Protein Expression in *P. pastoris*

Screening of *P. pastoris* Clones

Aim

Screening *P. pastoris* clones for protein expression after transformation with the pPICZα A vector.

Materials

- Clones with integrated plasmid (confirmed by colony PCR)
- BMGY medium
- BMMY medium
- Methanol
- 500X copper sulfate

Procedure

1. Using a single colony, inoculate 20 mL of BMGY medium in a 100 mL baffled flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD600 = 2–6 log-phase growth, approximately 16–18 hours).
2. Harvest the cells by centrifuging at 1,000 × g for 5 minutes at 4°C. Decant the supernatant and resuspend the cell pellet to an OD600 of 1.0 in 20 mL BMMY medium to induce expression. Add 100-200µl of 100% methanol directly to culture flasks to reach a final concentration of 0.5-1%.
   Note: For expression of laccases, add 20-40µL of 500X copper sulfate for a final concentration of 0.1-0.2 mM.
3. Place the culture in a 100 mL flask and return it to the incubator to continue growth.
4. Add 100% methanol to a final concentration of 0.5-1% methanol every 24 hours to maintain induction. Be sure to check the volume of the culture, and add methanol accordingly. Evaporation may reduce the culture volume.
   Note: For expression of laccases, add 500X copper sulfate every 24 hours to a final concentration of 0.1-0.2 mM.
5. At each of the times indicated below, transfer 1 mL of the expression culture to a 1.5 mL microcentrifuge tube. These samples will be used to analyze expression levels and to determine the optimal time post-induction to harvest. Centrifuge the samples at maximum speed in a tabletop microcentrifuge at 4°C for 5 minutes.
   Time points (hours): 24 (1 day), 48 (2 days), 72 (3 days), 96 (4 days) and 120 (5 days).
6. For secreted expression, transfer the supernatant to a separate tube. Freeze the supernatant and the cell pellets quickly in liquid N_2 or a dry ice/alcohol bath, and store them at –80°C until ready to assay.

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For intracellular expression, decant the supernatant and store just the cell pellets at –80°C until ready to assay. Freeze quickly in liquid N_2 or a dry ice/alcohol bath.

7. Analyze the supernatants and the cell pellets for protein expression by Coomassie-stained SDS-PAGE and western blot (see separate protocols), or functional assay (see below).

Notes

Perform expression at 30°C, in a shaking incubator. It is critical that the temperature does not exceed 30°C. If your incubator temperature fluctuates, set the temperature at 28°C. If using a floor shaking incubator, shake at 225–250 rpm. If using a table-top shaker that sits inside an incubator, shake at 250–300 rpm.

ABTS activity assay

Aim

Screening supernatant of *P. pastoris* clones for laccase activity after induction of expression.

Materials

- Supernatant from clones
- ABTS™ buffer (11204530001 Roche)
- ABTS ™ (10102946001 Roche)
- Citric Acid – Na_2HPO_4 buffer pH 4.0

Procedure

Measuring absorbance in cuvettes

1. Dissolve 1 mg of ABTS in 1 mL of ABTS buffer to create an ABTS stock solution (1 mg/mL).
2. Add 800µl of citric acid-phosphate buffer to the cuvette, followed by 100µl of ABTS stock.
3. Add 100µl of supernatant to the cuvette. Seal the cuvette with parafilm and mix thoroughly by inverting three times.
4. Measure at 420 nm for 5-60 minutes right after mixing the content of the cuvette.
5. Calculate the slope (abs/min) of this continuous curve. Alternatively, calculate the difference in absorbance between the chosen time points.

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6. Repeat steps 2-5 for all clones and compare.

Notes

Different time points can be chosen for absorbance measurements, however the same time points should be used for all clones and screening days to make the data comparable.

Scale Up Expression

Aim

Scale up expression of proteins in *P. pastoris* after screening.

Materials

- Protein producing clone (confirmed by screening)
- BMGY medium
- BMMY medium
- Methanol
- 500X copper sulfate

Procedure

1. Using a single colony, inoculate 30 mL of BMGY medium in a 100 mL flask. Grow at 28–30°C in a shaking incubator (250–300 rpm) until the culture reaches an OD600 = 2–6 log-phase growth, approximately 16–18 hours).
2. Harvest the cells by centrifuging at 1,000 × g for 5 minutes at room temperature. To induce expression, decant the supernatant and resuspend the cell pellet in 100 mL BMMY medium. Add 1 mL of 100% methanol directly to culture flasks to reach a final concentration of 1%. **Note:** For expression of laccases, add 100 µL of 500X copper sulfate for a final concentration of 0.1 mM.
3. Place the culture in a 100 mL flask and return it to the incubator to continue growth.
4. Add 100% methanol to a final concentration of 1% methanol every 24 hours to maintain induction. Be sure to check the volume of the culture, and add methanol accordingly. Evaporation may reduce the culture volume.

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Note: For expression of laccases, add 500X copper sulfate every 24 hours to a final concentration of 0.1 mM.

5. Harvest cells by centrifuging at 1,000 × g for 5 minutes at 4°C.
6. For intracellular expression, decant the supernatant and store the cell pellets at –80°C until ready to process.
   For secreted expression, save the supernatant, chill it to 4°C, and concentrate it, if desired. Proceed directly to purification (see 'IMAC' protocol) or store the supernatant at –80°C until ready to process further.

Notes

Perform expression at 30°C, in a shaking incubator. It is critical that the temperature does not exceed 30°C. If your incubator temperature fluctuates, set the temperature at 28°C. If using a floor shaking incubator, shake at 225–250 rpm. If using a table-top shaker that sits inside an incubator, shake at 250–300 rpm.

References

This protocol is a modified version of the 'Expression of recombinant Pichia strains' and 'Scale up expression' protocols from the Pichia Expression Kit manual (MAN0000012) from Invitrogen.

The ABTS assay was designed based on information from 'Childs RE, Bardsley WG. The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid) as chromogen. Biochem J. 1975;145: 93–103'.