Bioproduction of Eriodictyol By *Escherichia Coli* Engineered Co-Culture

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Abstract

Eriodictyol is a flavonoid in the flavanones subclass. It is abundantly present in a wide range of medicinal plants, citrus fruits, and vegetables. In addition, eriodictyol owns numerous importantly medicinal bioactivities such as inhibition of proliferation, metastasis and induction of apoptosis in glioma cells or inhibition of glioblastoma migration, and invasion. This study described the heterologous production of eriodictyol by *E. coli* based co-culture engineering system from the initial substrate as D-glucose. Notably, the upstream module was composed of genes for synthesis of *p*-coumaric acid (pCA) from D-glucose. The downstream module consisted of genes for the synthesis of eriodictyol from *p*-coumaric acid. The maximal result of eryodictyol was achieved 51.5 mg/L using optimal culture conditions, while mono-culture was only achieved 21.3 mg/L. In conclusion, co-culture was the efficiently alternative approach for the synthesis of eriodictyol and other natural products.

Highlights

- Microbial *E. coli – E. coli* co-culture was used to synthesis of eriodictyol from D-glucose.
- Production of eriodictyol was evaluated using the mono-culture and co-culture, respectively.
- The maximum yield of eriodictyol was achieved of 51.5 mg/L by shake flask culture.

Introduction

Eriodictyol (ED) [IUPAC name (2S)-2-(3,4-dihydroxyphenyl)- 5,7-dihydroxy-2,3-dihydrochromen-4-one], is a flavonoid in the flavanones subclass abundantly found in various medicinal plants, citrus fruits, and vegetables (Islam et al. 2020). ED has multiple therapeutic effects such as anti-oxidant, anti-cancer, anti-flammatory, and neuro-protective bioactivity (Clavin et al. 2007; Deng et al. 2020; Islam et al. 2020; Li et al. 2020; Lv et al. 2021). Such numerous pharmacological and biological roles of ED have attracted much research interest for increasing titer and quality (Marin et al. 2017).

Traditionally, ED is mainly extracted from yerba Santa Clause (*Eriodictyon californicum*), a vascular plant locally distributed in North America (Marohn 2001). Additionally, this phytomedicine and its derivatives are also found in *Eupatorium arnottianum* (Clavin et al. 2007), *Rosa canina* (Hvattum 2002), and *Millettia duchesnei* (Ley et al. 2005).

Exception of plant extraction, ED was also produced via enzymatic synthesis using flavonoid 3’ hydroxylase (F3’H), a cytochromes P450. This enzyme could be used as purified form (in-vitro) (Brugliera et al. 1999) or recombinant for whole-cell biotransformation or reconstruction type of modular engineering. For example, ED was synthesized by biostranformation in the recombinant *S. cerevisiae* containing *Gerbera hybrid*-derived flavonoid 3’ hydroxylase (F3’H) gene. Particularly, hydroxylation-functionalized yeast was used for whole-cell bioconversion of NRN to ED. Moreover, the highest titer was 200 mg/L of ED in the selective media (Amor et al. 2010). In other studies, ED had been heterologously synthesized by various recombinant *E. coli* mono-culture. Firstly, *E. coli* was used to produce ED by

adding exogenous caffeic acid resulting in a titer of 11 mg/L (Leonard et al. 2007). Subsequently, the substrates as malonate and 2 mM caffeic acid were supplemented to the culture of recombinant *E. coli* to improve the production of ED up to 50 mg/L (Leonard et al. 2008).

However, researchers also showed several disadvantages of microbial mono-culture for producing complex natural products as a metabolic burden due to all genes in the biosynthetic pathway accumulated in heterologous production in a host. In addition, the application of various molecular biotechniques in a single host may inhibit its growth and formation of by-products. Recently, co-culture has been a crucial synthetic method and widely applied to the synthesis of different complex natural products. Different species hosts have been used as components in co-culture as *E.coli* – *Saccharomyces cerevisiae* (Yuan et al. 2020), *Synechococcus elongates–E. coli* (Liu et al. 2021) and *Streptomyces* sp. with *Bacillus* sp. (Shin et al. 2018) to produce of resveratrol, isoprene, and dentigerumycin E, respectively. Furthermore, *Clostridium* spp. was used with various microbial strains as *Rhodobacter sphaeroides* or *Thermoanaerobacterium thermosaccharolyticum* GD17 to produce bio-fuels and bio-solvents (Du et al. 2020). Based on those works, it is obviously clear that the co-culture microbial system positively affects chemicals and natural products production.

In this study, a co-culture system comprising two metabolically engineered *E. coli* strains was constructed to produce naringenin from D-glucose (Fig. 1). In order to achieve the optimal strain contributed to this system, both *E. coli* strains were rationally engineered by deletion and overexpression of some relevant genes, which play essential roles in the process of tyrosine biosynthesis, as shown in Figure 1. In addition, this study was designed to engineer a *p*-coumaric acid - producing upstream module and ED-producing downstream by optimizing the *E. coli* host strain, inoculation size, temperature, and inoculation ratio of two microorganisms to achieve a high efficiency platform.

Materials And Methods

Culture media, reagents, and chemicals

Luria-Bertani (LB) medium containing the appropriate antibiotics, ampicillin (100 µg/mL), chloramphenicol (30 µg/mL), streptomycin (100 µg/mL) or kanamycin (30 µg/mL) was utilized for cultivating the general strains for cloning, screening the recombinant plasmid or seed culture.

Minimal M9 was used for the synthesis of *p*-coumaric acid, naringenin, and eriodictyol. M9 mineral medium contained 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). In addition, 15 g/L D-glucose was extra added for the culture of the upstream module to test *p*-coumaric acid production. 2 gram/L malonate was also supplemented for the culture of the downstream module.

Extraction chemicals 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.

Plasmid and strain construction

Inactivation of pheA gene. A pair of primer: 5'-GGC CTC CCA AAT CGG GGG GCC TTT TTT ATT GAT AAC AAA AAG GCA ACA CTG TGT AGG TGT AGG CTG GAG CTG CTT C-3' (forward primer) and 5'-TGA AAA GGT GCC GGA TGA TGT GAA TCA TCC GGC ACT GGA TTA TTA CTG GCA TGG GAA TTA GCC ATG GTC C-3' (reverse primer) was used to knock-out *pheA* using PCR-based efficient method (Datsenko and Wanner 2000). This work resulted in the mutant *E. coli* BL21(DE3)/Δ*pheA*.

Cloning and expression of tktA and ppsA. tktA and *ppsA* were cloned and expressed using pairs of primers for cloning of *tktA* and *ppsA* as followed: For_tktA (*Nde*I): GTG CCC TTC ATC CATATG TCT GGA GTC AAA ATG / Rev_tktA (*Bgl*II): CCT TTT TAC CCG AGATCT AAA TGC TAA TTA and For_ppsA (*BamH*I): GAG GGATCC TGC ACT GTG ATG TTG TCA / Rev_ppsA (EcoRI) ACC CCG GAATTC ACG GGG TTG TGA TTA. Consequently, those genes were introduced into pETDuet-1 containing ampicilin-resistant gene to obtain pETD-ppsA-tktA. Pair of primers for PCR of *Rhodotorula glutinis - originated tal* gene included For_TAL (*Nco*I): CATG CCA TGG CGC CGC GCC CGA CTT CTC and Rev_TAL (*EcoR*I): CCG GAA TTC TTA TGC CAG CAT CTT CAG CAG AAC GTT GTT GAT. Subsequently, this gene was introduced into pACYC Deut-1 containing chloramphenicol resistant gene to construct the recombinant plasmid pAC-TAL. All those recombinant plasmids were transformed into *E. coli* BL21(DE3)/Δ*pheA* to construct *E. coli* MCA.

Cloning and expression of matB and matC. PCR cloning of *matB* and *matC* was used pairs of primers as followed: For_matB (*BamH*I): GGATCC AGGAGGGCGAAAATGAGCAAC / Rev_matB (*Hind*III): CCAAAGCTT GAGAGCGCGGTCGCCTTACGT and For_matC (*Nde*I): CATATG GGGAGGGGAATCATGGGTATT / Rev_matC (*Xho*I): GCTCTCGAGAGCAGCCCGTCGTCAAAC. Next, *Rhizobium tripolii* -originated *matB* (malonyl-CoA synthetase) and *matC* (dicarboxylate carrier protein) were cloned into the vector pCDF-Duet1 containing streptomycin resistant gene to generate the recombinant pCDF-*matB-matC*.

Cloning and expression of 4CL. CL gene was digested from pAC-4CL-FNSI using restriction enzymes *Nco*I/*Not*I and subsequently cloned into pET28a vector to construct pET28-CL (Thuan et al. 2018a). In additional, pAC-CHS-CHI contains CHS and CHI genes (chloramphenicol resistant gene) (Thuan et al. 2018a).

Bacillus megaterium cytochrome P450 BM3 (CYP450 BM3) enzyme was efficient function as a regio-specific 3-hydroxylation flavonoid and one of the mutant M13 was cloned to pCW(Ori^+) to generate pCW(Ori+)-mutant 13 (M13) (ampicilin resistant gene) (Chu et al. 2016; Kim et al. 2008). Those plasmids were transformed into *E. coli* BL21(DE3) to generate *E. coli* ED1. This strain was used to investigate the production of eriodictyol from *p*-coumaric acid.

E. coli MCA was transferred various recombinant plasmids as pCDF-matB-matC (Str), pET28-CL (4-coumaryl CoA ligase) (Km), pAC-CHS (chalcone synthase)-CHI (chalcone isomerase) (Cm) and pCW(Ori+)-mutant 13 (M13) (Amp) to generate *E. coli* MED.

All strains and DNA plasmids were listed in Table 1. DNA manipulation such as DNA plasmid extraction, purification, digestion, and ligation will be followed standard protocols as described (Sambrook et al. 2001).

Table 1
DNA plasmids and strains in this study.

Strains/plasmids	Descriptions	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.	This study
pAC-TAL (Cm)	Expression vector pACYCDuet-1, T7 promoter, chloramphenicol resistance. TAL: tyrosine amnonia lyase	This study
pET28-CL (Km)	Expression vector pET28a, T7 promoter, kanamycin resistance.	This study
pAC-CHS-CHI (Cm)	Expression vector pACYCDuet-1, behind the T7 promoter, streptomycin resistance.	This study
pCDF-matB-matC	Expression vector pCDF-Duet 1 containing *matB* and *matC*.	This study
pCW(Ori^+)-mutant 13 (M13)	Expression vector pCW(Ori+), *Tac* promoter, ampicillin resistance.	Kim et al., 2008; Chu et al., 2016
E. 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 MCA	*E. coli* BL21(DE3)/Δ*pheA* containing pETD-*ppsA-tktA* (Amp) and pET28-TAL (Km).	This study
E. coli ED1	*E. coli* BL21(DE3) containing pCDF-matB-matC (Str), pET28-CL (Km), pAC-CHS-CHI (Cm) and pCW(Ori+)-mutant 13 (M13) (Amp)	This study
E. coli MED	*E. coli* BL21(DE3)/Δ*pheA* containing pETD-*ppsA-tktA* (Amp), pET28-TAL (Km), pCDF-*matB-matC* (Str), pET28-CL (Km), pAC-CHS-CHI (Cm) and and pCW(Ori+)-mutant 13 (M13) (Amp)	This study

Whole-cell biotransformation process for a single population of E. coli

E. coli MCA, *E. coli* ED1, and *E. coli* MED strains were used as mono-culture for the production of *p*-coumaric acid from D-glucose, eriodictyol from *p*-coumaric acid, and eriodictyol from D-glucose, respectively. M9 medium plus 15 g/L D-glucose was used for *E. coli* MCA while *E. coli* ED1 were cultured in the minimal M9 plus 2 g/L melonate. *E. coli* MED was cultured in minimal M9 plus 15 g/L D-glucose and 2 g/L melonate. Particularly, the *E. coli* strains were firstly cultured overnight at 37 ^{o}C in LB medium as the seed cultures. Subsequently, it was centrifuged and re-suspended in a fresh M9 medium. For mono-cultures, the initial net cell density of each of re-suspended culture was inoculated into the minimal M9 medium with the value of 5 x 10^6 cell per mL.

Co-culture techniques for the production of eriodictyol

E. coli upstream and downstream cell ratios (100:1, 10:1, 1:1, 1:10 and 1:100 (v/v) with a constant initial net cells density of 5 × 10^6 cells per mL of culture), as well as temperatures (25, 30, 33.5 and 37°C) were adopted for production testing in test tubes containing 3 mL minimal M9 media.

To investigate the effect of increasing initial net cells density on the consortia's capacity for production of eriodictyol, initial net co-culture inoculum was increased from original 5 × 10^6 to 5 × 10^7 cells per mL of culture, and fermentations were performed at various temperatures (25, 30, 33 and 37 ^{o}C) while keeping the inoculation ratio constant at 1:1.

Time for culture in the range of 12-60 h. The formation of *p*-coumaric acid, naringenin and eriodictyol were intervals analyzed by HPLC.

Extraction and chromatographical analysis of p-coumaric acid, naringenin, and eriodictyol

This procedure was followed Zhu et al., 2013 with several modifications using dimethyl sulfoxide (DMSO) to obtain eriodictyol and intermediate metabolites. The Mightysil RP-C18 column (4.6 x 250 mm) (Kanto Chemical Co. Inc., Japan) was maintained at 25°C and used to separate eriodictyol and its intermediates. Acetonitrile (100%) (solvent B) and deionized distilled water containing 0.1% trifluoro acetic acid (TFA) (solvent A) were used as mobile phase on HPLC 1260, diode array detector (Agilent). The binary program using solvent B: 10% (0-8 mins), 10-35% (8-15 mins), 35-70% (15-20 mins) and 70-100% (20-25 mins) running at 1 mL/ min. was adapted for HPLC analysis. The UV absorption at 330 and 280 nm was measured. Under these conditions, the retention time for *p*-coumaric acid, NRN, and ED was 6.5, 13.3, and 12.5 min, respectively. For quantification of flavonoids, a calibration curve of authentic NRN was drawn using 10-, 20-, 30-, 40-, and 50 µg/ml concentrations. LC–ESI/MS analysis were carried out in the Center for Applied Spectroscopy, Institute of Chemistry, Vietnam Academy of Science and Technology (VAST).

Statistical analysis

Student's *t* test was employed to investigate statistical differences, and differences with *P* values of 0.05 were considered significant.

Results

Synthesis of pCA from D-glucose using mono culture E. coli MCA

To evaluate the ability of single *E. coli* strain to synthesize *p*-coumaric acid, this strain was cultured in minimal M9 plus 15 g/L glucose. In another report, we tested the production of pCA from different carbon sources as glucose and glycerol (Thuan et al. 2021) (submitted). As a result, D-glucose was allowed to produce better titer of pCA (25.3 mg/L) after 48 h. Hence it was used for further study (Fig. 2).

Synthesis of eriodictyol from pCA using mono-culture E. coli

Different *E. coli* host strains were constructed with genes in the biosynthetic pathway of ED (4CL, CHS, CHI, and M13) to assess their ability for protein expression and functional activity. Mainly, *E. coli* BL21(DE3) containing those genes were designated as *E. coli* ED1. Dependence of biosynthetic dynamic of ED using *E. coli* ED1 on incubation time was shown in Fig. 3. ED concentration was gradually increased from 12-48 h of culture and reached the highest amount (21.3 mg /L) after 48 h. Hence, the host *E. coli* ED1 was further used for the study.

Production of eriodictyol in the mono-culture E. coli MED strain

E. coli MED strain contained all genes that were to synthesize ED from D-glucose (Table 1). This work aimed to demonstrate that whole genes in the biosynthetic pathway of eriodictyol working well in *E. coli*. Production of ED and its intermediates was shown in Fig. 4. Moreover, the highest titer of ED was reached 18.1 mg/L after 48 h.

Co-culture for the synthesis of ED

Design of synthetic co-culture system

E. coli - based co-culture included two modules. Module 1 (*E. coli* MCA, upstream) contained genes for the biosynthetic pathway of L-tyrosine from D-glucose. Notably, gene doses of *ppsA* and *tktA* were increased to enhance the intracellular accumulation of main precursors, PEP and E4P. Furthermore, the inactivation of *pheA* was to restrict the bioconversion of L-tyrosine to L-phenylanaline (Fordjour et al. 2019; Patnaik et al. 2008). And it resulted in the mutant *E. coli* /ΔpheA/*ppsA/tktA.* On the other hand, this strain was introduced TAL gen for conversion of tyrosine to pCA. Module 2 (*E. coli* ED1) contained genes (4CL, CHS, CHI) for the synthesis of NRN from pCA and flavonoid-3'-hydroxylase type M13 to convert NRN to ED (Fig. 1).

Effect of initial inoculum ratio

Up and downstream strains were mixed at different ratio (100:1; 50:1; 10:1; 1:1; 1:10; 1:50 and 1:100) and cultured under the same condition (minimal M9 medium + 15 g/L glucose and 33 ^{o}C) with supplementation of D-glucose. The initial inoculum ratio affected the formation of intermediates (pCA, NRN) and ED, as shown in Fig. 5.

Effect of temperature

Various temperature ranges were used to set up culture conditions, including 25, 30, 33, and 37 ^{o}C. Production titer of ED was gradually increased at 25 ^{o}C and achieved 38.4 mg/L at 33 ^{o}C as the best result before decreasing (Fig. 6).

Effect of initial net inoculum

To investigate the impact of increasing initial net cell density of each module on the titer of ED production, the initial cell density of each up and downstream module strain was enhanced from 5x10^{6} to 5x10^{7} cells per mL and cultured at a temperature of 33^{o}C. Firstly, this work was tested on a test tube scale. As shown in Table 2, the highest concentration of ED was reached 44.5 mg/L at 33 ^{o}C after 48 h. Moreover, comparatively, it resulted in an increased titer 1.15-fold (44.5/38.4).

Table 2
Effect of initial net inoculum on production of eriodictyol.

Temperature (^{o}C)	pCA (mg/L)	NRN (mg/L)	ED (mg/L)
25	13.8	22.7	37.6
28	15.2	24.8	39.8
33	**16.5**	**26.9**	**44.5**
37	13.4	26.1	42.1

Scale up the co-culture for production of eriodictyol using shake flask

Finally, to evaluate the scalability of this synthetic consortium for the production of ED and its intermediates, shake flask culture was utilized under mentioned above conditions, including the ratio of MCA : ED1 = 1 : 1, 33 ^{o}C, and initial net inoculum density of 5x10^{7} cells per mL. The titer of ED was measured and achieved of 51.5 mg/L at 48 h as the highest amount (Fig. 7).

Discussions

It is demonstrated that different strains in the same species have similar growth rates and sustainable coordination due to their similar physiological, biochemical, and genetic properties. As a result, co-culture of those strains will minimize the competition in nutritional resources and avoid growth inhibition caused

by metabolites. Furthermore, the co-culture system was also meaningful for the production of valuable natural products with a long synthetic route, for instance, NRN, AGP, RES, acacetin, and 3-amino-benzoic acid (Thuan et al. 2018a; Thuan et al. 2018b; Wang et al. 2020; Zhang and Stephanopoulos 2016; Zhang et al. 2017). All those showed the improved titer of targeted compounds. It was the main reason that *E. coli* – based the up- and downstream modules were designed and investigated in this study.

To investigate the dynamic bioconversion, *E. coli* MCA, *E. coli* ED1 and *E. coli* MED were constructed and tested the synthesis of target compounds, respectively. As a result, ED1 and MED strains were proven to produce ED at different amounts at 48h of culture (21.3 and 29.5 mg/L, respectively), while MCA strain gave 25.3 mg/L. Next, *E. coli* MCA and *E. coli* ED1 were used to check how the effect of co-culture works.

The inoculum ratio of strains significantly affected the co-culture performance. As a result, various ratios were experimentally investigated and resulted in the combination of MCA:ED1 = 1:1 with a titer of 25.5 mg/L (Fig. 5). This ratio produced ED higher than 1.2 fold compared to mono-culture (25.5/21.3). To further improve the production of the co-culture system, a range of temperatures from 25 – 37 ^{o}C was applied to test with the ratio of MCA:ED1 = 1:1. And, this resulted in 38.4 mg/L of ED at 33 ^{o}C (Fig. 6).

Previous studies demonstrated that a high density of initial net inoculum could increase growth rate and volumetric productivity (Goers et al. 2014). For example, higher density of *S. cerevisiae* and *E. coli* as components in co-culture system in an optimal ratio of those strains led to enhance production titer of NRN and RES production (Yuan et al. 2020; Zhang et al. 2017). In this work, we tested to increase the initial inoculum ratio of strain from 5x10^{6} to 5x10^{7} cells per mL. As a result, the maximal titer of ED was achieved of 44.5 mg/L at 33 ^{0}C after 48h. This result was higher than 2.10-fold (44.5/21.3) and 1.16-fold (44.5/38.4) compared to the mono-culture and low initial inoculum density, respectively. Finally, the scalable possibility of the synthetic consortium was checked with a shake flask. As a result, an enhanced titer of eriodictyol was found and achieved 51.4 mg/L or 1.16-fold (51.5/44.5) higher than the test tube scale. This result was reasoned by improved volumetric productivity, mass transfer, dissolved oxygen, and aeration speed (Klockner and Buchs 2012; Yuan et al. 2020).

Conclusion

An artificial *E. coli – E. coli* engineered co-culture system was constructed to synthesize ED from D-glucose. Using this system, the highest titer of ED was 49.8 mg/L at 33 ^{o}C after 48h. Consequently, co-culture is an alternatively efficient approach to producing the natural product without much genetic or metabolic modification. Further modification to improve titer of a targeted compound may concentrate on (i) increased pool of intracellular cofactors (NADH, FAD, FMN) and precursors (malonate, pCA), (ii) proteins / genes of channel to import or export natural product via cell membrane, (iii) plug and play biological system performance using extracellular supplies as biosensors to control pH, temperature and substrate concentration.

Declarations

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Competing financial interests

The authors declare no competing financial interests.

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Figures

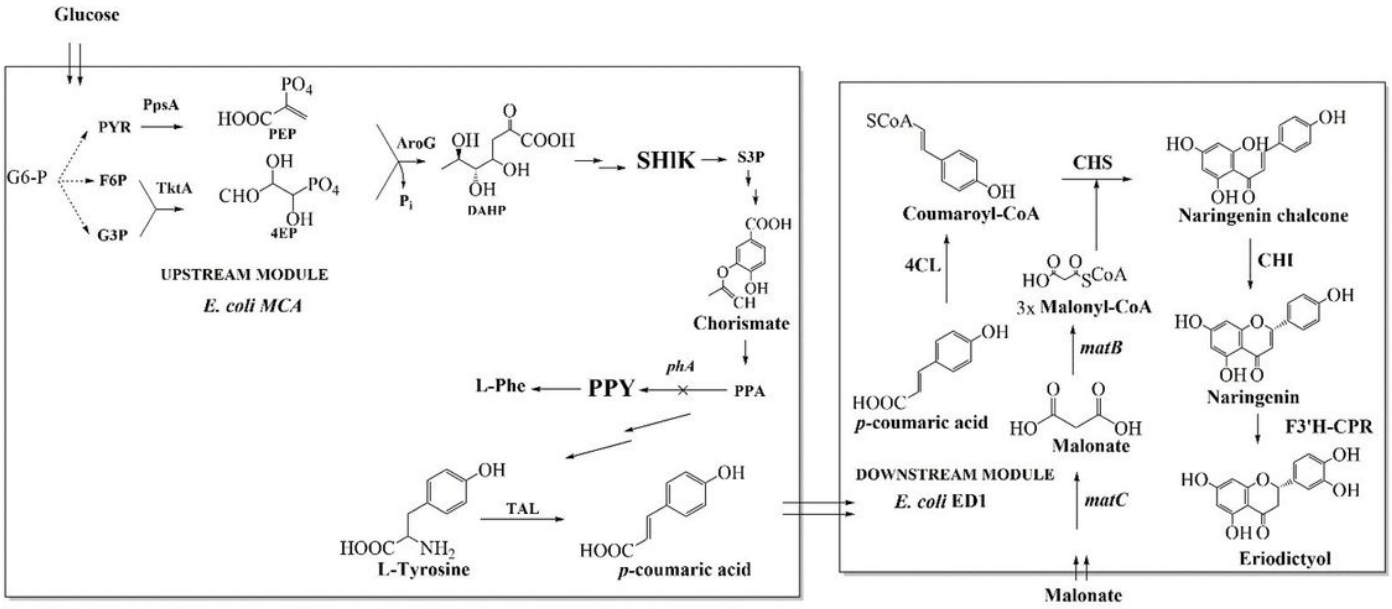

Figure 1

Design and application of co-culture engineering strategy for production of eriodictyol.

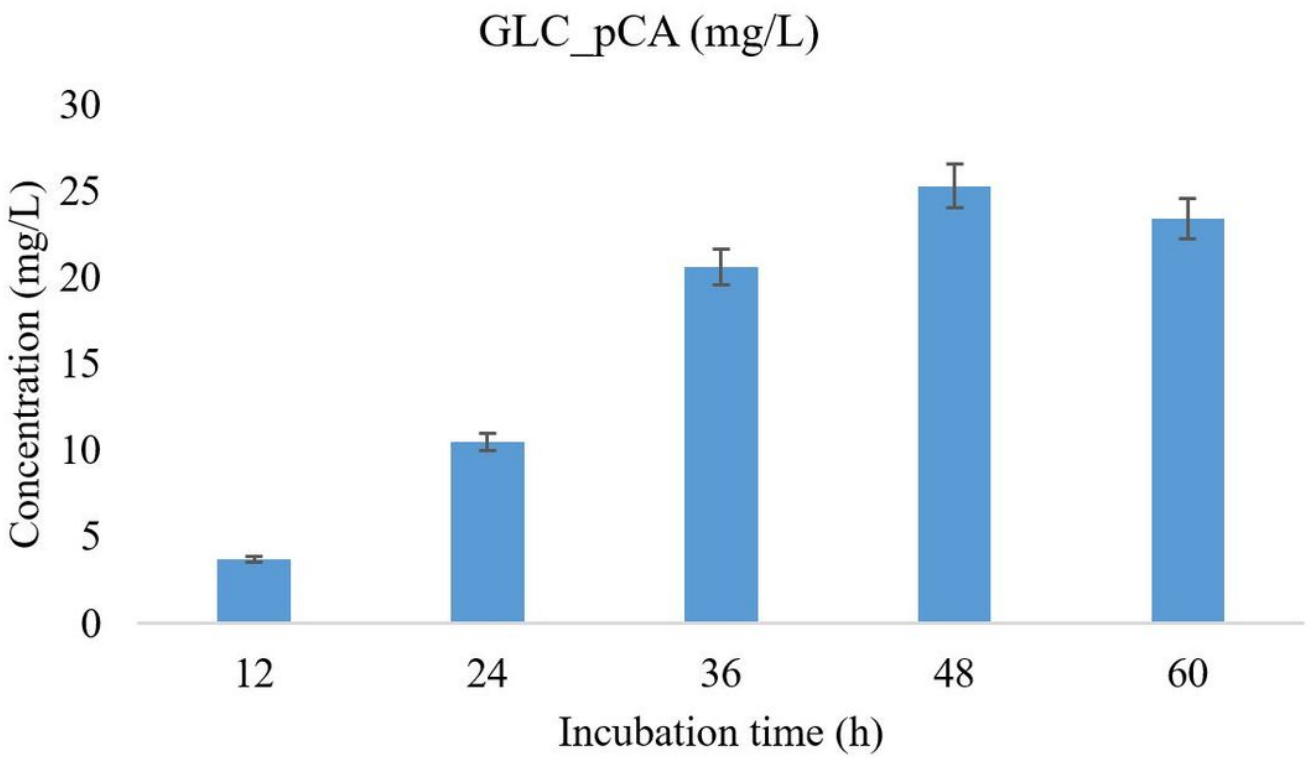

Figure 2

Production of pCA from D-glucose using E. coli MCA strain

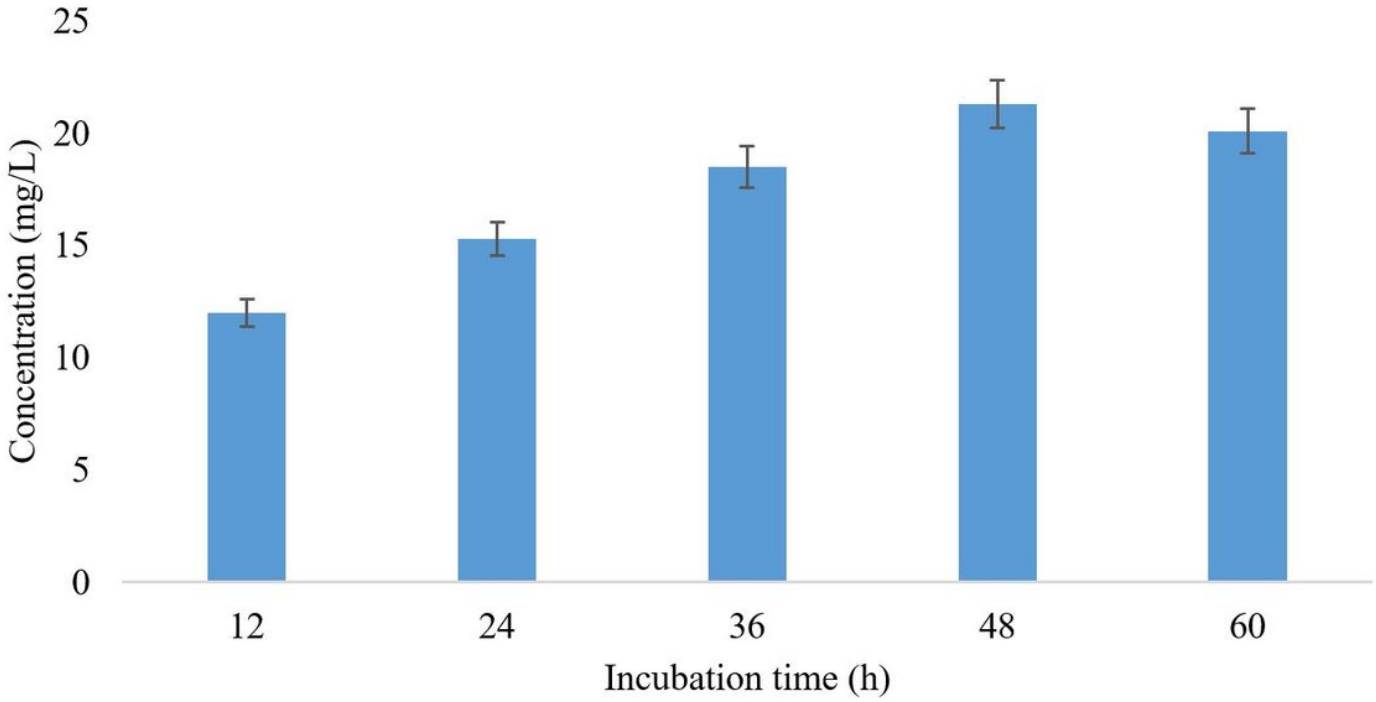

Figure 3

Synthesis of eriodictyol in the E. coli ED1.

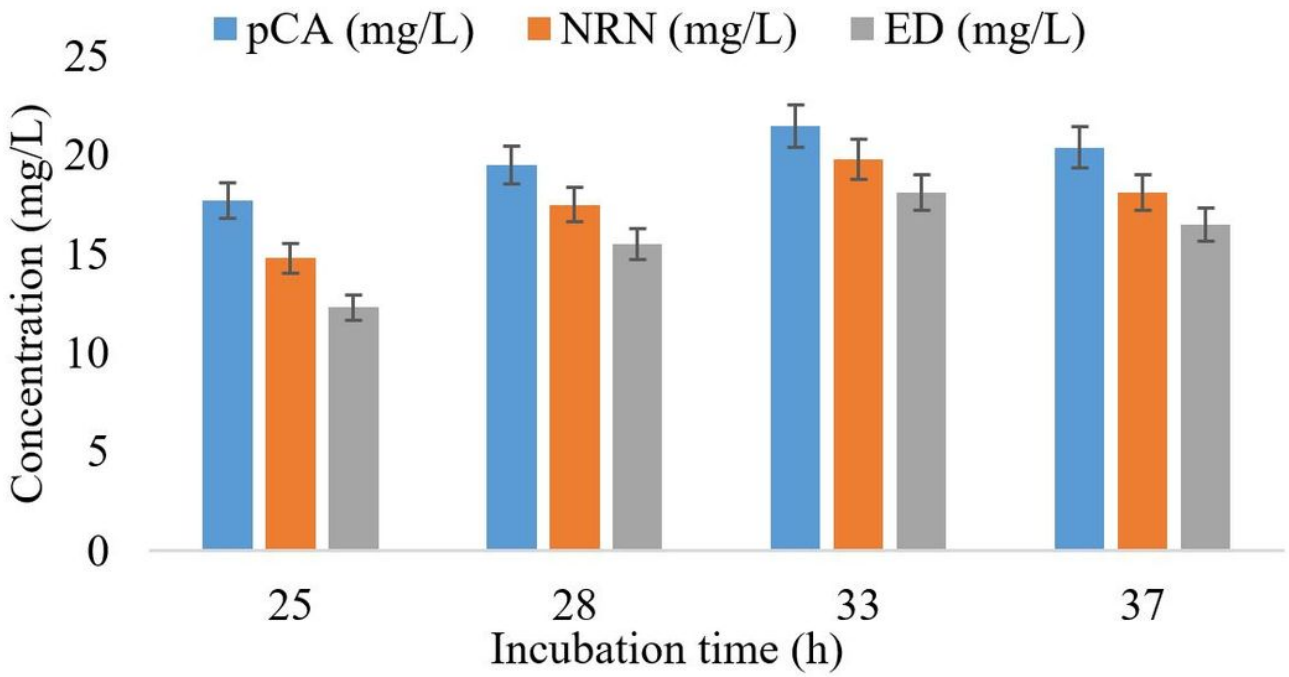

Figure 4

Synthesis of eriodictyol in the mono-culture E. coli MED

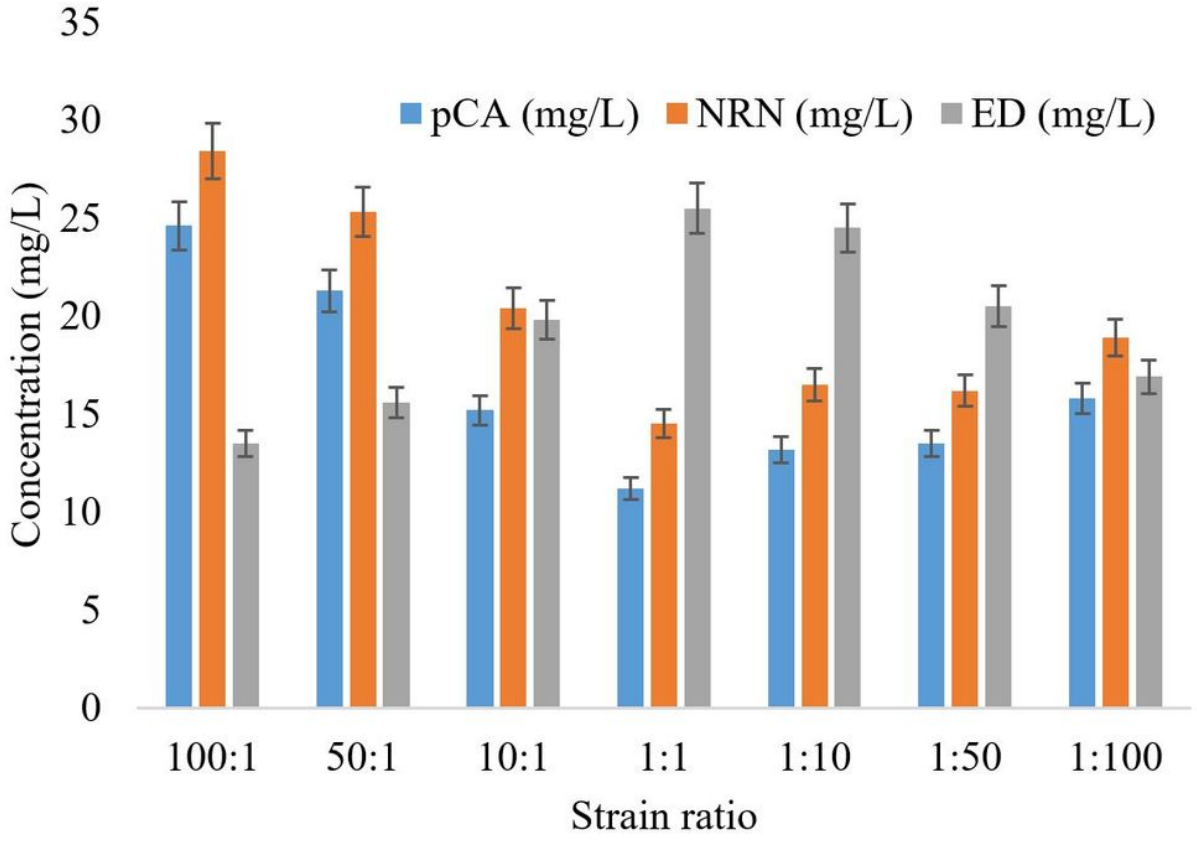

Figure 5

Comparison of eriodictyol bioproduction by different initial inoculum ratios of co-culture

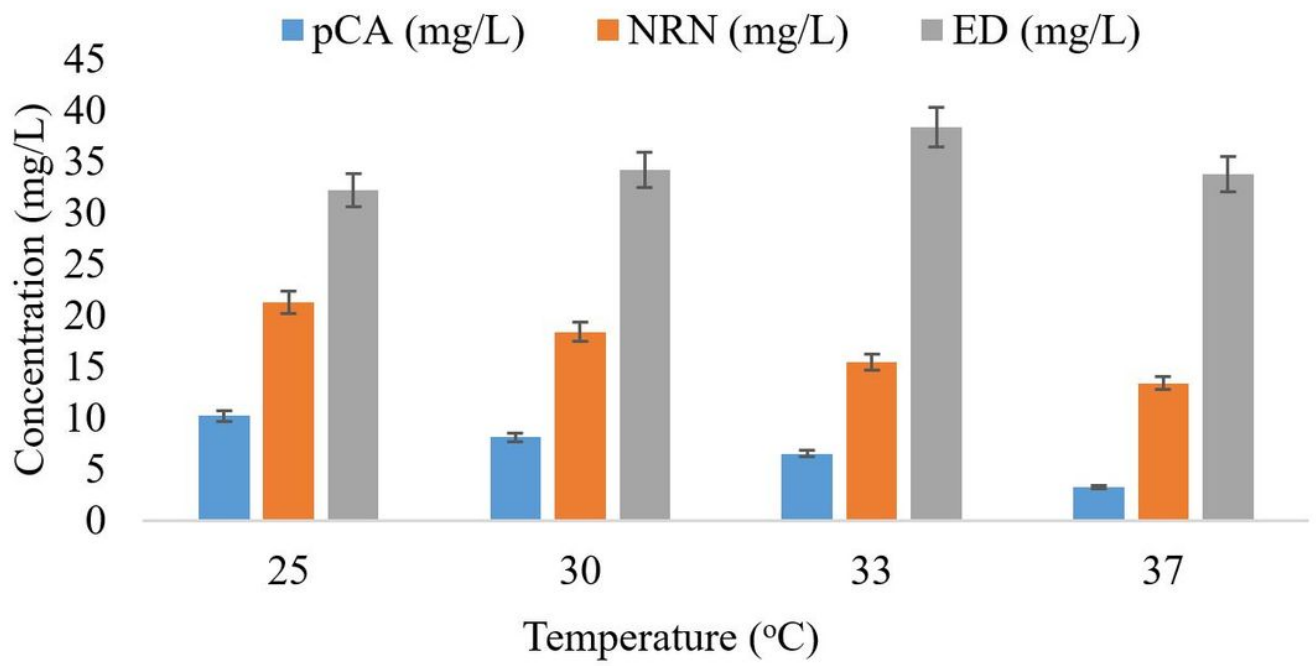

Figure 6

Effect of temperature on the production of eriodictyol by co-culture

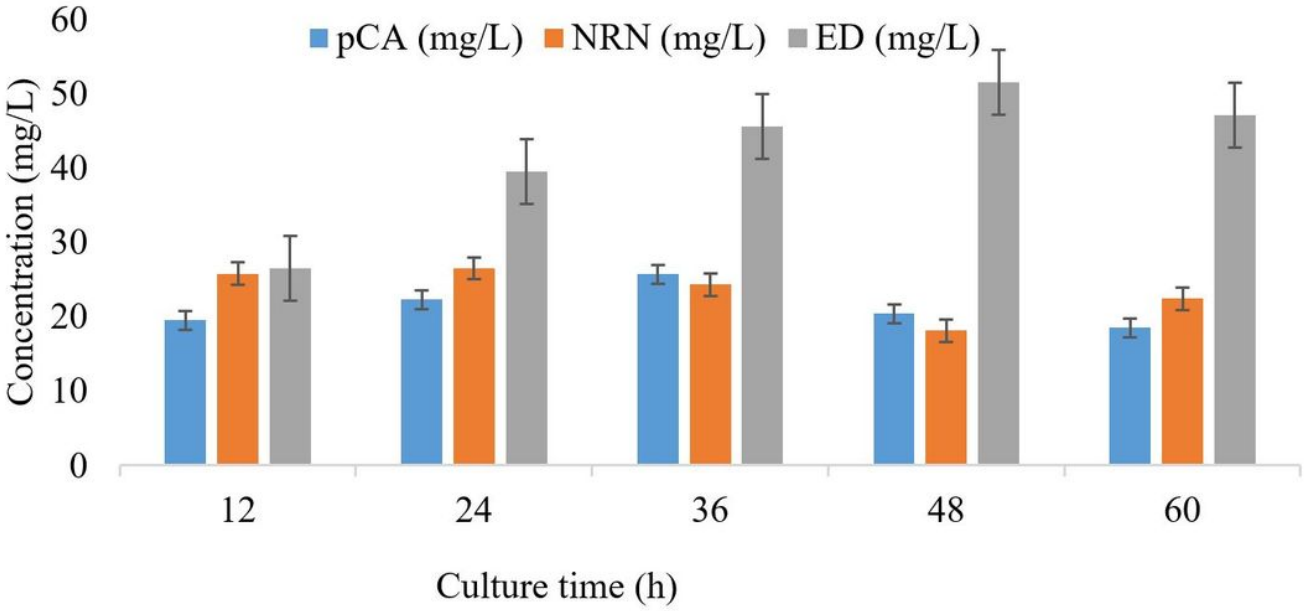

Figure 7

Eriodictyol productivity of the synthetic co-culture at a shake fask scale with inoculation ratio (1:1), initial net cells density (5x107 cells / mL of culture) and 33 °C.

Supplementary Files

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