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Recombinant Protein Expression in Yeast system

Introduction

As a eukaryote, Pichia pastoris has many of the advantages of higher eukaryotic expression systems such as protein processing, protein folding, and posttranslational modification, while being as easy to manipulate as *E. coli* or Saccharomyces cerevisiae. It is faster, easier, and less expensive to use than other eukaryotic expression systems such as baculovirus or mammalian tissue culture, and generally gives higher expression levels. As a yeast, it shares the advantages of molecular and genetic manipulations with Saccharomyces, and has the added advantage of 10- to 100-fold higher heterologous protein expression levels. These features make Pichia very useful as a protein expression system.

Pichia pastoris is a methylotrophic yeast, capable of metabolizing methanol as its sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. This reaction generates both formaldehyde and hydrogen peroxide. To avoid hydrogen peroxide toxicity, methanol metabolism takes place within a specialized cell organelle called the peroxisome, which sequesters toxic by-products from the rest of the cell. Alcohol oxidase has a poor affinity for O2, and Pichia pastoris compensates by generating large amounts of the enzyme. The promoter regulating the production of alcohol oxidase drives heterologous protein expression in Pichia.
The AOX1 and AOX2 genes code for alcohol oxidase in Pichia pastoris. The AOX1 gene product accounts for the majority of alcohol oxidase activity in the cell. Expression of the AOX1 gene is tightly regulated and induced by methanol to high levels, typically > 30% of the total soluble protein in cells grown with methanol as the carbon source. The AOX1 gene has been isolated and the AOX1 promoter is used to drive expression of the gene of interest. While AOX2 is about 97%homologous to AOX1, growth on methanol is much slower than with AOX1. This slow growth allows isolation of Mut^S strains.

Heterologous expression in Pichia can be either intracellular or secreted. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. While several different secretion signal sequences have been used successfully, including the native secretion signal present on some heterologous proteins, success has been variable. The secretion signal sequence from the Saccharomyces cerevisiae α factor prepro peptide has been used most successfully. The major advantage of expressing heterologous proteins as secreted proteins is that Pichia pastoris secretes very low levels of native proteins. That, combined with the very low amount of protein in the minimal Pichia growth medium, means that the secreted heterologous protein comprises the vast majority of the total protein in the medium and serves as the first step in purification of the protein. Note: If there are recognized glycosylation sites (Asn-X-Ser/Thr) in your protein's primary sequence, glycosylation may occur at these sites.

Experimental Outline

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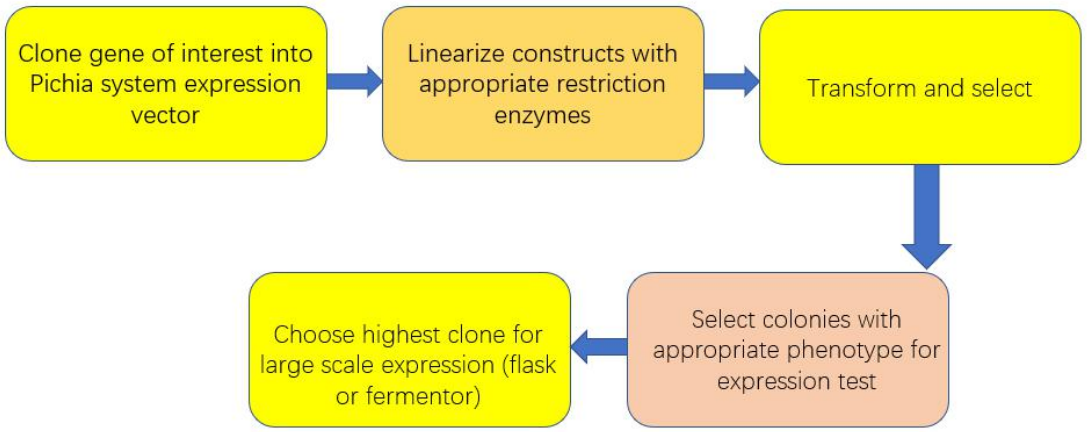

Protocol for Recombinant protein expression using the Pichia Expression System

The following protocol can be a reference for you to understand more details of the techniques utilized in our lab and workflow involved in producing the protein of interest using *P.pastoris*.

1. Select the appropriate Expression Vector for your target gene.

You may choose to express your protein intracellularly if your protein is cytosolic and non-glycosylated. You can also have your target protein secreted into the culture media if your protein is generally secreted, glycosylated or directed to an intracellular organelle.

2. Transformation into E.coli.

i. Insert the target gene in frame with the expression vector.
ii. Transform the E.coli with the ligation mixture by electroporation or chemical methods.
iii. Add LB medium to the cells for recovery after heat shock or electroporation.
iv. Plate on LB medium with appropriate antibiotics.
v. Incubate overnight.

3. Analyze transformants.

i. Select antibiotics -resistant colonies and inoculate into LB medium with appropriate antibiotics.
ii. Grow overnight and isolate plasmid DNA.
iii. Sequence the gene construct to confirm the correct insertion of gene within the vector.

4. Preparation for transformation

Prior to transformation and selection in Pichia, the plasmid should be linearized. Vector linearized within the 5' AOX1 region will integrate into the host 5' AOX1 region by gene insertion.

i. Digest the plasmid DNA with selected restriction enzymes.
ii. Check the digested mixture for complete linearization by agarose gel electrophoresis.
iii. Once the vector is confirmed to be completely linearized, purify DNA.

5. Electroporation of Pichia.

The method of electroporation is strongly recommended because it gives the highest transformation frequency in Pichia.

i. Grow the selected *P.pastoris* strain in yeast medium overnight.

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ii. Add fresh medium in the overnight culture. Grow overnight again.
iii. Centrifuge the cells and resuspend with ice-cold, sterile water. Repeat twice.
iv. Centrifuge the cells, then resuspend with ice-cold sorbitol. Repeat twice. Place the cells on ice before use.
v. Mix the cells with linearized DNA.
vi. Transfer the mixture to an ice-cold electroporation cuvette. Incubate on ice.
vii. Add ice-cold sorbitol to the cells, then transfer the cuvette content and incubate at 30°C.
viii. Spread the cells on plates for appropriate antibiotics or screening plate.
ix. Incubate the plates until colonies form.
x. Pick 10-20 colonies for further selection.

6. Analyze Pichia transformants

i. Inoculate yeast medium with the chosen single colony of Pichia strain. Grow overnight.
ii. Dilute the cells from the overnight culture and grow approximately 4-6 h.
iii. Centrifuge the cells and keep the pellet.
iv. Resuspend the cell pellet and get the competent cells.
v. The cells can be kept at room temperature and used for transformation assay at once or frozen for storage.

7. Transformation assay

The following procedure provides details of transformation of freshly prepared or frozen competent Pichia cells. Note that transformation efficiency may vary among different Pichia strains and expression vectors used.

i. Add linearized recombinant vector DNA to the competent cells.
ii. Add solution containing PEG into the DNA/cell mixture.
iii. Incubate the transformation reaction for 1h at 30°C. Mix the reaction solution to increase transformation efficiency.
iv. Treat with heat shock and split the cells into microcentrifuge tubes for incubation.
v. Centrifuge the cells and keep the pellet.
vi. Resuspend and combine the cells.
vii. Plate the cells for selection. Incubate the plates until colonies appear.

8. Determine the Mut (Methanol Utilization Slow) phenotype.

Identify the expression levels among several chosen Mut phenotypes by SDS-PAGE analysis and screen for the high-expression Pichia recombinant clones. This may help optimize the expression condition of the recombinant clone.

The effectiveness of expression conditions of Pichia strain with Mut phenotype can be tested as follows.

i. Include a control gene transformed with the parent vector as a control for background intracellular expression.
ii. Inoculate a single colony in a baffled shaking flask and grow at 28-30°C.
iii. Harvest the cells and centrifuge at room temperature.
iv. Discard supernatant and resuspend cell pellet. Cover the flask with 2 layers of sterile gauze and continue growth.
v. Add methanol every 24 hours to maintain induction.
vi. Transfer 1 ml of the expression culture to a fresh microcentrifuge tube to analyze expression levels at a series of time points (i.e., 0-96 h post-induction).

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vii. Determine the optimal time to harvest the cells. Centrifuge the cells at room temperature.

viii. For secreted expression, transfer the supernatant to a fresh tube and store until ready to assay. While, for intracellular expression, discard the supernatant and store the cell pellets until ready to assay.

ix. Analyze the supernatants and cell pellets respectively for protein expression by SDS-PAGE and Western blot.

9. Scale-up expression.

Once expression condition is optimized, larger baffled flasks or fermentation will be utilized to increase the culture volume for scale-up production of target protein.

Note that proteins secreted into the media are normally more than 50% homogeneous and need additional purification. Therefore, it is an optimal step to concentrate the protein before the purification process if the expression level is low. Commonly used methods for this step are ammonium sulfate precipitation, lyophilization and centrifuge concentrator for small volumes