Green Chemistry

Supplementary Materials for

Systematic Metabolic Engineering of *Bacillus licheniformis* for Hyperproduction of Antioxidant Hydroxytyrosol

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Table S1 Strains and plasmids used in this study

Strains	Characteristics	Sources
B. licheniformis		
DWc9n	DW2 with Cas9n integrated strain	[1]
DW3	DWc9n-*xkdE::yugJ*	Lab stock
DW4	DWc9nΔ*pyk-xkdE::yugJ*	Lab stock
DH1	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}	This study
DH2	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}	This study
DH3	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*	This study
DH4	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*	This study
DH5	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*	This study
DH6	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS*	This study
DH7	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS* Δ*dhbC*	This study
DH8	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS* Δ*dhbC*Δ*adhA*	This study
DH9	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS*Δ*dhbC*Δ*adhA-PbacA-glcU-PbacA-glcK*	This study
DH10	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS*Δ*dhbC*Δ*adhA-PbacA-glcU-PbacA-glcK-ParoK-ptsG*	This study
DH11	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*yvnA::tyrA*^{fbr}*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA* Δ*hisC*Δ*dhaS*Δ*dhbC*Δ*adhA-PbacA-glcU-PbacA-glcK-ParoK-ptsG*	This study
DH12	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*yvnA::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS* Δ*dhbC*Δ*adhA-PbacA-glcU-PbacA-glcK-ParoK-ptsG-PbacA-tkt*	This study
DH13	DWc9nΔ*pyk-xkdE::yugJ-ldh::tyrA*^{fbr}-*yvnA::tyrA*^{fbr}-*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS* Δ*dhbC*Δ*adhA-PbacA-glcU-PbacA-glcK-ParoK-ptsG-PbacA-tkt-PbacA-zwf*	This study

DH14	DWc9nΔ*pyk-xkdE::yugJ-ldh*::*tyrA*^{fbr}*-yvnA*::*tyrA*^{fbr} -*xkdG::aroG*^{fbr}-PbacA-*aroK*Δ*pheA*Δ*hisC*Δ*dhaS*Δ*dhbC*Δ*adhA-PbacA-glcU-PbacA-glcK-ParoK-ptsG- lanP-P43-Bbxfpk*^{opt}	This study
Strains	**Characteristics**	**Sources**
DHT1	DW3/pHY-*PbacA-kivD-P43-hpaBC*	This study
DHT2	DW3/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT3	DW4/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT4	DH1/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT5	DH2/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT6	DH3/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT7	DH4/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT8	DH5/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT9	DH6/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT10	DH7/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT11	DH8/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT12	DH10/pHY-*PbacA-kivD*^{VI}*-P43-hpaBC*	This study
DHT13	DH10/pHY-*PbacA-kivD*^{VI}*-PbacA-hpaBC*	This study
DHT14	DH10/pHY-*PbacA-kivD*^{VI}*-Pbay-hpaBC*	This study
DHT15	DH10/pHY-*P43-kivD*^{VI}*-P43-hpaBC*	This study
DHT16	DH10/pHY-*P43-kivD*^{VI}*-PbacA-hpaBC*	This study
DHT17	DH10/pHY-*P43-kivD*^{VI}*-Pbay-hpaBC*	This study
DHT18	DH10/pHY-*Pbay-kivD*^{VI}*-P43-hpaBC*	This study
DHT19	DH10/pHY-*Pbay-kivD*^{VI}*-PbacA-hpaBC*	This study
DHT20	DH10/pHY-*Pbay-kivD*^{VI}*-Pbay-hpaBC*	This study
DHT21	DH11/pHY-*P43-kivD*^{VI}*-Pbay-hpaBC*	This study
DHT22	DH12/pHY-*P43-kivD*^{VI}*-Pbay-hpaBC*	This study
DHT23	DH13/pHY-*P43-kivD*^{VI}*-Pbay-hpaBC*	This study
DHT24	DH14/pHY-*P43-kivD*^{VI}*-Pbay-hpaBC*	
DWc9n/pHY-*kivD*	DWc9n harboring pHY-*kivD*^{V461}, Tet^r	This study
DWc9n/pHY-*kivD*^{VY}	DWc9n harboring pHY-*PbacA-kivD*^{VY}, Tet^r	This study
DWc9n/pHY-*kivD*^{VT}	DWc9n harboring pHY-*PbacA-kivD*^{VT}, Tet^r	This study
DWc9n/pHY-*kivD*^{VF}	DWc9n harboring pHY-*PbacA-kivD*^{VF}, Tet^r	This study
DWc9n/pHY-*kivD*^{VH}	DWc9n harboring pHY-*PbacA-kivD*^{VH}, Tet^r	This study
DWc9n/pHY-*kivD*^{VR}	DWc9n harboring pHY-*PbacA-kivD*^{VR}, Tet^r	This study
DWc9n/pHY-	DWc9n harboring pHY-*PbacA-kivD*^{VA}, Tet^r	This study

Strains	Characteristics	Sources
kivD^{VA}		
DWc9n/pHY-*kivD*^{VG}	DWc9n harboring pHY-*PbacA-kivD*^{VG}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VI}	DWc9n harboring pHY-*PbacA-kivD*^{VI}, Tet^{r}	This study
Strains	**Characteristics**	**Sources**
DWc9n/pHY-*kivD*^{VM}	DWc9n harboring pHY-*PbacA-kivD*^{VM}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VS}	DWc9n harboring pHY-*PbacA-kivD*^{VS}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VN}	DWc9n harboring pHY-*PbacA-kivD*^{VN}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VK}	DWc9n harboring pHY-*PbacA-kivD*^{VK}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VD}	DWc9n harboring pHY-*PbacA-kivD*^{VD}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VE}	DWc9n harboring pHY-*PbacA-kivD*^{VE}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VW}	DWc9n harboring pHY-*PbacA-kivD*^{VW}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VP}	DWc9n harboring pHY-*PbacA-kivD*^{VP}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VQ}	DWc9n harboring pHY-*PbacA-kivD*^{VQ}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VC}	DWc9n harboring pHY-*PbacA-kivD*^{VC}, Tet^{r}	This study
DWc9n/pHY-*kivD*^{VL}	DWc9n harboring pHY-*PbacA-kivD*^{VL}, Tet^{r}	This study
DWc9n*Δadh8*	DWc9n with *adh8* deletion	Lab stock
DWc9n*ΔyimD*	DWc9n with *yimD*deletion	Lab stock
DWc9n*Δadh4*	DWc9n with *adh4* deletion	Lab stock
DWc9n*ΔyogA*	DWc9n with *yogA* deletion	Lab stock
DWc9n*ΔgbsB*	DWc9n with *gbsB* deletion	Lab stock
DWc9n*ΔybdH*	DWc9n with *ybdH*deletion	Lab stock
DWc9n*ΔadhE*	DWc9n with *adhE* deletion	Lab stock
DWc9n*ΔyfmJ*	DWc9n with *yfmJ* deletion	Lab stock
DWc9n*ΔadhB*	DWc9n with *adhB* deletion	Lab stock
DWc9n*ΔadhZ*	DWc9n with *adhZ*deletion	Lab stock
DWc9n*ΔyogA*	DWc9n with *yogA*deletion	Lab stock
DWc9n*ΔyugK*	DWc9n with *yugK*deletion	Lab stock
DWc9n*ΔyugJ*	DWc9n with *yugJ*deletion	Lab stock
DWc9n*ΔadhA*	DWc9n with *adhA* deletion	Lab stock
DWc9n/pHY-300	DWc9n harboring pHY-300, Tet^{r}	Lab stock

***Escherichia coli* DH5a**	F⁻ Φ80d/*lac*ZΔM15, Δ(*lacZYA-argF*) U169, *recA*1, *endA*1, *hsdR*17 (r_K^-, m_K^+), *phoA*, *supE*44, λ⁻, *thi*-1, *gyrA*96, *relA*1	Lab stock
BL21(DE3)	F^-, *omp*T, $hsdS_B(r_B^- m_B^-)$, *gal* (λ*cI*857, *ind*1, *Sam*7, *nin*5, *lac*UV5-T7 *gene*1), *dcm*(DE3)	Lab stock
Plasmids	**Characteristics**	**Source**
BL21(DE3)/pET-28a(+)	BL21(DE3) harboring pET-28a(+), Kan^r	Lab stock
BL21(DE3)/pET-28a(+)*kivD*	BL21(DE3) harboring pET-28a(+)*kivD*, Kan^r	This study
BL21(DE3)/pET-28a(+)*kivD*^{VI}	BL21(DE3) harboring pET-28a(+)*kivD*^{VI}, Kan^r	This study
T_2(2)-Ori	*E. coli* and *B. subtillis* shuttle vector, Kan^r	Lab stock
pHY300PLK	*E. coli-Bacillus* shuttle vector; Amp^r in *E. coli*, Tc^r in both *E. coli* and *B. licheniformis*	Lab stock
pHY-*kivD*	pHY300 containing *kivD*gene with PbacA	This study
pHY-*kivD^{VY}*	pHY300 containing *kivD^{VY}* gene with PbacA	This study
pHY-*kivD^{VT}*	pHY300 containing *kivD^{VT}* gene with PbacA	This study
pHY-*kivD^{VF}*	pHY300 containing *kivD^{VF}* gene with PbacA	This study
pHY-*kivD^{VH}*	pHY300 containing *kivD^{VH}* gene with PbacA	This study
pHY-*kivD^{VR}*	pHY300 containing *kivD^{VR}* gene with PbacA	This study
pHY-*kivD^{VA}*	pHY300 containing *kivD^{VA}* gene with PbacA	This study
pHY-*kivD^{VG}*	pHY300 containing *kivD^{VG}* gene with PbacA	This study
pHY-*kivD^{VI}*	pHY300 containing *kivD^{VI}* gene with PbacA	This study
pHY-*kivD^{VM}*	pHY300 containing *kivD^{VM}* gene with PbacA	This study
pHY-*kivD^{VS}*	pHY300 containing *kivD^{VS}* gene with PbacA	This study
pHY-*kivD^{VN}*	pHY300 containing *kivD^{VN}* gene with PbacA	This study
pHY-*kivD^{VK}*	pHY300 containing *kivD^{VK}* gene with PbacA	This study
pHY-*kivD^{VD}*	pHY300 containing *kivD^{VD}* gene with PbacA	This study
pHY-*kivD^{VE}*	pHY300 containing *kivD^{VE}* gene with PbacA	This study
pHY-*kivD^{VW}*	pHY300 containing *kivD^{VW}* gene with PbacA	This study
pHY-*kivD^{VP}*	pHY300 containing *kivD^{VP}* gene with PbacA	This study
pHY-*kivD^{VQ}*	pHY300 containing *kivD^{VQ}* gene with PbacA	This study
pHY-*kivD^{VC}*	pHY300 containing *kivD^{VC}* gene with PbacA	This study
pHY-*kivD^{VL}*	pHY300 containing *kivD^{VL}* gene with PbacA	This study
pHY-P43-*hpaBC*	pHY300 containing*hpaBC* with P43 promoter	This study
pHY-PbacA-*kivD^{VI}-P43-hpaBC*	pHY300 containing*kivD^{VI}* with PbacA promoter and *hpaBC* genes with P43 promoter, respectively	This study
pHYΔ*adhA*	pHY300 containingP43 (no RBS) +sgRNA (*adhA*)+*adhA* up arm+ down arm	Lab stock
pHYΔ*pheA*	pHY300 containingP43 (no RBS) +sgRNA (*pheA*) +*pheA* up arm+ down arm	This study
pHYΔ*hisC*	pHY300 containingP43 (no RBS) +sgRNA (*hisC*)	Lab stock

	+*hisC* up arm+ down arm	
pHYΔ*dhaS*	pHY300 containingP43 (no RBS) +sgRNA (*dhaS*)+*dhaS* up arm+ down arm	Lab stock
pHYΔ*dhbC*	pHY300 containingP43 (no RBS) +sgRNA (*dhbC*)+*dhbC* up arm+ down arm	This study

Plasmids	**Characteristics**	**Source**
T_2(2)-*ldh-tyrA*^{fbr}	T_2(2) containing *ldh* up arm+PylB+*tyrA*^{fbr}+*ldh* down arm	This study
T_2(2)-*yvnA-tyrA*^{fbr}	T_2(2) containing *yvnA* up arm+P43+*tyrA*^{fbr}+*yvnA* down arm	This study
T_2(2)-*xkdE-aroG*^{fbr}	T_2(2) containing *xkdE* up arm+P43+ *aroG*^{fbr}+TamyL+*xkdE* down arm	Lab stock
T_2(2)-PbacA-*aroK*	T_2(2) containing P*aroK* up arm+PbacA+P*aroK* down arm	Lab stock
T_2(2)-PbacA-*glcU*	T_2(2) containing P*glcU* up arm+PbacA+P*glcU* down arm	Lab stock
T_2(2)-PbacA-*glcK*	T_2(2) containing P*glcK* up arm+PbacA+P*glcK* down arm	Lab stock
T_2(2)-ParoK-*ptsG*	T_2(2) containing *PptsG* up arm+P*aroK*+*PptsG* down arm	Lab stock
T_2(2)-PbacA-*tkt*	T_2(2) containing *Ptkt* up arm+P*bacA*+*Ptkt* down arm	This study
T_2(2)-PbacA-*zwf*	T_2(2) containing *Pzwf* up arm+P*bacA*+*Ptkt* down arm	This study

Table S2 The varied metabolites between the DHT12 and DHT17 strains

	Ratio (DHT17/DHT12)		
Metabolites name	**24 h**	**36 h**	**48 h**
Up-regulation			
D-Fructose 6-phosphate	4.60	4.94	/
Xylose	22.70	4.47	1.91
Gluconic acid	/	45.11	3.83
D-Xylulose	9.63	/	6.00
Xylitol	20.82	6.40	3.03
Lyxose	17.30	6.05	3.06
4-Hydroxyphenylpyruvic acid	down	/	2.37
Maleamic acid	4.42	35.44	7.02
N-Acetyl-D-hexosamine	2.22	3.12	4.25
Tyrosol	2.92	2.33	1.91
Down-regulation			
L-Methionine	0.35	/	/
L-Leucine	0.26	0.50	0.24
L-Valine	0.19	0.20	0.14
L-Threonine	/	/	0.10
Malonic acid	/	/	0.17
D-Isocitric acid	0.29	/	/
D-(+)-Glucosamine	0.40	/	/
L-Isoleucine	0.44	/	0.23
N-alpha-Acetyl-L-ornithine	0.08	0.13	0.08
Oleic acid	0.36	/	/
Pantothenic acid	0.34	/	/
4-Hydroxyphenylpyruvic acid	0.36	/	up
D-Trehalose	/	0.45	
L-Iditol	/	/	0.30
Stearic acid	/	/	0.09
Gly-Gly	/	0.43	/

The varied metabolites between the DHT12 and DHT17 strains were listed based on ratios >2 and p-values < 0.05 for the Student's T-test. "/" means that the abundance of metabolite is not significant in this time point.

Table S3 Summary of hydroxytyrosol (HT) production from simple carbon sources by engineered microorganisms

Strains	Carbon source	Characteristics	HT titer (mg/L)	HT yield mg/g	Produc-tivity (mg/L/h)	Refer-ences
E. coli	Glucose	*TH, PCD, DHPR, DDC* and *TYO*, Δ*feaB*	12.3 shake flasks	1.2	0.17	[2]
E. coli	Glucose	*aroG^{fbr},tyrA^{fbr}, TDC, TYO, and hpaBC,* Δ*tyrR*Δ*pheA*Δ*feaB*	268.3 shake flasks	Not shown	8.9	[3]
E. coli	Glucose	*aroE, aroD,aroB^{OPT},aroG^{fbr}, ppsA, tktA, tyrB, tyrA^{fbr} ,aroC, aroA,aroL, PcAAS, RsTYR, yqhD*	270.8 shake flasks	27.1	6.0	[4]
E. coli	Glucose glycerol	*tyrA^{fbr}, ppsA, tktA, aroG^{fbr}, ARO10, ADH6, hpaBC*Δ*feaB*Δ*pheA*Δ*tyrA*	647.0 shake flasks	34.2	13.5	[5]
E. coli	Glucose	*ΔfeaB ΔpheAΔtyrBΔtyrRΔtrpE ΔpabB ΔpabAΔpykFΔpoxB, 5*ARO10*, EchpaBC*	1810.0 shake flasks	90.5	37.7	[6]
			2950.0 5L bioreactor	Not shown	122.9	
S. cerevisiae	Sucrose glycerol	*ARO4*^{K229L}, *ARO7*^{G141S}, *Bbxfpk*^{opt}, *Pcaas*^{opt}, *PahpaBC*^{opt}, ΔGAL80	308.7 shake flasks	Not shown	3.2	[7]
S. cerevisiae	Glucose	*ARO3*^{D154N}, *ARO4*^{K229L}, *ARO7*^{G141S}, *ARO10, EchpaBC,*	375.0 shake flasks	2.2	1.5	[8]
S. cerevisiae	Glucose	*ARO2, ARO3*^{D154N}, *ARO4*^{K229L}, *ARO7*^{G141S}, *ARO10, TKL1, RKI1, PahpaB, EchpaC, TRP2*↓, Δ*TRP1*	1120.0 shake flasks	56	13.3	[9]
			6970.0 5L bioreactor	36.5	79.2	
B. licheniformis	Glucose	*aroG^{fbr},2*tyrA^{fbr}, aroK, EchpaBC, yugJ, kivD*^{VI}, *glcU, glcK, tkt, zwf* Δ*pyk*Δ*hisC*Δ*pheA*Δ*dhaS*Δ*dhbC*Δ*adhA,ptsG*↓	6299.0 shake flasks	157.3	131.2	This study
			9475.0 5L bioreactor	135.4	395.0	

↓：means: Down-regulation of gene expression

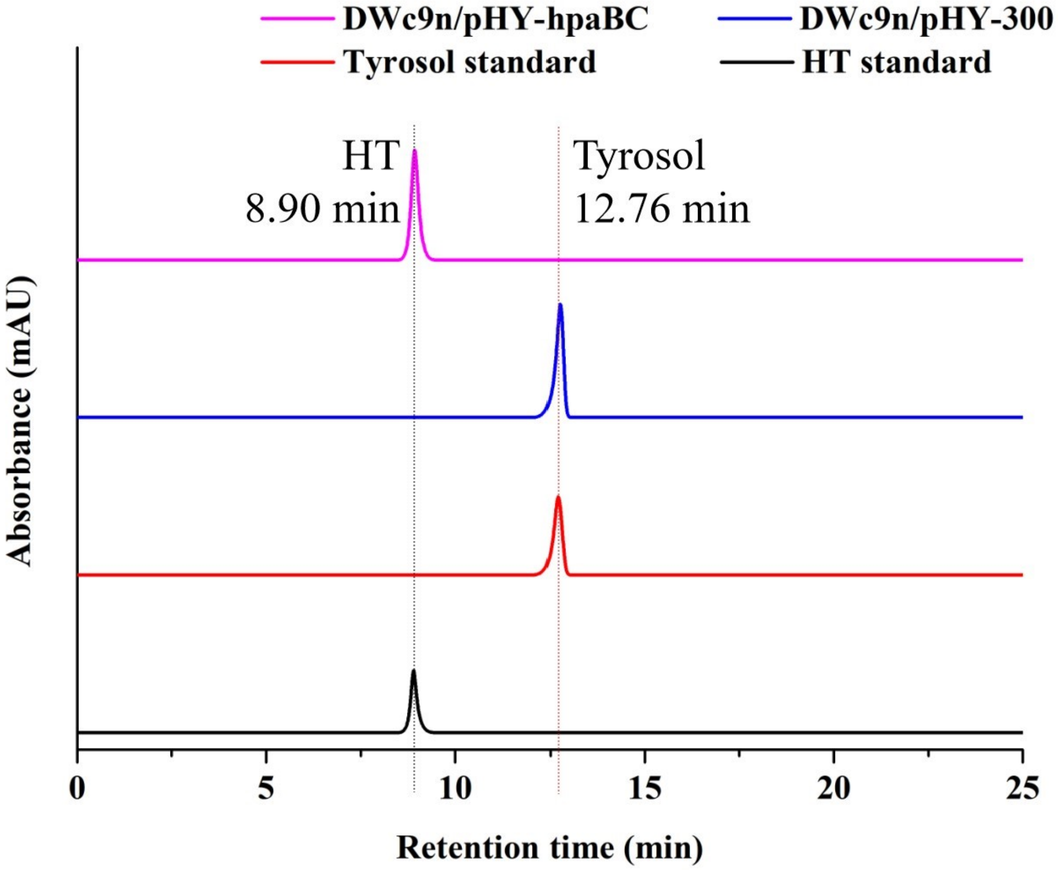
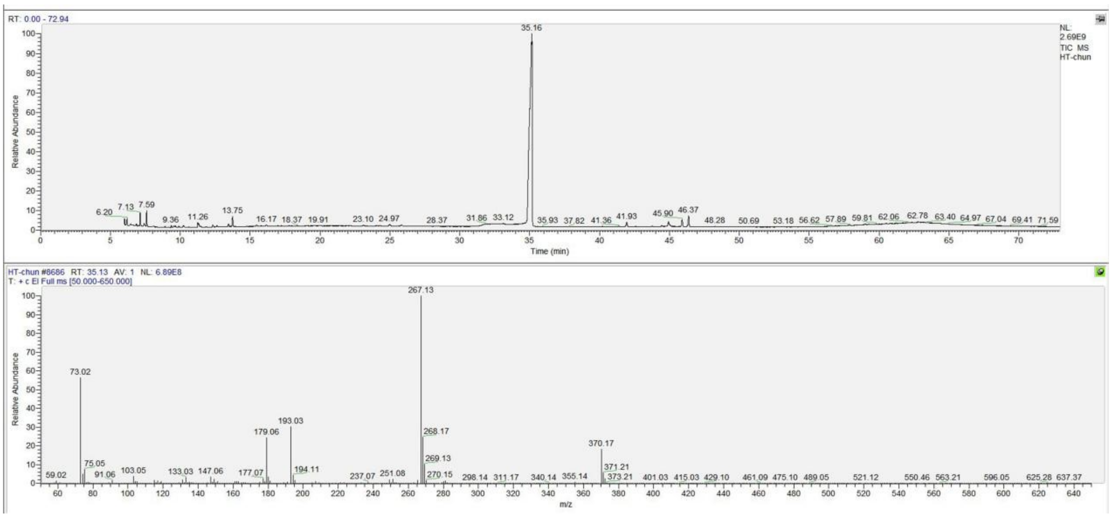


Fig. S1 HPLC spectra of HT and tyrosol

a HT sample



b HT standard

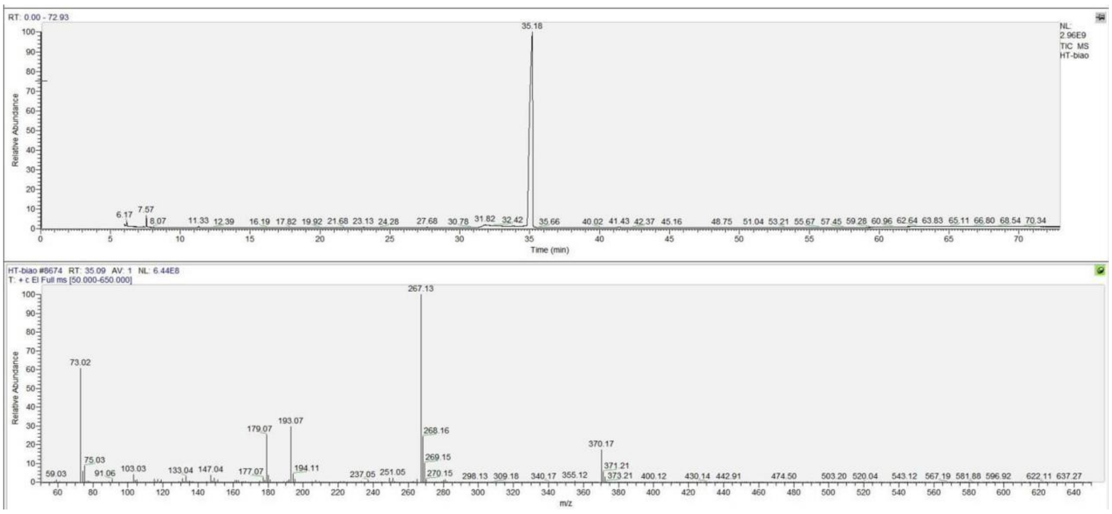
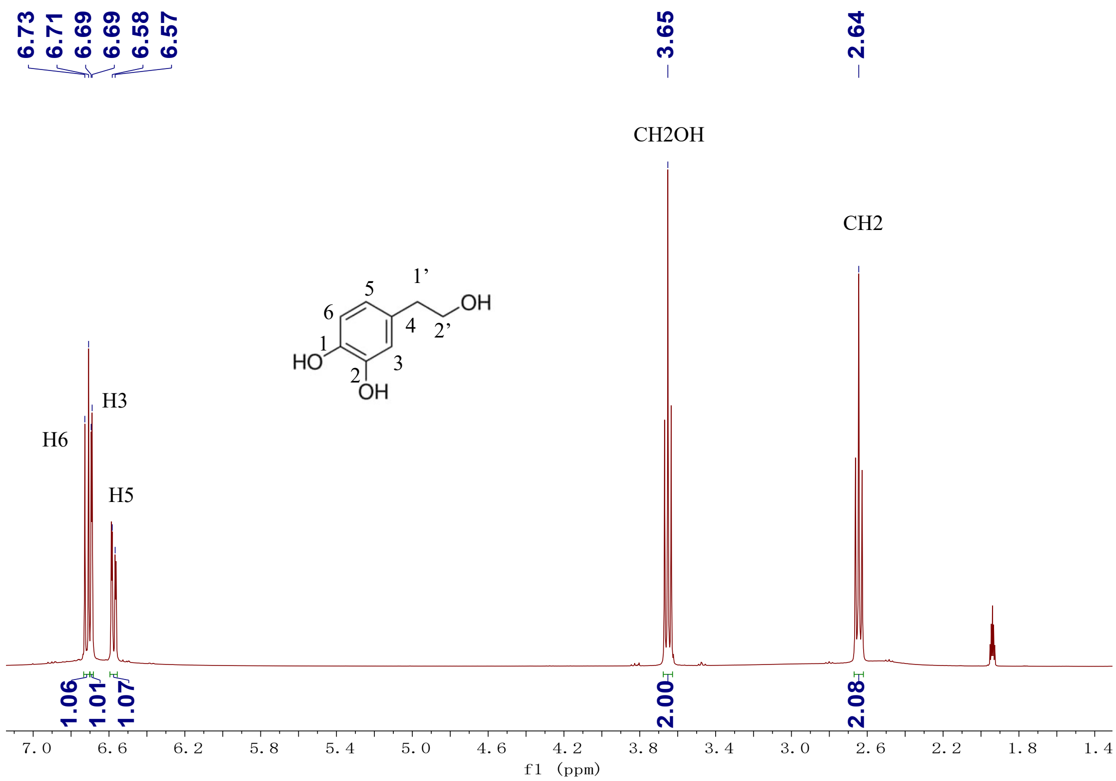


Fig. S2 The results of GC-MS of HT sample and standard

^{1}H NMR



^{13}C NMR

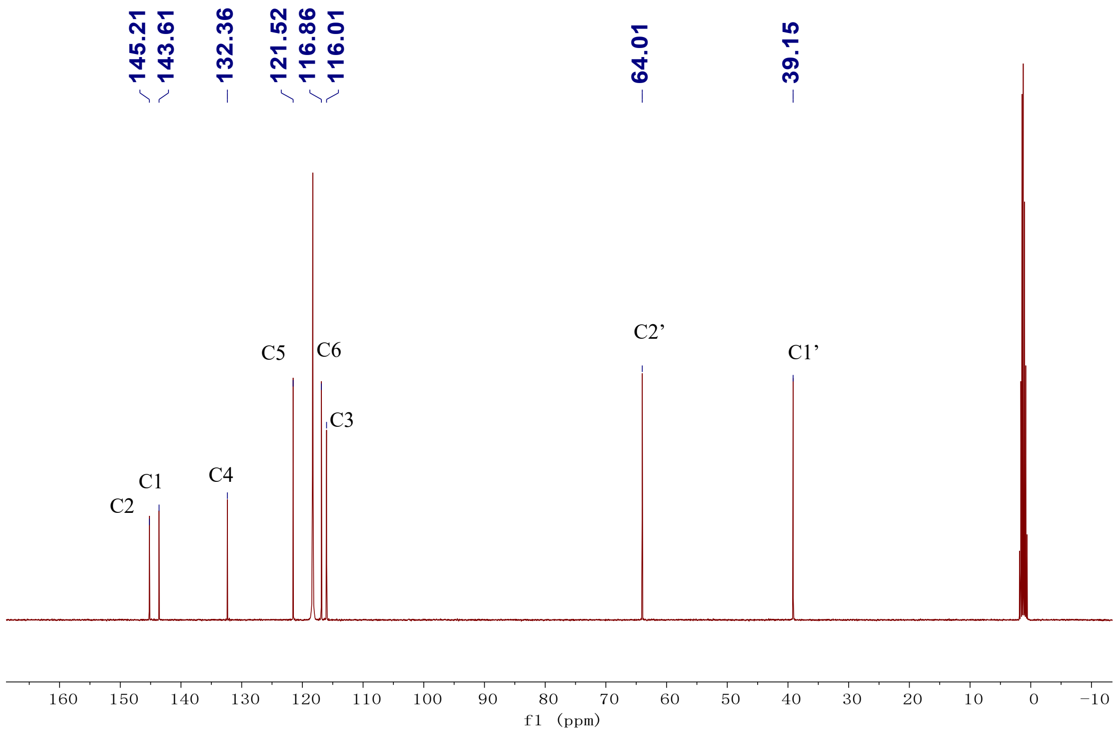


Fig. S3 ^{1}H and ^{13}C NMR spectra of purified HT.

The chemical shifts are in parts per million (Solvent:Acetonitrile-d3)

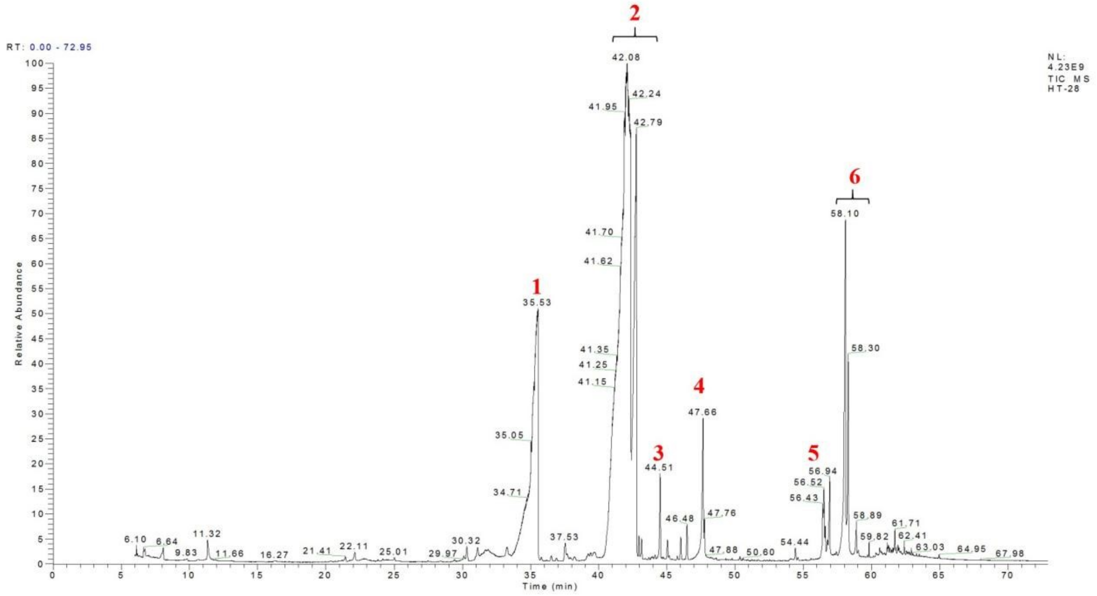


Fig. S4 GC-MS analysis results of the main extracellular metabolites of DHT23 strain at 24 h. 1: HT, 2: glucose, 3: 2, 5-dihydroxymandelic acid, 4: galactose, 5: 3,4-Dihydroxyphenylacetic acid, 6: N-Acetyl-D-galactosamine

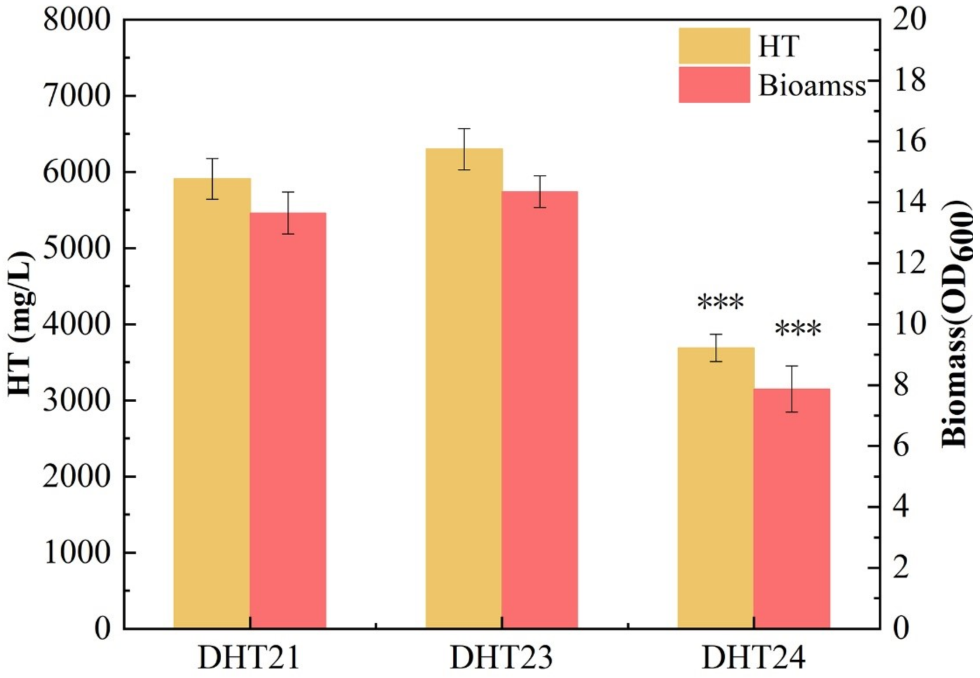


Fig. S5 Effects of *xfpK* introduction on HT production

***, p<0.001 indicate the significance levels between DHT21 and DHT24 introducing *xfpK* strains.

DNA sequence of TyrA^{fbr}

ATGGTTGCTGAATTGACCGCATTACGCGATCAAATTGATGAAGTCGATAA
AGCGCTGCTGAATTTATTAGCGAAGCGTCTGGAACTGGTTGCTGAAGTGG
GCGAGGTGAAAAGCCGCTTTGGACTGCCTATTTATGTTCCGGAGCGCGAG
GCATCTATCTTGGCCTCGCGTCGTGCAGAGGCGGAAGCTCTGGGTGTACC
GCCAGATCTGATTGAGGATGTTTTGCGTCGGGTGATGCGTGAATCTTACTC
CAGTGAAAACGACAAAGGATTTAAAACACTTTGTCCGTCACTGCGTCCGG
TGGTTATCGTCGGCGGTGGCGGTCAGATGGGACGCCTGTTCGAGAAGATG
CTGACCCTCTCGGGTTATCAGGTGCGGATTCTGGAGCAACATGACTGGGA
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DNA sequence of AroG^{fbr}

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DNA sequence of KivD^{VI}
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DNA sequence of AdhA
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