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METABOLIC ENGINEERING AND SYNTHETIC BIOLOGY - ORIGINAL PAPER

Engineering co-culture system for production of apigetrin in *Escherichia coli*

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Abstract

Microbial cells have extensively been utilized to produce value-added bioactive compounds. Based on advancement in protein engineering, DNA recombinant technology, genome engineering, and metabolic remodeling, the microbes can be re-engineered to produce industrially and medicinally important platform chemicals. The emergence of co-culture system which reduces the metabolic burden and allows parallel optimization of the engineered pathway in a modular fashion restricting the formation of undesired byproducts has become an alternative way to synthesize and produce bioactive compounds. In this study, we present genetically engineered *E. coli*-based co-culture system to the de novo synthesis of apigetrin (APG), an apigenin-7-*O*-β-D-glucopyranoside of apigenin. The culture system consists of an upstream module including 4-coumarate: CoA ligase (4CL), chalcone synthase, chalcone flavanone isomerase (CHS, CHI), and flavone synthase I (FNSI) to synthesize apigenin (API) from *p*-coumaric acid (PCA). Whereas, the downstream system contains a metabolizing module to enhance the production of UDP-glucose and expression of glycosyltransferase (PaGT3) to convert API into APG. To accomplish this improvement in titer, the initial inoculum ratio of strains for making the co-culture system, temperature, and media component was optimized. Following large-scale production, a yield of 38.5 µM (16.6 mg/L) of APG was achieved. In overall, this study provided an efficient tool to synthesize bioactive compounds in microbial cells.

Keywords Apigetrin · Co-culture · *E. coli* · Fermentation · Recombinant

Introduction

Metabolic engineering by remodeling metabolic system of the cultured organisms has become a critically important tool to synthesize bioactive secondary metabolites. There are indeed more substantial numbers of biofuels, drugs, and industrially essential chemicals being produced by metabolomics-modified organisms [2, 4, 25, 29, 41, 51]. Monoculture systems have generated most of these bioactive compounds under optimal conditions. Although monoculture system is an efficient way to produce platform chemicals, it suffers from several disadvantages including lack of an optimal environment for the functioning of all pathway-specific enzymes, the increment in metabolic burden due to reconstruction or heterologous expression of complex pathways, and the formation of undesired byproducts [56, 58].

Several secondary metabolites with high industrial and medicinal values are produced by microorganisms and plants [53, 54]. Among them, flavonoids are a class of ubiquitous metabolites found in different plant species with profound bioactivity. Most of them exist in their glycosylated form with more stability, bioactivity, and solubility. However, aglycones are also present in large quantities [21]. Among various flavonoid reported, apigenin (API) and its derivative have been proven to be a well-known anti-aging, anti-fungal, anti-tumor, and

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anti-inflammatory agents [27, 28, 44]. Moreover, its derivative apigenin-7-*O*-β-D-glucopyranoside, also known as apigetrin (APG), has shown additional anti-proliferative and anti-oxidant activity against reactive oxygen species (ROS) [36, 44]. Besides glycosylation, hydroxylation, malonylation, methylation, and sulfation are common modifications that diversify flavonoids. Therefore, there is an increasing demand for these molecules in clinics and food-processing practices. Since lower solubility in water, short retention time in the intestine, and lower absorption rate restrict the consumption of flavonoids, the development of water-soluble flavonoids has been implicated for the treatment of many medical problems [15]. Glycosylation is one of the ways to enhance water solubility, the stability, and pharmacokinetic properties of flavonoids [21, 22, 46]. The biological activities of most natural products are attributed to the sugar moieties attached to them [11]. While chemical synthesis is used to synthesize such derivatives, the use of site-specific glycosyltransferases (GTs) that catalyzes the formation of the glycosidic bond are often used for the in vitro and in vivo synthesis of glycosides [5, 6, 8, 14, 47, 49]. Due to the development of chemical and molecular biology techniques, APG has been synthesized by chemical, semi-, and biosynthetic approaches [26, 32, 38, 50]. Among them, most biological approaches for APG production were carried out using plant-originated genes including TAL, 4CL, CHS, CHI, FNSI (or FNSII), and GTs via reconstruction in recombinant plasmids and hosts such as *E. coli, Bacillus, and Saccharomyces cerevisiae* to construct a monoculture system [13, 19, 23, 35].

Recently, the co-culture system simultaneously culturing two distinct metabolically engineered strains for production of desired metabolites has become an exciting and alternative tool. Numerous studies have reported the use of *E. coli*, *Bacillus*, and *S. cerevisiae* as a co-culture system to produce plenty of known bioactive compounds such as flavonoids, alkaloids, terpenoids, etc. [7, 10, 18, 30, 55–57]. Therefore, in the present study, we described the use of a co-culture system by genetically engineered *E. coli* for the production of APG. To achieve this endeavor, the culture system was compartmentalized into two parts: upstream strain containing biosynthetic pathway for production of API and downstream strain for overproduction of UDP-glucose and heterologous expression of a glycosyltransferase (Fig. 1).

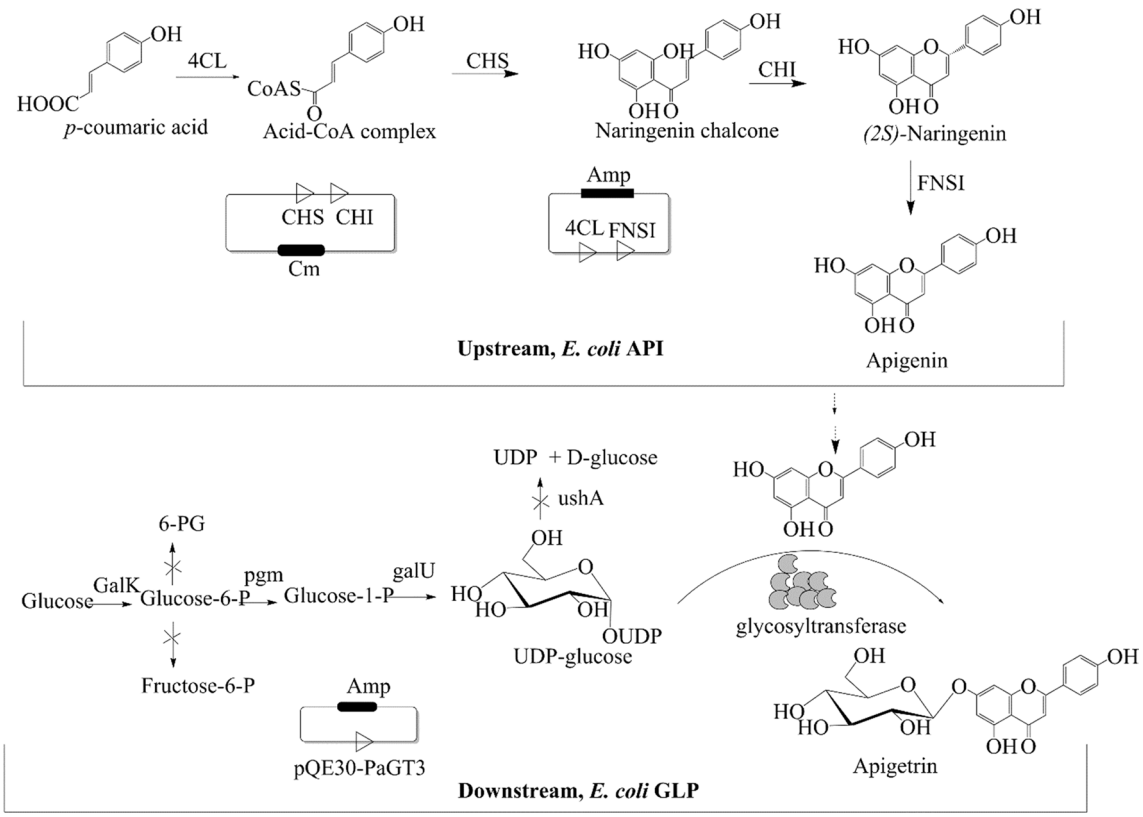

Fig. 1 Scheme of *E. coli* co-culture for production of APG

Materials and methods

Culture media and chemicals

LB (Luria–Bertani) and M9 minimal medium (per liter, 6 g Na_2HPO_4, 3 g K_2HPO_4, 0.5 g NaCl, 0.1% NH_4CL, 1% glucose, 1 mM $MgSO_4{\cdot}7H_2O$, 100 µM $CaCl_2$, 1.46 mg biotin, 1 mg thiamine-HCl) containing 2% glucose were used for seed culture and substrate fermentation, respectively. Antibiotics including ampicillin, kanamycin, and chloramphenicol (Biobasics, Canada) were used in the final concentration of 100, 50 and 30 µg/mL, respectively. Ethyl acetate, methanol, acetonitrile, and dimethyl sulfoxide (DMSO) were purchased from Merck (Germany) or Sigma (USA). Nanodrop 2000 UV–Vis Spectrophotometer (Thermo, USA) and HPLC–PDA (Agilent, USA) were used for chromatographic analysis.

DNA manipulation, bacterial transformation, recombinant plasmid reconstruction

DNA manipulation such as DNA plasmid extraction, purification, digestion, and ligation followed standard protocols as described by [43]. pACYC-Deut-1 containing chalcone synthase (CHS) and chalcone flavanone isomerase(CHI) from *Petunia hybrida* was previously described [24, 31]. 4CL-2 [4-coumarate: CoA ligase (4CL2) from *Nicotiana tabacum* cv. Samsun] gene was digested from the pAC-4CL-STS plasmid by *Nco*I/*Not*I restriction enzyme and then transferred to the multicloning site 1 (MCS1) of pCDFDuet-1 bearing streptomycin resistance gene (*Str*) [3]. Similarly, flavone synthase I (FNSI) from parsley *Petroselinum crispum* cv was digested with *Nde*I/*Xho*I from pET15b [33] and cloned into MCS2 of this duet vector. Those vectors were then introduced into *E. coli* BL21(DE3) to construct the recombinant *E. coli* API to produce API. In another way, *E. coli* MA3 strain, previously constructed for enhancing production of UDP-glucose [40, 48], was transformed with the recombinant pQE-30 vector containing glycosyltransferase-encoded gene (PaGT3) [37, 39] to generate *E. coli* GLP. All strains and DNA plasmids were listed in Table 1.

Use of *E. coli* GLP strain for the synthesis of apigetrin

Escherichia coli GLP strain was cultured in 3 mL of LB medium, 220 rpm, and 37 °C for overnight. Then, 500 µL of this seed culture was transferred to 250 mL flask containing 50 mL minimal M9 medium plus 2% glucose and maintained at 37 °C, 220 rpm until its optical density (OD) at 600 nm achieved 0.6. Total 1 mM IPTG was then added and continuously cultured under the same condition for 3–4 h for induction of protein. Total 100 µM purified API was supplemented to the induced broth and continuously maintained until 60 h. Bioconversion of substrate was checked at 12 h of interval time.

Table 1 Genes and strains in this study

Strains/plasmids	Descriptions	Sources/references
Genes		
CHS	*Petunia hybrida*-derived chalcone synthase	[25, 31]
CHI	*Medicago sativa*-derived chalcone flavanone isomerase	[25, 31]
PaGT3	Glycosyltransferase, GeneBank accession number: AB458517	[37, 39]
FNSI	*Parsley* flavone synthase I, GeneBank accession number: AY230247	[33]
4CL-2	4-Coumarate: coenzyme A (CoA) ligase (4CL) (EC 6.2.1.12)	[3]
Plasmids		
pAC-4CL-FNSI	Expression vector pETDuet-1 containing the T7 promoter and containing ampicillin resistance gene	Novagen, USA
pAC-CHS_CHI (Cm)	Expression vector pACYCDuet-1 bearing the T7 promoter and chloramphenicol resistance gene	Novagen, USA
pQE30-PaGT3	Expression vector pCDFDuet-1 containing the T7 promoter and ampicillin resistance gene	Novagen, USA
E. coli strains		
E. coli XL1Blue	General cloning bacterial host	Promega, USA
E. coli BL21(DE3)	*ompT hsdT hsdS*(r_B^- m_B^-) *gal* (DE3)	Promega, USA
E. coli BL21(DE3) Δ*zwf*/Δ*pgi*/Δ*ushA*	Triple deletion *E. coli* mutant of *zwf*, *pgi* and *ushA*	[40, 48]
E. coli API	*E. coli* BL21(DE3) containing pAC-4CL-FNSI (Amp), pAC-CHS_CHI (Cm)	This study
E. coli APG	*E. coli* BL21(DE3) Δ*zwf*/Δ*pgi* containing pAC-4CL-FNSI (Amp), pAC-CHS_CHI (Cm), pQE30-PaGT3 (Amp)	This study
E. coli GLP	*E. coli* BL21(DE3) Δ*zwf*/Δ*pgi*/Δ*ushA* containing pQE30-PaGT3(Amp)	This study

Synthesis of apigetrin and apigenin from *p*-coumaric acid

Escherichia coli API was used to produce API by feeding various concentration of *p*-coumaric acid (PCA). Similarly, minimal M9 plus 2% glucose was used for culture with 37 °C and 220 rpm. To increase the bioconversion of substrate, PCA was supplemented gradually into the induced *E. coli* API culture broth to avoid its growth inhibition by excessive substrate, i.e., 20, 20, 30, and 30 µM of PCA. Moreover, the sample was taken out at 12-h intervals for production testing.

In another way, the *E. coli* MA2 strain [40, 48] was transformed with various recombinant vectors (pAC-4CL-FNSI, pAC-CHS_CHI, and pQE30-PaGT3) to generate *E. coli* APG strain and used for the synthesis of APG. PCA was also gradually supplemented to culture to study the dependence of substrate bioconversion upon a time. Mainly 20, 20, 30, and 30 µM PCA was gradually added at 12, 24, 36 and 48 h, respectively, and the broth culture was maintained until 60 h.

Experimental co-culture for production of apigetrin

Effect of initial inoculum ratio on the production of apigetrin

Escherichia coli API and GLP were separately cultured by 3 mL of LB broth medium containing appropriate antibiotics at 37 °C and 220 rpm for overnight. Five hundred µL (unless stated) of each strain was subsequently diluted into 50 mL of M9 minimal media supplementing necessary antibiotics in 250 mL flasks; then the flasks were incubated at 37 °C and 220 rpm till the OD_{600nm} of 0.6. The culture broths were centrifuged, and the cell pellets were resuspended in 50 mL of M9 media plus 2% glucose. The two populations were mixed in different ratios by OD, i.e., various ratios of API/GLP = 1: 1; 2:1; 4:1; 6:1; 8:1 and 10:1 (v/v) to generate a co-culture system. The broth culture was added with 1 mM IPGT and continuously cultured for 3–4 h at 37 °C, and 220 rpm to induce protein expression. Subsequently, the system was supplemented by total 100 µM PCA, i.e., 20, 20, 30, 30 µM at 12, 24, 36, and 48 h. Formation of NRN, API, and APG was investigated at interval time of 12 h until 60 h of culture. Similarly, inverse various ratios (in which API was kept in constant, but GLP was varied) of API/GLP, i.e. 1:2, 1:4, 1:6, 1:8, and 1:10 were also used for evaluating the production of APG under the same conditions and substrate feeding as described above.

Effect of culture temperature

Various range of culture temperature from 25 to 37 °C, i.e., 25, 27, 30, 32, and 37 °C was used to investigate the effect on the production of API and its derivatives. The total PCA concentration of 100 µM was supplemented to the culture under 220 rpm. The converted naringenin, API, and APG concentration were checked after interval time of 12 h until 60 h of culture.

Analysis and quantification

HPLC–DAD analysis was performed by injecting 20 µL of the samples on an Agilent 1260 HPLC system equipped with a photodiode array detector (DAD), degasser, an autosampler. An Agilent Zorbax SB C18 column (250 mm × 4.6 mm i.d., 5 µm; Agilent, Santa Clara, CA, USA) was used. The mobile phase of 0.1% trifluoroacetic acid (TFA) aqueous solution (solvent A) and acetonitrile (solvent B) were used with 1 mL/min flow rate. The concentration of acetonitrile during the binary gradient condition was as: 0–10 min, 0–60%; 10–20 min, 75%; 20–35 min, 75–90%. Peak detection was carried out at UV absorbance at 295 nm whereas.

Naringenin, API, and APG were purified using an MPLC instrument equipped the silica gel RP-packed column (YMC gel ODS-A, AA12SA5, Japan). The corresponding peaks were collected and then lyophilized by freeze drier (FDU-1200, Eyela, Japan). All chemicals were dissolved in DMSO-d6 and subjected to NMR analysis. A series of concentrations ranging from 10 to 100 mg/L of the product were prepared to construct a calibration curve. The molecular mass of the compounds was determined in LC–ESI–MS using Phenomenex Synergi Polar-RP column (150 × 4.6 mm, 4 µm), positive-ion mode.

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.

Results

Production of apigenin from *p*-coumaric acid using *E. coli* API strain

Since glucose increases the intracellular acetyl-CoA concentration via glycolysis and acts as the primary carbon source for flavonoid production [45], instead of LB, M9 minimal media with 2% glucose was used to restrict the rapid degradation of PCA [31]. The recombinant *E. coli* harboring biosynthetic gene cluster for API production was constructed using 4-coumarate: CoA ligase (4CL2) gene from *Nicotiana tabacum* cv. Samsun [3], chalcone synthase (CHS) and chalcone flavanone isomerase(CHI) from *Petunia hybrida* and

Medicago sativa, respectively, as described early, and flavone synthase I (FNSI) from parsley *Petroselinum crispum* cv. [33]. When cultured at 37 °C, 220 rpm, and fed with 20 µM of PCA, the formation of naringenin (NRN) and API were observed by HPLC analysis (Fig. 2b). Further analysis by LC–ESI–MS resulted in the mass – charge ratio (*m*/*z*) of $[M + H]^+$ of 272.9 and 270.8 for NRN and API in positive mode, respectively (Fig. 3a, b). Also, the time and concentration-dependent study showed highest concentration of API (31.8 µM) at 60 h, when 100 µM PCA was fed entirely (Fig. 4). Moreover, the yield of conversion was not improved when a higher concentration of PCA was supplied into the broth culture.

Synthesis of apigetrin using *E. coli* APG strain using PCA as initial substrate

To avoid growth inhibition of *E. coli* by extra-cellular addition of PCA [51], 10 µM PCA was initially supplemented to the induce broth culture. Post substrate addition after 5 h,

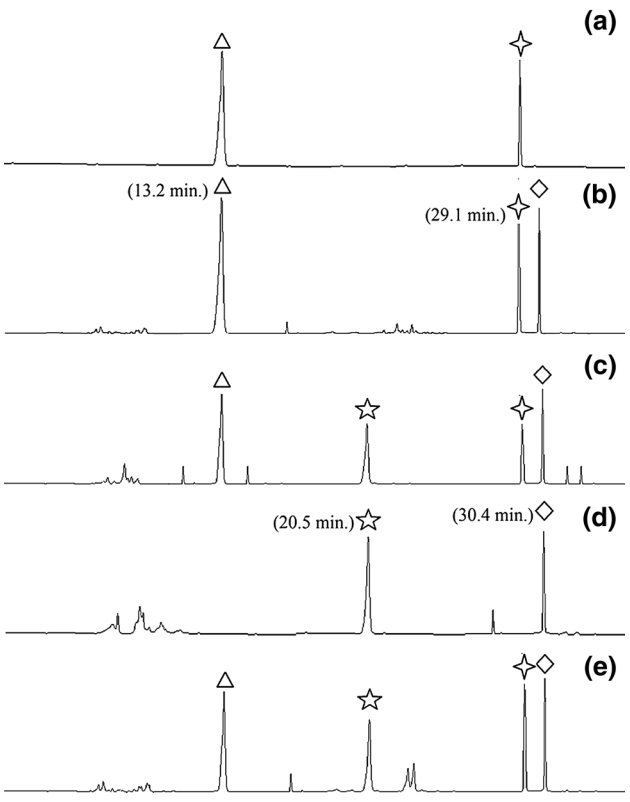

Fig. 2 HPLC trace analysis of the biotransformation products of recombinant *E. coli* recombinant strains. **a** authentic *PCA* and API standard compounds, **b** *E. coli* API supplemented with the substrate PCA for production of API, **c** synthesis of APG by *E. coli* APG strain utilizing PCA as initial substrate, **d** *E. coli* GLP used for bioconversion of API to APG, and **e** co-culture of API and GLP (ratio of 1:2) for production of APG. triangle, PCA peak; star, APG peak; diamond, API peak; and 4 pointed star shape, naringenin peak

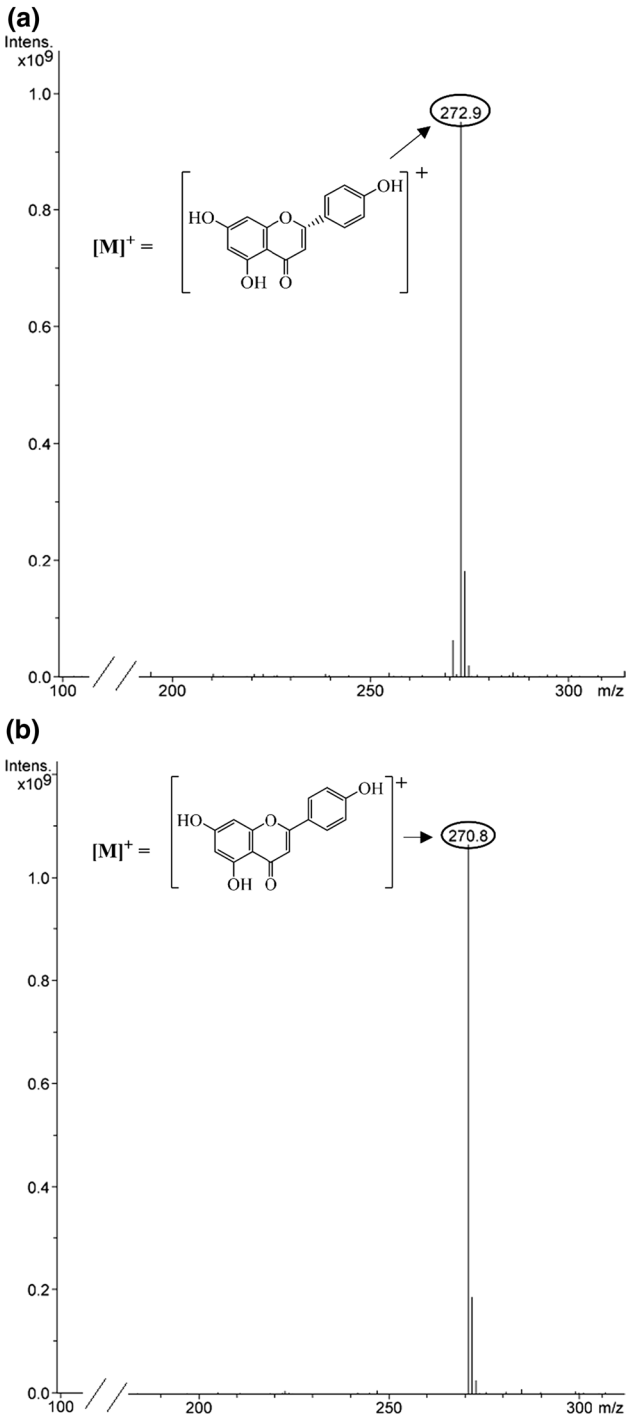

Fig. 3 LC-ESI–MS profile of products in positive mode: **a** naringenin, mass-to-charge ratio (*m*/*z*) of $[M + H]^+ = 272.9$; **b** API, *m*/*z* of $[M + H]^+ = 270.8$; **c** APG, *m*/*z* of $[M + H]^+ = 433.0$

the production of NRN, API, and APG were analyzed. As evident from Fig. 3c, we observed the presence of APG peak with retention time of 20.50 min in HPLC chromatogram (Fig. 2c) with *m*/*z* of $[APG + H]^+ = 433.0$. Further analysis of bioconversion at 12 h interval showed 77.7% PCA

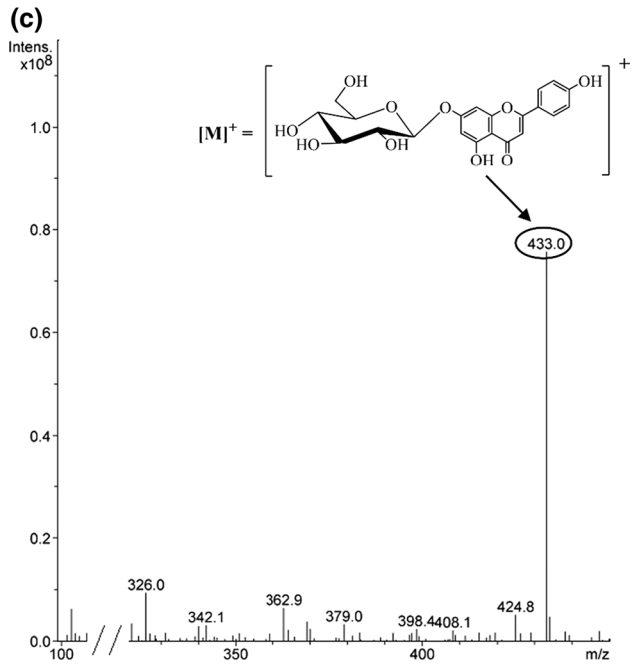

Fig. 3 (continued)

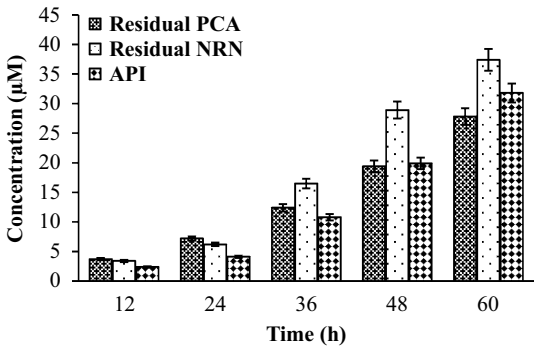

Fig. 4 Production of API by *E. coli* API strain, during 60 h of culture, using PCA as the substrate

to be consumed, while the maximum yield of APG, NRN, and API reached 15.5, 32.8, and 27.4 μM after 60-h culture, respectively (Fig. 5).

Biotransformation of API to APG by *E. coli* GLP

To compare the yield of APG production, 10 μM API was initially supplemented to induced culture broth, which gradually increased up to 100 μM in total at 48 h maintaining till 60 h. As evident from HPLC chromatogram monitored at the 12-h interval, the maximum accumulation of APG reached to 45.8 μM (19.8 mg/L) after 60 h (Figs. 2d, 6), where the rate of biotransformation of API to APG was higher than a synthesis of APG from PCA.

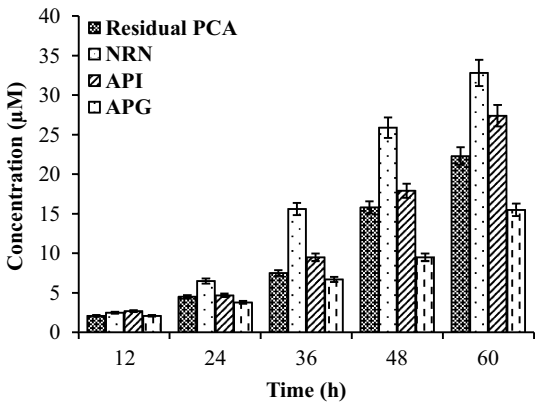

Fig. 5 Production of APG in *E. coli* APG strain, during 60 h of culture, using PCA as the substrate

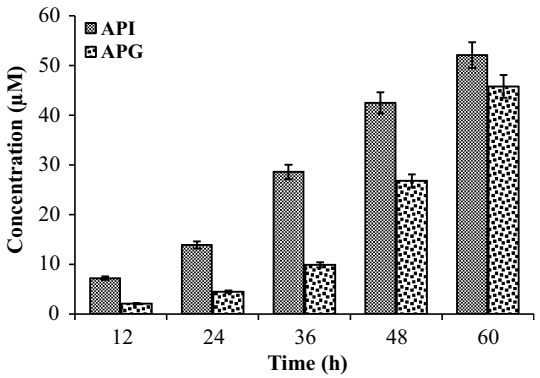

Fig. 6 Bioconversion of API to APG using *E. coli* GLP as the whole-cell biocatalyst

De novo co-culture for production of APG

Optimization of inoculum ratio in co-culture

Co-culture for the production of natural products requires testing proper ratio of initial inoculum because each strain was designed to carry specific work. Mainly, *E. coli* API and GLP were stepwise reconstructed to synthesis API and APG.

By changing the ratio of API/GLP from 1:1 to 10:1, the production of APG concentration was achieved to maximum, yielding of 32.5 μM (14.1 mg/L) or 2.1-fold compared to the use of mono-culture *E. coli* APG (Figs. 2e, 7). Hence, *E. coli* GLP expressed the strong bioactivity of PaGT3 again as proved by biotransformation of API to APG too. Moreover, the higher the ratio of *E. coli* API the more production of APG. Concurrently, the more production of API the more consumption of initial substrate, PCA.

In contrast, the ratio of API/GLP was changed from 1/2 to 1/10 the maximum concentration of APG has achieved the highest yield of 23.4 μM at a ratio of 1/10 and gradually

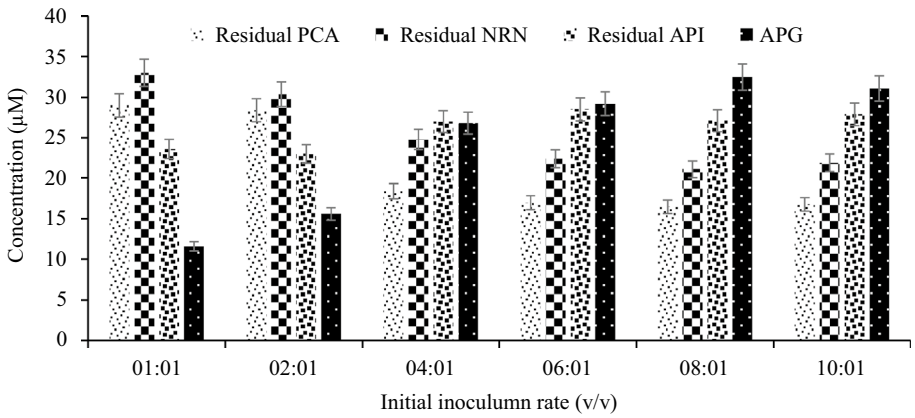

Fig. 7 Effect of primary inoculum culture on the production of APG by various types of *E. coli* co-culture

decreased to 15.6 μM at the ratio of 1/2. Therefore, it was observed that higher APG concentration in accompanies with a higher ratio of GLP/API. However, the ratio of *E. coli* API was constant then the maximum yield of APG just achieved 72% (23.4/32.5 × 100) compared to the previous case. Based on above result analysis the ratio of API/GLP was selected between 8 and 10 for further experiments.

Effect of temperature on co-culture for production of APG

Temperature is one of the most important factors that affect bacterial growth and heterologous expression of enzymes. It has been proven that 35–37 °C is a suitable range for *E. coli* culture. However, glycosyltransferases are optimally expressed at a lower temperature. Thereby, various range of temperature was tested for co-culture to obtain higher production of APG. As a result, API was strongly synthesized, PCA concentration was fast consumed resulting in the highest yield of APG of 38.5 μM (16.6 mg/L) (Fig. 8) when the range of temperature maintained between 25 and 32 °C. However, the production of API was not increased when the temperature reached 37 °C with a maximal yield of APG was 32.5 μM.

Structural confirmation of NRN, API, and APG

The structures of API, APG, and NRN were confirmed by 1D and 2D NMR. We observed two *meta*-coupled [δ_H 6.19 (H-6) and 6.48 (H-8), each 1H, d, J = 2.0 Hz] and four *ortho*-coupled [δ_H 7.92 (H-2′ and H-6′) and 6.92 (H-3′ and H-5′), each 2H, d, J = 9.0 Hz] aromatic protons of API in ^{1}H NMR spectrum. Also, the signals of one olefinic [δ_H 6.76 (1H, s, H-2)] and one chelated hydroxyl [δ_H 12.95 (1H, s, 5-OH)] protons were observed. The ^{13}C NMR spectrum of API exhibited signals of 15 carbons for a flavone skeleton (Table 2). Comparison of the ^{1}H and ^{13}C NMR data of API with the reported values [1, 49] as well as detailed analysis of its HMBC correlations (Fig. 9) elucidated its structure as 5,7,4′-trihydroxyflavone. Similarly, the other compounds were characterized as APG (apigenin-7-*O*-β-D-glucoside) [12, 49] and 2*S*-5,7,4′-trihydroxyflavanone (naringenin) [52], which is in good agreement with their ^{1}H and ^{13}C NMR chromatograms, earlier report, and combination with their HMBC evidence (Figs. S2, S3, and S4, supporting information).

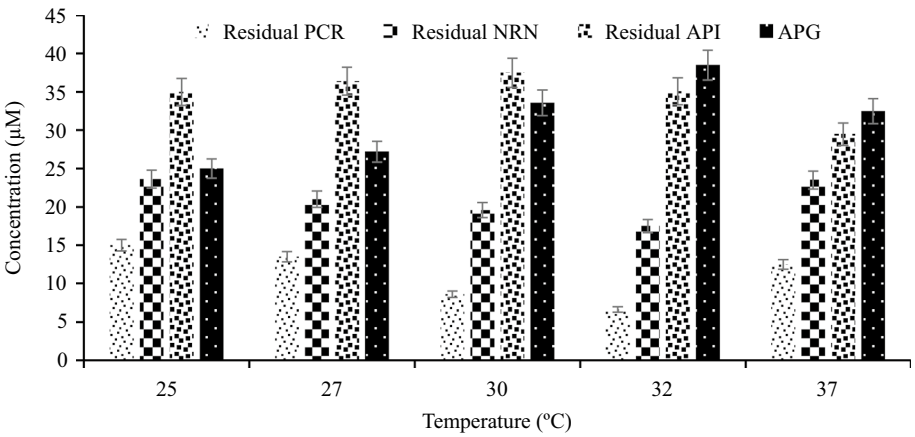

Fig. 8 Effect of various types of culture temperature on production of APG by *E. coli* co-culture

Table 2 1H NMR (500 MHz, DMSO-*d6*) and 13C NMR (125 MHz, DMSO-*d6*) data of API, APG and NRN

Position	Apigenin		Apigetrin		Naringenin	
	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)	δ_C	δ_H mult. (*J* in Hz)
2	163.71	–	164.24	–	78.36	5.44 dd (3.0, 13.0)
3	102.81	6.76 s	103.08	6.86 s	41.93	2.68 dd (3.0, 16.5) 3.25 dd (13.0, 16.5)
4	181.70	–	181.96	–	196.31	–
5	161.41	–	161.08	–	163.43	–
6	98.79	6.19 d (2.0)	99.50	6.44 d (2.0)	95.73	5.88 s
7	164.08	–	162.94	–	166.58	–
8	93.92	6.48 d (2.0)	94.83	6.83 d (2.0)	94.91	5.88 s
9	157.27	–	156.91	–	162.89	–
10	103.67	–	105.31	–	101.72	–
1′	121.15	–	121.00	–	128.81	–
2′, 6′	128.42	7.92 d (9.0)	128.58	7.95 d (8.5)	128.26	7.31 d (8.5)
3′, 5′	115.92	6.92 d (9.0)	115.97	6.94 d (8.5)	115.11	6.79 d (8.5)
4′	161.12	–	161.33	–	157.67	–
5-OH	–	12.95 s	–	12.95 s	–	12.14 s
glc						
1″			99.90	5.06 d (7.5)		
2″			73.08	3.27[a]		
3″			76.42	3.29[a]		
4″			69.55	3.19 t (9.0)		
5″			77.15	3.45 m		
6″			60.59	3.72 dd (4.5, 12.0) 3.48 dd (2.0, 12.0)		

[a]Overlapped signals

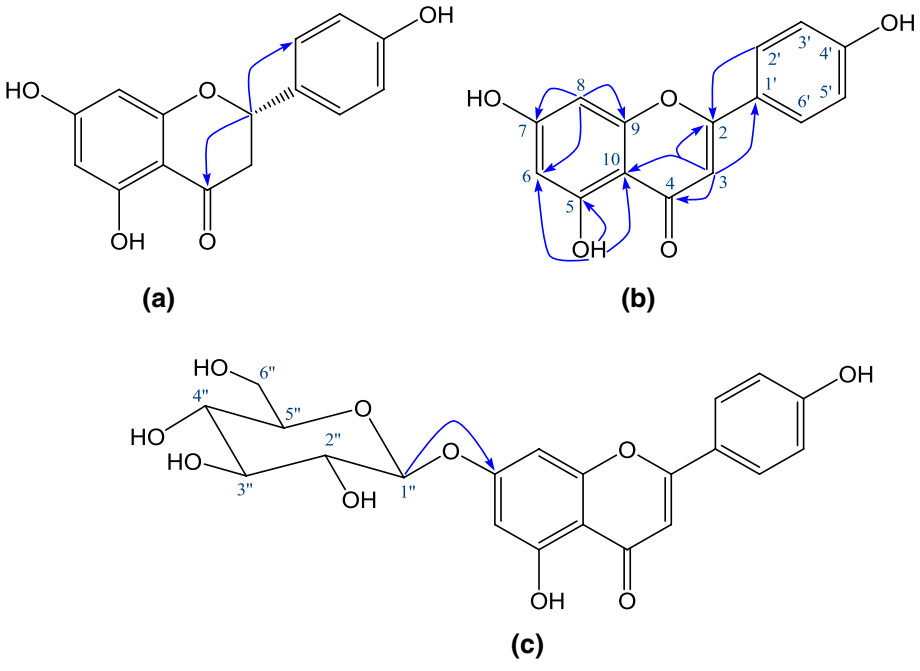

Fig. 9 Structures and critical HMBC (H → C) correlations of NRN (**a**), API (**b**) and APG (**c**)

Discussion

It is observed that flavonoids are stable in the form of glycosides (O-, C- or N-glycosidic linked derivatives) in vascular plants. Till today, synthesis of flavonoid glycosides was carried out by a chemical approach with several disadvantages such as protection of un-reacted moiety, generation of various derivatives under severe conditions [50]. However, glycosyltransferase acts as a biocatalyst to catalyze the attachment of glycosyl moiety to various flavonoid acceptor in vitro and in vivo. Microbial synthesis of glycosides by mono-culture requires the construction of recombinant plasmids with numbers of genes, inhibition of bacterial chemo-physiological process, and challenge to achieve optimal culture conditions. For example, biological synthesis of flavanones (naringenin, pinocembrin) by recombinant mono-culture *E. coli* has been carried out by [16, 19, 24, 35]. Also, flavones (API, chrysin) have been synthesized by FNSI [34] including its derivative, genkwanin [23]. Therefore, as an alternative approach, co-culture system was designed in this study to fix the bottlenecks of monoculture. For example, resveratrol, afzelechin, muconic acid, and 3-aminobenzoic acid have been successfully produced by co-culture approach [9, 18, 55, 57]. Here, we have experimentally biosynthesized API and APG from PCA by monoculture system, where the maximal yield of each metabolite was 31.8 (8.6 mg/L) and 15.5 µM (6.7 mg/L), respectively. Besides, API substrate was also used for direct biotransformation by *E. coli* GLP and resulted in 45.8 µM of APG (19.8 mg/L). Furthermore, co-culture based on the modular system including upstream (*E. coli* API) and downstream (*E. coli* GLP) was optimized by the ratio of inoculum and temperature condition. By changing the various ratio of inoculum API/GLP, it resulted in the maximal yield of APG of 32.5 µM. Furthermore, the best yield of APG was achieved (38.5 µM or 16.6 mg/L) under optimal temperature (32 °C). Thereby, it meant increment in 2.5-fold of APG compared to mono-culture *E. coli* APG (16.6 mg/L), however, still lower than direct biotransformation of API by *E. coli* GLP (19.8 mg/L). For reference, the yield of API was mentioned in different records such as 30 mg/L [23] or 13 mg/L [34] via mono-culture. In comparison to other literature, the titer of afzelechin and resveratrol was achieved 40.7 ± 0.1, and 22.6 mg/L by *E. coli* co-culture; our yield of APG was in comparative level.

To further increase the titer of APG, several works need to be continuously carried out including strain improvement of *E. coli* API such as over-expression of malonyl-CoA [25, 34] or biosynthesis pathway of naringenin from the direct amino acid by the introduction of tyrosine ammonium lyase (TAL) or phenylalanine ammonia-lyase (PAL) [34, 35]. Similarly, improvement of *E. coli* GLP can be achieved by strengthening of PaGT3 via site-directed mutagenesis [20, 42]. Finally, it is necessary to optimize the culture conditions for co-culture system [17, 18].

Conclusions

Bioactive compounds are becoming essential in clinical practices, production of cosmetics, adjuvant, antibiotics as well as functional foods. Therefore, biosynthesis of bioactive compounds has evolved from simple to sophisticated approaches. Combination of genetic engineering, recombinant protein, metabolic engineering, and fermentation technology creates a novel ways for biosynthesis of these valuables including co-culture system. Our observations showed co-culture as an applicable and reproductive approach, which can be used to solve the bottlenecks of monoculture, yielding higher production of APG under simple conditions.

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Author contributions NHT and ACK conceived the study, designed experiments, analyzed data, and wrote the manuscript. NHT, DVC, and NXC performed the experiments, and NXC performed NMR study and analyzed the NMR data. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing financial interests.

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