

Recombinant Allergens Production in Yeast

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Abstract

The methylotrophic yeast *Pichia pastoris* has been extensively used in large-scale production of properly folded recombinant proteins. As an eukaryotic organism *P. pastoris* presents a series of advantages at expression and processing of heterologous proteins such as post-translational modifications, protein processing, and a reasonably sophisticated quality control of protein folding when compared against *Escherichia coli*. In this chapter, we describe the modified lab procedure for cloning and expression in *Pichia pastoris* of common food allergens sequences from the raw fruit to the fully folded biotinylated protein product.

Key words Food allergens, In vivo biotinylation, *Pichia pastoris*, Protein expression, Protein tagging, Yeast expression

1 Introduction

Pichia pastoris has become an interesting alternative to *E. coli* in recombinant antibody production. It presents the advantages of single-celled organisms, such as ease of handling and cost-effectiveness, combined with the benefits of eukaryotic systems that include post-translational modifications, protein processing, and a reasonably sophisticated quality control of protein folding. Additionally, it is a more manageable and sturdy system than higher eukaryotes, such as insects and mammalian tissue culture cells systems, that besides being auxotrophic they can employ cheap methanol as the sole source of carbon and energy [1, 2].

In this work, we describe the modified lab procedure for cloning and expression in *Pichia pastoris* of common food allergens. As an example of this application, the following steps describe the amplification of Pru p 3 gene from the raw fruit (*Prunus persica*), sequencing, and the target DNA sequence cloning into yeast expression vector driven by methanol. In this system, the AOX promoter drives the alpha-mating type pre-prosequence that directs the fusion product to the ER and subsequent GOLGI and cell secretion. The fusion protein is then cleaved from the

pre-prosequence by an internal protease system (Kex II) [3–6]. Most of the general steps of this procedure are described in details in the Pichia expression system brochures produced by INVITROGEN using the vector pPICZ that is easily accessible on line.

In addition to the procedure described by the manufacturer we added few essential selection steps (dot blots) that make an effective contribution toward a successful expression. Further, we describe our latest generic tagging system where a biotinylation C-terminal target sequence was engineered [7, 8] for in vivo biotinylation of the target protein [9–12]. The procedure described here has been successfully used in the production of biotinylated scFv [13, 14] and others not yet published, and is now routine in our lab.

2 Materials

2.1 Gene Amplification

1. RNA Extraction: RNeasy mini Kit (QIAGEN, UK).
2. Complementary DNA: M-MLV reverse transcriptase and Random Primers.
3. End-point PCR: ZymoTaq DNA Polymerase Set including enzyme, buffer and dNTPs (Zymoresearch Cambridge Biosciences, UK or similar). Synthetic DNA oligonucleotides (5 μ M each).

2.2 Agarose Gel Electrophoresis

1. 1% Agarose gel: Weigh 1 g agarose and dissolve in 100 mL of 1 $\times$ TAE Buffer.
2. Tris-acetate EDTA running buffer (TAE Buffer; 1 $\times$): 40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA.
3. Gel electrophoresis apparatus.
4. Power pack (Bio-Rad, UK).
5. DNA Stain: Midori Green Direct DNA Stain.

2.3 PCR Product Purification and Cloning

1. QIAquick PCR Purification Kit (QIAGEN, UK).
2. TOPO TA Cloning Kit with pCR2.1 vector (Life Technologies, UK).
3. Purified PCR product.
4. TOP 10 Chemically Competent *E. coli*.
5. DNA midi kit Plasmid purification (QIAGEN, UK).

2.4 General Media

1. Luria-Bertani medium (LB): 1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl. Autoclave at 121 $^{\circ}$ C for 15 min cycle. Store at 4 $^{\circ}$ C.
2. Luria-Bertani agar plates (LBA): 1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl, 1.5% agar pH 7.5. Autoclave at 121 $^{\circ}$ C for 15 min cycle. Cool down before adding the selective antibiotic.

3. Yeast Extract Peptone Dextrose (YPD) medium: 1% yeast extract, 2% peptone, 2% dextrose. Store at 4 °C. Autoclave at 121 °C for 15 min cycle. Cool down before adding the selective antibiotic.
4. Yeast Extract Peptone Dextrose Sorbitol (YPDS) agar plates: 1% Yeast Extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar with 100 µg/mL of Zeocin.
5. Buffered Glycerol-complex Medium (BMGY): 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base (YNB), 1% glycerol, $4.10^{-5}\%$ biotin.
6. Buffered Methanol-complex Medium (BMMY): 1% yeast extract, 2% peptone, 100 mM potassium phosphate pH 6.0, 1.34% YNB, 1% methanol, $4.10^{-5}\%$ biotin.
7. Glycerol stocks: Luria-Bertani medium with 25% final concentration of glycerol.

2.5 *Pichia* Protein Production

1. Plasmid DNA linearization: SacI restriction enzyme.
2. *Pichia* Electrocompetent cells: pMJA180-GS115 *Pichia*, cold (4 °C) sterile 1 M sorbitol.
3. Electroporator: BioRad MicroPulser electroporation apparatus and 0.2 cm cuvettes (Bio-Rad, UK).
4. Selection antibiotics: 100 µg/mL Zeocin, 500 µg/mL Blasticidin.

2.6 Dot Blot Analysis

1. Acrylic Dot blot microfiltration unit.
2. PVDF membrane.
3. Phosphate-buffered saline (PBS): One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C.
4. Tris Buffered Saline (TBS; 10×): 1.5 M NaCl, 0.1 M Tris-HCl, pH 7.4.
5. Blocking solution: 3% BSA (w/v) TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5).
6. Washing buffer: TBST (0.05% v/v Tween-20).
7. Streptavidin Alkaline Phosphatase dilution: (1:5000 v/v) in 1% BSA (w/v) TBST.
8. Develop agent: a ready-to-use solution of 5-bromo-4-chloro-3-indolyl-1-phosphate and nitroblue tetrazolium.

2.7 SDS-PAGE

1. NuPAGE XCell SureLock System Mini-cell electrophoresis system.
2. Mini Gel: NuPAGE Bis-Tris, Novex Pre-Cast Gel 12%.

3. NuPAGE LDS Sample Buffer 4×.
4. Reducing agent: 1 M DTT.
5. Running Buffer; 1×: 50 mL of 20× NuPAGE MES Running buffer diluted in 950 mL of distilled water.
6. NuPAGE Antioxidant: contains Dimethylformamide and Sodium bisulfite.
7. Protein Molecular marker: Precision Plus All Blue Pre-stained Protein Marker.
8. Staining solution: 0.1% Coomassie Brilliant Blue R250 in 50% Methanol (v/v), 10% Glacial acetic acid (v/v), and 40% water.
9. Destaining solution: 40% Methanol (v/v), 10% Glacial acetic acid (v/v).

2.8 Western Blotting

1. Xcell II Blot Module.
2. Transfer Buffer: 1× NuPAGE Transfer Buffer with 10% Methanol and 0.1% Antioxidant.
3. Blotting pads.
4. PVDF membrane (Immuno-Blot, Bio-Rad).
5. Filter paper.

3 Methods

The following procedure describes the cloning and expression of peach allergens from the native fruit to the final protein expression in *Pichia*. All procedures should be carried out at room temperature and under sterile conditions unless otherwise specified.

3.1 RNA Extraction and Amplification

1. The peach (*Prunus persica*) RNA is isolated from 30 mg of frozen raw fruit using RNA extraction kit essentially as described by the manufacturer (QIAGEN, UK).
2. Complementary DNA: Each reverse transcription (RT) reaction is carried out in duplicate, one containing the Reverse Transcriptase enzyme (+RT) and one where RT is substituted with water (−RT) (*see Note 1*). Complementary DNA (cDNA) is synthesized from 0.5 µg of total RNA incubated with 200 units of the M-MLV reverse transcriptase enzyme, primers in 5× reaction buffer in a final volume of 25 µL, for 1 h at 42 °C.
3. End-point Polymerase Chain Reaction (PCR): The peach-specific genes are amplified by PCR using specific primers containing the PstI site in frame with the alpha-mating restriction site at 5′ end and a NotI site at 3′ end. All primers are designed based on the mature protein and with a T_m of ca. 58 °C (*see Note 2*). The amplification is carried out at 30 cycles of

94 °C 30 s, 58 °C 30 s, 72 °C 30 s using the cDNA produced in **step 2** (2 µL), ZymoTaq DNA Polymerase (2 units/50 µL), enzyme buffer and dNTPs according to the manufacturer's instructions (Zymoresearch Cambridge Biosciences, UK or similar).

4. The PCR product size is confirmed by gel sizing in a 1% agarose gel electrophoresis (*see Note 3*). Running conditions are 90 V for 45 min.

3.2 TA Cloning TOPO and Screening

1. The correct size amplified product is excised from the gel or if single intense band, purified directly essentially as described in QIAquick PCR Purification Kit.
2. The purified product (2 µL) is then ligated to the TOPO pCR 2.1vector (0.5 µL) in a 30 min room temperature reaction essentially as described by the manufacturer (*see Note 4*).
3. The ligated mixture is then transformed into either electro or chemically competent *E. coli* cells and spread on pre-warmed low salt Luria-Bertani agar plates containing 50 µg/mL Ampicillin.
4. Plates are incubated overnight at 37 °C.

3.3 Plasmid Purification

1. PCR colony screening: In a sterile flow cabinet, 5–10 growing colonies are picked and inoculated into 100 µL of LB medium containing 50 µg/mL Ampicillin and grown for 1 h at 37 °C with 200 rpm shaking. The positive clones are confirmed by PCR using the LB culture (2 µL) as template, M13 oligonucleotides specific for TOPO-pCR 2.1vector at 30 cycles of 94 °C 30 s, 58 °C 30 s, 72 °C 30 s using DyNazyme II DNA Polymerase (0.5 units/25 µL), enzyme buffer and dNTPs according to the manufacturer's instructions (*see Note 5*).
2. Positive *E. coli* clones are then inoculated into either 10 mL or 100 mL LB containing 50 µg/mL Ampicillin accordingly to the amount of plasmid required by the plasmid purification Kits following the manufacturer's instructions (*see Note 6*).
3. Purified plasmids are then sent for sequencing.

3.4 pPICZ Cloning

1. Once the sequence is confirmed the TOPO vector (5 µg) produced in **step 3** is digested for 2 h at 37 °C with PstI and NotI (10 U/each) in 100 µL reaction (*see Note 7*).
2. The digested product size is confirmed by 1% agarose gel to electrophoresis.
3. The correct size fragment is then excised from the gel and purified using the QIAquick PCR Purification Kit according to the manufacturer's instructions.

4. The purified fragment (10–20 ng) is then ligated to the PstI/NotI digested plasmid pMJA167 (90 ng) for 2–4 h at 15 °C using T4 Ligase (5 U) in a 20 µL reaction mixture containing enzyme buffer and ATP.
5. The ligated mixture is then transformed into *E. coli* competent cells and spread on pre-warmed low salt LB agar plates containing 50 µg/mL of Zeocin and incubated O/N at 37 °C.
6. The positive clones are again selected by PCR essentially as described in Subheading 3.3 step 1 but using the primer-specific oligonucleotides.
7. The positive clones are then inoculated in 100 mL LB medium containing 50 µg/mL Zeocin and grow O/N at 37 °C with 200 rpm shaking for Midi or Maxi purification according to the Kit's manufacturer.

3.5 *Pichia* Transformation [15]

1. Inoculate pMJA180-GS115 into 100 mL of YPD containing 500 µg/mL of Blasticidin and grow O/N at 30 °C with 200 rpm shaking.
2. Measure the OD₆₀₀ density of the overnight culture until reaching 1.3–1.5. Then centrifuge cells (3000 g, 10 min), wash pellet in 30 mL of cold (4 °C) sterile water. Vortex the cell pellet to dissolve and centrifuge again (3000 g, 5 min).
3. Repeat once the wash step then resuspend cell pellet in 2 mL of cold (4 °C) sterile 1 M sorbitol and centrifuge (3000 g, 5 min). Finally, resuspend cell pellet in about 200 µL cold (4 °C) sterile 1 M sorbitol (*see* Note 8).
4. The pPICZ plasmid DNA (10 µg) from each protein of interest is linearized by SacI digestion followed by ethanol precipitated [15].
5. The plasmids are then transformed into *P. pastoris* BirA-GS115 competent cells (step 3 above) by electroporation, following the electroporator manufacturer's instructions.
6. Transformed cells are spread on Yeast Extract Peptone Dextrose Sorbitol Medium (YPDS) agar plates containing 100 µg/mL Zeocin and 500 µg/mL Blasticidin and incubate for 72 h at 30 °C.
7. 24 isolated colonies from each construct plate are selected and grown again on an YPDS 96-well plate containing 100 µg/mL Zeocin and 500 µg/mL Blasticidin and incubate for 48 h at 30 °C (*see* Note 9).
8. 24 colonies from the backup plate are inoculated in 2 mL of Buffered Glycerol-complex Medium (BMGY) in 24-well plates containing 100 µg/mL Zeocin and 500 µg/mL Blasticidin and incubate for 72 h at 30 °C with 200 rpm shaking.
9. Plates are then centrifuged (1800 g, 10 min, 4 °C), the supernatant removed and cells resuspended in Buffered Methanol-

complex Medium (BMMY) to induce the production of protein of interest incubated for 72 h at 30 °C with 200 rpm shaking.

10. Methanol (1% v/v) is replenished every 12 h for 72 h.
11. Finally, plates are centrifuged (1800 g, 10 min, 4 °C) and the supernatant is analyzed by dot blot analysis in search for the expression of each protein.

3.6 Dot Blot Analysis

1. 400 µL of supernatant from 24 selected *P. pastoris* clones are filtered through a dot blot microfiltration unit containing a PVDF membrane (*see Note 10*).
2. Membrane is then blocked with 3% BSA (w/v) TBS at 37 °C for 1 h (*see Note 11*).
3. After washing the membrane three times for 5 min with TBST, incubate with Streptavidin Alkaline Phosphatase (1:5000 v/v) at 37 °C for 2 h (*see Note 12*).
4. Membrane is then washed three times with TBST and once with distilled water.
5. Finally, add a ready-to-use solution of 5-bromo-4-chloro-3--indolyl-1-phosphate and nitro blue tetrazolium to detect the alkaline phosphatase enzyme activity.
6. Reaction is stopped by rinsing the membrane with water.
7. Figure 1 shows a typical dot blot analyses.

3.7 SDS-PAGE

1. Sample preparation: samples from Subheading 3.5 step 11 are dialyzed (3.5 K cutoff) against water overnight followed by freeze-drying.
2. Dried samples are then resuspended in LDS Sample buffer (*see Note 13*).
3. Samples are separated by SDS electrophoresis using a NuPAGE Bis-Tris, Novex Pre-Cast Gel 12% essentially according to the manufacturer's protocol (*see Note 14*).
4. The gel is fixed and stained in Coomassie blue into a staining container overnight on a shaking platform. Next morning the gel is destained completely until the bands easily visualized.

3.8 Western Blotting

1. The blotting procedure is performed essentially according to the manufacturer's protocol (Novex). Briefly, the SDS-PAGE gel was blotted onto a PVDF membrane at 30 V/1 h using the dedicated Transfer buffer.
2. The membrane is washed three times in TBST and water and the biotinylated proteins detected essentially as described in Subheading 3.6 for dot blot analysis or through the use of specific antibody.
3. Figure 2 shows a typical Western blotting for the biotinylated protein Pru p 3.

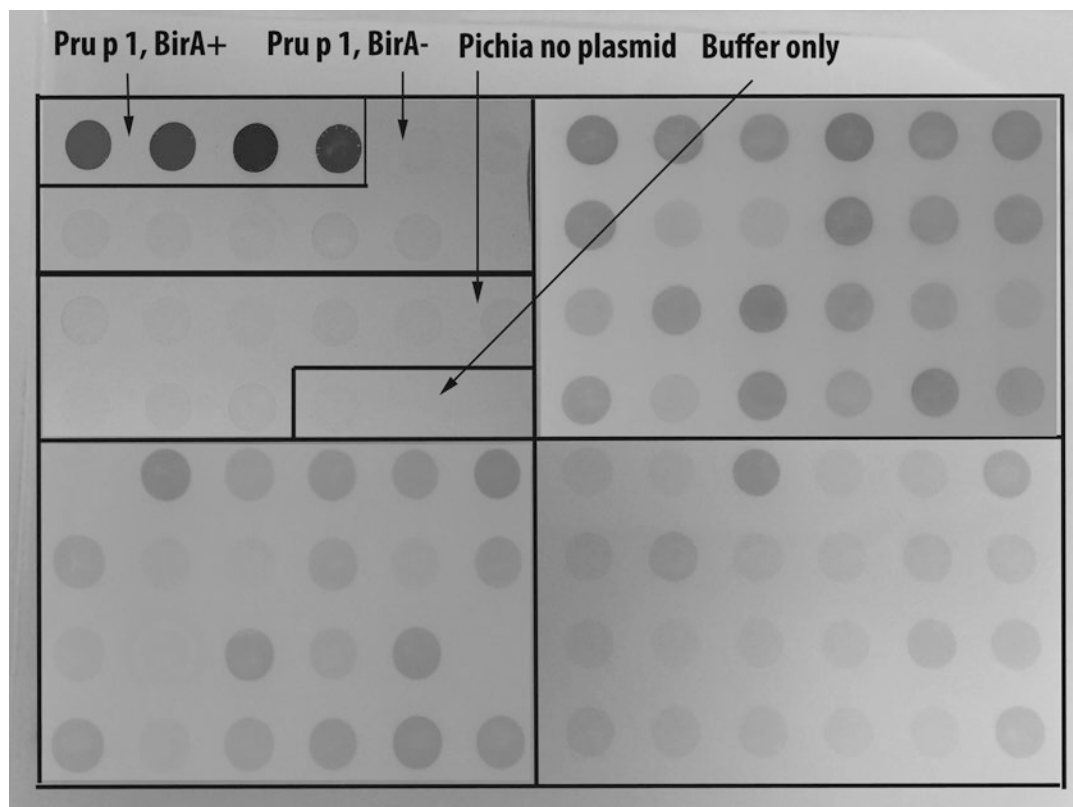


Fig. 1 Representative screening of 24-well expression plates after methanol induction. After induction for 72 h the supernatants were dot blotted into PVDF membrane and probed with avidin-Alkaline Phosphatase. Pru p 1, a peach allergen was used as positive control. No to very low expression of Pru p 1 was detected in the absence of BirA. The other three panels are representative expression of different scFv clones under the BirA+ background. The selection of the higher expressers is carried out visually

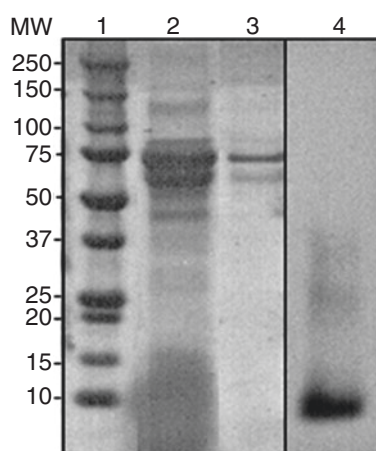


Fig. 2 SDS-PAGE and Western Blot of the biotinylated protein Pru p 3. *Lane 1* shows pre-stained SDS-PAGE molecular weight standards; *Lane 2* shows Pru p 3 supernatant extract; *Lane 3* shows Pru p 3 supernatant diluted 1:10; *Lane 4* shows Western Blot of Pru p 3 supernatant diluted 1:10 using Streptavidin-AP as detection in a 1:1000 dilution

4 Notes

1. The Reverse Transcriptase enzyme is substituted with water (–RT) to control for any genomic contamination in our RNA samples as well as other reaction components. RNA denaturation is carried out at 70 °C for 5 min and then on ice for 1 min. Random primers or specific gene primers can be used at this stage.
2. More information and tips for the primer design can be found in the INVITROGEN pPICZ manual.
3. Midori Green Dye is a safer replacement for the ethidium bromide. Mix samples and DNA markers with Midori Green Direct stain at 1:10 (dye:samples) dilution rate. If you expect only one or few DNA bands in a lane do not use more than 0.5 µL Midori Green Direct per sample even if you use more than 5 µL sample volume. If you apply DNA ladders or samples with a lot of bands use 1 µL of Midori Green Direct. After the electrophoresis, view the results on the FastGene Blue LED Illuminator.
4. For the ligation reaction use half of the recommended amount, i.e. 0.5 µL of TOPO vector, 0.5 µL of Salt solution, and 2 µL of purified PCR product. Incubate the maximum recommended time.
5. DyNazyme II DNA Polymerase diluted and added separately after the second cycle in a conventional hot-start procedure. We find that way give us better results. By using M13 external primers, the absence of ligated band will result in a 100–120 bp product so the successful amplification can be easily monitored.
6. On the step where applying the supernatant that contains the plasmid DNA into the QIAGEN-tip filter, pass it through a Whatman paper first. This is to make sure that the tip will not be blocked from any precipitation that might be leftover in supernatant.
7. We use PCR machine for all the incubation reactions.
8. BirA Pichia cells contain the *E. coli* biotinylation enzyme that will recognize the C-terminal Tag sequence in the construct. Dissolve cell pellet in a suitable volume of cold (4 °C) sterile 1 M sorbitol, depending on the size of cell pellet.
9. We use that 96-well plate as a backup plate by adding Glycerol at final concentration of 20%. The plates are then stored at –80 °C.
10. The dot blots are carried out in duplicates, with one dot from neat supernatant and another diluted 100× in PBS [16]. The dot blot step is essential to this procedure as it ensures that the highest expresser clones are selected.

11. We find that it is best to prepare this fresh each time.
12. Streptavidin Alkaline Phosphatase is used for the detection of biotinylated molecules.
13. LDS sample buffer is used to prepare protein samples for denaturing gel electrophoresis. Heat the samples at 70 °C for 10 min and make sure there is a hole on the top of the lid to avoid any lid explosion from the high temperature.
14. NuPAGE® Antioxidant is added to the running buffer in the upper (cathode) buffer chamber when performing protein gel electrophoresis under reducing conditions.

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