



Unlock Pichia<sup>®</sup> - *Pichia pastoris* Protein Expression Excellence

## Case Study

Boosted expression of different VHH antibody formats through application of VALIDOGEN's UNLOCK PICHIA toolbox

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## Introduction

VHHs, derived from camelid species, the so-called “third generation” of antibodies and also known as sdAbs or Nanobodies®, feature a number of intriguing characteristics that make them superior for many applications. Compared to mAbs and antibody fragments (Fab and scFv), VHHs exhibit equally high specificities and affinities in antigen binding and show further advantages due to their small size, high solubility, thermal stability, design flexibility & modularity, good tissue penetration in vivo, ease of genetic engineering and expression in microbial systems.

As a consequence, VHHs are currently widely used in research and are being developed for an increasing number of different applications including pharmaceutical use as APIs (first approval of a Nanobody® - Caplacizumab - in 2018) and in diagnostics as well as for diverse applications in other industries.

### UNLOCK PICHIA protein expression strategies

The case study aimed to show, among others, the benefits of applying a higher number of **PAOX1 promoter variants from VALIDOGEN’s UNLOCK PICHIA toolbox** in a ‘pool approach’ which typically leads to a significant productivity boost, at the same time enabling more rapid generation and identification of high-level production strains.

This approach leverages broad random diversity of genetic arrangements and expression profiles, with concomitant fine-tuning and optimization of gene dosage, applying promoter variants and auxiliary proteins (helper factors) simultaneously in pools of expression constructs.

The targeted application of VALIDOGEN's full toolbox by identification of optimum combinations with different elements such as **strain background, helper factors, secretion signals** or **production regimes** further boosts productivity.

The case studies applied a range of approaches, using different UNLOCK PICHIA toolbox elements and expression strategies:

### Case Study 1: Applying more promoter variants

The first case study focused on bi-modular VHH yield optimization through application of a higher number of UNLOCK PICHIA promoter variants, analyzing 90 transformants in an initial small screening to reach a product yield of 7g/L of culture supernatant in both methanol-free and methanol-induced production.

A subsequent large screening used a higher number of UNLOCK PICHIA promoter variants yielding 15 g/L under methanol-free conditions versus 18 g/L with methanol induction (analyzing 6800 transformants each).

The UNLOCK PICHIA promoter library includes methanol-inducible promoter variants for maximum output and methanol-free promoter variants for improved process safety and economy. The library allows more effective fine-tuning of gene expression and significant productivity boost through an increase in promoter variants used in pool transformation (small screening vs. large screening).

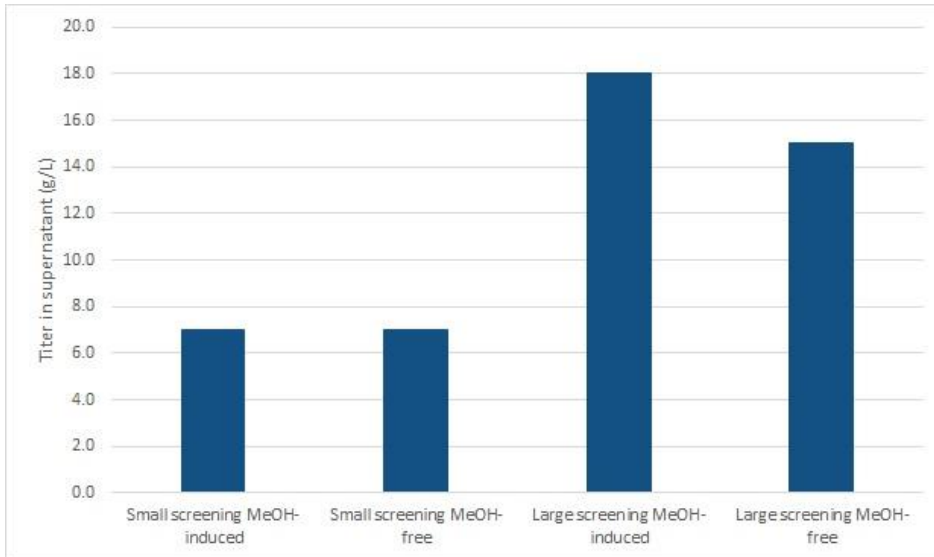


Figure 1: Case study 1 - Yield optimization through application of a higher number of UNLOCK PICHIA promoter variants

### Case Study 2: Helper factors with diverse functions

The second case study sought bi-modular VHH product yield improvement through timely adjusted expression of helper proteins acting at multiple sites within the production host.

Studying various helper factors (HFs) acting at different locations in the cell resulted in following specific improvements:

- Cytosolic chaperone - improvement factor: 2.0-fold
- Translocation engineering - improvement factor: 2.5-fold
- ER-resident helper - improvement factor: 2.7-fold
- All helper factors combined - improvement factor: 3.5-fold.

UNLOCK PICHIA includes a broad range of exclusive helper factors that enhance protein production and improve product quality by timely adjusted co-expression, further boosting product yields.

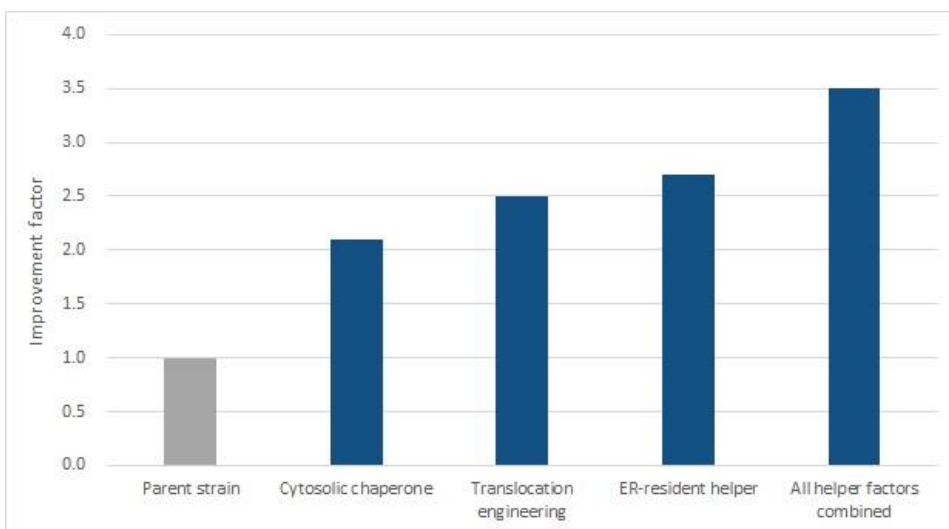


Figure 2: Case Study 2 - VHH product yield improvement through timely adjusted expression of helper proteins acting at multiple sites within the production host

### Case Study 3: MeOH-free process optimization

Case study number 3 reviewed single VHH yield optimization through increasing the number of promoter variants combined with the application of HFs followed by process optimization for methanol-free production.

After ARMs-free cloning (antibiotic-resistance marker-free), the feasibility study used 90 transformants to achieve 0.8 g/L in a small screening and helper factor screening (on top of best clone from feasibility study) to increase yield to 1.3 g/L.

Production strain development (screening of 6,200 clones), combining higher number of UNLOCK PICHIA promoter variants and expression of the most promising HF reached 2.7 g/L while lean process optimization across four runs in 5L-scale achieved a further yield increase to 3.9 g/L.

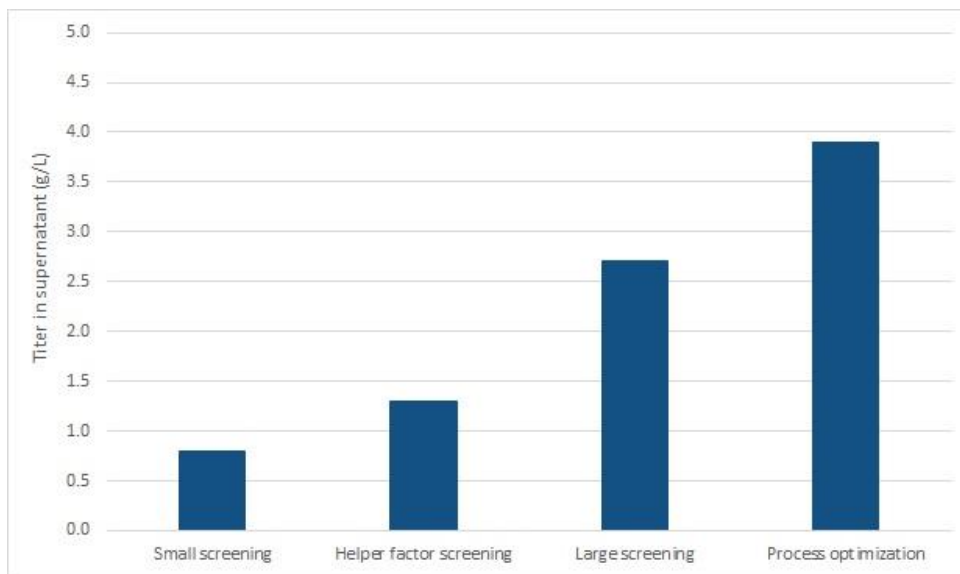


Figure 3: Case Study 3 - Yield optimization through application of the UNLOCK PICHIA toolbox and process optimization

### Case Study 4: Novel secretion signals

The fourth case study focused on bi-modular VHH yield improvement through application of a novel UNLOCK PICHIA secretion signal in methanol-induced cultivation.

While use of a standard secretion signal achieved 8 g/L, use of the novel UNLOCK PICHIA secretion signal under the same conditions resulted in more than double the yield at 16.5 g/L, in both cases in only a small screening with 90 transformants.

This demonstrated how novel UNLOCK PICHIA secretion signals can outperform standard leader sequences. The toolbox also includes hybrid versions of novel and standard secretion leader sequences.

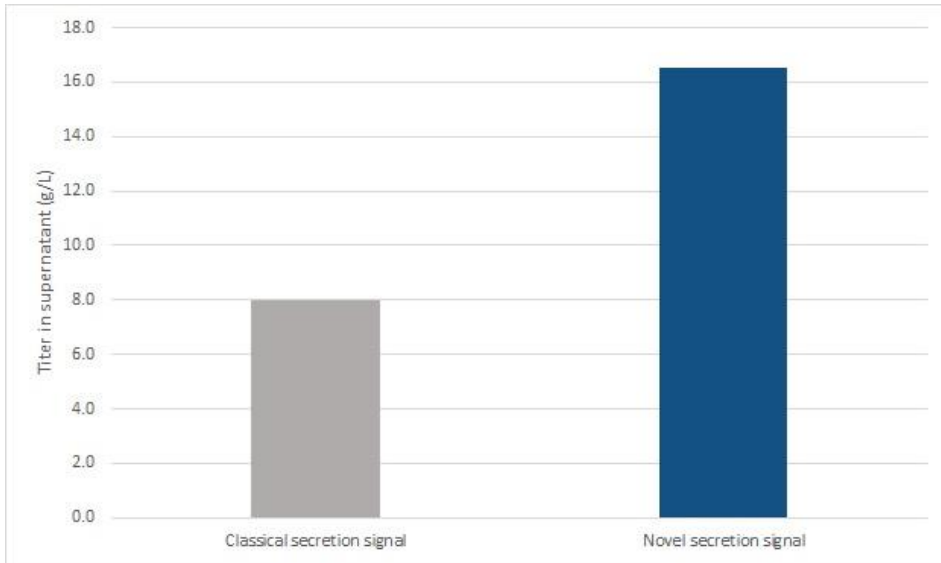


Figure 4: Case Study 4 - Yield optimization through application of a novel UNLOCK PICHIA secretion signal

### Case Study 5: Novel platform strains

The final case study looked at tri-modular VHH yield optimization through application of a novel engineered UNLOCK PICHIA platform strain in methanol-free and methanol-induced cultivation.

In small screening, using 44 transformants, use of the novel UNLOCK PICHIA platform strain yielded 3.0 g/L compared with 1.8 g/L for a "standard" strain in methanol-induced cultivation.

In methanol-free cultivation, using the UNLOCK PICHIA platform strain yielded an even larger proportional increase to 1.8 g/L versus a "standard" strain at 1.0 g/L.

UNLOCK PICHIA incorporates a variety of different platform strains including antibiotic resistance free host-vector systems.

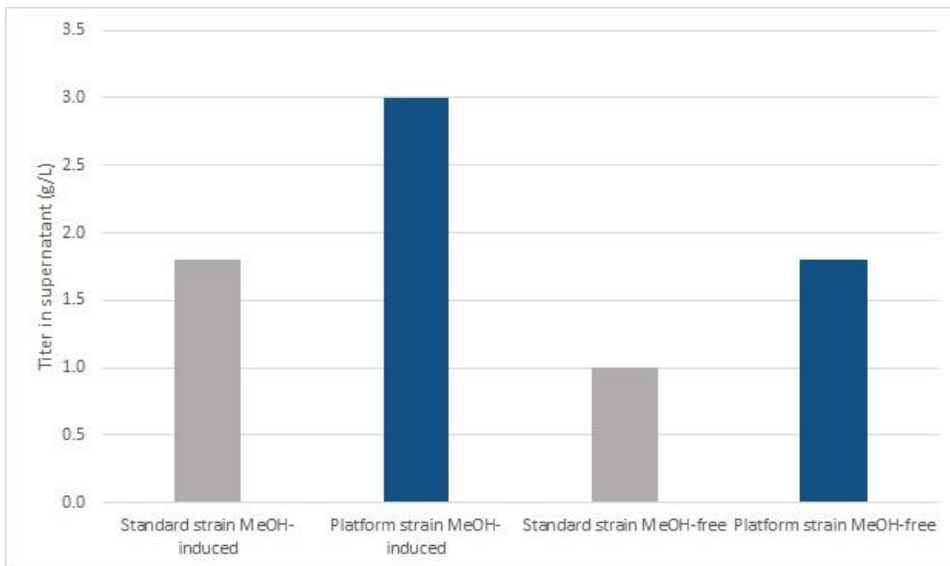


Figure 5: Case Study 5 - Yield optimization through application of a novel UNLOCK PICHIA platform strain

## The power of diversity

The case studies demonstrate how VALIDOGEN is able to apply the UNLOCK PICHIA toolbox elements to generate a large number of different genetic constellations in thousands of strains for fine-tuning of expression, proving the power of *Pichia pastoris* for successful production of fully active, high quality proteins.

In the VALIDOGEN laboratories, expression constructs comprising UNLOCK PICHIA promoter variants and the gene of interest or potentially also genes encoding for different auxiliary proteins are transformed simultaneously in pools into selected Pichia platform strains. Generating a broad diversity of genetic arrangements in the host cells results in diverse expression profiles with the potential to significantly boost protein production in *Pichia pastoris*.

Following specifically optimized protocols, thousands of clones can be screened in minimum time with the most promising clones being reliably identified at the microscale screening stage. Screening results are transferable into bioreactors, rendering obsolete those laborious intermediate steps that have been conventionally used for clone comparison.

## UNLOCK PICHIA advantages

The case studies confirm the advantages of UNLOCK PICHIA as a very broad range Pichia-based platform incorporating numerous expression tools and different strategies for the enhancement of production yields and product quality.

Advantages include:

- Boosted expression levels for many proteins (more than 20g/L) including monovalent- and multivalent VHH antibodies: up to 16.5 g/l
- Secreted target proteins providing a beneficial starting point for protein purification
- Complete absence of methanol through VALIDOGEN's methanol-free promoters
- Stable, robust and scalable processes for commercial manufacturing.