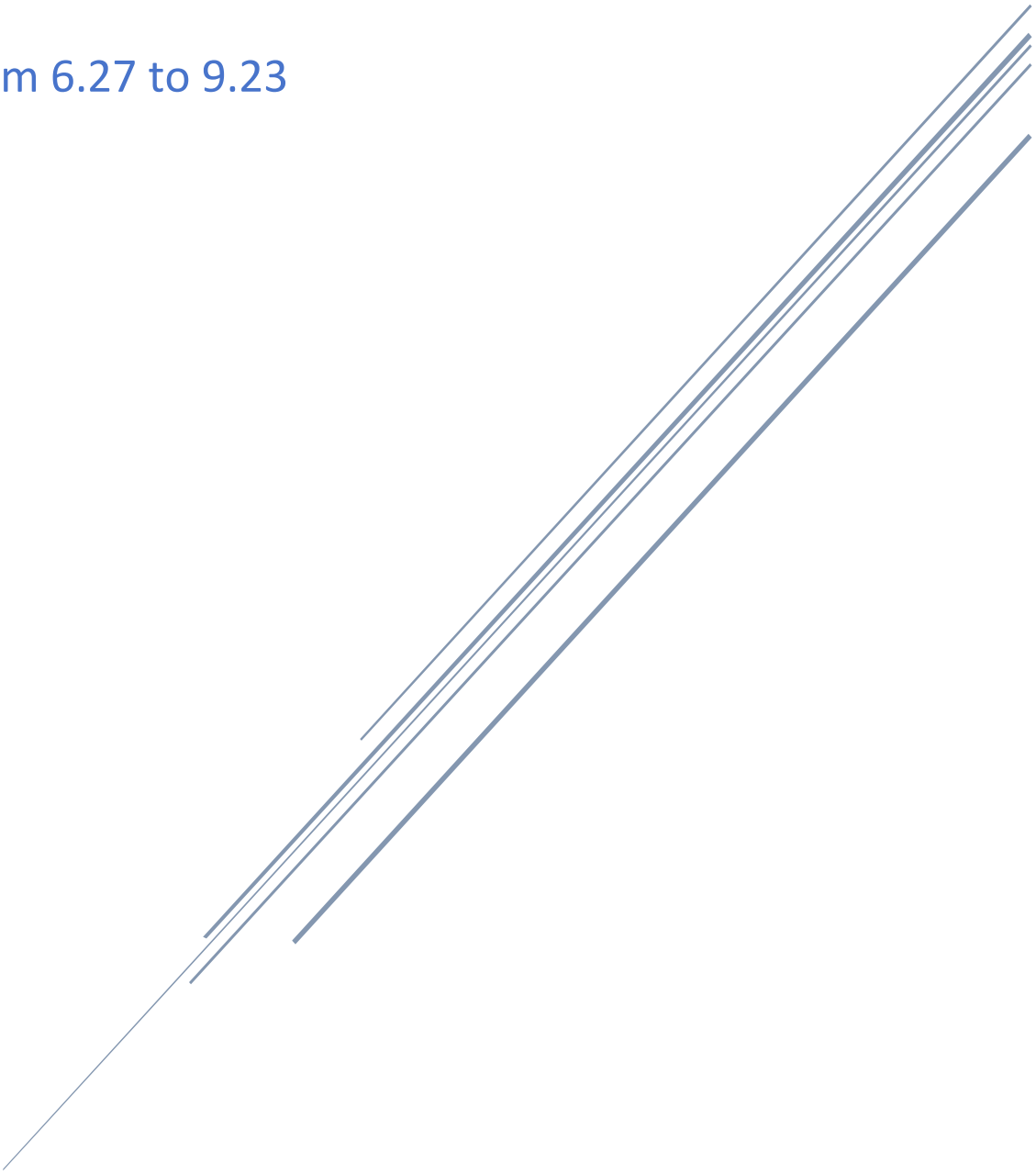
NOTEBOOK

iGEM-HUST China

From 6.27 to 9.23



Contents

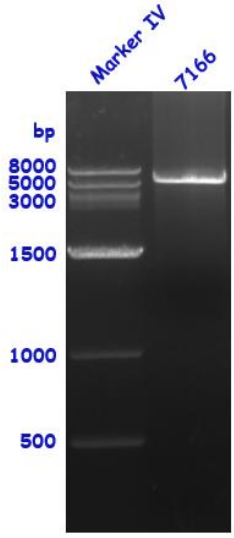
1. Molecular Experiments

6.27-6.29
members：zwt,zsz,zsj,cyl
Prepared reagents required for the experiment (YNB reagent, Tris-HCl, LB solid and liquid media, YPD solid and liquid media, MD liquid medium, MD-Leu medium, olive oil emulsion, oleic acid medium, linoleic acid medium, linolenic acid medium) and organized basic experimental protocols (including homologous recombination, E. coli heat shock transformation, yeast competent cell preparation and transformation, double enzyme digestion, ligation, PCR, agarose gel electrophoresis, SDS-PAGE, yeast tag recovery, yeast genome extraction, gel extraction, plasmid extraction).
Summary: Protocols were printed into hard copies; basic required reagents were correctly prepared and sterilized.

6.30
members:szx,wyx,wh
(1) Inoculated Po1f-WT and conducted expanded culture.
(2) Extracted the yeast genome, verified it by agarose gel electrophoresis, and observed the DNA bands.
Summary: Yeast genome extraction failed.

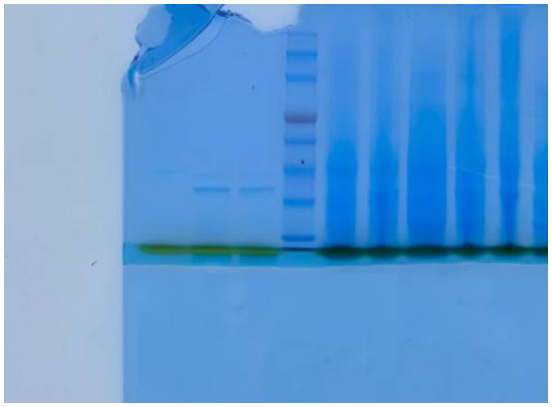
7.2
members:ldd,myc
Re-extracted the yeast genome and performed verification.
Summary: The yeast genome was successfully extracted.

7.3-7.5
members:zwt,wjr
Obtained the 7166 empty vector and the 7166 vector carrying the tll gene from the professor. Transformed the 7166 empty vector into E. coli for overnight culture, extracted the plasmid the next day, and verified it by agarose gel electrophoresis.
Summary: Successfully extracted the 7166 empty vector and the 7166-tll vector.

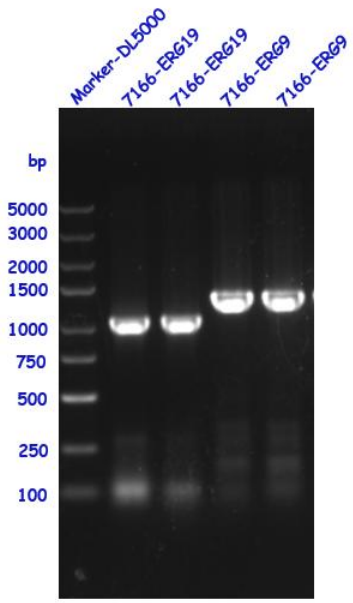


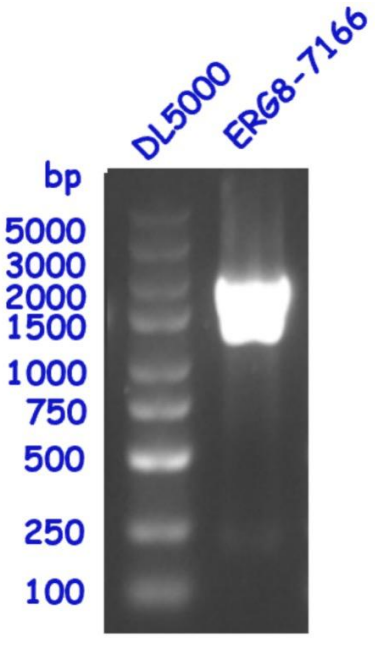
7.6-7.8
members:zwt,szx,zyy
(1) Prepared yeast competent cells, transformed the 7166-tll vector into yeast, performed two rounds of screening by plating after overnight culture, then recovered the yeast tag to obtain the target strain and preserved it.

(2) Extracted the fermentation supernatant and verified lipase secretion by SDS-PAGE.
Summary: Successfully obtained yeast with the tll gene inserted, which can secrete [lipase] extracellularly.



7.9-7.12
members:myc,zyy,wyx,cyl
(1) Used primers to obtain gene fragments (including ERG8, ERG9, ERG19, ERG20, ERG12, ERG10, ERG13, IDI) from the Po1f-WT yeast genome. Prepared a double enzyme digestion (NotI, ClaI) system to obtain digested linear plasmids and genes. Constructed complete vectors using T4 ligase, then transformed the corresponding plasmids into E. coli DH5α respectively. The next day, randomly selected 3 colonies from the plate, detected bands by colony PCR, and sent the bacterial solutions with bands for sequencing.
(2) Preserved the verified correct strains in glycerol and stored them in a -20°C refrigerator. Prepared LB (amp+) solid medium and LB liquid medium.
Summary: Successfully constructed overexpression vectors for ERG9, ERG19, and ERG8, but failed to obtain gene fragments of ERG10, ERG12, and ERG20.

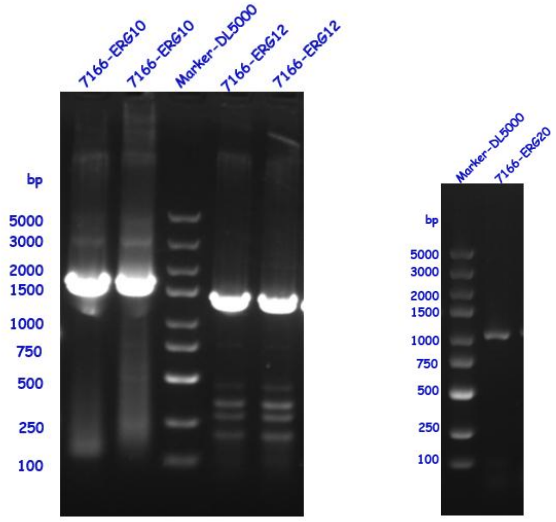


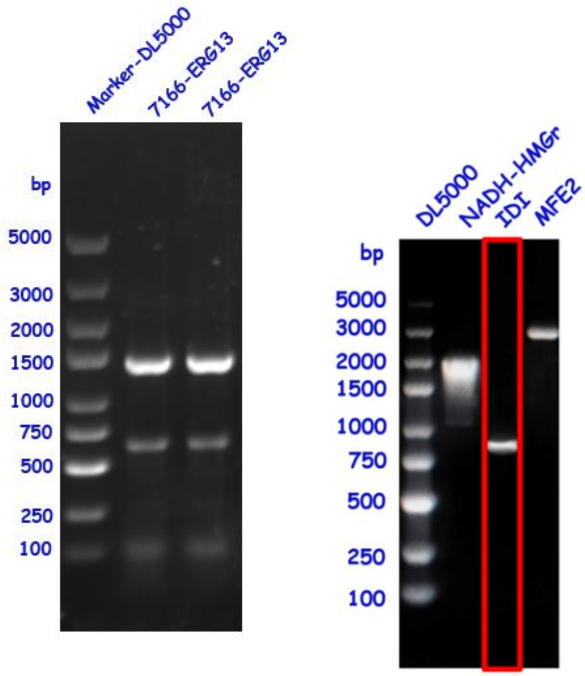


7.13-7.14
members：cyl,wyx,wh
(1) Re-selected 3 other colonies from the remaining plates for colony PCR verification, and sent the bacterial solutions with correct bands for sequencing.

(2) Transformed ERG9, ERG19, and ERG8 (returned by the company) into E. coli DH5α respectively. The next day, picked colonies from the plate for expanded culture, preserved the verified correct strains in glycerol, and stored them in a -20°C refrigerator.
(3) Obtained the upstream and downstream sequences of the MFE2 and POX2 genes, and the complete POT1 sequence from the Po1f-WT yeast genome.
Summary: Plasmids with correct sequences were still not obtained; successfully obtained the upstream and downstream sequences of the MFE2 and POX2 genes, and the complete POT1 sequence.

7.15-7.18
members: szx,zwt,zyy
(1) Re-prepared a double enzyme digestion (NotI, ClaI) system to obtain digested linear plasmids and genes (ERG13, IDI). Adjusted the annealing temperature to obtain the remaining genes (ERG10, ERG12, ERG20) from the genome. Constructed complete vectors using T4 ligase, then transformed the corresponding plasmids into E. coli DH5α respectively. The next day, randomly selected 3 colonies from the plate, detected bands by colony PCR, and sent the bacterial solutions with bands for sequencing.
(2) Extracted plasmids from the expanded cultures of ERG9, ERG19, and ERG8.
(3) Performed fusion PCR using the upstream and downstream sequences of the MFE2 and POX2 genes.
Summary: Successfully constructed yeast strains carrying ERG10, ERG12, ERG20, ERG13, and IDI respectively, but failed to fuse the upstream and downstream sequences of the MFE2 and POX2 genes.

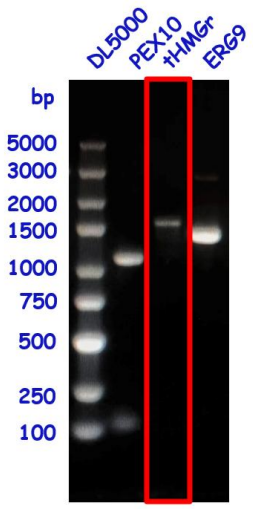




7.19-7.21
members:szx,zsj,zsz,ldd
(1) Used primers to obtain gene sequences with homologous arms required for homologous recombination from the vectors for all MVA pathway genes (including ERG9, ERG19, ERG20, ERG12, ERG10, ERG13, ERG8, IDI). Prepared a homologous recombination system for plasmid construction (ERG10-ERG13-ERG8, IDI-ERG20-ERG10, ERG12-ERG19-ERG20) and transformed into E. coli. The next day, randomly selected 3 colonies from each plate and sent them for sequencing.
(2) Transformed the plasmids of ERG10, ERG12, ERG20, ERG13, and IDI (returned by the company) into E. coli DH5α respectively. The next day, picked colonies from the plate for expanded culture, preserved the verified correct strains in glycerol, and stored them in a -20°C refrigerator.
(3) Performed fusion PCR again using the upstream and downstream sequences of the MFE2 and POX2 genes.
Summary: All homologous recombinant plasmids had deletions or errors; the target plasmids were not successfully constructed; the upstream and downstream sequences of the MFE2 and POX2 genes still failed to fuse.

7.22-7.25
members:zwt,ldd,wyx,zsz,myc
(1) Re-performed homologous recombination and attempted to construct three types of large plasmids (ERG10-ERG13-ERG8, IDI-ERG20-ERG10, ERG12-ERG19-ERG20) again. Transformed the homologous products into E. coli DH5α. The next day, randomly selected 3 colonies from each plate and sent them for sequencing.
(2) Extracted plasmids from the expanded cultures of ERG10, ERG12, ERG20, ERG13, and IDI.
(3) Prepared LB (amp+), MD-Leu, MD solid and liquid media, and poured plates.
Summary: All homologous recombinant plasmids had deletions or errors; the target plasmids were not successfully constructed.

7.26-7.27
members: myc,zyy,wyx,wjr
Prepared competent cells of yeast Po1f-lipase and transformed all MVA pathway genes (including PEX10, ERG9, ERG19, ERG20, ERG12, ERG10, ERG13, ERG8, IDI) respectively. Performed two rounds of screening by plating, followed by yeast tag recovery.
Summary: Suspected successful construction of yeast strains carrying individual MVA pathway genes (including PEX10, ERG9, ERG19, ERG20, ERG12, ERG10, ERG13, ERG8, IDI) respectively.



8.1-8.3
members:zyy,ytx,zwt,szx,ldd,wh
(1) Used primers to obtain gene sequences with NotI and ClaI restriction sites from the synthetic NADH-HMGr and HMGr puC57 vectors. Prepared a double enzyme digestion system for digestion, followed by ligation. Transformed the ligation products into E. coli. The next day, randomly selected 3 colonies from the plate, verified by colony PCR, and sent the bacterial solutions with DNA bands for sequencing.
(2) Expanded the culture of NADH-HMGr and HMGr bacterial solutions and extracted plasmids.
(3) Linearly digested the pURC knockout vector with BlnI (AvrII) single enzyme, then connected sgRNA to the vector via homologous recombination to construct the ERG1-sg knockout vector, and transformed it into E. coli DH5α for sequencing.
Summary: Successfully constructed overexpression vectors carrying NADH-HMGr and HMGr, and the ERG1-sg knockout vector.

8.4-8.5
members: ytx,wjr,zyy
(1) Designed overlap extension primers for PCR to amplify Up500 and Dn500 respectively. After purification, used the up and dn homologous arms as templates and Up-F and Dn-R as primers to amplify Dor-DNA. Digested the ERG1-sg plasmid with PacI/SpeI double enzymes, connected Dor-DNA to the digested plasmid via homologous recombination, transformed into E. coli DH5α, and sent for sequencing.
(2) Transformed and cultured the ERG1-sg, NADH-HMGr, and HMG vectors (returned by the company) for expanded culture, and extracted plasmids the next day.
(3) Stored the bacterial solutions with successful sequencing in a -20°C refrigerator.
Summary: Failed to successfully construct the ERG1-sg-DorDNA knockout vector.

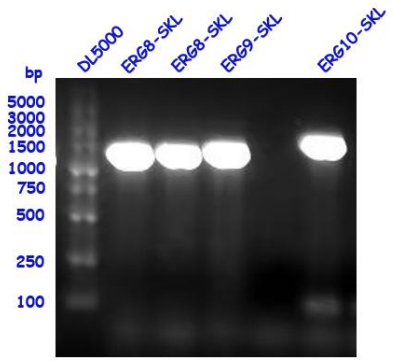
8.6
members:zwt,szx,myc
Prepared competent cells of yeast Po1f-lipase, and transformed the ERG1 knockout vector, NADH-HMGr, and HMGr into yeast Po1f-lipase respectively. Performed two rounds of screening using plates, followed by yeast tag recovery to obtain the target strain, which was then preserved.
Summary: Suspected successful construction of yeast strains carrying the overexpression vectors of NADH-HMGr and HMGr, as well as the ERG1-sg knockout vector.

8.7
members:ldd
(1) Obtained the GFP gene sequence carrying SKL from the pUC57 vector synthesized by the company using PCR.
(2) Transformed GFP-SKL into E. coli DH5α and plated the culture.
Summary: Successfully obtained the gene sequence carrying GFP-SKL.

8.8-8.9
members: wjr,wyx

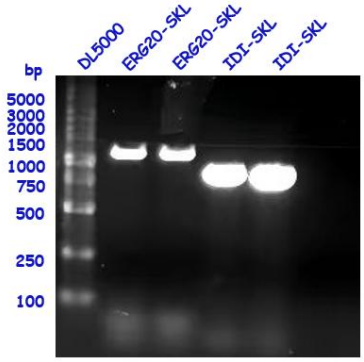
(1) Used primers with the SKL signal peptide sequence to obtain all MVA pathway genes with signal peptides (including PEX10-SKL, ERG9-SKL, ERG19-SKL, ERG20-SKL, ERG12-SKL, ERG10-SKL, ERG13-SKL, IDI-SKL) from the Po1f-WT yeast genome. Prepared a double enzyme digestion (NotI, ClaI) system for gene digestion, ligated them to the 7166 linear vector, and transformed into E. coli DH5α, but no colonies appeared the next day.
(2) Verified the previously extracted yeast genome by electrophoresis; inoculated and expanded the culture of Po1f-lipase.
(3) Picked colonies from the GFP-SKL plate, verified by colony PCR, and sent for sequencing.
Summary: The previously extracted yeast genomes had degraded; the constructed GFP-SKL overexpression vector plasmid was successful.

8.10-8.12
members:zyy,wh,ldd
(1) Re-extracted the genome of yeast Po1f-lipase.
(2) Re-prepared a double enzyme digestion (NotI, ClaI) system for digestion, ligated the genes (including ERG8-SKL, ERG9-SKL, ERG19-SKL, ERG20-SKL, ERG12-SKL, ERG10-SKL, ERG13-SKL, IDI-SKL) to the 7166 linear vector, transformed into E. coli DH5α. The next day, randomly selected 3 colonies from the plate, verified by colony PCR, and sent for sequencing.
(3) Prepared LB (amp+) solid medium and poured plates.
Summary: Successfully constructed overexpression vectors for ERG8, ERG9, and ERG10 genes carrying SKL.

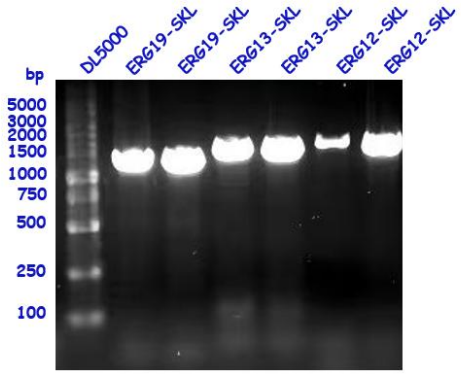


8.11-8.13
members:zwt,szx，ldd
(1) Ligated the genes (including ERG19-SKL, ERG20-SKL, ERG12-SKL, ERG13-SKL, IDI-SKL) to the 7166 linear vector again, transformed into E. coli DH5α. The next day, randomly selected 3 colonies from the plate, verified by colony PCR, and sent for sequencing.
(2) Prepared competent cells of yeast Po1f-lipase, transformed the GFP-SKL overexpression vector, and no screening was performed.
Summary: No bands were detected in gene PCR; no vectors were successfully constructed.

8.15-8.16
members:cyl,zsj
(1) Reduced the annealing temperature, re-performed PCR to obtain target genes (including ERG19-SKL, ERG20-SKL, ERG12-SKL, ERG13-SKL, IDI-SKL) from the yeast genome, transformed into E. coli DH5α. The next day, randomly selected 3 colonies from the plate, verified by colony PCR, and sent for sequencing.
(2) Transformed the ERG8-SKL, ERG9-SKL, and ERG10-SKL plasmids returned by the company into E. coli for expanded culture, and extracted the plasmids the next day.
Summary: Successfully constructed ERG20 and IDI genes carrying SKL, but no bands were detected in PCR for the remaining genes.



8.17-8.18
members:wyx,wjr
(1) Further reduced the annealing temperature, re-performed PCR to obtain target genes (including ERG12-SKL, ERG19-SKL, ERG13-SKL) from the yeast genome, transformed into E. coli DH5α. The next day, randomly selected 3 colonies from the plate, verified by colony PCR, and sent for sequencing.
(2) Transformed the ERG13-SKL, ERG19-SKL, and ERG12-SKL plasmids returned by the company into E. coli for expanded culture, and extracted the plasmids the next day.
Summary: Successfully constructed ERG13, ERG19, and ERG12 genes carrying SKL, but no bands were detected in PCR for the remaining genes.



8.19-8.20
members:zyy,myc,yyt
(1) Prepared competent cells of yeast Po1f-lipase and transformed the ERG12-SKL, ERG19-SKL, ERG20-SKL, and ERG9-SKL vectors. Performed two rounds of screening via plates and conducted tag recovery.
(2) Prepared LB (amp+) solid medium and MD-Leu liquid medium.
Summary: Screening failed; the transformed yeast could grow on both auxotrophic and non-auxotrophic plates.

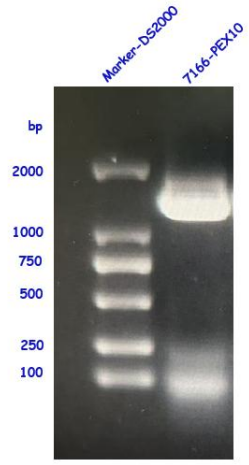
8.21-8.23
members:zyy,ldd
Re-prepared competent cells of yeast Po1f-lipase and re-transformed the ERG12-SKL, ERG19-SKL, ERG20-SKL, and ERG9-SKL vectors. Performed two rounds of screening via plates and conducted tag recovery.
Summary: Screening failed again; the transformed yeast could grow on both auxotrophic and non-auxotrophic plates.

8.24-8.25
members: myc,zwet
(1) After communicating with the professor, re-prepared competent cells of yeast Po1f-lipase and transformed the ERG12-SKL, ERG19-SKL, ERG20-SKL, and ERG9-SKL vectors. Performed two rounds of screening using liquid medium, and tag recovery was no longer conducted.
(2) Prepared MD plates, MD-Leu plates, and LB liquid medium.
Summary: The yeast was suspected to be successfully transformed but requires further verification.

8.26-8.27
members:szx,yyt
Used newly designed primers for MFE2 and POX2 to obtain the corresponding gene fragments from the genome of yeast Po1f-lipase. Abandoned fusion PCR and adopted homologous recombination instead. Using the plasmid carrying POT1 as the vector, ligated MFE2 and POX2, transformed the recombinant plasmid into E. coli DH5α. The next day, randomly selected 3 colonies from the plate, verified by colony PCR, and sent for sequencing.
Summary: Successfully constructed overexpression vectors for MFE2 and POX2.

8.28-8.31
members:zwt,zyy
(1) Transformed the MFE2-POX2-POT1 plasmid returned by the company and expanded the culture. Extracted the plasmid the next day, and preserved the correct strain in glycerol at -20°C.
(2) Prepared competent cells of yeast Po1f-lipase, transformed the MFE2-POX2-POT1 plasmid, and performed two rounds of screening using liquid medium.
Summary: Yeast suspected to have successfully transformed with the large MFE2-POX2-POT1 plasmid, but further verification is required.

9.5-9.6
members:wyx,wjr
（1）(1) Obtained the PEX10 sequence from the genome of yeast Po1f-lipase, performed enzyme digestion, followed by ligation. Transformed the ligation product into E. coli. The next day, randomly selected 3 colonies from the plate, verified by colony PCR, and sent the bacterial solutions with DNA bands for sequencing.
(2) Linearly digested the pURC knockout vector with BlnI (AvrII) single enzyme, then connected sgRNA to the vector via homologous recombination to construct the PEX23-sg knockout vector, and transformed it into E. coli DH5α for sequencing.
Summary: Successfully constructed the overexpression vector carrying PEX10; failed to construct the PEX23-sg knockout vector.



9.7-9.8
members:zwt,yyt

(1) Transformed the plasmids returned by the company and expanded the culture. Extracted the plasmids the next day, and preserved the correct strains in glycerol at -20°C.
(2) Expanded the culture of the preserved strains (including ERG8-SKL, ERG9-SKL, ERG19-SKL, ERG20-SKL, ERG12-SKL, ERG10-SKL, ERG13-SKL, IDI-SKL) and extracted the plasmids the next day.
Summary: Successfully extracted the overexpression vectors of ERG8-SKL, ERG9-SKL, ERG19-SKL, ERG20-SKL, ERG12-SKL, ERG10-SKL, ERG13-SKL, and IDI-SKL.

9.9-9.11
members:zwt,zyy,yyt
Re-prepared competent cells of yeast Po1f-lipase and transformed the ERG8-SKL and ERG19-SKL vectors. Performed two rounds of screening using liquid medium and conducted tag recovery.
Summary: The yeast still failed to successfully recover the tags.

2.Lipase

7.31-8.1
members:szx,wyx
Prepare YNB solid medium and spread-inoculate Po1f-lipase.
Summary: Po1f-lipase cannot grow normally; a complete medium must be used.

8.2-8.3
members:szx
Prepare tributyrin plates and spread-inoculate Po1f-lipase.
Summary: Po1f-lipase grew normally, but the hydrolysis zones were difficult to identify with the naked eye.

8.4-8.6
members:wyx
Prepare phenol red-tributyrin plates and spread-inoculate Po1f-lipase.
Summary: The hydrolysis zones covered the entire plate, which did not meet the expected results.

8.7-8.8
members:szx
(1) Re-prepared phenol red-tributyrin plates and inoculated Po1f-lipase using two methods: spot injection and spreading.
(2) Extracted the fermentation supernatant of Po1f-lipase for alkaline titration.
Summary: Circular hydrolysis zones formed after spot injection inoculation, which met the expected results.

9.14-9.16
members:szx
(1) Re-prepared phenol red-tributyrin plates and inoculated Po1f-lipase and Po1f-WT respectively using two methods: spot injection and spreading.
(2) Extracted the fermentation supernatants of Po1f-lipase and Po1f-WT respectively for alkaline titration.
Summary: Successfully confirmed that Po1f-lipase has a better ability to decompose fats.

3.MVA pathway

7.28-7.31
members:ytx

(1) Used a vacuum freeze dryer and steel beads to prepare freeze-dried powder of yeast Po1f-lipase cells (including WT, ERG9, ERG19, ERG20, ERG12, ERG10, ERG13, ERG8, IDI).
(2) Performed extraction on a shaker, removed impurities and dissolved the next day, filtered with an organic filter, stored in brown gas chromatography vials, and sent for testing.
Summary: Successfully measured the content of squalene.

8.8-8.10
members:zyy,ytx,zwt
(1) Used a vacuum freeze dryer and steel beads to prepare freeze-dried powder of yeast Po1f-lipase cells (including tHMGr, NADH-tHMGr, tHMGr).
(2) Performed extraction on a shaker, removed impurities and dissolved the next day, filtered with an organic filter, stored in brown gas chromatography vials, and sent for testing.
Summary: Successfully measured the content of squalene and verified the effects of NADH-tHMGr and tHMGr.

8.15
members:ytx,zwt
(1) Stained yeast GFP-SKL with Nile Red.
(2) Observed yeast GFP-SKL using a confocal microscope, detected the distribution of green fluorescent protein, and compared the co-stained regions of Nile Red and green fluorescence.
Summary: Successfully demonstrated the targeting ability of the leader peptide.

9.2-9.4
members:szx
(1) Cultured the presumably successfully transformed MFE2-POX2-POT1 yeast strain (BO), Po1f-WT, and Po1f-lipase overnight in YPD medium, then collected the bacterial cells by centrifugation respectively.
(2) Resuspended the collected engineered bacterial cells with olive oil emulsion respectively, and after 24 days of culture, let them stand at room temperature for 30 minutes.
(3) Mixed well, sampled, extracted one group with petroleum ether respectively, and performed alkaline titration on the other group.
Summary: The MFE2-POX2-POT1 yeast strain was successfully constructed and exhibited better lipid degradation ability.

9.12-9.15
members:ytx,zwt
(1) Overnight cultured Po1f-lipase yeast presumably successfully transformed with ERG9-SKL, ERG19-SKL, ERG20-SKL, and ERG12-SKL.
(2) Used a vacuum freeze dryer and steel beads to prepare freeze-dried powder of yeast Po1f-lipase cells (including Po1f-lipase, ERG9, ERG19, ERG20, ERG12).
(3) Performed extraction on a shaker, removed impurities and dissolved the next day, filtered with an organic filter, stored in brown gas chromatography vials, and sent for testing.
Summary: Successfully measured the squalene content, but the error was large, making the data unusable.

9.20-9.23
members:ytx
(1) Overnight cultured Po1f-lipase yeast in lipid medium and YPD liquid medium respectively.
(2) Used a vacuum freeze dryer and steel beads to prepare freeze-dried powder of Po1f-lipase cells grown in the two aforementioned media.
(3) Performed extraction on a shaker, removed impurities and dissolved the sample the next day, filtered it with an organic filter, stored it in a brown gas chromatography vial, and sent it for testing.
Summary: The squalene content was successfully measured and consistent with expectations, proving that the modified yeast can efficiently utilize waste oil.

4.Growth Curve

7.9-7.12
members:zsz
(1) Prepared different types of lipid-based media (including those containing linolenic acid, linoleic acid, and oleic acid) were prepared. An equal amount of Po1f-lipase was inoculated into each medium and cultured for 3 days. During this period, samples were taken at regular intervals, and the absorbance of the bacterial suspension at OD600 was measured using a spectrophotometer.
(2) The obtained data were plotted into curves.
Summary: Po1f-lipase was able to grow in large quantities in all these media.

7.8-7.12
members:cyl,zsj
(1) Prepared lipid media with different glycerol-to-fatty acid ratios (including 1:1, 1:3, 1:5), inoculated an equal amount of Po1f-WT, and cultured for 3 days. During this period, samples were taken at regular intervals, and the absorbance of the bacterial suspension at OD600 was measured using a spectrophotometer.
(2) Plotted the obtained data into curves.
Summary: The obtained data were unusable, as the lipids themselves interfered with the absorbance measurement, and this issue needs to be avoided.

7.15-7.19
members:zwt,cyl
(1) Prepared canteen waste oil media with different pH values (including pH=4.0, 5.0, 6.0, 7.0) respectively, inoculated an equal amount of Po1f-lipase into each medium, and placed them in incubators at 28°C, 30°C, and 37°C respectively for 3 days of culture. During this period, samples were taken at regular intervals, and the absorbance of the bacterial suspension at OD600 was measured using a spectrophotometer.
(2) Plotted the obtained data into curves.
Summary: The yeast grew best under the conditions of pH=6.0 and temperature of 28°C.

7.31-8.1
members:zsz
(1) Prepare lipid media with different carbon-to-nitrogen (C/N) ratios (including C/N=10, 20, 40, 60, 80), inoculate an equal amount of Po1f-lipase, and culture for 3 days. During this period, take samples at regular intervals and measure the absorbance of the bacterial suspension at OD600 using a spectrophotometer.
(2) Plot the obtained data into curves.
Summary: Po1f-lipase grows best when the C/N ratio is 10.

8.6-8.8
members:zyy
(1) Prepare YPD medium, inoculate an equal amount of Po1f-lipase with the ERG1-sg knockout vector transferred in, and culture for 3 days. During this period, take samples at regular intervals and measure the absorbance of the bacterial suspension at OD600 using a spectrophotometer.
(2) Plot the obtained data into curves.
Summary: When the culture was approaching the end stage, the absorbance of the bacterial suspension decreased significantly, indicating a large number of bacterial cell deaths.