Supplementary Information (SI) for Green Chemistry.
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Supplementary Information

De novo biosynthesis of the 4,6-dihydroxycoumarin in *Escherichia coli*

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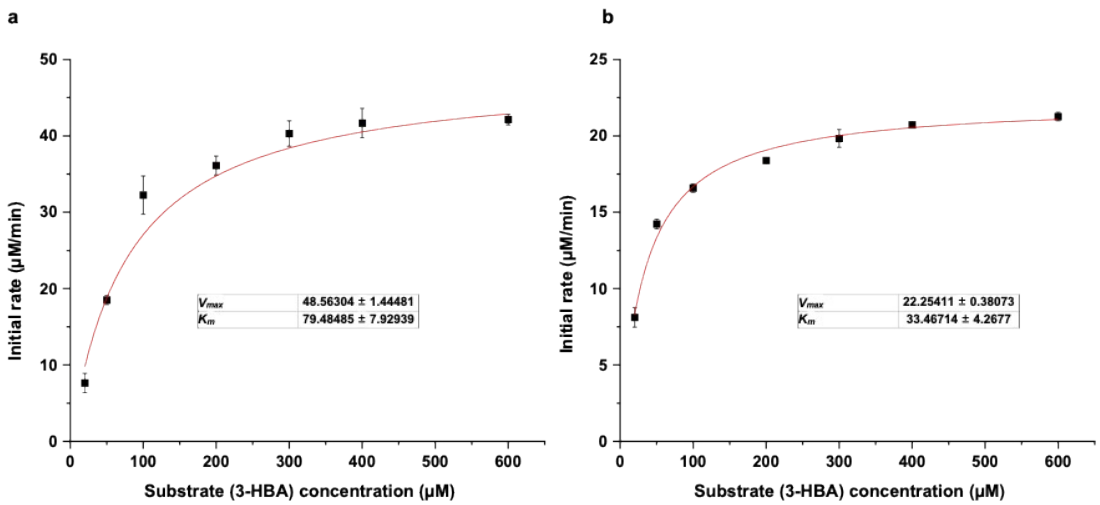
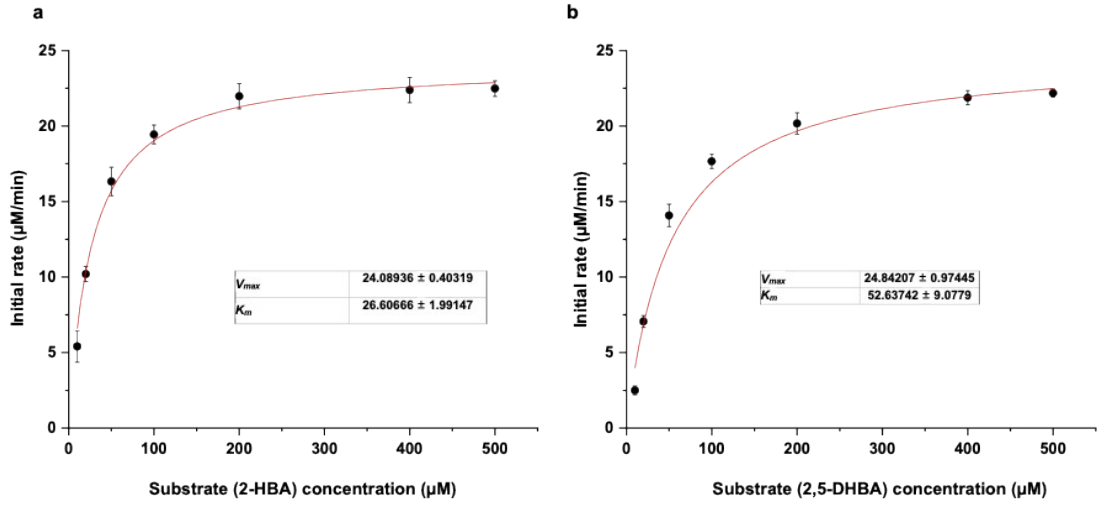
[δ]These authors contributed equally to this work.

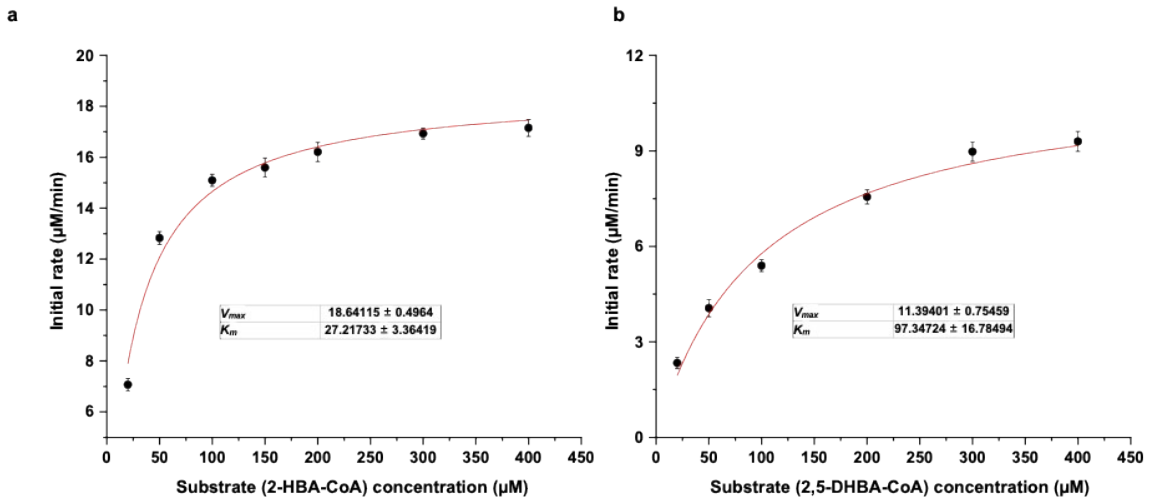


Supplementary Fig. 1 Kinetic parameters of (a) 3HB6H and (b) Re3HB6H towards 3-HBA. The K_m and V_{max} values were estimated with OriginPro2020 through non-linear regression of the Michaelis-Menten equation. Protein concentration [E] in the reaction systems is 0.25 μM for 3HB6H and 0.65 μM for Re3HB6H. The turnover number (k_{cat}) values were subsequently calculated using the formula $k_{cat}= V_{max}$ /[E]. The data presented represents the mean ± standard deviation (SD) from three independent experiments, with error bars indicating the SD.

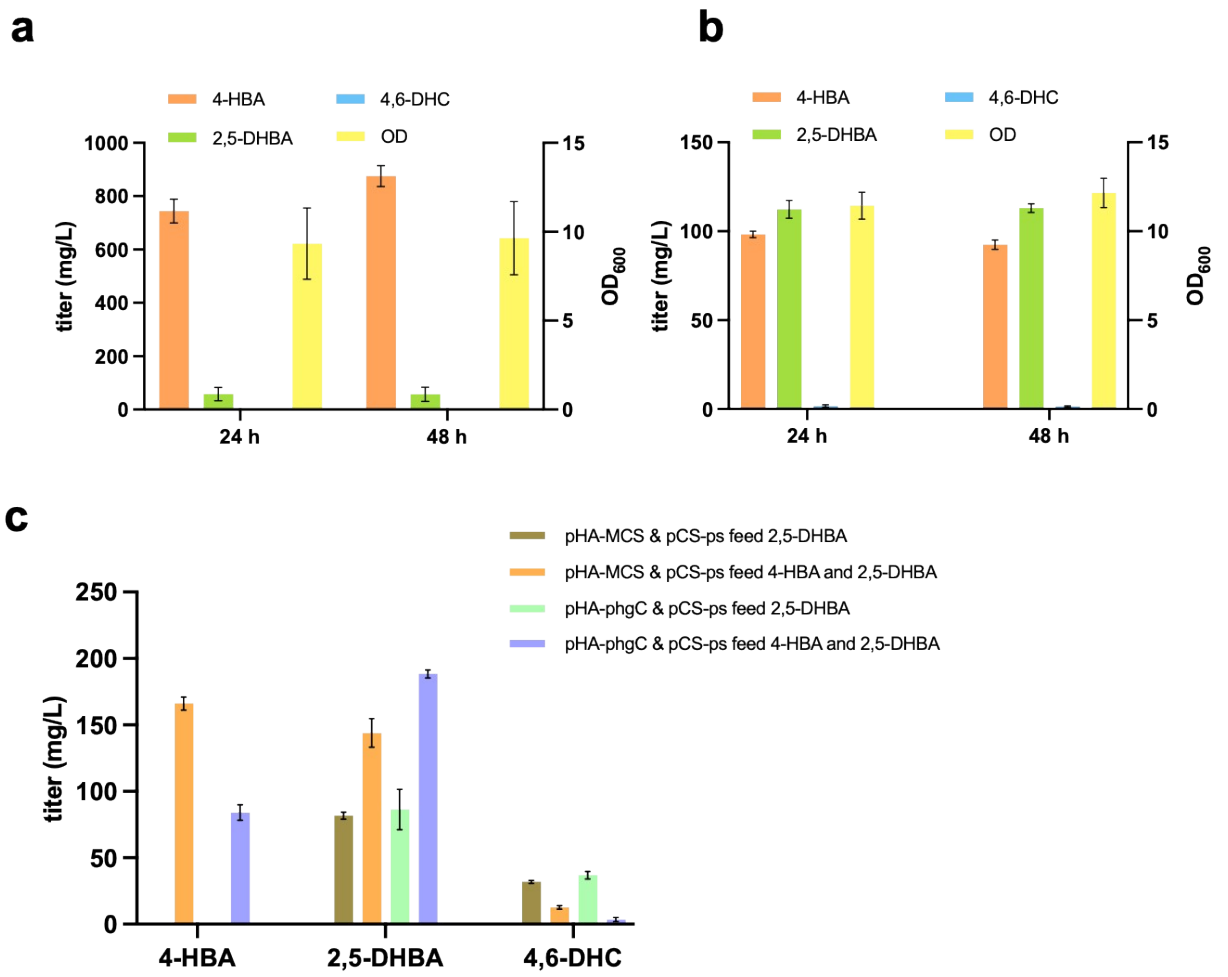


Supplementary Fig. 2 The kinetic parameters of sdgA towards (a) 2-HBA and (b) 2,5-DHBA were determined. The Michaelis-Menten equation was fitted to experimental data using OriginPro2020 to estimate the K_m and V_{max} values. The protein concentration [E] in the reaction systems was 0.03 μM. The turnover number (k_{cat}) values were

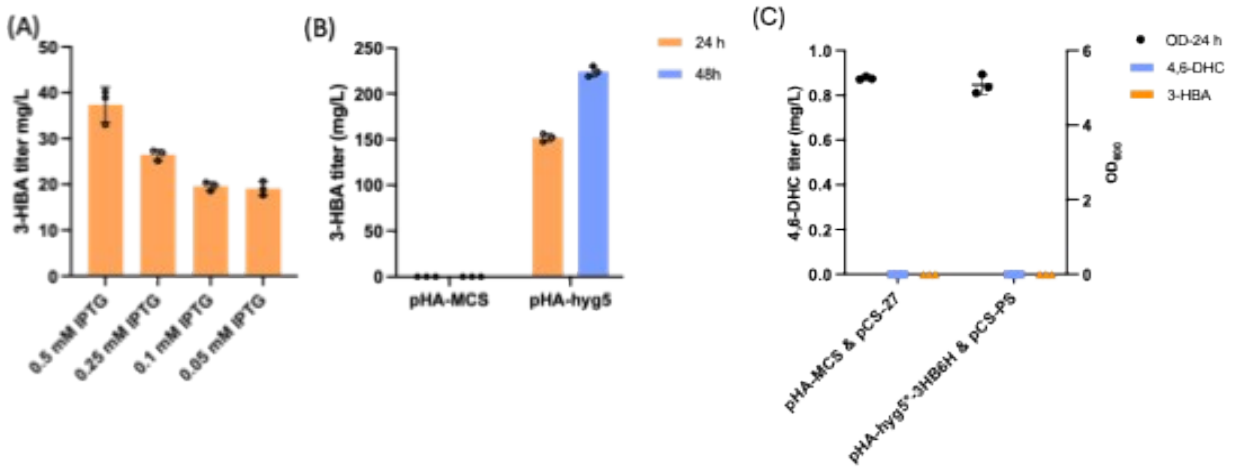
subsequently calculated using the formula $k_{cat}= V_{max}$ /[E]. The data presented represents the mean±standard deviation (SD) from three independent experiments, with error bars indicating the SD.



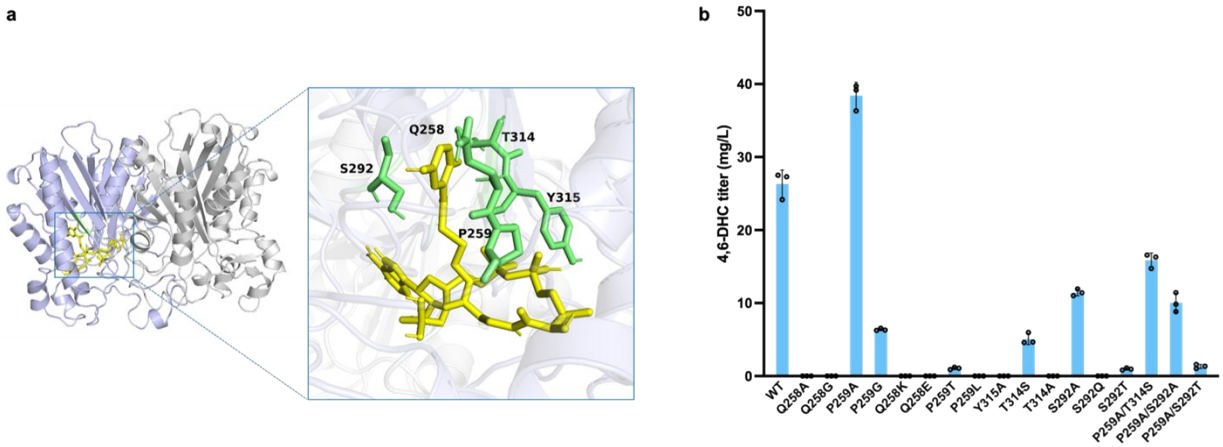
Supplementary Fig. 3 Activity of PqsD towards (a) 2-HBA-CoA and (b) 2,5-DHBA-CoA. The Michaelis-Menten equation was fitted to experimental data using OriginPro2020 to estimate the K_m and V_{max} values. The protein concentration [E] in the reaction systems was 0.18 μM. The turnover number (k_{cat}) values were subsequently calculated using the formula $k_{cat}= V_{max}$ /[E]. The data presented represents the mean ± standard deviation (SD) from three independent experiments, with error bars indicating the SD.



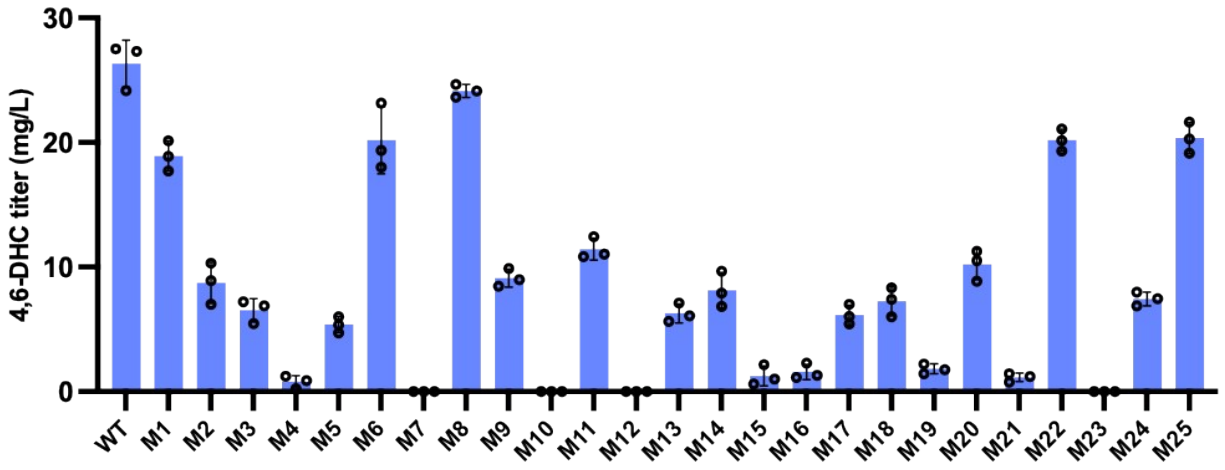
Supplementary Fig. 4 The conversion from 2,5-DHBA-CoA to 4,6-DHC though the route III (4-HBA pathway). **a.** The titer of Strain DX7 (BW25113 (F') co-expressing plasmids pCS-PS and pHA-PhgA-PhgB-PhgC) by feeding 1g/L 4-HBA. **b.** The titer of strain DX7S (BW25113 (F') expressing plasmids pHA-PhgC-PhgA-PqsD) by feeding 200 mg/L 4-HBA. **c.** The titer of 4-HBA, 2,5-DHBA and 4,6-DHC of different strains in the feeding experiment. *E. coli* BW25113 (F') expressing different plasmids were tested, to investigate the influence of 4-HBA-CoA on the conversion of 2,5-DHBA-CoA to 4,6-DHC. The data presented represents the mean±standard deviation (SD) from three independent experiments, with error bars indicating the SD.



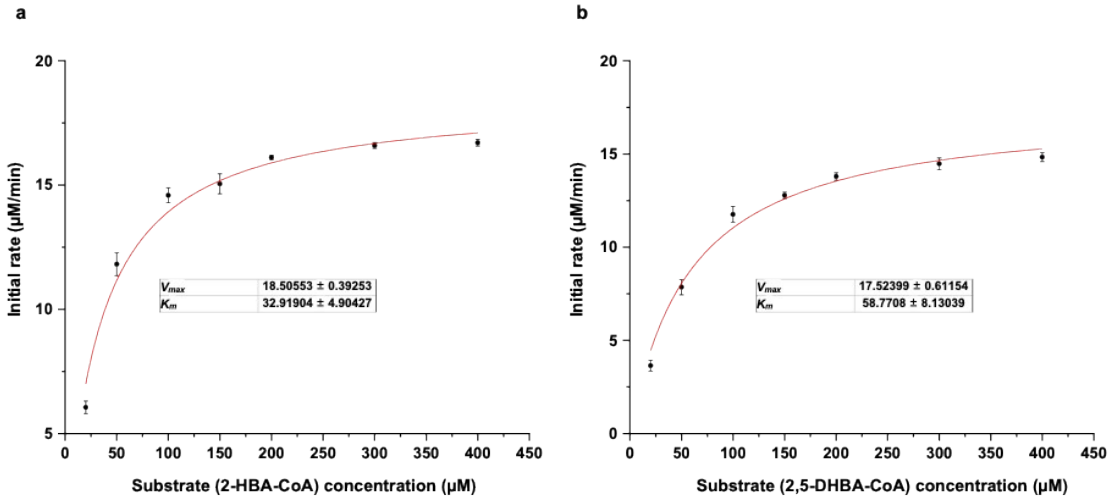
Supplementary Fig. 5 Strategies applied to improve 3-HBA production. (A) 3-HBA titer induced by different IPTG concentration. (B) 3-HBA titer after introducing pHA-Hyg5 into the strain *E. coli* ATCC 31884 *ΔpheA ΔtyrA*, (C) the titer of 4,6-DHC and 3-HBA and the OD_{600} after introducing different plasmids into the strain *E. coli* ATCC 31884 *ΔpheA ΔtyrA*. The data presented represents the mean ± standard deviation (SD) from three independent experiments, with error bars indicating the SD.



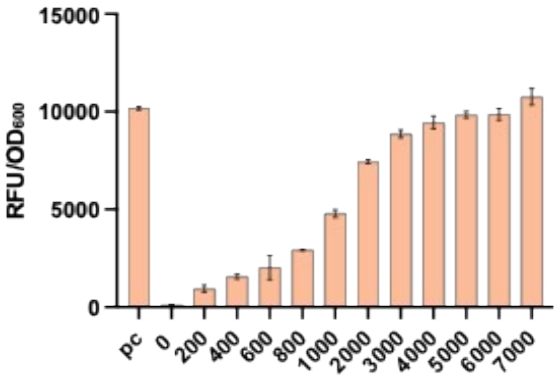
Supplementary Fig. 6 Protein engineering of PqsD to enhance 4,6-DHC production. **a.** Binding model illustrating the interaction of 2,5-DHBA-CoA with PqsD. The model was constructed using PqsD from *Pseudomonas aeruginosa* PAO1 as a template, with potential five target residues for enzyme engineering highlighted in green. **b.** Concentrations of 4,6-DHC produced in flask cultures of the DX1 strain (*E. coli* BW25113 (F') carrying pCS-PqsD-SdgA) and its derivative strains (*E. coli* BW25113 (F') carrying pCS-PqsD*-SdgA), each containing one of the 17 different PqsD mutants. The data presented represents the mean ± standard deviation (SD) from three independent experiments, with error bars indicating the SD.



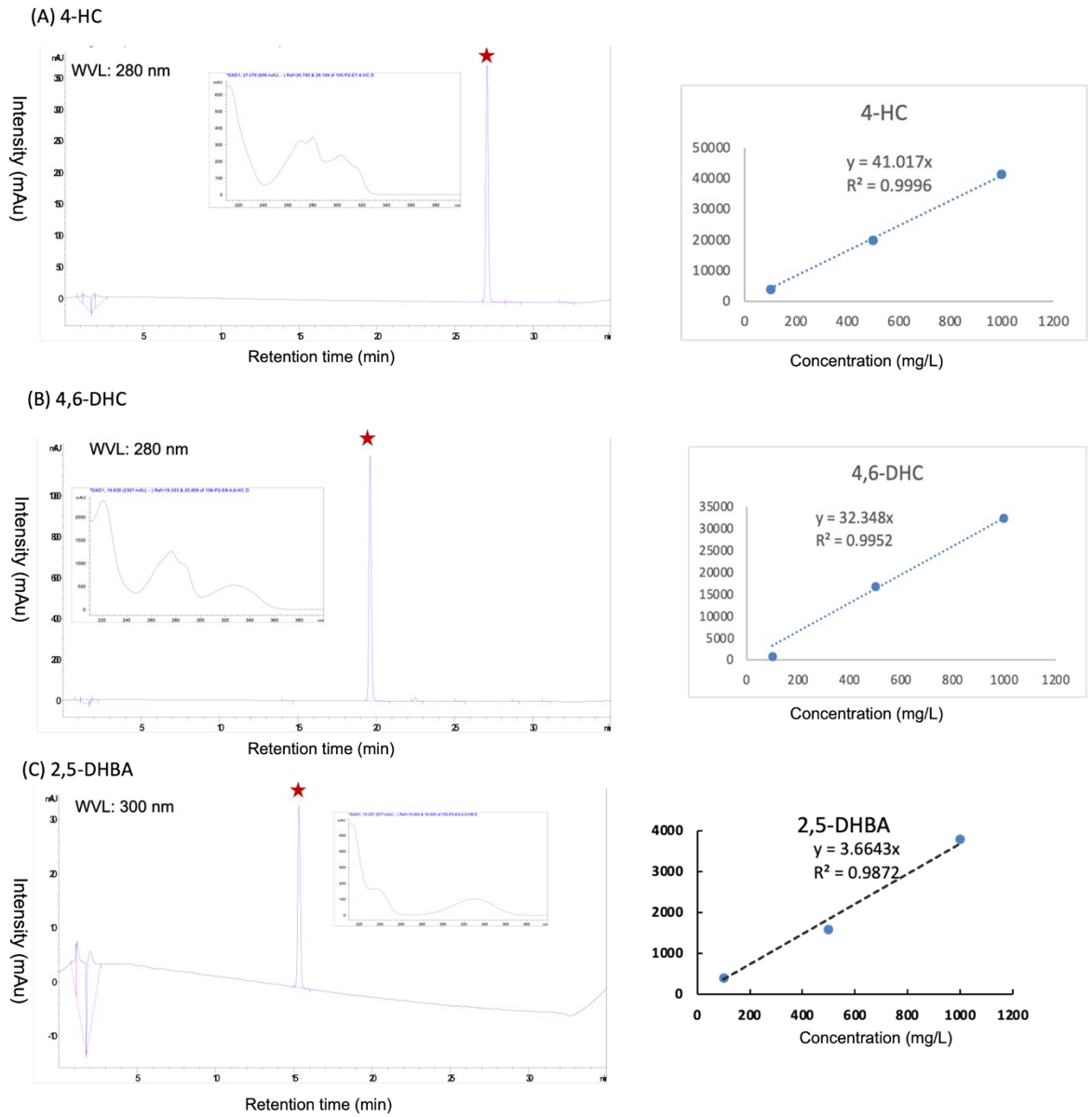
Supplementary Fig. 7 Enzyme screening for the random mutagenized variants based on pqsD(P259M$_x$)-sdgA. Concentrations of 4,6-DHC produced in flask cultures of the strain *E. coli* BW25113 (F') carrying pCS-PqsD(M$_x$)-SdgA. Note: WT (Wide Type): Refers to the wild-type (unmodified) version of the pqsD enzyme. Mx (Variants): Represents the specific variants of pqsD with mutations at the P259 site. The data presented represents the mean ± standard deviation (SD) from three independent experiments, with error bars indicating the SD.



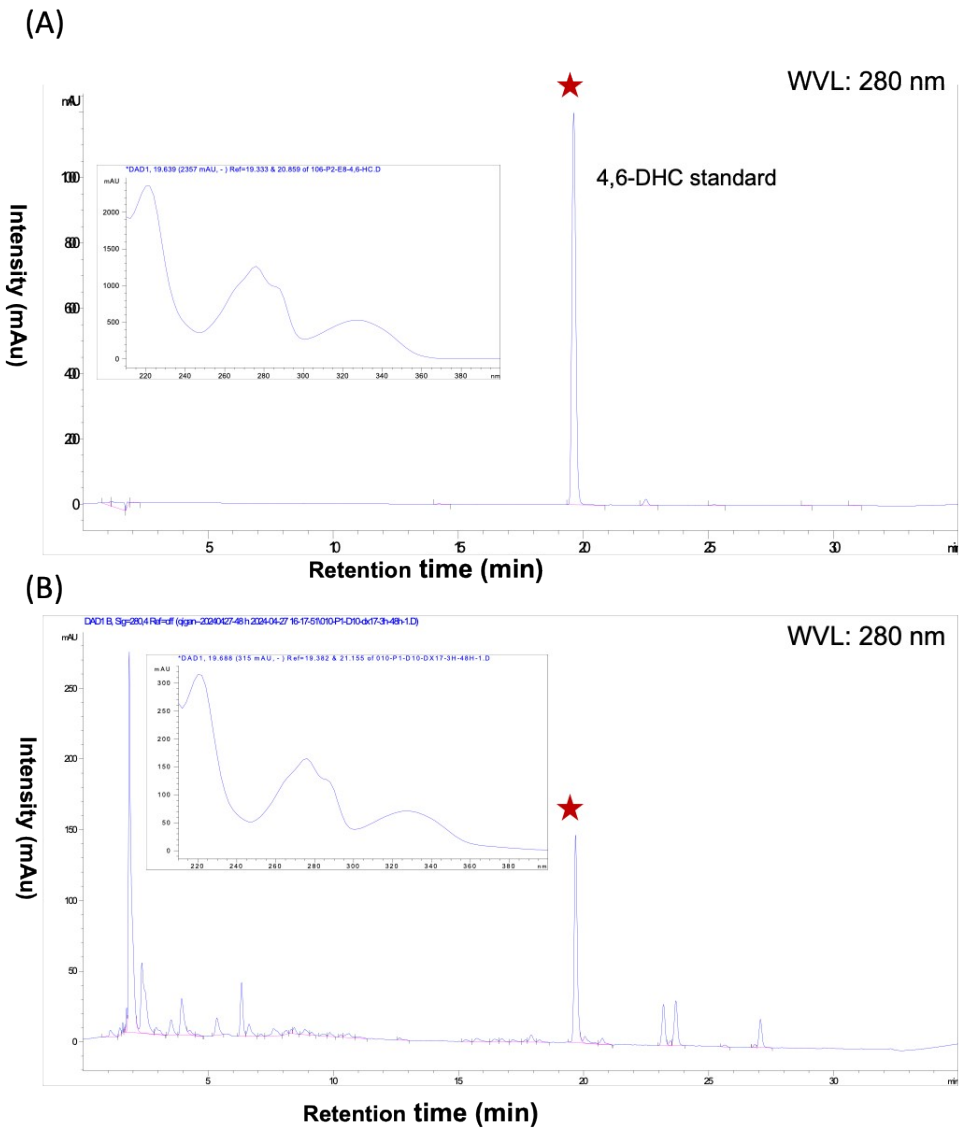
Supplementary Fig. 8 Kinetic parameters of PqsD-P259A towards (a) 2-HBA-CoA and (b) 2,5-DHBA-CoA. The K_m and V_{max} values were estimated with OriginPro2020 through non-linear regression of the Michaelis-Menten equation. Protein concentration [E] in the reaction systems is 0.12 μM. The turnover number (k_{cat}) values were subsequently calculated using the formula $k_{cat}= V_{max}$ /[E]. The data presented represents the mean±standard deviation (SD) from three independent experiments, with error bars indicating the SD.



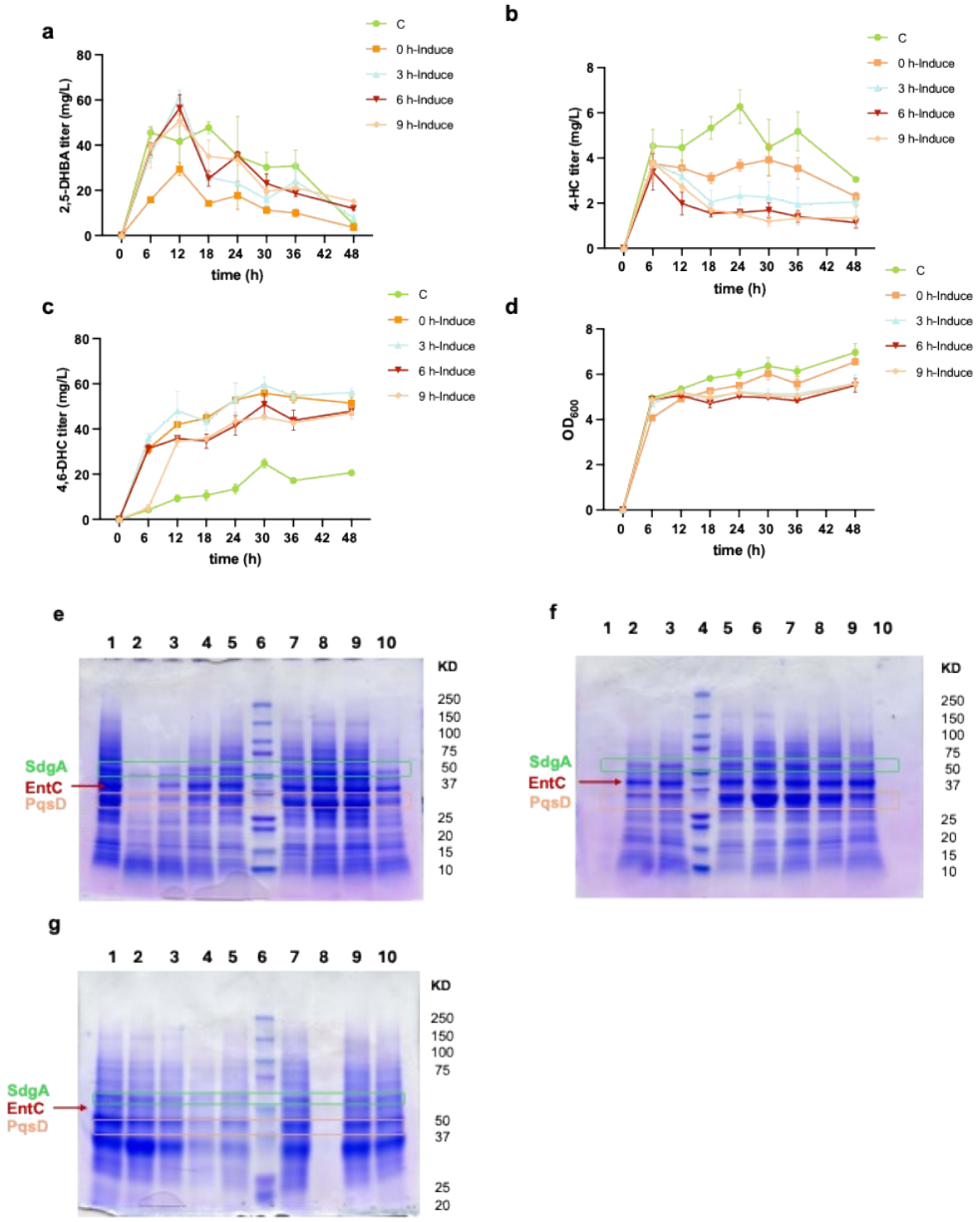
Supplementary Fig. 9 Tetracycline Inducible concentration test. This test was carried out using strain P1, BW25113/F′ carrying pZE-EP-P_{teto1}-egfp and pCS-SalABCD-$_{Plpp0.2}$-tetR, the positive control was tested using BW25113/F′ carrying pZE-EP-P_{laco1}-egfp and pCS-SalABCD-$P_{lpp0.2}$-tetR.



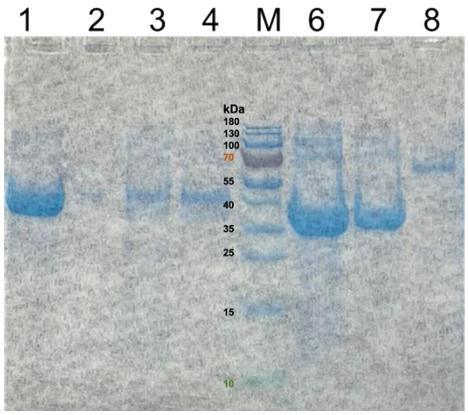
Supplementary Fig. 10 HPLC analysis of critical products in this research. (A) the 4-HC standard and its standard curve. (B) the 4,6-HCstandard and its standard curve. (C) the 2,5-DHBA standard and its standard curve. UV absorbance profiles are shown beside the peaks.



Supplementary Fig. 11 HPLC analysis of 4,6-DHC produced by strain DX17. (A) A sample taken from the cell culture after 48 hours. (B) 100 mg/L 4,6-DHC standard. The retention time of 4,6-DHC was about 19.4 min. UV absorbance profiles are shown beside the peaks.



Supplementary Fig. 12 Inducible regulation of de novo biosynthesis of 4,6-DHC in strain DX17. **a.** The titer of 2,5-DHBA by using different induction time. **b.** The titer of 4-HC by using different induction time. **c.** The titer of 4,6-DHC by using different induction time. **d.** The OD_{600} by using different induction time. **e.** SDS-PAGE analysis was performed to assess protein expression levels. The bands corresponding to specific enzymes are highlighted in different colors and marked with distinct arrows. 1: c-6h, 2: 0h-6h, 3: 3h-6h, 4: 6h-6 h, 5: 9h-6h, 6: marker, 7: c-12 h, 8: 0h-12 h, 9:3 h-12 h, 10:6 h-12 h. **f.** SDS-PAGE analysis was performed to assess protein expression levels. The bands corresponding to specific enzymes are highlighted in different colors and marked with distinct arrows. 2: 6h-12h, 3: 9h-12h, 4: marker, 5: c-18 h, 6: 0h-18 h, 7:3 h-18 h, 8:6 h-18h, 9:9 h-18h. **g.** SDS-PAGE analysis was performed to assess protein expression levels. The bands corresponding to specific enzymes are highlighted in different colors and marked with distinct arrows. 1: c-24 h, 2: 0h-24 h, 3: 3h-24 h, 4: 6h-24 h, 5: 9h-24 h, 6: marker, 7: c-30 h, 8: 0h-30 h, 9:3 h-30 h, 10:6 h-30 h. (Note: X_1 h–X_2 h indicates detection at X_2h, where tetracycline was added at X_1h after IPTG induction. "c" refers to the control group, which genes sdgA and pqsD are uder Placo1 promoter) The data presented represents the mean ± standard deviation (SD) from three independent experiments, with error bars indicating the SD. The SDS-PAGE analysis was performed using samples from triplicate cultures.



Supplementary Fig. 13 SDS-PAGE analysis of purified proteins. 1: 3HB6H, 2: Re3HB6H, 3: Hyg5, 4: Hyg5*, 5: Marker, 6: PqsD, 7: PqsD –P259A, 8: SdgA.

Table S1. Strains and plasmids used in this study

Strains	Description	Reference
E. coli BW25113/F′	*rrnBT14 ΔlacZWJ16 hsdR514 ΔaraBADAH33 ΔrhaBADLD78 F' [traD36 proAB lacIqZΔM15 Tn10(Tetr)]*	1
E. coli XL1-blue	*recA1 endA1gyrA96thi-1hsdR17supE44relA1lac [F' proAB lacIqZDM15Tn10 (TetR)]*	Stratagene
E. coli BL21 Star (DE3)	*F−OMPT HSDSB (RB-MB-) GAL DCM (DE3)*	Invitrogen
E. coli ATCC 31884 ΔpheA Δtyr	*E. coli ATCC31884 ΔpheLA ΔtyrA*	2
DX1	BW25113/F′ carrying pCS-PS	This study
DX2	BW25113/F′ carrying pZE-SalABCD	This study
DX3	BW25113/F′ carrying pHA-3HB6H	This study
DX4	BW25113/F′ carrying pHA-PhgC-PhgB-PhgA	This study
DX5	BW25113/F′ carrying pZE-SalABCD and pCS-PS	This study
DX5’	BW25113/F′ carrying pZE-$P_{lpp0.2}$-tetR-SalABCD and pCS-P_{tetO1}-PqsD-SdgA	This study
DX6	BW25113/F′ carrying pHA-3HB6H and pCS-PS	This study
DX7	BW25113/F′ carrying pHA-PhgC-PhgB-PhgA and pCS-PS	This study
DX7S	BW25113/F′ carrying pHA-PhgC-PhgA-PqsD	This study
DX8	BW25113/F′ carrying pZE-EP	This study
DX9	BW25113/F′ carrying pHA-Hyg5	This study
DX10	BW25113/F′ carrying pHA-Hyg5*	This study
DX11	BW25113/F′ carrying pHA-Ubic	This study
DX12	BW25113/F′ carrying pZE-EPPS and pCS-SalABCD	This study

DX13	BW25113/F′ carrying pHA-Hyg5-3HB6H and pCS-PS	This study
DX14	BW25113/F′ carrying pHA-PhgC-PhgB-PhgA and pCS-PS-Ubic	This study
DX15	BW25113/F′ carrying pHA-Hyg5*-3HB6H and pCS-PS	This study
DX16	BW25113/F′ carrying pZE-EP-P_{teto1}-PqsD-SdgA and pCS-SalABCD-$P_{lpp0.2}$-tetR	This study
DX17	BW25113/F′ carrying pZE-EP-P_{teto1}-PqsD (P259A)-SdgA and pCS-SalABCD-$P_{lpp0.2}$-tetR	This study
P1	BW25113/F′ carrying pZE-EP-P_{teto1}-egfp and pCS-SalABCD-$P_{lpp0.2}$-tetR	This study
Plasmaids	**Description**	**Reference**
pZE12-luc	P_{LlacO1}; *luc; ColE1 ori; Amp*^{R}	3
pCS27	P_{LlacO1}; *p15A ori; Kan*^{R}	4
pMK-eGFP-MCS	P_{LlacO1}; eGFP; multiple cloning site; *p15A ori*; *Kan*^{R}	5
pHA-eGFP-MCS	P_{LlacO1}; eGFP; multiple cloning site; *ColE1 ori*; *Amp*^{R}	6
pHA-MCS	P_{LlacO1}; multiple cloning site; *ColE1 ori*; *Amp*^{R}	6
pMK-MCS	P_{LlacO1}; multiple cloning site; *p15A ori*; *Kan*^{R}	5
pETDuet-1	two T7 promoters; two MCS; *pBR322 ori; Amp*^{R}	Novagen
pCS-PS	pCS-27 containing *pqsD-sdgA* under the promoter P_{LlacO1} in one operon	7
pZE-SalABCD	pZE12-luc containing *salAB*, *salCD* from *Ralstonia eutropha*	8
pHA-3HB6H	pHA-MCS containing *3hb6h* from *Rhodococcus jostii* RHA1 under the control of promoter P_{LlacO1}	This study
pHA-PhgC-PhgB-PhgA	pHA-MCS containing *phgC*, *phgB*, *phgA* from *Brevibacillus laterosporus* PHB-7a under the control of promoter P_{LlacO1} in separate operon	This study
pZE-EP	pZE12-luc containing *entC-pfpchB*	7
pHA-Hyg5	pHA-MCS containing *hyg5* from *Streptomyces rapamycinicus* under the control of promoter P_{LlacO1}	This study
pHA-Hyg5*	pHA-MCS containing *hyg5** under the control of promoter P_{LlacO1}	This study
pHA-Ubic	pHA-MCS containing *ubic* from *Escherichia coli*	This study

	MG1655 under the control of promoter P_{LlacO1}	
pZE-EPPS	pZE12-luc containing *entC-pfpchB-pqsD-sdgA*	7
pCS-SalABCD	pCS-27 containing *salABCD*	This study
pHA-Hyg5-3HB6H	pHA-MCS containing *hyg5* and *3hb6h* under the control of promoter P_{LlacO1} in separate operon	This study
pHA-Hyg5*-3HB6H	pHA-MCS containing *hyg5** and *3hb6h* under the control of promoter P_{LlacO1} in separate operon	This study
pCS-PS-Ubic	pCS-27 containing *pqsD-sdgA* and *ubic* under the promoter P_{LlacO1} in separate operon	This study
pMK-Ubic	pMK-MCS containing *ubic*	This study
pHA-PhgC-PhgA-PqsD	pHA-MCS containing *phgA, phgC* and *pqsD* under the promoter P_{LlacO1} in separate operon	This study
pZE-P_{teto1}-PqsD-SdgA	pZE12-luc contaning *pqsD-sdgA* under the control of promoter P_{tetO1}, respectively	This study
pZE-EP-P_{teto1}-PqsD-SdgA	pZE12-luc contaning *entC-pfpchB* and *pqsD-sdgA* under the control of promoter P_{LlacO1} and P_{tetO1}, respectively	This study
pZE-EP-P_{teto1}-PqsD (P259A)-SdgA	pZE12-luc contaning *entC-pfpchB* and *pqsD(P259A)-sdgA* under the control of promoter P_{LlacO1} and P_{tetO1}, respectively	This study
pCS-PqsD(M_x)-sdgA	pCS-27 containing *pqsD(Mx)-sdgA* under the promoter P_{LlacO1} in one operon	This study
pZE-PtetO1- eGFP	pZE12-luc, tetO1; eGFP	This study
pCS-lpp0.2-RBS1.0-K127Y	pCS27 carrying the promoter $P_{lpp0.2}$, RBS1.0, and regulator K127Y	5
pCS-$P_{lpp0.2}$-tetR	pCS27 carrying the promoter $P_{lpp0.2}$ and regulator tetR	This study
pCS-$P_{lpp0.2}$-tetR-SalABCD	pCS-27 contaning *salABCD* and *tetR* under the control of promoter of P_{LlacO1} and $P_{lpp0.2}$, respectively	This study
pZE-EP-P_{laco1}-egfp	pZE12-luc contaning *entC-pfpchB* and *egfp* under the control of promoter P_{LlacO1}	This study
pZE-EP-P_{teto1}-egfp	pZE12-luc contaning *entC-pfpchB* and *egfp* under the control of promoter P_{LlacO1} and P_{tetO1}, respectively	This study
pET-SdgA	pETDuet-1containing *sdgA*	This study
pET-3HB6H	pETDuet-1 contaning *3hb6h*	This study
pET-Re3HB6H	pETDuet-1contaning *Re3hb6h*	This study

pET-PqsD	pETDuet-1 containing *pqsD*	This study
pET-PqsD (P259A)	pETDuet-1 containing *pqsD* (P259A)	This study
pET-Hyg5	pETDuet-1 containing *hyg5*	This study
pET-Hyg5*	pETDuet-1 containing *hyg5**	This study

Table S2 DNA sequences used in this study

Name	Sequence (5'-3')
P_{LlacO1} promoter	**AATTGTGAGCGGATAACAATTGACATTGTGAGCGGATAACAAGATACTGAGCACATCAGCAGG ACGCACTGACC**
P_{tetO1} promoter	**TCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACATCAGCAGG ACGCACTGACC**
$P_{lpp0.2}$ promoter	**ATCAAAAAAATATTGACAACATAAAAAACTTTGTGTTGCTGGTGTAACG**
3hb6h	**ATGTCGAACCTGCAGGACGCACGTATCATTATTGCCGGAGGAGGAATCGGAGGCGCGGCCAA TGCGTTGGCCCTGGCGCAGAAAGGTGCAAATGTAACGTTATTTGAACGCGCCTCTGAGTTCGG CGAAGTAGGTGCCGGACTTCAAGTCGGACCACATGGAGCGCGTATCCTGGATTCCTGGGGAG TCCTGGATGACGTGTTATCACGCGCCTTTCTGCCAAAAAACATCGTCTTTCGTGACGCCATTAC TGCGGAAGTTTTGACGAAGATCGATCTGGGGTCGGAATTTCGCGGACGTTATGGCGGACCATA TTTTGTTACTCATCGTAGCGACCTGCACGCCACCTTAGTGGATGCAGCGCGCGCTGCTGGAGC CGAATTACATACCGGGGTTACCGTTACCGATGTCATCACGGAGGGCGACAAAGCAATCGTCAG CACTGATGATGGGCGCACCCACGAGGCTGATATCGCTCTTGGCATGGACGGTCTTAAGTCCCG TTTACGCGAAAAGATCTCGGGAGATGAACCTGTGAGCAGCGGATATGCTGCGTACCGCGGCA CAACGCCGTACCGTGATGTAGAACTGGATGAGGATATCGAGGATGTTGTAGGTTACATCGGCC CGCGTTGTCACTTCATCCAGTACCCACTGCGCGGCGGAGAGATGCTTAATCAAGTTGCCGTCT TTGAATCACCGGGCTTCAAGAATGGCATCGAAAACTGGGGAGGACCGGAAGAATTAGAACAG GCATACGCACACTGCCACGAAAACGTGCGTCGCGGTATCGATTATTTGTGGAAGGATCGTTGG TGGCCTATGTACGACCGCGAACCAATCGAAAATTGGGTCGACGGTCGTATGATTCTTTTGGGC GATGCTGCTCACCCTCCACTGCAATATTTGGCGTCTGGGGCGGTTATGGCTATTGAGGACGCA AAATGCCTGGCCGACTATGCGGCAGAGGACTTTAGCACCGGTGGAAACAGCGCCTGGCCGCA GATTCTTAAGGAAGTAAACACAGAGCGCGCTCCGCGCTGCAATCGCATCTTAACGACCGGCCG CATGTGGGGAGAGTTGTGGCACCTTGACGGGACGGCCCGTATTGCACGCAACGAACTTTTTCG TACTCGTGATACCAGTTCCTATAAATACACCGATTGGCTGTGGGGATATAGTTCGGACCGCGC**

TTCGTAA

Re3hb6h **ATGAGCAGTACCAACAACGCGACCGGCAAGGTCCTCATCATCGGCGGCGGCATCGGCGGCCT**
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TGAACCAGGCGGATCTGTTCCCTCTATTAGAAATCAAATTGAAACCATTTGGGGAGCTACTCT
TTTTGACCATGTAGGCATGACTGAAATGGGTGCCTACGGGTATTCCTGCTCGGAGCATAATGG

ACTTCATATCAATGAATCAGAATTCATTGCGGAGGTAGTTAATCCAGACACTCTTAAATCTGTA
GAAATTGGTGAACGCGGGGAATTGGTACTAACAAACTTAGGTCGTTATGGATATCCACTTATT
CGCTATAGGACTGGAGATATTGTAATTCACTCTAGTGAATCCTGTCCTTGCGGAAATACTTATA
AACTTCTTCCAAAGGGGATTATCGGCCGCAAAGATGACATGGTCGTCATTCGCGGAATCAATA
TTTACCCTTCCTCCATTGAAGCCATTGTACGTGAATTTTCAGCAATAACGGAATACCGCATTAT
ATATTATACAAAAGGCGGCATGGATCAAATCAAGCTGCAAATTGAAACAAATGTAGATATCTC
CTCCATTGTAGCCACACATTTAAGAGATCGTGTCGGTTTAAGAATTGATGTAGAGGTCGTAGA
AACCAACATTCTTCCACGTTTCGATATGAAGGCAAGGCGTGTTTTGGACAAAAGAGACGCCTT
CTAA

phgB **ATGGGTGCAGTTACTTATGATTTTCAAGTGAAATGGGGAGATACGGATGCAGCAGGAATCGTA**
TATTACCCGAATTTTTATAGCTGGATGGATGAAGCTACGCATCATTTTTTCAAAACACTTGGCC
ATCCTACATCTAAATTATTTACAGAGAATCGAATAGGGGTTCCTTTACTTGAAGCACATTGCAG
CTTTAGAATCCCCTTATTTCATGAAGATGATGTCCAGATTCAAACAGAAGCGATAGAAATCCGT
GATAAGGTTTTTAAGCTGATCCATACTTTTATGAGAGATAATGACGTCATCGCGGAAGGATAT
GAGCTACGCGCCTATGCATCTGTAGCAGATCATAAACCAAAGGCGCAGTCCATTCCAGAGGAA
TTGAAATCGAAAATGGAAATAGGCGCATAA

phgA **ATGCAAACGAATCAAACGGATCATTTATGCCGTCAGAGCATAGTTATTGGGGCTGGCCCTGTT**
GGCTTAACAGCTGCGTTGGCATTGAAAAACCTAGGACTGGAAGTAGCAGTTTTAGAAGGAGAG
CCTAAGGATAGACAAAGACCAGGAAGTCGTGCCATTTATCTACATAAAGCCACACTCGGCCTT
ATAGATGAAATTTCTCCGGGACTTGGATATAAACTTGCTGAAAATGGAGTGGTTTGGCCTGTT
AAAAGAACGTTATTCCGTGGGAAAGAAGTGTATGTACGTAACTACGGTGCACCAAAAACGGAA
GGATTACCTCCTTTTACAAGCTTACCTCAAGTAGAGGCAGAAAAATATCTTTATGAAGCTTGTA
TTAAAGCGGGCATAGAATTCTTCTACGATAAGCCGGTAACTGACGTTAAGACTAGTGAAAATG
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GCGGGCGATCAGCTGTACGCCAGGCTATAGGTGTAGAGCTTGAAGGTCCTCGAACGAAAGAT
ACCTTCTTAGTGGTTGATGTGATTGAAGATAAAGAAAATCCGCTTCCCCTTGAAAGGATCTTTC
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ATACATTCCACCAAGCGGTTTCAAGTTCTTATACTGATGAAAACAATCGTGTACTTCTTGCTGG
AGAAGCAGCGCATTTATTCGCTCCATTTGGTGCTCGGGGTTTGAATTCAGGTGTACCCGATGC
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ATACGGCCCTTCATCATATTCAAGGATCATCTTCTTATATGAACAGAAAAAAAGAGGTGGCTG
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GATCTGGTCCGCCGGAATTGACAACAAAATACTAA

hyg5 **ATGAACCCGTCCTCGTTGGTTCTTAACGGACTGACAAGCTATTTCGAGAATGGCCGCGCCCGC**
GTAGTACCCCCCGTGGGACGTAACATCCTGGGTGTGGTGAATTATGCCTCTGTTTGCGAATAC
CCAACACTGGATCACGGATACCCAGAGTTAGAGATTAACATGGTTGCCCCGACAGCGGAGCCA
TTCGCAGAGGTATGGGTTACCGATGCTGAGTCCGAACATGGTGAGCGTGACGGAATCACATAT
GCTCACGACGGGGAGTACTTTTTTTGTGCAGGCCGCGTGCCCCCTACGGGTCGTTATACTGAA
GCTACTCGTGCTGCGTACGTTACGATGTTTGAACTGCTGGAGGAGTTTGGGTATTCTAGCGTG
TTTCGCATGTGGAACTTTATCGGTGATATTAATCGCGATAACGCGGAGGGGATGGAAGTCTAC
CGCGATTTCTGCCGTGGTCGCGCCGAGGCGTTTGAACAGTGCCGTCTGGAGTTCGATCAATTT
CCTGCGGCCACAGGCATCGGATCACGTGGAGGAGGTATTGCCTTCTACCTTTTAGCGTGCCGC
AGTGGGGGTCACGTACACATCGAGAACCCGCGCCAAGTCCCAGCGTACCATTACCCCAAGCG
TTACGGACCCCGCGCTCCGCGTTTTGCTCGCGCGACATATCTTCCTTCGCGCGCGGCTGATGG
AGTAGGTGGTCAAGTCTTCGTCTCGGGAACTGCCTCAGTGCTTGGCCATGAGACAGCACACGA
AGGCGACCTTGTAAAACAATGTCGCCTGGCACTGGAGAACATCGAATTAGTTATTTCGGGCGG
CAACTTGGCTGCCCATGGCATTTCCGCCGGTCATGGTTTAACTGCTTTGCGTAACATTAAGGT
ATATGTGCGCCGTTCTGAAGACGTACCTGCAGTTCGTGAAATCTGTCGCGAGGCCTTCTCTCC
AGACGCGGACATTGTATATTTAACAGTAGACGTGTCACGCTCTGACTTACTTGTTGAGATTGA
GGGCGTCGTCATGTAA

*hyg5** **ATGAACCCGTCAAGCCTGGTCCTGAATGGGCTGACGTCGTACTTTGAGAACGGTCGTGCTAGG**
GTAGTGCCCCCGGTTGGCAGAAACATTCTGGGCGTGGTGAATTACGCGTCCGTTTGCGAATAT
CCGACCCTGGACCATGGGTATCCGGAACTCGAAATCAACATGGTCGCGCCTACCGCAGAACCC
TTCGCAGAAGTGTGGGTGACAGATGCCGAGAGTGAACACGGTGAACGCGATGGGATTACGTA
TGCCCATGATGGCGAGTACTTCTTTTGCGCGGGACGTGTTCCGCCCACTGGCCGCTATACCGA
AGCCACACGCGCAGCGTACGTAACGATGTTCGAACTCCTGGAAGAGTTTGGCTATAGCAGTGT
GTTTCGGATGTGGAACTTCATTGGCGACATCAATCGCGATAATGCCGAAGGGATGGAAGTGTA
TCGCGACTTTTGCCGCGGACGTGCGGAAGCGTTTGAACAGTGTCGCCTGGAGTTCGACCAGTT
TCCGGCTGCAACCGGGATTGGTTCTCGTGGCGGTGGTATCGCGTTTTATCTGCTGGCCTGTCG
TTCGGGTGGGCACGTCCATATCGAGAATCCGCGTCAAGTCCCAGCGTATCACTATCCGAAACG
CTATGGTCCTCGTGCTCCCCGCTTTGCCCGTGCGACCTACTTGCCGAGTCGTGCGGCTGATGG
CGTAGGAGGCCAAGTGTTCGTATCAGGCACCGCATCAGTACTGGGACACGAAACTGCCCATG
AAGGTGATCTGGTTAAACAGTGTCGATTAGCCCTTGAGAACATTGAGTTGGTCATTAGTGGCG

	GGAATTTAGCGGCTCATGGCATAAGCGCTGGTCATGGCCTCACGGCATTACGCAACATCAAGG TTTACGTTCGTCGCTCCGAGGATGTGCCTGCAGTTCGGGAAATTTGTCGCGAAGCGTTCTCGC CAGATGCCGACATTGTGTACCTTACCGTTGACGTCTGCCGTAGCGATCTCTTGGTGGAAATCG AAGGTGTCGTGATGTAA
ubic	**ATGTCACACCCCGCGTTAACGCAACTGCGTGCGCTGCGCTATTGTAAAGAGATCCCTGCCCTG GATCCGCAACTGCTCGACTGGCTGTTGCTGGAGGATTCCATGACAAAACGTTTTGAACAGCAG GGAAAAACGGTAAGCGTGACGATGATCCGCGAAGGGTTTGTCGAGCAGAATGAAATCCCCGA AGAACTGCCGCTGCTGCCGAAAGAGTCTCGTTACTGGTTACGTGAAATTTTGTTATGTGCCGA TGGTGAACCGTGGCTTGCCGGTCGTACCGTCGTTCCTGTGTCAACGTTAAGCGGGCCGGAGCT GGCGTTACAAAAATTGGGTAAAACGCCGTTAGGACGCTATCTGTTCACATCATCGACATTAAC CCGGGACTTTATTGAGATAGGCCGTGATGCCGGGCTGTGGGGGCGACGTTCCCGCCTGCGAT TAAGCGGTAAACCGCTGTTGCTAACAGAACTGTTTTTACCGGCGTCACCGTTGTACTAA**

Table S3 Key Primer sequences used in this study

Primer	sequence	use
KpnI-3HB6H-F	GGAAggtaccATGTCGAACCTGCAGGACGCACG	to amplify 3hb6h gene for pHA-3HB6H
HindIII-3HB6H-R	GGAAaagcttTTACGAAGCGCGGTCCGAACTATATC	
KpnI-Re3HB6H-F	GGAAggtaccatgagcagtaccaacaacgcg	to amplify Re3hb6h gene for pHA-Re3HB6H
HindIII-Re3HB6H-R	GGAAaagcttttacagccacggcgtggggctc	
KpnI-PhgA-F	TGTACAttagaaggcgtctcttttgtccaaaac	to amplify phgA gene for pHA-phgA
SalI-PhgA-R	GGAAgtcgacttagtattttgttgtcaattccggcggaccaga	
KpnI-PhgB-F	GGAAggtaccatgggtgcagttacttatgatttt	to amplify phgB gene for pHA-phgB
XbaI-PhgB-R	GGAAtctagagtcattattatgcgcctatttccattttcgatttcaa	
KpnI-PhgC-F	GGAAggtaccatggtagcaagtcaacagcaacccggatta	to amplify phgC gene for pHA-phgC
BsrGI-PhgC-R	ggaaTGTACAttagaaggcgtctcttttgtccaaaac	
XhoI-PLlacO1-F	GGAActcgagaattgtgagcggataacaattgacatt	to amplify phgB operon for pHA-phgC-phgB
BsrGI-ter-R	ggaaTGTACAtactcaggagagcgttcaccgacaaacaacaga	
XbaI-PLlacO1-F	ggaaTCTAGAaattgtgagcggataacaattgacattgtgagc	to amplify phgA operon for pHA-phgC-phgB-phgA
SacI-Ter-R	gggaaaGAGCTCcaggagagcgttcaccgacaa	
KpnI-hyg5-F	GGAAggtaccATGAACCCGTCCTCGTTGGTTC	to amplify hyg5 gene for pHA-Hyg5
SalI-hyg5-R	GGAAgtcgacTTACATGACGACGCCCTCAATCTC	
KpnI-Hyg5*-F	GGAAggtaccATGAACCCGTCTAGCCTGGTC	to amplify hyg5* gene for pHA-Hyg5*
SalI-Hyg5*-R	GGAAgtcgacTTACATCACGACACCTTCGATTTCCA	
KpnI-ubiC-F	GGAAggtaccatgTCACACCCCGCGTTAACG	to amplify ubic gene for pHA-ubic
SalI-ubiC-R	GGAAgtcgacttaGTACAACGGTGACGCCGG	

SpeI-PLlacO1-F	GGAAactagtaattgtgagcggataacaattg	to amplify 3hb6h operon for pHA-Hyg5-3HB6H and pHA-Hyg5*-3HB6H; to amplify ubic operon for pCS-PS-ubic; to amplify salABCD operon for pCS-Plpp0.2-tetR- SalABCD
SacI-Ter-R	gggaaaGAGCTCcaggagagcgttcaccgacaa	
XhoI-PLlacO1-F	GGAActcgagaattgtgagcggataacaattgacatt	to amplify phgA operon for pHA-phgC-phgA
BsrGI-ter-R	ggaaTGTACAtactcaggagagcgttcaccgacaaacaacaga	
SacI-XbaI-pHA-F	GGAAgagctcgtaTCTAGAgtctgacagttaccaatgcttaatcagtga	introduce XbaI site to pHA-phgC-phgA
ScaI-pHA-R	GGAAagtactcaccagtcacagaaaagcatc	
SacI-PLlacO1-F	GGAAgagctcaattgtgagcggataacaattgac	to amplify pqsD operon for pHA-phgC-phgA-pqsD
XbaI-Ter-R	ggaaTCTAGAcaggagagcgttcaccgacaaacaac	
ptetO1-SpeI-F	gggaaaACTAGTTCCCTATCAGTGATAGAGATTGACA	to amplify Pteto1-PS operon for pZE-EP-Pteto1-pqsD-sdgA
SacI-Ter-R	gggaaaGAGCTCcaggagagcgttcaccgacaa	
tetR-KpnI-F	gggaaaGGTACCATTCATTTTTCAGATCCTGTGTTAA	to amplify tetR gene for pCS-lpp0.2-tetR
tetR-BamHI-R	gggaaaGGATCCttaagacccactttcacatttaag	
P259A FL	tgccatcaaGcgaacctgcgcatcctcgatgcggtgcagga	to get pET-PqsD (P259A) and pZE-EP-Pteto1-PqsD (P259A)-SdgA based on pET-PqsD and pZE-EP-Pteto1-PqsD-SdgA
P259A FS	cgcatcctcgatgcggtgcagga	
P259A RL	caggttcgCttgatggcagatcacatggtcgatgtcgt	
P259A RS	gatcacatggtcgatgtcgt	
P259G FL	tgccatcaaGGCaacctgcgcatcctcgatgcggtgcagga	to get pqsD mutant
P259G FS	caggttGCCttgatggcagatcacatggtcgatgtcgt	
Q258A FL	tgccatGCaccgaacctgcgcatcctcgatgcggtgcagga	
Q258A RL	caggttcggtGCatggcagatcacatggtcgatgtcgt	
Q258G FL	tgccatggaccgaacctgcgcatcctcgatgcggtgcagga	
Q258G RL	caggttcggtCCatggcagatcacatggtcgatgtcgt	
Y315A FL	ctggtcctgaccGCCggctccggcgcgacctggggcgcgg	
Y315A FS	ggcgcgacctggggcgcggc	
Y315A RL	ggagccGGCggtcaggaccagcacccgctgtcccggctgga	
Y315A RS	cacccgctgtcccggctgga	
T314S FL	ctggtcctgAGCtacggctccggcgcgacctggggcgcgg	
T314S RL	ggagccgtagctcaggaccagcacccgctgtcccggct	
Q258K FL	tgccatAAGccgaacctgcgcatcctcgatgcggtgcagga	
Q258K RL	caggttcggCTTatggcagatcacatggtcgatgtcgt	
Q258E FL	tgccatGAGccgaacctgcgcatcctcgatgcggtgcagga	
Q258E RL	caggttcggCTCatggcagatcacatggtcgatgtcgt	
P259T FL	tgccatcaaACCaacctgcgcatcctcgatgcggtgcagga	
P259T RL	caggttGGTttgatggcagatcacatggtcgatgtcgt	
P259L FL	tgccatcaaCTGaacctgcgcatcctcgatgcggtgcagga	
P259L RL	caggttCAGttgatggcagatcacatggtcgatgtcgt	
T314A FL	ctggtcctgGCAtacggctccggcgcgacctggggcgcgg	

T314A RL	ggagccgtatgccaggaccagcacccgctgtcccggct	
S292A FL	atggcttcggccgccaccccggtcacgctggcgatgttct	
S292A FS	gtcacgctggcgatgttctg	
S292A RL	cggggtggcggccgaagccatgttgcccagacgatcca	
S292A RS	gttgcccagacgatccacggtca	
S292Q FL	atggcttcggcccagaccccggtcacgctggcgatgttct	
S292Q RL	cggggtctgggccgaagccatgttgcccagacgatcca	
S292T FL	atggcttcggccaccaccccggtcacgctggcgatgttct	
S292T RL	cggggtggtggccgaagccatgttgcccagacgatcca	
BamHI-hyg5-F	GGAAggatccGATGAACCCGTCCTCGTTGGTTC	to amplify hyg5 gene for pET-Hyg5
HindIII-hyg5-R	GGAAaagcttTTACATGACGACGCCCTCAATC	
BamHI-3HB6H-F	GGAAggatccGATGTCGAACCTGCAGGACGC	to amplify 3hb6h gene for pET-3HB6H
HindIII-3HB6H-R	GGAAaagcttTTACGAAGCGCGGTCCGAACTATATC	
BamHI-Ru3HB6H	GGAAggatccGatgagcagtaccaacaacgc	to amplify Re3hb6h gene for pET-Re3HB6H
HindIII-Re3HB6H-R	GGAAaagcttttacagccacggcgtggggctc	
BamHI-sdgA-His-F	gggaaaGGATCCGacgcgtgagggattcgtgccct	to amplify sdgA gene for pET-SdgA
PstI-sdgA-R	gggaaactgcagtcacaccgcctcgacggagtct	
BamHI-pqsD-F	GGAAggatccGatgggtaatccgatcctggc	to amplify pqsD gene for pET-PqsD
HindIII-pqsD-R	GGAAaagctttcaacatggccggttcacctcct	

Supplementary Results

Enzyme Engineering to Increase the Pathway Efficiency

pqsD engineering

To address by-product formation in the route I, the PqsD enzyme was rationally engineered to improve its substrate selectivity and activity for 2,5-DHBA-CoA. The feeding experiment indicated that PqsD showed relatively low efficiencies in converting 2,5-DHBA-CoA to 4,6-DHC, which was a limiting step for 4,6-DHC generation (**Fig. 3a**). Kinetic studies revealed PqsD displayed a greater substrate preference for 2-HBA-CoA (K_{cat} = 103.56±2.72 min^{-1}, K_m =27.2±3.4 μM) over 2,5-DHBA-CoA (K_{cat} = 63.28±4.17 min^{-1}, K_m = 97.3±16.7 μM) (**Table 1**). Rational protein engineering was applied to enhance PqsD selectivity and catalytic activity towards 2,5-DHBA-CoA to eliminate the byproduct production. Docking simulations were operated though AutoDock Vina[9]. The crystal structure of PqsD from *Pseudomonas aeruginosa* PAO1 (PDB code: 3H76) was selected for our work. The binding mode was found to be like the co-crystal structure of PqsD, which involves the binding of the ligand anthranilate (**Fig. 5a**)[10]. Due to the larger size of 2,5-DHBA-CoA compared to initial substrates such as anthranilate-CoA and 2-HBA-CoA, replacing residues in the active site with smaller residues will enlarge the pocket's cavity volume, potentially enhancing the interaction between the active site and 2,5-DHBA-CoA. To verify this hypothesis, residues at positions 258, 259, 292, 314 and 315 of PqsD, situated within the catalytic pocket, were selected as potential sites for optimization. Firstly, variants Q258A/G, P259A/G, S292A, T314A, Y315A were designed with the rationale to reduce the size of these locations by introducing small residues. The feeding experiment showed that only the P259A mutant produced increased titer of 4,6-DHC compared to the wild-type PqsD (1.45-fold, 38.4±1.8 mg/L *vs* 26.3±1.9 mg/L) (**Fig. 5b**). In addition to this, we postulated that hydrophobic interactions within the active pocket could play a significant role in maintaining enzyme activity. Therefore, we muted the P259 into leucine. Additionally, we assumed optimizing residues within the binding pocket, such as introducing some polar residues may enhance the possibility to form hydrogen bond between 2,5-DHBA-CoA and the receptor, thereby improving its catalytic activity. So, the following 6 single mutations were constructed: Q258K, Q258E, P259T, T314S, S292T and S292Q. The feeding assays were also carried out to test these mutants' activity. Based on the current result, we further added three combinational mutants (P259A/T314S, P259A/S292A, P259A/S292T) to potentially yield better variants. Additionally, we conducted random mutation on P259 of PqsD to further enhance catalytic activity and 25 single colonies were selected

and tested, though no better variants were selected (**Fig. 5b, Supplementary Fig. 6**). Kinetic studies with PqsD-P259A (K_{cat} = 140.92 ± 10.16 min^{-1}, K_m = 58.8 ± 8.1 μM) (**Table 1, Supplementary Fig. 7**) showed an increase in K_{cat} and a decrease in K_m toward 2,5-DHBA-CoA compared to wild-type PqsD (K_{cat} = 63.28 ± 4.17 min^{-1}, K_m = 97.3±16.7 μM). The combination of increased K_{cat} and K_m signifies PqsD-P259A mutation improves both the binding affinity and the catalytic activity of the enzyme toward 2,5-DHBA-CoA.

Supplementary References

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