2023. 12. 2–2023. 12. 31

1. **Prepare the competent cells of *Z. mobilis* ZM4 and ZMNP，then Store them in the refrigerator at -80℃ after liquid nitrogen quick freezing.**

2. **T4 Ligase-mediated**

(1) Mix 30 μL of no-load edited plasmid pL2R with BsaI enzyme and digested at 37℃ for 5-8 hours, recovering the digested products to obtain

(2) the digested vector. Annealing forward and reverse primers, gRNA-F and gRNA-R at 95℃ for 5 minutes to obtain gRNA fragment.

(3) Link gRNA fragments and vector with T4 enzyme under T4 ligase system, react at 22℃ for 4-5 hours to obtain plasmid pL2R-gRNA.

3. **Transformation**

Transfer the plasmid pL2R-gRNA into the competent cells of E. coli and put the bacteria into a 37℃ shaker for 45 minutes to 1 hour culture after transformation, then coat on LS (LB medium with spectinomycin) medium plates in super-clean bench and put the medium plates into a 37℃ incubator for overnight culture.

4. **Monoclonal colony PCR**

(1) Select single colonies from the *E. coli* culture plates that have successfully transformed the plasmid for PCR analysis. Preparing the PCR system according to the proportion of the monoclonal colony PCR system, mix primer pEZ15A-F/gRNA-R evenly, and absorb 9 μL of the PCR system with a pipette gun and add it into the eight-row PCR tube. Add 10 μL of sterile water to the sterilized eight tubes, pick out the single colony on the plate, and then put it into the corresponding eight tubes, and blow it with the pipette gun until uniform. Then absorb 1 μL of water-soluble bacteria into the eight tubes containing the PCR system, cover the eight-row PCR tube cover, make a mark, and mix well, set the PCR instrument program, and start PCR.

(2) Result: There are bands and the size is correct.

5. **Culture bacterias.**

(1) Select correct strains after verifying through agarose gel electrophoresis to inoculate in 10 mL tube with 8 mL LS medium, and put into 37℃ shaker for overnight culture.

(2) Cryopreservation DH5a pL2R-gRNA strain, and send it for sequencing.

6. **Plasmid extraction** DH5a pL2R-gRNA extract plasmids.

7. **Polymerase chain reaction**

(1) Primers:15A double enzyme digestion inverse expansion -F/15A double enzyme digestion and reverse expansion -R

(2) DNA:DH5a pL2R-gRNA

(3) Result: The bands and the size are correct. The concentration of purified PCR products is 21.35 ng/μL.

8. **Link the Reverse amplification vector with DNA fragment containing 500 - 1200 bp of upstream and downstream and large fragments of target genes through T5 enzyme.**

Then transfer to the competent cells of E. coli. Coating the bacteria on LS medium plate in super-clean bench after putting in a 37℃ shaker for 45minutes-1hour culture, and then placed in a 37℃ incubator for overnight culture. 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 LS medium, and put into 37℃ shaker for overnight culture.

9. **Preserve the bacteria and send it for sequencing.**

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.

10. **Plasmid extraction** Extract the plasmids with kit after culturing.

11. **Electroporation**

Transfer the extracted plasmids in competent cells of ZMNP. Set up the transformation program of the electroporator (capacitance: 25uF, 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 bacterias 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 (RMG5 medium with Spe)medium, and put into 30℃ shaker for overnight culture.

12. 3-HB tolerance test of Z. mobilis.

(1) At first, absorb 100 μL glycerobacteria to cryopreservation with 1 mL of RS medium, and then activated to turbidity in an incubator at 30℃.

(2) Vaccinat the activated bacteria liquid into the Bioscreen C microcellular panel (containing 300 μL RMG2 medium with 1 g/L-40 g/L concentration of 3-HB), and control the initial OD_{600nm} of the transformed seed liquid is 0.1.

(3) Finally, use Bioscreen C to measure the growth curves of the recombinant strains.

13. Construction of strains with the exogenous 3-HB synthesis pathway

(1) Design primers: Design primers basicing the sequence of target gene, control the Tm of primers to be 58-62℃, the length to be 18-40 bp, and the GC content to be 40%-60%.

(2) The target gene fragments of the key enzymes in the 3-HB biosynthesis process, namely phaA, phaB, fadM, yciA, tesB, as well as the tetracycline-inducible promoter P*tet* and the pEZ15A vector fragment, are obtained through polymerase chain reaction technology.

① Primers: phaA-R10-F/phaA-R10-R Result: There are bands and the size is correct. The concentration of recovered plasmid is 30.5 ng/μL.

② Primers: phaB-R10-F/phaB-R10-R Result: There are bands and the size is correct. The concentration of recovered plasmid is 27.5 ng/μL.

③ Primers: phaB-R10-F/phaB-R10-R Result: There are bands and the size is correct. The concentration of recovered plasmid is 24.15 ng/μL.

④ Primers: yciA-F/yciA-phaA-R Result: There are bands and the size is correct. The concentration of recovered plasmid is 23.45 ng/μL.

⑤ Primers: tesB-mwy-F/tesB-R10-R Result: There are bands and the size is correct. The concentration of recovered plasmid is 39.7 ng/μL.

⑥ Primers: P*tet*-F/P*tet*-R Result: There are bands and the size is correct. The concentration of recovered plasmid is 37.65 ng/μL.

⑦ Primers: pEZ15A-F/pEZ15A-R Result: There are bands and the size is correct. The concentration of recovered plasmid is 17.65 ng/μL.

14. Overlap PCR

The target fragments obtained by PCR are connected in series through Overlap PCR, and the target gene fragments in series are P*tet*-fadM-phaA-phaB (PtF), P*tet*-yciA-phaA-phaB (PtY) and P*tet*-tesB-phaA-phaB (PtT1).

15. T5 exonuclease assisted cloning

① Plasmid fragment PtF 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 0.5 μL of buffer and 0.5 μL of diluted T5 enzyme are added. Under the condition of T5 exonuclease assisted cloning reaction, it is connected by T5 enzyme and transferred into DH5α competent cells to form plasmid:

PtF+pEZ15A——pEZ15A-PtF

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

② Plasmid fragment PtY 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 0.5 μL of buffer and diluted T5 enzyme are added. Under the condition of T5 enzyme link reaction, it is connected by T5 enzyme and transferred into DH5α competent cells to form plasmid:

PtY+pEZ15A——pEZ15A-PtY

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

③ Plasmid fragment PtT1 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 DH5α competent cells to form plasmid:

PtT1+pEZ15A——pEZ15A-PtT1

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

16. Monoclonal colony PCR

Preparing PCR system according to the proportion of monoclonal colony PCR system, mix primer pEZ15A-F/gRNA-R evenly, and absorb 9 μL PCR system with pipette gun and adding into the eight-row PCR tube. Add 10 μL sterile water to the sterilized eight tubes, pick out the single colony on the board, and then put it into the corresponding eight tubes, and blow it with the pipette gun until uniform. Then absorb 1 μL of water-soluble bacteria into the eight tubes with PCR system, cover the eight-row PCR tube cover, make a mark and mix well, set the PCR instrument program and start PCR.

17. Culture bacterias.

Select correct strains after verifying through agarose gel electrophoresis to inoculate in 10 mL tube with 8 mL LS medium, and put into 37℃ shaker for overnight culture.

18. Cryopreservation DH5α pEZ15A-PtF、DH5α pEZ15A-PtY、DH5α pEZ15A-PtT1 strain, and send it for sequencing.

19. Plasmid extraction

Extract the plasmids of DH5α pEZ15A-PtF、DH5α pEZ15A-PtY、DH5α pEZ15A-PtT1 with kit after culturing.

20. Electroporation

Transfer the extracted plasmids into the competent cells of ZMNP. Set up the transformation program of the electroporator (capacitance: 25μF, resistance: 200Ω, voltage: 1600 V).

(1) Thaw the competent cells and cool the electroporator on ice.

(2) Absorbe the plasmid into 50 μL comoetent cells, and calculate the amount according to the concentration of the extracted plasmid, the total mass is less than 450 ng.

(3) Immediately absorb the mixture of plasmids and competent cells into the electroporator.

(4) Wipe the water outside the rotating cup and start the program;

(5) Absorb the bacterial liquid in the electroporator into the RM liquid culture meduim and mark it on the EP tube;

(6) The EP tube is sealed with the sealing film and cultured in a shaker at 30℃ for 4-6 hours;

(7) Coat on RS solid medium plate in super-clean bench, 3 tubes of bacterial liquid, each tube is absorbed 100μL and 200μL, marked with the label, a total of 6 plates, and put into a 30℃ incubator for upside down culture.

21. Monoclonal colony PCR

Transfered bacterias are made monoclonal colony PCR. Result: There are bands and the size is correct.

22. Culture bacterias

Inoculated the correct strains of water-soluble bacteria ZMNP pEZ15A-PtF, ZMNP pEZ15A-PtY and ZMNP pEZ15A-PtT1 into 50 mL centrifuge tubes with 8 mL RS liquid culture meduim and cultured overnight in an incubator at 30℃.

23. Cryopreservation ZMNP pEZ15A-PtF、ZMNP pEZ15A-PtY、ZMNP pEZ15A-PtT1 strain, and send it for sequencing.

24. Fermentation

(1) Firstly, 100 μL of glycerol bacteria of recombinant strains ZMNP-PtF、ZMNP-PtY、ZMNP-PtT1 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.

25. 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 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).