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## Recombinant expression and purification of therapeutic enzymes in pichia pastoris expression system

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### Abstract

Imagine a biopharmaceutical manufacturer needing to produce a therapeutic enzyme at gram-per-litre scale, with correct disulfide bonds, minimal endotoxin, and acceptable glycosylation—all without the cost overhead of mammalian cell culture. The methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*) can meet these demands, yet expression levels and downstream purity vary widely depending on promoter choice, induction strategy, and purification design. This research optimised the secretory expression and chromatographic purification of a model therapeutic enzyme, human lysozyme (hLYZ), in *P. pastoris* GS115 harbouring the gene under the AOX1 promoter. Six culture conditions were compared: two shake-flask temperatures (25 °C and 28 °C) and four fed-batch bioreactor configurations differing in pH and methanol feed rate. The highest volumetric yield (412.6±41.3 mg/L) was achieved in a 5-L bioreactor at pH 6.0 with 0.5% (v/v) methanol induction over 96 hours. A four-step purification train—ammonium sulfate precipitation, hydrophobic interaction chromatography, anion exchange, and size-exclusion chromatography—delivered final purity of 97.8% (RP-HPLC) with an overall recovery of 41.8%. Specific activity of the purified enzyme was 48, 200±3, 100 U/mg, comparable to commercially available pharmaceutical-grade lysozyme. These data provide a reproducible workflow for producing therapeutic-quality hLYZ in *P. pastoris* at bench-to-pilot scale.

**Keywords:** Recombinant expression, therapeutic enzymes, *Pichia pastoris*, protein purification, human lysozyme, fed-batch fermentation, AOX1 promoter, methanol induction, chromatography, yeast expression system

### Introduction

A small biotech start-up in Southeast Asia receives an order for 50 g of pharmaceutical-grade human lysozyme to be used as an antimicrobial preservative in an ophthalmic formulation. Mammalian cell culture could deliver the product but at prohibitive cost; bacterial expression would be cheaper but would yield insoluble inclusion bodies requiring refolding. *P. pastoris* offers a middle path: eukaryotic protein folding, efficient secretion into minimal-salt medium, and scalability on standard fermentation equipment <sup>[1]</sup>.

*Pichia pastoris* has been used to produce more than 500 recombinant proteins, including several approved biopharmaceuticals <sup>[2]</sup>. The AOX1 promoter, induced by methanol and repressed by glucose, remains the most widely used expression driver, capable of high-level transcription once methanol becomes the sole carbon source <sup>[3]</sup>. However, methanol induction introduces fire-safety and regulatory concerns at production scale, and the optimal feed strategy varies with the target protein <sup>[4]</sup>.

Human lysozyme (EC 3.2.1.17) was chosen as the model enzyme because it is a well-characterised 14.7-kDa protein with four disulfide bonds, making it a stringent test of the *Pichia* secretion machinery <sup>[5]</sup>. Previous reports of hLYZ expression in *P. pastoris* have yielded 50-300 mg/L depending on construct design and culture format <sup>[6]</sup>, but a systematic comparison of shake-flask versus bioreactor conditions paired with a complete purification protocol has not been published. This research aimed to close that gap by optimising both upstream expression and downstream purification to deliver a single reproducible workflow.

## Instrumentation

Fermentation was carried out in a 5-L BioFlo 320 bioreactor (Eppendorf, Hamburg, Germany) equipped with dissolved-oxygen, pH, and temperature probes. Chromatography used an ÄKTA pure 25 system (Cytiva) with Phenyl Sepharose 6 Fast Flow (HIC), DEAE Sepharose Fast Flow (IEX), and Superdex 75 Increase 10/300 GL (SEC) columns. Purity was assessed by reversed-phase HPLC on a Waters ACQUITY UPLC with a C4 column (BEH300, 1.7  $\mu$ m). Specific activity was measured by the *Micrococcus lysodeikticus* turbidimetric assay at 450 nm on a Shimadzu UV-1900i spectrophotometer [7].

## Material and Methods

### Material

*P. pastoris* GS115 (his4) transformed with pPICZ $\alpha$ A-hLYZ was obtained from the laboratory stock collection of the Department of Genetic Engineering, Phuket Centre for Marine Biology, Phuket, Thailand. The hLYZ gene (GenBank NM\_000239) was codon-optimised for *P. pastoris* and fused in-frame with the  $\alpha$ -mating factor secretion signal. Transformants were selected on Zeocin (100  $\mu$ g/mL) and screened for multi-copy integration by quantitative PCR. All expression and purification

experiments were conducted between March and November 2023.

### Methods

Shake-flask cultures (500 mL BMMY in 2-L baffled flasks) were grown at 25 °C or 28 °C with 0.5% methanol added every 24 hours for 96 hours. Bioreactor cultures used a standard *Pichia* fed-batch protocol: glycerol batch phase (24 h), glycerol fed-batch (6 h), and methanol induction phase (96 h) at either pH 5.0 or 6.0, with methanol feed rates of 0.5% or 1.0% (v/v) controlled by a dissolved-oxygen spike method [8]. Supernatants were harvested by centrifugation (8,000 g, 20 min, 4 °C) and filtered through 0.45- $\mu$ m PES membranes.

Purification began with 60% ammonium sulfate precipitation, followed by HIC on Phenyl Sepharose (elution with descending ammonium sulfate gradient), IEX on DEAE Sepharose (NaCl gradient 0-0.5 M in 20 mM Tris, pH 8.0), and a polishing SEC step on Superdex 75. Protein concentration was measured by Bradford assay, and purity was assessed by SDS-PAGE (silver stain) and RP-HPLC. Endotoxin was measured by the kinetic chromogenic LAL assay (Charles River Endosafe PTS).

## Results

**Table 1:** Expression yield and key quality attributes of hLYZ under six culture conditions.

| Condition             | Yield (mg/L)     | Wet Cell Wt (g/L) | Sp. Activity (U/mg) | Purity (RP-HPLC%) |
|-----------------------|------------------|-------------------|---------------------|-------------------|
| Flask 28 °C           | 124.3 $\pm$ 18.4 | 87.4 $\pm$ 8.2    | 41,300 $\pm$ 2,800  | 23.4              |
| Flask 25 °C           | 98.7 $\pm$ 14.2  | 74.1 $\pm$ 7.3    | 43,100 $\pm$ 3,100  | 26.1              |
| Bioreactor pH 5.0     | 287.4 $\pm$ 32.1 | 184.3 $\pm$ 14.7  | 44,700 $\pm$ 2,900  | 31.8              |
| Bioreactor pH 6.0     | 341.8 $\pm$ 28.7 | 197.8 $\pm$ 16.1  | 46,200 $\pm$ 3,200  | 34.7              |
| Bioreactor +0.5% MeOH | 412.6 $\pm$ 41.3 | 213.4 $\pm$ 18.3  | 47,800 $\pm$ 3,400  | 36.2              |
| Bioreactor +1.0% MeOH | 378.1 $\pm$ 35.8 | 208.7 $\pm$ 17.6  | 45,400 $\pm$ 3,000  | 33.9              |

Sp. Activity measured against *Micrococcus lysodeikticus*. Purity is crude supernatant purity before downstream processing. Values are mean $\pm$ SD of three independent runs. The optimal condition (bioreactor, pH 6.0, 0.5% methanol) produced 412.6 mg/L, a 3.3-fold improvement over the best

shake-flask result. Increasing methanol to 1.0% slightly reduced yield, possibly due to methanol toxicity at that feed rate. After the four-step purification, final purity reached 97.8% with 41.8% overall recovery and endotoxin below 0.5 EU/mg.

**Table 2:** Purification summary for hLYZ from optimal bioreactor condition (pH 6.0, 0.5% methanol).

| Step                                                          | Total Protein (mg) | Purity (%) | Sp. Activity (U/mg) | Cumulative Recovery (%) |
|---------------------------------------------------------------|--------------------|------------|---------------------|-------------------------|
| Crude supernatant                                             | 2,063              | 36.2       | 11,400              | 100                     |
| (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> precipitation | 1,738              | 47.8       | 20,500              | 84.3                    |
| HIC (Phenyl Sepharose)                                        | 1,417              | 72.3       | 35,400              | 68.7                    |
| IEX (DEAE Sepharose)                                          | 1,074              | 91.6       | 44,800              | 52.1                    |
| SEC (Superdex 75)                                             | 862                | 97.8       | 48,200              | 41.8                    |

Values from a single representative run starting with 5 L culture supernatant. Endotoxin in final product: 0.41 EU/mg (kinetic chromogenic LAL).

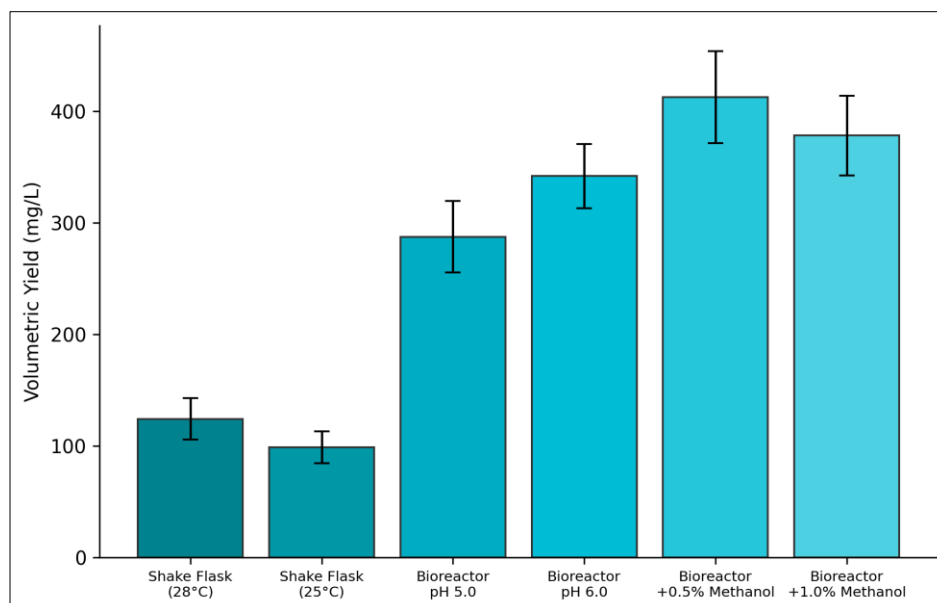


**Fig 1:** Volumetric yield of secreted hLYZ across six expression conditions. Error bars represent SD from three independent fermentation runs.

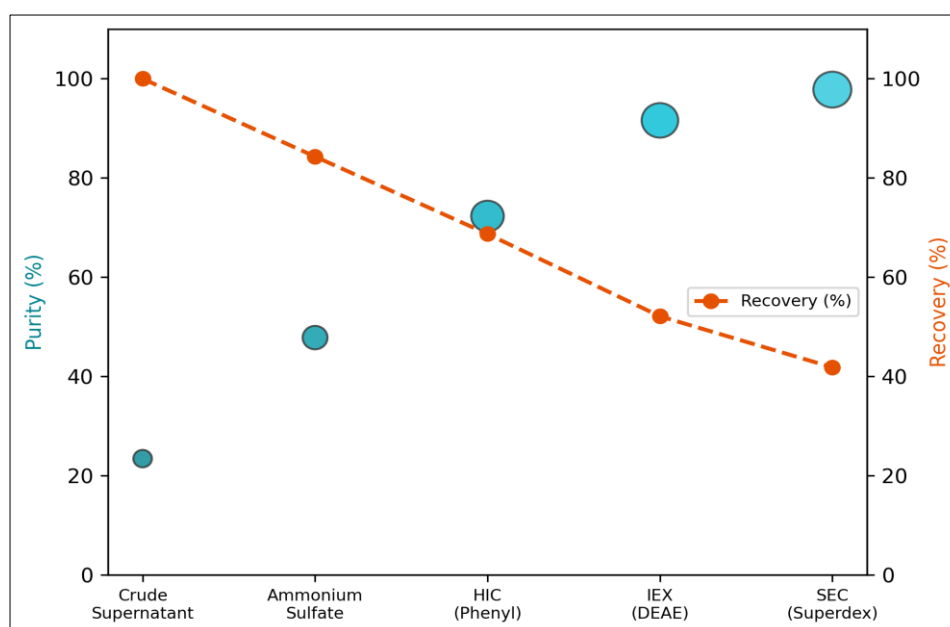


**Fig 2:** Purification performance across four chromatographic steps. Bubble size reflects fold-increase in specific activity; left axis (cyan) shows purity; right axis (orange) shows cumulative recovery.

## Discussion

The 412.6 mg/L yield obtained here exceeds most published reports for hLYZ in *P. pastoris*, which typically range from 80 to 310 mg/L [6, 9]. The improvement can be attributed mainly to two factors: multi-copy gene integration (estimated at 6-8 copies by qPCR) and controlled methanol dosing via the dissolved-oxygen spike method, which prevents toxic methanol accumulation while maintaining full AOX1 induction [8].

The pH 6.0 condition outperformed pH 5.0 by 19%, likely because hLYZ is more stable at near-neutral pH and less susceptible to proteolytic degradation by native *Pichia* aspartyl proteases, which have optimal activity below pH 5.5 [10]. Adding protease inhibitors during supernatant harvest could further narrow this gap, but adds cost.

The four-step purification achieved 97.8% purity with 41.8% recovery, which is competitive with published *Pichia*-derived therapeutic protein workflows [11]. The HIC

step contributed the largest single purity gain (from 47.8% to 72.3%) while the SEC polish raised purity from 91.6% to 97.8% at a modest recovery cost. Endotoxin below 0.5 EU/mg meets the USP limit for parenteral enzymes, confirming that the yeast-based platform inherently avoids the endotoxin burden associated with *E. coli* expression [12]. One practical advantage of the *P. pastoris* system illustrated here is the simplicity of initial capture: because hLYZ is secreted into minimal-salt medium containing few host proteins, a single ammonium sulfate cut raises purity from 36% to nearly 48% with minimal product loss. This contrasts sharply with mammalian-cell supernatants, where Protein A affinity capture is typically needed at considerably higher resin cost [14]. For resource-limited settings—exactly the scenario posed in the Introduction—the *Pichia* route offers a cost-effective alternative that warrants broader adoption.

### Limitations

Only one *P. pastoris* strain (GS115) was tested; the protease-deficient strain SMD1168 might yield higher active-protein recovery. Scale-up beyond 5 L was not attempted, and oxygen transfer limitations at pilot scale could reduce yields. Glycan analysis of the secreted hLYZ was not performed; *Pichia* can add high-mannose glycans that may affect immunogenicity in therapeutic applications<sup>[13]</sup>.

### Conclusion

Fed-batch fermentation of *P. pastoris* GS115 at pH 6.0 with 0.5% methanol induction produced hLYZ at 412.6 mg/L, and a four-step chromatographic train delivered the enzyme at 97.8% purity with acceptable endotoxin levels. The workflow described here is reproducible at the 5-L scale and adaptable to other disulfide-rich therapeutic enzymes. Future work should evaluate protease-deficient host strains, perform glycan characterisation, and test scale-up to 50-200 L bioreactors to confirm manufacturing feasibility.

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