ENGINEERING *E.COLI-E.COLI* CO-CULTURES FOR THE PRODUCTION OF NARINGENIN

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Written under the direction of

Dr. Haoran Zhang

and approved by

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ABSTRACT OF THE THESIS

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Naringenin is an important natural product of the flavonoid family and possesses various biological activities. It has a complex chemical structure and is mainly produced by plants. However, naringenin production by either plant extraction or chemical synthesis is challenging, due to low productivity and yield associated with these methods. Microbial co-culture engineering has been found to be a robust approach for production of a wide range of biochemical products and holds strong potential for efficient complex natural product biosynthesis. In this project, novel *E.coli-E.coli* co-cultures were constructed to produce naringenin from simple carbon substrates, such as glucose and glycerol. The cultivation conditions for the *E.coli- E.coli* co-culture approach was systematically studied for bioproduction optimization. It was found that the *E.coli-E.coli* co-culture approach showed superior production performance compared to the mono culture approach. The findings of this project demonstrate the potential of engineering microbial co-cultures for heterologous biosynthesis of complex molecules.

Acknowledgements

I would like to express my gratitude to Dr.Haoran Zhang for his guidance and support in my research. He helped me in understanding about research work and also encouraged me to be a better researcher. He is a brilliant mentor and I am grateful for his encouragement for me to perform research work. I could not have imagined having a better adviser for my thesis research work

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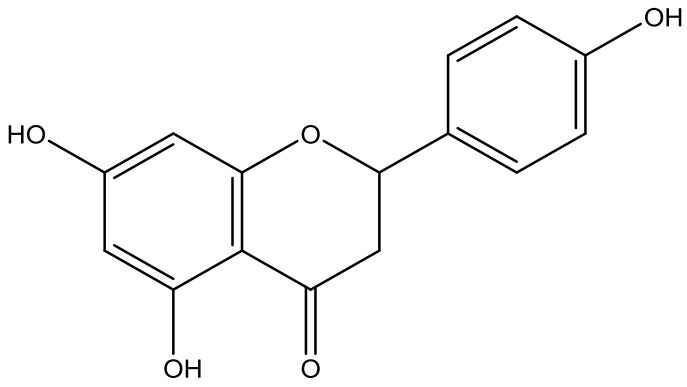
I am always grateful to my parents for their love and support.

Table of contents

List of Figures and tables

1. INTRODUCTION

Naringenin is an important natural product with identified biological activities such as anti-oxidative, anti-cancer, anti-obesity and anti-inflammatory activities [1, 2, 3, 4] and thus holds the potential for being used as high-value nutraceutical molecule. Naringenin is a member of the broad flavonoid family. Flavonoids belong to the class of plant and fungus metabolites. Flavanoids are further classified as anthoxanthis, flavanones, flavanonols, flavans and anthocyandins. Naringenin specifically belongs to the category of flavanones and is mostly found in grape fruit[5,6]. The chemical structure of naringenin has been well characterized. Naringenin possesses a 15-carbon skeleton and three ring structures [7]. The full chemical name for naringenin is 2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one.



naringenin

Fig 1. Chemical structure of naringenin ($C_{15}H_{12}O_5$).

Naringenin is mainly produced as a secondary metabolite by plants, although it has also been reported to be produced by prokaryote *Streptomyces clavuligerus* [9]. The characterization of the naringenin biosynthetic pathway, including the key enzymes and their catalytic activities, has been completed]. As shown in Fig 1, simple carbon source,

such as glucose and glycerol, is first used to produce amino acid tyrosine through aromatic amino acid biosynthetic pathway. Tyrosine is then converted to *p*-coumaric acid by tyrosine ammonia lyase (TAL), which is further utilized to make *p*-coumaroyl-CoA through by 4-coumarate: CoA ligase (4CL). The condensation of *p*-coumaroyl-CoA and malonyl-CoA leads to the formation of naringenin by two serial enzymatic reactions catalyzed by chalcone synthase (CHS) and chalcone isomerase (CHI). The heterologous biosynthesis of naringenin has been achieved through functional reconstitution of the complex biosynthetic pathway in selected microbial hosts, such as *E. coli* and *S. cerevisiae*.[10,11,12]. However, these previous efforts relied on utilization of the mono-culture of the engineered microbial strains, and the resulting naringenin biosynthesis performance was found sub-optimal, as the associated bioproduction processes either needed addition of exogenous substrate of malonate or suffered from low product titers [13, 14]. In particular, the low conversion of the key intermediate tyrosine to the final product has been found to be the major challenge for high-efficiency naringenin biosynthesis [15].

On the other hand, designed modularization of biosynthetic pathways in the context of microbial co-cultures, referred to as modular co-culture engineering, has been shown to be a robust method for production of a variety of value-added compounds[16,17,18,19,20,21,22,23]. Several reports have demonstrated that this approach improved the biosynthesis of natural products with complex structures. Zhou et al. employed an *E. coli-S. cerevisiae* co-culture system to biosynthesize oxygenated isoprenoids with significantly higher titers than the mono-culture approach[24]. Minami et al. developed *E. coli-S. cerevisiae* co-cultures to accommodate complex biosynthetic

pathways for benzylisoquinoline alkaloids and successfully produced 7.2 mg/L magnoflorine and 8.3 mg/L scoulerine[25]. Using an *E. coli-E. coli* co-culture, Jones et al. achieved the 960-fold improvement of flavan-3-ols production from exogenous *p*-coumaric acid and caffeic acid substrates[26].Although naringenin was included in the co-culture system in this study, it was only produced as a pathway intermediate from an exogenous metabolic precursor. In fact, so far there were no established microbial co-cultures specifically dedicated to de novo naringenin biosynthesis. In this work, we aim to investigate the applicability of microbial co-cultures in the naringenin microbial biosynthesis.

Specifically, the naringenin biosynthetic pathway is divided into two separate portions: the upstream portion for intermediate tyrosine formation via *E. coli*'s native metabolism and its conversion to *p*-coumaric acid by TAL, and the downstream portion for tyrosine and *p*-coumaric acid conversion to naringenin via expression of the associated heterologous genes. Each portion is accommodated in a specialized *E. coli* strain specifically engineered for designed biosynthesis task. *E.coli* has been chosen for the biosynthesis of naringenin because it can grow in simple media and the nutritional requirements are very low compared to other organisms. [30, 31]. The upstream portion is accommodated by a strain capable of over producing tyrosine. This strain, *E. coli* P2H, is capable of overproducing tyrosine from simple carbon sources (Table 1) [27]. For the downstream portion, four heterologous enzymes, including TAL, 4CL, CHS and CHI, are simultaneously expressed. Notably, different *E. coli* strains were screened to identify the best strain to produce functional heterologous proteins to support naringenin production [29]. Recently, resveratrol was produced through a similar co-culture engineering

approach [28]. This strategy significantly improved the production of resveratrol and demonstrated the potential of co-culture engineering for biosynthesis of naringenin.

This co-culture design splits the biosynthetic labor between two *E. coli* strains and thus reduces the metabolic burden on each strain. Importantly, through adjusting the ratio between the upstream and downstream strains, the balance between different portions of the pathway can be fine-tuned for enhancing the conversion of the intermediates to product naringenin. The production of naringenin through the co-culture approach was subsequently compared with the mono-culture approach in which all the pathway enzymes were introduced into a single strain for naringenin production.

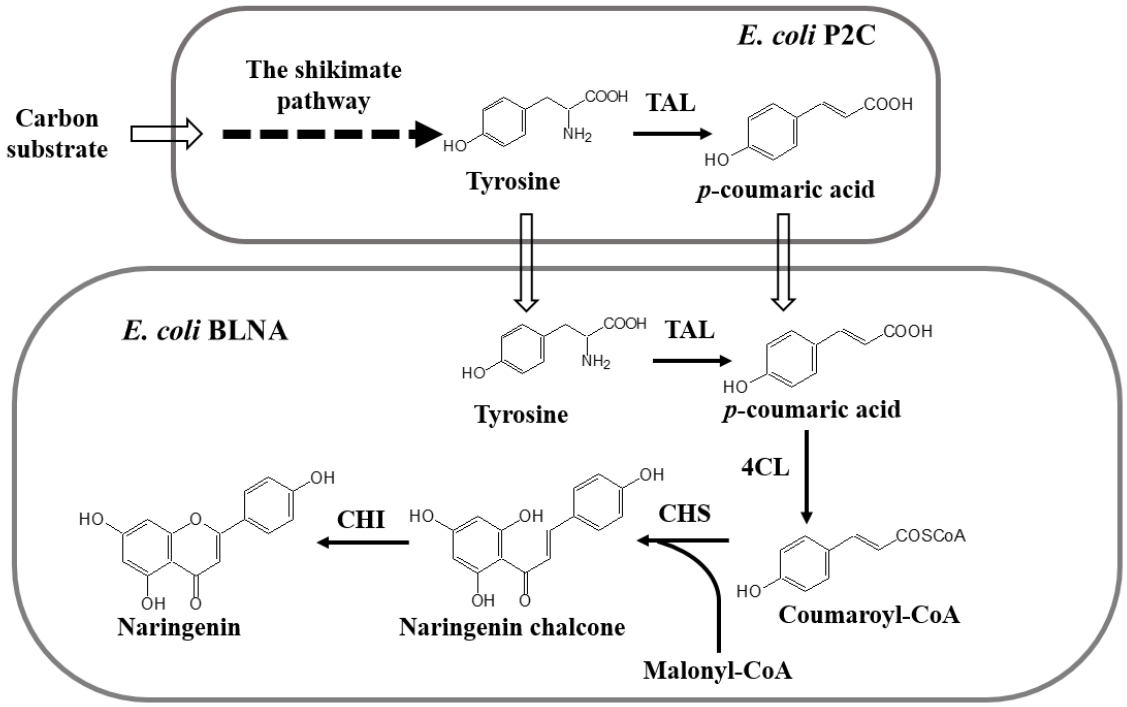


Fig 2. Design of the co-culture approach for naringenin biosynthesis

2. MATERIALS AND METHODS:

2.1 NARINGENIN BIOSYNTHESIS:

For *E.coli-E.coli* co-culture experiments, P2C and BLNA were first inoculated into LB media containing 50 mg/L streptomycin and 100 mg/L ampicillin at 37 ^{0}C or 30 ^{0}C . Appropriate amount of P2C overnight culture and BLNA overnight culture was centrifuged using and the supernatant was discarded. The cell pellets were suspended in 5 mL of glycerol/ glucose medium containing 50 mg/L of streptomycin and 100 mg/L of ampicillin. The initial cell density of P2C was set to OD_{600}=0.15 and the cell density of BLNA was varied according to the inoculation ratio. 1mM IPTG was added either the time of inoculation or at designated time point after inoculation. The temperature was varied according to the experiments and fermentation was carried out for 72 hours.

For the monoculture experiments, PMC was initially inoculated into LB media containing 50 mg/L streptomycin and 100 mg/L ampicillin at different temperatures. The overnight culture was centrifuged and the cell pellet was suspended into 5 mL glycerol or glucose medium . 1mM IPTG was used for induction either at the time of inoculation or at the designated time point after inoculation. The initial cell density of PMC mono culture was varied according to the coculture inoculation ratios. The cell density was determined by measuring the absorbance at 600 nm with a UV-Visible Spectrophotometer (VWR, Radnor).

2.2 HPLC ANALYSIS:

For the analysis of tyrosine, culture supernatants were filtered through 0.45 um PTFE membrane syringe filters(VWR) and used for HPLC with Shimadzu HPLC connected

with Shimadzu Photodiode Array detector at 280 nm. Separation was done on Waters C18 column with 95% Water (Solvent A) and 5 % Acetonitrile (Solvent B) as the mobile phase at a flow rate of 0.3 ml/min at 4 minutes.

To analyze *p*-coumaric acid and naringenin, 1 mL of culture supernatant was extracted with 1 mL of ethyl acetate. After vortexing and centrifugation, the top layer was evaporated to dryness and dissolved with 1 mL of ethanol by vortexing. Different range of standards were prepared by dissolving the standard sample in ethanol. *p*-coumaric acid and naringenin were separated with Waters C18 column with an isocratic method of 65 % Water and 35% Acetonitrile at a flow rate of 0.3 ml/min. The wavelength used was 290 nm for naringenin and 300 nm for *p*-coumaric acid. The retention time of naringenin was 11.2 minutes whereas the retention time of coumaric acid was 3.6 minutes. Concentrations were determined by using calibration curves correlating the concentration of the standards and the HPLC peak areas.

2. 3 STRAINS AND CULTIVATION MEDIUM:

All strains used in the study were cultivated in 50 mL culture tubes containing 5 mL medium at 275 rpm. The media used in the work are as follows. One liter of glycerol medium contained 2 g of NH4Cl, 5 g of (NH4)2SO4,3 g of KH2PO4, 7.3 g of K2HPO4, 8.4 g of 3-(N-morpholino)propanesulfonic acid, 0.5 g of NaCl, 0.24 g of MgSO4, 0.5 g of yeast extract ,40 mg of tyrosine, 40 mg of tryptophan, 10 mg of 4-HB and 5g of glycerol. 5 g of glucose was used instead of glycerol for glucose medium.

The cells were inoculated in a New Brunswick scientific shaker at 250 rpm.

Table 1 Strains and Plasmids used in this study:

Plasmid	Description	Source
PTrcHis2B	Trc promoter, pBR322 ori, Amp^R	Invitrogen
pCDFDuet-1	Double T7 promoters, CDF ori, Sp^R	Novagen
pOM	Vector from Evonik (derived from pUC18), contains pGAP promoter, rrnB terminator, Amp^R and ColE1 origin	[15]
pCA1	pTrcHis2B carrying codon optimized *R.glutinis* TAL	[15,18]
pCDF-trc-RgTAL^{syn}-Pc4CL^{syn}	pCDFDuet-1 carrying codon-optimized *R. glutinis* TAL and codon-optimized *P. crispus* 4CL-1 with trc promoter	[15]
pOM-PhCHS-MsCHI	pOM carrying *P. hybrida* CHS and *M. sativa* CHI with a single GAP (constitutive) promoter	[15]

Strains	Description	Source
P2H	*E. coli* K12 Δ*pheA* Δ*tyrR* *lacZ*::PLtetO-1-*tyrA*^{fbr}*aroG*^{fbr} *tyrR*::$P_{LtetO-1}$-*tyrA*^{fbr}*aroG*^{fbr} *hisH*(L82R)	[33] (Santos CNS (2010) PhD thesis. Massachusetts Institute of Technology.)

PMC	P2H carrying pCDF-trc-RgTAL^{syn}- Pc4CL^{syn} and pOM-*Ph*CHS-*Ms*CHI	This study
P2C	P2H carrying pCA1 and pCDFDuet-1	This study
BL21(DE3)	F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3)	Invitrogen
BLNA	BL21(DE3) carrying pCDF-trc-RgTAL^{syn}-Pc4CL^{syn} and pOM-PhCHS-MsCHI	This study
BL21 Star(DE3)	F– *omp*T *hsd*SB(rB–, mB–) *gal dcm rne131*(DE3)	Life technologies
StarNA	BL21 Star(DE3) carrying pCDF-RgTALsyn-Pc4CLsyn and pOM-PhCHS-MsCHI	This study
JM109(DE3)	F-traD36 proA+B+lacIq Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14- gyrA96 recA1 relA1 endA1 thi hsdR17 (DE3)	NEB
JMNA	JM109(DE3) carrying pCDF-trc-RgTAL^{syn}-Pc4CL^{syn} and pOM-PhCHS-MsCHI	This study
K12(DE3)	*E. coli* K12 carrying the gene for T7 RNA polymerase	[15]
K12NA	K12(DE3) carrying pCDF-trc-RgTAL^{syn}-Pc4CL^{syn} and pOM-PhCHS-MsCHI	This study

3. SPECIFIC AIMS:

3.1 To utilize the *E.coli-E.coli* co-culture approach for biosynthesis of naringenin from glycerol :

Glycerol has been touted as an inexpensive substrate for the production of value-added compounds. However, there has been no research on the biosynthesis of naringenin or other flavonoids from glycerol. This study aims to produce naringenin using glycerol as a main carbon source and investigate the production performance by the *E. coli-E. coli* co-culture.

3.2 To utilize the *E. coli-E. coli* co-culture approach for biosynthesis of naringenin from glucose:

Glucose is a commonly used carbon substrate for bioproduction of various biochemical. This study aims to analyze the naringenin production on glucose using a traditional mono-culture approach and the *E.coli-E.coli* co-culture approach. The production performance will also be compared with that on glycerol to provide insight on which carbon substrate is more suitable for co-culture-based naringenin bioproduction.

4. RESULTS AND DISCUSSION:

GLYCEROL AS A MAIN CARBON SOURCE:

4.1 Impact of temperature on the production of naringenin:

In plants, the formation of metabolite naringenin involves steps catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), P450 reductase (CPR), 4-coumaric acid-CoA ligase (4CL), chalcone synthase (CHS) and chalcone isomerase (CHI) (Figure1). These enzymes may have preference for low temperature for enzymatic activities. However, *E. coli* has better growth rate at 37 ^{0}C . Therefore, this study compared the production under 37 ^{0}C and 30 ^{0}C to identify the most suitable temperature.

a)37 ^{0}C **:**

Fig.3, 4 and 5 show the naringenin titer, *p*-coumaric acid accumulation and tyrosine accumulation at 37 ^{0}C for the biosynthesis using the mono-culture and co-culture approach. 37 ^{0}C was selected because the growth rate of *E.coli* is highest at 37 ^{0}C . Naringenin was produced at 37 ^{0}C using the mono-culture as well as the co-culture approach. Production of naringenin increased as the inoculation ratio of P2C: BLNA decreased (more BLNA than P2C). The improvement in the naringenin titre can be correlated to the decrease in the accumulation of *p*-coumaric acid. At the ratio 1:5, naringenin titre was 4.3 mg/L which was twice the naringenin titre for PMC strain. The accumulation of *p*-coumaric acid was high for the mono-culture, indicating the production advantage of the co-culture approach over the mono-culture approach. The

accumulation of tyrosine also decreased as the production of naringenin increased. The decrease in the tyrosine and *p*-coumaric acid can be attributed to the increase in the biosynthetic strength of the downstream strains to convert the intermediates to naringenin.

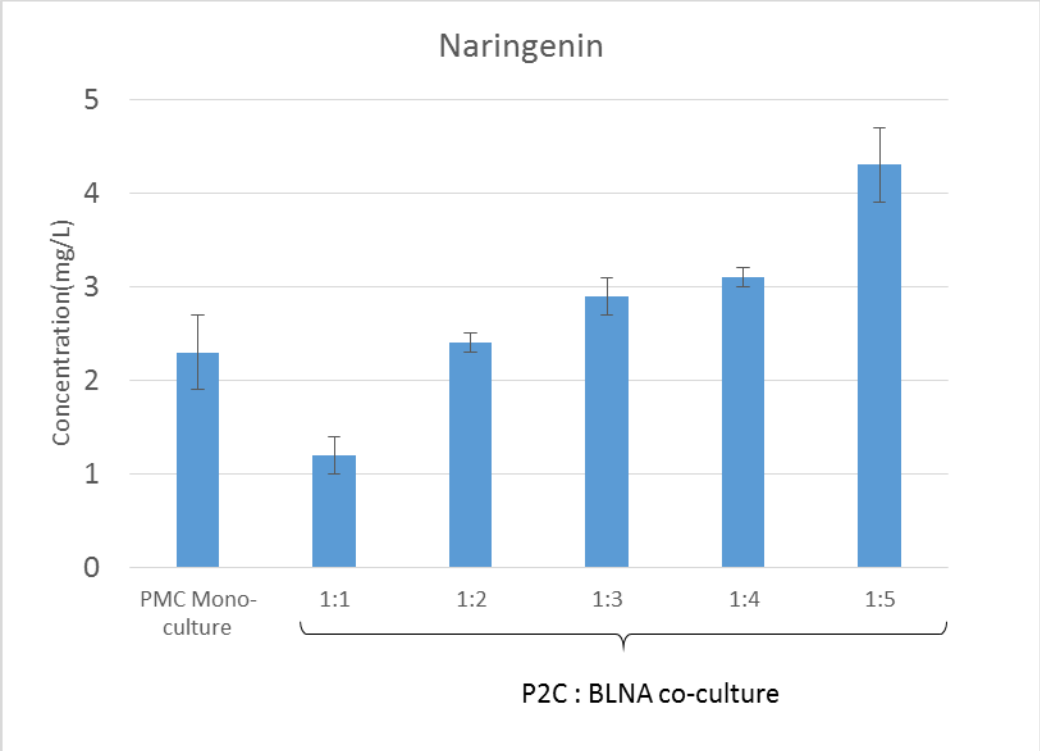


Fig 3. Naringenin production at 37 ^{0}C when glycerol was used as the carbon substrate

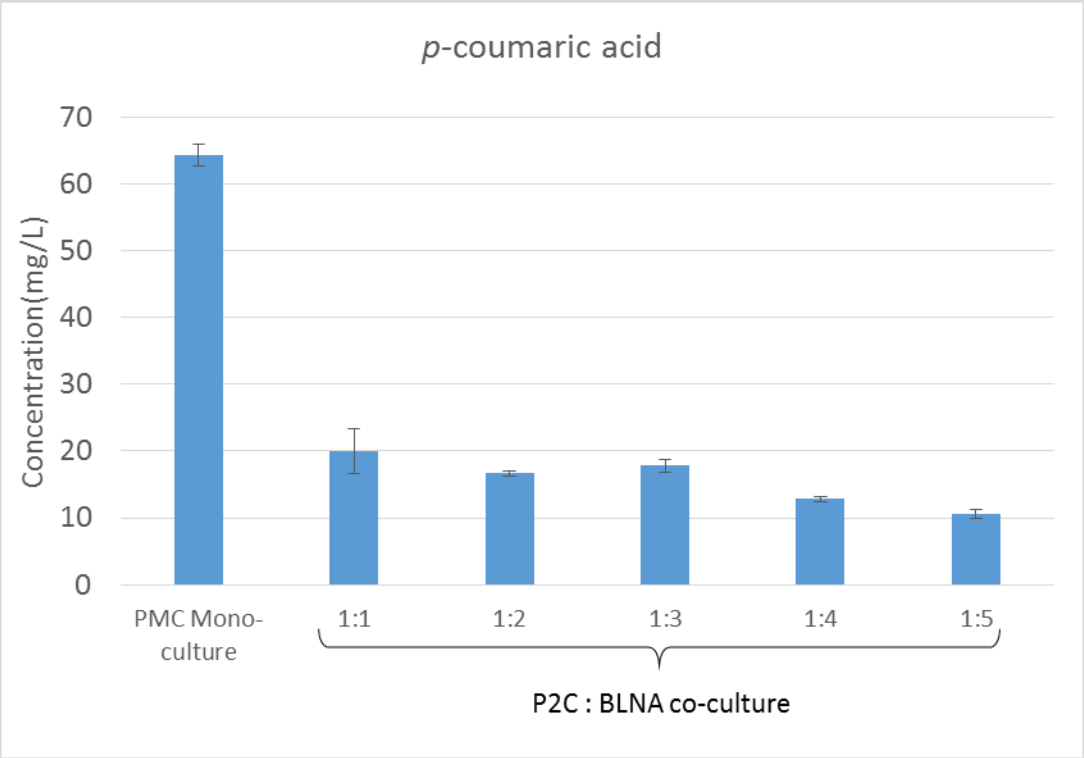


Fig 4. *p*-coumaric acid accumulation at 37 ^{0}C when glycerol was used as substrate

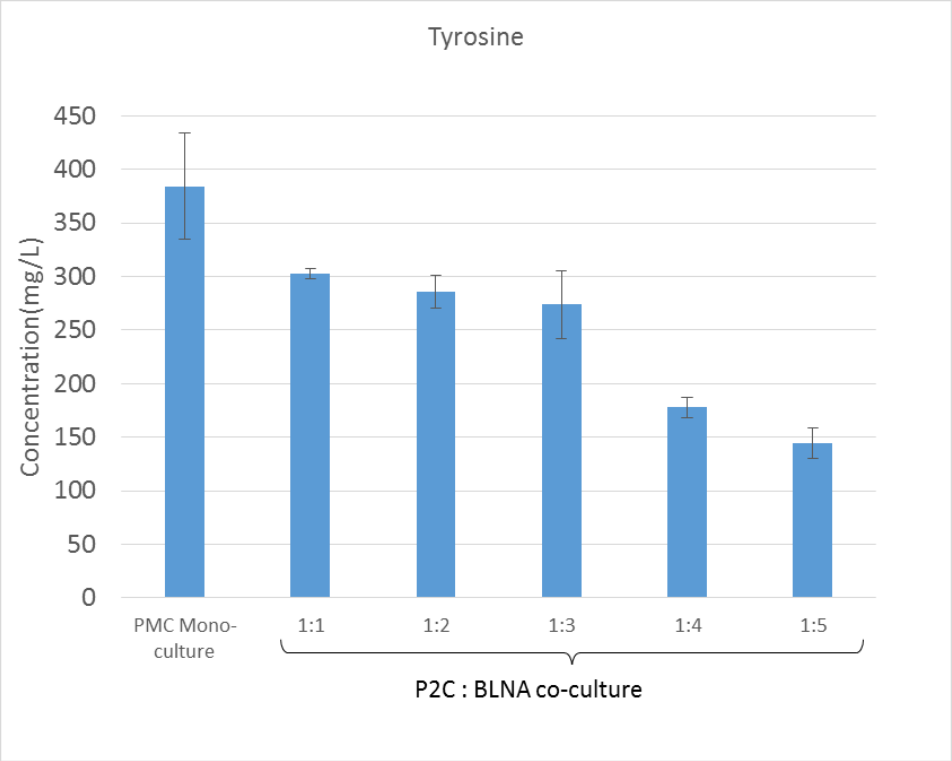


Fig 5. Tyrosine accumulation at 37 ^{0}C when glycerol was used as the carbon substrate

The total amount of naringenin produced was 4.3 mg/L at the inoculation ratio of 1:5. At the inoculation ratio of 1:5, the initial cell density of the upstream strain(P2C) was 0.15 and the initial cell density of the downstream strain(BLNA) was 0.75. For the inoculation ratio 1:1, the initial cell density of the upstream strain was 0.15 and the initial cell density of the downstream strain was 0.15. The production of naringenin as well as the intermediates varied as the inoculation ratio of upstream strain and the downstream strain changed. For the ratio of 1:2, the naringenin produced was 2.4 mg/L. For 1:3 inoculation ratio, the naringenin produced was 2.9 mg/L. The maximum production of naringenin was observed at the ratio 1:5. The initial cell density of the mono-culture strain was 0.3, which produced 2.3 mg/L naringenin. This shows that naringenin was better produced using the co-culture approach compared to the mono-culture approach. The accumulated *p*-coumaric acid was still high (64.3 mg/L) for the mono-culture strain. In comparison, the intermediate accumulation was reduced in the co-cultures. At the inoculation ratio of 1:2, 16.7 mg/L of *p*-coumaric acid was produced. As the inoculation ratio of downstream: upstream cells increased, the amount of *p*-coumaric acid accumulation also decreased. At the ratio of 1:5, the accumulated coumaric acid was only 10.6 mg/L. The accumulation of coumaric acid was about 6 times lower compared to the mono-culture approach. Similar trend was observed for the accumulation of tyrosine using the co-culture approach and the mono-culture approach. For the mono-culture approach, the tyrosine accumulation was about 384.3 mg/L. For co-culture inoculated at the ratio of 1:5, the accumulation of tyrosine was only 144.2 mg/L. The accumulation of tyrosine at the inoculation ratio 1:5 was twice lower than the accumulation of tyrosine using the mono-culture approach. This

suggests the inability of the mono-culture to fully convert tyrosine to naringenin, largely due to the overwhelming metabolic stress

b)**30** ^{o}C **:**

30 ^{0}C was selected here, as a few previous studies used this temperature for flavonoids bioproduciton [15,28]. In fact, a lower temperature is beneficial for protein folding and thus the functional expression of the heterologous pathway enzymes Even though the growth of *E.coli* is lowered at 30 ^{0}C the overall biosynthesis performance may be improved.. It was found in this study that, as the temperature was decreased to 30 ^{0}C, production of naringenin along with the intermediates *p*-coumaric acid and tyrosine was changed.

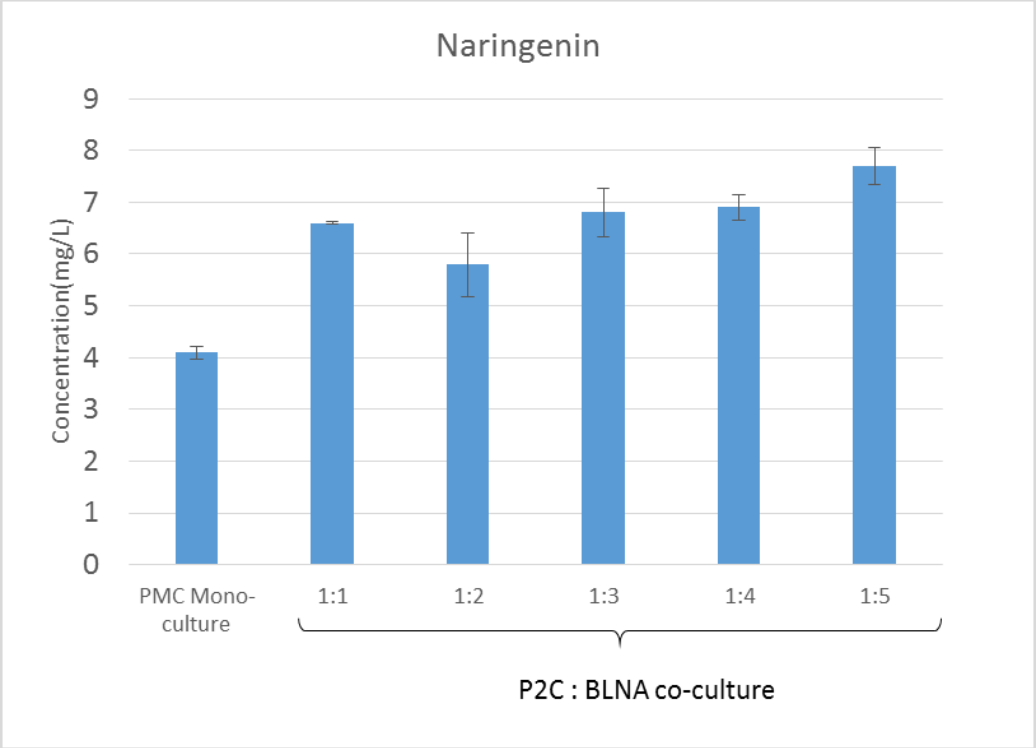


Fig 6. Naringenin production at 30 ^{0}C when glycerol was used as the carbon substrate

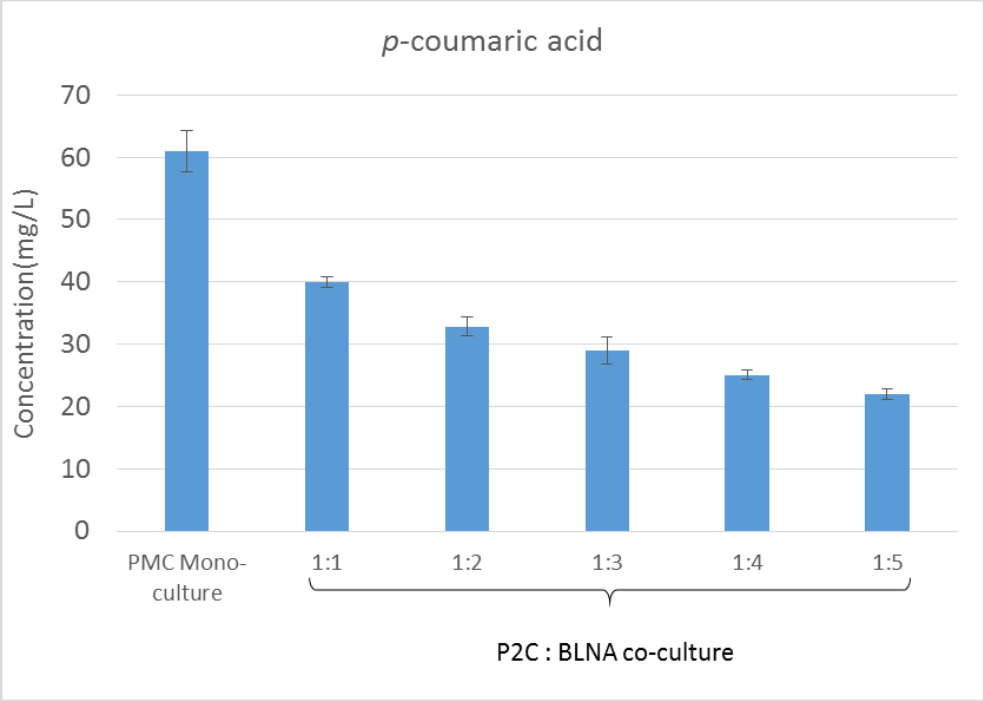


Fig 7. *p*-coumaric acid accumulation at 30 ^{0}C when glycerol was used as the carbon substrate

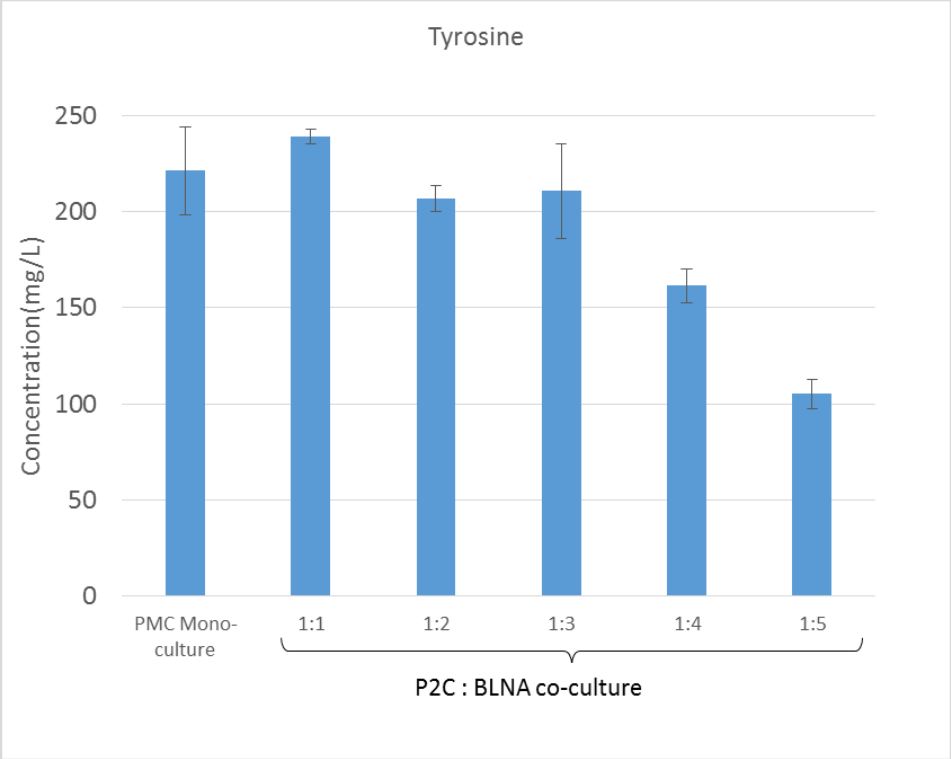


Fig 8. Tyrosine accumulation at 30 ^{0}C when glycerol was used as the carbon substrate

The results of Fig. 6 show that production of naringenin increased as the cultivation temperature decreased. In addition, the accumulation of *p*-coumaric acid also increased at lower temperature, as shown in Fig.7. Improvement in the accumulation of the intermediate *p*-coumaric acid indicates that the TAL's enzymatic activity was increased, which could be due to the improved TAL protein folding at 30 ^{0}C . The maximum production of naringenin (7.7 mg/) was observed at inoculation ratio of 1:5, which is significantly higher than the production with the ratio of 1:1. This shows that the downstream conversion of the pathway intermediates is the main rate limiting step for the overall biosynthesis. This trend of increased naringenin production with the varying inoculation ratio is similar to the observation at temperature 37 ^{0}C . The mono-culture produced 4.1 mg/L naringenin under the same condition. The accumulation of *p*-coumaric acid was found to be higher with the mono-culture approach. At the inoculation ratio 1:5, the co-culture produced 22 mg/L of *p*-coumaric acid. The accumulation was increased to 40 mg/L at the inoculation ratio of 1:1.In comparison, the mono-culture approach produced 61 mg/L of coumaric acid under the same cultivation conditions. This difference in the *p*-coumaric acid accumulation was also reflected in the naringenin production. Similar observation was found for tyrosine accumulation. For the co-culture inoculated at the ratio 1:5, tyrosine accumulated was 105.1 mg/L. For the inoculation ratio 1:1, the tyrosine accumulated was increased to 239 mg/L. The mono-culture accumulated 221.3 mg/L tyrosine, which is higher than most of the co-cultures . In comparison with the production under 37 ^{o}C, the tyrosine accumulation decreased at 30

^{o}C, suggesting that the lower temperature helped increase the conversion of tyrosine and *p*-coumaric acid to the final product.

4.2 Impact of IPTG induction time:

In this study, IPTG was used for the induction of the heterologous enzymes. There has been several studies which have reported the variation in the production of naringenin by varying the IPTG induction time points [15, 26]. Therefore, it is of research interest to investigate the production of naringenin using IPTG induction time points. The production was evaluated when four different time points was used, which resulted in different naringenin production as well as intermediate accumulation values. Specifically, IPTG induction was initialed at 0 hours 3 hours, 6 hours and 10 hours after the inoculation of the co-culture system.

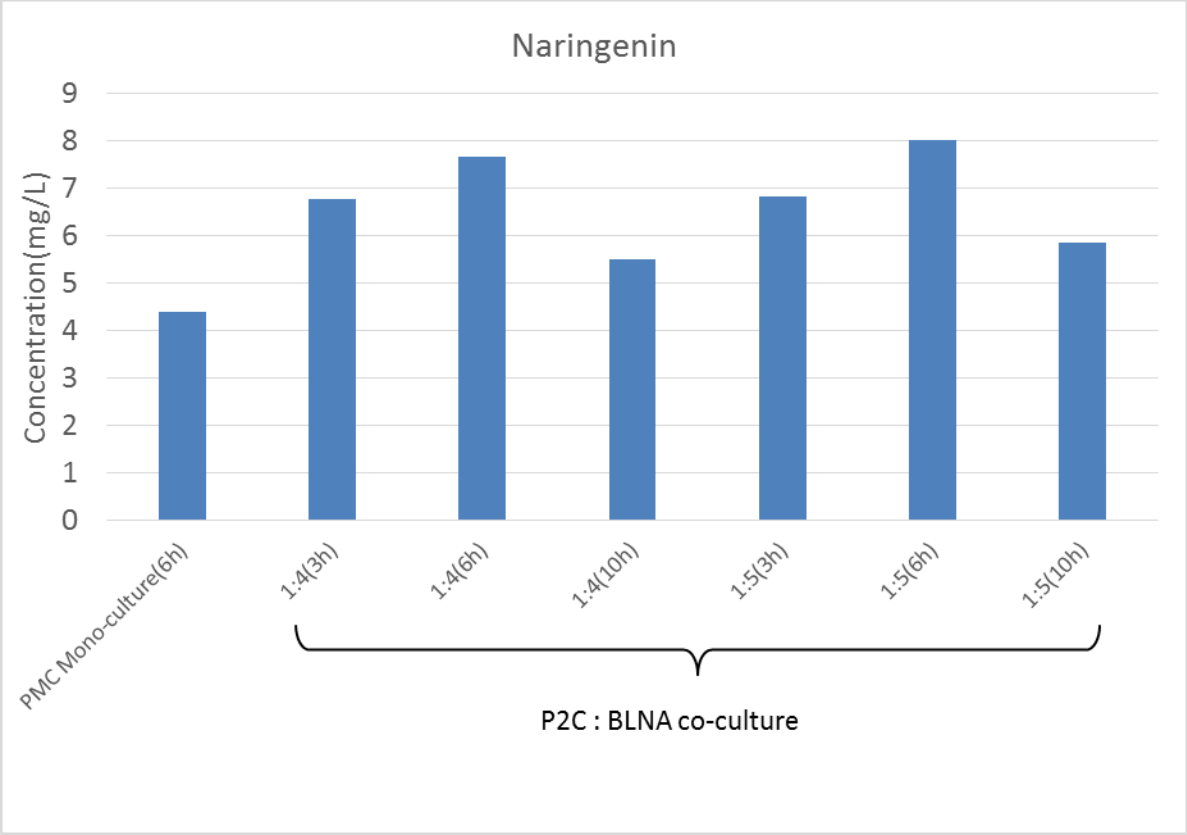


Fig 9. Effect of IPTG induction time points on naringenin biosynthesis at 37 ^{0}C when glycerol is used as the carbon substrate. Induction time is given in parenthesis.

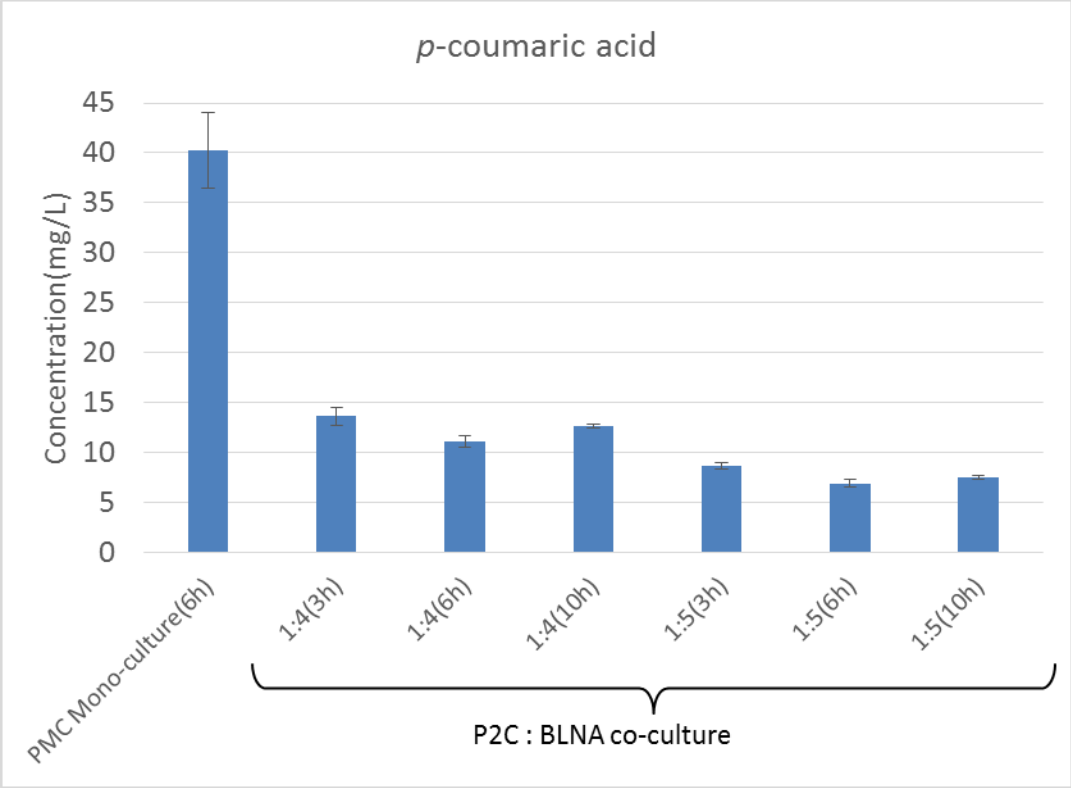


Fig 10.Effect of IPTG induction time points on *p*-coumaric acid accumulation at 37 ^{0}C when glycerol is used as the carbon substrate. Induction time is given in parenthesis.

For this study, two different inoculation ratios (1:4 and 1:5) were used to more systematically evaluate the impact of IPTG induction time points. The production results are shown in Fig 9 and Fig 10. It was found that IPTG induced at 6 hour produced 8 mg/L naringenin whereas the induction at 3 h produced 6.8 mg/L naringenin. Induction at 10 h after the inoculation produced 5.8 mg/L naringenin. For the same IPTG induction time, the titre of naringenin was less for the 1:4 ratio compared to the ratio 1:5. For example, for the 1:4 ratio, naringenin produced at the 6 hour IPTG induction time point

was 7.7 mg/l which is less than the naringenin produced at the same IPTG induction time point for the ratio 1:5. For the mono-culture production, it was also found that IPTG induction at 6 hour was an optimal for the production of naringenin.

Similarly, the accumulation of intermediates was also evaluated for different IPTG induction time points at 30 ^{0}C. It was shown in Fig 10 that *p*-coumaric acid accumulation fluctuated as the time points for the IPTG induction varied. 40.3 mg/L of *p*-coumaric acid was produced by the mono-culture when IPTG induction was initiated at 6 hour. For the same IPTG induction time point, the co-culture inoculated at the ratio 1:5 produced 6.9 mg/L *p*-coumaric acid. For the inoculation ratio 1:4, 11.1 mg/L coumaric acid was produced. Similar trend was also observed for production at other induction time points.

As shown in Fig.13, The tyrosine accumulation was also observed for different IPTG induction time points. There was a decrease in the tyrosine accumulation as IPTG induction was delayed. . The results were consistent for both the inoculation ratios 1:4 and 1:5. These results demonstrate that IPTG induction at 6 h was optimal for the production of naringenin.

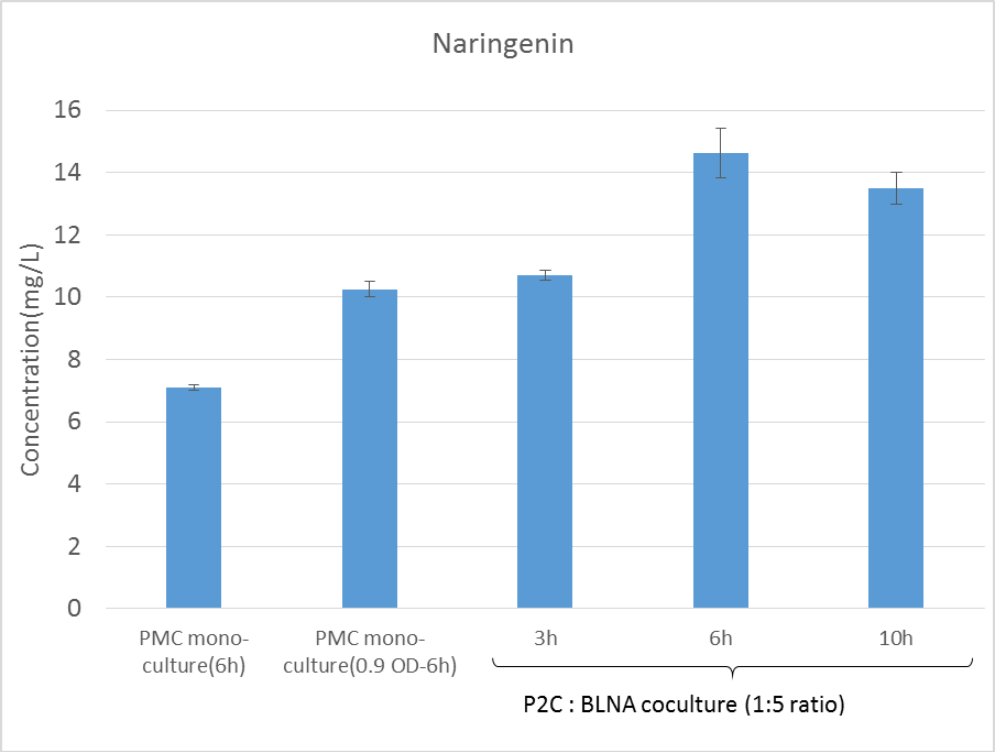


Fig 11. Effect of IPTG induction time points on the production of naringenin at 30 ^{0}C when glycerol was used as the carbon substrate.Induction time is given in parenthesis.

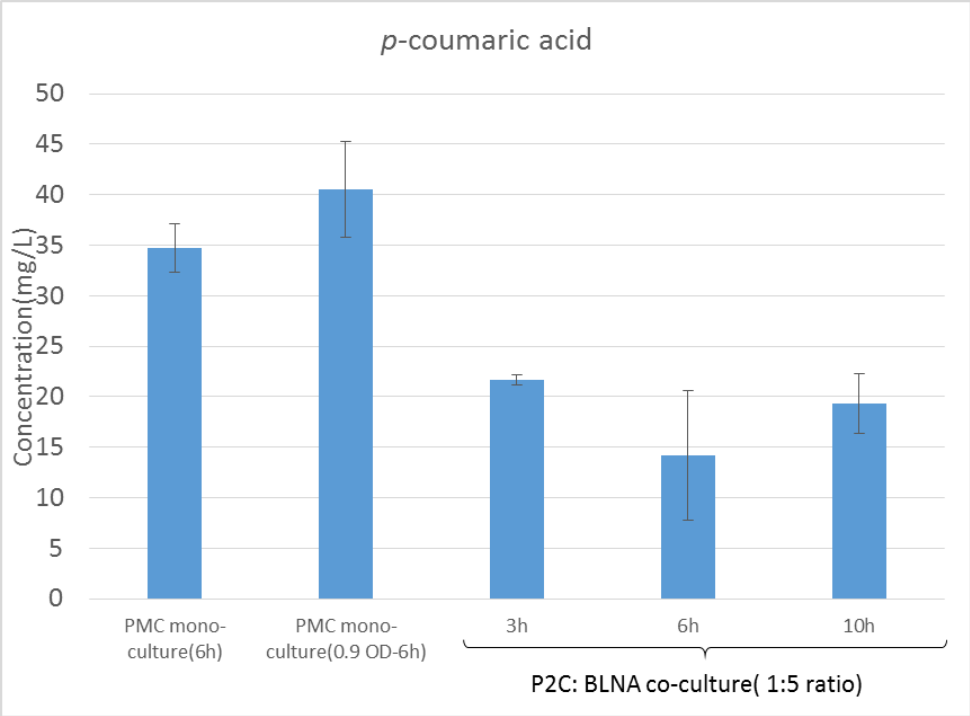


Fig 12. Effect of IPTG induction on *p*-coumaric acid accumulation at 30 ^{0}C when glycerol was used as the carbon substrate. Induction time is given in parenthesis

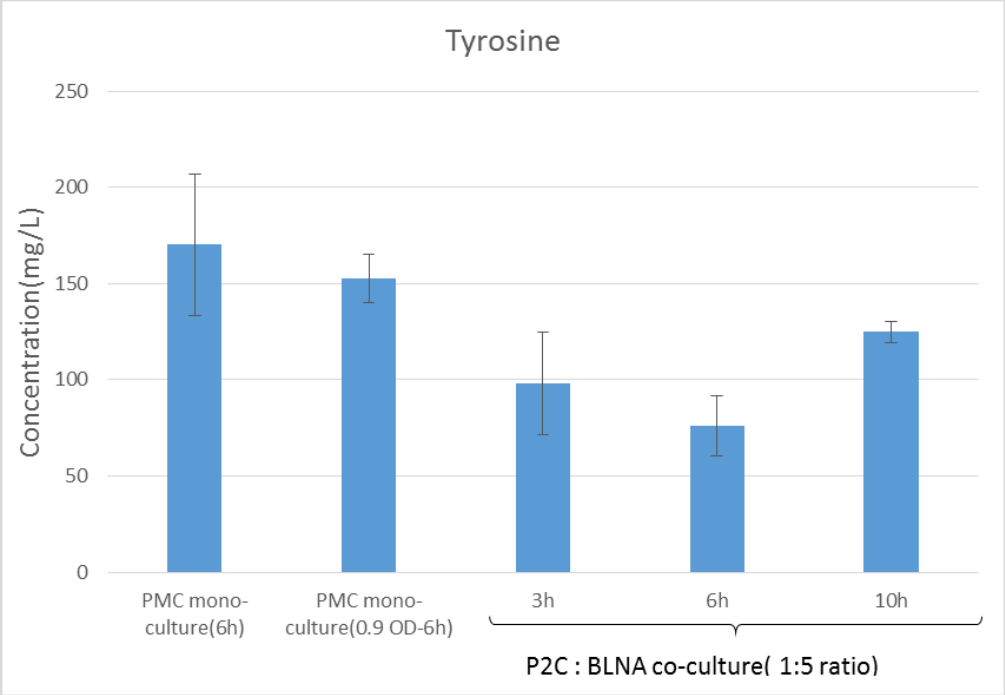


Fig 13. Effect of IPTG induction time points on tyrosine accumulation at 30 ^{0}C when glycerol was used as a carbon source. Induction time is given in parenthesis.

Fig.11, 12 and 13 show the naringenin titre and the accumulation of intermediates under 30 ^{o}C at different IPTG induction time points. The production was improved to 14.2 mg/L from 7.8 mg/L when the induction time was changed from 0 h to 6 h. The decrease in the intermediates accumulation was also consistent with the previously observed trend for both tyrosine and coumaric acid. For comparison, the mono-culture of the PMC strain was induced at 6h and it showed a production of 10.2 mg/L naringenin, 152.6 mg/L tyrosine and 40.6 mg/L p-coumaric acid, respectively.

B) GLUCOSE AS A CARBON SOURCE:

For all the experiments using glucose as the carbon source, the protocols for production using glycerol were followed.

4.3 Optimization of the inoculation ratio:

The inoculation ratio between the co-culture strains plays a key role in naringenin bioproduction. Here, the inoculation ratio for the naringenin production was optimized for the P2C:BLNA co-culture. The PMC strain was used as a mono-culture control strain for the production of naringenin. The accumulation of intermediates was also measured to determine the correlation between the naringenin production and accumulation of intermediates. The inoculation ratio for the co-culture was varied from 1:1 to 1:5. This experiment was performed at the IPTG induction time of zero hour and 30 ^{0}C

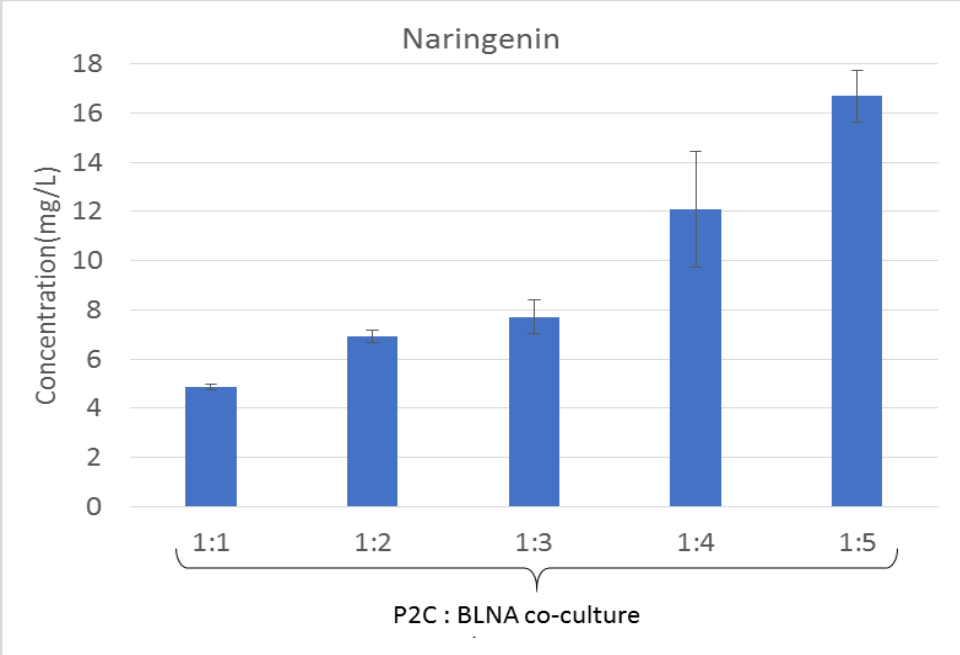


Fig.14 Naringenin production using P2C: BLNA co-culture when glucose is used as the carbon substrate

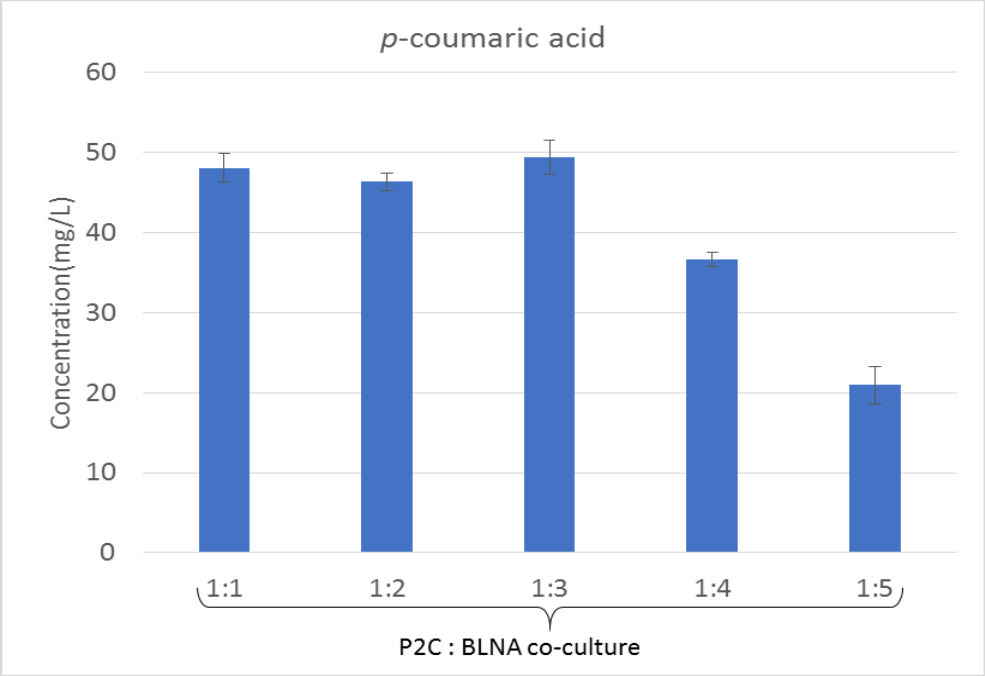


Fig 15. *p*-coumaric acid accumulation using P2C:BLNA co-culture when glucose is used as the carbon substrate

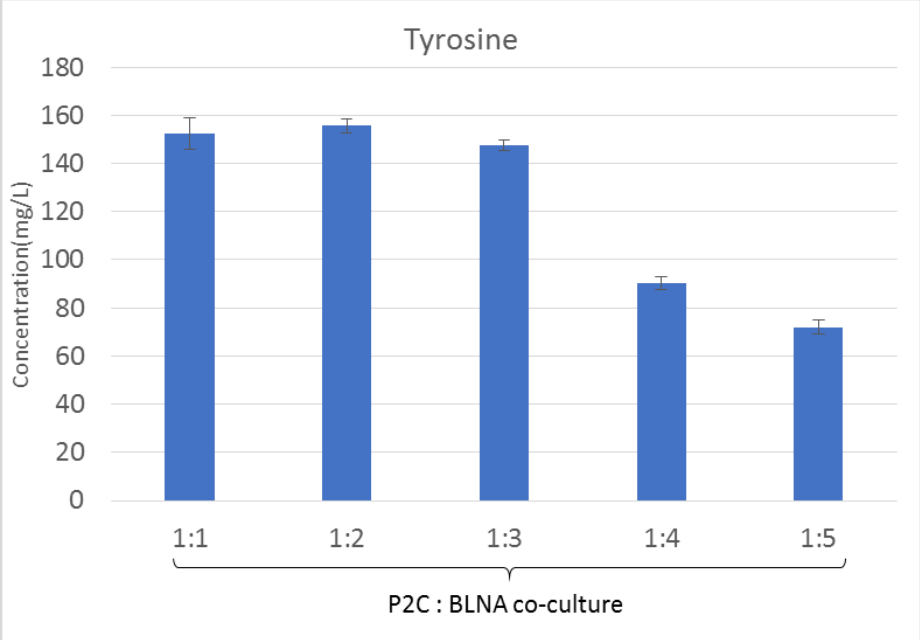


Fig 16. Tyrosine accumulation using P2C: BLNA co-culture when glucose is used as the carbon substrate

The production of naringenin was found to vary with the inoculation ratio. Naringenin production increased from 4.2 mg/L to 16.7 mg/L as the ratio of the upstream: downstream cells was changed from 1:1 to 1:5. As shown in fig 15, the P2C: BLNA co-culture at the ratio 1:5 accumulated 22 mg/L of *p*-coumaric acid. In comparison, 48.1 mg/L *p*-coumaric acid was accumulated at the inoculation ratio 1:1. Therefore, the reduction in *p*-coumaric acid accumulation suggests that naringenin production improvement can be attributed to the increase of *p*-coumaric acid conversion to naringenin by increased population of the downstream strain.

4.4 Naringenin synthesis for different cell densities:

For the co-cultures used in this study, the initial cell density changed with the inoculation ratio. In fact, the inoculation of the upstream strain P2C was fixed, and the downstream strain BLNA was added at different amount for achieving the desired inoculation ratio. In order to rule out the possibility that the production improvement by the co-cultures was due to the increased inoculation amount, this section of study used different initial cell density for the mono-culture and evaluate its impact on the naringenin production. Glucose was used as the carbon source and the IPTG induction was initiated at the beginning of the cultivation.

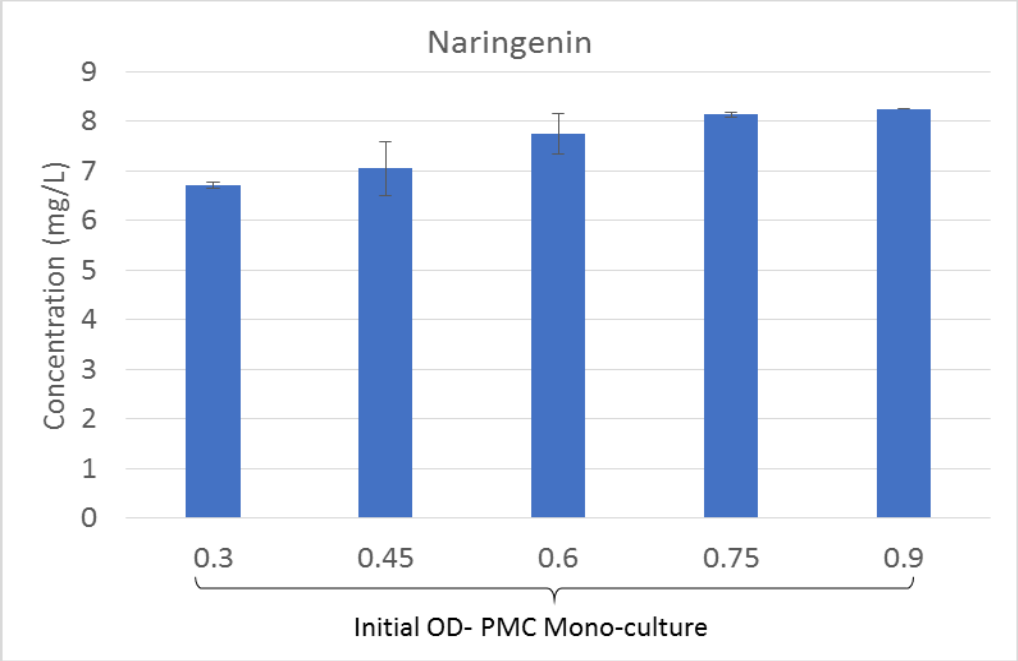


Fig.17 Naringenin production for different cell densities from mono-culture PMC strain when glucose is used as the carbon substrate

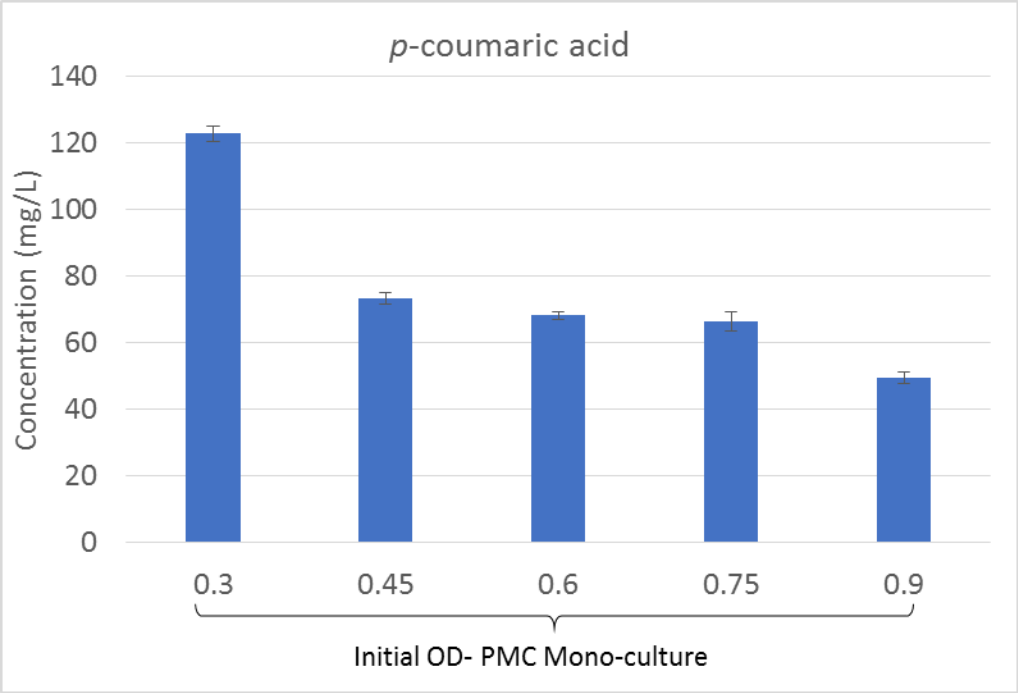


Fig.18 Coumaric acid accumulation for different cell densities from mono culture PMC strain when glucose is used as a carbon source

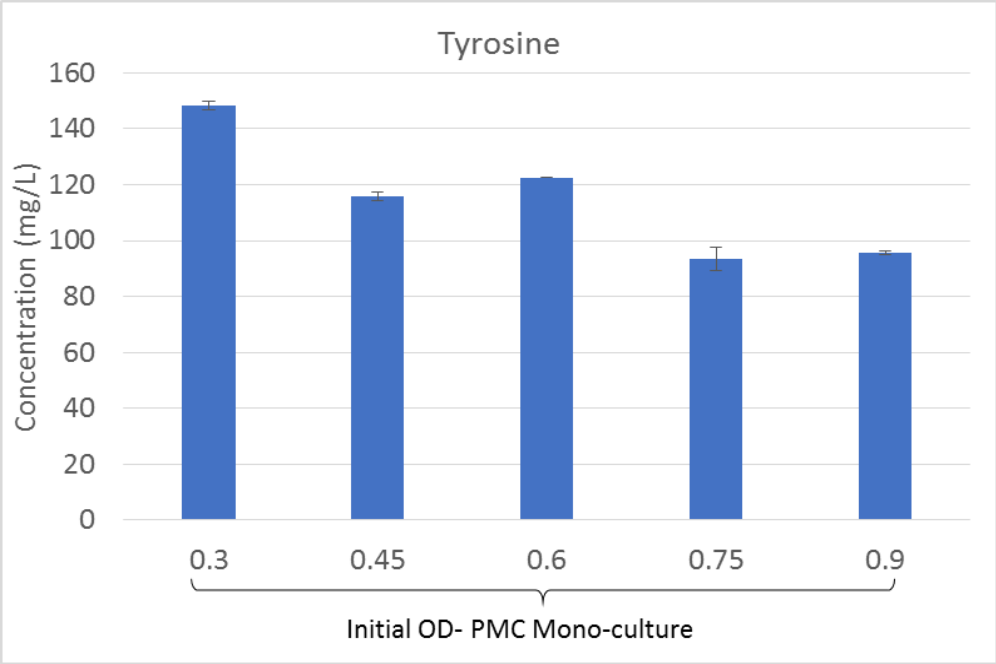


Fig.19 Tyrosine accumulation for different cell densities from mono-culture PMC strain when glucose is used as the carbon substrate

As shown in Fig.17, the production of naringenin varied with the initial cell density. At the cell density OD_{600}=0.3, 6.7 mg/L naringenin was observed. At the cell density OD_{600}=0.9, 8.3 mg/L naringenin was observed. There was a steady improvement in the production of naringenin. However, the production difference between different initial cell density conditions was not significantly different. In fact, even the production of 8.3 mg/L was still much lower that what was achieved by the co-culture under the same initial cell density (1:5 inoculation ratio). As such, it is demonstrated that the production improvement by the co-culture is largely due to the stronger bioconversion capability, rather than the change in the initial cell density. On the other hand, the accumulation of coumaric acid and tyrosine also varied as the cell density varied. At 0.3 OD, 122.3 mg/L coumaric acid and 145 mg/L tyrosine was observed. As the cell density increased, the accumulation of coumaric acid decreased, whereas there was no linear relationship between the accumulation of tyrosine and cell density. At the cell density OD_{600}=0.9, 95.6 mg/L tyrosine and 49.4 mg/L coumaric acid was observed.

4.5 Screening of downstream strains:

One of the main advantages of the co-culture approach is the ability to screen different downstream strains for identification of the best candidate for the naringenin production. Since the interactions between the upstream and the downstream strains are not predictable, different downstream strains were screened for the production of naringenin. *E. coli* P2C was selected as the upstream strain because of its ability to overproduce the pathway intermediate tyrosine. Different downstream strains were chosen based on their metabolic characteristics, which determines the capability of supporting the heterologous enzymes activity for converting the pathway intermediates to the final product

naringenin. Specifically, *E. coli* K12(DE3), JM109(DE3), BL21(DE3), and BL21 Star(DE3), were individually investigated for their performance as the downstream strain for the naringenin bioproduction. Among these strians, K12 and JM109 are considered commonly utilized strain for cloning, but both strains lack the ability to better produce recombinant proteins.[32]. BL21(DE3) is an established strain for heterologous protein expression, whereas BL21 Star(DE3) is a modified strain of BL21(DE3) which is engineered to enhance mRNA stability. BL21(DE3) and BL21 Star(DE3) have relatively little tolerance to the variation in cultivation conditions. The introduction of the downstream pathway genes, namely, TAL, 4CL, CHI, CHS, in these strains resulted in the new strains K12NA, JMNA, BLNA and StarNA, respectively. The constructed strains were co-cultivated with the upstream strain P2C to constitute four new co-cultures. Three different ratios (1:3, 1:4 and 1:5) were investigated for the production of naringenin.

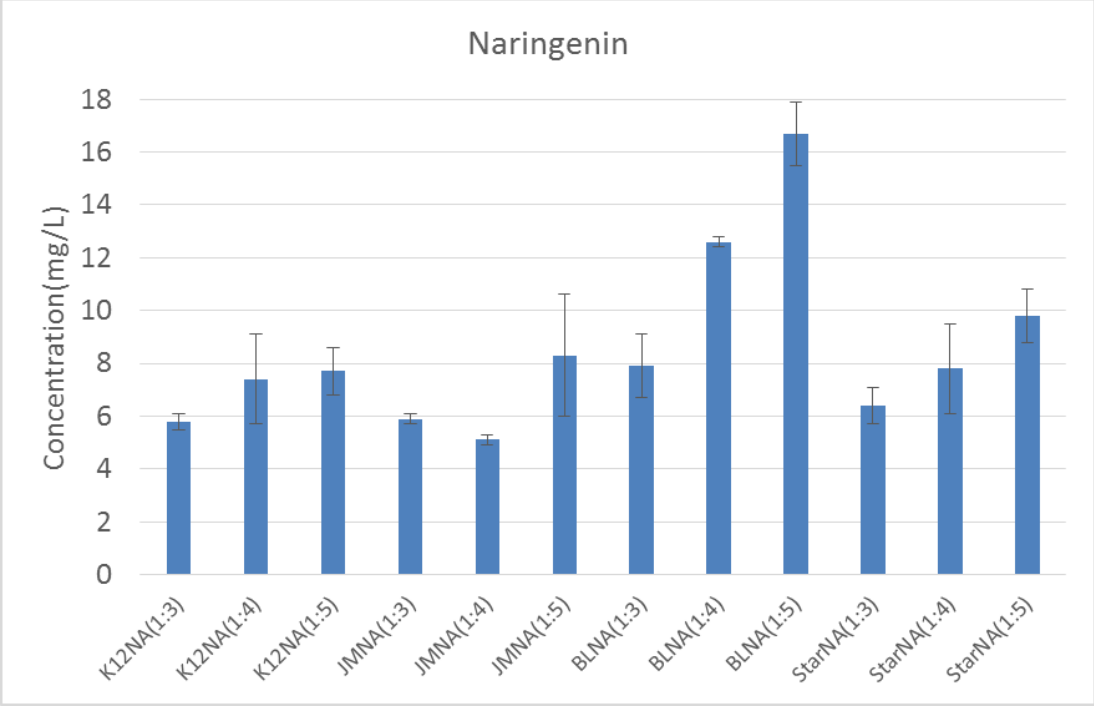


Fig 20. Biosynthesis of naringenin by using different *E.coli* strains as a host for the

downstream portion

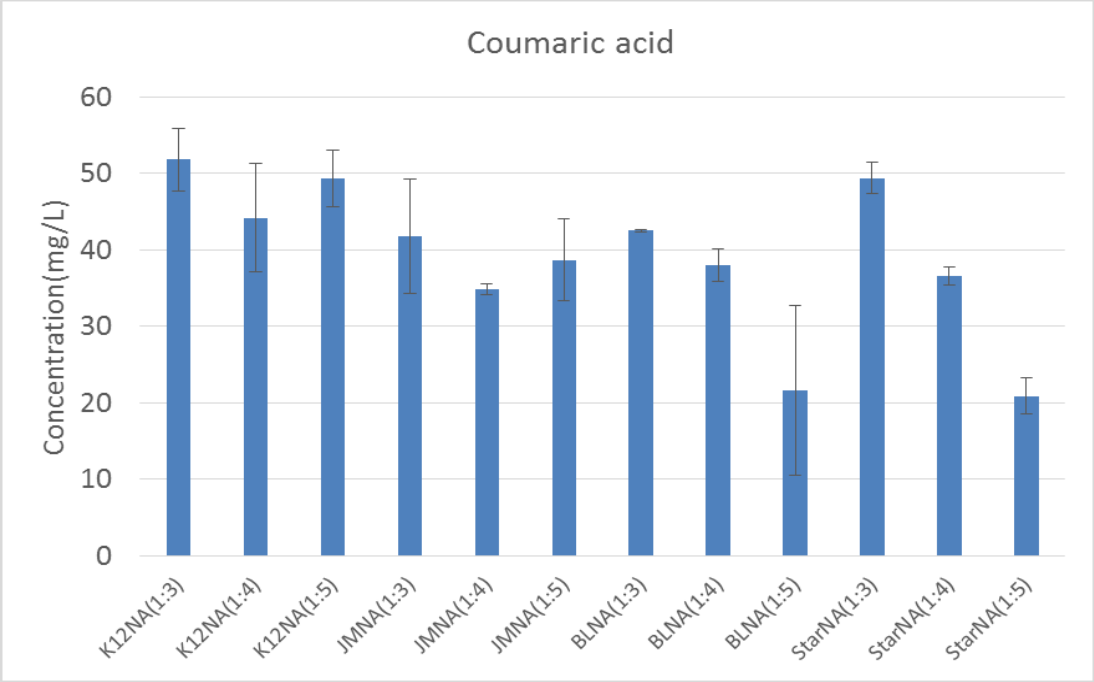


Fig 21.Accumulation of *p*-coumaric acid for different E.coli strains as a host for the downstream portion

As shown in Fig.22, different downstream strains gave different naringenin production values. BLNA (BL21(DE3) containing the downstream pathway enzymes) showed better production profile compared to other strains. Specifically, the co-culture of P2C and BLNA produced the highest amount of naringenin compared to the co-culture consisting of P2C and the other strains. At the inoculation ratio of 1:5, the P2C-BLNA co-culture produced 14.5 mg/L of naringenin. In comparison, the inoculation ratio 1:3 and 1:4 for the P2C: BLNA produced 7.9 and 12.6 mg/L of naringenin, respectively. These results are consistent with the results obtained in the previous sections.

The co-culture of P2C:StarNA produced 9.8 mg/L of naringenin at the inoculation ratio of 1:5. The inoculation ratio 1:3 and 1:4 produced even lower amount of naringenin

compared to the co-culture of P2C and BLNA. Even though StarNA was considered close to the BLNA strain in terms of the phenotype characteristics, the production of naringenin was still lower than thePC2:BLNA co-culture. Similarly, the co-culture of P2C: K12NA at the inoculation ratio of 1:5 produced 7.8 mg/L naringenin whereas the co-culture of P2C: JMNA produced 8.3 mg/L of naringenin at the inoculation ratio of 1:5. This suggests that BL21(DE3) is indeed capable of better supporting the expression of the recombinant proteins.

On the other hand, *p*-coumaric acid concentration was also evaluated to give a better idea about the accumulation of intermediates. The P2C:K12NA co-culture produced 49.3 mg/L of *p*-coumaric acid at the inoculation ratio of 1:5 whereas P2C:BLNA co-culture produced 24.9 mg/L p-coumaric acid at the same inoculation ratio. The P2C:StarNA produced the lowest amount of p-coumaric acid of 21.7 mg/L. Notably, the screening experiments here were performed at the IPTG induction time of zero hour and the temperature 30 ^{o}C. From these screening experiments, it was evident that BLNA is the best downstream strain for the production of naringenin. Therefore, it was used for all the following sections.

4.6 Impact of IPTG induction on the production of naringenin:

Previous experiments were conducted when IPTG induction was initiated at inoculation. As shown by the section XX (glycerol part of IPTG timing), the IPTG induction timing can decide the production. Here, the effect of IPTG induction timing on naringenin production on glucose was further investigated. Four different IPTG induction time points, including 0, 3, 6, and 10 h after inoculation, were tested. P2C and BLNA strains

were used for upstream and downstream respectively. The inoculation ratio of 1:5 was used for the IPTG induction timing experiments.

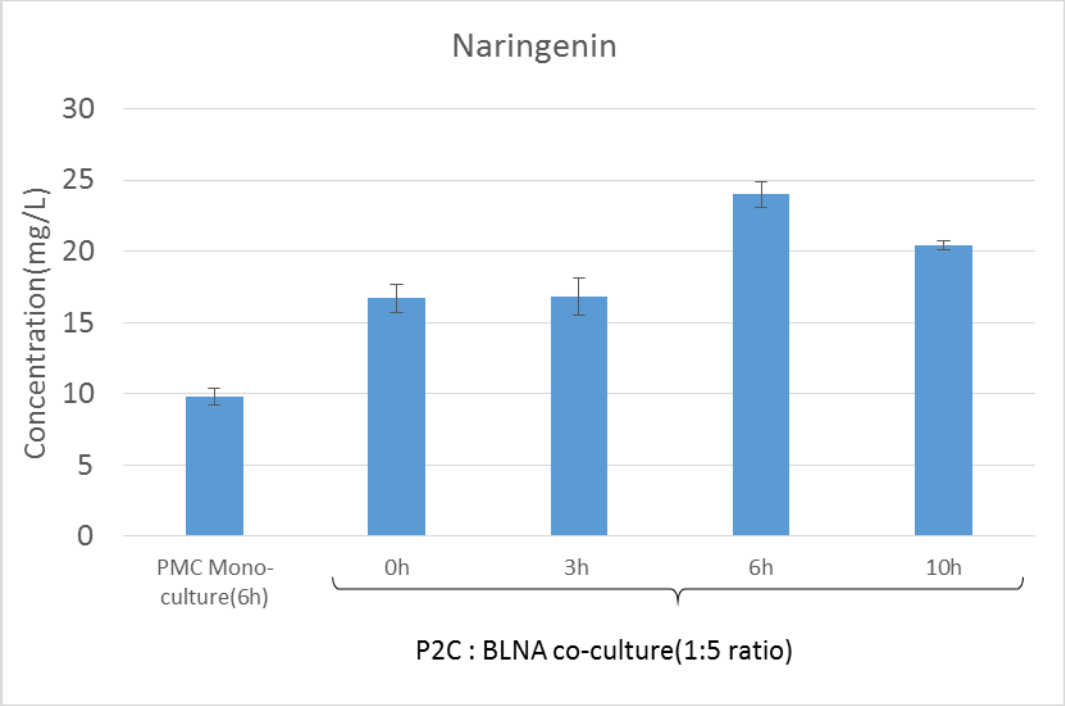


Fig 22. Effect of IPTG induction time points for the production of naringenin when glucose is used as the carbon substrate. Induction time is given in the figure

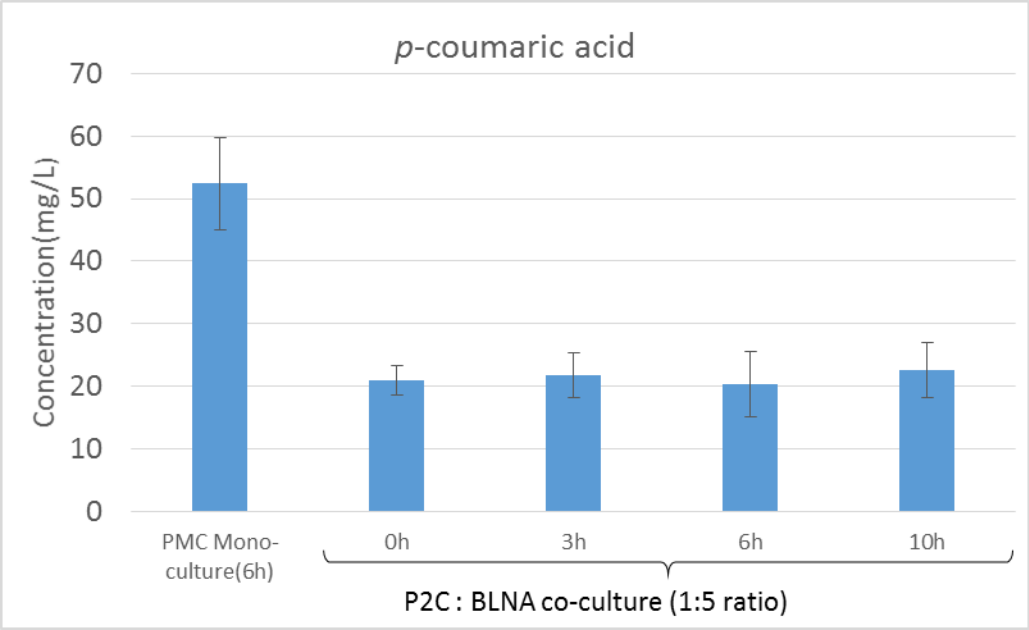


Fig 23.Effect of IPTG induction time points for the accumulation of *p*-coumaric acid when glucose is used as the carbon substrate. Induction time is given in the figure.

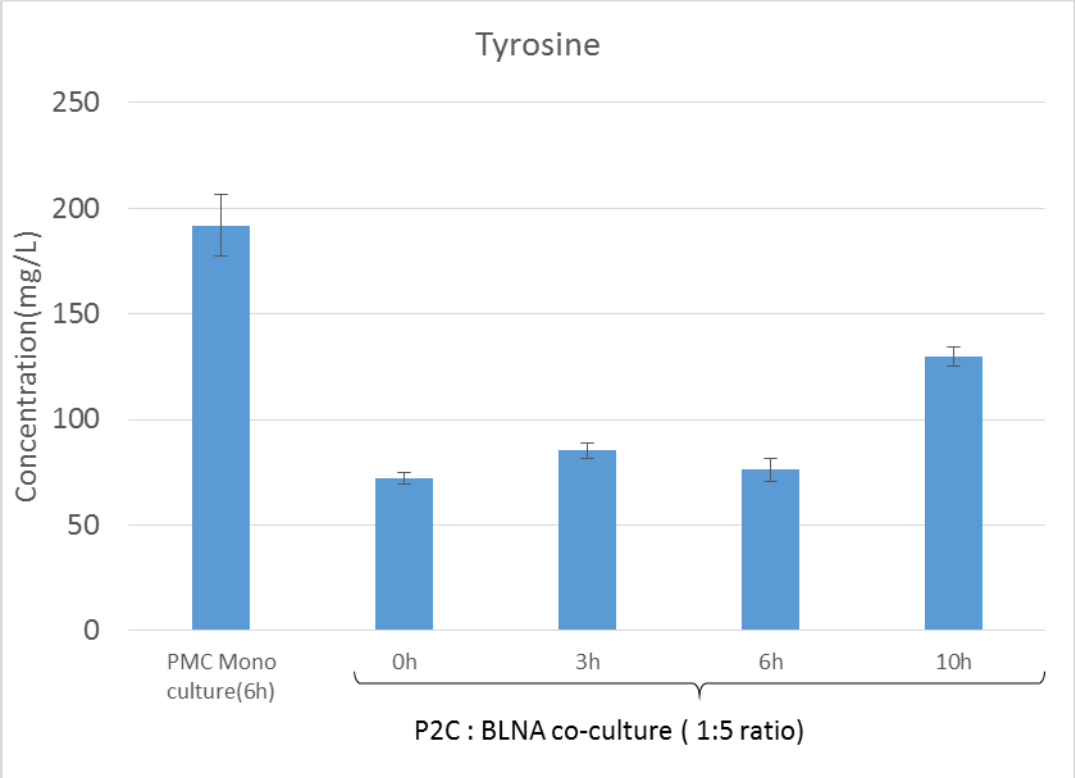


Fig 24.Effect of IPTG induction time points for the accumulation of tyrosine when glucose is used as the carbon substrate. Induction time is given in the figure.

The results of this section were shown in Fig 22. As the IPTG induction time points were varied, the production of naringenin also varied. Similar to the observation of production on glycerol, naringenin production was highest when the induction was started 6h after inoculation. Under this condition, the co-culture of P2C and BLNA produced 24 mg/L of naringenin. The production of naringenin at 0, 6 and 10 hour IPTG induction was 16.7, 16.8 mg/L and 20.4 mg/L respectively. At 6 hour IPTG induction, the PMC mono-culture produced 9.8 mg/L naringenin. For the monoculture PMC strain, the accumulation of coumaric acid was high. For the PMC mono-culture, 52.4 mg/L *p*-coumaric acid was accumulated. The *p*-coumaric acid accumulation was lower for the P2C and BLNA co-culture. At the inoculation ratio of 1:5 and the induction at 6 h, 20.3 mg/L *p*-coumaric acid was accumulated. The low accumulation of *p*-coumaric acid was due to the fact that most of the *p*-coumaric acid was converted to naringenin in the co-culture system. Similarly, the accumulation of tyrosine was found to be higher in the PMC mono-culture. For the co-culture, only 76.8 mg/L tyrosine was accumulated.

4.7 SHAKE FLASK BIOPRODUCTION:

Previous bioproduction was performed using a small scale culture tube. In this section, shake flasks was used for the production of naringenin to accommodate a higher volume (50 mL) of culture and also to improve the dissolved oxygen in the culture. For the shake flask experiment, glucose concentration was increased from 5 g/L to 20 g/L, and the cultivation time was accordingly increased to 72 h. As determined by the previous sections, the P2C: BLNA co-culture was cultivated under 30^{o}C and the induction was

carried out 6 h after the inoculation. Samples were taken every 12 hours to generate the time profile of naringenin *p*-coumaric acid and tyrosine concentrations.

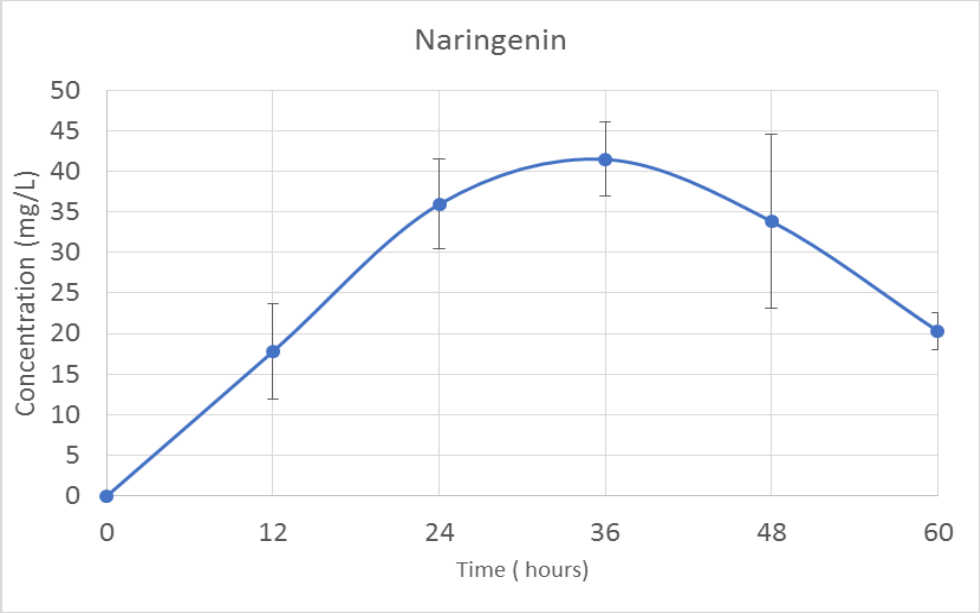


Fig 25.Naringenin production for P2C : BLNA co-culture using shake flasks at different time intervals

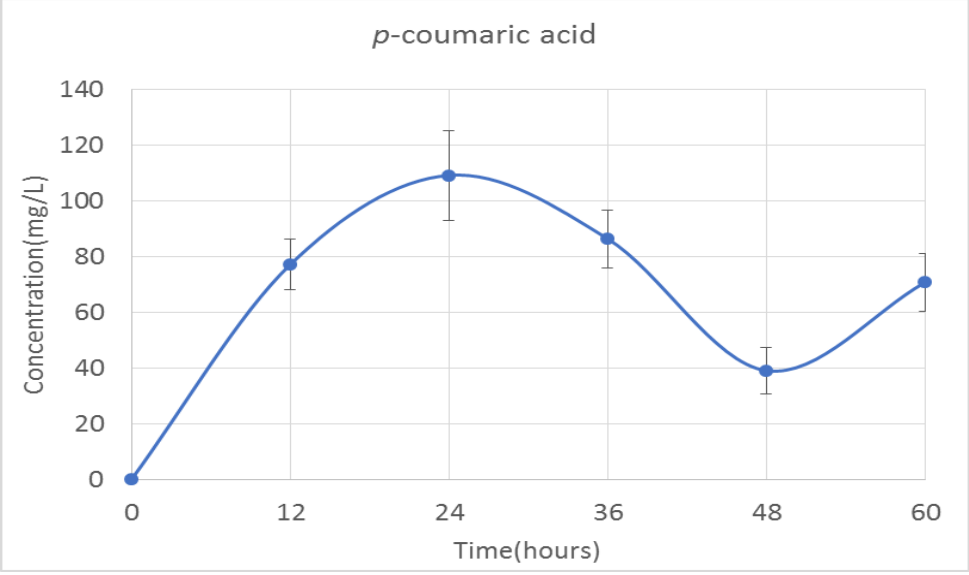


Fig 26. *p*-coumaric acid accumulation for P2C : BLNA co-culture using shake flasks at

different time intervals

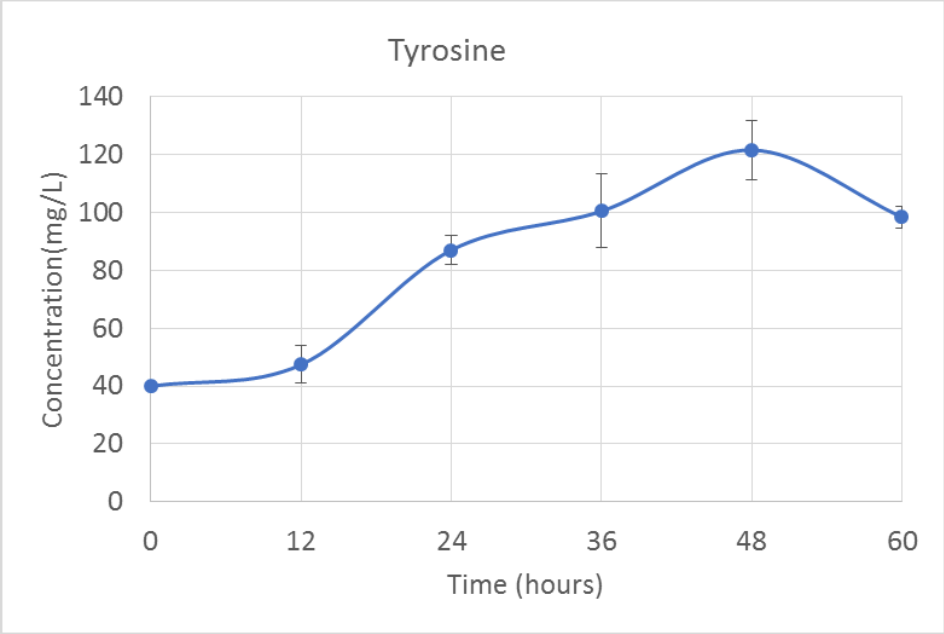


Fig.27 Tyrosine accumulation for P2C : BLNA co-culture using shake flasks at different time intervals

As shown in Fig 25, naringenin production fluctuated with time. The maximal production of 41.5 mg/L was observed at 36 hours followed by a decrease towards the end of cultivation process. The final naringenin concentration was only 20 mg/L at 60 hours. Such results suggest that there was degradation of the naringenin product, although the exact mechanism is still unknown. On the other hand, the accumulation of *p*-coumaric acid first increased to 109.2 mg/L at 24 hours and then further fluctuated at 36 h and 60 h. In contrast, there was a linear increase for the tyrosine accumulation up to 48 hours. Tyrosine concentration then decreased to 98.5 mg/L at 60 hours. These findings suggest that there are dynamic change of the pathway intermediate concentration due to the relative biosynthetic power change between the upstream supply and downstream

consumption. In addition, there appears to be a degradation mechanism for p-coumaric acid, as p-coumaric acid and naringenin concentrations both decreased over time.

Blue white screening was also done to differentiate between the upstream and downstream stains in the co-culture. Specifically, the upstream P2C strain does not have the *lacZ* gene and thus can only show white color on the X-gal plate, whereas the downstream BLNA strain can utilize its chromosomal *lacZ* gene to generate blue colonies. Fig. 28 shows the results obtained from the blue white screening.

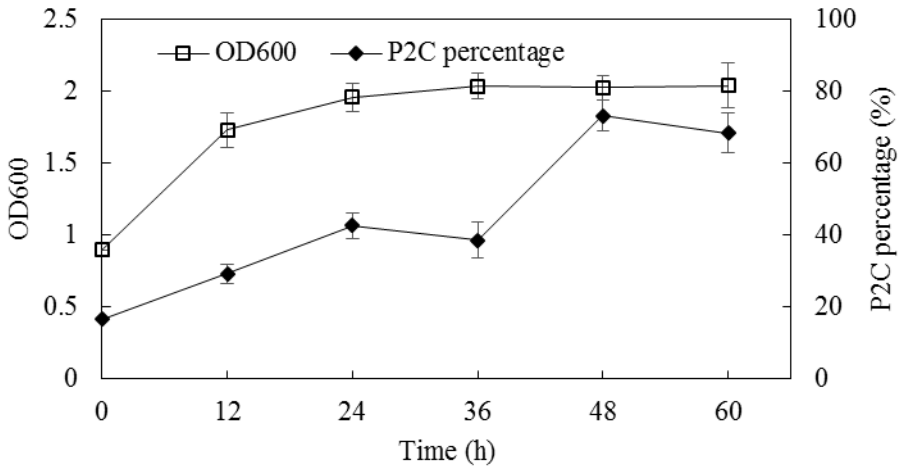


Fig 28. Profiles for co-culture overall cell density and the P2C strain percentage in the co-culture population.

It was found that the relative growth of P2C strain to the BLNA strain changed dynamically with respect to time. Overall, the growth of P2C strain was higher than the BLNA strain. In fact, the P2C strain only took 16% of the whole co-culture population. After 60 h cultivation, 68 % of the cells in the co-culture was P2C. As a result, there was no enough downstream strain for pathway intermediate conversion, especially towards

the end of the cultivation. Nonetheless, the shake flask experiment showed that the co-culture approach could produce 41.5 mg/L naringenin at the optimal condition and it provides insight regarding the strain-to-strain ratio change over the course of co-culture production.

5. CONCLUSION:

In this study, biosynthesis of naringenin was explored using the novel co-culture engineering approach. The naringenin production was optimized by fine-tuning different cultivation parameters such as carbon source, bioproduction temperature, inoculation ratio, IPTG induction timing, and downstream strain selection. The results of this study demonstrate that the constructed *E. coli*-*E. coli* co-cultures were able to produce more naringenin product without less intermediates than the mono-culture. Therefore, this study paves the way for further application of microbial co-cultures in complex natural product biosynthesis.

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