RUTGERS
THE STATE UNIVERSITY OF NEW JERSEY

SOAR | Scholarly Open Access at Rutgers

SOAR showcases Rutgers scholarship and makes it freely accessible to the world

Establishing microbial co-cultures for 3-hydroxybenzoic acid biosynthesis on glycerol

Rutgers University has made this article freely available. Please share how this access benefits you.
Your story matters. [https://rucore.libraries.rutgers.edu/rutgers-lib/59997/story/]

This work is an **ACCEPTED MANUSCRIPT (AM)**

This is the author's manuscript for a work that has been accepted for publication. Changes resulting from the publishing process, such as copyediting, final layout, and pagination, may not be reflected in this document. The publisher takes permanent responsibility for the work. Content and layout follow publisher's submission requirements.

Citation for this version and the definitive version are shown below.

Citation to Publisher Version: Zhou, Yiyao, Li, Zhenghong, Wang, Xiaonan & Zhang, Haoran. Establishing microbial co-cultures for 3-hydroxybenzoic acid biosynthesis on glycerol. *Engineering in Life Sciences*.

Citation to *this* Version: Zhou, Yiyao, Li, Zhenghong, Wang, Xiaonan & Zhang, Haoran. Establishing microbial co-cultures for 3-hydroxybenzoic acid biosynthesis on glycerol. *Engineering in Life Sciences*. Retrieved from http://dx.doi.org/doi:10.7282/t3-856k-et51.

Terms of Use: Copyright for scholarly resources published in RUcore is retained by the copyright holder. By virtue of its appearance in this open access medium, you are free to use this resource, with proper attribution, in educational and other non-commercial settings. Other uses, such as reproduction or republication, may require the permission of the copyright holder.

Article begins on next page

SOAR is a service of RUcore, the Rutgers University Community Repository
RUcore is developed and maintained by Rutgers University Libraries

Short communication

Establishing microbial co-cultures for 3-hydroxybenzoic acid biosynthesis on glycerol

Yiyao Zhou, Zhenghong Li, Xiaonan Wang, Haoran Zhang*

Department of Chemical and Biochemical Engineering

Rutgers, the State University of New Jersey

98 Brett Rd, Room Piscataway, NJ 08854

* corresponding author. Haoran.Zhang@Rutgers.edu

Abstract

Converting renewable feedstocks to aromatic compounds using engineered microbes offers a robust approach for sustainable, environment-friendly and cost-effective production of these value-added products without the reliance on petroleum. In this study, rationally designed *E. coli*-*E. coli* co-culture systems were established for converting glycerol to 3-hydroxybenzoic acid (3HB). Specifically, the 3HB pathway was modularized and accommodated by two metabolically engineered *E. coli* strains. The co-culture biosynthesis was optimized by using different cultivation temperatures, varying the inoculum ratio between the co-culture strains, recruitment of a key pathway intermediate transporter, strengthening the critical pathway enzyme expression, and adjusting the timing for inducing pathway gene expression. Compared with the *E. coli* mono-culture, the optimized co-culture showed 5.3-fold improvement for 3HB biosynthesis. This study demonstrated the applicability of modular co-culture engineering for addressing the challenges of aromatic compound biosynthesis.

Keywords

Modular co-culture engineering, Microbial biosynthesis, 3-hydroxybenzoic acid, *E. coli*

Introduction

Production of aromatic compounds at present is mainly based on utilization of petroleum resources, which often brings about economic, environmental and sustainability concerns. Microbial biosynthesis has been developed as a viable method for converting renewable feedstocks to a wealth of aromatic products, offering an alternative approach for effective production and thus reducing the reliance on the use of petroleum. To date, significant success has been achieved for microbial biosynthesis of various aromatic compounds [1-4]. On the other hand, novel engineering approaches, such as modular co-culture engineering, have recently been developed for improving microbial biosynthesis performance [5]. Modular co-culture engineering employs microbial co-cultures composed of multiple microbial strains of the same or different species to accommodate the modularized biosynthesis pathways of the target products. This strategy allows for the pathway modularization in the context of a microbial co-culture, as each co-culture strain is only assigned with one module of the entire pathway. As a result, it facilitates the reduction of the metabolic burden on the recruited microbial strains, which has been considered to a major challenge metabolic engineering [6]. In addition, balancing of the biosynthesis strengths between individual pathway modules harbored by the corresponding co-culture strains can be achieved by straightforward manipulation of the strain inoculum ratio, offering an effective way for addressing the need of pathway balancing [5, 7]. To date, the modular co-culture engineering approach has been utilized for production of a variety of biochemicals ranging from simple biofuel molecules to complex natural products [5, 8, 9].

In the present study, we designed and constructed *E. coli-E. coli* co-cultures for producing 3-hydroxybenzoic acid (3HB) from renewable and abundant carbon substrate glycerol [10, 11]. 3HB is structurally similar to 2-hydroxybenzoic acid, the key component of aspirin. It has been found

to possess stress response desensitizing, anti-radical, and antioxidant activities [12-15]. It also has potential beneficial effect for maintaining normal blood lipid level [16]. In addition, 3HB can be used for synthesis of industrially important chemicals such as polymer, herbicide and plasticizer [17-20]. 3HB biosynthesis using the mono-cultures of engineered *E. coli* and *C. glutamicum* has been reported, both of which utilized glucose as the carbon substrate [21, 22]. For this study, we chose to use glycerol as the pathway substrate for 3HB bioproduction for a few reasons. First, glycerol is an important byproduct of the biodiesel industry and there has been increasing interest in using glycerol for microbial biosynthesis applications. To this end, 3HB bioproduction on glycerol has not been reported before. Moreover, there are relatively few studies using glycerol as the carbon substrate for co-culture engineering, which leaves extensive unexplored areas for further investigation.

The 3HB biosynthetic pathway is illustrated in Fig. 1A. Carbon substrate, such as glycerol used in this study, is first converted to 3-dehydroshikimic acid (DHS) via the central metabolism and the shikimate pathway [23]. DHS is then utilized to produce chorismic acid (CHR), which is subsequently converted to 3HB by 3-hydroxybenzoate synthase [24]. For constructing the co-cultures in this study, two *E. coli* strains were individually engineered to accommodate the upstream (Glycerol to DHS) and downstream (DHS to 3HB) pathway modules, respectively. The co-cultivation of the two strains in one consolidated culture presented a complete biosynthetic system for production of 3HB from Glycerol. The biosynthesis performance by the co-cultures was investigated and compared with the mono-culture control under different conditions to demonstrate the effectiveness of the modular co-culture engineering for 3HB biosynthesis.

Materials and Methods

87 Chemicals and medium

88 The chemicals and culture medium used in this study were purchased from Sigma-Aldrich (St.
89 Louis, MO, United States) or Thermo Fisher Scientific (Waltham, MA, United States). The DNA
90 cloning agents, including Q5 High-Fidelity DNA polymerase, restriction enzymes, T4 ligase, were
91 obtained from New England Biolabs (Ipswich, MA, United States). PCR primers were obtained
92 from Sigma-Aldrich (St. Louis, MO, United States). LB medium was used for all DNA cloning
93 manipulations. The 3HB biosynthesis was conducted using a glycerol medium. 1 L glycerol
94 medium contained 5 g glycerol, 2 g of NH_4Cl, 5 g $(NH4)_2SO_4$, 3 g KH_2PO_4, 7.3 g K_2HPO_4, 8.4 g
95 3-(N-morpholino) propanesulfonic acid (MOPS), 0.5 g NaCl, 0.24 g $MgSO_4$, 0.5 g yeast extract,
96 40 mg of tyrosine, 40 mg of tryptophan, 100 mg phenylalanine and trace elements (0.4 mg
97 Na_2EDTA, 0.03 mg H_3BO_3, 1 mg thiamine, 0.94 mg $ZnCl_2$, 0.5 mg $CoCl_2$, 0.38 mg $CuCl_2$, 1.6 mg
98 $MnCl_2$, 3.77 mg $CaCl_2$, and 3.6 mg $FeCl_2$). When needed, 50 mg/L kanamycin, 34 mg/L
99 chloramphenicol, and 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) were supplemented
100 into the medium.

101 **Table 1** Plasmids and strains used in this study

Plasmids	Description	Source
pET28a	T7 promoter, Kan^R	Novagen
pACYCDuet-1	two T7 promoters, Cm^R	Novagen
pET21c	T7 promoter, Amp^R	Novagen
pYZ1	pUC57(Kan) carrying *E. coli aroE, aroL, aroA, aroC* and codon optimized *S. hygroscopicus hyg5* genes under the control of the constitutive *Zymomonas mobilis* pyruvate decarboxylase promoter (Ppdc)	This study
pYZ2	pET21c carrying codon optimized *S. hygroscopicus hyg5* gene	This study

pCS5	pACYCDuet-1 carrying the *E. coli aroE, aroL, aroA* and *aroC* genes, as well as the *shiA* gene that is under the control of a constitutive lacuv5 promoter	[25]

Strains	Description	Source
BL21(DE3)	F^- ompT $hsdS_B$ (r_B^-, m_B^-) gal dcm (DE3)	Invitrogen
P5	*E. coli* K12 *ΔpheA ΔtyrR lacZ*::PLtetO-1-*tyrA*^{fbr} *aroG*^{fbr} *tyrR*::PLtetO-1-*tyrA*^{fbr} *aroG*^{fbr} *hisH*(L82R) *ΔaroE ΔydiB*	[25]
BH2	*E. coli* BL21(DE3) Δ*xylA* Δ*tyrA* Δ*pheA*	[25]
PUN1	P5 harboring pET28a	This study
PUN2	P5 harboring pET28a and pACYCDuet-1	This study
PUN3	P5 harboring pET21c and pACYCDuet-1	This study
PMH1	P5 harboring pYZ1	This study
PMH2	P5 harboring pYZ1 and pCS5	This study
BDZ1	BH2 harboring pYZ1	This study
BDZ2	BH2 harboring pYZ1 and pCS5	This study
BDZ4	BH2 harboring pYZ2 and pCS5	This study

Plasmid and strain construction

The plasmids and strains used in this study are listed in Table 1. All plasmids were constructed using *E. coli* NEB5-alpha high-efficiency competent cell (New England Biolabs, Ipswich, MA, United States). Commercial kits (Zymo research, Irvine, CA, United States) were used for DNA purification, gel recovery and miniprep. The *hyg5* gene encoding 3-hydroxybenzoate synthase was codon-optimized and synthesized by Bio Basic Inc (Amherst, NY, United States). To construct plasmid pYZ1, the *hyg5* gene was PCR amplified using primers pHyg5-up (CGGTCGACTTAAGGAGGAACAGACATGTTGAACCCGTCTAGC) and pHyg5-down (ATGCGGCCGCCTAGTTTACATAACAACACCTTCA), then cloned into a previously constructed plasmid pBA5 (pUC57-Kan carrying *E. coli aroE, aroL, aroA, aroC* and *ubiC* genes,

unpublished data) to replace the *ubiC* gene using SalI/NotI restriction sites. Similarly, the PCR amplified *hyg5* gene was inserted into plasmid pET21c using SalI/NotI restriction sites to generate plasmid pYZ2.

3HB biosynthesis by engineered mono-cultures and co-cultures

For monoculture biosynthesis, *E. coli* PMH1 and PMH2 were initially grown overnight in the LB medium at 275 rpm under 37 ℃. The cell culture was then centrifuged at 3500 rpm for 5 min, and the cell pellets were re-suspended in 5mL glycerol medium to reach an initial OD of 0.5. The inoculated culture was grown at 250 rpm under 30 ℃ for 72h. When needed, 0.1 mM IPTG was added at the beginning of the cultivation.

For *E. coli*-*E. coli* co-culture biosynthesis, *E. coli* PUN1, PUN2, PUN3, BDZ1, BDZ2 and BDZ4 were cultivated separately in the LB medium. The overnight cultures were centrifuged and re-suspended in the fresh glycerol medium in desired combinations (PUN1 and BDZ1, PUN2 and BDZ2, PUN3 and BDZ4). The total initial OD_{600} of the co-cultures was set to be 0.5, although the inoculum ratio between the upstream and downstream strain was varied. Except the PUN1:BDZ1 co-culture, 0.1 mM IPTG was added at the beginning of the cultivation. For the IPTG induction time point experiment, 0.1 mM IPTG was added to the PUN3:BDZ4 co-culture at 0, 2, 4, 6 and 8 h after the co-culture inoculation.

Analytical method for 3HB quantification

To quantify 3HB, 1 mL culture samples were centrifuged at 13,000 rpm for 5 min. The supernatant was then filtered through 0.45 μm PVDF syringe filter (Whatman Inc., NJ, United States). The filtered samples were analyzed by Agilent HPLC equipped with a Waters C18 column HyperSelect ODS 5μ 150 mm X 4.6 mm (Milford, MA , United States) and a UV detector set to a wavelength of 293 nm. The following water-acetonitrile gradient elution program was used at a flow rate of 0.5 mL/min: 0 min, 25% acetonitrile; 4 min, 50% acetonitrile; 6 min, 99% acetonitrile; 8 min, 25% acetonitrile; 10 min, 25% acetonitrile.

Results and Discussion

In this study, we used glycerol, an important byproduct of the biodiesel industry and renewable raw material, as the carbon substrate for biosynthesis of valuable compound 3HB. In order to construct the first co-culture system, two *E. coli* strains were utilized for harboring the upstream and downstream 3HB pathway modules, respectively, as shown in Fig. 1A. The upstream strain PUN1 was derived from a previously constructed strain for over-production of metabolite dehydroshikimate (DHS) [25, 26]. Notably, the genes *aroE* and *ydiB* involved in the competing pathway for DHS were deleted to improve the DHS availability. The downstream strain was engineered for establishing the DHS-to-3HB conversion. Specifically, plasmid pYZ1 was constructed to over-express *E. coli*'s *aroE*, *aroL*, *aroA*, *aroC* genes for enhancing the formation of CHR from DHS. In addition, this plasmid contained the codon-optimized *hyg5* gene encoding *S. hygroscopicus* 3-hydroxybenzoate synthase for converting CHR to 3HB [22, 24]. Strain BDZ1 containing plasmid pYZ1 was used as the downstream stream for the co-culture.

The PUN1:BDZ1 co-culture was then grown on 5 g/L glycerol for the 3HB production. Two strains were inoculated at a ratio of 1:1. Strain PMH1 over-expressing the selected shikimate pathway genes and 3HB synthase gene *hyg5* was employed as the mono-culture control. The mono-culture and co-culture were cultivated at three different temperatures, including 25, 30 and 37 ^{o}C, for 72h before subjecting to HPLC analysis. As shown in Fig. 1B, the change of the cultivation temperature caused significant biosynthesis performance variation. For all temperatures, the engineered co-culture showed higher production than the mono-culture. For both mono-culture and co-culture, the optimal 3HB production was achieved at 30 ^{o}C. At this temperature, the co-culture produced 74 mg/L 3HB and the mono-culture produced 55 mg/L 3HB. It was therefore indicated that 30 ^{o}C was the best temperature for meeting the need of cell growth and heterologous 3HB biosynthesis, and this temperature was thus employed for all the following experiments. To our knowledge, this is the first report utilizing glycerol as the carbon substrate for biosynthesis of value-added 3HB using the engineered *E. coli* mono-culture and co-culture.

Next, the 3HB biosynthesis by the PUN1:BDZ1 co-culture was optimized by changing the inoculum ratio between the upstream and downstream strains, which helped to balance the biosynthetic strengths of the corresponding pathway modules carried by these strains. As shown in Fig. 2A, when the downstream BDZ1 strain was inoculated at low ratios (19:1, 9:1 4:1), the DHS-to-3HB conversion and 3HB production were relatively low. With the increase of the BDZ1 inoculum, the 3HB concentration gradually increased. The highest production of 99 mg/L was achieved at the PUN1:BDZ1 ratio of 1:19. These results indicated that the DHS conversion in the downstream strain was the limiting step for the overall 3HB biosynthesis. The specific 3HB production, defined by 3HB concentration over the culture cell density, showed similar trend with the inoculum ratio change. Notably, the 3HB concentration and specific production of the mono-

culture control PMH1 were merely 55 mg/L and 17.2 mg/L/OD, respectively. The 3HB biosynthesis improvement by the co-culture could be largely due to (1) reduction of metabolic burden on each strain improved their fitness and biosynthetic capabilities and (2) flexible increase of the downstream strain's sub-population strengthened the DHS-to-3HB conversion. Although the DHS-to-3HB conversion can also be enhanced in the context of mono-culture, it requires laborious optimization of the gene expression promoter, ribosomal binding site, plasmid copy number, etc. in a trial-and-error manner. In contrast, the co-culture engineering approach offers a new perspective to circumvent these technical barriers for pathway balancing, and thus holds considerably potentials for wider application in the broad field of microbial biosynthesis.

To further improve 3HB biosynthesis, a new co-culture PUN2:BDZ2 was constructed in which the *E. coli* membrane-bound transporter ShiA of the pathway intermediate DHS was employed to strengthen the DHS assimilation by the downstream strain. In fact, pathway intermediate mass transfer between the co-culture strains is a common challenge for modular co-culture engineering. The recruited ShiA transporter has been found to facilitate the importation of extracellular DHS into the *E. coli* cell and has been used for microbial biosynthesis of different compounds [25-27]. For this study, the gene *shiA* encoding the transporter was over-expressed using plasmid pCS5 in the downstream strain. pCS5 also carried additional copies of *aroE*, *aroL*, *aroA*, *aorC* genes for attracting stronger metabolic flux towards CHR formation. Notably, due to the modular nature of the co-culture system, such metabolic engineering modification in the downstream strain did not interfere with the upstream strain's biosynthesis capability, highlighting the advantage of the modular co-culture engineering. As shown in Fig. 2B, the 3HB concentration and specific production of the PUN2:BDZ2 co-culture were increased dramatically when the inoculum ratio was changed from 19:1 to 1:4, suggesting that the enhancement of the downstream

pathway module for DHS-to-3HB conversion was critical for the overall bioproduction performance. No significant production improvement was observed when inoculum ratio was further changed to 1:9 and 1:19. 137 mg/L 3HB was produced at the inoculum ratio of 1:9, which was 38 % higher than the optimal production by the PUN1:BDZ1 co-culture. Interestingly, although the ShiA over-expression was also expected to take up the secreted DHS for biosynthesis enhancement for mono-culture, the mono-culture control strain PMH2 engineered using the same strategy did not show any biosynthesis improvement over PMH1 (55 vs 52 mg/L). Therefore, it was demonstrated that the same engineering strategy could result in different outcomes in mono-cultures and co-cultures.

Next, we further modified the co-culture design by adapting a dedicated plasmid for expressing 3-hydroxybenzoate synthase Hyg5 using the strong T7 promoter, rather than the mild constitutive promoter Ppdc. Also, other downstream pathway genes (*aroE*, *aroL*, *aroA*, *aorC*) were solely expressed by plasmid pCS5.The correspondingly constructed strains PUN3 and BDZ4 showed slightly compromised DHS production and improved DHS uptake capabilities, respectively (Fig. S1). Also, these strains' glycerol uptake rates did not show significant difference from the co-culture strains constructed early on (Fig. S2). The PUN3:BDZ4 co-culture was then grown in the glycerol medium for 3HB biosynthesis. As shown in Fig. 2C, the 3HB concentration and specific production both varied depending on the inoculum ratio between PUN3 and BDZ4. Importantly, the 3HB concentrations at all tested inoculum ratios were increased to different levels, compared with the production by the PUN2:BDZ2 co-culture. The biosynthesis improvement hereby indicated that improving the expression strength of the key pathway enzyme Hyg5 played a critical role in strengthening the overall biosynthesis performance. In the PUN3:BDZ4 co-culture, this strategy resulted in 229 mg/L 3HB production after the inoculum ratio optimization, which

was significantly higher than the previous mono-cultures and co-cultures. Notably, compared with the previous co-cultures, the optimal inoculum ratio of PUN3:BDZ4 was shifted to 1:1. This finding showed that the modification of the pathway genes expression strategy altered the relative biosynthetic capabilities of the corresponding co-culture strains and thus changed the optimal strain-to-strain ratio for 3HB bioproduction. Compared with the inoculum ratio, the endpoint strain-to-strain ratios showed dramatic changes at the end of the co-culture cultivation (Table S1). Interestingly, the observed endpoint ratios converged to the range between 1.2:1 and 0.2:1, which is much smaller than the inoculum ratio range. This finding suggests that the co-culture strains' growth competitions under different starting situations eventually stabilized at a similar level.

We then investigated the influence of IPTG induction timing on 3HB production using the PUN3:BDZ4 co-culture inoculated at the ratio of 1:1. In fact, it was found that the use of IPTG to induce strong gene expression could generate a negative impact on cell growth (Fig. S3). On the other hand, although adding the inducer, e.g. IPTG, at the beginning of cultivation is a convenient and straightforward way for initiating pathway gene expression, it may not be necessarily the best strategy for maximizing the bioproduction. As shown in Fig. 2D, early induction at the beginning of the co-culture cultivation was found to be a sub-optimal strategy for 3HB biosynthesis. As the induction timing was delayed to 2 and 4 h, the 3HB concentration gradually increased. Further delay of the induction timing to 6 and 8 h resulted in reduction of the bioproduction performance. The highest production of 294 mg/L achieved at 4h induction was 5.3-fold higher than the original mono-culture strain PMH1 constructed for 3HB biosynthesis. The specific 3HB production also peaked at induction time point of 4 h. In fact, early induction of pathway genes' overexpression often results in overwhelming metabolic burden on the host cells at early growth phases and can lead to reduced growth profile and sub-optimal bioproduction performance. On the other hand,

overly late induction cannot give the host cells sufficient time to dedicate metabolic resources on target production biosynthesis. As such, varying time point of induction is an effective way for balancing the need of cell growth and biosynthesis. Overall, the achievement of our study showed significant 3HB biosynthesis improvement in a step-wise manner and demonstrated the general applicability of modular co-culture engineering for de novo biosynthesis of various biochemicals.

Practical application

The establishment of the *E. coli-E. coli* co-cultures in this study offers an innovative and effective approach for converting renewable glycerol to value-added compound 3-hydroxybenzoic acid. After several rounds of engineering efforts, the optimized co-culture system biosynthesized 294 mg/L 3HB from 5 g/L glycerol. The mass yield of 0.059 g/g was achieved in the test tube scale, showing strong potentials for further bioproduction improvement using the bioprocess engineering approaches. In addition, the constructed co-cultures can be recruited for utilization of other renewable feedstock molecules such as glucose and xylose, as *E. coli* has native capabilities for consuming these molecules as carbon substrates. More broadly, the co-culture engineering methodology developed in this work can be adapted for biosynthesis of other aromatic compounds derived from the same or similar pathways.

Acknowledge

This material is based upon work supported in part by the National Science Foundation under Grant No. 1706058. This work is also supported by startup research funds from Rutgers, The State University of New Jersey. Zhenghong Li is a recipient of CSC Ph.D. fellowship.

The authors have declared no conflict of interest.

Reference

1. Rodriguez, A., Martnez, J.A., Flores, N., Escalante, A. et al., Engineering Escherichia coli to overproduce aromatic amino acids and derived compounds. *Microbial Cell Factories.* 2014, *13(1)*, 126.
2. Bongaerts, J., Krämer, M., Müller, U., Raeven, L., Wubbolts, M., Metabolic Engineering for Microbial Production of Aromatic Amino Acids and Derived Compounds. *Metabolic engineering.* 2001, *3(4)*, 289-300.
3. Gosset, G., Production of aromatic compounds in bacteria. *Current Opinion in Biotechnology.* 2009, *20(6)*, 651-658.
4. Jiang, M., Zhang, H., Engineering the shikimate pathway for biosynthesis of molecules with pharmaceutical activities in E. coli. *Curr Opin Biotechnol.* 2016, *42*, 1-6.
5. Zhang, H., Wang, X., Modular co-culture engineering, a new approach for metabolic engineering. *Metabolic engineering.* 2016, *37* ,114-121.
6. Wu, G., Yan, Q., Jones, J.A., Tang, Y.J. et al., Metabolic Burden: Cornerstones in Synthetic Biology and Metabolic Engineering Applications. *Trends in biotechnology.* 2016, *34(8)*, 652-664.
7. Jones, J.A., Toparlak, O.D., Koffas, M.A., Metabolic pathway balancing and its role in the production of biofuels and chemicals. *Curr Opin Biotechnol.* 2015, *33*, 52-59.
8. Chen, T., Zhou, Y., Lu, Y., Zhang, H., Advances in heterologous biosynthesis of plant and fungal natural products by modular co-culture engineering. *Biotechnology letters.* 2018.
9. Jones, J.A., Wang, X., Use of bacterial co-cultures for the efficient production of chemicals. *Curr Opin Biotechnol.* 2017, *53*, 33-38.
10. Yang, F., Hanna, M.A., Sun, R., Value-added uses for crude glycerol--a byproduct of biodiesel production. *Biotechnology for Biofuels.* 2012, *5(1)*, 13.
11. Pradima, J., Kulkarni, M.R., Archna, Review on enzymatic synthesis of value added products of glycerol, a by-product derived from biodiesel production. *Resource-Efficient Technologies.* 2017, *3(4)*, 394-405.

12. Khadem, S., Marles, R.J., Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: occurrence and recent bioactivity studies. *Molecules (Basel, Switzerland).* 2010, *15(11)*, 7985-8005.
13. Sroka, Z., Cisowski, W., Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food and Chemical Toxicology.* 2003, *41(6)*, 753-758.
14. Ford, C.M., Hoj, P.B., Multiple glucosyltransferase activities in the grapevine Vitis vinifera L. *Australian Journal of Grape and Wine Research.* 1998, *4(2)*, 48-58.
15. Khan, S.A.C., Shyam, Kumar, Vikas, Potential anti-stress, anxiolytic and antidepressant like activities of mono-hydroxybenzoic acids and aspirin in rodents: A comparative study. *Austin Journal of pharmacology and therapeutics*. 2015, *3(3)*, 1073.
16. Juurlink, B.H.J., Azouz, H.J., Aldalati, A.M.Z., AlTinawi, B.M.H., Ganguly, P., Hydroxybenzoic acid isomers and the cardiovascular system. *Nutrition Journal*. 2014, *13(1)*, 63.
17. Kricheldorf, H.R., Zang, Q-Z., Schwarz, G., New polymer syntheses: 6. Linear and branched poly(3-hydroxy-benzoates). *Polymer*. 1982, *23(12)*, 1821-1829.
18. Kricheldorf, H.R., Löhden, G., New polymer syntheses, 79. Hyperbranched poly(ester-amide)s based on 3-hydroxybenzoic acid and 3,5-diaminobenzoic acid. *Macromolecular Chemistry and Physics.* 1995, *196(6)*, 1839-1854.
19. Kirk-Othmer, *Kirk-Othmer encyclopedia of chemical technology*, Wiley, 2004.
20. Franck, Heinz-Gerhard, Stadelhofer, Jürgen W., *Industrial Aromatic Chemistry: Raw Materials · Processes · Products*, Springer-Verlag Berlin Heidelberg, 2012.
21. Kallscheuer, N., Marienhagen, J., Corynebacterium glutamicum as platform for the production of hydroxybenzoic acids. *Microb Cell Fact*. 2018, *17(1)*, 70.
22. Noda, S., Shirai, T., Oyama, S., Kondo, A., Metabolic design of a platform Escherichia coli strain producing various chorismate derivatives. *Metabolic engineering.* 2016, *33*, 119-129.
23. Averesch, N.J.H., Krömer, J.O., Metabolic Engineering of the Shikimate Pathway for Production of Aromatics and Derived Compounds—Present and Future Strain Construction Strategies. *Frontiers in Bioengineering and Biotechnology*. 2018, *6(32)*.
24. Andexer, J.N., Kendrew, S.G., Nur-e-Alam, M., Lazos, O. et al., Biosynthesis of the immunosuppressants FK506, FK520, and rapamycin involves a previously undescribed

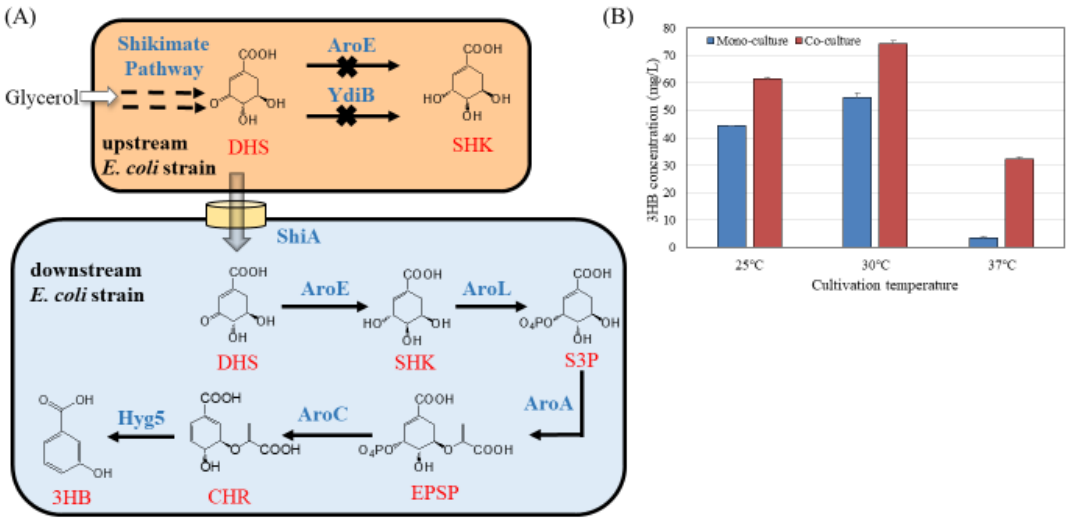
family of enzymes acting on chorismate. *Proceedings of the National Academy of Sciences of the United States of America.* 2011, *108(12)*, 4776-4781.

25. Zhang, H., Pereira, B., Li, Z., Stephanopoulos, G., Engineering Escherichia coli coculture systems for the production of biochemical products. *Proceedings of the National Academy of Sciences of the United States of America*. 2015, *112(27)*, 8266-8271.

26. Zhang, H., Li, Z., Pereira, B., Stephanopoulos, G., Engineering E. coli–E. coli cocultures for production of muconic acid from glycerol. *Microbial Cell Factories.* 2015, *14(1)*, 134.

27. Zhang, H., Stephanopoulos, G., Co-culture engineering for microbial biosynthesis of 3-amino-benzoic acid in Escherichia coli. *Biotechnology journal.* 2016, *11(7)*, 981-987.

Figure Captions



347

348 Figure 1. (A) The 3HB biosynthetic pathway and the *E. coli-E. coli* co-culture design for Glycerol-
349 to-3HB conversion. The ratio between the co-culture strains determined the relative biosynthetic
350 strengths of the upstream and downstream pathway modules. DHS: 3-dehydroshikimic acid, SHK:
351 shikimic acid, S3P: shikimate 3-phosphate, EPSP: 5-enolpyruvoyl-shikimate 3-phosphate, CHR:
352 chorismic acid, 3HB: 3-hydroxybenzoic acid. (B) Effect of the cultivation temperature on 3HB
353 biosynthesis using the engineered mono-culture PMH1 and co-culture PUN1:BDZ1. Specific 3HB
354 production is defined by 3HB concentration over the culture cell density.

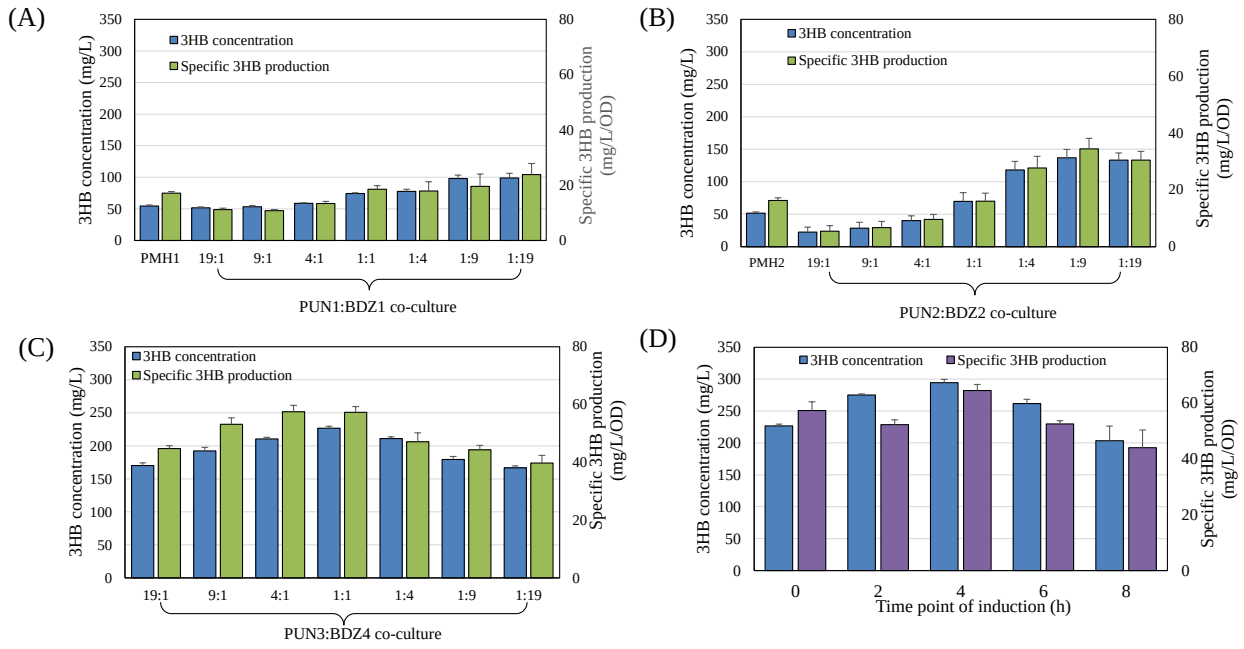


Figure 2. Optimization of the 3HB biosynthesis by employing engineered microbial co-cultures of (A) PUN1:BDZ1, (B) PUN2:BDZ2, (C) PUN3:BDZ4. *E. coli* PMH1 and PMH2 were used as the mono-culture controls. (D) The effect of IPTG induction time point on 3HB biosynthesis using the PUN3:BDZ4 co-culture inoculated at 1:1 ratio.

Short communication

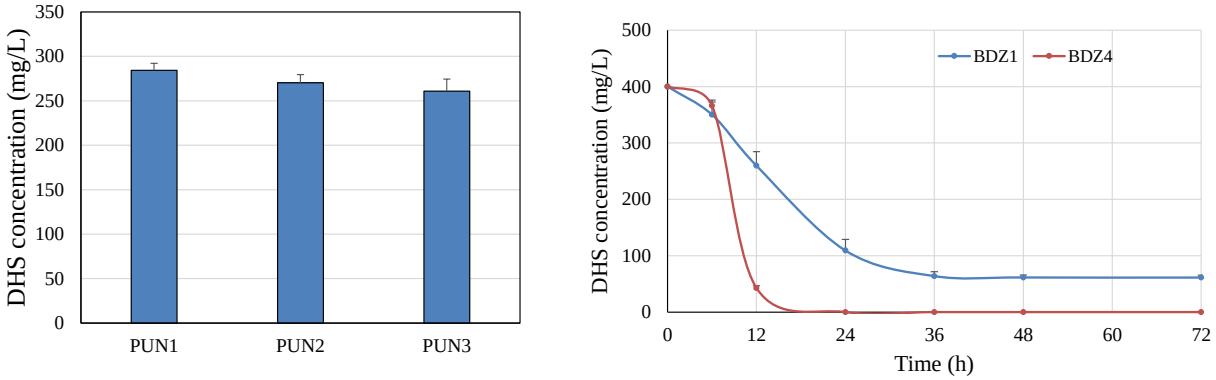
Establishing microbial co-cultures for 3-hydroxybenzoic acid biosynthesis on glycerol

Yiyao Zhou, Zhenghong Li, Xiaonan Wang, Haoran Zhang

Department of Chemical and Biochemical Engineering

Rutgers, the State University of New Jersey

98 Brett Rd, Room Piscataway, NJ 08854



(A) (B)

Fig. S1 Characterization of the DHS biosynthesis and bioconversion capabilities by the co-culture strains. (A) DHS accumulation by the engineered upstream strains PUN1, PUN2 and PUN3. Strains are grown in the glycerol medium for 72 h before the DHS quantification. (B) DHS consumption by downstream co-culture strains BDZ1 and BDZ4. BDZ4 harboring the DHS transporter ShiA showed higher DHS uptake rate.

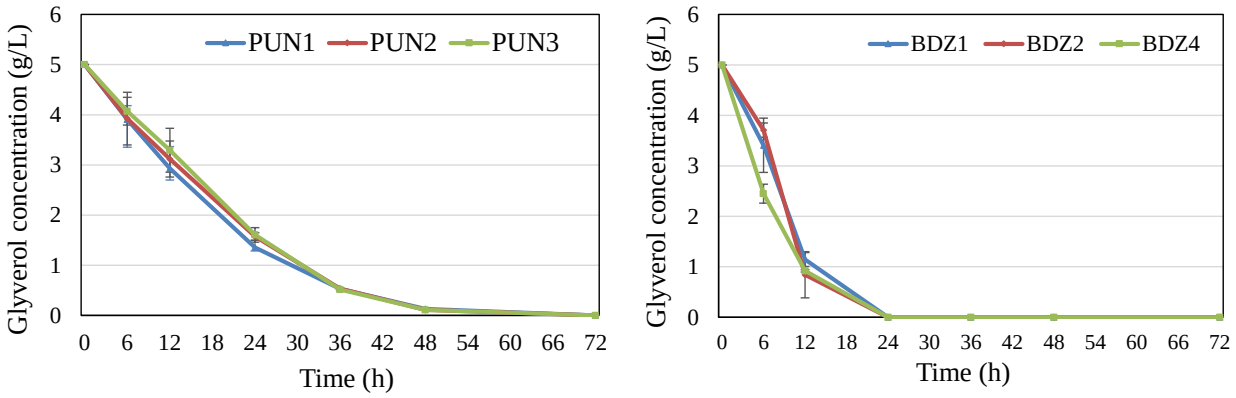


Fig. S2 Characterization of glycerol uptake by (A) the upstream strains PUN1, PUN2 and PUN3 and (B) downstream strains BDZ1, BDZ2 and BDZ4. Strains are grown in the glycerol medium and residual glycerol concentration was measured using EnzyChrom™ Glycerol Assay Kit (VWR, Radnor, PA). The culture samples were centrifuged and the supernatant was diluted to appropriate folds by the glycerol quantification.

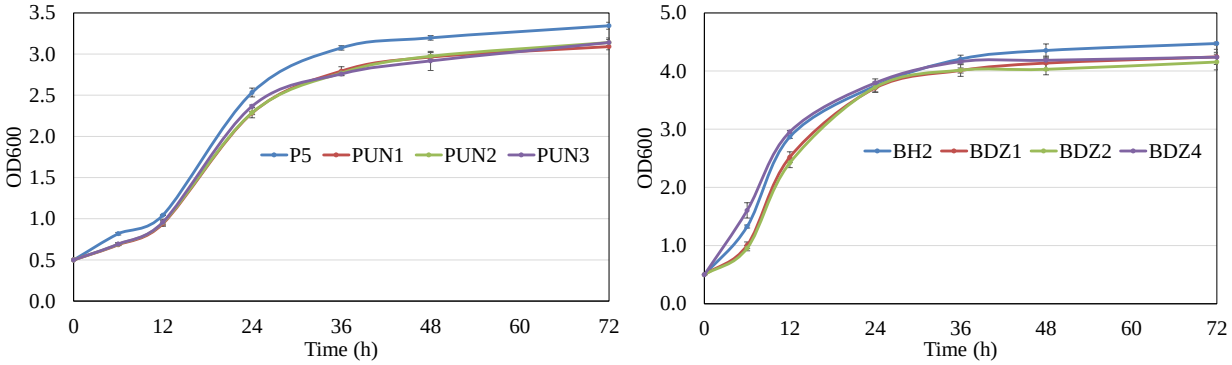


Fig. S3 Growth profiles of the engineered upstream (A) and downstream (B) co-culture strains in glycerol medium at 30 ºC. Compared with the control strains P5 and BH2, the strains with the T7 promoter –controlled gene expression did not show dramatic growth difference in the presence of the inducer IPTG.

Table. S1 Comparison of the strain-to-strain ratios for the PUN1:BDZ1 co-culture before and after the cultivation. The endpoint strain-to-strain ratios were measured by using a blue-white colony differentiation methods described previously [25]. The upstream strain PUN3 did not have the *lacZ* gene, whereas the downstream strain BDZ4 had an intact chromosomal *lacZ* gene. These two strains showed white and blue colonies on the X-gal plates, respectively.

Inoculation ratio	**19:1**	**9:1**	**4:1**	**1:1**	**1:4**	**1:9**	**1:19**
Endpoint ratio	(1.20± 0.21):1	(0.81± 0.05):1	(0.72± 0.24):1	(0.78± 0.29):1	(0.32± 0.07):1	(0.32± 0.19):1	(0.26± 0.12):1