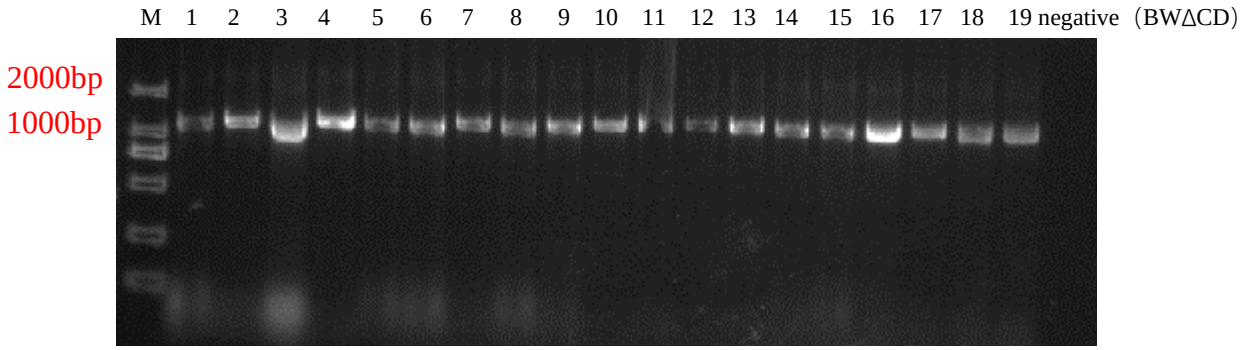
1. screening of random mutant library

- Pre-experiment: screening concentration settings

The concentration of HPP was set to 0.6 g/L, which was above the metabolisable threshold of the wild type, and the amount of solvent added to the medium (the amount of anhydrous ethanol) did not inhibit the growth of E. coli to achieve a certain level of stress. At the same time, we configured screening plates with 10^{-2}, 10^{-1}, and 100 mg/L 5-FC concentration gradients, and applied equal amounts of wild-type gy9s-hmas(Am,scpa1)-bio177 mixed with the bacterial solution of Bio177 to the plates.

Cultivate at 37°C for 17 h. Observe the growth of the colonies and verify the bacterial PCR amplification. The colony PCR system was as follows.

PCR system (50 μL)	
Bio177-F	2 μL
Bio177-R	2 μL
gy9s(scpa1)-177-hmas3	2 ng
2 x Mix	25 μL
DDW	20 μL



According to the bacterial PCR amplification results obtained, colonies containing HmaS could be distinguished from Bio177 strains not containing HmaS under screening conditions of 0.01 mg/L 5-FC, and wild-type gy9s-hmas(Am,scpa1)-bio177 was unable to grow under conditions of 0.1 mg/L 5-FC, which could be used as a starting screening concentration to distinguish between mutant and wild-type individuals.

0.01 mg/L 5-FC **0.05 mg/L 5-FC** **0.1 mg/L 5-FC**

2. Screening of random mutant libraries

Transferred the gy9s-hmas(Am,scpa1)-bio177 random mutant library plasmids into to the expression strain BWΔCD and cultured at 37°C for 12 h. The recovered bacterial solution was then centrifuged at 4000 rpm for 10 minutes to enrich the bacterial. Configure ZY M5052 medium to resuspend the organisms, and induce protein expression at 30℃ for 20h.

The induced strain was transferred to the screening medium containing 0.6 g/L HPP and 0.1, 0.2, 0.3 mg/L 5-FC, and detected its growth. When the strain grew to logarithmic phase (OD600=0.6), drawn the line to isolate the single clone, and after 12 h of extended shaking culture at 37 ℃, the protein expression was induced for 20 h at 30 ℃, and took 6 OD bacterial fluid for whole-cell catalysis.

Screening medium:

Screening system (10 ml)	
50×5052	200 μL
5×M9	2 mL
1M $MgSO_4$	20 μL
1M $CaCl_2$	1 μL
Str	10 μL
HPP (40 g/L)	150 μL
5FC (2 g/L)	1.5 μL
DDW	To 10 mL

Group	environmental stress screening	fluorescence intensity	culture time	HMA concentration
A1	0.6g/L HPP，0.1mg/L 5-FC	33754	22 h	1h:3.4 mM
A2	0.6g/L HPP，0.2mg/L 5-FC	23772	22 h	1h:2.4 mM
A3	0.6g/L HPP，0.3mg/L 5-FC	19281	22 h	1h:4.4 mM

The whole-cell catalytic products were diluted 10-fold with pure water, and the product accumulation was detected using high performance liquid chromatography (HPP). After the colony fluorescence values and HMA yields, the individuals with increased enzyme activity could be obtained under the condition of 0.6 g/L HPP, 0.3 mg/L 5-FC, and this condition was applied in the subsequent screening.

In the new round of screening, we found that when we isolated the monoclones at OD600=0.6, the yield of 35% of the strains was improved compared to the wild type, and the improvement was large. However, at this time, a large number of escape phenomenon occurred, the isolated monoclones did not produce yield, guessing that at this time, 5-FC began to interfere with the growth and metabolism of the individuals with poor enzyme activity, and at the same time, E. coli achieved the purpose of survival and escape through the loss of part of the plasmid genes.

OD600=0.6 Catalytic 1h

Group	HMA concentration	Group	HMA concentration
0-1-1	1.86 mM	0-3-2	2.15 mM
0-1-2	2.69 mM	0-3-3	3.3 mM
0-1-3	no output	0-3-4	1.49 mM
0-1-4	no output	0-3-5	no output
0-1-5	3.78 mM	0-4-1	no output
0-1-6	no output	0-4-2	2.48 mM
0-1-7	4.05 mM	0-4-3	no output
0-1-8	no output	0-5-1	no output
0-3-1	no output	0-5-3	1.15 mM

Therefore, we isolated and tested the monoclonal strain at OD600=0.2 in a new round of screening.

Group	HMA concentration	Group	HMA concentration
1-1	1.55 mM	1-10	1.12 mM
1-2	1.43 mM	1-11	1.30 mM
1-3	1.79 mM	1-12	1.91 mM
1-4	1.23 mM	1-13	1.33 mM
1-5	0.92 mM	1-14	no output
1-6	1.74 mM	1-15	1.49 mM
1-7	1.48 mM	1-16	no output
1-8	0.98 mM	1-17	1.50 mM
1-9	1.98 mM	1-18	1.68 mM

When we went to isolate the monoclones at OD600=0.2, 60% of the strains had improved yield compared to the wild type, guessing that at this time, due to the short incubation time, 5-FC had not been involved in the growth and metabolic activities of the strains, and the stress pressure was small, and there was no strain escape occurring for this time.

We continued to conduct several rounds of screening under the condition of 0.6g/L HPP and 0.3mg/L 5-FC.