Chapter 21

Cloning and Expression of Hemicellulases from *Aspergillus nidulans* in *Pichia pastoris*

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Abstract

The methylotrophic yeast *Pichia pastoris* is increasingly used for heterologous expression of high quality proteins in laboratory-scale (milligram) quantities. Commercially available polysaccharide-active enzyme preparations have limited applications in plant cell wall research due to their heterogeneous mix of hydrolytic activities. *P. pastoris* provides an ideal in vitro expression system for producing monocomponent enzymes, since it lacks endogenous plant cell wall-active enzymes and can perform eukaryotic post-translational modifications (i.e., glycosylation). We have routinely prepared cDNA constructs from *Aspergillus nidulans* encoding a broad array of hydrolases active on various linkages contained in plant cell wall polysaccharides. The cDNAs were inserted into the pPICZα C shuttle vector (Invitrogen) in-frame with the *Saccharomyces cerevisiae* α-secretion factor and expressed under the transcriptional control of the highly inducible alcohol oxidase 1 (AOX1) promoter. The enzyme products were efficiently secreted into buffered complex methanol medium (BMMY) as C-terminal his-tagged proteins for simple one-step affinity purification. The insertion of the c-*Myc* epitope enabled easy immunodetection. Here we present the detailed protocols for primer design, cloning, expression, and activity assays for a representative set of xylan-acting hemicellulases produced in *P. pastoris*.

Key words: *Pichia pastoris*, *Aspergillus nidulans*, Protein expression, Plant cell wall hemicellulase, Enzyme assay, Xylanase, Xylosidase

1. Introduction

Pichia pastoris has become a well-established system for heterologous expression of quality proteins in high levels. *P. pastoris* is a single-celled eukaryotic methylotrophic yeast, capable of metabolizing methanol as the carbon source. Transformation in *P. pastoris* uses a similar set of well-defined molecular and genetic manipulation techniques established for *Saccharomyces cerevisiae*. However, unlike *S. cerevisiae*, *P. pastoris* can grow to very high cell densities

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in minimal medium owing to its respiratory growth rather than the fermentative growth (1). Additionally, *Pichia* can perform suitable post-translational modifications (i.e., glycosylation) and is thus generally regarded as simple, fast, and readily manipulated eukaryotic protein expression system (1, 2). The expression of protein is tightly regulated by the methanol-inducible alcohol oxidase 1 (AOX1) promoter, which improves protein yields. Furthermore, this expression system has high genetic stability (i.e., decreased gene deletion) and is capable of efficiently secreting correctly folded heterologous proteins to the culture medium (3). Extracellular secretion of heterologous protein is the preferred option for *Pichia* expression, rather than intracellular cytoplasmic accumulation (4), as it simplifies the downstream processing for protein recovery. The availability of a commercial kit (Invitrogen) also promotes the versatility of *Pichia* as an in vitro expression system.

Commercially available enzyme preparations can have limited applications in plant cell wall polysaccharide research due to their heterogeneous mix of hydrolytic activities (5). Since *P. pastoris* lacks endogenous plant cell wall active enzymes and is able to secrete expressed protein in high levels with significantly low level of endogenous proteins, they are suitable for recombinant expression of cell wall active enzymes (4, 5). Being a saprophytic filamentous ascomycete fungus, *Aspergillus nidulans* secretes a variety of cell wall-active enzymes to survive on dead and decaying plant materials. Thus *A. nidulans* is expected to contain genes encoding diverse hydrolases active on various plant cell wall polymers. The complete genome sequence of *A. nidulans* is now available (6, 7), and a comprehensive set of *A. nidulans* gene sequences (ORFs) were identified based on homology to known cell wall active enzymes (8). We successfully cloned respective complementary DNAs (cDNAs) from *A. nidulans* into *P. pastoris* and expressed them in frame with the *S. cerevisiae* α-secretion factor (mating factor) under the transcriptional control of highly inducible AOX1 promoter of the pPICZα C shuttle vector (8). The enzymes were secreted directly into buffered complex methanol medium and then purified efficiently by use of a C-terminal polyhistidine-tag with metal-chelate affinity chromatography media. The recombinant enzymes also contained a c-*Myc* epitope for easy immunodetection by dot-blot. The one-step purification using metal-chelation chromatography provided milligram quantities of high purity cell wall active enzymes (9). These enzymes were devoid of any contaminating side activities on the respective cell wall polymers, and are thus "monocomponent" (8–10). We present in this chapter the detailed protocols for genomic DNA and mRNA isolation from *A. nidulans*, primer design, cloning and expression in *P. pastoris*, and subsequent purification and activity assays for a representative set of xylan-acting enzymes.

2. Materials

1. *A. nidulans* FGSC A4 (Glasgow WT, from the Fungal Genomic Stock Center, University of Missouri, Kansas City, MO).
2. YPD: 1% Yeast extract, 2% peptone, and 2% glucose (dextrose).
3. Sorbitol buffer: 1 M sorbitol and 0.1 M Na_2 EDTA pH 8.0.
4. Zymolyase 100T (MP Biomedicals).
5. Yeast resuspension buffer: 50 mM Tris–HCl pH 7.4, 20 mM Na_2 EDTA pH 8.0.
6. 10% SDS: dissolve 100 g of electrophoresis-grade sodium dodecyl sulfate in 900 mL water by heating to 68°C, adjust pH to 7.2 with HCl and make up volume to 1 L.
7. 5 M potassium acetate: Mix 60 mL of 5 M potassium acetate (49.07 g potassium acetate in a total volume of 100 mL water), 11.5 mL of glacial acetic acid and 28.5 mL of water.
8. 1 M Tris–Cl: Dissolve 12.11 g Tris base [Tris(hydroxymethyl) aminomethane] in 80 mL of water, adjust pH to 7.4 or pH 8.0 with HCl (6 M) and make the volume to 100 mL.
9. 0.5 M Na_2 EDTA (pH 8.0): Add 18.61 g of Na_2 EDTA·2 H_2O in 80 mL of water, stir vigorously, and adjust the pH to 8.0 with NaOH pellets. Adjust volume to 100 mL.
10. TE buffer (pH 8.0 and pH 7.4): 10 m M Tris–HCl, pH 8.0 or pH 7.4, 1 mM Na_2 EDTA.
11. RNase (e.g., Invitrogen).
12. 3 M sodium acetate: Dissolve 408.3 g sodium acetate trihydrate in 800 mL of water. Adjust pH to 7.0 with dilute acetic acid and adjust the volume to 1 L. Sterilize by autoclaving.
13. Minimal medium: 1 L containing 6.0 g $NaNO_3$, 1.52 g KH_2PO_4, 0.52 g KCl, and 0.52 g $MgSO_4$.
14. Hutner's trace elements: 100 mL contained 5.0 g Na_2 EDTA·2 H_2O, 2.2 g $ZnSO_4 \cdot 7H_2O$, 1.14 g H_3BO_3, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 6H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, and 0.11 g $(NH_4)_6MO_7O_{24} \cdot 4H_2O$. Adjust pH to 6.5–6.8 with KOH.
15. Complete medium: Contained minimal medium supplemented with 0.5% yeast extract, 1% peptone, 2% glucose, and Hutner's trace elements (1 mL/L), pH 4.5.
16. Conical and baffled flasks (e.g., Pyrex).
17. Rotary shaking incubator (e.g., Innova 4000, New Brunswick Scientific).
18. Miracloth (Calbiochem).

19. Citrus pectin, larch wood xylan, gum arabic (e.g., Sigma-Aldrich).
20. RNase-free centrifugal tubes.
21. Centrifuges (e.g., Sorvall high speed, and Eppendorf microcentrifuge).
22. Liquid nitrogen.
23. Mortar and pestle.
24. TRIzol® reagent (e.g., Invitrogen).
25. Ethanol: 100% and 75% prepared with DEPC (diethylpyrocarbonate)-treated water.
26. RNAase-free water.
27. Temperature-controllable water bath.
28. Turbo DNA-free™ Kit containing Turbo DNase, 10× Turbo DNase buffer and DNase Inactivation Reagent (Applied Biosystems).
29. UV-Vis Spectrophotometer.
30. SuperScript® First-Strand Synthesis Kit for RT-PCR containing SuperScript™ II reverse transcriptase, 5× First strand buffer, DTT (dithiothreitol), RNaseOUT™, Oligonucleotide (dT) 12–18 primers, dNTP mix, 25 mM $MgCl_2$, DEPC-treated water, RNase H (Invitrogen).
31. PCR Purification Kit (Qiagen).
32. Primer design software "Oligo6" (current version is "Oligo7").
33. Taq polymerase (e.g., Invitrogen).
34. 10× PCR buffer: 750 mM Tris–HCl pH 8.5, 200 mM $(NH_4)_2SO_4$, and 0.1% Tween-20.
35. PCR ready-to-use mixture: 100 µL 10× PCR buffer, 80 µL 25 mM $MgCl_2$, 20 µL dNTP mix (10 mM each), and 800 µL of water.
36. Thermocycler.
37. DNA molecular mass ladder of known concentration (e.g., MassRuler™ DNA Ladder Mix, ready-to-use, 80–10,000 bp, Fermentas).
38. Electrophoresis assembly for DNA electrophoresis. (e.g., BioRad SUB® Cell GT and PowerPac™ Basic).
39. 50× TAE running buffer: 24.2% Tris–HCl pH 7.5, 5.71% (w/v) acetic acid and 3.72% EDTA·2 H_2O, adjust the pH to 8.0. Dilute 50 times (e.g., Research Organics).
40. Expression vector pPICZα C (Invitrogen).

41. 10× Buffer Y+(Fermentas): 330 mM Tris–acetate pH 7.9, 660 mM potassium acetate, 100 mM magnesium acetate, and 1 mg/mL bovine serum albumin (BSA).
42. Restriction enzymes *(Xba*I, *Pml*I, and *Pme*I).
43. Alkaline phosphatase (Shrimp AP, Fermentas).
44. Ligation Kit (Fermentas): 10× ligation buffer, 50% PEG 4000, and T4 ligase.
45. Chemically competent TOP10 *Escherichia coli* strain (Invitrogen).
46. SOC medium (Invitrogen): 2% Tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MnCl_2$, 10 mM $MgSO_4$, 20 mM glucose.
47. Low salt LB plates: 1% Tryptone, 0.5% yeast extract, 0.5% NaCl, and 1.5% agar.
48. Zeocin™ (Invitrogen).
49. TE buffer: 10 mM Tris–HCl pH 8.0, 1 mM EDTA.
50. Centrifugal vacuum concentrator (e.g., Speed-Vac from Savant).
51. Big Dye® Terminator Kit (Applied Biosystems).
52. Alkaline lysis buffer (0.2 N NaOH, 1% SDS).
53. Phenol:chloroform:isoamyl alcohol (25:24:1, as a ready-to-use mixture saturated with 100 mM Tris–HCl pH 8.0; contains ~0.1% 8-hydroxyquinoline, e.g.,, from Sigma-Aldrich).
54. Centri-Sep™ spin columns (Princeton Separations).
55. 10× Buffer B+(Fermentas): 100 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$ and 1 mg/mL BSA.
56. *P. pastoris* X-33 (Invitrogen).
57. YPDS plates: 1% Yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar and 100 μg/mL of Zeocin.
58. Electroporation cuvettes: Gene Pulser/Micro Pulser, 0.2 cm gap (BioRad).
59. Electroporation device *E. coli* Pulser™ Transformation Apparatus (BioRad).
60. Falcon conical tubes with screw-cap lid, 50 mL, sterile.
61. Nitrocellulose membrane (e.g., Schleicher & Schuell).
62. Instant non-fat dry milk powder (e.g., Carnation from Nestlé).
63. TBS buffer: 1.21 g Tris–HCl (10 mM) pH 7.4, 8.77 g NaCl (150 mM) in 1 L.

64. Anti-c-*Myc* antibody produced in rabbit (Sigma-Aldrich), ImmuniPure® Antibody, host: Goat Anti-, Antigen: Rabbit lgG (H + L), label: horseradish peroxidase (Thermo Scientific).
65. SuperSignal West Pico Chemiluminescence Kit (Thermo Scientific).
66. Imaging system (e.g., LAS-4000, Fujifilm).
67. Yeast nitrogen base with ammonium sulfate (YNB, Difco).
68. BMGY: 10 g Yeast extract, 20 g peptone, 13.4 g yeast nitrogen base, 10 mL of glycerol and 100 mM phosphate buffer pH 6.0 in 1 L.
69. BMMY: BMGY where glycerol was replaced with 5 mL of 100% methanol.
70. MMY: Is BMMY, where phosphate buffer was omitted.
71. BMM: Is BMMY without yeast extract and peptone.
72. MM: Is BMM where phosphate buffer was omitted.
73. Centrifuge bottles, 250 mL.
74. Stirred cell ultrafiltration unit with 10 kDa membrane (e.g., Amicon).
75. Nickel-chelation affinity chromatography media (e.g., His Bind Resin, Novagen).
76. Centricon centrifugal ultrafiltration devices, 10 kDa NMWCO, 2 mL (Millipore).
77. NuPAGE electrophoresis system consisting of Bis-Tris gels, LDS sample loading buffer, reducing agent, antioxidants, MOPS buffer (Invitrogen).
78. SimplyBlue™ Safestain (Invitrogen).
79. Lowry reagent A: 2.0 g NaOH and 10.0 g Na_2CO_3 in 500 mL of water.
80. Lowry reagent B: 1.0 g $CuSO_4 \cdot 5H_2O$ and 2.0 g sodium citrate in 100 mL of water.
81. F-C reagent (Folin Ciocalteu phenol reagent, Sigma-Aldrich).
82. BSA (bovine serum albumin): Pre-diluted protein assay standards (Pierce).
83. Various *p*NP-α-glycosides or *p*NP-β-glycosides substrates (Sigma-Aldrich).
84. Bicinchonic acid (BCA) Reagent A: 10.856 g Na_2CO_3, 4.84 g $NaHCO_3$ and 0.3884 g BCA sodium salt in 200 mL of water.
85. BCA Reagent B: 0.2496 g $CuSO_4 \cdot 5H_2O$ and 0.2524 g L-serine in 200 mL of water.

3. Methods

The standard molecular cloning methods (11) are used as required throughout this chapter in combination with the *Pichia* protocols (12) and protocols in the Invitrogen manuals (13, 14).

3.1. Preparation of Genomic and cDNA from A. nidulans

3.1.1. Preparation of Genomic DNA from A. nidulans

1. Grow *A. nidulans* in 10 mL YPD medium at 37°C with shaking at 150 rpm overnight.
2. Centrifuge 5 mL of the culture media at 2,000 × *g* for 5 min and remove supernatant.
3. Resuspend cells in a microfuge tube with 0.5 mL sorbitol buffer and add 20 µL of Zymolyase 100T (2.5 mg/mL in sorbitol buffer). Incubate for 1 h at 37°C.
4. Centrifuge at 16,800 × *g* for 1 min and remove supernatant.
5. Resuspend cells in 0.5 mL yeast resuspension buffer, and add 50 µL of 10% SDS.
6. Invert the closed tube several times rapidly and incubate at 65°C for 30 min.
7. Add 0.2 mL of 5 M potassium acetate and store the tube on ice for 1 h.
8. Centrifuge at 16,800 × *g* for 5 min at 4°C to pellet the cell debris and transfer supernatant to a fresh microfuge tube using a wide-bore pipet (e.g., cut the tip of a plastic pipet tip).
9. Add an equal volume of isopropanol, mix well, and keep at room temperature (RT) for 5 min to precipitate DNA. Do not allow the reaction to go more than 5 min!
10. Centrifuge at 16,800 × *g* for 10 s to recover the precipitated DNA. Remove the supernatant by aspiration and air-dry the pellet for 10 min.
11. Dissolve pellet in 0.3 mL TE buffer pH 8.0 containing 20 µg/mL RNase, and incubate at 37°C for 30 min.
12. Add 30 µL of 3 M sodium acetate pH 7.0, mix the solution, and add 0.2 mL isopropanol. Mix again and centrifuge at 16,800 × *g* for 20 s.
13. Remove supernatant by aspiration and air-dry pellet for 10 min. Dissolve in 150 µL TE buffer pH 7.4.

3.1.2. Preparation of cDNA from A. nidulans mRNA

1. Inoculate *A. nidulans* mycelia from a culture plate into 1 L of complete medium pH 4.5 in 2.8 L Fernbach flasks.
2. Incubate at 37°C for 3 days with shaking at 200 rpm.
3. Recover mycelium by filtering with sterile Miracloth.
4. Transfer mycelium to minimal medium pH 4.5 containing Hutner's trace elements (1 mL/L) and 0.5% (w/v) pectin,

0.5% (w/v) larch wood xylan or 2% (w/v) gum arabic as the carbon source (see Note 1).

5. Incubate at 37°C for 24 h with shaking at 200 rpm.
6. Pellet cells in aliquots by centrifugation in RNase-free centrifuge tubes at 12,000 × *g* for 5 min at 4°C (see Note 2). Do not wash the pellet.
7. Freeze the pellet in centrifuge tube using liquid nitrogen and grind pellet with mortar and pestle, which was cooled in liquid nitrogen before use.
8. Add TRIzol® reagent (ten times the volume of the tissue mass) and incubate for 5 min at RT.
9. Add 0.2 mL of chloroform for each 1 mL of TRIzol reagent used, mix capped tubes vigorously by hand for 15 s and incubate for 3 min at RT.
10. Centrifuge at 12,000 × *g* for 10 min at 4°C and transfer aqueous (upper) phase to a fresh tube.
11. Add 0.5 mL of isopropanol for each 1 mL of TRIzol reagent used, mix, and incubate for 10 min at RT followed by centrifugation at 12,000 × *g* for 10 min at 4°C.
12. Remove supernatant and wash RNA pellet with 1 mL of 75% ethanol for each 1 mL TRIzol reagent used, centrifuge at 7,000 × *g* for 10 min at 4°C.
13. Discard supernatant, briefly dry pellet for 10 min and dissolve in RNase-free water.
14. Remove DNA using the Turbo DNA-free™ Kit by mixing 87 μL of RNA solution, 10 μL of 10× Turbo DNase buffer, 3 μL of Turbo DNase, and incubate at 37°C for 60 min.
15. Add 20 μL of DNase Inactivation Reagent and incubate at RT for 5 min, with intermittent mixing (two to three times) during this period.
16. Centrifuge at 10,000 × *g* for 1.5 min and transfer supernatant containing RNA into new tube, then immediately proceed to the next step (RT-PCR) or store at −80°C.
17. Determine RNA concentration by measuring A260/280 ratio of the solution using a spectrophotometer. The ratio should usually be around 2.0.
18. To perform first-strand cDNA synthesis, set up first-strand cDNA synthesis reaction mixture in a 0.2 mL nuclease-free microfuge tube. Mix 1–5 μg of RNA with 1 μL of Oligo(dT) 12–18 primer (500 μg/mL) and 1 μL of dNTP mix (10 mM each of dATP, dGTP, dCTP, and dTTP), and add DEPC-treated water to 12 μL. Mix well by shaking. For convenience, all the following heating steps (steps 19–23) can be performed in the thermocycler.

19. Incubate at 65°C for 5 min and place immediately on ice.
20. Add the following to each tube on ice: 4 µL of 5× First-Strand Buffer, 2 µL of 0.1 M DTT, 1 µL of RNaseOUT™ (40 U/µL). Mix the contents of the tube gently by shaking and incubate at 42°C for 2 min.
21. Add 1 µL (200 U) of SuperScript™ II Reverse Transcriptase, mix gently by pipetting up and down, and incubate at 42°C for 50 min.
22. Inactivate the reaction by heating at 70°C for 15 min.
23. To remove RNA complementary to the cDNA, add 1 µL (2 U) of *E. coli* RNase H and incubate at 37°C for 20 min.
24. Use PCR purification Kit (Qiagen) according to the manufacturer's instruction to purify the genomic DNA or cDNA products.

3.2. Primer Design

Based on the sequence homology (available from the NCBI taxonomy browser) to known fungal cell wall hydrolase genes (7), we identified a large set of sequences from the *A. nidulans* genome database (8). Those which are significantly best hits to known cell wall hydrolases were selected and used to design primers for cloning these enzymes into *P. pastoris* (5, 8). We chose pPICZα C (see Note 3) (13) to clone all the cell wall hydrolases. You can similarly identify your gene homolog of interest from *A. nidulans.*

For primer design, we use the primer design software Oligo6, which allows the analysis of existing restriction sites within the target sequence as well as possible intra- or intermolecular primer–primer interactions and other criteria (see Note 4). Since most genomic DNA (from Subheading 3.1.1) and cDNA (from Subheading 3.1.2) sequences lacked *Pml*I and *Xba*I sites, these restriction enzyme sites can be used to clone most genes of interest into the multiple cloning site (MCS) of pPICZα C.

The complete coding sequence of each target gene is cloned, including any signal sequence. For this the 5′ (forward or upper) primer sequence is annealed at the ATG start codon. Additional nucleotides matching the template sequence of the genes are appended at the 3′ end of the primer to reach the desired target melting temperature range of 56–60°C. In order to be in line with the reading frame, the restriction site for *Pml*I (CACGTG) requires two nucleotides be added before the ATG codon. The nucleotides A and T are used since their contribution to the melting temperature was lowest. For enzyme recognition, five additional nucleotides (GAAAG) adjacent to the restriction site are attached. As a result, the common design for a primer using *Pml*I restriction site is GAAAG**CACGTG**AT**ATG**X_n, where X_n is 12–21 nucleotides from the target template's 5′ end.

A similar strategy is followed for the 3′ (reverse or lower) primer containing the *Xba*I site (TCTAGA). The stop codon in the target sequence is deleted, including one additional nucleotide from the preceding codon triplet, in order to be in line with the reading frame and to be contiguous with the c-*Myc* and 6× histidine tag coding sequence. The primer sequence therefore ends 1 nucleotide before the stop codon of the target sequence. Again, the primer sequence matching the target sequence consists of 15–24 nucleotides so that the resulting melting temperature will be in the range of 56–60°C, matching the upper primer. The *Xba*I primer has the general structure TACAG**TCTAGA**Y_n, where Y_n is 15–24 nucleotides from the target template's 3′ end, excluding the four last nucleotides.

3.3. Polymerase Chain Reaction

Genomic DNA or cDNAs are amplified by polymerase chain reaction (PCR) using gene specific primers generating *Pml*I and *Xba*I restriction sites at 5′ and 3′ ends, respectively. PCR is performed using the designed primers. Store all PCR solutions at –20°C and keep on ice when thawed. The PCR reaction mixture contains 18.2 µL Ready-to-use mixture, 0.8 µL forward primer (10 µM), 0.8 µL reverse primer (10 µM), 0.1 µL genomic DNA or cDNA, and 0.12 µL Taq polymerase. To obtain maximum efficiency, it is important to work with an optimal PCR program. A typical Touchdown PCR program used is detailed below (see Note 5):

1. 96°C for 2 min.
2. 94°C for 20 s.
3. 65°C for 15 s (–1°C/cycle).
4. 72°C for 1 min (extend 1 min for each additional kb product).
5. 9× to step 2 (i.e., repeat step 2–5, nine times).
6. 94°C for 20 s.
7. 55°C for 20 s.
8. 72°C for 1 min (extend 1 min for each additional kb product).
9. 30× to step 6 (i.e., repeat step 6–9, 30 times).
10. 72°C for 30 min.
11. 4°C ∞.

Check and confirm the size of the PCR product by running a 1% agarose gel electrophoresis (see Note 6). Once the presence of a selected sequence has been confirmed in a genomic or cDNA preparation, set up 12 additional PCR reactions targeting this sequence, then combine after completion of the cycles.

3.4. Cloning into E. coli

1. Use PCR Purification Kit to purify the PCR products. PCR results can be checked on a 1% agarose gel.

2. Set up restriction digest reaction mixture (20 μL reaction) by mixing 10–16 μL of purified PCR product or pPICZα C vector with 2 μL of buffer Y + and 1 μL each of *Xba*I and *Pml*I. Add water to make up to 20 μL.
3. Mix well by pipetting gently up and down several times (important!); briefly centrifuge to collect the liquid at the bottom and incubate overnight at 37°C.
4. For dephosphorylation of pPICZα C plasmid, add 2.5 μL of 10× reaction buffer and 2 μL shrimp alkaline phosphatase (2 U) only to the digest containing the pPICZα C vector (20 μL), incubate at 37°C for 1 h and heat inactivate at 65°C for 20 min. This helps in reducing self-ligation due to incomplete restriction reaction.
5. Use PCR Purification Kit to purify all reactions, check aliquots on 1% agarose gel and estimate the concentration by comparing to the DNA molecular weight ladder.
6. To set up ligation reaction, mix 2 μL of 10× ligation buffer, 2 μL of 50% PEG 4000, 3 μL of T4 DNA ligase with 13 μL of water containing purified PCR product (from step 3 of this section) and pPICZα C vector (from step 4 of this section). The molar ratio of PCR product (50–400 ng) to vector should be around 1 : 3. Incubate at RT for 1 h and heat inactivate at 65°C for 10 min.
7. For transformation, pipet 5 μL of the ligation mixture into a vial containing 100 μL of TOP10 chemically competent *E. coli* cells, and place on ice for 30 min. Incubate at 42°C (heat shock) for exactly 30 s and immediately place the cells back on ice. Add 200 μL of SOC medium and incubate the vial at 37°C for 3 h with moderate shaking.
8. Spread 10, 50 and 200 μL of this media on to different low salt LB plates (pre-heated to 37°C) containing Zeocin (25 μg/mL) and incubate at 37°C overnight or until colonies are visible.
9. For each transformation, transfer 24–48 colonies to a new LB plate (daughter plate) containing Zeocin and perform colony PCR (see Note 7) using 5′AOX1 and lower PCR primer, respectively.
10. Estimate the size of the PCR product on a 1% agarose gel (Fig. 1a).

3.5. Plasmid Extraction and Sequencing

1. Pick candidates (inoculate aseptically using a sterile toothpick or 10 μL micropipet tip) showing expected size of the insert (and thus having the correct orientation) from the daughter plate and grow in 5 mL of low salt LB media containing Zeocin (25 μg/mL) at 37°C overnight with vigorous shaking.
2. Pellet the cells by centrifugation (16,800 × *g* for 10 min) and extract plasmid DNA by alkaline lysis. Resuspend pellet in

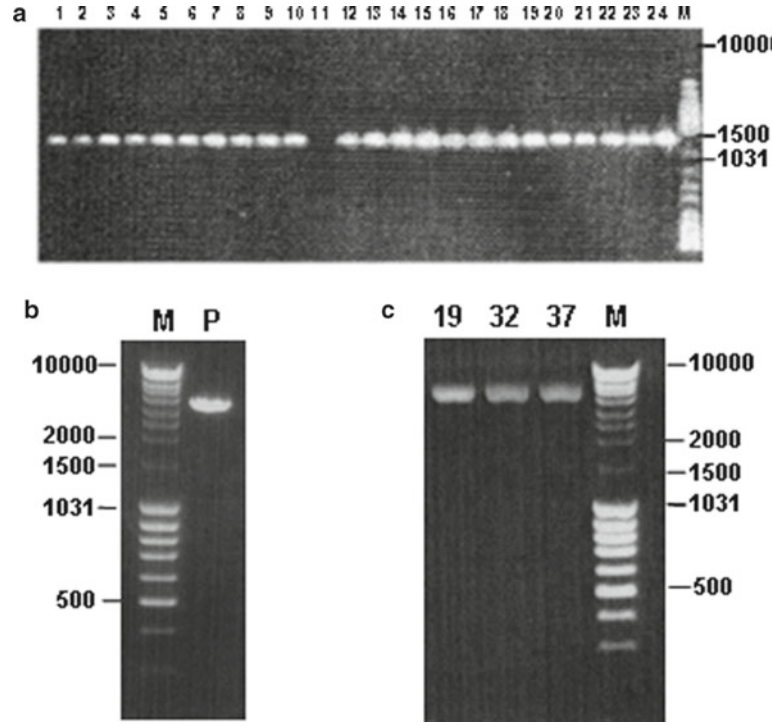

Fig. 1. Example of a colony PCR and confirmation for a typical construct. (**a**) Verification of correct transformants by colony PCR. One out of twenty-four colonies is negative. (**b**) Plasmid pPICZα C (P) digested with the restriction enzymes; *Pml*I and *Xba*I. (**c**) Based on the screening of 24–48 colonies for each construct, the best three colonies selected were grown overnight in low salt LB media containing Zeocin (25 μg/mL). Mini-prep DNA extractions (alkaline extraction/phenol/chloroform) were made and pellets dissolved in 25 μL of RNase water (20 μg/mL). DNA extracted (1 μL) from three different colonies (lanes 19, 32, 37) was loaded. Molecular weight ladders (M) are used to determine the correct size of the recombinant pPICZα C plasmid containing inserts. The increase in the size of the construct is observed.

100 μL 25 mM Tris–HCl pH 8.0 containing 10 mM EDTA and 150 mM glucose. Add 200 μL of freshly prepared lysis buffer (0.2 N NaOH, 1% SDS) and gently mix by inverting the tube several times (do not vortex!) and place on ice for 5 min.

3. Add 150 μL of ice-cold potassium acetate (pH 4.8), gently mix by inverting the tube several times, and let the tube stand on ice for 5 min. Centrifuge at 16,800 × *g* for 10 min and collect the supernatant.
4. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and mix by vortexing; then centrifuge (16,800 × *g* for 10 min) to achieve phase separation. Collect aqueous upper phase and add an equal volume of chloroform. Vortex and centrifuge (16,800 × *g* for 10 min) and collect the upper phase.
5. Add 0.1 volumes of 3 M sodium acetate pH 5.2 and 2 volumes of 100% ethanol and incubate at –20°C for 30 min. Centrifuge

at 16,800 × *g* for 10 min, discard supernatant, and wash the pellet with 200 µL of 70% ethanol by inverting the tube several times.

6. Pellet the cells by centrifugation (16,800 × *g* for 10 min) and air-dry the pellet for 10 min at RT and resuspend pellet in 50 µL of TE buffer containing 20 µg/mL RNAse. Check and verify the size and concentration of DNA on a 1% agarose gel using 2 µL solution (Fig. 1b–c). Store the DNA solution at –20°C if not used immediately.
7. Set up 20 µL of sequencing reaction in PCR tube using the Big Dye® Terminator Kit. Mix 8 µL Terminator Ready Reaction Mix, 2 µL primer (1.6 µM), 200–500 ng plasmid and water up to 20 µL.
8. Perform the PCR reaction. A typical PCR reaction temperature profile is 25 cycles of 96°C for 30 s, 50°C for 15 s, 60°C for 4 min, followed by cooling to 4°C until ready to purify.
9. Use Centri-Sep™ spin columns according to manufacturer's instruction to purify sequencing products and dry sample using a Speed-Vac. Samples are then submitted for sequencing using the dye-terminator sequencing method (Big Dye® Terminator Kit, see Note 8).

3.6. Isolation of Target Plasmid and Linearization

1. Grow *E. coli* clone showing an error-free insert in 50 mL of low salt LB containing 25 µg/mL zeocin at 37°C overnight with vigorous shaking. Extract plasmid according to the above extraction protocol (see steps 2–7 in Subheading 3.5). Increase volumes used to resuspend and lysis by a factor of 3.
2. Mix 20 µL of isolated plasmid (template), 5 µL of 10× buffer B+, 22 µL of water and 3 µL of *Pme*I to linearize plasmid by incubating overnight at 37°C.
3. Use PCR Purification Kit to purify and elute with water, check aliquots on 1% agarose gel, and estimate the size by comparing to the DNA mass ladder.

3.7. Preparation of Competent Cells and Transformation by Electroporation

1. Grow wild type *P. pastoris* X-33 (a Mut$^+$ His$^+$ prototropic strain) in 10 mL YPD medium in a 50 mL flask at 30°C with shaking at 280 rpm overnight.
2. Transfer 0.5 mL of this culture into 500 mL YPD medium in a 2.5 L flask and grow at 30°C and 280 rpm overnight until OD_{600} 1.3–1.5.
3. Centrifuge at 1,500 × *g* for 5 min at 4°C to pellet the cells, and then resuspend cells in 500 mL ice-cold water.
4. Centrifuge at 1,500 × *g* for 5 min at 4°C to pellet the cells, and then resuspend cells in 250 mL ice-cold water.
5. Centrifuge at 1,500 × *g* for 5 min at 4°C to pellet the cells, and then resuspend cells in 20 mL ice-cold 1 M sorbitol.

6. Centrifuge at 1,500 × *g* for 5 min at 4°C to pellet the cells, and then resuspend cells in 1 mL ice-cold 1 M sorbitol resulting in around 2 mL final volume. Keep competent cells on ice and use same day for electroporation to ensure high efficiency transformation. Using frozen competent cells, which are stored at −80°C, is not recommended.
7. Mix 80 μL of competent cells with 5–10 μg linearized plasmid (from step 3 in Subheading 3.6), transfer into an ice-cold electroporation cuvette (0.2 cm gap), then place the cuvette on ice for 5 min.
8. Pulse the cells with 1.5 kV charging voltage (use a capacitance of 25 μF and a resistance of 200 Ω, or use protocol according to manufacturer's instructions for *S. cerevisiae*) and immediately add 1 mL of ice-cold 1 M sorbitol.
9. Transfer content of cuvette into a sterile 15 mL tube and incubate at 30°C without shaking for 2 h.
10. Spread 10, 50, and 200 μL each of this solution on YPDS plates containing 100 μg/mL zeocin and incubate plates at 30°C until colonies are visible (usually 3–5 days).
11. Isolate 16 colonies on YPDS plates with 100 μg/mL zeocin (streak for single colony).

3.8. Screening for Protein Expression by Immunodetection (Dot-Blot)

1. Inoculate each zeocin-resistant clone (from step 11 in Subheading 3.7) aseptically using a sterile toothpick or 10 μL micropipet tip to 5 mL of BMGY (growth medium) in 50 mL sterile conical tubes with lids, and grow overnight at 30°C in a shaking incubator at 280 rpm.
2. Pellet cells (1,500 × *g*, 5 min) and resuspend in 5 mL of induction media (MM, MMY, BMM, or BMMY) in 50 mL conical tubes and grow at 28°C at 280 rpm overnight.
3. Centrifuge cells at 3,000 × *g* for 5 min and take 1 mL of supernatant and store on ice.
4. Spot 2 μL of each supernatant on nitrocellulose membrane and let air-dry. Block membrane with 5% fat-free milk in TBS buffer containing 0.1% Tween-20 at RT for 2 h.
5. Add first antibody (anti-c-*Myc*, 20 μL in 10 mL of 5% fat-free milk in TBS buffer containing 0.1% Tween-20) and incubate for 3 h at RT or at 4°C overnight.
6. Wash membrane 7 times (5 min each) with TBS buffer containing 0.1% Tween-20.
7. Add secondary antibody (anti-rabbit IgG peroxidase conjugated, 5 μL in 15 mL of 5% fat-free milk in TBS buffer containing 0.1% Tween-20) and incubate for 3 h at RT or at 4°C overnight.

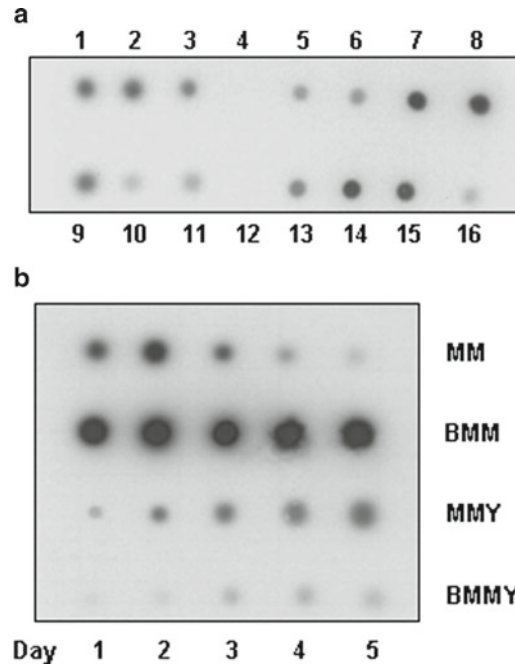

Fig. 2. Reprsentative immuno dot-blot detection of *Pichia*-expressed protein using the c-*Myc* epitope. (**a**) Protein expression levels for 16 clones from the same transformation are assessed after 24 h of induction to select the best expressing clone (i.e., clones 7, 8, 14, or 15). (**b**) Protein expression level of a selected clone grown with different induction media (MM, BMM, MMY, BMMY) over 5 days induction time (see Note 9). Differences in protein expression levels are clearly observed by intensities for different media.

8. Wash membrane 7 times (5 min each) with TBS buffer containing 0.1% Tween-20.
9. Transfer membrane into a ziplock bag and add 1–2 mL of SuperSignal West Pico Chemiluminescence mixture.
10. Wait for 5 min and visualize chemiluminescence using imaging system, LAS-4000, Fujifilm (Fig. 2) (see Note 9).

3.9. Protein Expression in Induction Media (Small-Scale Trial)

The transformants showing the highest protein expression are selected for small-scale expression and purification of recombinant proteins.

1. Inoculate a single colony aseptically using a sterile 10 µL micropipet tip to 1 mL of YPD medium in 5 mL sterile tubes, and grow at 30°C at 280 rpm overnight.
2. Transfer aseptically this 1 mL of YPD culture medium directly into 200 mL BMGY (growth) medium in 500 mL baffled flasks, and grow at 30°C at 280 rpm overnight.
3. Pellet the cells by centrifugation at 5,000×*g* for 5 min at 4°C in 250 mL sterile centrifuge bottles, and resuspend cell pellet in 200 mL induction medium (BMM, MM, MMY, or BMMY based on dot-blot analysis) in 500 mL flasks to induce expression.

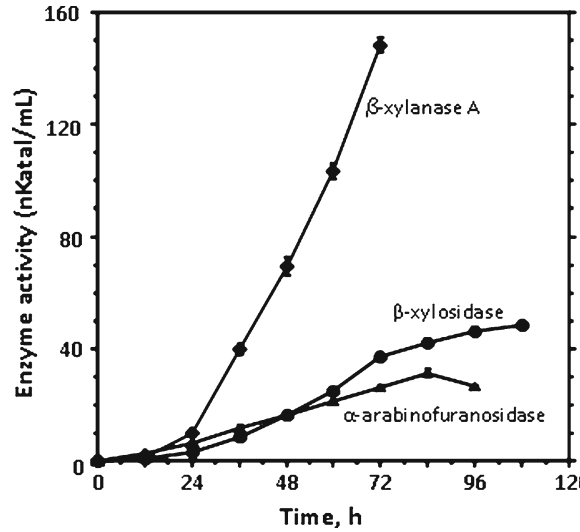

Fig. 3. Enzyme activity profiles for three *Aspergillus nidulans* xylanolytic hemicellulases expressed in *Pichia* cultures during induction in BMMY media. Representative enzymes are: β-xylanase A (AN3613.2), β-xylosidase (AN2359.2), and α-arabinofuranosidase (AN1571.2).

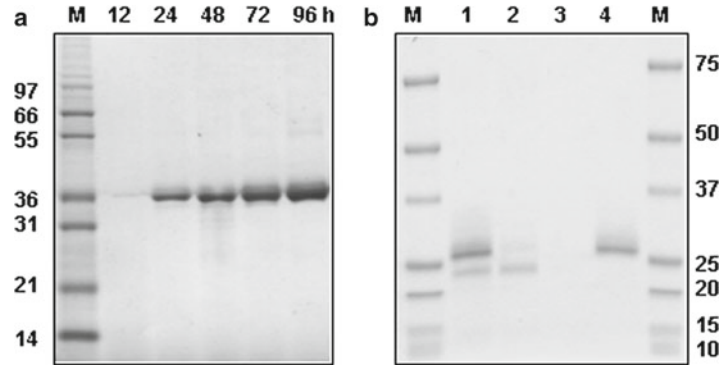

Fig. 4. Representative SDS-PAGE gels for expressed (secreted) and purified xylanolytic enzymes. (**a**) SDS-PAGE of culture supernatant (20 μL) expressing recombinant β-xylanase (AN1818.2) during induction (*lanes* indicate 12, 24, 48, 72, and 96 h of induction) in BMMY media. (**b**) SDS-PAGE profile of a typical enzyme purification using metal (Ni) chelation chromatography: 1, Supernatant concentrate. 2, Unbound column flow-through. 3, Buffer wash. 4, Eluted pure protein. *M* molecular weight markers (molecular masses are indicated in kDa).

4. Add 1 mL of 100% methanol (0.5% final concentration) to each flask and shake well and grow at 28°C at 280 rpm for 3–6 day.
5. Add 1 mL of 100% methanol to each flask every 24 h and pipet 1 mL aliquot every 12 h (0, 12, 24, 36 … and so on), preferably prior to methanol addition.
6. Analyze growth (OD_{600}) and protein expression levels [by the respective enzyme activities (Fig. 3) and SDS-PAGE (Fig. 4a)]

to determine the optimal time to harvest. Store the aliquot at 4°C until ready to analyze.

7. Once the culture reaches the maximum induction level (usually 3–6 days of induction), pellet the cells (centrifuge at 5,000 × *g* for 5 min at 4°C) in 250 mL centrifuge bottles.
8. Collect the supernatant and add sodium azide to a final concentration of 0.02%. Store at 4°C until use. Discard the pellet/cells according to safety protocol (see Note 10).

3.10. Purification of Recombinant Enzymes

3.10.1. Concentration of Protein by Ultrafiltration or Ammonium Sulfate Precipitation

1. Concentrate the supernatant (induction medium containing expressed enzymes, stored at 4°C) at least tenfold using an ultrafiltration device, such as an Amicon stirred cell, equipped with a 10-kDa NMWCO membrane, according to the manufacturer's instruction (see Note 11).
2. Alternatively precipitate protein with $(NH_4)_2SO_4$. For this add $(NH_4)_2SO_4$ to supernatant to 80% saturation (at 4°C) with constant stirring. After overnight incubation at 4°C, centrifuge the suspension (10,000 × *g* for 30 min at 4°C), collect the protein pellet, and dissolve in 10–20 mL of sodium acetate buffer (0.02 M, pH 5.0) (see Note 12).

3.10.2. Purification of Recombinant Enzymes Using Metal (Nickel) Chelation Column

1. Wash the His-Bind Resin (Novagen) (see Note 13) column (0.7 × 5 cm) extensively with water.
2. Charge with nickel by flushing the column with 10 mL of 400 mM $NiSO_4$.
3. Wash and equilibrate the column with 10 mL of the starting buffer (Buffer A: 20 mM Tris–HCl pH 7.9 and 500 mM NaCl) containing 10 mM imidazole (see Note 14).
4. Adjust the pH of the concentrated supernatant to pH 7.9 with NaOH (see Note 14) and load on to the Ni^{2+}-charged His-Bind column at 4°C.
5. Wash the column with 10 mL of Buffer B (Buffer A containing 50 mM imidazole).
6. Elute the bound His-tagged protein with 10 mL of Buffer C (Buffer A containing 250 mM imidazole).
7. Repeatedly wash the eluted fraction with 0.02 M acetate buffer pH 5.0 in order to remove imidazole and concentrate using Amicon stirred-cell ultrafiltration units. Further concentration to small volumes can be achieved by 10-kDa NMWCO Centricon centrifugal ultrafiltration devices.
8. Sample aliquot is assayed for protein content and enzyme activities and stored in the freezer. Purity of the protein is assessed by SDS-PAGE (Fig. 4b).

3.11. Analyses of Growth and Protein Expression (SDS-PAGE, Protein Content, and Enzyme Activities)

3.11.1. Determine Culture Growth by Absorbance (Optical Density) at 600 nm

Growth is monitored by measuring absorbance of the culture media at 600 nm.

1. Pipet 100 µL culture medium to clean glass test tube and add 900 µL fresh induction medium. Mix by pipetting up and down.
2. Blank spectrophotometer with induction medium then read absorbance of suspended cell solution at 600 nm.

3.11.2. SDS-PAGE Analysis (15)

Protein expression is monitored by SDS-PAGE. We use the NuPAGE Bis-Tris gel electrophoresis system by Invitrogen, but the traditional Laemli buffer system is suitable.

1. To 13 µL of media supernatant of different time points, or suitably diluted purified samples, add 5 µL of 4× LDS sample loading buffer and 2 µL of 10× sample reducing agent and incubate at 70°C for 10 min.
2. Load 10–20 µL of these samples to 10 or 12% Bis-Tris gels (1 mm thickness), and perform electrophoresis at 200 V (constant) for 55 min using MOPS buffer system, according to the manufacturer's instruction (15).
3. Stain the gels by SimplyBlue Safestain™ to visualize the proteins (see Note 15).
4. Transfer the gel to ziplock bag for scanning (Fig. 4) and store at 4°C.

3.11.3. Protein Estimation by Lowry's Method (16)

1. Pipet 10 µL of pre-mixed standards (10–100 µg BSA) or samples into different glass test tubes and make up the final volume to 200 µL using deionized water.
2. Add 1 mL of Lowry reagent (mix Lowry reagent A and B; 49:1, v/v) into these tubes, shake well, and incubate for 10 min at RT.
3. Add 0.1 mL of F-C reagent (diluted 1:1 with deionized water) into the tubes and shake to mix. Allow the test tubes to stand at RT for 45 min.
4. Blank spectrophotometer with water-reagent blank and read absorbance at 650 nm.

3.11.4. Micro-assays for Glycosidase Activities (α-/β-Arabinofuranosidases, α-/β-Xylosidases)

1. Pipet 14 µL of *p*NP substrate (*p*NP-α arabinofuranoside, *p*NP-β-arabinofuranosidase, *p*NP-α-xylosidase, or *p*NP-β-xylosidase) prepared as 3 mg/mL solution into a microcentrifuge tube.
2. Add 5 µL of 0.2 M sodium acetate buffer pH 5.0 and 1 µL of enzyme solution (culture supernatant).
3. Incubate the tubes at 37°C for exactly 15 min.

4. Stop the reaction immediately by adding 980 µL of 0.2 M Na_2CO_3.
5. Blank spectrophotometer with water-reagent blank and read absorbance at 420 nm.

3.11.5. Micro-assays for Glycanase Activities (β-Xylanases)

1. Pipet 10 µL of different 10 mg/mL substrate solutions (larch wood xylan).
2. Add 5 µL buffer 0.2 M sodium acetate buffer pH 5.0, then 5 µL of suitably diluted enzyme solution (culture supernatant) into these tubes.
3. Incubate the tubes at 37°C for exactly 20 min.
4. Stop the reaction immediately by adding 980 µL BCA working reagent (reagent A, reagent B, and water, 2:2:1 (v/v/v)).
5. Incubate the tubes at 80°C for 30 min. Cool to RT for about 20 min.
6. Blank spectrophotometer with water-reagent blank and read absorbance at 560 nm.

4. Notes

1. Different carbon sources are used to induce transcription of specific mRNAs in *A. nidulans*. We tried several other carbohydrate sources (e.g., carboxymethyl cellulose, oat spelt xylan, tamarind xyloglucan), but we observed better transcription of mRNA in the above-mentioned media.
2. Use sterile, disposable, certified RNase-free centrifuge tubes and pipet tips, automatic pipettors dedicated for RNA preparation, and wear disposable gloves in order to avoid contamination with RNase from labware and human skin. Avoid polycarbonate tubes for RNA precipitation as they are not resistant to isopropanol. Add solvents at RT to minimize co-precipitation of salts. The centrifugation steps should be performed at 4°C to prevent warming of the sample.
3. The expression vector (pPICZα C) is a shuttle vector of 3.6 kb, which propagates both in *E. coli* and *P. pastoris*. Important features of the plasmids include (1) the promoter of AOX1 gene, as the homologous sequence with the yeast genome for recombination, (2) the transcriptional termination sequence of AOX1 gene for efficient processing and polyadenylation of mRNAs, (3) the secretion signal sequence from the *S. cerevisiae* α-factor prepro-peptide sequence, which leads to secretion of the recombinant protein into external medium, (4) the MCS with 10 unique restriction sites for the insertion of the foreign gene

between the two AOX regions, (5) the C-terminal (c-) *Myc* epitope and polyhistidine tag downstream of MCS for detection and purification of the recombinant protein, (6) the pUC *ori* for maintenance and replication in bacterial hosts, (7) the unique restriction sites *Bgl*II, 5′ to the AOX1 promoter sequence, and *Bam*HI, 3′ to AOX1TT sequence, facilitate the generation of in vitro multimers. Additionally, restriction enzymes that cut one time in 5′ AOX1 region (*Pme*I, or *Sac*I or *BstI*) can be used to linearize the plasmid, and finally (8) the single, small, dominant selectable marker gene, (*Sh ble* gene) that confers resistance to Zeocin, which functions as selectable marker in both bacteria and yeast. The expression is driven and controlled by the EM7 promoter (for *E. coli*), TEF1 promoter (for yeast), and CYC1TT transcription termination regions in the plasmid. The gene of interest is inserted in the yeast genome by a single cross-over type insertion via shared sequences (5′ AOX1 region). Pichia Expression Kit (Invitrogen) contains 20 μg (40 μL) of pPICZα C plasmid. For long-term storage, transform TOP10 *E. coli* strain with pPICZα C (according to Subheading 3.4).

4. In general, the melting temperature (T_m) of the primers should be between 55 and 65°C. The simple formula for estimating T_m is T_m (°C) = 2 × (A + T) + 4 × (G + C). For best results, both primers used in PCR should have a melting temperature within 5°C of each other. The primers should not form hairpin loops, have no tendency to dimerize, and ideally lack a secondary binding site within the template. Further considerations involve the introduction of a GC clamp at the 3′, where the sequence ends in either G or C, but should not have more than 3 G or C in the last five nucleotides. Poly G, poly C, poly A, poly T as well as polypyrimidine (T & C) and polypurine (A & G) should also be avoided since this could cause either non-specific annealing or lower the efficiency of amplification. Ideally, the primer should consist of a random mix of nucleotides with a GC content of 45–55%.
5. When T_m is not known with certainty, a Touchdown PCR is used to optimize yields of amplified DNA, because choosing annealing temperatures too low or too high can lead to primer-dimer formation, non-specific products, or reduced yield due to poor primer annealing. Touchdown PCR uses a cycling program with varying annealing temperatures. The annealing temperature in the initial cycle is chosen to be 5–10°C above the T_m of the primers. This favors only accumulation of duplexes where primer–template complementation is highest. In subsequent cycles, the annealing temperature is decreased in steps of 1°C/cycle until the temperature is around 2–5°C below the

T_m of the primers. In this way touchdown PCR enhances the specificity of the initial primer–template duplex formation and hence the specificity of the final PCR product.

6. To cast 1% agarose gels, dissolve 1 g agarose (electrophoresis grade) in 100 mL of 1× TAE running buffer, and boil the solution until all agarose has dissolved completely. Though the solution appears clear, boil for another 30 s and let it cool for approximately 60–70°C. Add 5 μL of ethidium bromide (10 mg/mL) and mix thoroughly. Pour to the gel tray according to the manufacturer's guide. DNA electrophoresis is run at a voltage of 140 V for 45–60 min or 150 V for 30–45 min.
7. Colony PCR is used to check the presence of cloned insert in the transformants. By using a primer binding inside the vector sequence (5′AOX1), only inserts having the right orientation will show the expected size on the agarose gel. A convenient way of performing colony PCR is to use sterile 10 μL pipet tips to touch the parent colony and transfer the cells onto new numbered YNB plates containing 25 μg/mL zeocin, followed by pipetting into a 20 μL PCR set-up mixture. For 24 reactions, we usually prepare a 500 μL PCR mixture containing the respective primer pair, Ready-to-use mixture, and Taq polymerase in the proportions mentioned above. The PCR mixture is aliquoted into wells of a 96-well plate placed on ice.
8. We have used a dye-terminator sequencing method (Big Dye Terminator Kit, ABI PRISM™ Big Dye™ dideoxy nucleotide termination sequencing, Applied Biosystems) as it was the protocol recommended by the core facility. Good sequencing for around 600–700 base pairs was obtained downstream of each primer. Sequence information of the first ~30 bp is inaccurate, so place primers 40–50 bp upstream from sequence of interest. For inserts around 1.0–1.2 kb only 5′AOX1 and 3′AOX1 primers are used as sequencing primers. Larger inserts require additional primers designed to anneal around 400 bp apart from these primers to cover entire sequence.
9. Immunodetection by dot-blot is used to monitor the protein expression level of each clone. Based on the protein expression levels of 16 different clones of the same transformation (same construct) determined after 24 h of induction, the best expressing clone is selected. It is grown in BMGY media overnight and the recovered cell pellet is suspended in different induction media (MM, BMM, MMY, and BMMY) for a 1–5-day induction period. The best induction medium is then determined for each clone (Fig. 2b). Note that BMMY may not always be the best medium for highest protein expression. Select the best media for expressing the protein of interest based on dot-blot results.

10. Inactivate/sterilize the cells by autoclaving or by chemical bleaching, as recommended by your institution's biosafety protocol.
11. Amicon stirred cell ultrafiltration units with 10- or 3-kDa NMWCO membranes (such as YM) also work fine for concentration. Do not use regenerated cellulose membrane for concentrating xylanases and cellulases, as they may degrade the membrane.
12. Aliquots (pellets and final supernatant) are set aside for determining the protein content and assay for enzyme activities. For long-term storage, enzyme solutions are made to 25% or 50% glycerol, and stored in 50 mL Falcon conical tubes at –20°C.
13. We have tried different metal chelation columns Ni charged His-Bind column (Novagen), Ni-NTA column (Qiagen), Ni charged IDA column (POROS-M®, Boehringer Mannheim), and His-link protein purification resin (Promega). All worked well for most of the His-tagged proteins. A typical purification protocol is presented (Fig. 4b). Resin can be regenerated following the manufacturer's instructions.
14. The pH of the buffer should be between pH 7.5 and pH 7.9 to keep the histidine (pKa is 6.0) in ionized form to bind chelated nickel ions. In buffered media (BMMY or BMM) a precipitate of phosphate salts may be formed after pH adjustment. Remove by centrifugation or filtration. All buffers tested for affinity binding supported good binding. These include Tris buffer (20 mM Tris–HCl pH 7.9, 500 mM NaCl containing 10 mM imidazole), phosphate buffer (50 mM potassium phosphate pH 7.9, 500 mM NaCl containing 10 mM imidazole), and HEPES buffer (100 mM HEPES–HCl, pH 7.5, 500 mM NaCl containing 10 mM imidazole). Binding can be performed by either flow column chromatography or by batch column chromatography, where the resin is pre-mixed with protein solution prior to column elution. If the protein is not binding to the column, reduce the imidazole concentration in wash buffer. Elution can be performed either by single-step elution with 100 mM, 250 mM, and 500 mM imidazole or with continuous gradient elution (from 10 to 750 mM imidazole) in starting buffer.
15. The microwave technique involves washing the gel with 100 mL water in a loosely closed container using a microwave (for ca. 1 min) and shaking at RT for 1 min, three times each. After the third washing, gels are stained with 30 mL of SimplyBlue Safestain by microwaving for 1 min and shaking for 10 min at RT, after which they are de-stained with 100 mL water for several hours. While microwaving gel in solution, switch power off and on as needed to avoid boiling and

damaging the gels. Gels can be stored at 4°C for short-term storage until they can be processed for peptide mass fingerprinting analysis by MALDI-TOF MS with trypsin digestion, as described in Chapter 2 of this book.

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