Supporting Information

Highly efficient biosynthesis of heliotropin by engineered *Escherichia coli* co-expressing trans-anethole oxygenase and formate dehydrogenase

Peng Wen, Dan Wu, Pu Zheng,* Pengcheng Chen, Siqin Liu and Yin Fu

The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China

*Corresponding author: The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, People's Republic of China.

Tel./fax: +86 510 8591 8156.

E-mail address: zhengpu@jiangnan.edu.cn (P. Zheng).

Supplementary materials

Supplementary Table 1. Strains and plasmids used in this study

Supplementary Table 2. Primers used in this study.

Supplementary Fig. 1A. Construction of the recombinant plasmid pETDuet-1-*tao*.

Supplementary Fig. 1B. Construction of the recombinant plasmid pCDFDuet-1-*fdh*.

Supplementary Fig. 1C. Construction of the recombinant plasmid pETDuet-1-*fdh*.

Supplementary Fig. 1D. Construction of the co-expression recombinant plasmid pETDuet-1-*tao-fdh*.

Supplementary Fig. 1E. Construction of the co-expression recombinant plasmid pETDuet-1-*fdh-tao* or pETDuet-1-*fdh* -tao_{3g2}.

Supplementary Fig. 2. The process of high throughput screening mutants for directed evolution.

Supplementary Fig. 3. The homogenization of insoluble substrate by Tween-80.

Supplementary Fig. 4. Coloration of aldehyde and DNPH and full wavelength scanning of benzoquinone. (A) Coloration of heliotropin and DNPH. 1: heliotropin; 2: phenylhydrazone; 3: benzoquinone; 4: DNPH; 5: DNPH and alkalines; (B) Wavelength scanning of benzoquinone.

Supplementary Fig. 5. Optimization of benzoquinone chromogenic method in high-throughput screening. (A) Effects of KOH and NaOH on color development. (B) Effects of concentrations of NaOH on color development. (C) Effects of methanol and ethanol on color development. (D) Effects of concentrations of ethanol on color development.

Supplementary Fig. 6. Preliminary screening and rescreening colonies of the mutant library. (A) Primary screening of 96-well plates. (B) Re-screening of shake flasks. The red horizontal line in (A) indicates the value of the OD_{470} obtained by the original, and the line in (B) indicates that the original was set as a control. Error bars represent the SD of the mean calculated for three replicates.

Supplementary Fig. 7. Extraction and confirmation of heliotropin in biotransformation by DE3/FDH-TAO_{3G2} whole cells. (A) Transformed supernatant by the conversion of isosafrole. (B) Obtaining the crystals heliotropin. (C) Confirmation of the sample and standard of heliotropin by Fourier infrared spectroscopy (NEXUS, USA). (D) The purity of the extracted heliotropin analyzed by HPLC.

Supplementary Table 1. Strains and plasmids used in this study.

Name	Description	Rources
Strains		
DE3/TAO	BL21(DE3) carrying pETDuet-1-*tao*	This study
DE3/TAO_{3G2}	BL21(DE3) carrying pETDuet-1-tao_{3g2}	This study
DE3/TAO_{4C7}	BL21(DE3) carrying pETDuet-1-tao_{4c7}	This study
DE3/TAO_{5F4}	BL21(DE3) carrying pETDuet-1-tao_{5f4}	This study
DE3/TAO_{5D1}	BL21(DE3) carrying pETDuet-1-tao_{5d1}	This study
DE3/FDH	BL21(DE3) carrying pCDFDuet-1-*fdh*	This study
DE3/TAO+FDH	BL21(DE3) carrying pETDuet-1-*tao* and pCDFDuet-1-*fdh*	This study
DE3/TAO-FDH	BL21(DE3) carrying pETDuet-1-*tao-fdh*	This study
DE3/FDH-TAO	BL21(DE3) carrying pETDuet-1-*fdh-tao*	This study
DE3/FDH-TAO+FDH	BL21(DE3) carrying pETDuet-1-*fdh-tao* and pCDFDuet-1-*fdh*	This study
DE3/ FDH-TAO_{3G2}	BL21(DE3) carrying pETDuet-1-fdh-tao_{3g2}	This study
Plasmids		
pETDuet-1	Double T7 promoters, pBR322 ori, Amp^R	Novagen
pCDFDuet-1	Double T7 promoters, CloDF13 ori, Sm^R	Novagen
pET-28a-*fdh*	pET-28a carrying codon-optimized FDH gene	GENEWIZ，Nanjing
pETDuet-1-*tao*	pETDuet-1 carrying TAO gene	This study
pETDuet-1-tao_{3g2}	pETDuet-1 carrying TAO_{3G2} gene	This study
pCDFDuet-1-*fdh*	pCDFDuet-1 carrying codon-optimized FDH gene	This study
pETDuet-1-*fdh*	pETDuet-1 carrying codon-optimized FDH gene	This study
pETDuet-1-*tao-fdh*	pETDuet-1 carrying TAO gene and codon-optimized FDH gene	This study

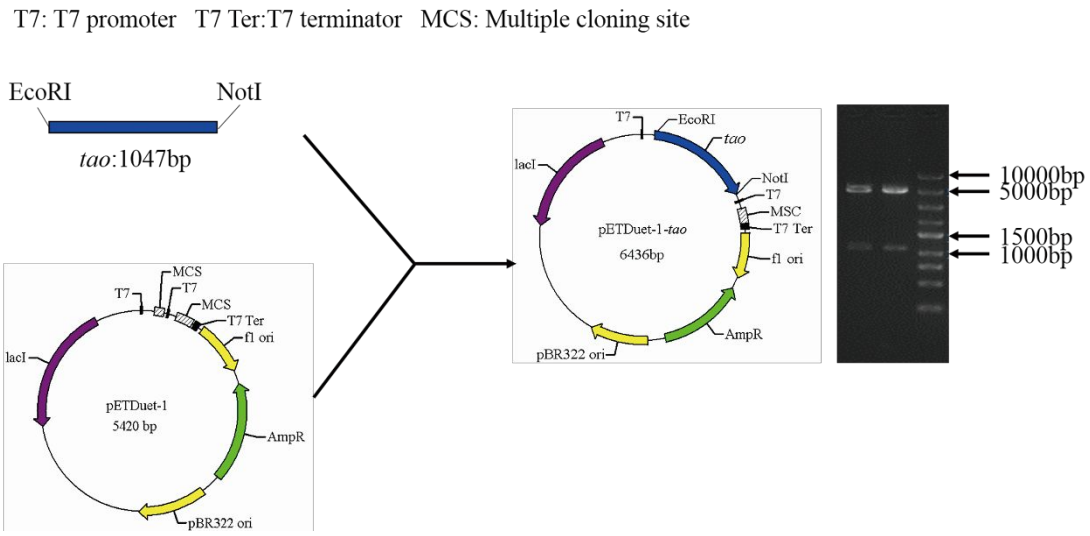
pETDuet-1-*fdh-tao*	pETDuet-1 carrying codon-optimized FDH gene and TAO gene	This study
pETDuet-1-*fdh-tao*$_{3g2}$	pETDuet-1 carrying codon-optimized FDH gene and TAO$_{3G2}$ gene	This study

The GenBank ID of codon-optimize FDH gene is MN180062. And the GenBank ID of four mutants TAO genes, named tao$_{4c7}$, tao$_{5f4}$, tao$_{5d1}$ and tao$_{3g2}$, are MN180063, MN180064, MN180065, and MN180066, respectively.

Supplementary Table 2. Primers used in this study.

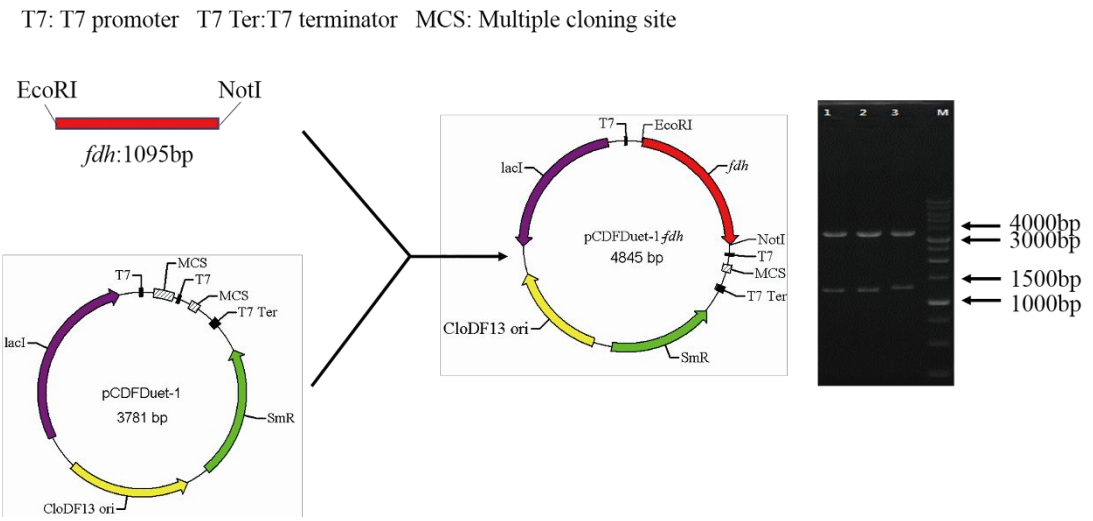
Name	Sequence
taoCF	CCAGCAAGTATATAGCATGGCCTTTGC
taoCD	CCGCTTACAGACAAGCTGTGAC
taoU1	CCACAGCCAGGATCCGAATTCATGGAGGACATCATGCAAGGC（*EcoR*I）
taoB1	CGACTTAAGCATTATGCGGCCGCTCAGTTAGTCCTCAAGTCGGAATTG（*Not*I）
fdhU1	AGATATACATATGGCAGATCTATGAAAATTGTGCTGGTGCTGTA（*BgI*II）
fdhB1	GGTTTCTTTACCAGACTCGAGTTATTTCTTATCATGTTTGCCATAGGC（*Xho*I）
taoU2	AGATATACATATGGCAGATCTATGGAGGACATCATGCAAGGC（*BgI*II）
taoB2	GGTTTCTTTACCAGACTCGAGTCAGTTAGTCCTCAAGTCGGAATTG（*Xho*I）
fdhU2	CCACAGCCAGGATCCGAATTCATGAAAATTGTGCTGGTGCTGTA（*Eco*RI）
fdhB2	CGACTTAAGCATTATGCGGCCGCTTATTTCTTATCATGTTTGCCATAGGC（*Not*I）

Supplementary Fig. 1A



Supplementary Fig. 1A. Construction of the recombinant plasmid pETDuet-1-*tao*. The recombinant strains were verified by enzyme digestion of Quick EcoRI and NotI. The strip above was the linearized pETDuet-1 and the strip below was *tao* gene.

Supplementary Fig. 1B

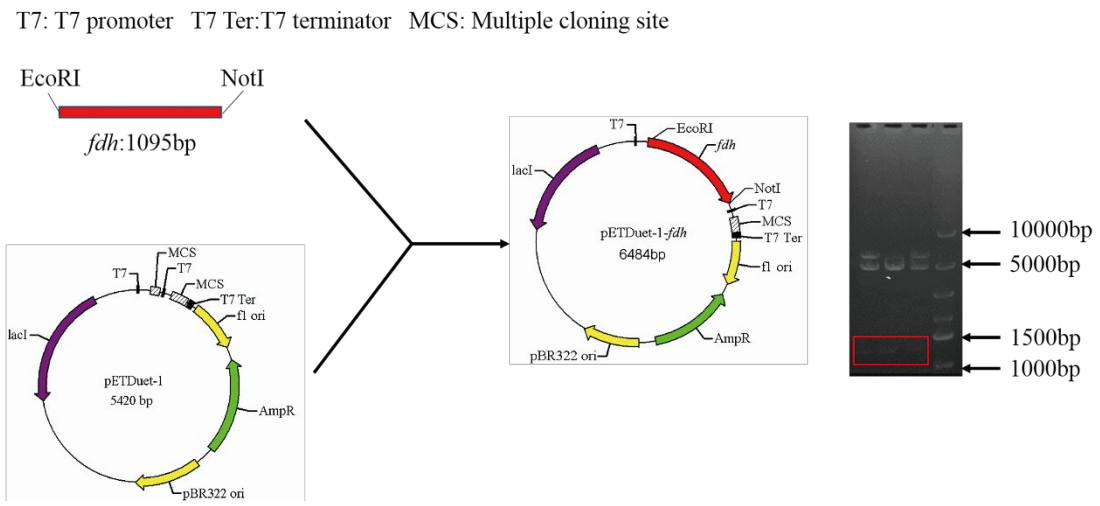


Supplementary Fig. 1B. Construction of the recombinant plasmid pCDFDuet-1-*fdh*.

The recombinant strains were verified by enzyme digestion of Quick EcoRI and NotI.

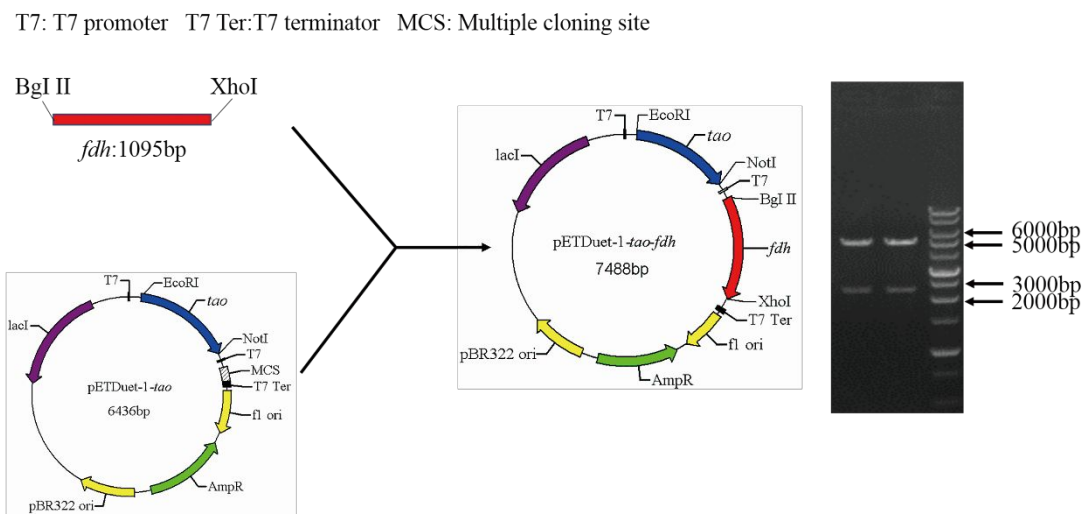
The strip above was the linearized pCDFDuet-1 and the strip below was *fdh* gene.

Supplementary Fig. 1C



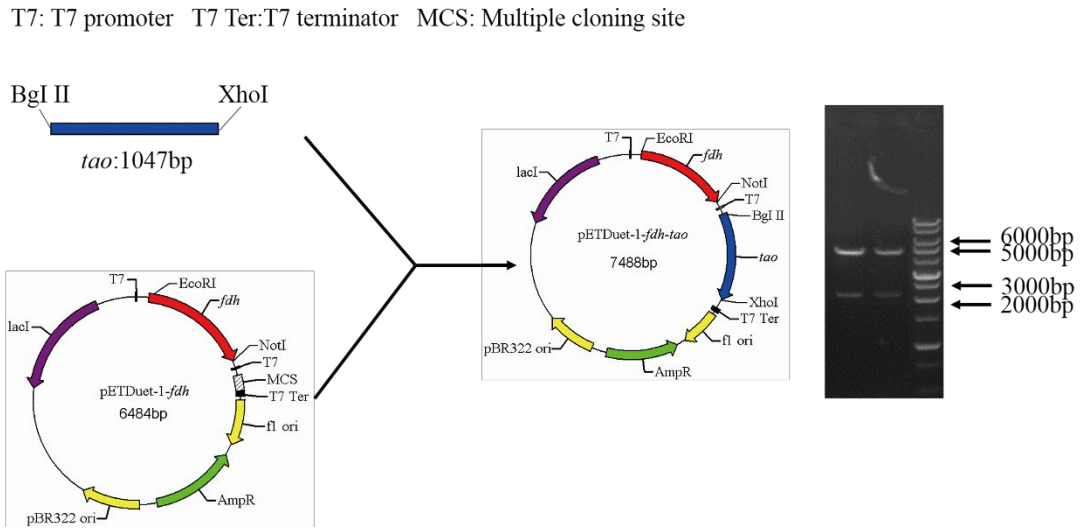
Supplementary Fig. 1C. Construction of the recombinant plasmid pETDuet-1-*fdh*. The recombinant strains were verified by enzyme digestion of Quick EcoRI and NotI. The strip above was the recombinant plasmid pETDuet-1-*fdh*, the middle strip was the linearized pETDuet-1 and the strip below was *fdh* gene.

Supplementary Fig. 1D



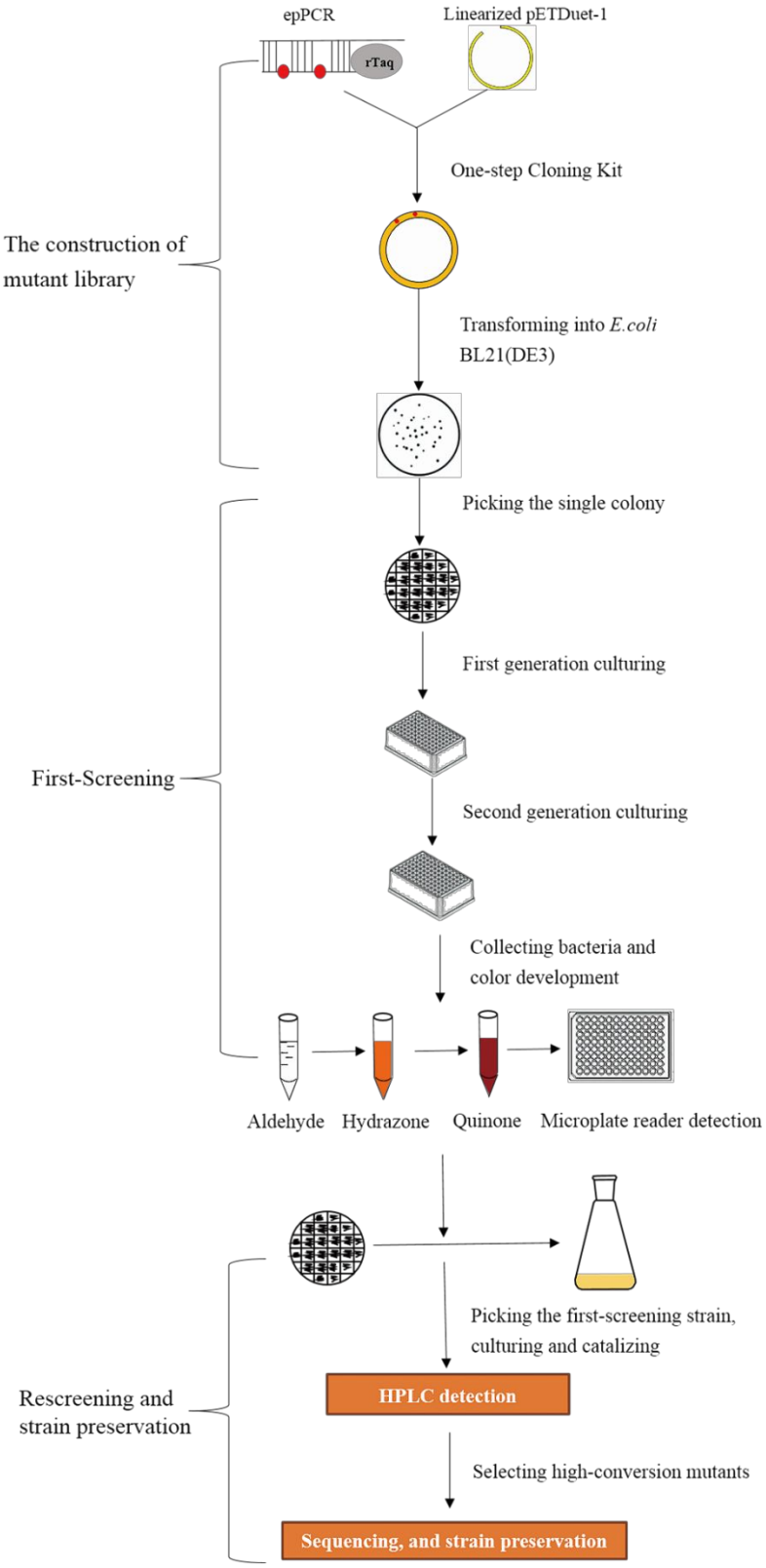
Supplementary Fig. 1D. Construction of the co-expression recombinant plasmid pETDuet-1-*tao-fdh*. The co-expression recombinant strains were verified by enzyme digestion of Quick EcoRI and XhoI. The strip above was the linearized pETDuet-1 and the strip below was the combination of *tao* gene and *fdh* gene.

Supplementary Fig. 1E



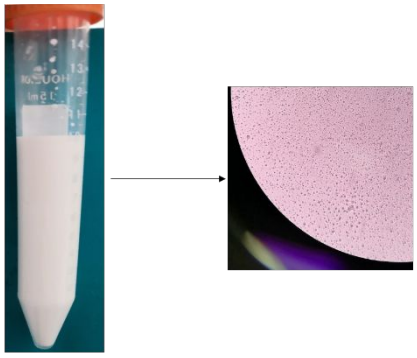
Supplementary Fig. 1E. Construction of the co-expression recombinant plasmid pETDuet-1-*fdh*-*tao* or pETDuet-1-*fdh* -tao_{3g2}. The co-expression recombinant strains were verified by enzyme digestion of Quick EcoRI and XhoI. The strip above was the linearized pETDuet-1 and the strip below was the combination of *fdh* gene and *tao* gene.

Supplementary Fig. 2



Supplementary Fig. 2. The process of high throughput screening mutants for directed evolution.

Supplementary Fig. 3

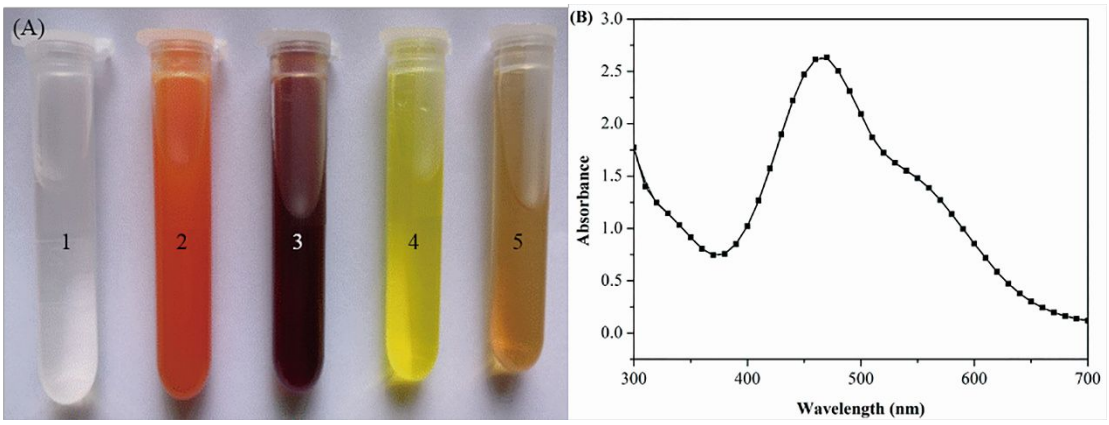


Emulsion

Substrate particles under the microscope

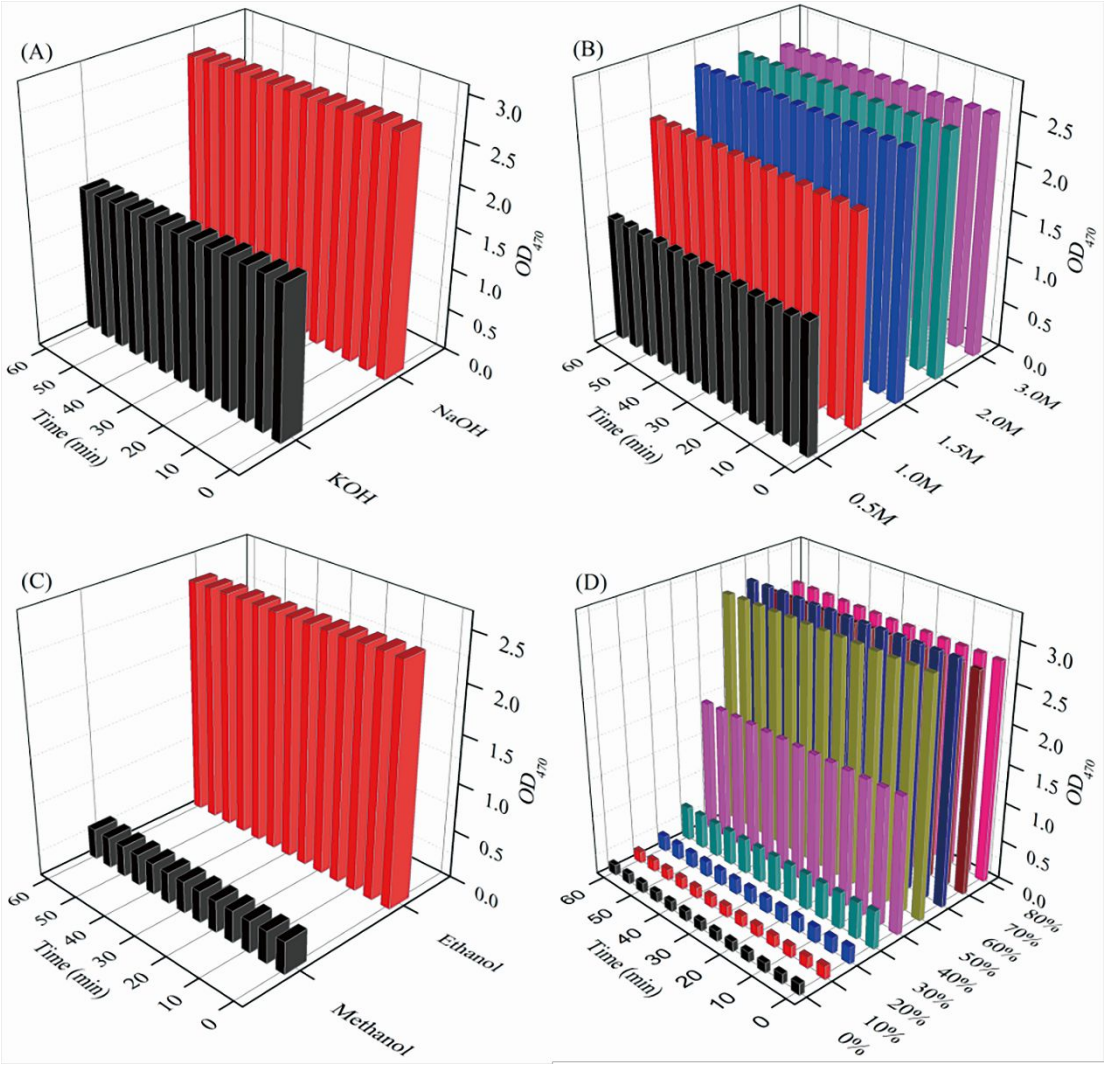
Supplementary Fig. 3. The homogenization of insoluble substrate by 1% of Tween-80. The left picture showed that isosafrole emulsion was obtained by adding Tween-80 and glass beads with small diameter, remixed and homogenized by a shaker for 15min. And the state of emulsified mixture can be stable for a long time. The right picture is one that homogenized isosafrole particles were observed under the microscope (100×).

Supplementary Fig. 4



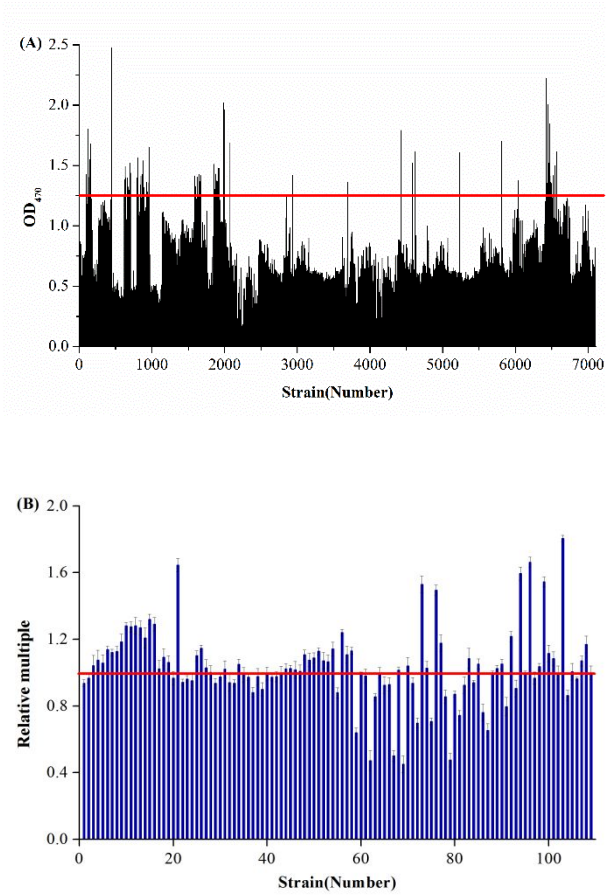
Supplementary Fig. 4. Coloration of aldehyde and DNPH and full wavelength scanning of benzoquinone. (A) Coloration of heliotropin and DNPH. 1: heliotropin; 2: phenylhydrazone; 3: benzoquinone; 4: DNPH; 5: DNPH and alkalines; (B) Wavelength scanning of benzoquinone.

Supplementary Fig. 5



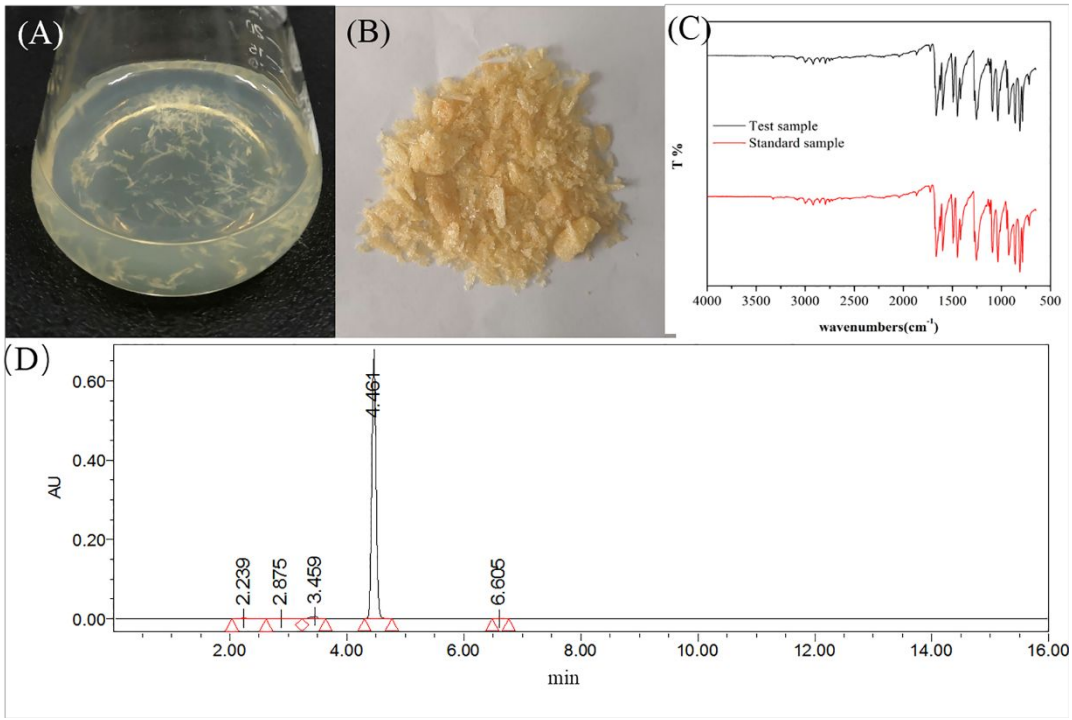
Supplementary Fig. 5. Optimization of benzoquinone chromogenic method in high-throughput screening. (A) Effects of KOH and NaOH on color development. (B) Effects of concentrations of NaOH on color development. (C) Effects of methanol and ethanol on color development. (D) Effects of concentrations of ethanol on color development.

Supplementary Fig. 6



Supplementary Fig. 6. Preliminary screening and rescreening colonies of the mutant library. (A) Primary screening of 96-well plates. (B) Re-screening of shake flasks. The red horizontal line in (A) indicates the value of the OD_{470} obtained by the original, and the line in (B) indicates that the original was set as a control. Error bars represent the SD of the mean calculated for three replicates.

Supplementary Fig. 7



Supplementary Fig. 7. Extraction and confirmation of heliotropin in biotransformation by DE3/FDH-TAO_{3G2} whole cells. (A) Transformed supernatant was obtained after conversion and the white acicular crystal (heliotropin) was observed under 4 °C overnight. (B) The helitrotropin crystals were obtained after washing by water and drying by vacuum. (C) The sample and standard sample of helitrotropin were confirmed by Fourier infrared spectroscopy (NEXUS, USA). There are absorption peaks at 2850 cm^{-1} and 1720 cm^{1}, which indicate aldehyde-based C-H and aldehyde-based C=O stretching vibrations. Peaks with different intensities appear at 1600 cm^{-1}, 1500 cm^{-1}, and 1450 cm^{-1}, indicate the presence of an aromatic ring. An absorption peak at 1250 cm^{-1} indicates stretching vibration at the C=O bond. An absorption peak at 1030 cm^{-1} indicates two C=O stretching vibrations on the benzene ring. The absorption peak at 864 cm^{-1}, 810 cm^{-1} indicates the stretching vibration of 1, 2, and 4 substitutions on the

benzene ring. The above data fully proved that the product was heliotropin. And two samples (experimental samples and standards) were tested and two infrared images are the same, further confirming the product was heliotropin. (D) The purity of the extracted heliotropin analyzed by HPLC. The purity of heliotropin can reach 97.74%, compared to the standard (Fig. 5A). The retention time of heliotropin above was around 4.4 min, which is consistent with the standard (Fig. 5A). Some unknown substances of which retention time was not 4.4 min, and occupied a few proportion about 2%.