 and MY1 media. It showed MY1 as the preferred medium and resulted in the maximal titer of 28.1 ± 1.8 mg/L. This result was consistent with the use of the complex medium for the production of different natural products such as resveratrol, sakuranetin, and acacetin (Yuan et al., 2020; Wang et al., 2020a, 2020b). This work demonstrated that all genes could work well in an *E. coli* host; however, the yield of the product is still lower than expected. The limitation in heterologous overexpression of a large number of genes in the artificial biosynthetic pathway of genkwanin and maintenance of metabolic flux balance in monoculture *E. coli* host might be the reason for this low titer.

In light of the low genkwanin production by R1E strain monoculture, the multivariate co-culture approach was used to improve its production. Indeed, co-culture has been an efficient alternative approach to improve the yield of products because it can restrict the burden of metabolisms by dividing the long and complex biosynthetic pathway into different modules and allowing optimization of each module, providing optimal environment for functional overexpression of all pathway genes, and enhancing substrate utilization and the yield of target compounds (Zhang & Wang, 2016; Wang et al., 2020c). Until now, *E. coli*-based co-culture has been applied to synthesize different value-added compounds such as sakuranetin, acacetin, apigenin, curcumin glycosides, etc. (Fang et al., 2018; Thuan et al., 2018a; Wang et al., 2020a, 2020b). In rational design, previous studies revealed that *p*-coumaric acid, a small molecule, can be simply transported via a cellular membrane (Wang et al., 2020a). In addition, it was demonstrated that *TAL* is feedback inhibited by 4-coumaroyl CoA (Santos et al., 2011). Consequently, *4-CL* and *TAL* should be spatially separated by integration in each module (Fig. 2). In the design concept, the entire biosynthetic pathway of genkwanin was divided into two consecutive strains in which *E. coli* R1 (upstream) contained genes for synthesizing *p*-coumaric acid from D-glucose and *E. coli* F3

contained genes for synthesizing genkwanin from *p*-coumaric acid. Experimental results showed that *E. coli* R1 used D-glucose as primary nutrition with a titer of 32.2 ± 1.5 mg/L *p*-coumaric acid (Fig. 3b). Concomitantly, the F3 strain was also tested to produce genkwanin using *p*-coumaric acid as a substrate. Consequently, it resulted in 32.7 ± 0.9 mg/L genkwanin at a 48 hr time point.

One challenge for co-culture engineering is the maintenance of compatible coexistence of the constituent co-culture strains. In so doing, the initial inoculum ratio of strains was optimized and resulted in the titer of 34.5 ± 2.7 mg/L genkwanin at an optimal ratio of R1:F3 = 5:5. Furthermore, RSM–BBD is used to optimize and predict the performance of biological systems, such as culture optimization for protein heterologous expression and strain improvement. Indeed, it provides a large amount of information based on the minimal trials (Ferreira et al., 2007; Xu et al., 2019). Subsequently, the regression model equation described the interaction of independent factors, for example, inoculum strain ratio and IPTG concentration, induction time and temperature, etc. Importantly, this equation model also allowed the prediction of culture parameters (i.e., R1 strain [%], IPTG concentration, induction time, and temperature of 62.4%, 0.6 mM, 2.5 hr, and 31.5°C, respectively) influencing cell growth (OD_{600}) and genkwanin production (45.1 mg/L). Interestingly, the subsequent experiments were carried out to validate the model, showing the maximal titer of 48.8 ± 1.3 mg/L genkwanin under those optimized conditions. Finally, scaling up the production of genkwanin was done by using a bioreactor. Consequently, the maximal yield of genkwanin was achieved at 68.5 ± 1.9 mg/L at the 48 hr time point of culture. This result proved that RSM–BBD is a cost-efficient multivariate statistical technique to optimize culture conditions (Wang et al., 2017).

Previously, Jeon et al. successfully synthesized genkwanin by heterologous expression of flavone synthase (*PFNS-1*) that converts naringenin into apigenin and flavone 7-*O*-methyltransferase (*POMT-7*) that converts apigenin into 7-*O*-methyl apigenin in *E. coli*. Subsequently, *E. coli* harboring genes of the genkwanin biosynthetic pathway (*TAL*, *4CL*, *CHS*, *CHI*, *FNS*, and *POMT7*) produced about the maximal titer of 41 mg/L (Lee et al., 2015). In comparison, the R1E strain produced less genkwanin than this (28.1 ± 1.8 < 41 mg/L) because of differences in culture method, reconstruction of the artificial biosynthetic pathway, gene heterologous expression, and extraction method. Recently, co-cultures of *E. coli* and *Streptomyces* were used to synthesize genkwanin. Specifically, strain *E. coli* (upstream module) carries genes encoding the biosynthesis of compounds including pterostilbene, naringenin, and apigenin. At the same time, the strain *Streptomyces venezuelae* carrying the *O*-methyltransferase (*SaOMT2*) gene was used as a downstream module. As a result, co-culture of *E. coli* strain BL21/APG (synthetic apigenin) and strain *S. venezuelae/SaOMT2* obtained about 27–42 mg/L of genkwanin (Cui et al., 2019). These above results were less than our biosynthetic titer (41 < 42 < 48.8 ± 1.3 mg/L). The differences between those results are due to (i) differences in gene expression and type of the reconstructed biosynthetic pathway to obtain the target compound; (ii) *S. venezuelae* and *E. coli* have different abilities to heterologous overexpression of protein/enzyme; and (iii) they are different in the nutrient requirement, growth rate, etc. (Zhang & Wang, 2016; Thuan et al., 2022b). Our results proved that the combination of metabolic engineering, co-culture techniques, and statistical experimental optimization could build *E. coli* as an efficient platform and working system to improve the productivity of genkwanin. To our knowledge, this is the highest bioproduction concentration that has been achieved up to now for *de novo* genkwanin biosynthesis by means of the heterologous host *E. coli*.

Conclusion

In this study, the genetically engineered *E. coli* monoculture and co-culture were designed and tested for *de novo* synthesis of genkwanin. The inoculation ratio between both strains and other fermentation parameters of the co-culture system were successfully optimized by RSM–BBD, and the optimum co-culture system could produce 48.8 ± 1.3 mg/L or 1.7-fold compared to the monoculture under the same conditions. Furthermore, the co-culture system was scaled up to produce genkwanin using high cell density fed-batch fermentation, proving the importance of strain-to-strain ratio and induction time at any scale. These results indicated that the combination of metabolic engineering, co-culture techniques, and a statistical multivariate variable experimental design system fully supports an efficient bioprocess to *de novo* produce genkwanin from only glucose, malonate, and L-methionine. Hence, this is proven to be a useful and feasible approach to synthesizing other value-added compounds.

Acknowledgments

We are grateful to Dr. Alex Toftgaard Nielsen and Dr. Sailesh Malla (The Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Hørsholm, Denmark), Dr. Mattheos A. G. Koffas (Rensselaer Polytechnic Institute, Troy, New York, USA), and the Technical University of Denmark for kindly providing *FjTAL*, *CHS*, and *CHI* genes, respectively.

Supplementary material

Supplementary material is available online at *JIMB* (*www.academic.oup.com/jimb*).

Author contributions

N.H.T. and V.B.T. designed the study. All authors discussed the results. N.H.T., V.B.T., N.V.G., and N.T.T. performed the experiments. N.H.T. and V.B.T. drafted the manuscript.

Funding

This research is funded by Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number: 106.02-2018.326.

Conflict of interest

No potential conflict of interest was reported by the authors.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

References

Adams, A. M., Kaplan, N. A., Wei, Z., Brinton, J. D., Monnier, C. S., Enacopol, A. L., Ramelot, T. A., & Jones, J. A. (2019). *In vivo* production of psilocybin in *E. coli*. *Metabolic Engineering*, 56, 111–119.

Bracalente, F., Sabatini, M., Arabolaza, A., & Gramajo, H. (2022). *Escherichia coli* co-culture for *de novo* production of esters derived of methyl-branched alcohols and multi-methyl branched fatty acids. *Microbial Cell Factories*, 21(1), 10. https://doi.org/10.1186/s12934-022-01737-0

Camacho-Zaragoza, J. M., Hernández-Chávez, G., Moreno-Avitia, F., Ramírez-Iñiguez, R., Martínez, A., Bolívar, F., & Gosset, G. (2016). Engineering of a microbial coculture of *Escherichia coli* strains for the biosynthesis of resveratrol. *Microbial Cell Factories*, 15(1), 163.

Christensen, A. B., Gregersen, P. L., Olsen, C. E., & Collinge, D. B. (1998). A flavonoid 7-*O*-methyltransferase is expressed in barley leaves in response to pathogen attack. *Plant Molecular Biology*, 36(2), 219–227. https://doi.org/10.1023/a:1005985609313

Cui, H., Song, M. C., Ban, Y. H., Jun, S. Y., Kwon, A. S., Lee, J. Y., & Yoon, Y. J. (2019). High-yield production of multiple *O*-methylated phenylpropanoids by the engineered *Escherichia coli–Streptomyces* cocultivation system. *Microbial Cell Factories*, 18(1), 67. https://doi.org/10.1186/s12934-019-1118-9

Du, J., Yang, D., Luo, Z. W., & Lee, S. Y. (2018). Metabolic engineering of *Escherichia coli* for the production of indirubin from glucose. *Journal of Biotechnology*, 267, 19–28.

Fang, Z., Jones, J. A., Zhou, J., & Koffas, M. A. G. (2018). Engineering *Escherichia coli* co-cultures for the production of curcuminoids from glucose. *Biotechnology Journal*, 13(5), e1700576.

Ferreira, S. L., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandão, G. C., da Silva, E. G., Portugal, L. A., dos Reis, P. S., Souza, A. S., & dos Santos, W. N. (2007). Box–Behnken design: An alternative for the optimization of analytical methods. *Analytica Chimica Acta*, 597(2), 179–186. https://doi.org/10.1016/j.aca.2007.07.011

Fordjour, E., Adipah, F. K., Zhou, S., Du, G., & Zhou, J. (2019). Metabolic engineering of *Escherichia coli* BL21 (DE3) for *de novo* production of L-DOPA from D-glucose. *Microbial Cell Factories*, 18(1), 74. https://doi.org/10.1186/s12934-019-1122-0

Gao, Y., Liu, F., Fang, L., Cai, R., Zong, C., & Qi, Y. (2014). Genkwanin inhibits proinflammatory mediators mainly through the regulation of miR-101/MKP-1/MAPK pathway in LPS-activated macrophages. *PLoS ONE*, 9(5), e96741. https://doi.org/10.1371/journal.pone.0096741

Horvath, C., Houriet, J., Moser, C., Balazova, L., Balaz, M., Dong, H., Horvath, A., Efthymiou, V., Rutz, A., Marcourt, L., Kitalong, C., Graz, B., Yano, V., Queiroz, E., Wolfender, J., & Wolfrum, C. (2022). Genkwanin glycosides from the *Phaleria nisidai* extract improve glucose homeostasis by stimulating insulin-independent glucose uptake. *Cell Metabolism*, https://ssrn.com/abstract=3996393

Jendresen, C. B., Stahlhut, S. G., Li, M., Gaspar, P., Siedler, S., Förster, J., Maury, J., Borodina, I., & Nielsen, A. T. (2015). Highly active and specific tyrosine ammonia-lyases from diverse origins enable enhanced production of aromatic compounds in bacteria and *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology*, 81(13), 4458–4476. https://doi.org/10.1128/AEM.00405-15

Jeon, Y. M., Kim, B. G., & Ahn, J. H. (2009). Biological synthesis of 7-*O*-methyl apigenin from naringenin using *Escherichia coli* expressing two genes. *Journal of Microbiology & Biotechnology*, 19(5), 491–4. https://doi.org/10.4014/jmb.0807.402

Jones., J. A., Vernacchio, V. R., Sinkoe, A. L., Collins, S. M., Ibrahim, M. H. A., Lachance, D. M., Hahn, J., & Koffas, M. A. G. (2016). Experimental and computational optimization of an *Escherichia coli* co-culture for the efficient production of flavonoids. *Metabolic Engineering*, 35, 55–63. https://doi.org/10.1016/j.ymben.2016.01.006

Katsuyama, Y., Miyahisa, I., Funa, N., & Horinouchi, S. (2007). One-pot synthesis of genistein from tyrosine by coincubation of genetically engineered *Escherichia coli* and *Saccharomyces cerevisiae cells*. *Applied Microbiology and Biotechnology*, 73, 1143–1149.

Koirala, N., Pandey, R. P., Thuan, N. H., Ghimire, G. P., Jung, H. J., Oh, T. J., & Sohng, J. K. (2015). Metabolic engineering of *Escherichia coli* for the production of isoflavonoid-4′-*O*-methoxides and their biological activities. *Biotechnology & Applied Biochemistry*, 66(4), 484–493. https://doi.org/10.1002/bab.1452

Koma, D., Kishida, T., Yoshida, E., Ohashi, H., Yamanaka, H., Moriyoshi, K., Nagamori, E., & Ohmoto, T. (2020). Chromosome engineering to generate plasmid-free phenylalanine- and tyrosine-overproducing *Escherichia coli* strains that can be applied in the generation of aromatic-compound-producing bacteria. *Applied and Environmental Microbiology*, 86(14), e00525–20.

Kumarasamy, Y., Cox, P. J., Jaspars, M., Nahar, L., & Sarker, S. D. (2006). Bioactivity of hirsutanolol, oregonin and genkwanin, isolated from the seeds of *Alnus glutinosa* (Betulaceae). *Natural Product Communications*, 1(8), 641–644.

Lee, H., Kim, B. G., Kim, M., & Ahn, J. H. (2015). Biosynthesis of two flavones, apigenin and genkwanin, in *Escherichia coli*. *Journal of Microbiology & Biotechnology*, 25(9), 1442–1448. https://doi.org/10.4014/jmb.1503.03011

Leonard, E. & Koffas, M.A. (2007). Engineering of artificial plant cytochrome P450 enzymes for synthesis of isoflavones by Escherichia coli. *Applied and Environmental Microbiology*, 73(22), 7246–7251.

Li, H., van den Driesche, S., Bunge, F., Yang, B., & Vellekoop, M. J. (2019). Optimization of on-chip bacterial culture conditions using the Box–Behnken design response surface methodology for faster drug susceptibility screening. *Talanta*, 194, 627–633. https://doi.org/10.1016/j.talanta.2018.10.048

Li, Y., Hong, J., Li, H., Qi, X., Guo, Y., Han, M., & Wang, X. (2017). Genkwanin nanosuspensions: A novel and potential antitumor drug in breast carcinoma therapy. *Drug Delivery*, 24(1), 1491–1500. https://doi.org/10.1080/10717544.2017.1384519

Liu, X., Hua, K., Liu, D., Wu, Z. L., Wang, Y., Zhang, H., Deng, Z., Pfeifer, B. A., & Jiang, M. (2020). Heterologous biosynthesis of type II polyketide products using *E. coli*. *ACS Chemical Biology*, 15(5), 1177–1183. https://doi.org/10.1021/acschembio.9b00827

Malla, S., Koffas, M. A. G., Kazlauskas, R. J., & Kim, B. G. (2012). Production of 7-*O*-methyl aromadendrin, a medicinally valuable flavonoid, in *Escherichia coli*. *Applied and Environmental Microbiology*, 78(3), 684–694.

Nakai, Y., Hayashi, H., & Kagamiyama, H. (1996). Cloning and characterization of the tyrB gene from *Salmonella typhimurium*. *Biochimica et Biophysica Acta*, 1308(3), 189–92. https://doi.org/10.1016/0167-4781(96)00113-3

Patnaik, R., Zolandz, R. R., Green, D. A., & Kraynie, D. F. (2008). L-tyrosine production by recombinant *Escherichia coli*: Fermentation optimization and recovery. *Biotechnology & Bioengineering*, 99, 741–752.

Pontrelli, S., Chiu, T. Y., Lan, F. Y., Chen, F. Y., Chang, P., & Liao, J. C. (2018). *Escherichia coli* as a host for metabolic engineering. *Metabolic Engineering*, 50, 16–46.

Porras, G., Bacsa, J., Tang, H., & Quave, C. L. (2019). Characterization and structural analysis of genkwanin, a natural product from *Callicarpa americana*. *Crystals*, 9(10), 491. https://doi.org/10.3390/cryst9100491

Sambrook, J. & Green, M. R. (2012). *Molecular cloning: A laboratory manual* (4th ed.). Cold Spring Harbor Laboratory Press.

Santos, C. N. S., Koffas, M., & Stephanopoulos, G. (2011). Optimization of a heterologous pathway for the production of flavonoids from glucose. *Metabolic Engineering*, 13(4), 392–400.

Takemura, M., Tanaka, R., & Misawa, N. (2017). Pathway engineering for the production of β-amyrin and cycloartenol in *Escherichia coli*: A method to biosynthesize plant-derived triterpene skeletons in *E. coli*. *Applied Microbiology and Biotechnology*, 101(17), 6615–6625.

Thuan, N. H., Chaudhary, A. K., Cuong, D. V., & Cuong, N. X. (2018a). Engineering co-culture system for production of apigetrin in *Escherichia coli*. *Journal of Industrial Microbiology and Biotechnology*, 45(3), 175–185.

Thuan, N. H., Tatipamula, V. B., Canh, N. X., & Giang, N. V. (2022b). Recent advances in microbial co-culture for production of value-added compounds. *3 Biotech*, 12(5), 115. https://doi.org/10.1007/s13205-022-03177-4

Thuan, N. H., Tatipamula, V. B., Viet, T. T., Tien, N. Q. D., & Loc, N. H. (2022a). Bioproduction of eriodictyol by *Escherichia coli* engineered co-culture. *World Journal of Microbiology & Biotechnology*, 38(7), 112.

Thuan, N. H., Trung, N. T., Cuong, N. X., Cuong, D. V., Quyen, D. V., & Malla, S. (2018b). *Escherichia coli* modular coculture system for resveratrol glucosides production. *World Journal of Microbiology and Biotechnology*, 34(6), 75.

Wang, R., Zhao, S., Wang, Z., & Koffas, M. A. (2020c). Recent advances in modular co-culture engineering for synthesis of natural products. *Current Opinion in Biotechnology*, 62, 65–71.

Wang, X., Li, Z., Policarpio, L., Koffas, M. A. G., & Zhang, H. (2020a). *De novo* biosynthesis of complex natural product sakuranetin using modular co-culture engineering. *Applied Microbiology and Biotechnology*, 104(11), 4849–4861.

Wang, X., Shao, A., Li, Z., Policarpio, L., & Zhang, H. (2020b). Constructing *E. coli* co-cultures for *de novo* biosynthesis of natural product acacetin. *Biotechnology Journal*, 15(9), e2000131.

Wang, Y., Wang, Q., Wang, Y., Han, H., Hou, Y., & Shi, Y. (2017). Statistical optimization for the production of recombinant cold-adapted superoxide dismutase in *E. coli* using response surface methodology. *Bioengineered*, 8(6), 693–699. https://doi.org/10.1080/21655979.2017.1303589

Wu, G., Yan, Q., Jones, J. A., Tang, Y. J., Fong, S. S., & Koffas, M. A. G. (2016). Metabolic burden cornerstones in synthetic biology and metabolic engineering applications. *Trends in Biotechnology*, 34(8), 652–664. https://doi.org/10.1016/j.tibtech.2016.02.010

Xu., Y., Yang, L., Zhao, S., & Wang, Z. (2019). Large-scale production of tauroursodeoxycholic acid products through fermentation optimization of engineered *Escherichia coli* cell factory. *Microbial Cell Factories*, 18(1), 34. https://doi.org/10.1186/s12934-019-1076-2

Yang, D., Prabowo, C. P. S., Eun, H., Park, S. Y., Cho, I. J., Jiao, S., & Lee, S. Y. (2021). *Escherichia coli* as a platform microbial host for systems metabolic engineering. *Essays in Biochemistry*, 65(2), 225–246. https://doi.org/10.1042/EBC20200172

Yuan, S. F., Yi, X., Johnston, T. G., & Alper, H. S. (2020). *De novo* resveratrol production through modular engineering of an *Escherichia coli–Saccharomyces cerevisiae* co-culture. *Microbial Cell Factories*, 19(1), 143. https://doi.org/10.1186/s12934-020-01401-5

Zhang, H. & Wang, X. (2016). Modular co-culture engineering, a new approach for metabolic engineering. *Metabolic Engineering*, 37, 114–121.

Zhang, W., Liu, H., Li, X., Liu, D., Dong, X. T., Li, F. F., Wang, E. X., Li, B. Z., & Yuan, Y. J. (2017). Production of naringenin from D-xylose with co-culture of *E. coli* and *S. cerevisiae*. *Engineering in Life Sciences*, 17(9), 1021–1029. https://doi.org/10.1002/elsc.201700039

Received: August 4, 2023. **Accepted:** September 18, 2023