ENGINEERING FOR 4-HYDROXYSTYRENE BIOSYNTHESIS BY E. COLI

By

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Written under the direction of

Haoran Zhang

And approved by

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New Brunswick, New Jersey

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ABSTRACT OF THE THESIS

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Thesis Director: Dr. Haoran Zhang

4-hydroxystyrene is a phenolic chemical compound used in polymer and flavor industries. Microbial biosynthesis of this compound has been studied over recent years as a sustainable green alternative to synthetic chemical production. In this current study, 4-hydroxystyrene was biosynthesized using engineered *E. coli*. To improve the hydroxystyrene biosynthesis utilizing the carbon source following strategies were adopted. The pathway for bioproduction was bifurcated, and the co-culture of E. coli was implemented over monoculture. An amino acid exporter protein PhpCAT was utilized in combination biosensor-assisted cell selection mechanism to maximize the pathway intermediate tyrosine's biosynthesis. The exporter improved tyrosine's mass transfer between the co-culture members, and the resulting co-culture produced 298 mg/L 4-hydroxystyrene from 5 g/L glucose, which is 96% higher than the control co-culture without the exporter. The achieved yield of 0.06 g/g glucose is the highest among previous studies on de novo microbial biosynthesis. It shows that the exporter protein's utilization and biosensors enhance the pathway intermediate in a co-culture study and boost the hydroxystyrene biosynthesis performance. This strategy can pave the path for microbial biosynthesis for all the potential aromatic-derived compounds.

DEDICATION

To my father,

who encouraged me to study in States,

I promised him my research

before he left this world.

ACKNOWLEDGEMENT

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I want to acknowledge the Department of Chemical and Biochemical Engineering at Rutgers. My graduate experience benefitted greatly from the courses I took. Special thanks to former graduate program director Charles M. Roth, who guided me during my difficult time; I value the help you have given me, and current program director Fuat E. Celik, whom I frequently troubled him with my questions. Thank you for sharing your advice with me.

Finally, I would like to acknowledge my family, my mother and siblings who supported me during my time here. Their support has been unconditional. Thank you for everything.

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Introduction

4-Hydroxystyrene, also known as 4-vinyl phenol or p-vinyl phenol, is a phenolic compound as styrene with a hydroxy group at the 4th position. It has a vanilla-like odor and is naturally found in asparagus, ciders, beer, wine, blueberry, cloudberry (Sjöborg et al., 1984). 4-Hydroxystyrene is valuable in making flavoring agents and polymer industries such as petroleum-based feedstock for resins, elastomers, and adhesives. It is also used to manufacture photoresists, photolithography, and semiconductor (Bernini et al., 2007; Flanagin et al., 1999; Kang et al., 2015; Qi et al., 2007). This compound's microbial biosynthesis using renewable materials is of significant research interest due to its advantages in sustainability, process economy, and environmental friendliness.

1.1 Recent studies on 4-Hydroxystyrene biosynthesis

Researchers have studied 4-Hydroxystyrene for improving the production titer in recent years. Kang et al. reported 355 mg/L of 4-hydroxystyrene from 15 g/L glucose in a shake flask study after 36h of incubation (Kang et al., 2015). W.W. Qi et al. studies report 0.4 g/L of 4-hydroxystyrene from 15g/L glucose in a 14 L fermenter under limited phosphate conditions (Qi et al., 2007). Verhoef et al. established the 4-Hydroxystyrene pathway in *P. putida.* They utilized this species' high tolerance of toxic organic solvent to develop a two-phase fermentation process, leading to 21 mM 4-hydroxystyrene from glucose (Verhoef et al., 2009). The highest yield using this engineered strain reached 6.7% (C-mol/C-mol). Also, the 4-hydroxystyrene pathway was reconstituted in *Streptomyces lividans*, which resulted in the production of 390 and 250 mg/L 4HB from glucose and phosphoric acid

swollen cellulose, respectively (Noda et al., 2015). Using a biphasic reaction system, Jung and coworkers alleviated the toxicity of 4-hydroxystyrene against the recombinant *E. coli* and achieved 88.7 % conversion of exogenous p-coumaric acid to 4-hydroxystyrene (Jung et al., 2013). Sariaslani, F. S. discussed previously that at the scale of 200L, 50g/L of tyrosine when produced from glucose, can be converted by immobilized catalyst to give 39±4 g/L p-coumaric acid after more than 250 consecutive hours. Furthermore p-coumaric acid can be chemically converted to p-hydroxystyrene at 150°C; 2M concentration of p-coumaric acid yielded 98.6% p-hydroxystyrene in 150mins (Sariaslani, 2007). Fujiwara et al. genetically engineered *S. mobaraense* and produced 273 mg/L 4-hydroxystyrene from 10 g/L glucose (Fujiwara et al., 2016). Similarly, Salgado et al. used a two-phase extractive fermentation strategy to make 4-hydroxystyrene from corn cob alkaline hydrolysate using engineered *E. coli* (Salgado et al., 2014). Despite these recent achievements, the overall 4-hydroxystyrene production yield remains low and can hardly meet large-scale industrial production requirements.

1.2 Pathway for bioproduction of 4-Hydroxystyrene

The present study investigates 4-Hydroxystyrene biosynthesis using endogenously synthesized L-tyrosine as a precursor, converted to 4-hydroxystyrene through the following enzymatic steps shown in Figure 1. Here, carbon sources such as glucose are feed as starting material converted to phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P). PEP and E4P are converted to 3-deoxy-D-arabino heptulosonate-7-phosphate (DAHP) by *aroG*^{fbr} (Phospho-2-dehydro-3-deoxyheptonate aldolase), converted to shikimate with the help of aroB (3-dehydroquinate synthase), aroD (3-dehydroquinate

dehydratase), and shikimate dehydrogenase aroE. Shikimate is converted to Chorismate by shikimate kinase 2 (aroL), 3-phosphoshikimate 1-carboxy vinyl transferase (aroA), and chorismate synthase (aroC). Finally, tyrA^{fbr} converts chorismite to tyrosine (Averesch & Krömer, 2018; Gosset, 2009). Then the tyrosine is converted to p-Coumaric acid (pCa) using enzyme tyrosine-ammonia lyase TAL. This phenolic acid undergoes an enzymatic non-oxidative decarboxylation reaction by ferulic acid decarboxylase FDC1 to make 4-hydroxystyrene (Bhuiya et al., 2015).

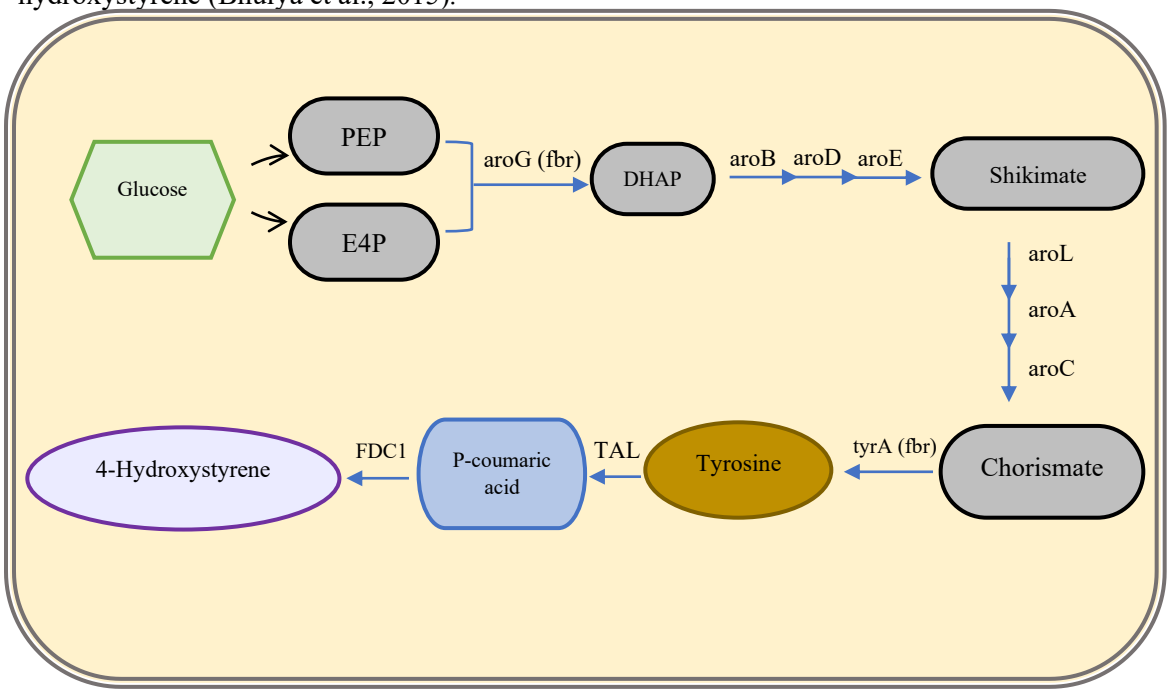


Figure 1: Schematic representation of metabolic pathway for 4 Hydroxystyrene biosynthesis

1.3 Modular co-culture and metabolite exporter

Modular co-culture, which is an engineered microbial co-culture with modularized pathways for microbial biosynthesis, has been used for a wide range of biomolecules like simple biofuel compounds to involved pharmaceutical agents such as drugs (oxygenated

taxanes) or chemicals commodities (flavan-3-ols, curcuminoids, and anthocyanins) (Roell et al., 2019; R. Wang et al., 2020; Zhang & Wang, 2016). The co-culture distributes the biosynthetic work between upstream and downstream E. coli cells. This reduces the metabolic burden on each strain and improves production. However, modular co-culture engineering requires an effective mass transfer of pathway intermediates between different co-culture members to connect the corresponding pathway modules. In fact, most of the microbial co-culture studies so far relied on the native metabolite transportation mechanism, including passive diffusion, and facilitated transportation, to move pathway intermediates from one co-culture strain to another. Although this strategy has shown to be successful in many instances, further improvement of the microbial biosynthesis performance calls for more advanced strategies to minimize the pathway intermediate accumulation caused by the mass transfer limitation. For example, a pathway intermediate produced by the upstream co-culture strain should be efficiently secreted out of the producing cells for bioconversion by the downstream strain. To demonstrate its feasibility, Widhalm et al. constructed *E. coli-E. coli* co-culture to produce 4-hydroxystyrene via amino acid tyrosine. An aromatic amino acid exporter from *Petunia hybrida* was adopted to facilitate the tyrosine relocation for the 4-hydroxystyrene biosynthesis. This exporter is a plastidial cationic amino-acid transporter gene PhpCAT that transports all three aromatic amino acids (Widhalm et al., 2015). Metabolite exporter can effectively reduce the intracellular concentration. It helps shift the reaction equilibrium towards the formation of product and creates a strong selection force to improve the bioproduction (van der Hoek & Borodina, 2020). There exists a dynamic environment in the medium, the uptake of tyrosine by the downstream strain, and conversion to 4-hydroxystyrene. The upstream cells

produce the tyrosine, after which the downstream cell began to grow and uptake this exported tyrosine, which further encourages the production of tyrosine by upstream cells. With this method, we can ensure the upstream strain can over-produce tyrosine and enhance the output of 4-hydroxystyrene without increasing the stress on the strain. To further optimize production, the ratio between the two E. coli cells is adjusted to improve tyrosine conversion to 4-hydroxystyrene. The co-culture is compared with the monoculture approach containing all the enzymes to produce 4-hydroxystyrene. The co-culture design pathway is shown in Figure 2. Thus, using metabolite exporters provides additional benefits for adopting advanced engineering strategies for microbial biosynthesis.

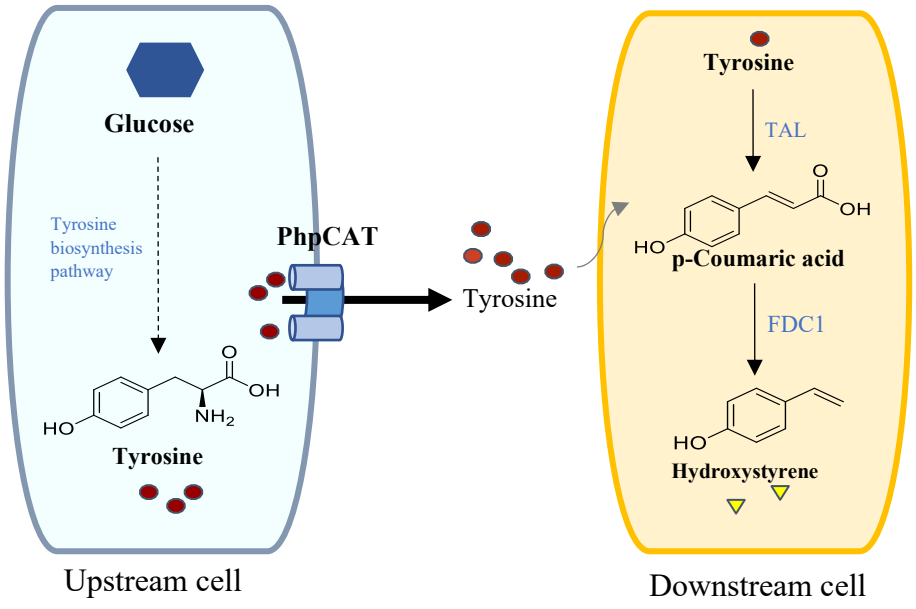


Figure 2:Design for co-culture approach for 4-Hydroxystyrene biosynthesis. TAL: tyrosine-ammonia lyase, FDC1: ferulic acid decarboxylase, PhpCAT: tyrosine exporter protein.

1.4 Use of toxin-as biosensor

Efforts have been previously made to use biosensors to promote the population of microbes that are high-performing growth of engineered strain. Such a strategy uses a metabolite-specific biosensor hipA (**hi**gh **p**ersistence factor **A**), which is a serine/threonine protein kinase toxin, for the selection of high-performing cells based on the intracellular concentration of the target metabolite (Guo et al., 2019; Li et al., 2020; Rugbjerg et al., 2018; Wang et al., 2019; Xiao et al., 2016).

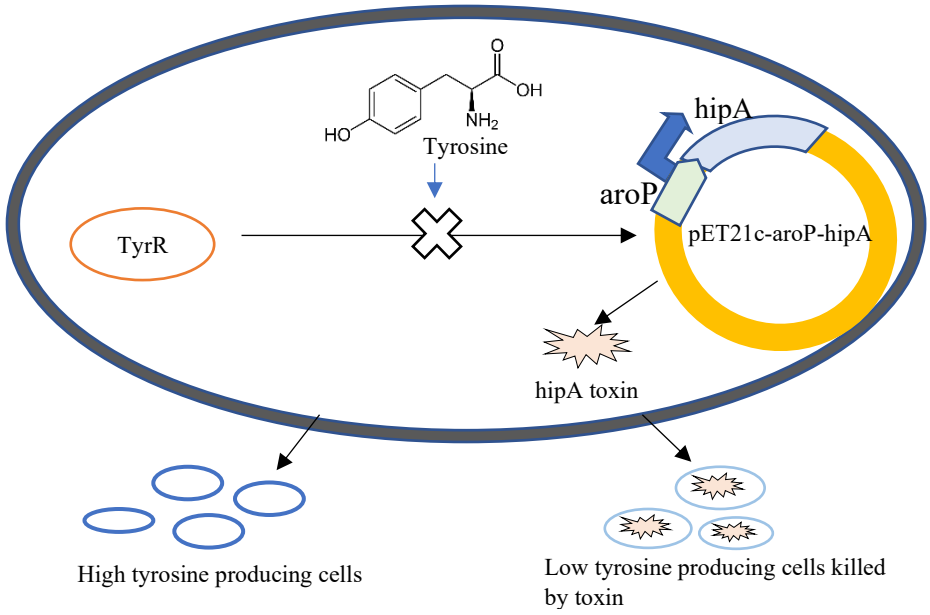


Figure 3:Biosensor mechanism to select high performing cells for tyrosine production

An overview of the mechanism is shown in Figure 3. TyrR is a regulatory dimer protein in E.coli that is involved in transcription. In the presence of a tyrosine molecule, the TyrR protein aggregates and forms a hexamer, inhibiting the initiation of transcription or promoter activity (Yang et al., 2002). Whereas in the absence of tyrosine, the TyrR binds to the aroP promoter region (Wang et al., 2003; Yang et al., 1999) and initiates expression of hipA gene to produce hipA toxin. HipA toxin then kills the cells as they have low

tyrosine production. In this way, the hipA toxin helps eliminate low-performance cells and retains only the high-performing cells, i.e., tyrosine over-producing *E.coli*.

1.5 Goal

This study aims to produce 4 Hydroxystyrene using suitable carbon source and investigate the production performance by the *E. coli-E. coli* co-culture. The intention is to optimize the production using a sensor, exporter and co-culture and compare it with the monoculture.

2. Materials and Methods

2.1 Chemicals and Medium

The chemicals used in this study were purchased from VWR Life Science (Radnor, PA, United States), Thermo Fisher Scientific (Waltham, MA, United States), and Sigma-Aldrich (St. Louis, MO, United States). The MY1 medium was used for the 4-hydroxystyrene biosynthesis. 1 L MY1 medium contained 5 g glucose, 2 g of NH4Cl, 5 g (NH4)2SO4, 3 g KH2PO4, 7.3 g K2HPO4, 8.4 g 3-(N-morpholino) propane sulfonic acid (MOPS), 0.5 g NaCl, 0.24 g MgSO4, 0.5 g yeast extract, 40 mg of tyrosine, 40 mg of tryptophan, 40 mg phenylalanine, 10mg 4-hydroxybenzoic acid and trace elements. The working concentrations of trace elements were 0.4 mg/L Na2EDTA, 0.03 mg/L H3BO3, 1 mg/L thiamine, 0.94 mg/L ZnCl2, 0.5 mg/L CoCl2, 0.38 mg/L CuCl2, 1.6 mg/L MnCl2, 3.77 mg/L CaCl2, and 3.6 mg/L FeCl2. When needed, 50 mg/L kanamycin, 34 mg/L chloramphenicol, 100 mg/L for ampicillin, 50 mg/L streptomycin, and 0.2 mM isopropyl

β-D-1-thiogalactopyranoside (IPTG) were supplemented into the medium. Similarly, for Glycerol special medium, 5g glycerol is used instead of glucose in the medium.

Table 1: Plasmids and strains used in the present study

PLASMIDS	DESCRIPTION	SOURCE
PET21C	T7 promoter, Amp^R	Novagen
PET28A	T7 promoter, Kan^R	Novagen
PRSFDUET-1	double T7 promoters, Kan^R	Novagen
PTRCHIS2B	trc promoter, pBR322 ori, Amp^R	Invitrogen
PACYCDUET-1	double T7 promoters, Cm^R	Novagen
PCDFDUET-1	double T7 promoters, Sp^R	Novagen
PCA1	pTrcHis2B carrying codon optimized *R. glutinis* TAL	(Santos et al., 2011)
PRSF-FDC1	pRSF carrying codon optimized *S. cerevisiae* FDC1	This study
PBS9	pET21c carrying *hipA* gene under the control of the *aroP* promoter (ParoP)	(Guo et al., 2019)
PBD	pACYCDuet-1 carrying the E. coli aroB and aroD genes with a proD promoter (PproD)	(Guo et al., 2019)
PBDEXP	pACYCD carrying *E. coli aroB*, *aroD* and codon optimized *P. hybrida PhpCAT* genes under the control of the *proD* promoter (PproD)	This study
PBS2	pET28a carrying the E. coli aroE, aroL, aroA, aroC, $tyrA^{fbr}$ and $aroG^{fbr}$ genes with a proD promoter (PproD)	(Li et al., 2019)
PARFP	pACYCDuet-1 carrying the red fluorescence protein *DsRed* gene	(Guo et al., 2019)
PCDF-TRC-RGTAL	pCDFDuet-1 carrying the codon-optimized RgTAL gene under the control of trc promoter	(Zhang & Stephanopoulos, 2013)

PSG1	pCDF-trc-RgTAL carrying FDC1 gene under the control of T7 promoter	This study
STRAINS	**Description**	**Source**
BL21(DE3)	F$^-$ *omp*T *hsdSB (rB-,* mB$^-$) gal dcm (DE3)	Invitrogen
BH2	E. *coli* BL21(DE3) ΔxylA ΔtyrA ΔpheA	(Zhang et al., 2015)
K12 (DE3)	F$^-$ λ$^-$ *ilvG$^-$ rfb-50 rph-1* (DE3)	(Santos et al., 2012)
JM109(DE3)	F' *traD*36 *proA$^+$B$^+$lacI^q* Δ(*lacZ*)*M15/Δ(lac-proAB) glnV44 e14$^-$ gyrA96 recA1 relA1 endA1 thi hsdR17* (DE3)	New England Biolabs
BL21STAR (DE3)	F$^-$*omp*T *hsdSB* (rB$^-$, mB$^-$) *gal dcm rne*131 (DE3)	Life technologies
BLP1	BL21(DE3) containing pCA1 and pRSF-FDC1	This study
KD1	K12 (DE3) containing pCA1 and pRSF-FDC1	This study
JMP1	JM109 (DE3) containing pCA1 and pRSF-FDC1	This study
BLSP1	BL21star containing pCA1 and pRSF-FDC1	This study
BLP2	BL21(DE3) containing pCA1, pRSF-FDC1 and pARFP	This study
KD2	K12 (DE3) containing pCA1, pRSF-FDC1 and pARFP	This study
N1	K12 (DE3) containing pBS9, pBS2 and pBD	This study
P1	K12 (DE3) containing pBS9, pBS2 and pBDexp	This study
N7	BL21(DE3) containing pBS9, pBS2 and pBD	This study
P7	BL21(DE3) containing pBS9, pBS2 and pBDexp	This study
N8	BH2 containing pBS9, pBS2 and pBD	This study
P8	BH2 containing pBS9, pBS2 and pBDexp	This study
N8M	BH2 containing pBS9, pBS2, pBD and pSG1	This study
P8M	BH2 containing pBS9, pBS2, pBDexp and pSG1	This study

2.2 Plasmid and strain construction

The DNA cloning agents, NEB5-alpha high-efficiency competent cell, restriction enzymes, T4 ligase, and SOC medium were obtained from New England Biolabs (Ipswich,

MA, United States). DNA purification, gel recovery, and plasmid isolation kits from Zymo Research (Irvine, CA, USA). To construct plasmid pSG1, pRSF-FDC1 was first digested with BamHI-HF and BsaI to isolate the FDC1 gene fragment, followed by its ligation with pCDF-trc-RgTAL treated with the same restriction enzymes. The strains were constructed by transforming with desired plasmids using the Eppendorf electroporator.

2.3 Cultivation conditions for 4-hydroxystyrene biosynthesis

For 4-hydroxystyrene biosynthesis using monoculture, *E. coli* N8M, and P8M, were first grown overnight in LB medium at 37°C. The seed cultures were centrifuged at 10,000 rpm for 1 min and resuspended in 2 mL MY1 medium such that the initial OD_{600} was 0.5. After the addition of 0.2mM IPTG, the resulting cultures were incubated at 37 °C for 48 h.

For 4-hydroxystyrene biosynthesis using co-culture, the upstream and downstream strains were cultivated separately in the LB medium. The overnight cultures were centrifuged and resuspended in fresh MY1 medium. After the measurement of the cell density, desired amounts of the upstream and downstream strain cultures were added into fresh MY1 medium at different inoculation ratios of 9:1, 4:1, 1:1, 1:4, 9:1. The total initial O.D600 was set to 0.5, and 0.2 mM IPTG was added before the initiation of the co-culture cultivation at 37 °C for 48 h.

2.4 Analytical method for Hydroxystyrene quantification

Quantification of tyrosine, p-coumaric acid, 4-hydroxystyrene was conducted using Agilent 1100 HPLC sets with a DAD detector. 1.0 mL culture sample was centrifuged at 10000 rpm for 5 min, and the supernatant was syringe filtered through 0.45 µm

polytetrafluoroethylene membrane (VWR International). 10 µL filtered sample was injected into a Waters C18 HPLC column and eluted using solvent A (0.05 % acetic acid in HPLC water) and solvent B (99.9 % acetonitrile) run at the flow rate of 0.7 mL/min. The following gradient was used for elution: 0 min, 100 % solvent A; 5 min, 95 % solvent A; 6 min, 75 % solvent A; 10 min, 10 % solvent A; 11-13 min 100 % solvent A. The metabolite concentration was determined by measuring the absorbance at wavelength 280 nm. For determining the intracellular product concentration, the cells pellet was collected by centrifugation at 10,00 rpm and resuspended in 1mL of HPLC water, followed by sonication using microtip at 30 hertz pulsed on and off for every 5 seconds for 5 min. The sonicated solution was then centrifuged at 10,00 rpm, and the supernatant was analyzed using the HPLC method described above. The measured metabolite amounts were then divided by the whole culture volume for calculation of the intracellular metabolite accumulation's contribution for the overall metabolite production

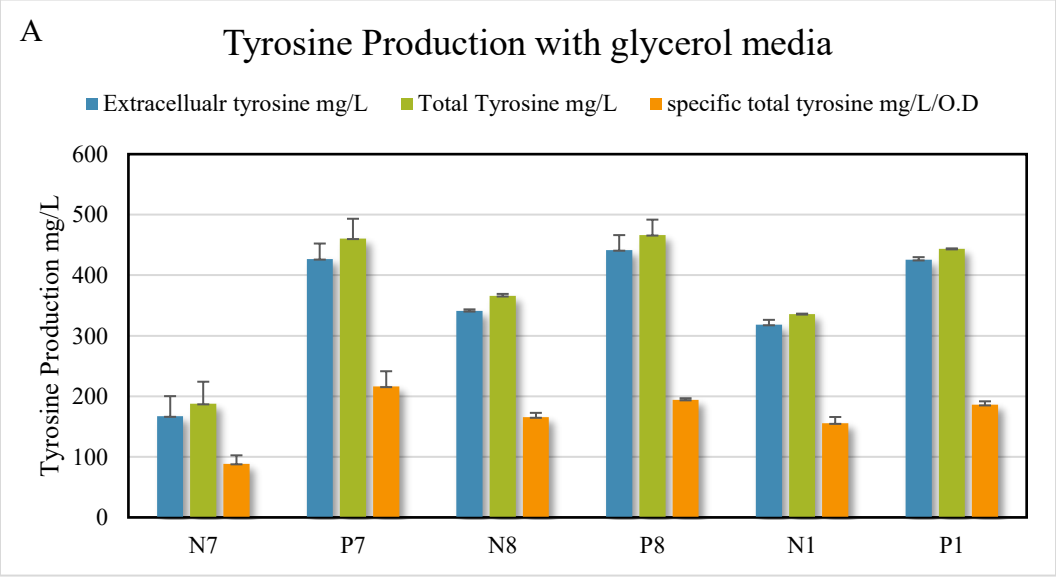
3. Results and discussion

3.1 Effect aromatic amino acid exporter and carbon source on tyrosine production

This study uses *Petunia hybrida* plastidial cationic amino-acid transporter PhpCAT that has been found to functionally secrete tyrosine in *E. coli* (Widhalm et al., 2015). The PhpCAT gene was codon-optimized and over-expressed in a previously constructed tyrosine-overproducing *E. coli* strain. This strain contains a tyrosine-biosensor-assisted cell selection system that promotes tyrosine production by removing the low-performing cells

from the culture population (Wang et al., 2019; Xiao et al., 2016). At high tyrosine concentration, tyrosine sensor protein TyrR represses the expression of the toxin gene *hipA* under the control of the *aroP* promoter and enables the cell to grow normally; at low tyrosine concentration, the *hipA* gene expression is expressed and the cell growth is accordingly inhibited.

The over-expression of PhpCAT in this biosensor-assisted strain facilitates the tyrosine exportation and thus stimulates the cell to produce more tyrosine in the presence of the selective pressure of ampicillin. The resulting strains were tested for tyrosine biosynthesis. Strains BL21(DE3), BH2, and K12 containing non-exporter (pBD) or exporter (pBDexp) plasmid along with plasmid having a sensor (pBS9) and tyrosine (pBS2) were constructed to give N7, P7, N8, P8, N1, and P1 respectively. Additionally, to improve metabolite production, the source of carbon is crucial. Glucose and glycerol have been known as suitable carbon substrates contributing to pyruvate and shikimate pathways to give tyrosine. Tyrosine production was compared using the above strains grown in glucose and glycerol MY1 medium with 5g/L carbon source for 48 hours.



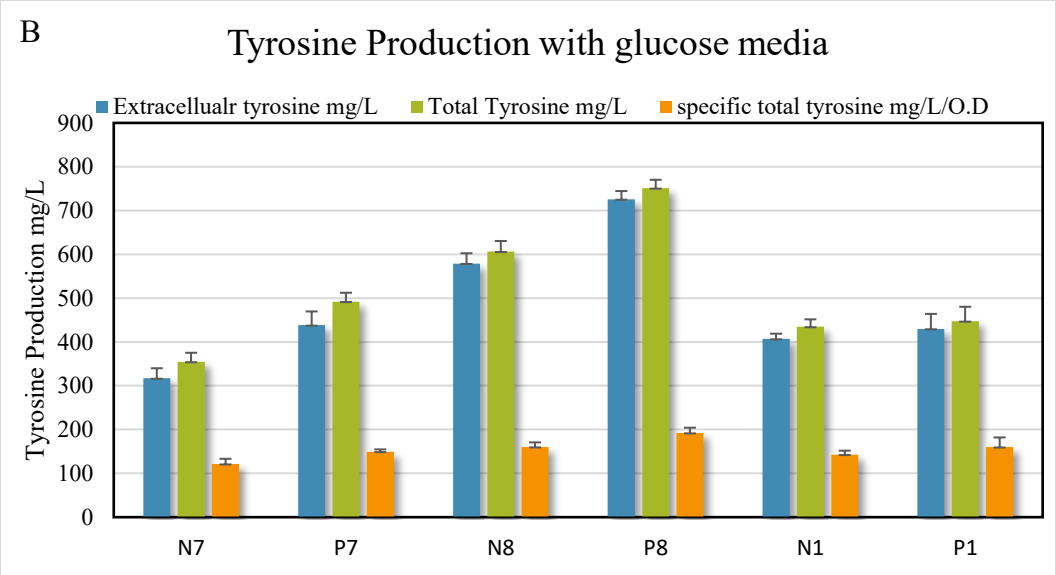


Figure 4:Comparison of the tyrosine production by E. coli N8 (without the tyrosine exporter) and P8 (with the tyrosine exporter). A) Glycerol media B) Glucose media, all strains cultivated under same inoculation O.D 0.5, at 37C for 48h.

Figure 4. shows a significant improvement in tyrosine when glucose is used in medium instead of glycerol. BL21 strain P7 produces a maximum of 492 mg/L tyrosine on glucose and 460.7mg/L on glycerol. Similarly, for BH2 strain P8, 751 mg/L on glucose medium and 466.5mg/L on glycerol medium tyrosine is formed. For the K12 strain P1, the tyrosine production is very similar, 447.3mg/L on glucose and 443.7 mg/L on glycerol. The trend of better tyrosine production on glucose is found true for all the strains BL21(DE3), BH2, and K12. The specific tyrosine production mg/L/OD is better for BH2 strains than K12 and BL21 on glucose medium, indicating biomass can effectively produce tyrosine on glucose medium. Also, it is evident that BH2 strains are better at extracellular tyrosine production.

Extracellular tyrosine accumulation of P8 strain reached 725.6 mg/L after 48 h cultivation, which is 25% higher than the control strain N8 without the exporter. The highest

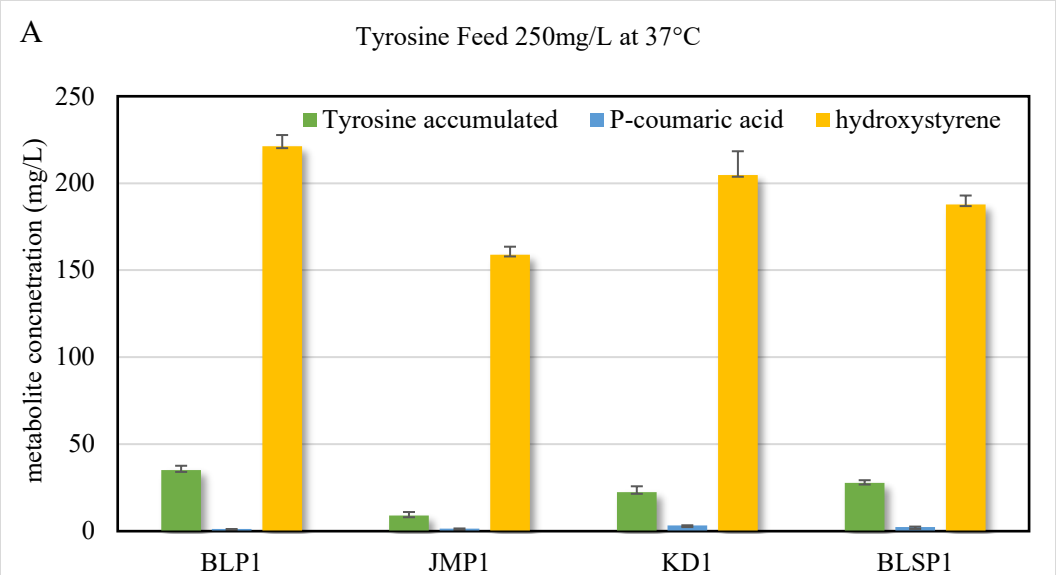
extracellular tyrosine production for BL21 strain P7 483 mg/L is lesser than the non-exporter BH2 strain N8, which gives 579.1 mg/L of extracellular tyrosine. Interestingly, the intracellular tyrosine concentration did not change much between the strains without and with the PhpCAT over-expression. These results suggest that *E. coli's* intracellular tyrosine concentration is quite stable even when the introduced exporter functionally works to secrete the produce tyrosine. The exact mechanism for this is still unknown. The total tyrosine production (including both intracellular and extracellular accumulation) was also improved in the strains P8, P7, and P1 with the PhpCAT exporter. This pattern is also found true for specific tyrosine production for the strains with and without the exporter. Nonetheless, it is confirmed that the tyrosine exporter's over-expression helped the biosensor-assisted cell selection system improves the tyrosine production, and the resulting strain P8 was utilized as the tyrosine provider for the following co-culture study using glucose as the carbon source.

3.2 Construct an *E. coli* strain for tyrosine-to-4-hydroxystyrene conversion

To construct an *E. coli-E. coli* co-culture for 4-hydroxystyrene biosynthesis. The tyrosine-overproducing strain developed in section 3.2 can be used as the co-culture upstream strain; it is required to identify a suitable *E. coli* strain for converting tyrosine to 4-hydroxystyrene. It has been found that strains with different metabolic characters showed varied biosynthesis capabilities (Zhang & Stephanopoulos, 2016) four different *E. coli* strains were engineered to over-express TAL derived from *R. glutinis (Santos et al., 2011; Vannelli et al., 2007)* and ferulic acid decarboxylase FDC1 derived from *S. cerevisiae* (McKenna & Nielsen, 2011; Mukai et al., 2010). *E. coli* strains BL21(DE3), JM109, K12

and BL21star (DE3) were engineered to give BLP1, JMP1, KD1, BLSP1 having two plasmids pCA1 and pRSF-FDC1 respectively and fed with 250 and 500 mg/L tyrosine for 4-hydroxystyrene production at two temperatures 32 °C and 37°C.

The two plasmid-containing strains were compared for two-level feed 250mg/L and 500mg/L at two temperatures, 32°C, and 37°C. From Figure 5, A, B, C, D for 250mg/L feed experiment, BLP1 was the highest producing 4-hydroxystyrene strain for both the temperatures amongst the five strains, i.e., 221.2mg/L at 37°C and 255.7mg/L at 32°C. It appears that at 250mg/L feed, production of 4-hydroxystyrene at 32°C is better than at 37°C for all five strains. Further comparing, as shown in Figure 5, E, F, G, H for the 500mg/L feed experiment at two temperatures, BLP1 is again the highest producing strain 413.1mg/L at 37°C. Also, it was found that specific production for BLP1 was higher for 32°C than 37°C, suggesting that less biomass at 32°C. KD1 was second highest in production of 4-hydroxystyrene 349 mg/L at 500 mg/L tyrosine feed at 37°C. It was observed that the tyrosine remaining (unutilized) was highest at 32°C for 500mg/L feed, indicating that this temperature is not suitable for 4-hydroxystyrene production when the tyrosine concentration is high. Notably, only small amounts of tyrosine and *p*-coumaric acid were observed, indicating high enzymatic activities of the TAL and FDC1 enzymes. The other strains showed lower tyrosine conversion capability under the same cultivation conditions.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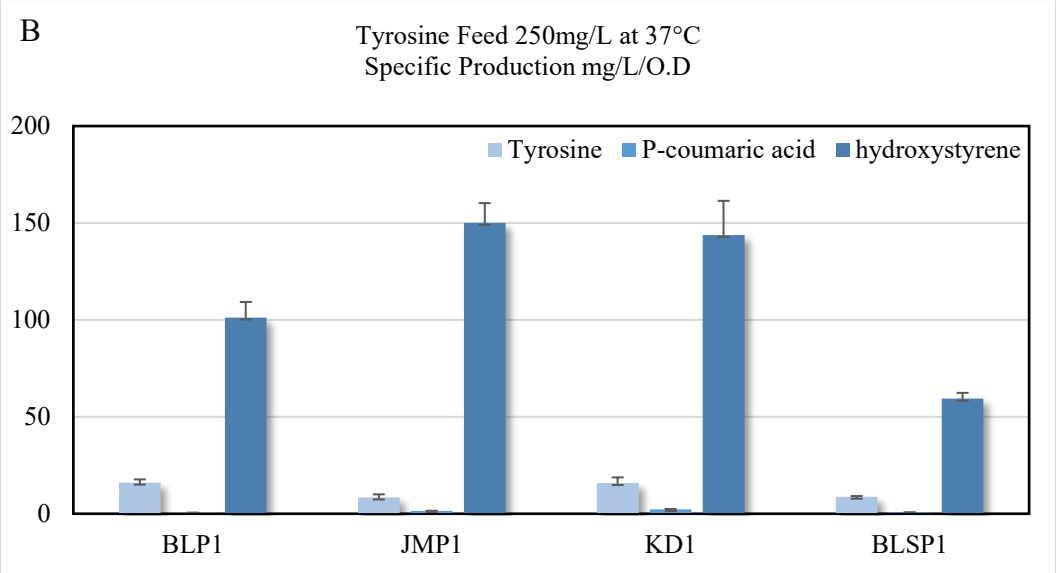
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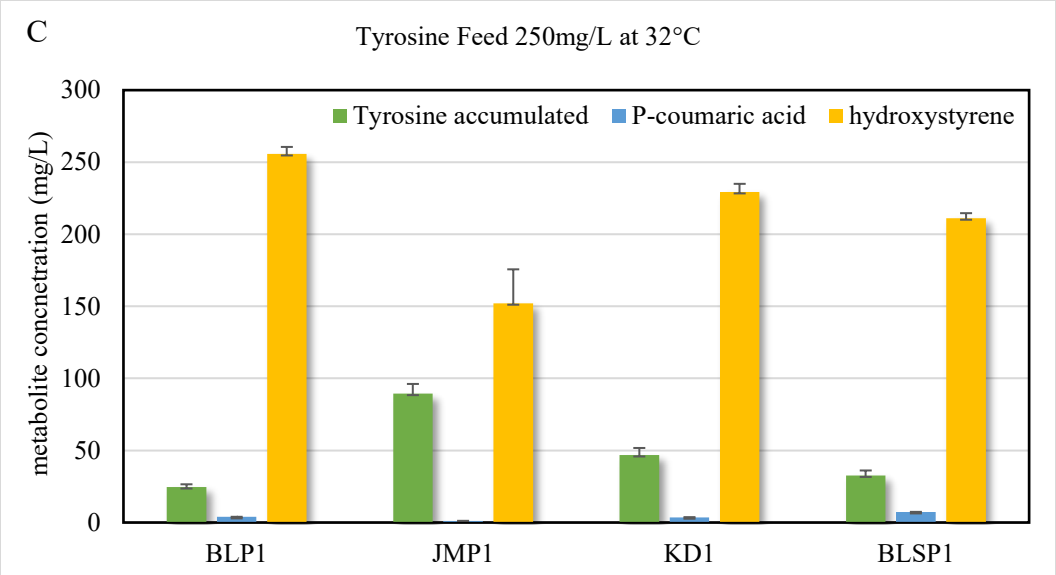
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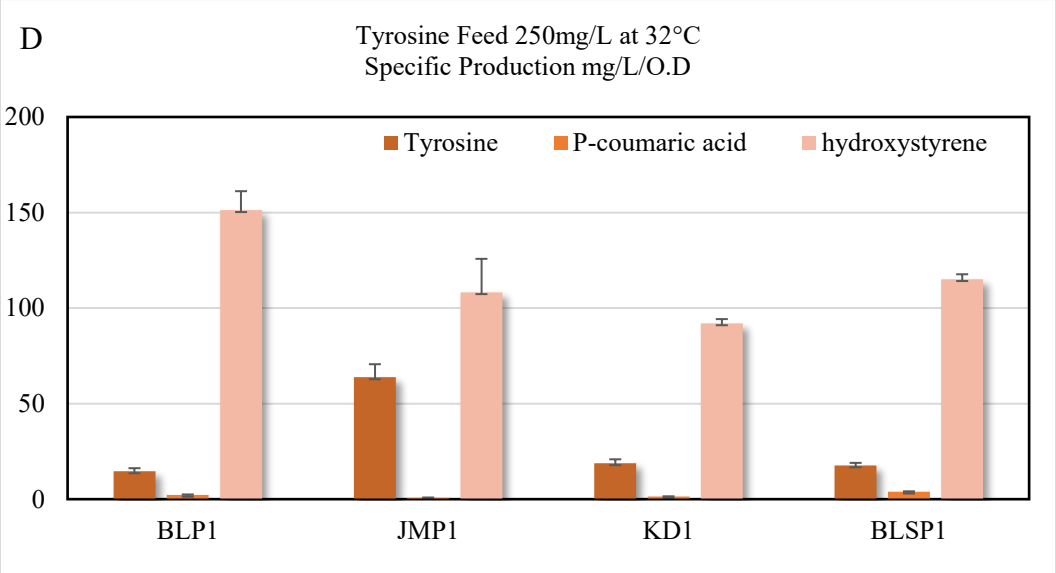
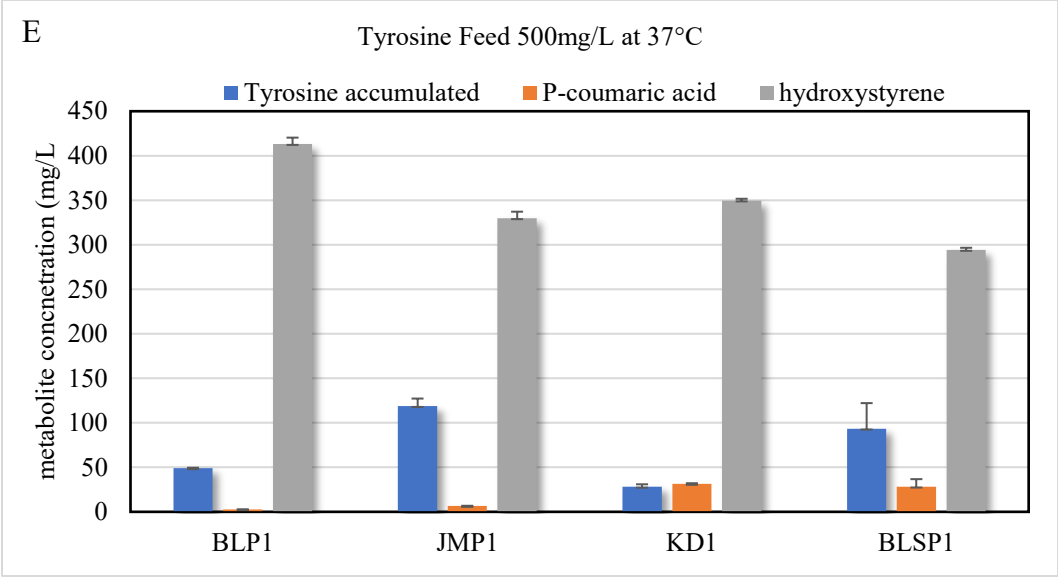
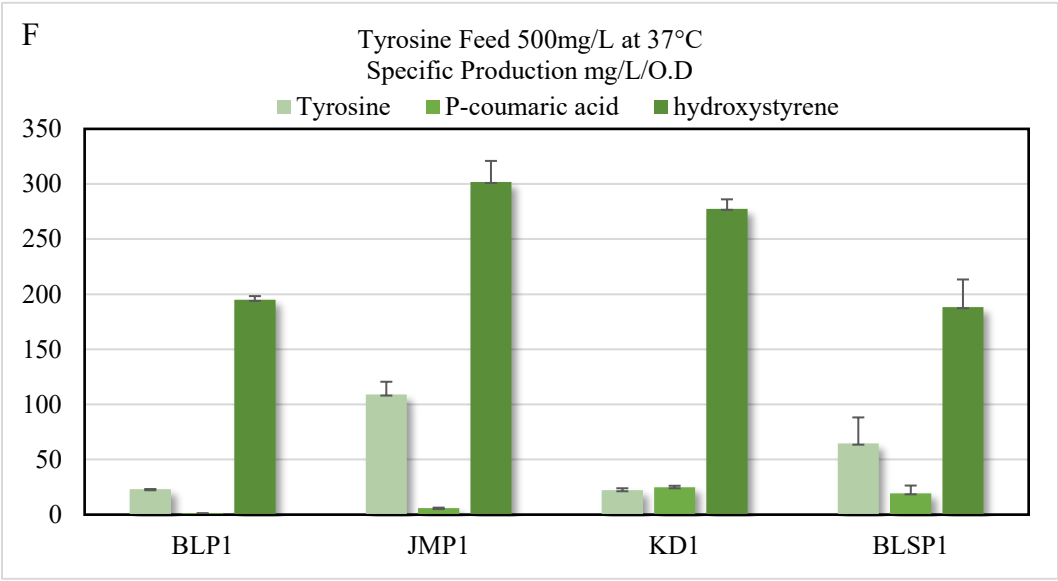

D
Tyrosine Feed 250mg/L at 32°C
Specific Production mg/L/O.D
200
150
100
50
0
Tyrosine
P-coumaric acid
hydroxystyrene
BLP1
JMP1
KD1
BLSP1

E


Tyrosine Feed 500mg/L at 37°C
Tyrosine accumulated
P-coumaric acid
hydroxystyrene
metabolite concnetration (mg/L
450
400
350
300
250
200
150
100
50
0
BLP1
JMP1
KD1
BLSP1

F


Tyrosine Feed 500mg/L at 37°C
Specific Production mg/L/O.D
Tyrosine
P-coumaric acid
hydroxystyrene
350
300
250
200
150
100
50
0
BLP1
JMP1
KD1
BLSP1

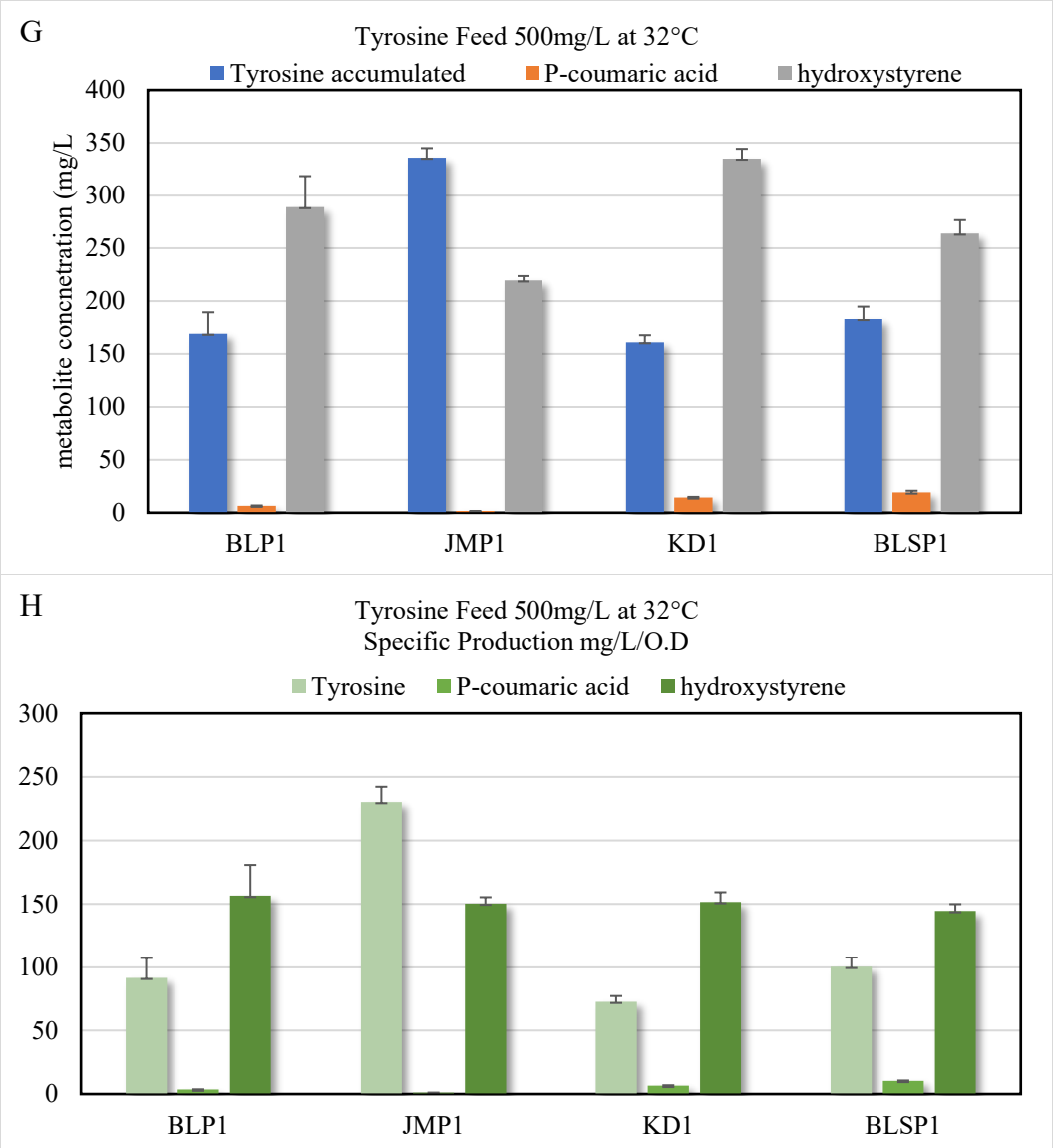


Figure 5: Conversion of tyrosine to 4-hydroxystyrene at 37 °C and 32 °C. Cultures were grown on MY1 medium with addition of 250 mg/L and 500 mg/L tyrosine. The error bars represent the standard error of at least three biological replicates. Metabolite concentration mg/L and its specific production mg/L/OD is as follows

A and **B)** Tyrosine feed 250mg/L at 37°C, **C** and **D**) Tyrosine feed 250mg/L at 32°C
E and **F**) Tyrosine feed 500mg/L at 37°C, and **G** and **H**) Tyrosine feed 500mg/L at 32°C

Two-way ANOVA analysis was performed for each feed at Factor A: two different temperatures 32°C and 37°C and Factor B: Four *E. coli* strains for hydroxystyrene production mg/L. Based on the results, at 250mg/L tyrosine feed experiment it was found

that results are statistically significant and means of observations grouped by factor A and B are not same. But it was found that there is no interaction between the Factor A and B at low level of tyrosine feed. At 500mg/L tyrosine feed the Two-way ANOVA results show that all the null hypothesis is rejected, and results are statistically significant with interaction between the factors A and B. This matches with the observation that for high tyrosine content role of temperature is significant. Therefore, the further feed experiments were conducted at 37°C at 500mg/L using BLP1 and KD1.

The BLP1 and KD1 strain with two plasmids was transformed to BLP2 and KD2 respectively, to contain a third plasmid pARFP with chloramphenicol resistance so that all the plasmids have the same antibiotic resistance as the upstream strain P8.

From Figure 6, the best strain BLP2 was derived from BL21(DE3) produced 244.9 mg/L 4-hydroxystyrene from 495.7 mg/L tyrosine after 48 h. The tyrosine remaining in the medium was 113mg/L out of 500mg/L; that is to say, 387 mg/L of tyrosine is converted to P-coumaric acid 9.3mg/L and 245mg/L of 4-hydroxystyrene. Total conversion of 387mg/L tyrosine should give 256mg/L 4-hydroxystyrene, but it was observed that 11.5 mg/L tyrosine was converted to 9.3mg/L P-coumaric acid, and 375mg/L is translated to 245mg/L 4-hydroxystyrene. This calculation is agreeable with the theoretically expected value. Since BL21(DE3) is a popularly used strain for heterologous protein expression, it is not surprising that it can best support the 4-hydroxystyrene production in vivo. Additionally, the bioconversion efficiency was higher at 37 °C, which indicates that the temperature played an important role in the bioconversion. Also, the optimal temperature was also in good agreement with the previous study on 4-hydroxystyrene biosynthesis in *E. coli* (Kang

et al., 2015). According to the bioconversion analysis above, strain BLP2 was used as the tyrosine conversion strain for 4-hydroxystyrene biosynthesis.

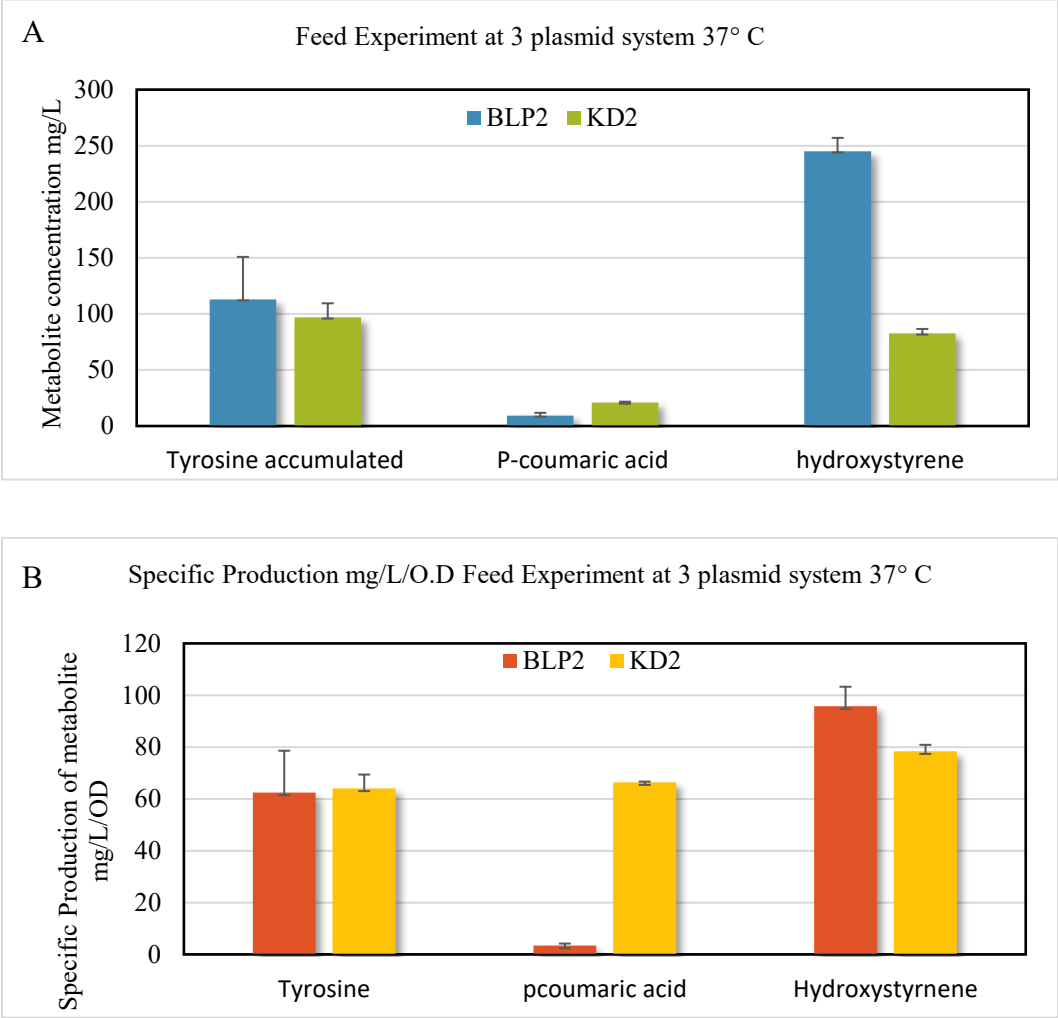
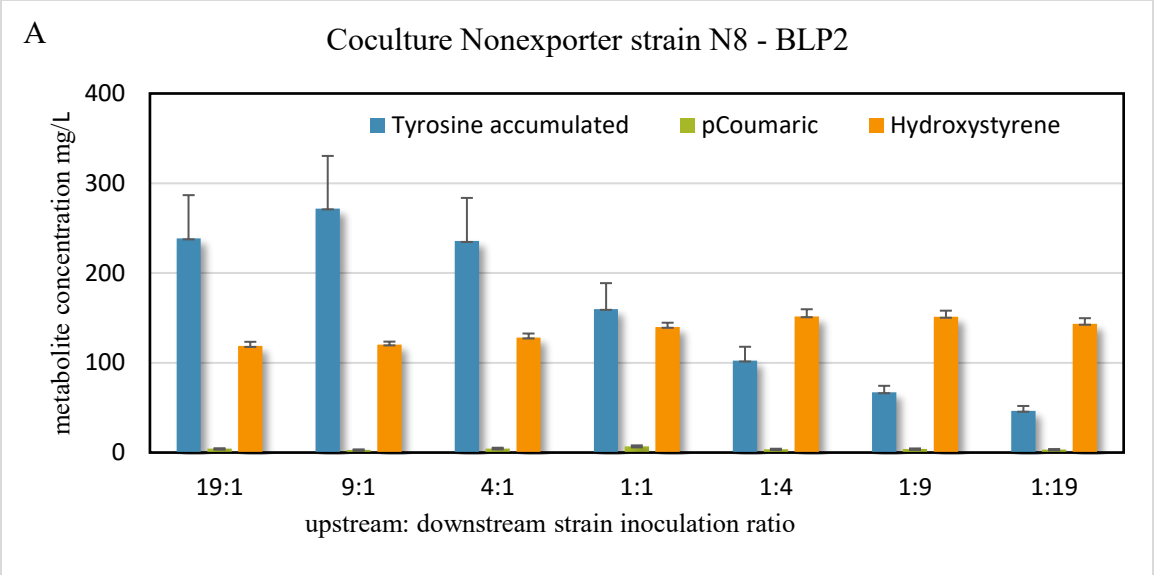


Figure 6:Tyrosine feed experiment 3 plasmid system 37° C BLP2 and KD2 strain A) Metabolite concentration mg/L B) Specific production of metabolite mg/L/O.D. Error bars represent standard error of at least 9 biological replicates.

3.3 Establishing the co-culture system for de novo 4-hydroxystyrene biosynthesis

To use modular co-culture engineering for de novo 4-hydroxystyrene from glucose, as illustrated in Fig. 1. *E. coli* N8 and BLP2 were recruited to establish a co-culture system without using the tyrosine exporter. As shown in Figure 7, the biosynthesis performance

was strongly dependent on the co-culture strains' inoculation ratio. Overly high or low ratio led to an imbalance between the biosynthetic pathway modules and in turn reduced the biosynthesis. The highest 4-hydroxystyrene production of 152 mg/L was achieved at the ratio of 1:4. As more downstream strains was used in the inoculum, the relative strengths for tyrosine conversion and provision were changed and tyrosine accumulation was decreased. In the meantime, we only observed minimal accumulation of p-coumaric acid. On the other hand, when *E. coli* P8 harboring the tyrosine exporter was used as the upstream strain, the co-cultures biosynthesis performance was improved significantly. The tyrosine accumulation was significantly increased for all tested inoculation ratios, clearly showing that the exporter's use greatly enhanced the tyrosine production as expected. Also, at the optimal inoculation ratio of 1:4, the 4-hydroxystyrene production reached 298 mg/L (approx. 300mg/L), 96% higher than the control co-culture without the exporter. Based on Two-way ANOVA results between factor A: type of strain non-exporter or exporter and factor B: different inoculation ratios, with 9 replicates each, for all Factor A and Factor B and their interaction, it was found that $P<0.05$ and $F>F_{crit}$, which means the results are statistically significant and the Null hypothesis is rejected. This demonstrates that the exporter's incorporation into the co-culture improved the tyrosine provision and the overall 4-hydroxystyrene production. In the meantime, there was minimal accumulation of the other pathway intermediate *p*-coumaric acid. Notably, the de novo production yield of 0.06 g/g glucose achieved in this study is higher than the previous studies (Kang et al., 2015; Noda et al., 2015; Qi et al., 2007; Verhoef et al., 2009).



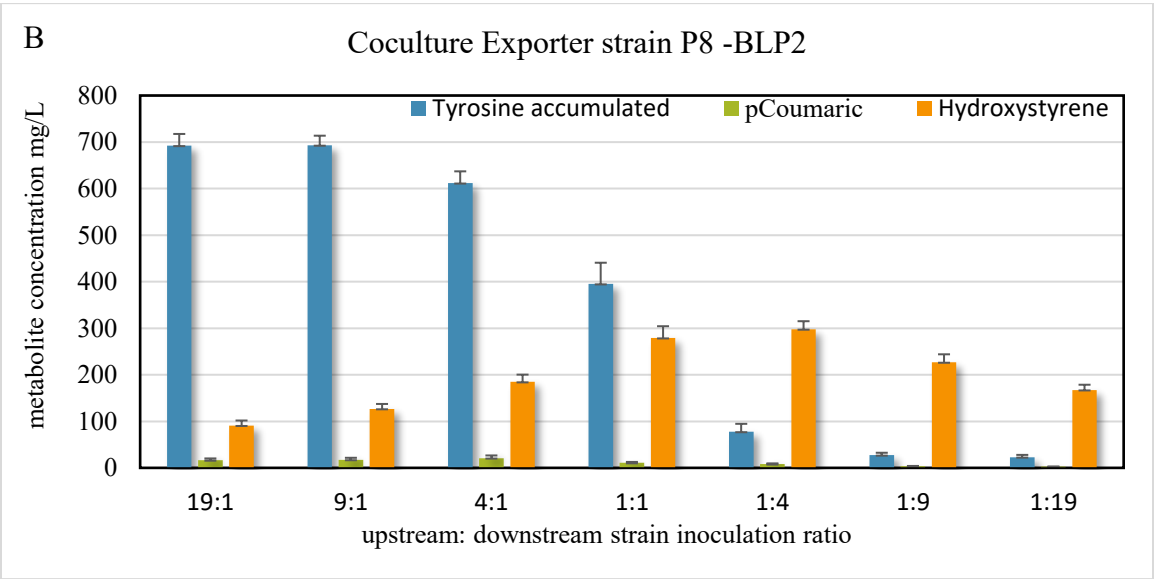


Figure 7: De novo 4-hydroxystyrene biosynthesis using (A) the N8:BLP2 co-culture without the PhpCAT exporter and (B) the P8:BLP2 co-culture with the PhpCAT exporter. The error bars represent the standard error of at least nine biological replicates

Table 2: Two-way ANOVA for co-culture between exporter and non-exporter with different inoculation ratios for 4-hydroxystyrene production mg/L

Anova: Two-Factor for hydroxystyrene production mg/L								
SUMMARY	19.1	9.1	4.1	1.1	1.4	1.9	1.19	Total
Non exporter								

Count	9	9	9	9	9	9	9	63
Sum	1068.039	1083.164	1153.111	1260.028	1365.786	1360.603	1290.436	8581.167
Average	118.671	120.3516	128.1234	140.0031	151.754	151.1781	143.3817	136.209
Variance	197.5914	93.51267	180.2315	194.5742	561.3115	427.4431	358.5361	427.571
Exporter								
Count	9	9	9	9	9	9	9	63
Sum	819.034	1141.1	1662.101	2511.679	2680.755	2041.322	1506.072	12362.06
Average	91.00378	126.7889	184.6779	279.0754	297.8617	226.8135	167.3414	196.2232
Variance	1038.757	991.3649	2200.067	5731.036	2673.2	2651.943	1147.324	7199.72
Total								
Count	18	18	18	18	18	18	18	
Sum	1887.073	2224.264	2815.212	3771.706	4046.541	3401.925	2796.508	
Average	104.8374	123.5702	156.4007	209.5392	224.8078	188.9958	155.3616	
Variance	784.437	521.4996	1966.779	7908.229	7172.917	2963.432	860.5981	

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	113453.8	1	113453.8	86.10409	1.52E-15	3.925834
Columns	209321.6	6	34886.94	26.47693	1.94E-19	2.180564
Interaction	115995.2	6	19332.54	14.67215	2.63E-12	2.180564
Within	147575.1	112	1317.635			
Total	586345.8	125				

3.4 Monoculture for the 4-Hydroxystyrene biosynthesis and comparison using exporter and non-exporter

Based on the constructed tyrosine-producing strains, we established the entire 4-hydroxystyrene pathway in *E. coli*. To this end, the codon-optimized TAL and FDC1 genes were introduced into *E. coli* N8 and P8, respectively. The biosynthesis performance of the resulting strains N8M and P8M, including the intracellular and total concentration of the pathway metabolites, is characterized. From the Figure 8 for monoculture under the same cultivation conditions, the strain N8M, one with the sensor but no exporter, produced 167.4 mg/L Total 4-hydroxystyrene, specific Hydroxystyrene of 116.6 mg/L/O.D. and 545 mg/L total tyrosine accumulation with specific tyrosine 395 mg/L/O.D. The strain P8M has both

sensor and exporter that exports tyrosine outside the cell, produced 124 mg/L 4-hydroxystyrene, specific Hydroxystyrene of 55 mg/L/O.D. and 648.2 mg/L tyrosine accumulated with specific tyrosine 287 mg/L/O.D. P8M accumulates more tyrosine compared to N8M. However, the use of the tyrosine exporter in P8M reduced the 4-hydroxystyrene biosynthesis by 24%, although the tyrosine accumulation was much higher. Some intracellular tyrosine was found to be accumulated but almost no intracellular 4-hydroxystyrene and p-Coumaric acid remained inside the cell. The efficiency of conversion of tyrosine to 4-hydroxystyrene is limited mostly by metabolic stress for monoculture strains.

It is also noteworthy that tyrosine exporter's use in the context of the monoculture is highly challenging. For the monoculture, tyrosine's secretion can reduce its accessibility to the downstream pathway enzymes in the cytosol for further conversion. The 4-hydroxystyrene production confirmed this by the monoculture strain constructed to harbor the PhpCAT exporter. Also, it has been found that using the biosensor-assisted cell selection strategy for improving the intermediate biosynthesis accumulation can reduce the production of the final product (Guo et al., 2019; X. Wang et al., 2020; Xiao et al., 2016). The results hereby show that the utilization of the pathway intermediate exporter is not an ideal strategy for mono-culture-based biosynthesis.

Importantly, the co-cultures' optimal 4-hydroxystyrene biosynthesis with the exporter was consistently higher than all the monoculture strains cultivated under the same conditions. It is, therefore, clearly demonstrated that modular co-culture engineering is an effective approach with outstanding advantages for microbial biosynthesis.

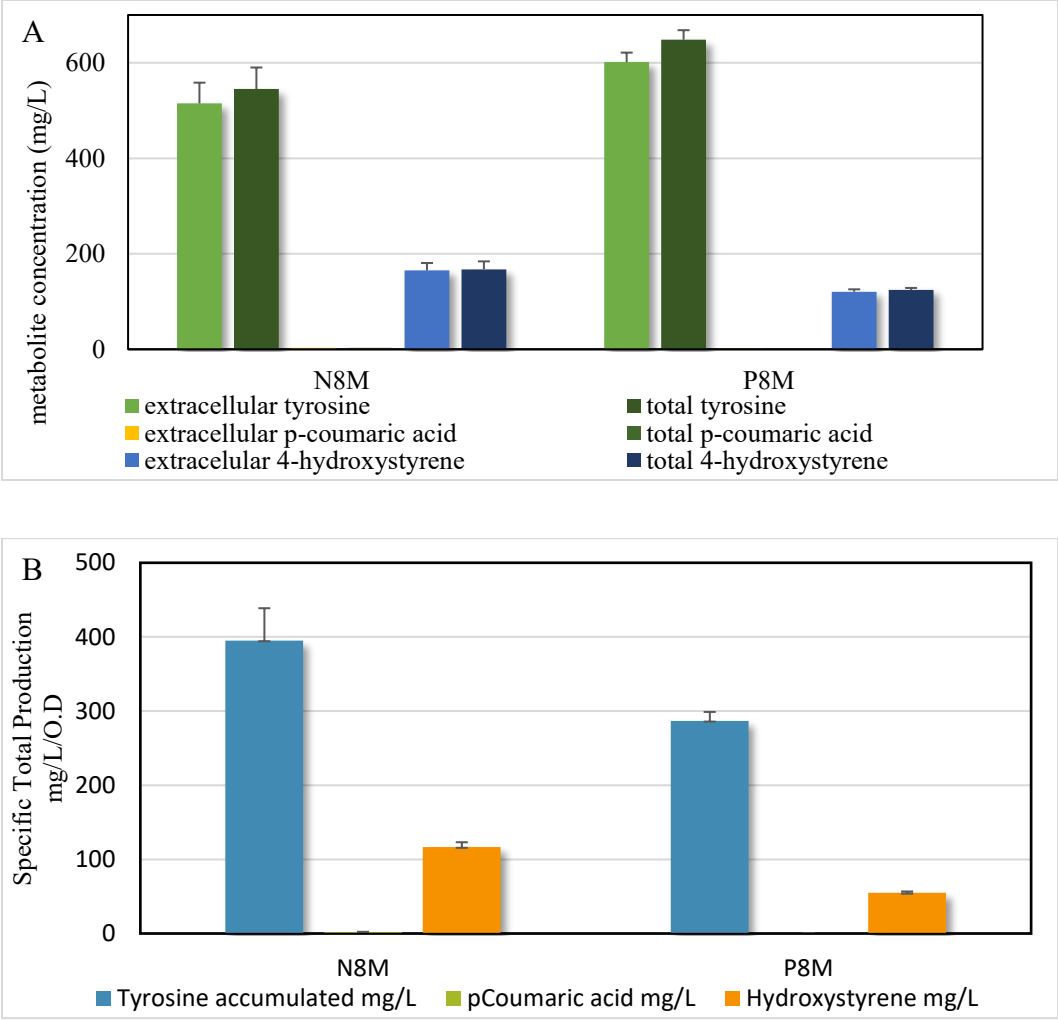


Figure 8: De novo 4-hydroxystyrene biosynthesis using E. coli strains containing the entire pathway. The error bars represent the standard error of at least three biological replicates. Monoculture strains N8M and P8M for producing 4-hydroxystyrene from glucose. A) Total 4-hydroxystyrene produced mg/L B) Specific production of Tyrosine, p-coumaric acid and 4-hydroxystyrene mg/L/O.D.

4. Conclusion

In this study, tyrosine exporter PhpCAT is utilized to facilitate the cross-membrane secretion of tyrosine. Such a strategy effectively stimulated the biosensor-assisted cell selection system for over-producing tyrosine in *E. coli*. In the meantime, tyrosine secretion helped the material transfer between co-culture strains for streamlined glucose conversion to 4-hydroxystyrene via tyrosine. Based on these two effects, the microbial co-culture harboring the exporter system showed enhanced biosynthesis performance. In principle, the same strategy can be adopted for the biosynthesis of other bioproducts using other aromatic amino acids as the pathway intermediate. More broadly, this study's accomplishment paves the way for future application of other exporter proteins in junction with biosensor techniques and modular co-culture engineering approach for microbial biosynthesis enhancement.

On the other hand, 4-hydroxystyrene biosynthesis performance is outstanding compared with previous reports. So far, the production yield of 0.076 g/g is the highest yield reported so far. However, to suit the need for large-scale bioproduction, the cultivation of the developed microbial co-culture system will need to be investigated to improve the final product concentration, which can be future research focus. Nevertheless, this work's findings demonstrate that the combination of exporter engineering and biosensing techniques is an up-and-coming and powerful tool for boosting the performance of rationally designed microbial co-cultures, which will find broader applications in the future.

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