iGEM
Methods in
Molecular Biology

Yeast Handbook

Yeast alliance

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HUST-China
CPU_CHINA
HiZJU-China
Jiangnan_China
NWU-CHINA-B

First Edition

Yeast Handbook

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(2021.10)

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UESTC-China

UM_Macau

HUST-China

CPU_CHINA

HiZJU-China

Jiangnan_China

NWU-CHINA-B

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Yeast Alliance

Content

Preface

Part 1 Reasons for choosing yeast

1.1 HUST-China's reasons

Because the source of many of our genes is eukaryotic, it is best to choose a eukaryotic expression system (correct folding and modification) if you want to obtain an active protein; Pichia pastoris is a more mature eukaryotic expression system. According to the data, Its over-glycosylation of protein is not as serious as that of Saccharomyces cerevisiae, and we also chose Pichia pastoris as the chassis in 2019 iGEM. We thought it is safe and we also have certain operating experience, so we finally chose it.

1.2 UESTC-China's reasons

We choose eukaryotes this year. For one reason that we choose cellulase and xylanase as the main deinking enzymes, we consider eukaryotes as the chassis organisms to make the enzymes work better, which can post-translationally process proteins. Another reason that we choose eukaryotes is most of the studys on cellulosome were in eukaryotes.
We found that Saccharomyces cerevisiae and Pichia pastoris were usually used as chassis organisms in eukaryotic expression system. Both of them are IGEM risk level 1, so there are no safety concerns.
However, as S. cerevisiae could not overcome the problem of glycosylation, we finally chose Pichia pastoris. Pichia pastoris can secrete and express exogenous proteins at a high level, and because there are few proteins secreted by pichia pastoris itself into the culture medium, it is convenient to purify and can produce soluble recombinant proteins with correct folding.

1.3 CPU_CHINA's reasons

The critical functional protein Manganese peroxidase (MnP) we used in our program is a highly glycosylated, heme-containing lignin peroxidase, which is derived from white-rot fungi. *Pichia pastoris* GS115 is reported be used to express MnP due to its predominant glycosylation ability and mature eukaryotic expression system, so in our design, *Pichia pastoris* GS115 is selected as one of our chassis.

1.4 UM_Macau's reasons

Our project focused on producing low purine and an ex tioxidation beer, and *Saccharomyces cerevisiae* is the organism used to brew beer, so our team chose to use *S. cerevisiae* as the chassis organism.

1.5 HiZJU-China's reasons

Differing from Saccharomyces cerevisiae, Pichia pastoris has no free expression vector. Outside vector is integrated into genome of Pichia pastoris in the form of single copy or multiple copies. However, HiZJU-China adopted yeast two-hybrid technique for detection design, which required stable expression vectors. Therefore, Saccharomyces cerevisiae is more appropriate than Pichia pastoris.

1.6 Jiangnan_China's reasons

Since the natural origin of the substance Gadusol, what we wanted to produce, was zebrafish eggs, we first excluded prokaryotic chassis and chose eukaryotic chassis, and because we wanted to use it for cosmetics production, we chose *Saccharomyces cerevisiae* as the starting strain after inquiring the *Measures for Genetic Engineering Safety*. In addition to the heterologous expression ability of vertebrates' substance and safety issues, yeast also has irreplaceable advantage in cosmetics production. It not only reduces the pollution from chemical reagent extraction, but also

reduces the production costs of purification since cosmetics ingredients produced by *Saccharomyces cerevisiae* could be added into cosmetics in the form of yeast lysate.

Part 2 Choose a strain you prefer

2.1 HiZJU-China

Y2H yeast was selected and modified to have two reporter genes: green fluorescent GFP and red fluorescent mCherry to prepare for the diversity of detection results.

2.2 CPU_CHINA

In the references cited, there have been reported cases of MnP secretion using GS115 and its use in the degradation of polyethylene, and samples of GS115 wild-type strain are kept in our laboratory.
In addition, after comparing the advantages and disadvantages of using *S. cerevisiae* and *Pichia pastoris to express our enzymes* we chose *Pichia pastoris* GS115 as the final chassis.

2.3 UESTC-China

We selected GS115 as the candidate specie according to foreign and domestic studies on the heterologous expression of cellulase. GS115 strain is the most mature strain in Pichia pastoris. It is histidine deficient type and has its own marker gene, which is convenient for screening. It can be expressed by adding histidine. However the other choice —— X33 is wild type without nutritional deficiency (After repeated comparisons, we summarized the following advantages and disadvantages of these two kinds of yeast in Table.4). Considering the effective screening in subsequent experiments, we finally selected GS115 as our chassis organism.

Table 1. Saccharomyces cerevisiae & Pichia pastoris

<table>
<tr><th></th><th>Saccharomyces cerevisiae</th><th colspan="2">Pichia pastoris</th></tr>
<tr><td rowspan="6">Advantages</td><td colspan="3">Eukaryotes can post-translationally process proteins.</td></tr>
<tr><td rowspan="3">1. Saccharomyces cerevisiae is safe and easy to cultivate in laboratory, and has been widely used in the food industry for a long time. It does not produce toxins, and has been identified as a safe organism by the FDA of the United States, and the expression products do not need to undergo a lot of host safety experiments.</td><td rowspan="3">1. The function of expression and secretion is strong.</td><td>Its secreted protein is less, which is conducive to the purification of the target protein.</td></tr>
<tr><td>The signal peptide suitable for target protein can be quickly obtained through existing research.</td></tr>
<tr><td>Good folding ability.</td></tr>
<tr><td>2. Its product is not easy to produce inclusion body and the secretion of it is stable.</td><td colspan="2">2. Defective and phenotypic strains have been studied extensively and can be constructed on demand.</td></tr>
<tr><td>3. It can absorb heavy metal ions, which is beneficial to expand the influence of the project.</td><td colspan="2">3. High degree of commercialization.</td></tr>
<tr><td>4. The genetic background is clear, the transformation is more simple with many ways.</td><td colspan="2">4. It has been proved that glycerol and glucose can be used as carbon sources, and rhamnose can also be induced after modification.</td></tr>
<tr><td>5. The substrate is simple and easy to operate.</td><td colspan="2"></td></tr>
<tr><td rowspan="4">Disadvantages</td><td>1. Low yield, difficult to carry out high density fermentation (difficult to secrete, protein above 30K Dalton is difficult to secrete, and low expression efficiency).</td><td colspan="2">1. The fermentation period is long, and the protease produced in the process is easy to decompose the target protein. There is also evidence that exogenous proteins are easy to stay in the endoplasmic reticulum and cannot be secreted during pichia pastoris expression under certain conditions.</td></tr>
<tr><td>2. The promoter is unstable (only TATAbox and its location is unstable, and the spacing between the sequence used to enhance expression and the gene is required not too far away).</td><td colspan="2">2. The AOX12 promoter must be induced with methanol, but after modification, rhamnose can be used as induction.</td></tr>
<tr><td>3. Target proteins are susceptible to superglycosylation.</td><td colspan="2">3. It is complicated and difficult to carry out renovation.</td></tr>
<tr><td>4. The process of cell division is not stable (plasmid transmission is not stable) with many mutations.</td><td colspan="2">4. Methanol induction involves product safety issues (if we are going to use bacteria directly to deink).</td></tr>
</table>

2.4 UM_Macau

FY4 is a most commonly used MATa type strain derived from S288C. It is safe to use and suitable for our project.

2.5 Jiangnan_China

BY4743 is a five-growth factor defective strain. It has potential to be subsequently introduced more synthetic pathways of high value-added natural products and engineered it into a safe yeast strains that can be used in the cosmetics industry.

Part 3 Choose a vector you prefer

3.1 NWU-CHINA-B （*pPIC9K and pPICZαA）

Within a certain range, with the increase of copy number, the expression level of target protein increased correspondingly, in a direct proportion relationship, but when it reached a certain value, the expression level of target protein would decrease if the copy number continued to increase.

The genes copy number in pPIC9K and pPICZαA were determined by fluorescence quantitative PCR.

△△CT=(CT, BGLY-CT,GAP)Test-(CT,
Bgly-ct (GAP)Calibrator calculated the mean CT values of target gene and reference gene and the expression level of target gene SS-BGLY in transforant GS115/9K-SS-BGLY 6# and transforant GS115/9K-Zα A-SS-BGLY 4#.

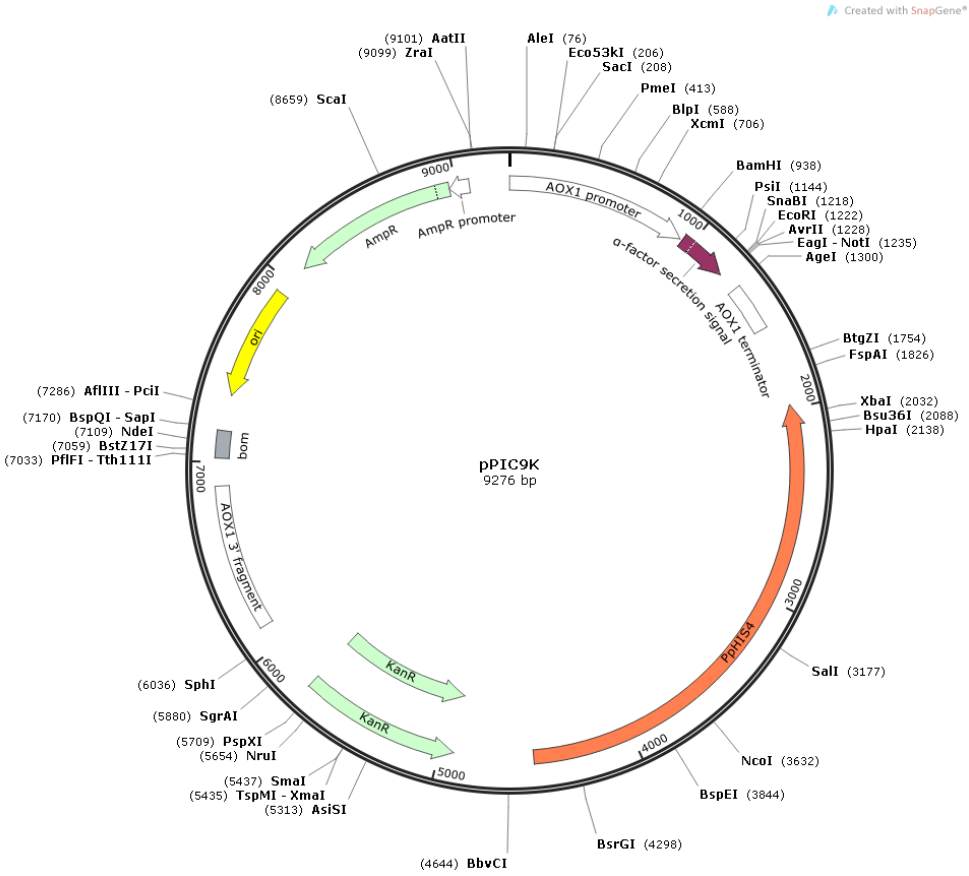
Using the plasmid pPIC9KSS-BGly as A single copy, the copy numbers of SS-BGLY gene in gs115/9K-SS-BGLY 6# and GS115/9K-ZαA- SS-BGLY 4# were 1.89 and 4.03, respectively. Rounded to 2 and 4.

It was found that expression effect of pPICZαA plasmid was higher

than that of pPIC9K.

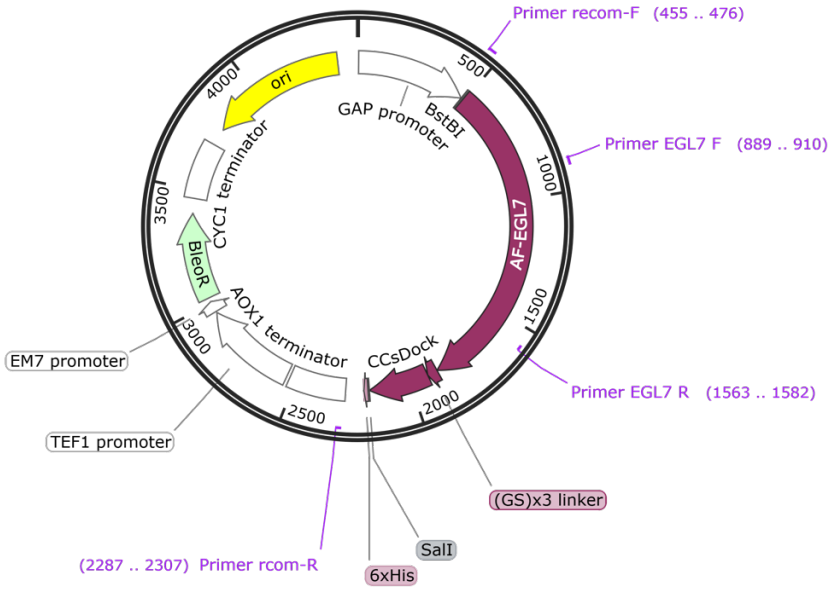
3.1 HUST-China

Because the chassis we chose is Pichia GS115, among its commonly used plasmids, pPIC9K is a multi-copy plasmid and is suitable for extracellular expression. In terms of our project design, what we expect is that the protein produced by the gene pathway can enter the culture medium and finally synthesize the substances outside the cell.



3.2 UESTC-China

Most expression vectors rely on the *AOX1* promoter to drive expression, but there are times where this promoter may not be ideal. Use of methanol induction protocols may not be appropriate for the food industry because the petroleum-related compounds used are health and fire hazards. Alternative promoters include the glucose-inducible strong constitutive *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase (*GAP)* gene promoter. So, we chose another pGAPZαA without methanol induction, which is a constitutive expression vector and can shuttle freely. It can be first stored, replicated and amplified in Escherichia coli, and then linearized and introduced into yeast cells to undergo homologous recombination with its chromosomes, and the gene expression framework of the target protein was integrated into the chromosome to realize the expression of foreign genes.



And there is a tip needed mention--**the codon optimization**:

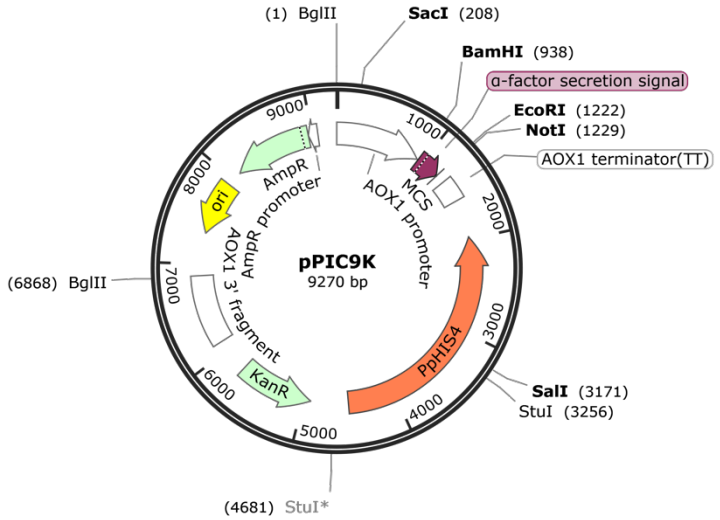
One of the first considerations in expressing any gene in a foreign system is the structure of the gene itself (i.e., is it compatible with the host's translational machinery). A number of reports on expression in *P. pastoris* have suggested that changing codons in a foreign gene to those of codons more frequently used by the yeast (as judged from the predicted amino acid sequences of highly expressed *P. pastoris* genes) significantly improves the levels of expression of those foreign genes . In fact, the jury is still out on such a conclusion.

——《Pichia Protocols by James M. Cregg (ed.)》

Gurkan, C., and Ellar, D. J. (2003) Expression of the Bacillus thuringiensis Cyt2Aa1 toxin in *Pichia pastoris* using a synthetic gene construct. *Biotechnol.Appl. Biochem*. **38,** 25–33.

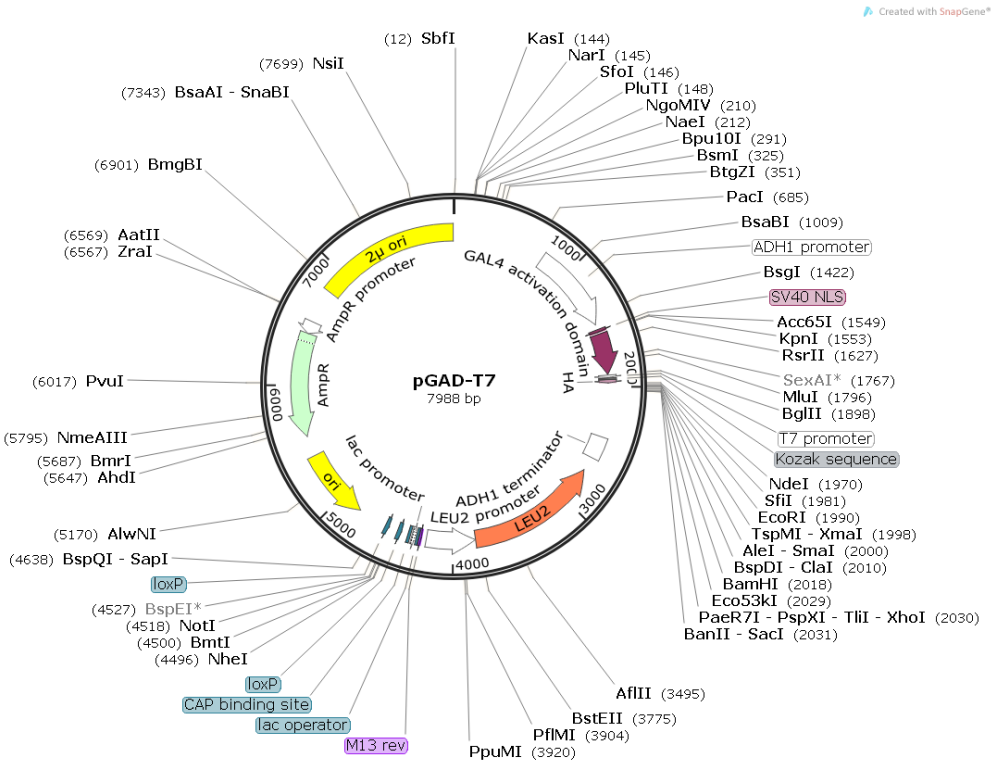
3.3 CPU_CHINA

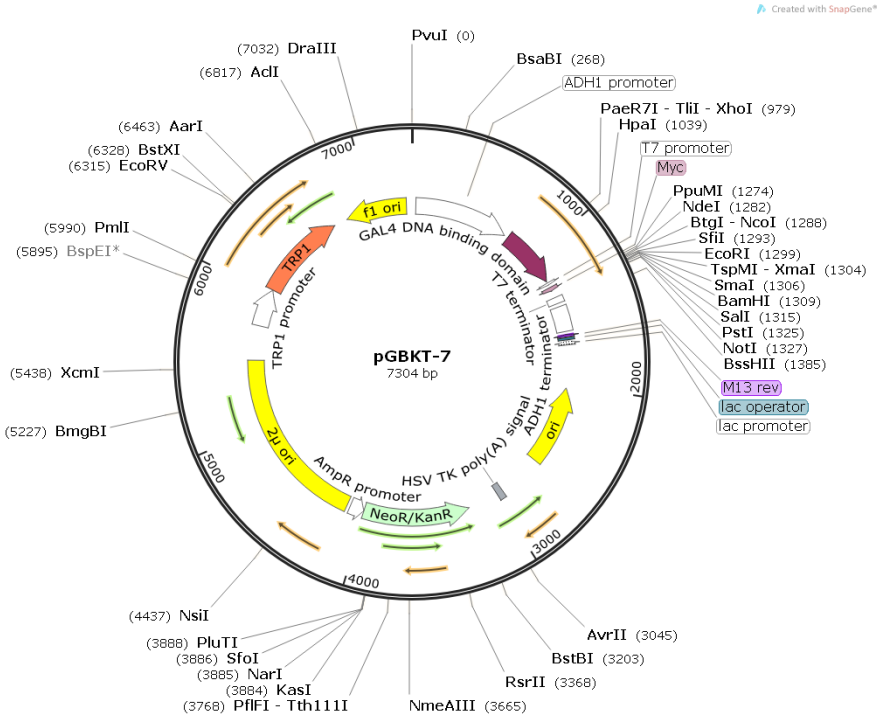
PPIC9K is a commonly used plasmid in *Pichia pastoris* GS115, and it carries a signal peptide which can meet the requirement of obtaining standardized protein components directly from culture medium supernatant.



3.4 HiZJU-China

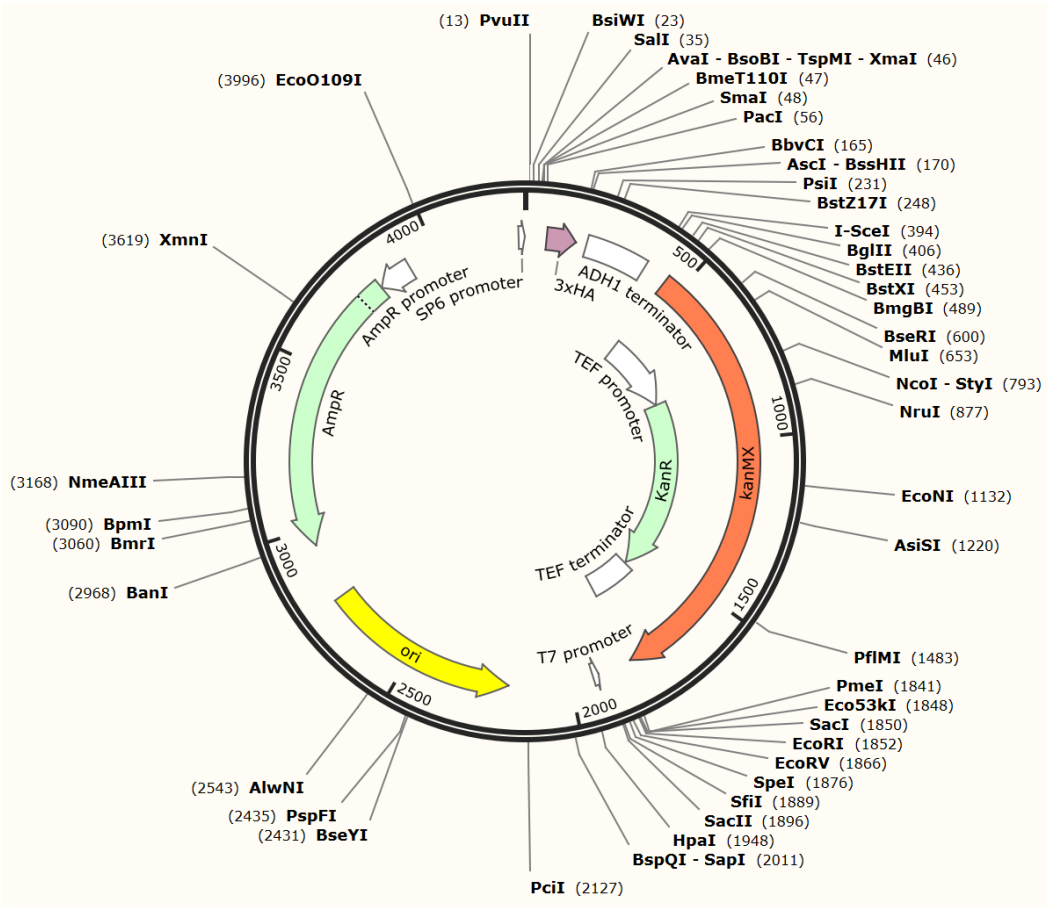
We chose pGAD-T7-GRIP1 and pGBKT-7-ERα-421F as our expression vectors. pGAD-T7 and pGBKT-7 are commonly used in yeast two-hybrid experiments. Both of them has suitable restriction sites for gene editing. The two plasmids were combined with GRIP1 gene and ERα-421F gene, which has receive codon optimization, and introduced into yeasts.





3.5 UM_Macau

pFA6a is a precursor to plasmids carrying kanMX and other selector modules(HA tag, ADH1 terminator). This plasmid is a commonly used backbone plasmid for generating epitope tagging vectors. We choose pFA6a as the backbone because it contains the HA tag, KanMX, and ADH1 terminator. HA tag is a general epitope tag, which doesn't influence the bioactivity and helps the detection of western blot. KanMX is an antibiotic resistance gene, we use it to detect plasmid-containing bacteria with selective media. The ADH1 terminator is a natural terminator sequence for Saccharomyces cerevisiae which also functions as a terminator for foreign genes expressed in yeast. Besides, this backbone is also specially designed for yeast expression.



3.6 Jiangnan_China

In our experiments, we used two vectors: pYEP352 and pY15TEF1. The former one is a high-copy plasmid with *URA3* genes that we used it to ensure that Gadusol synthetic pathway can effectively express in *Saccharomyces cerevisiae*. And we used the latter one, a low-copy plasmid with *LEU2* genes, to ensure that the expression of Xylose utilization pathway is appropriate, which not only can prevent from co-factors imbalance, but also prevent the metabolic pressure of *Saccharomyces cerevisiae* causing by introducing two plasmids (especially two high-copy vectors).

Chapter 1 Pichia pastoris

1 Activate strains

1. Take Pichia pastoris GS115 preserved at -80°C， or commercial strains.
2. After dipping with an inoculating loop, inoculate on MDH and MD plates by streaking.
Since GS115 is a histidine-deficient strain, it cannot grow on MD plates. And can grow on MDH plates containing histidine.
3. Compare the growth of yeast on the two plates to verify.
4. Pick a single colony from the MDH plate and inoculate it in 5ml YPD liquid culture in 50ml conical flask.
5. Cultivate at 30°C, 250rpm, when the OD value is 1.5
6. Take 1ml of bacteria liquid into 2ml of sterilized EP tube, add 1ml of 50% glycerin, and store at -20°C

2 Preparing the competent Pichia pastoris & Electroporation

2.1 Amplification and linearization of recombinant plasmids

1. The 50μL system of linearization reaction is: (the enzyme is added at the end)

10xL Buffer 5 uL (If only the restriction enzyme digestion verification is performed, a colored buffer can be added; if other operations are to be performed, a transparent buffer can be added)
Recombinant plasmid 5-10ug (usually 5-6ug) in order to make the total volume not exceed 50ul, the plasmid concentration is generally above 140ng/ul)

(Leave at least 2ul of the undigested plasmid and run the electrophoresis verification together with the digested plasmid)
Enzyme: digest 0.5-1ul overnight
ddH2O allocated to 50ul

2. After mixing thoroughly, use a 37°C water bath (the time can be longer to ensure sufficient digestion)
(Then take 2μL of the digested product and perform agarose gel electrophoresis to observe whether it is completely linearized)

3. Agarose gel electrophoresis to verify complete linearization
Restriction digestion verification system 6ul Restriction digestion verification control system 6ul
3ul ddH2O
2ul plasmid after digestion
1ul Loading Buffer （For specific enzymes)
Compare the two to see if the linearization is complete

Notes
1.selected linearizing enzyme must not have a digestion check point in the target gene
2.Enzymatic digestion time must be sufficient to linearize most plasmids.
3.Enzymes used for single digestion of several plasmids

Validation：agarose gel eletrophoresis

2.2 Ethanol precipitation of linearized DNA

Steps
1. DNA precipitation: place the system in a 65°C water bath for 20 minutes after digestion. Anhydrous ethanol, 80% ethanol pre-cooling
2. Add 1/10 (system after enzyme digestion) volume of 3M pH5.2 NaAC and 2.5 times volume of pre-cooled absolute ethanol, mix well, and place at -20°C for more than 35 minutes.

3. Centrifuge at 12000rpm for 15min, discard the supernatant
4. Wash the pellet with about 300-400ul pre-cooled 80% ethanol (resuspend), centrifuge at 12000rpm for 10min
5. Aspirate the supernatant (ethanol) and air dry for some time, (for example, 10 minutes, until there is no ethanol in the tube).
6. Resuspend in 10ul ddH2O

Note
The precipitate may not be seen, and the DNA is stuck on the wall of the EP tube.

2.3 Preparation of Pichia pastoris

Steps
1. Pick a single yeast colony and inoculate it into a 50ml Erlenmeyer flask containing 5ml YPD medium at 30°C, 250-300 revolutions/min, and cultivate overnight.
2. Take 100~500μl (one-thousandth) of the culture and inoculate it into a 200ml triangle shaker containing 100ml (reagents generally use 110-120ml liquid medium, and the excess is used for -80°C preservation and OD measurement) of fresh medium. In the bottle, incubate overnight at 30°C and 250-300rpm until the OD600 reaches 1.3-1.5 (note the record); pre-cool the sterile water and sorbitol solution.
3. Centrifuge the cell culture at 4°C and 7000 rpm for 6 min, and discard the supernatant.Resuspend the bacterial pellet with 50 mL of ice-cold sterile water.
4. Centrifuge according to step 3, and resuspend the bacterial pellet with 25ml of ice-cold sterile water.
5. Centrifuge according to step 3, and resuspend the bacterial pellet with 5 ml ice-cold 1 M sorbitol solution.
6. Centrifuge according to step 3, and resuspend the bacterial pellet with 160ul ice-cold 1M sorbitol solution, the final volume of which is about 240ul.
7. (Optional) It can be divided into 80μl aliquots and frozen at -80°C and

used within 2 weeks, but it may affect its conversion efficiency.

2.4 Electrotransformation

Pre-cooled 2mm electro-rotor cup, sorbitol solution
Steps：

1. 10ul + 80ul competent Pichia pastoris solution with linearized DNA (mix in the EP tube first and then ice bath), transfer to a 2mm electrotransformation cup (added to the gap of the electroporation cup) that has been pre-cooled for at least 5 minutes. Take out the dry electrode (the voltage is extremely high to avoid explosion).
2. Electric shock: (parameters have been set, no need to change) select fungus of igem or protols; parameter setting: voltage 1.5kV; capacitance 25μF; resistance 200-400Ω. The shock time is 5+-msec.
3. After the electric shock is finished, immediately add 1ml of 4°C pre-cooled 1M sorbitol solutionMix gently with a pipette tip and transfer to a 1.5ml EP tube; do not shake!!
4. Place the EP tube in a thermostat at 30°C and incubate for 1-2h. Don't shake it!!
5. Spread the bacterial suspension gradient (200ul) on the YPDZ plate. If the bacterial concentration is high, coat multiple plates; place the plate at 30°C and incubate until a single colony appears, about 1-2 days.

Note

1. Be careful to wipe the metal surface clean during electric shock, otherwise it may explode.
2. When inserting the electro-rotor, pay attention to the electrode surface and the metal surface of the electro-rotor.
3. Pay attention to "upside down" when placing the plate to prevent condensation from dripping into the culture medium.

3 Screening transformant and Expression

3.1 Using pPIC9K (a kind of plasmid)

3.1.1 Resistance screening/multi-copy screening（）

Method 1 (low reliability, but simple):

Prepare 10 YPD plates, each with geneticin concentrations of 0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0 and 4.0 mg/ml.
1) Pipette 1-2ml of sterilized water on all HIS+ transformant plates.
2) Resuspend the HIS+ transformant with a sterile scraper, do not scratch the agar.
3) Transfer the cell suspension to a sterile 50ml centrifuge tube and vortex (5-10S) a little.
4) Measure the concentration with a spectrophotometer (1 OD600=5×107 cells/ml), Note: mixed with agar will interfere with readings.
5) Spread 105 cells on each YPD plate containing geneticin.
(It is necessary to verify the titer of the cells on the YPD plate without geneticin, and calculate the proportion of geneticin-resistant clones on each geneticin-resistant plate to determine whether the multiple copies you get account for the transformants on the plate. Dilute the collected transformants to a concentration of 10-5, 10-6, 10-7, add 100-200 ul per plate)
6) Incubate the plate at 30 °C, check every day, and the geneticin-resistant clone is approximately Appears on 2.5 days, and it takes 2-3 days for clones to appear on YPD plates without geneticin, and then analyze the results later.

Note: If you suspend all cells in the above method, add 15% sterilized glycerol and store at -80 degrees, you can do geneticin resistance screening in the future.

Method 2 (high reliability and complexity):

Preparation: Three sets of two microassay plates (6 in total) are required to screen 180 HIS+ recombinants.
(Through continuous inoculation, obtain the same concentration of gram gonad to ensure that the number of cells on the geneticin plate is equal. Remember to have a strain background control and a single copy gene control. For every 180 clones, 1-10 antibodies can be selected Geneticin clone.)

1) Add 200ul YPD to each microassay plate under aseptic conditions.

2) Use a sterile toothpick to inoculate a single HIS+ transformant in each wReaction conditions: pre-denaturation for 5 min at 94 °C; 35 cycles were carried out at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min. Extension at 72 °C for 10 min;
3) Store at 4 °C. ell of the first group of plates, gently stir to suspend the cells.
4) Cover the microassay plate and incubate at 30°C for 2 days (no shaking required).
5) After 2 days, take a new microassay plate and add 190 ul YPD samples to indicate the well where the cells are located.
6) Use a multi-channel pipette to pipette 10 ul of the culture medium into the second set of assay plates. Make sure that the second set of plates is marked and placed so that the wells of the cells can be indicated.
7) Cover the second set of plates and incubate overnight at 30°C.
8) Repeat step 5.6 on the third set of assay plates on the second day. Note: Continuous growth and passage can make the clones reach the same cell concentration.
9) After incubation, take the third set of plates and pipette up and down with a 100ul multichannel pipette to resuspend the cells.
10) Take 10ul of the liquid in each well and place it on the YPD plate with geneticin concentration of 0, 0.25, 0.5...4.0 mg/ml. Use a multi-channel pipette or draw a line under the plate to ensure that the

columns are selected in a regular manner.

11) After the liquid is absorbed, put the plate at 30 degrees, 2, 3, 4, 5 days later to test the anti-geneticin, and then analyze result.

3.1.2 Screening of positive transformants（NWU-CHINA-B）

(1) Select some converters on the MD plate, mark them with marker pen, and then select the selected convertersA small amount of the germ was applied to the bottom of the PCR tube, and then 50 μℓ enzyme-free water was added, and beaten and mixed;

(2) It was heated at 95 °C for 10 min in the PCR instrument, and then immediately frozen in the ultra-low temperature refrigerator for 10 min, repeated 4-5 times;

(3) Centrifugation at 12000 RPM for 1 min, 3 μℓ supernatants were taken for PCR reaction.

Reagent	15 μℓ
systemTemplate	3 μℓ
2 x San Taq PCR Mix	7.5 μℓ
5 'AOX1 (10 μm)	0.5 μℓ
3 'AOX1 (10 μm)	0.5 μℓ
Enzyme free water	supplement to 15 μℓ

Reaction conditions: pre-denaturation for 5 min at 94 °C; 35 cycles were carried out at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min. extension at 72 °C for 10 min; store at 4 °C.

According to the results of colony PCR, a small number of bacteria corresponding to the number of inverters on the MD plate were selected with sterile spear head, and the spear head could be directly shot into 20 mL BMGY medium for culture. The culture condition was controlled at 30 °C and 220 rpm.

During the culture process, samples were continuously sampled until OD600 reached 4-10.The culture was stopped. According to the measured OD600 value, an appropriate amount of bacterial liquid was absorbed and centrifuged to obtain the thunb. Then the thunb was suspended in 20 mL BMMY medium, and its initial OD600 was 1.The

activity of OD600 and β -glycosidase was determined by a small amount of bacteria solution every 24 h, and then 200 $\mu\ell$ methanol was added to keep the final concentration of methanol in the medium at about 1%.

When β -glycosidase activity decreased continuously, the culture could be stopped.

3.1.3 Methanol induced expression（HUST-China）

1.Pick a single colony into 5 ml YPD and culture for 24 hours.
2.Inoculate 5ml into BMGY liquid medium and culture for 16-18 h, the OD is about 4-6;
3.BMGY is induced in advance by adding 0.2% methanol 2h before transferring to BMMY/BMM
4. Collect all the bacteria by centrifugation at 5000 rpm for 3 min, resuspend the bacteria in BMMY/BMM liquid medium, culture, and add 1% of the fermentation volume of methanol every 24 hours.

3.2 Using pGAPZa

3.2.1 Screening of recombinant in Pichia pastoris GS115 after electric transformation

In order to obtain multiple copies of transformants, the transformed single colonies shall be screened by G418.
(for more details, you can search:Scorer, C. A., Clare, J. J., McCombie, W. R., Romanos, M. A., and Sreekrishna, K. (1994) Rapid selection using G418 of high copy number transformants of *Pichia pastoris* for high-level foreign gene expression. *Bio/Technol* **12,** 181–184.)

1. Take a 96-well cell culture plate and add 200μL YPD to each well.

2. Use a sterile toothpick to pick a single colony (His+transformant) from the MD plate, stir and resuspend in the well which corresponds to a colony.
3. Cultivate at 30°C for two days.
4. Take the second 96-well cell culture plate, add 190μLYPD to each well, and then add 10μL of the bacterial solution from the first 96-well cell culture plate to each well, cover the lid and cultivate overnight at 30°C.
5. Repeat step 4. (Note: Continuous growth and passage can make the cell density of each transformant reach the same).
6. Resuspend the bacterial solution with a multi-channel gun, and take 1μL of bacterial solution from each well to grow on the YPD plate with a G418 concentration of 1.75mg/mL.
7. Cultivate and observe at 30°C.

3.2.2 Pichia pastoris Expression

1. Cultivate the verified transformants in the 250ml triangular flask containing 25mL BMGY medium at 28°C/200 rpm until OD600 equals 2-6(usually 16-18h).
2. Centrifuge at 6000g for 5min at room temperature, collect the bacteria, and resuspend the bacteria with 50mLBMMY to make OD600 = about 1.0.
3. Put the bacterial solution obtained in step 2 in a 500mL shake flask, seal it with cheesecloth, and place it on a shaker at 28-30°C/200 rpm to continue culturing.
4. Take 1 mL of bacterial liquid samples at different time points at 0, 24, 48, 72, 96 and centrifuge them in a 1.5 mL EP tube at maximum speed for 2 to 3 minutes, and collect the supernatant and bacterial cells respectively.
5. Detect protein concentration (Bradford method) and enzyme activity in the supernatant.

4 Expression and validation

4.1Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

4.1.1 Preparation of polyacrylamide gel

Steps

1. Installation of the plastic plate model: wash the glass plate, air dry, and spare. When making glue, choose the right glass plate and assemble the plastic plate model.
2. The separation glue solution (20ml, available for two plates) is prepared, and the mixture is shaken gently by hand. The mixture is carefully injected into the gap of the prepared glass plates, leaving enough space (~ 2.5cm) for the concentrated glue. The 0.5ml deionized water (or isopropanol) is gently added to the top layer to cover it, so as to prevent the inhibition of the condensation by oxygen in the air. It can be seen that there is an interface between the water and the gel when the water is first added, then it gradually disappears, and then the interface appears again soon, which indicates that the gel has polymerized. The whole process takes about 30 minutes (at 25°C).
3. Preparation of concentrated gel: first, the water on the upper layer of the polymerized separation gel is absorbed, and then the filter paper is used to blot out the residual liquid.

Reagent Name	10% separation glue (10mL)	5% concentration glue (6mL)
ddH2O	4ml	4.1ml
Acr/Bis30%	3.3ml	1ml
1.5mol/LTris-HClpH8.8(分离胶缓冲液)	2.5ml	0
0.5mol/LTris-HClpH6.8(浓缩胶缓冲液)	0	0.75ml
10% SDS	0.1ml	0.06ml
10% AP	0.1ml	0.06ml
TEMED	4ul	4ul

Preparation of concentrated gel solution. After mixing, it is injected into the top of the separating glue and inserted into the comb to avoid the appearance of bubbles.

4. During the concentrated gel polymerization, the protein sample was mixed with 4X sample buffer in equal volume, denaturated in 95°C water bath or metal bath for 10min, and cooled to room temperature for use.
5. After the concentrated gel polymerization is complete, put the gel template into the electrophoresis tank and fix it. Add 1X electrophoresis buffer to both the upper and lower tanks.
6. Carefully pull out the comb, check for leaks and remove any bubbles at the bottom of the gel between the glass panels.

4.1.2 Sampling and electrophoresis:

Steps:

1. Sample 20 ul/ well was added to each comb hole, and molecular weight marker 5ul was added to the first hole to react the protein size and monitor the electrophoresis process.
2. Electrophoresis: the voltage at the beginning is about 100V. After the dye is concentrated into a line and begins to enter the separation adhesive, the voltage is increased to about 160V, and the electrophoresis continues until the dye (bromophenol blue) reaches the bottom of the separation adhesive, and the power is disconnected.
3. Cut glue. Cut the separating glue into a large plate with staining solution, stain for 1~2h, and then pour back the staining solution for repeated use. Then wash with UP first, and then decolorize with decolorizing solution for 4h (even overnight).

4.2 Immunoblotting

4.2.1 Dot blot

A technique for detecting,analyzing, and identifying proteins,similar to the western blot technique but differing in that protein samples are not separated electrophoretically but are spotted through circular templates directly onto the membrane or paper substrate.
Concentration of proteins in crude preparations (such as culture supernatant) can be estimated semi-quantitatively by using"Dot Blot"method if you have both purified protein and specific antibody against it.

Materials:
1.TBS buffer:20 mM PH=7.5 Tris-HCl buffer

2.TBST solution:0.05% Tween20 in TBS
3.BSA/TBST solution:0.1%BSA in TBST
4.Nitrocellulose membrane

Steps:

1. Take a nitrocellulose membrane and draw a grid on the acetate fiber membrane with a pencil.
2. The standard product will be diluted by multiple ratio, sample, negative control, positive control point in the middle of the grid on the membrane, and air dry.
3. Soak 5% BSA in TBST, 0.5-1hr, RT, and place in a 10cm petri dish.
4. Incubate with primary Antibody (0.1-10 ug/ml for Antibody,1:1000 to 1:100000 Dilution for antisera, 1:100 to 1:10000 for hybridoma supernatant) dissolved in BSA/TBS-T for 30 min at RT.
5. Wash the nitrocellulose membrane twice with TBST buffer, and discard the solution as far as possible after each wash. Then wash with TBST buffer 3 times, 5 minutes each time.
6. After discarding the liquid, 30mL TBST buffer containing the second antibody (antibody dilution 1:2000) was added and incubated by shock at room temperature for 1 hour.
7. Fully wash the nitrocellulose membrane with TBST buffer twice, and discard the solution as far as possible after each wash. Then wash with TBST buffer 3 times, 5 minutes each time.
8. Remove the nitrocellulose membrane, slightly remove the excess solution on the membrane, and add an appropriate amount of fluorescent chromogen A and B(common with Western blot kit, generally 1/2 A film the size of 96-well plate plus 4mLA and B mixture, and liquid A: liquid B = 1:1).
9. Cover the nitrate cellulose film with cling film, as smooth as possible to avoid bubbles, and put it on the exposure clip, according to the brightness of the sample and the standard, initially determine the exposure time (under normal circumstances, if the sample brightness is visible to the naked eye and strong, then the first exposure time should be short, generally 3-5 seconds; If the brightness of the sample is not visible to the naked eye, the initial exposure time should be

slightly longer, generally 1 minute), then take the appropriate size of the film covering the film, close the clip exposure.

10. After exposure, remove the film immersed in solution (ECL), and observed under red light until the sample point on the film no longer change, remove the film rinsed in water it (this step avoid solution mixed with fixing bath, reduce the fixing bath use efficiency), add the fixing bath, after the blue background transparent film, the film can be removed in running water flushing, Wash and let dry.

4.2.2 Western blot

Polyacrylamide gel electrophoresis is a method of protein or nucleic acid separation using polyacrylamide gel as supporting medium. Polyacrylamide gel is a three dimensional network structure gel formed by the polymerization of acrylamide monomer and crosslinking agent N, N-methylene bisacrylamide under the action of catalyst. By changing the ratio of monomer concentration to crosslinker, gels with different pore sizes can be obtained for separating substances with different molecular weights.

SDS, or sodium dodecyl sulfate, is an anionic surfactant, which can combine with protein in a certain proportion to form a SDS-protein complex. At this time, the protein has a large amount of negative charge, and far more than its original charge, so that the charge difference between the natural protein molecules is reduced or eliminated. At the same time, the structure of proteins becomes loose and the shape tends to be uniform under the action of SDS, so the differences in the electrophoretic mobility of various SDS-protein complexes during electrophoresis only depend on the molecular weight of the protein.

Materials:

1. 30% gel storage solution: ACR 30g + Bis 0.8g was dissolved in 100 mL deionized water, filtered into brown bottles with filter paper, and stored at 4°C to avoid light.
2. 1.5mol/L Tris-buffer,pH8.8; 1 mol/L Tris HCl, pH6.8.

3. 10% SDS: dissolve 10 grams of SDS in 100mL deionized water and store at room temperature.
4. TEMED: 10% concentration, 20 mL, 4°C storage.
5. 10% AP: 10 mL, freshly prepared, divided into 1.5mL centrifuge tube, stored at -20°C for later use.
6. 4X protein loading buffer:

Tris (200mM, pH6.8) 10mL of 1M Tris (pH6.8)
SDS(8%) 20ml of 20% SDS
Bromphenol blue (0.4%), 0.2 mg
Glycerol (40%) 20ml
Add water up to 50ml. Stored at RT

7. Electrophoretic buffer:

Tris 3 g
Glycine 18.8 g
20% SDS 5 ml
Add H2O up to 1L
Stored at RT.

8. Ponceau S staining solution: 10X Ponceau S solution was diluted to 1X by adding H2O.
9. Western transfer buffer:

Tris 3 g
Glycine 18.8 g
methonal 200 ml Add H2O up to 1L
Cool at 4°C.

10. 10XTBST:

Tris 24g
NaCl 80g
Adjust pH to 7.5 and add H2O up to 1L. Stored at RT.

11. Protein blocking solution: 5% milk in TBST
12. Nitrocellulose membrane, primary antibody, secondary antibody, color substrate solution

Equipment:
Trace pipetting device, 4°C / - 20 °C freezer, ice machine, desktop frozen high-speed centrifuge, multifunctional enzyme mark all wavelengths, gel imaging analysis system, protein electrophoresis, electrophoresis system capillary, transblot system, chest, transfer protein membrane system, automatic chemiluminescence image analysis system, high pressure sterilization pot, constant temperature electric heating oven, magnetic stirrer, decoloring shaking table.

4.2.2.1 Preparation of polyacrylamide gel:

Steps:

1. Installation of the plastic plate model: wash the glass plate, air dry, and spare. When making glue, choose the right glass plate and assemble the plastic plate model.
2. The separation glue solution (20ml, available for two plates) is prepared, and the mixture is shaken gently by hand. The mixture is carefully injected into the gap of the prepared glass plates, leaving enough space (~ 2.5cm) for the concentrated glue. The 0.5ml deionized water (or isopropanol) is gently added to the top layer to cover it, so as to prevent the inhibition of the condensation by oxygen in the air. It can be seen that there is an interface between the water and the gel when the water is first added, then it gradually disappears, and then the interface appears again soon, which indicates that the gel has polymerized. The whole process takes about 30 minutes (at 25°C).
3. Preparation of concentrated gel: first, the water on the upper layer of the polymerized separation gel is absorbed, and then the filter paper is used to blot out the residual liquid.

Reagent Name	10% separation glue (10mL)	5% concentration glue (6mL)
ddH2O	4ml	4.1ml
Acr/Bis30%	3.3ml	1ml
1.5mol/LTris-HClpH8.8(分离胶缓冲液)	2.5ml	0
0.5mol/LTris-HClpH6.8(浓缩胶缓冲液)	0	0.75ml
10% SDS	0.1ml	0.06ml
10% AP	0.1ml	0.06ml
TEMED	4ul	4ul

Preparation of concentrated gel solution. After mixing, it is injected into the top of the separating glue and inserted into the comb to avoid the appearance of bubbles.

4. During the concentrated gel polymerization, the protein sample was mixed with 4X sample buffer in equal volume, denaturated in 95°C water bath or metal bath for 10min, and cooled to room temperature for use.

5. After the concentrated gel polymerization is complete, put the gel template into the electrophoresis tank and fix it. Add 1X electrophoresis buffer to both the upper and lower tanks.

Carefully pull out the comb, check for leaks and remove any bubbles at the bottom of the gel between the glass panels.

4.2.2.2 Sampling and electrophoresis:

Steps:

1. Sample 20 ul/ well was added to each comb hole, and molecular weight marker 5ul was added to the first hole to react the protein size and monitor the electrophoresis process.
2. Electrophoresis: the voltage at the beginning is about 100V. After the dye is concentrated into a line and begins to enter the separation adhesive, the voltage is increased to about 160V, and the electrophoresis continues until the dye (bromophenol blue) reaches the bottom of the separation adhesive, and the power is disconnected.
3. Stripping: remove the rubber board, gently pry open the glass board from the bottom side, cut the concentrated glue with a matching plastic knife, and cut off a corner for marking, and soak in the pre-cooled electrotransfer buffer for balance.

4.2.2.3 Transmembrane:

Cut one sheet of NC film of 4.5cm×8cm (cutting Angle marking direction) and six sheets of filter paper of 5cm×9cm, and soak them with the electrotransfer buffer precooled with ice in advance.

Steps:

Connect the converter to the power supply, 105V for 1 hour.

4.2.2.4 Antibody incubation and detection:

1. The transfer membrane was cleaned twice with 1X TBS for 5min, and then the blocking solution was added for antibody sealing. The room temperature was 1 hour.
2. Primary antibody was incubated overnight at 4°C (primary antibody was prepared with blocking solution).
3. On the second day, primary antibody was eluted; Secondary antibody was added and incubated in room temperature shaking table for 1h.
4. Eluting the secondary antibody, 5min each time, 5 times; Add chromogenic substrate for chemiluminescence chromogenic and image acquisition.
5. Exposure.

4.3 Specialized methods for specific expressed proteins

For example, Congo staining, enzyme assay.

5 The problems we met & how we tackle them.

5.1 A Sensitive Assay is Critical (UESTC-China)

A major difference between the P. pastoris system and E. coli is the level of recombinant protein typically observed in the early phases of development. With E. coli, one can typically count on 10% or greater of total protein being a recombinant product. As a consequence, one can expect to observe the product as a band on a stained gel. This is typically not true for P. pastoris, especially in the early development phases where intracellular protein yields of less than 1% of total protein are common for secreted proteins and where proteinases may initially degrade most products before it can be visualized. Thus, one must have a sensitive assay for observing the product in these early stages. This can be a

functional (enzymatic) assay or a polyclonal antibody preparation against the protein product or the presence of an epitope tag fused to the product for visualization by a western blot-based technique. However, going into an expression effort with P. pastoris without such an assay is almost certainly a recipe for frustration and disappointment.

5.2 contaminative microbes (UESTC-China)

From July to August, we have had fight with contaminative microbes for a long time, they even had **multi-drug resistances** including Zeocin, Kanamycin, Ampicillin.

After research, we find the ways of the invasion of contaminative microbes are nothing more than five aspects: raw materials, strains, air, equipment, and operations.

(1) In terms of culture medium, the raw materials used for fermentation, such as water and natural agricultural products, are rich in nutrients.
(2) In terms of strains, the strains used for fermentation are all obtained from the preservation of pure breeds and after a large amount of cultivation. Seeds with germs and the reasons why it is impossible to spread the germs;
(3) In the aspect of inhaling air, the expansion in the air is full of vitality. The expansion in the air is vitality, yeast spores, expanded spores and periodic excitement. If the air cannot be prepared, the oil and water are not separated cleanly, causing filtration and drainage, or equipment leakage, etc., the entire air is not completely sterilized and carries bacteria.
(4) In terms of equipment, if there are many dead spots in the structure of the fermentation tank, and the pipeline installation is unreasonable, these may lead to the contamination of bacteria.
(5) Possibly the foundation of the operation is not thorough, the operation may be incorrect, or the misoperation during cultivation or fermentation, etc.

However, we have found that contamination by bacteria still occurs frequently under the conditions of extremely strict sterilization and aseptic operation, as shown in the figure below. Our experiments have not progressed in the July, and the bacteria that were successfully expressed in the early stage have also been contaminated, which can be described as **a waste of all previous efforts**. With the advice and help of the coach and advisor, we carried out the laboratory **formaldehyde fumigation**. After the fumigation, the contamination of laboratory bacteria is still inevitable, but it is already relatively **controllable**. However, formaldehyde fumigation needs to be implemented after detailed consideration.

In addition, we found that by drawing the plate, relatively pure bacteria can be obtained from the contaminated bacterial liquid to a certain extent, and by switching between different plates (the target bacteria grow well, but the bacteria may have a preference), reduce the contamination of the bacteria Case.

For example, the GS115-pGAPZa we used can grow on both MDH and YPD plates, but the kind of cocci we found prefers to grow on YPD and hardly grows on MDH plates.

5.3 Low efficiency in purification (UESTC-China, helped by)

We have tried Nickel column purification method and his purification, but didn't get any promising answers. By communicating with Professor Cui and partners in Yeast Alliance, we find that protein content in fermentation broth is so low that it is impossible to use affinity chromatography to gain target proteins directly. Even after concentration, the proteins' own characteristics may result in failure during purification, such as affinity loss between his-tag and Nickel column and pigmentation. In addition, affinity chromatography is not commonly used for Yeast fermentation and there are some better methods. We can use ammonium sulfate precipitation for protein concentration and ion exchange chromatography for purification, and the product can be test by SDS-PAGE, and Western blot.

Materials:
① Ammonium Sulfate(saturated solution or solids)
② Fermentation Supernatant
③ Protein Resuspension Buffer: 20 mM HEPES, 10% glycerol, 500 mM NaCl, pH 7.5 (the isoelectric point of each protein is different, so the H needs to be set at the non-isoelectric point)

Pre-experiment:

Goal: Estimating the final concentration of ammonium sulfate required for protein precipitation.
Steps:
① Mix saturated ammonium sulfate solution with fermentation supernatant to final concentration of ammonium sulfate:10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%.
② Stand at 4 °C for 4~6h to precipitate the target protein.
Attention: standing for more than 6h would result in precipitating other proteins.
③ 4 °C, 12000rpm for 20 min in refrigerated centrifuge.
④ Discard supernatant and resuspend the precipitation using protein resuspension buffer.
⑤ If the target protein is an enzyme, its enzyme activity should be detected; If not, western blot is necessary to compare the amount of it and choose the best final concentration of ammonium sulfate.

Formal experiment:

Goal: precipitate large amount of fermentation supernatant.
Steps:
① Place the supernatant in a beaker filled with ice-water mixture and place it on a magnetic stirrer for continuous stirring.
② The ammonium sulfate solids required for the optimal final concentration of ammonium sulfate were uniformly added to the sample within 5-10min, and then stirred continuously for 20-30min。
Attention: the stirring should be mild and stable, and if a large amount of foam appears or the liquid heats up, the protein may have denatured.③ Placed at 4 °C for 4~6h.

③ Centrifuge at 4 °C for 20 min.
④ Discard the supernatant and add appropriate amount of protein resuspend buffer to resuspend the pellet.
⑤ Add PMSF and save at -80 °C. Avoid repeated freezing and thawing.

5.4 Colony Polymerase Chain Reaction(NWU-CHINA-B)

Several teams have mentioned that despite the use of magnetic beads and lysozyme for yeast genomic extraction, the final PCR results are still poor. Therefore, we provided our yeast colony PCR protocol:
Volume of the reaction system is 25 $\mu\ell$
2.5 $\mu\ell$ diluted colony sample as template source
0.5 $\mu\ell$ Primer - Forward
0.5 $\mu\ell$ Primer - Reverse
9.0 $\mu\ell$ ddH2O
12.5 $\mu\ell$ Enzyme mix(we used Green Taq mix)
*Preparation of diluted colony sample: simply picks a portion of one single colony and dilutes the cells with 10μl ddH2O
Program:
① Predenaturation 98°C, 10 min
② Denaturation 98°C, 30s
③ Annealing 59°C,15s
④ Elongation 72°C, 2.5min
30-35 cycles from step 2 to start the cycle
⑤ Complementary extension 72°C, 10 min

Chapter 2 Saccharomyces cerevisiae

1.Introduction

The species of yeast is called Saccharomyces cerevisiae. "Saccharomyces" derives from Latinized Greek and means "sugar-fungus". "cerevisiae" comes from Latin and means "of beer". And it is commonly called Brewer's yeast. People use Saccharomyces cerevisiae in winemaking, baking, and brewing since ancient times and this yeast is first obtained from bread bakers in the 19th century. It has been found to contribute to the smell of bread.

Saccharomyces cerevisiae can grow aerobically on glucose, maltose, and trehalose and fail to grow on lactose and cellobiose. Galactose and fructose are two best fermenting sugars. It can also use ammonia, urea, amino acids, small peptides, and nitrogen bases as nitrogen sources. Most strains of Saccharomyces cerevisiae require biotin.

Saccharomyces cerevisiae is one of the most intensively studied eukaryotic model organisms in molecular and cell biology, much like Escherichia coli as the model bacterium. It is a budding yeast to form a bud from the mother cell and the bud grows during the cell cycle and detaches, which is different from fission yeast and it's also used in cell cycle studies because its asymmetrical division during cytokinesis similar with stem cells when self-renewal and differentiation. It is diverged approximately 600 to 300 million years ago, and is a significant tool in the study of DNA damage and repair mechanisms. Saccharomyces cerevisiae is small with a short generation time and can be easily cultured. It divides with meiosis, allowing it to be used for sexual genetics research. It shares the complex internal cell structure of plants and animals without the high percentage of non-coding DNA that can confound research in higher eukaryotes. And it has been used to better understand aging and has contributed to identify mammalian genes affecting aging for more than 50 years. Saccharomyces cerevisiae can be transformed allowing for either the addition or deletion of new genes through homologous recombination.

Many proteins like cell cycle proteins, signaling proteins, were first discovered by studying their homologs in yeast.

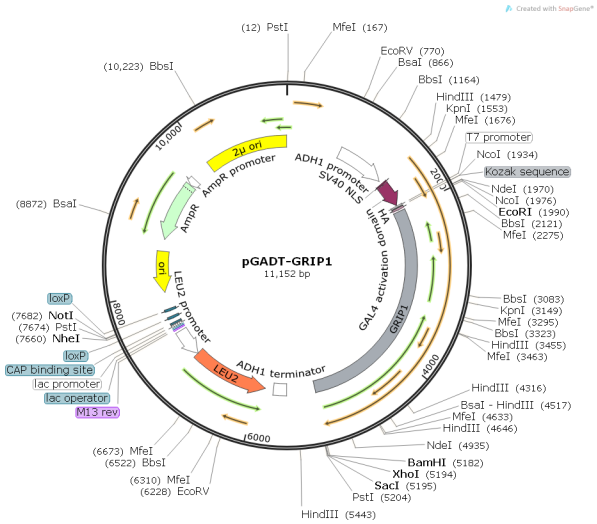
Saccharomyces cerevisiae was the first eukaryotic genome to be completely sequenced. The Saccharomyces cerevisiae genome is composed of about 12,156,677 base pairs and 6,275 genes, compactly organized on 16 chromosomes.

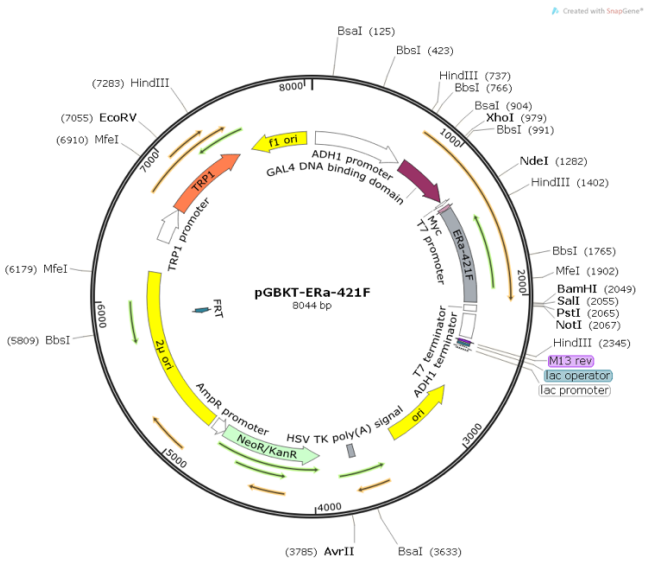
Saccharomyces cerevisiae research is a strong economic driver in industry. It can be used for some commercial applications: For example, Saccharomyces cerevisiae is the main source of nutritional yeast, which can be served as an ingredient in cheese substitutes, as a general food additive, or as a source of vitamins and minerals, especially amino acids and B-complex vitamins.

2.Construction of recombinant plasmid

2.1 HiZJU-China

ERα-LBD sequence and GRIP1 capture protein gene sequence of ERα promoter transcription were obtained from NCBI database. pGBKT7-ERα-AD sequence and PGAD7-GRIp1-BD sequence were constructed by restriction endonuclease EcoI,BamI.





2.2 UM_Macau

SOD1, PNP1, MF alpha, TPS1 promoter, and pGK1 promoter sequence come from Saccharomyces cerevisiae S288c. The chit42 sequence comes from Trichoderma harziana (CBS226.95). We chose the SmaIrestriction site on pFA6a for the construction of the plasmid.

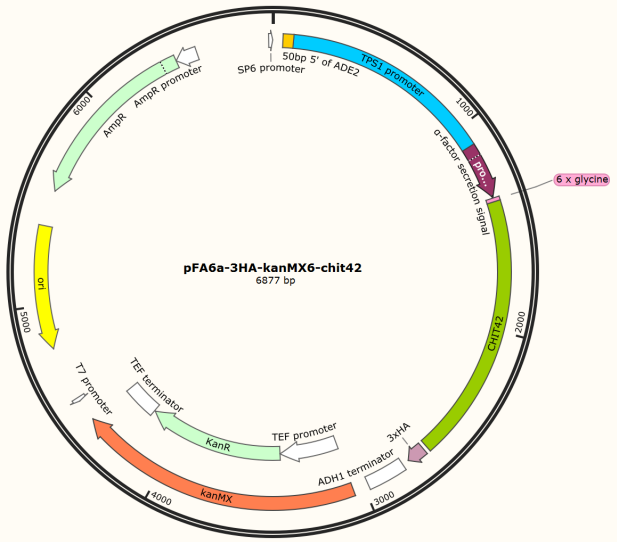
3.Problems and solutions

(1) Yeast cells grow slowly.

- Reason: The bait protein expressed by yeast is toxic to cells and affects the growth of yeast.
- Solution: select low-sensitivity yeast strains;use low-copy number expression vectors.

(2) Too many clones on the screening medium.

- Reasons: the bait protein has transcriptional activity and can initiate the expression of the screening gene;selection criteria is loose.
- Solution: Use stricter screening conditions to inhibit the self-activation activity of the bait protein.



(3) Too few clones on the screening medium.

- Reasons: low efficiency of yeast hybridization;strict screening conditions
- Solution: use fresh culture medium and fresh yeast clones with a diameter of about 2~3 mm to ensure the viability of the yeast; Use

ethanol to precipitate the plasmid to improve the concentration of plasmid; Extend hybridization time and improve hybridization efficiency; Relax screening conditions.

(4) The target gene is in the form of fragments, and the complete plasmid has not been synthesized

- Solution: Import yeast and wait for the organism to repair itself.

(5) The yeast transformation is not that well.

- Reasons: It might because of the yeast is too old to transform. Another reason is that we used the kanamycin but not G418.
- Solution: Change new yeast and use same strain to culture a new plate.

(6) The high absorption peak of Yeast Extract-Peptone-Dextrose (YPD) medium in solution (function assay)

- Solution: We decided to replace the YPD medium with the synthetic complete (SC) medium to culture our strain. The reason we choose to use the SC medium is that, firstly, the absorption peaks of the SC medium were much smaller in the 325 nm wavelength used in our pyrogallol assay. Secondly, the components of SC Media are all known. Compared with YPD containing yeast extract, it may have less background influence on the experiment itself.

(7) The secretion of SOD1 in the medium is too low due to the limitation of culture conditions. (function assay)

- Solution: We replaced the YPD medium with S.C. Medium to culture.

4.Notes

4.1 HiZJU-China

Due to the long and unstable sequence of GRIP1, there would be fracture in the process of enzyme digestion and recombination. At the

initial stage of the experiment, the full-length positive clone of the gene could not be obtained. (Therefore, the detection stage was carried out later than the degradation stage.) Accordingly, we introduced DNA fragments into yeasts and reconnected them through yeast culturing. Additionally, the integral plasmid structure was sequenced by using pGAD-7 standard primer sequence T7.

1. 10μL target plasmids pGBKT7-ERα-AD and PGAD7-GRIP1-BD were transformed into competent E.coli (BL21) respectively. Positive clones were selected through ampicillin selective plates. The plasmids were extracted, purified and identified by 0.8% agarose gel electrophoresis (AGE).
2. A single colony of Y187-GFP yeast, which was introduces into the reporter gene GFP regulated by GAL4, was selected and cultured in 25ml YPDA medium at 30°C.The culture was grown to the logarithmic growth stage (OD600 up to 0.4-0.6).The recombinant plasmids pGBKT7-ERα-AD and PGAD7-GRIp1-BD were simultaneously transfected into host cell Y187-GFP by electroconversion. Then the ERα-GRIP1 two-hybrid yeast was coated in YPD-His-Trp-Leu solid medium and grown at 30°C for 2-4 days.
3. The normal growing two-hybrid yeast single colony was selected and cultured overnight in YPD-His-Trp-Leu liquid medium at 30°C,shaken in 200rpm。 On the second day, part of the yeasts was added to 15% sterile glycerol and stored at -80 °C.
4. When the culture was grown to the logarithmic growth stage, add 5μL EE2 and DMSO with different gradients to 995μL yeasts solution to form exposure culture medium. Add the system into 96-well plate at 30°C,800rpm for 4h. Measure the optical density OD600.The supernatant was extracted from the culture after disruption. Cells from both samples, preinduction and postinduction, can be suspended in SDS sample loading buffer, and ran on an SDS-PAGE gel to verify protein expression (i.e.a prominent band at about 26 kDa). And the GFP protein expression was detected by HPLC-hv at 395 nm.
5. (1) The color of the yeast culture medium becomes brown after

sterilization.

Glucose in the culture medium will be carbonized during the sterilization process with long time or high temperature conditions. Carbonization of sugar result in a darker color. The degree of carbonization can be reduced by controlling the sterilization time and sterilization temperature.

(2) False positive phenomenon

A variety of reasons may cause false positive results, Two of the most common reasons are:

- Reason A. The protein has transcriptional activity and can initiate the expression of the reporter gene.
  Solution A: In this case, self-activation verification of the selected candidate protein is required.
- Reason B. Yeast may contain more than one library protein at the same time, and one of the library proteins can bind to the bait protein.
  Solution B: In this case, positive clones need to be streaked again 2~3 times to ensure that each yeast clone contains only one library protein and bait protein.

In addition, the number of streaking should not be too many, otherwise protein expression will occur The case of plasmid loss.

4.2. UM_Macau

1. Kanamycin was used in the previous experiment (TOP10 transformation) to select the vector, which is not G418 in yeast experiment, therefore we can't use kanamycin to select the yeast.

2. When we do the yeast transformation, the plating status, it may not grow well in the first day. Do not throw away simply because there is nothing grown in first day.

Appendix

1 Team profile

(In no particular order)

1.1 UESTC-China

Nowadays the recycling rate of office waste paper (OWP) in China is dramatically low. We find that cellulase, xylanase, lipase, and laccase will allow bio-deinking to occur, by helping the ink detach from the waste paper and decomposing hazardous substances. We also design a new surface assembly of a functional cellulosome by using Pichia pastoris. Otherwise, to bring our idea to the real world, we design a paper recycle machine (Deinker) ， which includes the transmission part, enzyme liquid smearing part, deinking part, and paper drying part. Through this device, the OWP will be reborn. In addition, 5 mathematical models are built for improving experiments, hardware, and implementation. Moreover, Human Practice has been constantly advancing as the project progresses, we design related activities from four aspects: practical issues, technology, market and industry, policy, and law to understand the exact needs of stakeholders.

1.2 HUST-China

The team relies on the platforms of College of Life Science and Technology of Huazhong University of Science and Technology, Qiming College, Institute of Microbial Technology of Environment and Resources, Institute of Energy Biotechnology and Ecology, Life Innovation Base, etc. And team invites the deputy secretary of the Party Committee of the School of Life Associate Professor Zhan Yi (Guiding various discipline competitions at home and abroad, more than ten gold and silver awards in the double innovation competition) and Professor Yan Yunjun, deputy dean of the Academy of Biological Sciences and deputy director of the Key Laboratory of Molecular Biophysics of the Ministry of Education (responsible for a number of major national strategic projects) as the guidance of the teacher team, respectively for the team to provide innovative entrepreneurial guidance and professional technical guidance. The two platforms, The Institute of Microbial Technology of Environment and Resources and the Institute of Energy Biotechnology and Ecology,

have complete and complete experimental equipment. The start-up capital is shared by the school, the experimental platform and IGEM team. The relatively favorable experimental conditions give us relatively free experimental space and a high fault tolerance. Under the guidance of the mentors, the experiment process of our team advanced in an orderly manner.

1.3 CPU_CHINA

Examplified by polyethylene, undegradable plastics have caused severe environmental crisis worldwide.

Manganese peroxidase (MnP) uses hydrogen peroxide (H2O2) to produce high-redox-potential Mn3+, possessing a PE-degrading potential. Our project takes MnP as our key PE-degrading enzyme, employs aryl alcohol oxidase (AAO) to provide H2O2 for MnP, and utilizes hydrophobin-1 (HFB1) to increase substrate accessibility, meanwhile applying SpyCatcher-SpyTag system with CRISPR/dCas9 system to anchor MnP, AAO and HFB1 onto one double-stranded DNA scaffold according to certain spatial order, distance and proportion. The final complex works as a molecular machine that

can adhere to and degrade PE in a green and swift way.

1.4 NWU-CHINA-B

Ginseng is the main source of ginsenosides, but unfortunately, its growth cycle is very slow and it is susceptible to seasonal climate and other factors, its yield is limited.

In order to overcome this problem, the SS-bgly gene derived from Sulfolobus solfataricus was highly expressed in pichia coli system after codon optimization, and the enzymatic reaction conditions of conversion of ginsenoside substrate Rb1 to CK from SS-bgly were explored.Under optimized enzymatic reaction conditions, the recombinant SS-bgly can transform ginsenoside Rb1 with higher substrate concentration into CK, which is conducive to the large-scale production of ginsenoside CK.

1.5 UM_Macau

Gout is a common rheumatic disease. Symptoms include swollen, red, hot, and stiff joints. The accumulated purine in beer is one of the reasons that cause gout after drinking beer. Low purine beer, therefore, can reduce the risk of beer drinkers suffering from gout. Meanwhile, oxidation in beer causes off-flavors and darkened color. Based on these problems, our team UM_Macau hence proposed to edit the yeast used in beer fermentation in order to produce a beer with low purine content and good antioxidant properties. We engineer the wild-type yeast by synthetic biology method such that the engineered yeast is able to produce and secrete three enzymes--purine nucleoside phosphorylase1 (PNP1), superoxide dismutase 1 (SOD1), and endochitinase (chit42). The PNP1 can convert purine polynucleotides into purine bases that can be directly used by yeast, thereby improving the utilization of purines by yeast and ultimately reducing the purine content in beer. The SOD1 and endochitinase will increase antioxidation in the final product.

1.6 HiZJU-China

The synthetic estrogen 17α-ethynylestradiol (EE2) is a micropollutant with strong endocrine disrupting effects. Due to the large-scale use of estrogen in contraceptives and feed as well as the shortage of effective degradation method, EE2 is accumulating in water environment and will induce serious diseases. Our project aims to solve the EE2 pollution problem. We designed genetically modified bacteria to degrade EE2 via catabolism in waste water. In order to detect EE2 concentration accurately and quickly, we developed a synthetic biological cascade amplification reaction based on yeast two-hybrid technique, which can easily co-relate the content of EE2 with portable readout, such as cell growth and fluorescence intensity.

1.7 Jiangnan_China

Save Coral Reefs at Risks: *De novo* synthesis of an eco-friendly bio-sunscreen by S*accharomyces cerevisiae*.
Every year, about 14,000 tons of sunscreen are deposited into the ocean, producing a large amount of chemical pollution, especially oxybenzone and octyl-methoxycinnamate. This has greatly worsened the phenomenon of coral bleaching, and will further lead to the collapse of marine ecosystem, as coral reefs are home to 25% marine species. To alleviate the current situation, we are developing an eco-friendly bio-sunscreen that is harmless to coral reefs and the marine ecosystem. Inspired by how marine species in nature protect themselves from UV rays, we have decided to produce Gadusol, a natural UV filter originated from zebrafish, as the main component of our bio-sunscreen product. We first introduced the synthetic pathway of Gadusol into *Saccharomyces cerevisiae*. Then, the utilization pathway of xylose was also introduced to engineer it into a high-yield cell factory. By doing this, we hope to balance the use of sunscreen and the protection of coral reefs.

2 Common medium for culturing

SCD-TRP medium：
YNB:0.51g/300ml
CSM-TRP:0.222g/300ml
Ammonium sulphate:1.5g/300ml
Agar:2%(2g/100ml)
Glucose:20g/L

YNB-G
YNB(Bacto yeast nitrogen base without amino acids):6.7g/L, glucose: 20g/L, (NH4)2SO4: 10g/L

YNB-G4A
YNB-G with 30mg/L histidine, 30mg/L lysine, 30mg/L methionine and 30mg/L leucine

YNB-G3A
YNB-G with 30mg/L histidine, 30mg/L lysine and 30mg/L methionine

YNB-X
YNB:6.7g/L, xylose: 20g/L, (NH4)2SO4: 10g/L

YNB-X3A
YNB-X with 30mg/L histidine, 30mg/L lysine and 30mg/L methionine

Synthetic Complete (SC) Medium
Reagents
Final concentration (1×)
Yeast nitrogen base without amino acids and with ammonium sulfate 6.7 g/L
Agar
2% (w/v)
Caution: Amino acid mix (10×)
1×
Glucose
2% (w/v)
Store synthetic complete medium for up to several months at 4°C.

Yeast Extract–Peptone–Dextrose (YPD) Medium (Liquid or Solid)

Reagent	Quantity
Yeast extract	10 g
Peptone	20 g
Dextrose	20 g
Agar (for plates only)	20 g

Dissolve reagents in 1 L of distilled water. Autoclave for 20 min at 121°C

and 0.5 bar. Store for several weeks at room temperature.

Screening medium YPDZ

In order to screen the successfully recombined Pichia pastoris GS115 after electroporation transformation,we use YPDZ.

1% yeast extract
2% peptone
2% glucose
1.5%-2% agar powder
100ug/ml zeocin

Glucose needs to be sterilized separately and mixed after sterilization.

MDH medium

MDH medium is not as rich in nutrients as YPD, and is used for the separation and purification of yeast.

13.4g/L YNB
20g/L glucose
0.4mg/L biotin
0.004% histidine
1.5%-2% agar powder

Glucose is sterilized separately, biotin and histidine are sterilized with a filter membrane and then added to the medium and mixed.

BMGY medium

BMGY medium is a complex buffer medium containing glycerol.

1% yeast powder
2% peptone
13.4g/L YNB
1% glycerol

It is used for intermediate culture in the early stage of fermentation.

Modified YPD medium

Modified YPD medium is the standard medium for pichia pastoris to

propagate.

1% yeast extract
2% peptone
6%NaCl
2% glycerin

It is usually used for yeast expression.But for some recombinant protein, we found it may inhibitory protein expression.