Sunflower

Application Note

Microbial Production

Keywords

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Improving production of recombinant proteins through yeast strain engineering

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Abstract

Robust, highly productive strains are foundational to achieving cost and quality targets in biomanufacturing. **Sunflower's comprehensive strain engineering platform has been used to produce >80 proteins and improve yields up to 150-fold.** By systematically and simultaneously optimizing protein sequences, expression vectors, and the host strain genome, we increase expression of diverse proteins from numerous therapeutic classes with broad biophysical properties. Our modular expression vector design allows for rapid iteration and our catalog of engineered *Pichia* strain backgrounds addresses key expression challenges, further improving yields for various protein classes. Sequence, vector, and strain improvements translate directly to enhanced performance in our Daisy Petal™ Perfusion Bioreactor system, as shown by a 150-fold increase in space-time yield for a vaccine antigen. Our flexible strain development approach enables robust, scalable, and cost-effective bioproduction of high-quality recombinant proteins for diverse applications.

INTRODUCTION

Achieving meaningful yields of high-quality recombinant protein requires robust expression from a capable host organism. The methylotrophic yeast *Pichia pastoris* (*Komagataella phaffii*) is an increasingly popular protein expression organism used in the biomanufacturing of multiple pharmaceutical, food, and industrial products currently on the market. Advantages of bioproduction in *Pichia* include its ability to secrete complex proteins, strong inducible gene regulation, short microbial doubling time, low expression of secreted host-cell proteins, and lack of contamination with viruses or endotoxins. These traits, combined with *Pichia*'s ability to grow to very high cell densities on low-cost raw materials, enable high space-time yields of proteins from *Pichia*-based fermentations, a critical lever for cost-effective manufacturing processes. Combining *Pichia*'s natural advantages with continuous perfusion fermentation using Sunflower's equipment empowers low-cost space-efficient manufacturing for a wide variety of important biomolecules.

The quality and productivity achieved from a protein-expressing strain greatly impact the cost and simplicity of biomanufacturing processes. The first step to cost-effective continuous and reliable manufacturing is creating stable recombinant protein-expressing strains. For *Pichia*, stable genomic integrants are created using vectors containing a recombinant gene of interest. Optimal protein expression and quality often requires vector optimization and strain engineering. Both the integration vector and the host strain genome can be modulated to maximize expression of any given recombinant protein.

When expressing a new recombinant protein at Sunflower, we use the protein sequence and its biophysical characteristics to determine a baseline set of expression vectors, engineered strains, and cultivation conditions to test (Figure 1). In only four weeks, we create an initial set of yeast strains for evaluation of protein expression and quality. Based on these results, we identify key levers for improved protein expression, if needed, and iterate on strain design to achieve optimized product titer and quality.

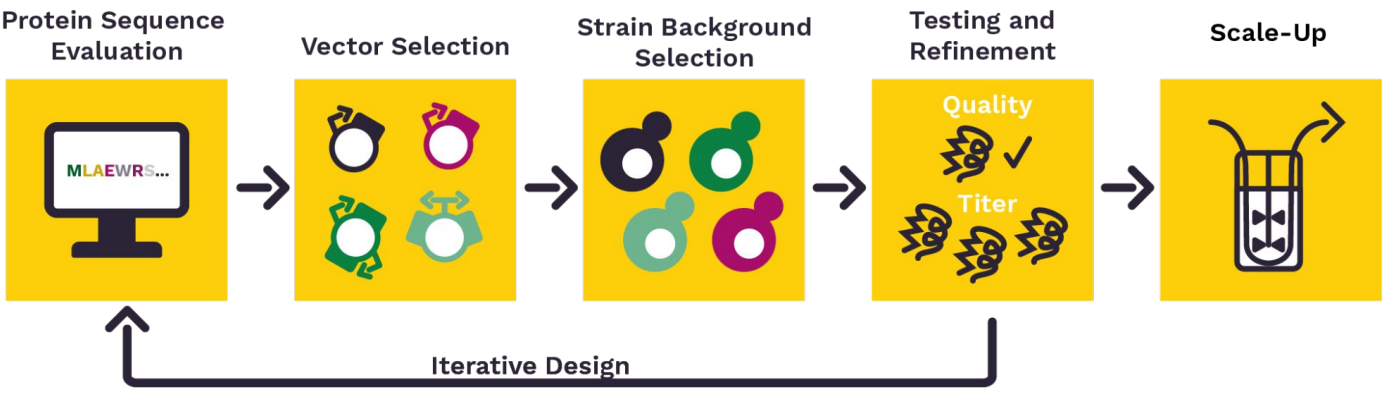

FIGURE 1. Sunflower's protein expression optimization process in Pichia.

To date, **Sunflower has developed a catalog of over 20 vectors and 30 engineered strain backgrounds designed to enhance expression and/or quality of a wide variety of proteins.** These, combined with our deep understanding of potential expression or quality flaws based on protein sequence analysis and a focus on protein design for manufacturability, has enabled us to successfully express over 80 proteins from multiple classes, including AI-designed proteins, cytokines, hormones, single-domain antibodies, fragment antigen-binding regions (Fabs), Fc-fusions, monoclonal antibodies (mAbs), enzymes, subunit vaccine antigens, and virus-like particles (VLPs). In the following sections, we highlight Sunflower's approach to each aspect of strain development, and present case studies demonstrating improved expression driven by our strain engineering capabilities.

MODULAR VECTORS

Sunflower's Modular Vector Approach

For any new target protein, we first evaluate the amino acid sequence for expressability based on biophysical characteristics. Key factors include molecular weight, pI, hydrophobicity (GRAVY score), potential for oligomerization, glycosylation sites, and potential cleavage sites. We also consider factors from our previous experience with other molecules in a similar product class (antibody, VLP, etc) from our library of over 80 proteins. Based on the biophysical data and our experience with similar proteins, we determine a set of initial protein sequences and vectors to test. Using our custom modular integration vector (Figure 2), we can test elements from our catalog of promoters, secretion signals, resistance markers, transcription terminators, and loci. Each vector element can meaningfully impact the transcription, translation, folding, and secreted expression level and quality of the recombinant protein. In addition to the vector components, we may test minimal protein sequence modifications for optimized expression and folding in *Pichia*. Our modular vector backbone enables straightforward assembly of the initial vector set, which is then transformed into *Pichia*. The vector is homologously recombined into the genome,[1] enabling long-term strain stability.[2]

Promoters enable robust inducible gene expression of a protein.

SS — **Secretion signals** can be tuned for reliable extracellular secretion of many proteins.

Transcription terminators can further tune gene expression levels.

abR — **Antibiotic resistance markers** enable selection of highly expressing strains.

Locus — **Locus of integration** enables stable integration into the Pichia genome.

Bicistronic expression vectors enable balanced expression of multi-domain proteins (such as mAbs or Fabs).

FIGURE 2. Map of Sunflower's modular vector elements and applications.

Vector Case Studies

Here, we present case studies using secretion signal modifications, vector background changes, and a bicistronic vector to improve protein expression. For secreted recombinant proteins, the secretion signal is vital to ensure efficient protein folding and export from the cell. The secretion signal is a short peptide leader that directs the recombinant protein from the ER to the Golgi and eventually out of the cell. By determining the optimal signal sequence from our catalog, we were able to increase expression of a SARS-CoV-2 subunit vaccine antigen (RBD) 4-fold (Figure 3A).

Selection of appropriate vector background elements can also be used to improve protein expression. Our set of modular vector backgrounds enables straightforward evaluation of the impact of multiple vector elements on titer for any given protein. Through changing the expression vector for a VLP, we improved expression over 4-fold (Figure 3B).

For multi-part proteins such as antibodies, the ratio of heavy chain and light chain is another key product quality attribute that can greatly impact intact protein titer. **Sunflower has developed bicistronic vectors that can be used to express two proteins from a single integrated genetic vector.** Using one vector for both protein elements ensures the copy number of each part can be kept consistent. The expression of each protein can be further tuned through careful selection of surrounding vector elements for each heterologous protein coding sequence. This enables more reliable expression of heavy and light chains, and

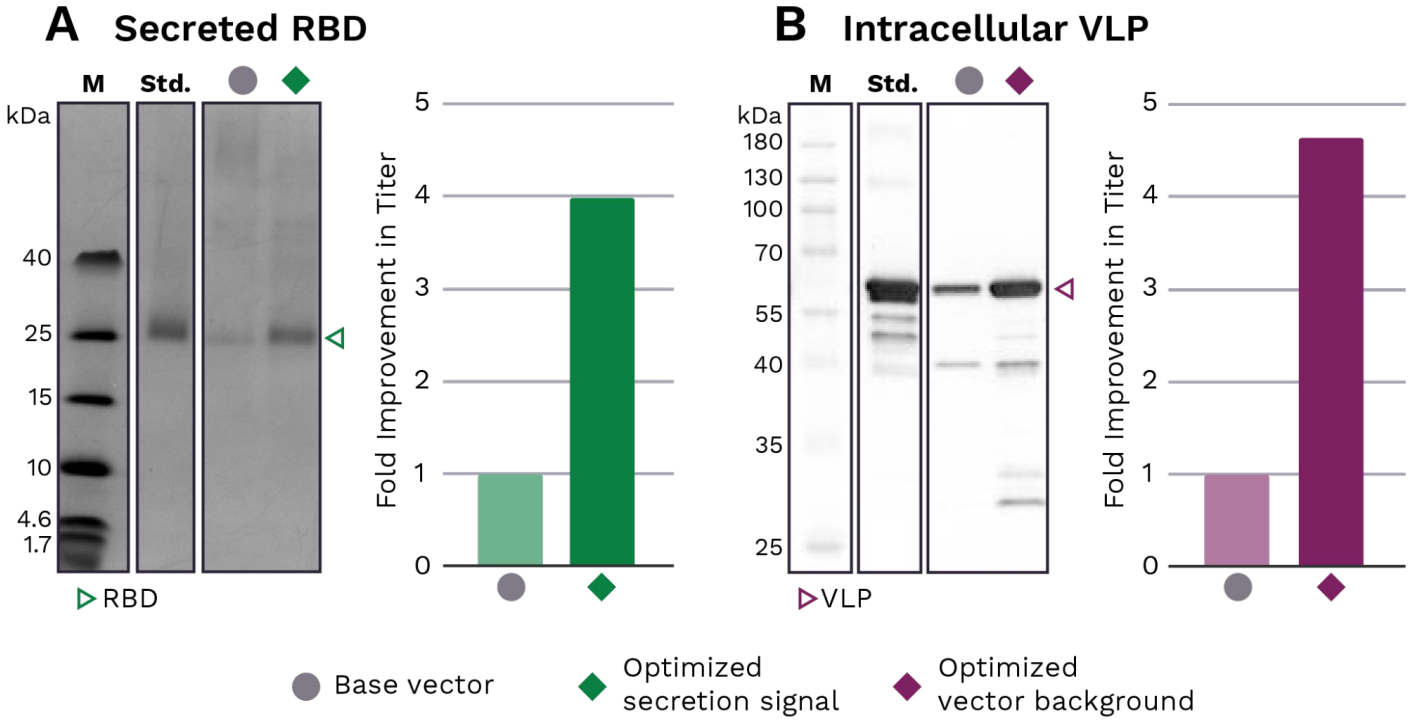

FIGURE 3. Vector element optimization improves expression of diverse proteins. (A) SDS-PAGE & titer analysis of unpurified SARS-CoV-2 RBD. (B) Western blot & titer analysis of unpurified VLP.

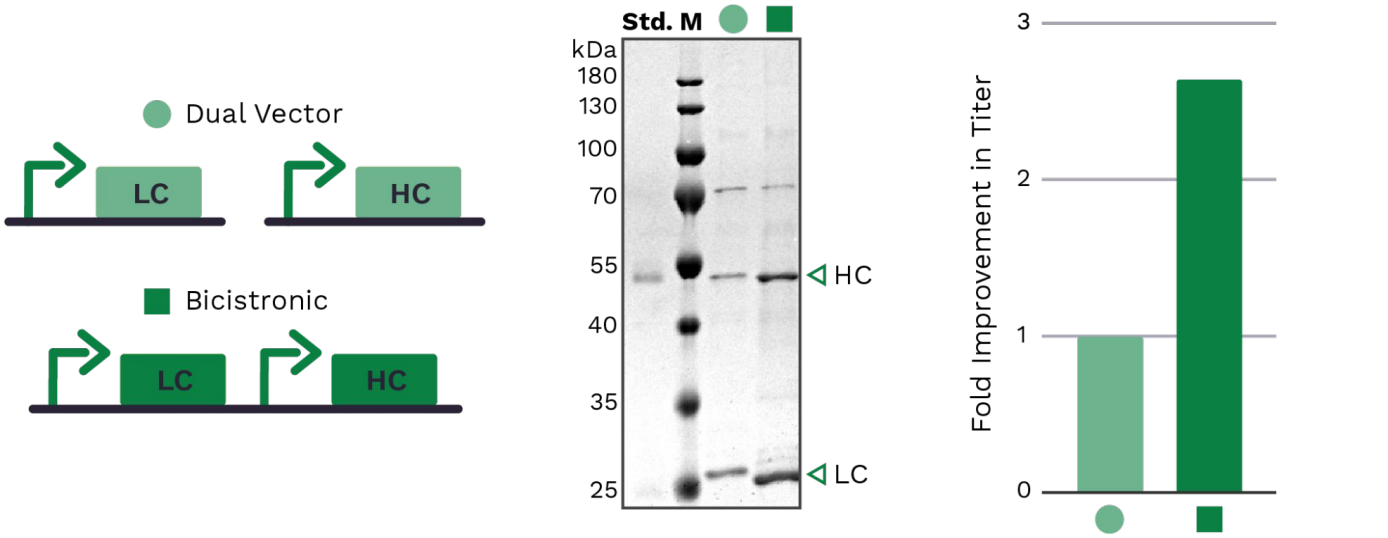

FIGURE 4. Use of a bicistronic integration vector improves titer of antibody heavy and light chains. SDS-PAGE and titer analysis of trastuzumab.

speeds up strain creation by removing the need for multiple rounds of transformation. By using our custom bicistronic vector, we improved the titer of trastuzumab by over 2-fold compared to strains expressing heavy and light chains integrated independently from separate vectors (Figure 4). Bicistronic vectors can also be applied to Fabs and other multi-domain proteins, and are a key part of Sunflower's recombinant protein expression toolbox.

Each of these examples demonstrates how combining the right genetic elements within our modular vector can improve protein expression. **By leveraging our catalog of over 20 base vectors with interchangeable elements, we can tune transcriptional, translational, folding, and secretory processes.** This optimization leads to robust protein production across a wide variety of protein classes. In tandem with developing an optimal vector, we can further improve expression through engineering the *Pichia* genome.

STRAIN BACKGROUNDS

Strain Engineering in *Pichia*

Different types of proteins require different expression conditions for optimal production. Many of these needs can be met through vector optimization, but others require fundamental changes to the host organism's capabilities. For example, complex, multi-domain proteins may require chaperones for proper folding, or cell wall changes to enable efficient secretion. Concurrent with vector optimization, we regularly test top vectors in engineered strain backgrounds to boost expression and relieve potential production bottlenecks. We use an IP-free technology to create engineered strains, either through gene knock-outs, knock-ins or gene upregulation. Through these edits, we create engineered strain backgrounds which can receive any recombinant protein of interest via transformation. **Sunflower has established a catalog of >30 engineered strain backgrounds**

specifically designed to enhance expression and/or quality for production of many different protein classes. Engineered backgrounds include cell wall and secretory pathway gene knock-outs to improve protein secretion, protease knock-outs to decrease undesirable proteolytic activity, and the addition of chaperones to improve recombinant protein expression and folding. Together with vector optimization, these engineered strain backgrounds enable robust expression of diverse target proteins.

Strain Case Studies

Our strain catalog combined with our *Pichia* host expertise enables us to rapidly select and test a variety of engineered strains to maximize quality and titer for a given target protein. Through strain engineering, we have significantly improved expression of a variety of protein types and complexities, including single-domain antibodies (sdAbs), albumins (human serum albumin; HSA), and monoclonal antibodies (mAbs) (Figure 5). Several of our engineered strain backgrounds, including the cell wall knock-out shown here, enable broad improvement across a variety of proteins, such as sdAbs and HSA. For more complex proteins with specific translation and folding requirements, including mAbs, customized engineered strain backgrounds often lead to greater yield improvements. Expression of mAbs specifically is regularly improved in strains that contain several modifications, including specific knock-outs and

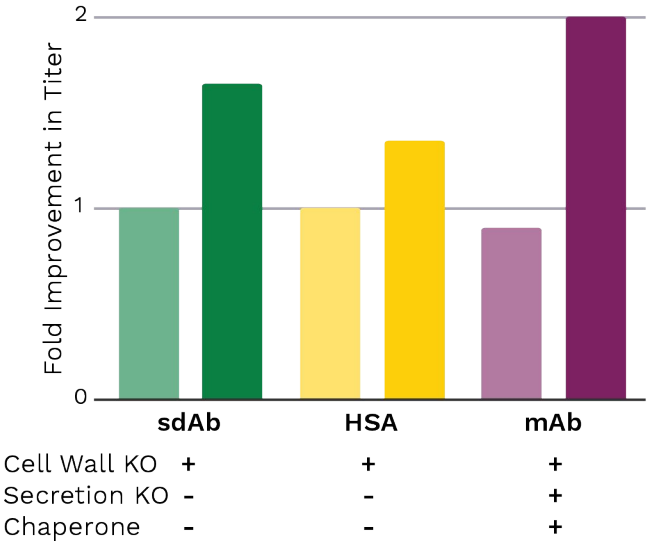

FIGURE 5. Use of appropriate engineered strain backgrounds increases protein production across classes, including for an sdAb, HSA, and a mAb.

knock-ins. Here, we see a 2-fold improvement in trastuzumab titer when expressed in a strain combining chaperone upregulation with cell wall and secretory pathway gene knock-outs. Identifying the best strain background for a given molecule regularly improves expression and quality, and is key to creating an optimized strain for recombinant protein production.

SCALE-UP TO PRODUCTION

Daisy Petal™ Perfusion Fermentation

Through combining sequence, vector, and strain optimization, a diverse range of proteins can be meaningfully expressed in *Pichia*. To date, **Sunflower has successfully expressed over 80 proteins in *Pichia*, with varied class, size, and complexity.** As expression strains are developed, they are tested at multiple

scales in batch fermentation from 1 mL to 200 mL. The data collected regarding protein titers, media component consumption, and growth rates at these scales are used to predict performance in our 1 L Daisy Petal™ Perfusion Bioreactor.

The Daisy Petal™ provides the next step in scaling recombinant protein production. By using continuous perfusion fermentation, we sustain higher cell densities for longer campaigns, leading to higher productivity than traditional batch or fed-batch bioreactors. Perfusion fermentations are evaluated by their space-time yield, which accounts for the total mass of protein produced per bioreactor volume per cultivation day. Further information on the Daisy Petal™ bioreactor system and space-time yield can be found in Sunflower's other application notes.[3,4]

Scale-Up Case Study

Here we show the impact of combined sequence, vector, and strain improvements on productivity at the bioreactor scale for a VLP. **Through four rounds of sequence, vector, and strain optimization, we improved space-time yield of this VLP in the Daisy Petal™ bioreactor by over 150-fold** compared to initial bioreactor expression—a massive increase in VLP produced per day. This result demonstrates the potential of iterative strain optimization to meaningfully improve product yield. Through precise sequence analysis, intentional selection of vectors, optimal base strains, and appropriate scale-up,

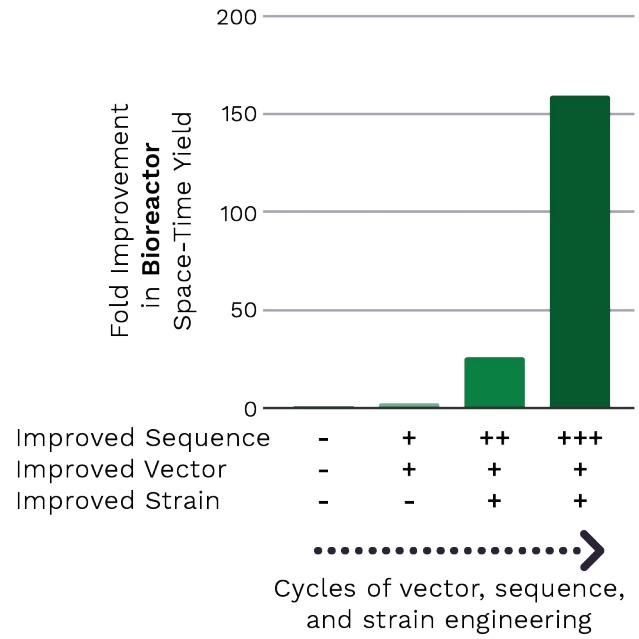

FIGURE 6. Through cycles of sequence, vector, strain and media optimization, Sunflower improved Daisy Petal™ bioreactor space-time yield of a VLP by over 150-fold.

Sunflower can demonstrate the full potential for recombinant protein expression in *Pichia*.

CONCLUSION

Highly productive strains are the cornerstone of efficient, low-cost bioproduction. Sunflower's strain engineering approach systematically improves protein expression through optimization of protein sequences, vectors, and strains. With our modular base vector using easily swappable parts, we can regulate genomic integration, translation, and secretion of single-part and multi-part proteins. Our straightforward expression system also enables short cloning cycles for rapid evaluation and iteration. By combining an

optimized vector with the right engineered strain background, we can further boost strain performance by improving *Pichia*'s native capabilities or relieving production bottlenecks. Ultimately, this toolset enables robust expression of diverse proteins with strong bioreactor-scale expression, driving meaningful improvements in productivity.

METHODS

Heterologous proteins were expressed in *Pichia pastoris* in Sunflower's proprietary defined media.[5] Plate and flask-based experiments were performed with a biomass accumulation phase followed by a production phase. Bioreactor runs were operated as previously described.[3] Cell supernatant was then collected for analyses including SDS-PAGE, Western blot, ELISA, and metabolite analysis. For intracellularly expressed proteins, cell pellets were collected and lysed before analysis. Base engineered strains were generated through an IP-free gene knockout method. Knock-outs and knock-ins were confirmed through sequencing of the relevant genomic regions.

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3. Crowell, L. et al., "Perfusion fermentation in the Daisy Petal™ bioreactor" (Sunflower Therapeutics 2024 Application Note).
4. Crowell, L. et al. "Space-Time Yield" (Sunflower Therapeutics 2024 Application Note).
5. Crowell, L. et al. "Chemically Defined Media for Yeast" (Sunflower Therapeutics 2024 Application Note).

Related Resources

Application Note: Perfusion fermentation in the Daisy Petal™ bioreactor

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