The experimental objective in July: Construct and validate shikimic acid sensor, test tryptophan in knockout strains, and evolve BWΔtrpRΔtnaAB to boost yield.

## Week1: 7.1-7.7:

### (A) pYB1a- hucR-eGFPPlasmid Construction:

### 1. pYB1a Vector PCR Amplification

The target carrier fragment was obtained via PCR. The RCR system is as follows:

| PCR system (50μL) | |
|---|---|
| 2×Mix | 25ul |
| pYB1a-F-0626 | 2ul |
| YB1a-R-0626 | 2ul |
| Template | 1ul |
| DDW | 20ul |

| PCR | | |
|---|---|---|
| 98℃ | 5min | |
| 98℃ | 30s | ×30 |
| 57℃ | 30s | |
| 72℃ | 136s | |
| 72℃ | 5min | |
| 25℃ | ∞ | |

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 4529 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

### 2. PCR Amplification of HucR Gene Fragments

The target fragment was obtained via PCR. The RCR system is as follows.

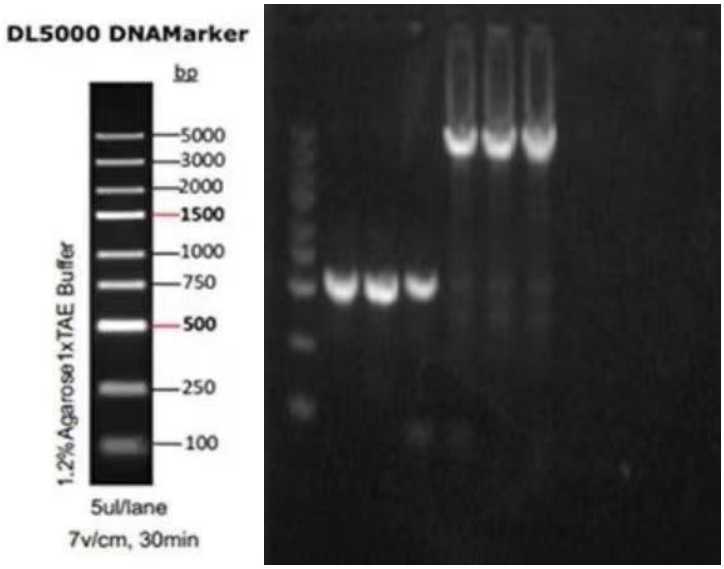
| PCR system (50μL) | |
|---|---|
| 2×Mix | 25ul |
| HucR-F | 2ul |
| HucR-R | 2ul |
| template | 1ul |
| DDW | 20ul |

| PCR | | |
|---|---|---|
| 98℃ | 5min | |
| 98℃ | 30s | ×30 |
| 52℃ | 30s | |
| 72℃ | 30s | |
| 72℃ | 5min | |
| 25℃ | ∞ | |

The PCR products were analyzed by agarose gel electrophoresis, revealing the correct target fragment length of 553 bp. We obtained the correct target fragment and proceeded with gel extraction and recovery of the sample.

### 3.Rubber Recycling

The correct band was excised from the gel and recovered. The PCR products were analyzed by agarose gel electrophoresis, yielding the following results:




**4. Use the Gibson assembly method to connect two fragments.**

The specific procedure is as follows:

Using Gibson (C117) ligation, the pYB1a-HUCR plasmid was obtained, with PYB1a being 4529 bp and HUCR being 553 bp.

| Gibson system | |
|---|---|
| 0.02×4529bp | ng |
| 0.04×553bp | ng |
| 2×c117 Mix | 5ul |
| DDW | to10ul |

| Gibson | |
|---|---|
| 50℃ | 15min |
| 4℃ | ∞ |

## 5.Chemical Conversion

Using chemical transformation, the ligation products were transformed into DH5α competent cells and then spread onto LB plates containing Amp resistance. The plates were incubated overnight at 37°C.

## 6.Selective Cloning Inoculation

After overnight incubation at 37°C, multiple colonies grew on the plate. One colony was randomly selected, and a single-colony inoculum was transferred to liquid medium for incubation at 37°C for 12 hours.

## Plasmid extraction

the sequencing results for pYB1a-HucR are correct.

## (B) pYB1a-hucR-eGFP Functional Validation:

## 1.Conversion

After transforming BW25113 competent cells with the correctly sequenced pYB1a-hucR-eGFP plasmid, plate the cells on LB agar containing ampicillin and incubate overnight at 37°C. Expected resistant single colonies will grow on the plates. Subsequently, select individual colonies for plasmid extraction and verification.

## 2.Inoculation

Select a single colony and inoculate it into liquid medium. Incubate at 37°C for 12 hours.

## 3.Prepare M9 medium

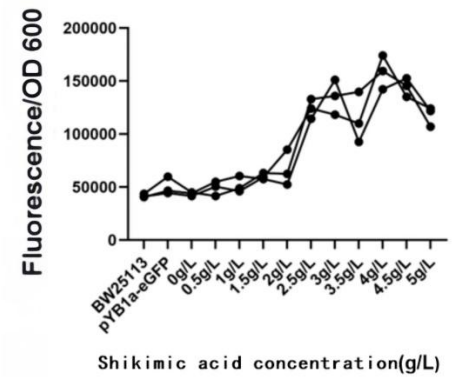
| M9 medium | |
|---|---|
| 5*M9 | 2mL |
| 20% Glycerin | 1mL |
| MgSO4 | 20μL |
| CaCl2 | 1μL |
| Amp | 10μL |
| ddH2O | To 10mL |

### 4.Induced culture

Configure 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 g/L. Set up BW parent strain and pYB1a-eGFP controls. Add 500 μL M9 medium, 500 μL shikimic acid solution, and 10 μL bacterial suspension to a 96-well deep-well plate. Incubate at 30°C for 18 hours. Three parallel replicates were performed for each concentration gradient. Transfer 200 μL from each well to a microplate and measure fluorescence and OD values using a microplate reader.

### 5.Enzyme-linked immunosorbent assay reader for measuring fluorescence and optical density (OD) values

Results: The data were unstable, and repeated testing revealed that the fluorescence intensity of the BW wild-type strain consistently exceeded that of pYB1a-eGFP. Analysis indicated that the fluorescence signal was not effectively detected, resulting in response distortion.




## （C）Exploration of Error prone PCR Conditions

### 1.Error prone PCR

Based on the principles of error-prone PCR, we used AtCOMT*** obtained from literature review as the template. By adding appropriate concentrations of $Mn^{2+}$ and $Mg^{2+}$ to the PCR system, we reduced process fidelity to obtain AtCOMT fragments with 2-3 mutation sites. Four different concentration ratios of $Mn^{2+}$:$Mg^{2+}$ were set: 1:30,1:20,1:15, and 1:10. The error-prone PCR was performed using the following PCR system:

| PCR system ($Mg^{2+}$：$Mn^{2+}$=10：1） | |
|---|---|
| 2×Mix Green Master | 25μl |
| $Mg^{2+}$(10mM) | 1μl |
| $Mn^{2+}$(1mM) | 1μl |
| AtCOMT-R | 2μl |
| AtCOMT-F | 2μl |
| Templete | 50ng |
| DDW | to 50μl |

| PCR system ($Mg^{2+}$：$Mn^{2+}$=15：1） | |
|---|---|
| 2×Mix Green Master | 25μl |
| $Mg^{2+}$(10mM) | 1.5μl |
| $Mn^{2+}$(1mM) | 1μl |
| AtCOMT-R | 2μl |
| AtCOMT-F | 2μl |
| Templete | 50ng |
| DDW | to 50μl |

| PCR system ($Mg^{2+}$：$Mn^{2+}$=20：1） | |
|---|---|
| 2×Mix Green Master | 25μl |
| $Mg^{2+}$(10mM) | 1μl |
| $Mn^{2+}$(1mM) | 0.5μl |
| AtCOMT-R | 2μl |
| AtCOMT-F | 2μl |
| Templete | 50ng |
| DDW | to 50μl |

| PCR system ($Mg^{2+}$：$Mn^{2+}$=30：1） | |
|---|---|
| 2×Mix Green Master | 25μl |
| $Mg^{2+}$(10mM) | 1.5μl |
| $Mn^{2+}$(1mM) | 0.5μl |
| AtCOMT-R | 2μl |
| AtCOMT-F | 2μl |
| Templete | 50ng |
| DDW | to 50μl |

**2.connection**

The obtained PCR fragments were purified and the plasmid pYB1a-AtCOMT**was constructed by Gibson connection. The ligation system is as follows：

| Gibson system | |
|---|---|
| 0.02×3600bp | 72ng |
| 0.04×1056bp | 42.24ng |
| 2×c115 Mix | 5μl |
| DDW | to 10μl |

| Gibson. | |
|---|---|
| 50° C | 15min |
| 4° C | ∞ |

**3.Transformation, plasmid quality and DNA sequencing**

The successfully constructed pYB1a-AtCOMT* plasmid was transformed into DH5α bacterial strain. After plate coating, the correct bacterial colony was picked and inoculated into 5 mL LB medium containing 0.1% ampicillin. Following 12-hour cultivation, the plasmid was extracted for sequencing. The results showed that when $Mg^{2+}$:$Mn^{2+}$ = 10:1, the mutation rate remained stable at 2-3. Therefore, the error-prone PCR conditions were determined as $Mg^{2+}$:$Mn^{2+}$ = 10:1.

**(D) In-depth Characterization: Quantifying Sensor Leakiness**

An excellent biosensor must have low "background noise" (i.e., low leakage expression), which is a prerequisite for ensuring the accuracy of screening results. Last week, we initially observed the leakage phenomenon; this week, we designed a more precise experiment to quantify its specific impact under different selection pressures.

**1.Solution Preparation**

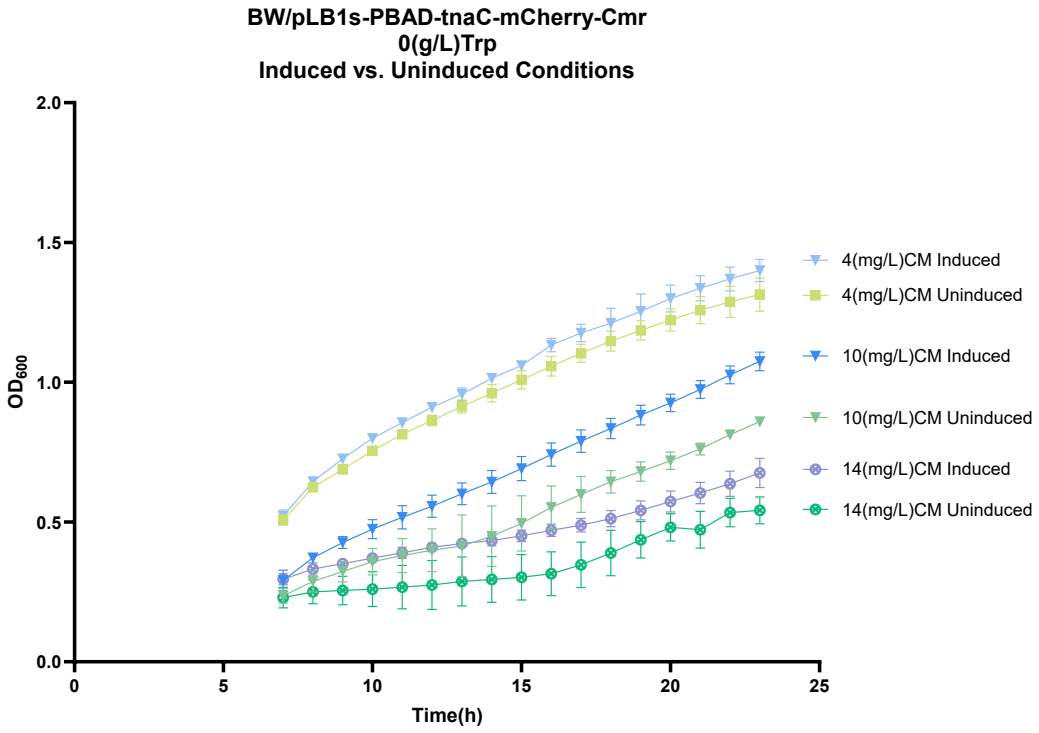
Prepare M9-Y solution according to the above formula.

## 2.Addition of Inducer and Chloramphenicol.No tryptophan was added.

Set up two control groups: one without arabinose addition, and the other with 1 mg/L arabinose addition. Each group included experimental subgroups with different chloramphenicol concentrations (4, 10, 14 mg/L) to observe the survival of the strain due to leakage expression under different selection pressures.

## 3.Growth Curve Plotting

Inoculate the strain into a 96-well plate and continuously monitor the 24-hour growth curve ($OD_{600}$) using a microplate reader.




Result: The growth curve of the induced group was slightly higher than that of the uninduced group, which confirmed that our PBAD promoter system has a low level of leakage expression. However, a key finding was that with the increase of CM concentration, this weak growth advantage caused by leakage was effectively inhibited. This indicates that although leakage exists, it is controllable. By applying sufficient selection pressure, we can effectively eliminate background noise and ensure that only real positive signals can survive.

## (E) Validating the Sensor's Dynamic Range and "Signal" Strength

The "signal" of the sensor refers to the growth advantage brought by the presence of tryptophan. We need to prove that this "signal" is strong enough to be clearly distinguished from the "noise" (leakage), thereby forming a reliable screening window.

## 1.Solution Preparation

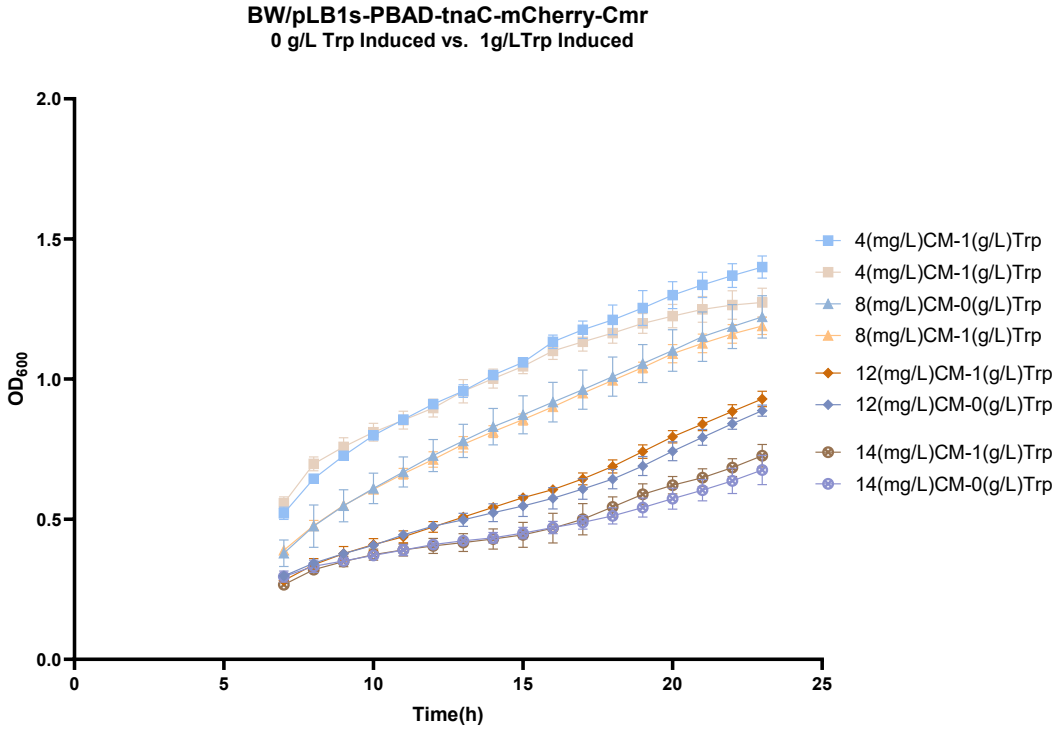
Prepare M9-Y solution using the above formula.

## 2.Addition of Inducer, Chloramphenicol, and Tryptophan

Add arabinose to a final concentration of 1 mg/L. Set up two experimental groups: one without tryptophan addition, and the other with 1 g/L tryptophan addition. Each group included experimental subgroups with different chloramphenicol (CM) concentrations (4, 8, 12, 14 mg/L). Each concentration was tested in triplicate.

## 3.Growth Curve Plotting

Inoculate the strain into a 96-well plate and continuously monitor the 24-hour growth curve ($OD_{600}$) using a microplate reader.




Result: At low CM concentrations, the strain could grow regardless of the presence or absence of tryptophan. However, with the increase of CM concentration (> 8 mg/L), the two growth curves began to separate significantly: the growth of the Trp-free group was gradually inhibited, while the Trp-containing group could maintain robust growth. This revealed a broad and effective "working window" for us. In the CM concentration range of 12-14 mg/L, the growth difference between the presence and absence of tryptophan reached the maximum. This is the ideal condition for future high-throughput screening experiments, as it can most effectively distinguish "positive" from "negative" strains.

# Week2: 7.8-7.14:

## (A) pYB1a-hucR-eGFP Functional Validation:

Exploring the Optimal Excitation and Emission Light for eGFP.

## 1.Inoculation

Inoculate the bacterial suspension containing the pYB1a-hucR-eGFP plasmid transformed into BW25113 competent cells from the bacterial culture tube into liquid medium and incubate at 37°C for 12 hours.

## 2.Prepare M9 medium

| M9 medium | |
|---|---|
| 5*M9 | 2mL |
| 20% Glycerin | 1mL |
| MgSO4 | 20μL |
| CaCl2 | 1μL |
| Amp | 10μL |
| ddH2O | To 10mL |

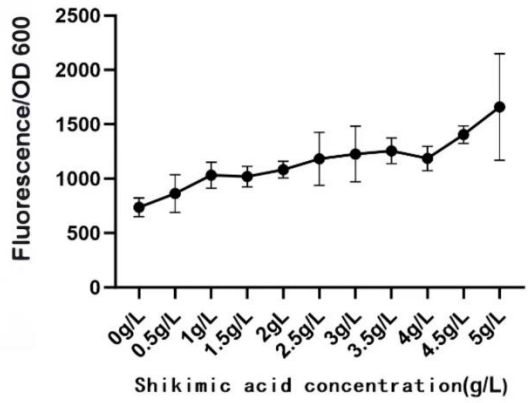
## 3. Induction Culture

Prepare 11 concentration gradients of shikimic acid solutions at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 g/L. Set up BW wild-type bacteria and pYB1a-eGFP as controls. Take 500 μL of M9 medium, 500 μL of shikimic acid solution, and 10 μL of bacterial suspension, and add them to a 96-well deep plate. Cultivate at 30°C for 18 hours. Perform three parallel experiments for each concentration gradient. Take 200 μL from each tube and transfer it to an enzyme-linked immunosorbent assay (ELISA) plate. Manually input the fixed excitation and emission wavelengths, and measure the fluorescence and OD values in the ELISA reader.

## 4. The enzyme labeler measures fluorescence and OD600 value.

Result: When the exciting light is at 488nm and the emission light is at 509nm, the original issue has been improved, meeting expectations. However, the trend of relative fluorescence intensity fluctuates with no clear pattern and the response is insufficient.

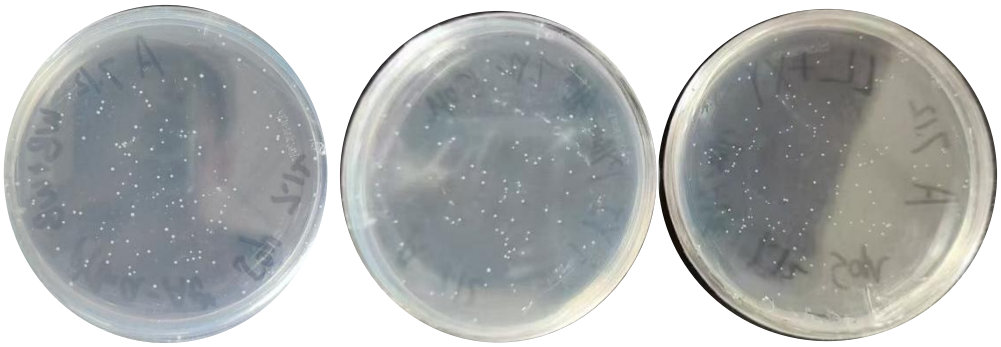
The analysis indicates that the gradient range of shikimic acid concentration is not wide enough.



## （B）Library capacity, positivity rate, mutation rate detection

### 1.Tank capacity detection

The successfully constructed pYB1a-AtCOMT* was transferred to DH5α strain, and 50μl of the bacterial liquid was taken for plate coating. The number of single colonies on the plate was recorded as shown in the figure：



135　　115　　120

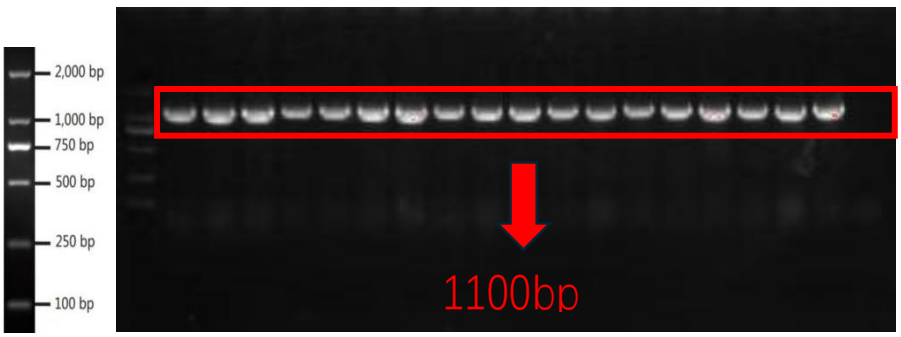
The library capacity was determined to be 2500 mutant monoclonal tubes for conversion

### 2.Positive rate tests

Eight colonies were picked from each plate for colony PCR detection of positive rate. The system is as follows：

| PCR system (10μL) | |
|---|---|
| 2×Taq | 5ul |
| AtCOMT*-F | 0.4ul |
| AtCOMT*-R | 0.4ul |
| DDW | 4.2ul |

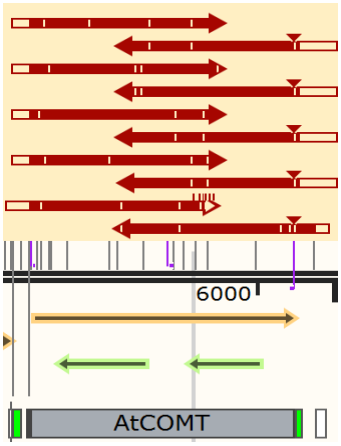
| PCR | |
|---|---|
| 98℃ | 5min |
| 98℃ | 30s |
| 57℃ | 30s |
| 72℃ | 35s |
| 72℃ | 5min |
| 25℃ | ∞ |




The detection result showed that the positive rate of bacterial colony PCR was 100%

### 3.Mutation rate testing

Five bacteria with correct results were selected for inoculation and plasmid transformation, and sent for sequencing. After removing synonymous mutations, it was found that the sequencing results of the five plasmids were all 2-3 mutations




## (B) Construction of MP6-K Plasmid

### 1.Amplification of the vector by PCR to replace CmR with KanR

The target vector fragment was obtained by PCR, using the following PCR program and system:

×30

| PCR | | |
|---|---|---|
| 98℃ | 5min | |
| 98℃ | 30s | |
| 65℃ | 30s | |
| 72℃ | 181s | |
| 72℃ | 5min | |
| 25℃ | ∞ | |

| PCR system (50μL) | |
|---|---|
| 2×Mix | 25ul |
| ZT-F | 2ul |
| ZT-R | 2ul |
| Template | 20ng |
| DDW | 20ul |

Agarose gel electrophoresis of the PCR product showed that the correct target fragment length was 6041 bp. The correct target fragment was obtained, and gel extraction was performed on the sample.

## 2.Amplification of the Kana fragment by PCR

The target fragment was obtained by PCR, using the following PCR program and system:

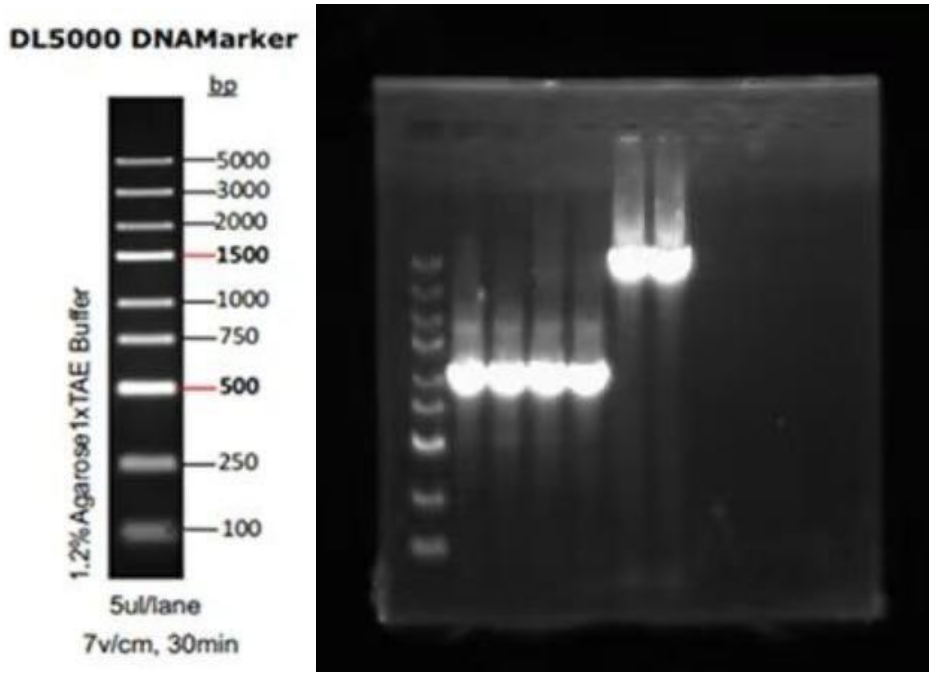
| PCR | | |
|---|---|---|
| 98℃ | 5min | |
| 98℃ | 30s | ×30 |
| 55℃ | 30s | |
| 72℃ | 25s | |
| 72℃ | 5min | |
| 25℃ | ∞ | |

| PCR system (50μL) | |
|---|---|
| 2×Mix | 25ul |
| Kana-F | 2ul |
| Kana-R | 2ul |
| template | 20ng |
| DDW | 20ul |

Agarose gel electrophoresis of the PCR product showed that the correct target fragment length was 836 bp. The correct target fragment was obtained, and gel extraction was performed on the sample.

## 3.Gel Extraction

The correct bands were excised for gel extraction, and the PCR products were verified by agarose gel electrophoresis.




## 4.Ligation of the Two Fragments

Using Gibson AssemblyThe MP6-K plasmid was obtained by ligation using Gibson Assembly (C116). The MP6 vector was 6041 bp, and the Kana fragment was 836 bp. The specific operation was performed using the following system and program:

| Gibson system | |
|---|---|
| 0.02×6041bp | ng |
| 0.02×836bp | ng |
| 2×c116 Mix | 5ul |
| DDW | to10ul |
| Gibson | |
| 50℃ | 15min |
| 4℃ | ∞ |

### 5.Chemical Transformation

The ligated product was transformed into DH5α competent cells using chemical transformation, and then plated on LB agar plates containing Kanamycin (Kana) resistance, followed by incubation overnight at 37°C.

### 6.Picking Single Clones for Inoculation

After overnight cultivation at 37°C, expected resistant single colonies grew on the plates. The single colonies were inoculated into liquid LB medium and cultured at 37°C for 12 hours.

### 7.Plasmid Extraction and Sequencing

Plasmids were extracted, and sequencing of MP6-K showed correct results

## Week3: 7.15-7.21:

### (A)pYB1a-hucR-eGFP Function Validation:

Expand the concentration gradient range of mortuarin, set a larger concentration gradient, and explore the response range of the biosensor.

### 1. Inoculation

Inoculate the BW25113 competent cell solution with the pYB1a-hucR-eGFP plasmid from the bacterial culture tube into liquid medium, and incubate at 37°C for 12 hours.

### 2.Prepare M9 culture medium

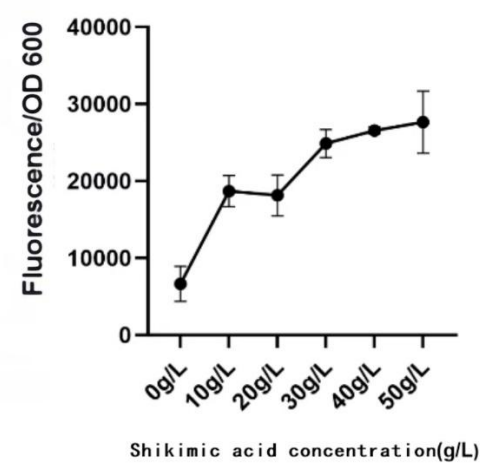
| M9 medium | |
|---|---|
| 5*M9 | 2mL |
| 20% Glycerin | 1mL |
| MgSO4 | 20μL |
| CaCl2 | 1μL |
| Amp | 10μL |
| ddH2O | To 10mL |

### 3.Induction and cultivation

Prepare 6 concentration gradients of shikimic acid solution at 0, 10, 20, 30, 40, and 50 g/L, and set up BW original bacteria and pYB1a-eGFP controls. Take 500 μL of M9 culture medium, 500 μL of shikimic acid solution, and 10 μL of bacterial solution, and add them to a 96-well plate. Incubate at 30°C for 18 hours, performing 3 parallel experiments for each concentration gradient. Transfer 200 μL from each tube into a microplate, and measure the fluorescence and OD600 values using a microplate reader.

### 4.The microplate reader measures fluorescence and OD600 values.

Result: The trend is relatively good and meets expectations. As the concentration of shikimic acid increases, the relative fluorescence intensity gradually increases. When the concentration of shikimic acid reaches a certain level, the growth of the colony is inhibited, leading to a decrease in the expression level of fluorescent proteins, and the increase in relative fluorescence intensity slows down.




### (B)pYB1a- hucR-eGFP Function Validation:

Perfect the concentration gradient range of malonic acid, set smaller concentration gradients to form low, medium, and high concentration gradients, and optimize the response range of the biosensor.

### 1. Inoculation

Inoculate the BW25113 competent cell solution with the pYB1a-hucR-eGFP plasmid from the bacterial culture tube into liquid medium, and incubate at 37°C for 12 hours.

### 2. Prepare M9 culture medium

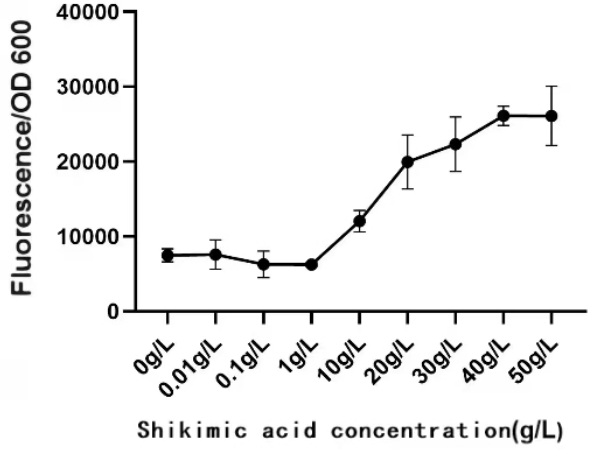
| M9 medium | |
|---|---|
| 5*M9 | 2mL |
| 20% Glycerin | 1mL |
| MgSO4 | 20μL |
| CaCl2 | 1μL |
| Amp | 10μL |
| ddH2O | To 10mL |

### 3. Induction and Cultivation

Prepare a total of 9 concentration gradients of shikimic acid solutions with concentrations of 0, 0.01, 0.1, 1, 10, 20, 30, 40, and 50 g/L, and set up controls with BW original strain and pYB1a-eGFP. Take 500 μL of M9 medium, 500 μL of shikimic acid solution, and 10 μL of bacterial liquid, add them to a 96-deep-well plate, and incubate at 30°C for 18 hours. Perform 3 parallel experiments for each concentration gradient, take 200 μL from each tube into the enzyme label plate, and measure fluorescence and OD values with the enzyme label instrument.

### 4. Fluorescence and OD values were measured by an enzyme-linked immunosorbent assay (ELISA) reader

Result: The response range was good and met expectations. The next step is to couple it with growth to further verify the function of the biosensor.




### (C) Fermentation test for tryptophan synthesis level (pre-experiment):

### 1. By chemical transformation, plasmids are transferred into competent cells that have had

**certain genes knocked out.**

| Chassis strain | The transferred plasmid |
|---|---|
| BW ΔtrpR ΔtnaAB | pBR322-trpEDCBA$^{fbr}$-aroG$^{fbr}$-serA$^{fbr}$ |
| BW ΔtrpR ΔtnaAB ΔptsG | pBR322-trpEDCBA$^{fbr}$-aroG$^{fbr}$-serA$^{fbr}$ |
| BW ΔtrpR ΔtnaAB ΔpykF | pBR322-trpEDCBA$^{fbr}$-aroG$^{fbr}$-serA$^{fbr}$ |
| BW ΔtrpR ΔtnaAB ΔpykA | pBR322-trpEDCBA$^{fbr}$-aroG$^{fbr}$-serA$^{fbr}$ |
| BW ΔtrpR ΔtnaAB ΔpheA | pBR322-trpEDCBA$^{fbr}$-aroG$^{fbr}$-serA$^{fbr}$ |

Using the chemical transformation method, the ligated products were transformed into the competent cells of the corresponding chassis strains and then spread on LB plates containing Tet and incubated at 37°C overnight. Since the strains usually grow poorly on LB plates with Tet, the concentration of Tet in the plates was appropriately reduced. After 12 hours of culture, the expected resistant single colonies grew on the plates.

## 2. Selective monoclonal inoculation:

After overnight culture at 37 ℃, the expected resistant single colonies grew on the plates. Inoculate the monoclonal into liquid LB medium and incubate at 37 ℃ for 12 hours.

## 3. Coating plate

The bacterial solution was dipped in three times from liquid LB medium with an inoculation ring and spread onto the corresponding resistant LB plates of each strain until paste-plate and incubated for 12h at 37℃.

## 4. Inoculate into seed culture medium:

The seed culture medium was used in 250 ml conical flasks, and the system ratio was as follows:

| Seed culture medium system (15ml) | |
|---|---|
| Seed culture medium | 13.5ml |
| Seed glucose | 1.5ml |
| Antibiotics corresponding to strains | 15ul |

The colonies on the plates were scraped off with an inoculation ring, plugged into seed medium, and incubated in a shaker at 37 ℃ for 18h until an OD of 5-6.

## 5. Inoculation into fermentation medium:

6 OD of the bacterial solution was taken into a 10ml EP tube, the centrifuge was precooled at 4 ° C, and the centrifugation program was set to 4200 RPM for 10min.

A 250 ml conical flask was used as a container in the ultra-clean table to configure the fermentation medium. The system ratio was as follows:

| Fermentation culture medium system (15ml) | |
|---|---|
| Fermentation medium | 12ml |
| Seed culture medium | 1.5ml |
| Fermented glucose | 1.5ml |
| Antibiotics corresponding to strains | 15ul |

In a super-clean environment, discard the supernatant from 10ml of EP tubes after centrifugation, draw 300 μ l of the prepared fermentation medium into the centrifuged EP tubes, and mix the colonies by pipetting. Then, pour all the liquid in the EP tubes back into the medium and place them in a 37 ° C shaker for cultivation.

## 6. Spectrophotometer for OD600 value measurement and liquid phase sample preparation

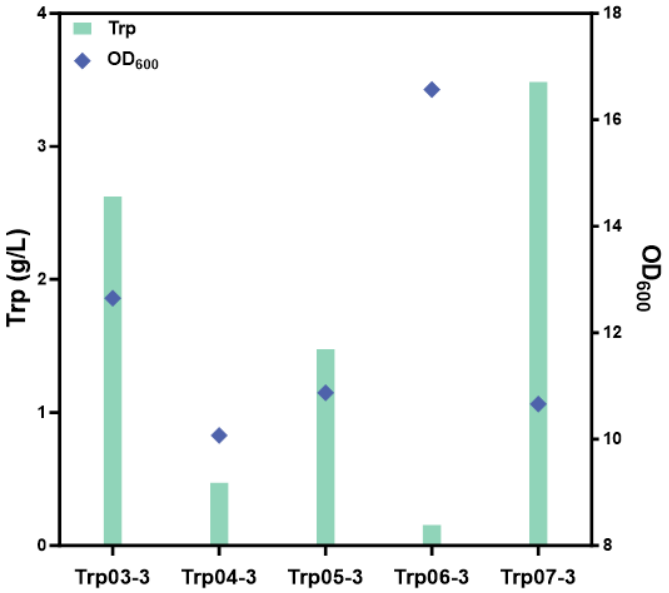
At the 28th hour after the fermentation medium began to be cultured, 500μL of the bacterial liquid was taken from each conical flask where the strain was located, and 200μL of the bacterial liquid was taken. The OD600 value was measured by a spectrophotometer. At 28 hours, it was judged by the OD600 value that the growth of each strain had basically reached the plateau period.

The remaining 300μL of the bacterial liquid was used for sample preparation. Centrifuge 10,000 g for 10 minutes, draw the supernatant with a syringe, filter the membrane, and prepare the sample.

## 7. High performance liquid chromatography (HPLC) detection of tryptophan production:

Using chassis strains as grouping criteria:

| **BWΔtrpRΔtnaAB** **Trp03** | **BWΔtrpRΔtnaABΔptsG** **Trp04** | **BWΔtrpRΔtnaABΔpykF** **Trp05** | **BWΔtrpRΔtnaABΔpykA** **Trp06** | **BWΔtrpRΔtnaABΔpheA** **Trp07** |
|---|---|---|---|---|
| PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$ Trp03 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$ Trp04 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$ Trp05 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$ Trp06 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$ Trp07 03 |




The tryptophan production of each strain was detected by HPLC, and it was found that the Trp07 03 strain produced 3.4848 g/L Trp at 28h of culture, which was the highest tryptophan production among the strains.

## Week4: 7.22-7.28:

### (A)pYB1a- hucR-CmR plasmid construction:

### 1. PCR amplification of HucR gene vector

The target vector fragment was obtained by PCR. The PCR system is as follows.

| PCR system (50μL) | |
|---|---|
| 2×Mix | 25ul |
| pYB1a-F | 2ul |
| pTB1a-R | 2ul |
| template | 1ul |
| DDW | 20ul |

| PCR | | |
|---|---|---|
| 98℃ | 5min | |
| 98℃ | 30s | ×30 |
| 61℃ | 30s | |
| 72℃ | 130s | |
| 72℃ | 5min | |
| 25℃ | ∞ | |

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 4322 bp was obtained. We obtained the correct target fragment and performed gel excision and recovery on the sample.

### 2.PCR amplification of CmR gene fragments

The target vector fragment was obtained by PCR. The PCR system is as follows.

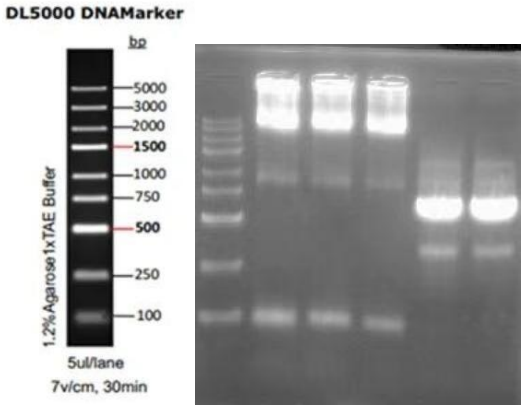
| PCR system (50μL) | |
|---|---|
| 2×Mix | 25ul |
| CmR-F | 2ul |
| CmR-R | 2ul |
| template | 1ul |
| DDW | 20ul |

| PCR | | |
|---|---|---|
| 98℃ | 5min | |
| 98℃ | 30s | ×30 |
| 62℃ | 30s | |
| 72℃ | 30s | |
| 72℃ | 5min | |
| 25℃ | ∞ | |

The PCR products were detected by agarose gel electrophoresis, and the correct target fragment length of 553 bp was obtained. We obtained the correct target fragment and performed gel recollection on the samples.

## 3. Cutting glue recycling

The correct bands obtained were cut and recovered, and the PCR products were detected by agarose gel electrophoresis as follows:




## 4. Connect the four segments using the Gibsion assembly method.

The specific operation is as follows:

The pYGRa1-mCherry plasmid was obtained using Gibson (C116) ligation, with a HucR loading of 4322 bp and a CmR loading of 533bp.

| Gibson system | |
|---|---|
| 0.02×4322bp | Ng |
| 0.04×533bp | ng |
| 2×c117 Mix | 5ul |
| DDW | to10ul |
| Gibson | |
| 50℃ | 15min |
| 4℃ | ∞ |

## 5.Chemical Conversion

Using chemical transformation, the ligation products were transformed into DH5α competent cells and spread onto LB plates containing Amp resistance, then incubated overnight at 37°C.

### 6.Selective Clonal Inoculation

After overnight incubation at 37°C, only one colony grew on the plate. The single colony was inoculated into liquid medium and incubated at 37°C for 12 hours.

### 7.Plasmid extraction and sequencing:

pYB1a-HucR-CmR sequencing results are correct.

## （B）PYB1a-HucR-CmRFunctional Validation

### 1.Conversion

After transforming the correctly sequenced pYB1a-HucR plasmid into BW25113 competent cells, plate the cells onto LB agar containing ampicillin and incubate overnight at 37°C. Expected resistant single colonies will grow on the plates. Subsequently, select individual colonies for plasmid extraction and verification.

### 2.Inoculation

Select a single colony and inoculate it into liquid medium. Incubate at 37°C for 12 hours.

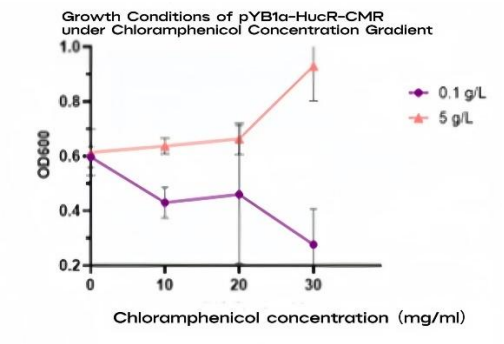
### 3.Establish a chloramphenicol concentration gradient to determine the optimal concentration of chloramphenicol.

Method: Take 500 μL of M9 medium and 500 μL of shikimic acid solution, add to a 96-well plate. Add 10 μL of bacterial suspension and 1 μL of chloramphenicol at different concentrations (0, 10, 20, 30 mg/mL). Perform 5 replicates per group and incubate at 30°C for 18 hours.

| M9 medium | |
|---|---|
| 5*M9 | 2mL |
| 20% Glycerin | 1mL |
| MgSO4 | 20μL |
| CaCl2 | 1μL |
| ddH2O | To 10mL |

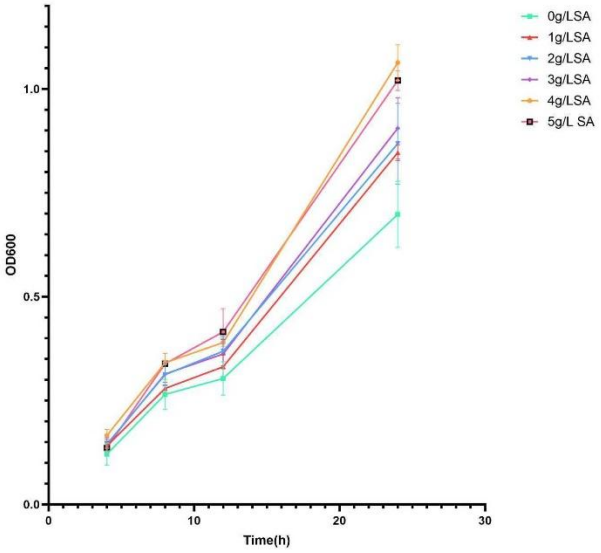
### 4.Outcome Evaluation

At a chloramphenicol concentration of 30 mg/mL, the strain exhibited the highest OD600 value, indicating that 30 mg/mL represents the optimal chloramphenicol concentration. Additionally, the strain's OD600 value at 5 g/L consistently exceeded that at 0.1 g/L, with test results aligning with expectations.




## 5.Investigating Strain Growth under Mangosteen Acid Concentration Gradients

The method is as follows: Set a concentration gradient of 0, 0.001, 1, 2, 3, and 4 g/L shikimic acid. Take 500 µL of M9 medium and 500 µL of shikimic acid solution, add to a 96-well plate, add 10 µL of bacterial suspension, 1 µL of 30 mg/mL chloramphenicol, with 5 replicates per group. Incubate at 30°C and perform 24-hour timed sampling. Measure the optical density (OD) using an enzyme-linked immunosorbent assay (ELISA) reader. 24 hours. Measure the optical density (OD) using a microplate reader.




## 6.Outcome Evaluation

There is already a clear trend, and the overall result is in line with expectations.

## (C) Re-fermentation test of tryptophan synthesis level:

Three groups of control experiments were set up.

## 1. Inoculation

The bacterial solution was drawn from the storage tube of each strain that had been transferred into the pBR322-trpEDCBA$^{fbr}$-aroG$^{fbr}$-serA$^{fb}$ plasmid, inoculated into liquid LB medium, and cultured at 37°C for 12 hours.

## 2. Plate coating:

The bacterial solution was dipped in three times from liquid LB medium with an inoculation ring and spread onto

the corresponding resistant LB plates of each strain until paste-plate and incubated for 12h at 37 ℃.

### 3. Inoculate into seed culture medium:

The seed culture medium was used in 250 ml conical flasks, and the system ratio was as follows:

| Seed culture medium system (15ml) | |
|---|---|
| Seed culture medium | 13.5ml |
| Seed glucose | 1.5ml |
| Antibiotics corresponding to strains | 15ul |

The colonies on the plates were scraped off with an inoculation ring, plugged into seed medium, and incubated in a shaker at 37 ℃ for 18h until an OD of 5-6.

### 4. Inoculation into fermentation medium:

6 OD of the bacterial solution was taken into a 10ml EP tube, the centrifuge was precooled at 4 ° C, and the centrifugation program was set to 4200 RPM for 10min.

A 250 ml conical flask was used as a container in the ultra-clean table to configure the fermentation medium. The system ratio was as follows:

| Fermentation culture medium system (15ml) | |
|---|---|
| Fermentation medium | 12ml |
| Seed culture medium | 1.5ml |
| Fermented glucose | 1.5ml |
| Antibiotics corresponding to strains | 15ul |

In a super-clean environment, discard the supernatant from 10ml of EP tubes after centrifugation, draw 300 μ l of the prepared fermentation medium into the centrifuged EP tubes, and mix the colonies by pipetting. Then, pour all the liquid in the EP tubes back into the medium and place them in a 37 ° C shaker for cultivation.

Three groups of experiments were set up when the inoculation to the fermentation medium.

### 5. Spectrophotometer for OD600 value measurement and liquid phase sample preparation

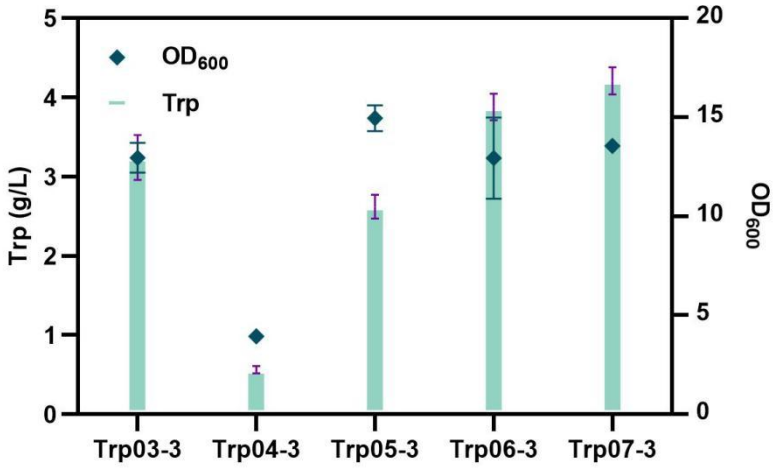
At the 12th hour after the fermentation medium began to be cultured, 500μL of the bacterial liquid was taken from each conical flask where the strain was located, and 200μL of the bacterial liquid was taken. The OD value was measured by a spectrophotometer. At 28 hours, it was judged by the OD value that the growth of each strain had basically reached the plateau period.

The remaining 300μL of the bacterial liquid was used for sample preparation. After centrifuging 10,000 g for 10 minutes, the supernatant was drawn with a syringe, filtered through a membrane, and injected into a liquid phase vial. At the 21st and 26th hours, repeat the above steps until the strain growth reaches the plateau stage.

### 6. Detection of tryptophan yield by high performance liquid chromatography (HPLC)

Plot based on the chassis strains as the grouping criterion:

| BWΔtrpRΔtnaAB<br>Trp03 | BWΔtrpRΔtnaABΔptsG<br>Trp04 | BWΔtrpRΔtnaABΔpykF<br>Trp05 | BWΔtrpRΔtnaABΔpykA<br>Trp06 | BWΔtrpRΔtnaABΔpheA<br>Trp07 |
|---|---|---|---|---|
| PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$<br>Trp03 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$<br>Trp04 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$<br>Trp05 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$<br>Trp06 03 | PBR322-trp$^{fbr}$EDCBA-aroG$^{fbr}$-serA$^{fbr}$<br>Trp07 03 |




During this fermentation, after 26 hours of culture, the Trp yield of the Trp0703 strain reached 4.2103g/L, which was further increased compared with the previous pre-experiment yield.

## (D) genome-wide mutation

### 1. Plasmid extraction:

Re-plasmid MP6-K, pLB1s-PBAD-tnaC-mcherry-cmr, pYB1a-P23119-tnaE$^{S40F}$DCBA

### 2. Chemical transformation:

The MP6-K, pLB1s-PBAD-tnaC-mcherry-cmr, pYB1a-P23119-tnaE$^{S40F}$DCBA plasmids were chemically transformed into BWΔtrpRΔtnaAB competent cells, and 100ng of each plasmid was added. After shaking at 37 ℃ for 1h, LB plates containing Amp, Kana, and Smr were coated and incubated at 30 ℃ for 24h, the expected resistant single colonies grew on the plates.

### 3. Inoculation

Single colonies were picked from the plate and inoculated into LB liquid medium. Two single colonies were selected for inoculation.

### 4. Induction culture in M9 glycerol-Y medium

Prepare M9 glycerol-Y medium, inoculate the bacterial liquid into M9-Y medium, and cultivate at 30 ℃ with a screening concentration of 10mg/L CM. The OD value is measured by a spectrophotometer to determine whether the logarithmic growth phase has been reached. When the bacteria are in the logarithmic growth phase (or before

reaching the plateau phase), they are transferred to the next round of M9-Y medium. This step is repeated in sequence for 10 rounds.

| M9-Y system （10ml） | |
|---|---|
| 5*M9 | 2ml |
| 20% glycerin | 1ml |
| $MgSO_4$ | 20ul |
| $CaCl_2$ | 1ul |
| Amp、Smr、Kana | 10ul each |
| 100g/L yeast | 50ul |
| L-Ara | 100ul |
| Cmr | 4ul |
| Bacterial liquid | 100ul |
| DDW | Fill to 10ml |

| Round | Time | OD600 |
|---|---|---|
| 1 | 30h | 0.2 |
| 2 | 30h | 0.8 |
| 3 | 37h | 0.26 |
| 4 | 24h | 0.67 |
| 5 | 25h | 2.04 |
| 6 | 9h | 0.46 |
| 7 | 8h | 0.46 |
| 8 | 9h | 0.50 |
| 9 | 8h | 0.24 |
| 10 | 26h | 4.45 |