

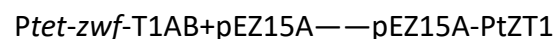
### 3-HB-producing strains overexpress cofactor related genes

#### 1. Overlap PCR

The internal *zwf* gene fragment of *Z. mobilis* is connected with T1AB through Overlap PCR, and the tandem target gene fragment *Ptet-zwf-T1AB* is obtained by *Ptet* drive.

#### 2. T5 Ligation

Plasmid fragment *Ptet-zwf-T1AB* and pEZ15A vector. According to the mole ratio fragment: vector  $\geq 3:1$ , and the total mass does not exceed 120 ng, it is calculated that the amount of fragment and vector needs to be added, and water is added to 4  $\mu\text{L}$  system, and then buffer and 0.5  $\mu\text{L}$  of diluted T5 enzyme are added. Under the condition of T5 enzyme link reaction, it is connected by T5 enzyme and transferred into DH5a competent cells to form plasmid pEZ15A-PtZT1:



Coat on the medium plate of LS in super-clean bench, and culturing for overnight in an incubator at 37°C.

#### 3. Monoclonal colony PCR

Select correct strains after verifying through agarose gel electrophoresis to inoculate in 50 mL centrifuge tube with 8 mL RS medium, and put into 37°C shaker for overnight culture.

#### 4. Plasmid extraction

Extract the plasmids of DH5a pEZ15A-PtZT1 with kit after culturing.

#### 5. Electroporation

Transfer the extracted plasmids in competent cells of ZMNP $\Delta$ 1 with *Pgap-T1AB* operon, and the strain constructed is ZMNP-PtT1. Seal the transferred bacteria with sealing film and culture bacteria in shaker at 30°C for 4-6 hours, then coated bacteria on RS solid medium plates in a clean workbench, make marks, and culture these plates upside down in incubator at 30°C.

6. Select correct strains after verifying through agarose gel electrophoresis to inoculate in 50 mL centrifuge tube with 8 mL RS medium, and put into 30°C shaker for overnight culture. Mix 1 mL of bacterial liquid with 1 mL of 60%

**glycerin in the cryopreservation tube in the super-clean bench, and store it in the refrigerator at -80°C.**

## **7. Fermentation**

- (1) 100  $\mu$ L of glycerol bacteria are inoculated into a cryotube containing 1 mL of RMG5 (containing 100  $\mu$ g/mL spectinomycin) medium and statically activated in a 30°C incubator until turbidity is achieved.
- (2) The activated bacterial liquid in the cryotube is poured into a 50 mL centrifuge tube containing an appropriate amount of RMG5 (with corresponding antibiotics) medium as the fermentation seed liquid and statically cultured in a 30°C incubator until the middle and late logarithmic phase. It is inoculated into the RMG5 (with corresponding antibiotics) medium with 80% bottling volume in a 50 mL Erlenmeyer flask, with the initial OD600nm controlled at 0.1, and fermented at 100 rpm and 30°C.
- (3) At fixed intervals, 1 mL of the sample is taken out in a super-clean bench for collection. The OD600nm of the bacterial liquid is detected using a UV-1800 UV spectrophotometer and recorded. The remaining samples are frozen at -80°C for subsequent detection.
- (4) The data graphs are plotted using Graphpad 9.0 (Insightful Science, CA, USA) software.

## **8. Analysis by High Performance Liquid Chromatography (HPLC)**

The samples are centrifuged at 12,000 rpm for 4 minutes, and the supernatant is collected and filtered through a 0.22  $\mu$ m filter. 400  $\mu$ L samples are taken into an HPLC injection vial for the detection of the contents of glucose, ethanol, and 3-HB in the HPLC (High Performance Liquid Chromatography).