2024.1.1-2024.1.18

1. Polymerase chain reaction

The plasmid is reverse-amplified in front of the promoter of pEZ15A-PtT1, and obtained the fragment without P*tet* promoter; P*gap* promoter is amplified using ZM4 as the template, and then assembled into pEZ15A-PgT1 plasmid by Gibson.

2. Electroporation

Transfer the extracted plasmids in competent cells of ZMNP. Set up the transformation program of the electroporation instrument (capacitance: 25μF, resistance: 200Ω, voltage: 1600 V). Seal the transferred bacteria with sealing film and culture bacteria in shaker at 30℃ for 4-6 hours, then coat bacteria on RS solid medium plates in super-clean bench,make marks, and culture these plates upside down in incubator at 30℃. For verification by 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 30℃ incubator for overnight culture.

3. Cryopreservation ZMNP pEZ15A-PgT1 strain, and send it for sequencing.

4. Replacing the RBS-10 sequence in the pEZ15A-PtT1 plasmid with RBS-10K, and the three genes tesB, phaA and phaB are connected in series to obtain the T1AB ' operon.

5. Overlap PCR

The promotor P*tet* and T1AB’ operon are connected in series through Overlap PCR to obtain P*tet*-T1AB’ operon.

6. T5 Ligation

Plasmid fragment P*tet*-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-PtT1’:

P*tet*-T1AB’+pEZ15A——pEZ15A-PtT1’

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

7. Monoclonal colony PCR

Transfered bacterias are made 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 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℃. At the same time, send 1 mL of bacterial liquid in 1.5 mL EP tube to the company for sequencing to verify the correctness of the transformants.

8. Plasmid extraction

Extract the plasmids of DH5α pEZ15A-PtT1'with kit after culturing.

9. Electroporation

Transfer the extracted plasmids in competent cells of ZMNPΔ0038， and the strain constructed is ZMNP-PtT1.

10. Fermentation

(1) Firstly, 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 OD_{600nm} 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 OD_{600nm} 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.

11. 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).