2024.2.1-2024.2.29

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 P*tet-zwf*-T1AB is obtained by P*tet* drive.

2. T5 Ligation

Plasmid fragment P*tet-zwf*-T1AB and pEZ15A vector. According to the mole ratio fragment: vector ≥ 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 μL system, and then buffer and 0.5 μL 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:

P*tet-zwf*-T1AB+pEZ15A——pEZ15A-PtZT1

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

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℃ 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 ZMNPΔ1 with P*gap*-T1AB operon, and the strain constructed is ZMNP-PtT1. Seal the transferred bacteria with sealing film and culture bacteria in shaker at 30℃ 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℃.

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℃ 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℃.

7. Fermentation

(1) 100 μL of glycerol bacteria are inoculated into a cryotube containing 1 mL of RMG5 (containing 100 μg/mL spectinomycin) medium and statically activated in a 30℃ 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℃ 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℃.

(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℃ 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 μm filter. 400 μL 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).