Pichia pastoris Protein Expression Excellence

WHITE PAPER

High level methanol-free phytase production in *Pichia pastoris*

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Introduction

Pichia pastoris is recognized as a highly competitive expression system for fast and economic production of recombinant proteins. It combines advantageous properties of prokaryotes and mammalian cells. On one hand, Pichia cultures grow fast and can reach high cell densities on inexpensive and chemically defined media, on the other hand, the organism has a subcellular protein processing machinery, which is required for post-translational modification and active secretion of proteins. Simplicity of genetic manipulation and high productivity paired with the ability to secrete recombinant proteins efficiently further underline the capabilities of *Pichia pastoris* for both R&D and commercial production. Levels of endogenous proteins in the culture supernatant are low, furnishing a raw product with high purity. Thus, Pichia not only enables cultivation processes with high volumetric productivity but also simplifies downstream processes rendering manufacturing of recombinant proteins even more economically viable.

Today, Pichia is used for the manufacture of numerous commercial products, including biologics, with a constantly growing list of clinical candidates, food & feed enzymes, research proteins and technical enzymes. A significant milestone for *Pichia pastoris* as a production host in food technology was when the U.S. FDA awarded the GRAS (generally recognized as safe) status for recombinant phospholipase C from Pichia applied for degumming of vegetable oils.

The AOX1 promoter (PAOX1) - in its native function controlling the expression of an enzyme from the methanol metabolism called alcohol oxidase 1 - is the promoter most often applied for recombinant protein production with *Pichia pastoris*. PAOX1 is strongly repressed in the presence of common carbon sources such as glucose and glycerol. Upon depletion of these carbon sources, the promoter is derepressed and elicits its full activity when induced with methanol. An important practical aspect of this tight regulation is during the initial phase of bioreactor cultivation in the presence of repressing carbon sources in order to avoid cellular metabolism being hampered by heterologous protein expression. Nevertheless, in some cases safety precautions are an obstacle for implementation of methanol-induced Pichia cultivation processes at large scale. As a consequence, alternative promoters, which do not require induction with methanol, while still exhibiting tight regulation patterns and furnishing high expression levels, are desired.

Based on Pichia´s inherent potential, VALIDOGEN established an exclusive and yield-enhancing protein production technology toolbox known as UNLOCK PICHIA - the core of which is the company´s cutting edge AOX1 promoter library. The library is composed of alterations of active sequence elements, such as transcription factor binding sites, resulting in variants with different strength and regulatory properties. After careful selection of the perfect match of a promoter variant with a particular gene of interest these differences allow for optimization of protein production by fine-tuning of expression levels.

At VALIDOGEN promoter variants are applied simultaneously in pools of expression constructs with the gene of interest and genes encoding for different auxiliary proteins, thereby generating a broad diversity of genetic arrangements and expression profiles. In this setting, protein production in *Pichia pastoris* is boosted significantly. Following specialized protocols, thousands of clones can be screened in minimum time and most promising clones will be reliably identified already at microscale. Results of microplate screening are transferable into much larger bioreactors making laborious intermediate steps for clone comparison obsolete.

Without Methanol

A subset of VALIDOGEN's AOX1 promoter variants has been found to elicit high productivities already during the derepression phase i.e. at low concentrations of glycerol or glucose. Thus, methanol feed for induction can be omitted and strong expression is facilitated even with just glycerol or glucose as the sole carbon source. Apart from abolishing toxic and explosive methanol as a substrate and retaining high expression levels, major additional advantages of this new technology are reduced oxygen consumption and therefore significantly reduced heat formation and cooling effort in bioreactor cultivation as well as a significant potential to reduce process time and cost of goods.

Case Study: Production of a Recombinant Phytase Using UNLOCK PICHIA - VALIDOGEN's Pichia Expression Technology

Phytases catalyze the stepwise removal of phosphate from phytic acid and its salt phytate, a storage compound of phosphorus in plants. Recombinant (native and engineered) phytases from different sources such as fungi and bacteria are applied as additives in animal feed for monogastric animals such as swine, poultry, and fish. The current annual phytase market is estimated to be about 350 million USD.

Harnessing VALIDOGEN's UNLOCK PICHIA technology and screening know-how in a case study, the production of an engineered variant of *Butiauxella sp*. phytase yielded 22 g/L of enzyme in a methanol-induced process and 20 g/L under methanol-free conditions, constituting the highest amounts of yeast-produced recombinant phytase reported so far.

Procedures

All media components used during expression strain generation as well as bioreactor cultivations are certified to be free of any animal derived components and contaminations resulting thereof. For regulatory purposes, no antibiotic selection markers were applied and full documentation is available for strains and all genetic elements used in a "ready to file" status.

Initially, two different PAOX1 variants for MeOH-induced and two different PAOX1 variants for MeOH-free production were tested in a screening of 400 clones each in order to assess the expressibility of the target phytase in Pichia (small screening). After simultaneous transformations (pool of two constructs each) and selection, clones were cultivated in 96-deep well plates according to VALIDOGEN´s specialized procedures. Supernatants were screened for phytase concentration and activity by microfluidic capillary electrophoresis (mCE) measurements and by DiFMUP (6,8-difluoro-4-methylumbelliferyl phosphate) assay.

Subsequently, in a second comprehensive development phase (extended screening), nine different PAOX1 variants (both MeOH-induced and MeOH-free) were applied in a screening of a total of 6000 clones each. After each microscale cultivation and screening step, the most productive clones were cultivated in 0.75 l bioreactors following VALIDOGEN´s established generic protocols without any further optimization of cultivation conditions.

Results and Discussion

As expected, the initial screenings after transformations with constructs carrying only two different AOX1 promoter variants for either MeOH-induced or MeOH-free production returned somewhat varying expression and activity levels. The most promising clones were assessed by cultivation in 0.75 l bioreactors. The final phytase concentrations in the cultures supernatants reached 6.2 g/l with MeOH induction and 4.2 g/l in the MeOH-free process. The relatively high productivities obtained in this initial stage of strain development indicated that *Pichia pastoris* is a well suited host for the production of recombinant *Butiauxella sp*. phytase.

The application of a higher number of promoter variants from VALIDOGEN´s UNLOCK PICHIA technology toolbox as a pool typically leads to a significant productivity boost leveraging random genetic diversity and concomitant fine-tuning and optimization of gene dosage. In this study with nine AOX1 variants in the large screening, a landscape of different expression levels was observed and productivity increased by a factor of around four. Phytase concentrations reached 22 g/l (MeOH-induced) and 20 g/l (MeOH-free) in the supernatants at the end of bioreactor cultivations of most promising clones identified in microscale (Figure 1).

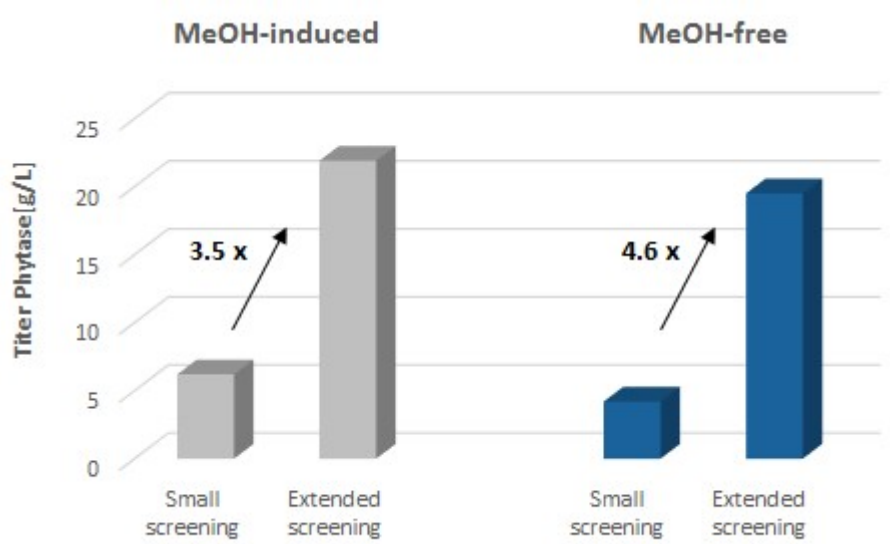

Figure 1: Methanol-induced and methanol-free production of *Butiauxella sp*. phytase in VALIDOGEN`s *Pichia pastoris;* small screening (low genetic diversity); extended screening (high genetic diversity); titers reached with final strains were 22 g/L for methanol-induced conditions and 20 g/L for the methanol free process. The improvement through increasing genetic diversity (increasing VALIDOGEN´s promoter variants and clone numbers) was 3.5 and 4.6-fold, respectively.

These results clearly underline the usefulness of VALIDOGEN´s AOX1 promoter library and the company´s entire expression toolbox as well as strain development and cultivation protocols to unlock Pichia as a powerful host for recombinant protein production. Using our approach, we could demonstrate that increasing the screening size by broadening the pool of AOX1 promoter variants in expression constructs used for transformation leads to a significant improvement of product yields.

Despite the advantages of tightly regulated PAOX1 driven expression, the necessity for induction with methanol is sometimes considered problematic because of safety concerns, especially for the large-scale production. This case study reveals that the appealing features of PAOX1 driven expression such as tight regulation of the production process while facilitating high-level protein production are equally effective with VALIDOGEN's MeOH-free AOX1 promoter variants.