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What about Pichia?

Latest product approvals and recent advances in optimising Pichia addressing recombinant protein expression limitations and challenges transform this yeast to an efficient expression system for biopharmaceutical manufacturing

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From the list of therapeutic protein approvals, two pre-eminent hosts appear to dominate the field of recombinant protein expression: the bacterial *Escherichia coli* (*E. coli*) system and mammalian cell lines, such as Chinese hamster ovary (CHO) cells. Combining the advantages of these, the versatile yeast *Pichia pastoris** (*P. pastoris*) is emerging to become a competitive alternative for biomanufacturing.

Major Player Potential

P. pastoris is an established, FDA and EMA approved, generally recognised as safe and highly competitive expression host. It has strong and effective secretory capacities, which often result in double-digit g/l levels of recombinant protein in the culture supernatant, while retaining most endogenous proteins within the cells. Delivering a secreted raw product with a high purity in the culture supernatant, *P. pastoris* enables simplified downstream procedures, which, when paired with cultivation processes with a high volumetric productivity, result in highly economic protein production.

This yeast combines the advantages of prokaryotes and mammalian cells: it is amenable to genetic manipulation. Pichia exhibits fast growth on cheap and chemically-defined media comparable to bacteria, together with eukaryotic features like a subcellular protein processing system that is required for post-translational modifications. Therefore, the host offers the speed and ease of highly efficient prokaryotic platforms and the secretion capabilities of mammalian expression systems,

reducing the struggle for protein purification. Unlike bacterial hosts such as *E. coli*, no cell lysis and time-consuming isolation from a crude lysate is required to obtain the target protein in pure form. Reaction steps, like refolding from inclusion bodies, are obsolete and unwanted enzymatic side activities are minimised.

In contrast to expensive, laborious and intricate mammalian cell line generation, establishing stable recombinant *P. pastoris* cells is a straightforward and time-saving process. Pichia produced proteins are not contaminated with endotoxins or viruses as bacterial and mammalian production systems might be, thus alleviating safety issues and further simplifying downstream processing in bioproduction. Among other aspects, the high genetic stability and robustness of Pichia cells against mechanical stress make process development and scale-up a clear-cut approach to scales of up to 200,000 litres.

Biomanufacturing Challenges

Today, about 27% of drugs approved per year by the FDA and EMA are biopharmaceuticals (1). According to market research, the biologics sector will grow at a compound annual growth rate of approximately 11%, more than doubling the revenues for biomanufacturers from \$209 billion to \$480 billion by 2020 (2).

Currently, about 1,300 recombinant pharmaceuticals are being developed – some with improved performance and new functionalities compared to the conventional, plain

protein species (3). Producing high-quality proteins for therapeutic applications at high yields is still an objective, as the bioprocessing industry faces many challenges in reaching its goals of increased efficiency and improved economics. Although considerable progress has been made over the past 20 years to raise the productivity and robustness of manufacturing processes for biopharmaceuticals, the cost and complexity of their development remain high (4). Advanced technological solutions in manufacturing and a deeper understanding of the processes, as demanded by the FDA in the QbD initiative, will lead to safer and more cost-effective drug products. Looking at the single components of a bioprocess, the expression host seems to be the most important ingredient.

Recognised Hosts

Several expression systems have been successfully established for industrial production of recombinant proteins in recent years. The most common of these use bacteria or yeast as hosts for production, as well as mammalian cells, which are typically utilised for generating more complex proteins.

E. coli is the preeminent microbial host for obtaining recombinant proteins in commercial and research settings. Culturing is then quick and inexpensive, making it ideal in many respects. However, reports indicate that expression of eukaryotic genes in this prokaryotic host often results in inclusion body formation and/or low yields (5). In contrast, more demanding protein structures, especially glycosylation, call for mammalian hosts like CHO cells.

Meanwhile, more than 50% of biopharmaceutical proteins are produced in mammalian cell lines, which is seemingly the production system of choice for complex (glyco-) proteins such as monoclonal antibodies. Although glycosylation patterns are similar to those in humans, glycans can be very heterogeneous and may lead to considerable batch-to-batch variation. Moreover, laborious, expensive and time-consuming mammalian cell line generation, low titres, long cultivation times, genetic instability and viral safety issues call for further optimisation or alternative expression systems.

Best of Both Worlds

Saccharomyces cerevisiae (*S. cerevisiae*), one of the most studied eukaryotes, is being used for the production of several biopharmaceuticals. Today, around half of the world supply of recombinant insulin is produced with *S. cerevisiae* (6). However, it shows strong fermentative metabolism and often limited recombinant protein productivity. Additionally, proteins produced by this eukaryote are reported to be frequently hyperglycosylated, and retention of the products within the periplasmic space is regularly observed with a consequent partial degradation (7).

Thus, other yeasts have been explored and *P. pastoris* is the most established and prominent host, with increasing popularity.

According to literature, the proportion of recombinant genes expressed in *P. pastoris* has steadily increased from 1995 (5). This finding suggests that researchers and industry are recognising its potential for challenging protein production, in which bacterial hosts often fail due to a lack of the eukaryotic protein processing machinery.

Indeed, *P. pastoris* continuously emerges as an alternative host for effective protein expression and secretion. The availability of convenient vector systems and different *Pichia* strains, an increasing repertoire of molecular tools and the option to generate glycoproteins with tailored glycans using engineered strains as well as significantly higher secretion levels in the double digit g/L range, position *P. pastoris* as an attractive choice for the manufacture of recombinant proteins for numerous applications. For example, Biocon, the fourth largest supplier of insulin products, utilises *Pichia* for insulin production (6).



A recent study comparing the manufacture of recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF) with *P. pastoris* and *S. cerevisiae* further reveals the effectiveness of this expression host. Sargramostim, rhGM-CSF from *S. cerevisiae*, is an FDA-approved biologic for the treatment of neutropenia and leukaemia, in combination with chemo- or radiotherapy. Productivity in *P. pastoris* was significantly higher than in *S. cerevisiae*, and, furthermore, rhGM-CSF overexpressed with *Pichia* was more active (8). In comparative studies, *Pichia* has been shown to outperform other yeast systems in terms of yield and activity of recombinant proteins (8,9).

Production Tools and Strategies

In the past, the *Pichia* system has been developed mainly in the areas of expression vector design, host strain engineering and screening for high-level expression strains and methods for strain selection (10). However, more recent research has brought about new exciting technologies, increasing the potential of this already powerful yeast production host.

Particularly, challenges in protein folding and secretion have been further addressed, with the extension of the molecular toolbox by versatile discharge signals, as well as aiding factors helping the expression of more complex proteins (11).



Elaborated screening techniques allow for a time-saving and efficient strain development programme, and optimised cultivation strategies maximise space-time yields without compromising quality.

The most progressive developments, which have made *Pichia* a competitive expression host for recombinant protein production, rely on innovations in promoter technologies, as well as glycoengineering approaches realising the production of homogeneous and human-like glycoproteins with *Pichia*.

The success of *P. pastoris* in the generation of recombinant proteins is inseparably linked to the strong, inducible promoter of the gene-encoding the first enzyme of the methanol utilisation pathway, alcohol oxidase1 (AOX1). The AOX1 promoter (PAOX1) is repressed by substances such as glucose, glycerol or ethanol and strongly induced by methanol, making *P. pastoris* a tightly controlled expression system with many advantages. Typically, higher expression levels for heterologous proteins in *P. pastoris* can be obtained, with PAOX1 clearly outperforming constitutive promoter systems for *Pichia* (12).

Based on this strong promoter, PAOX1 variants have been constructed to have advantageous properties for improving protein production (13). A library of synthetic promoter variants has been developed, composed of active sequence elements alterations, such as transcription factor-binding sites. This has resulted in variants with different strength and regulatory properties for fine-tuning of protein expression, allowing their production and secretion at double-digit levels – in some cases greater than 20 g/L. Additionally, this library has been evolved to encompass new PAOX1 variants, which are active even without methanol induction. This novel regulation system, based on de-repression under limited carbon source feed, is thus addressing large-scale processing issues associated with the use of methanol, such as process safety, excessive heat generation and ultimately process and equipment costs (14).

Another methanol-independent induction system is based on promoters identified by DNA microarray analysis and allows for expression in glucose-based protein production processes (15).

Carbon-source independent innovations – such as the use of thiamine-responsive promoters – have been reported, as well as strategies to engineer host cells allowing AOX1-based methanol-free protein manufacture (16,17).

As the glycosylation patterns from mammalian systems closely resemble those on human endogenous proteins, mammalian expression hosts such as CHO cells are applied for the manufacturing of the majority of biopharmaceuticals on the market. However, the glycosylation pattern of mammalian hosts is typically very heterogeneous and can be influenced by many factors during production, with potential for considerable batch-to-batch variation.

In light of problems sometimes associated with high mannose structures and glycosylation heterogeneity in *Pichia*, researchers have developed ways to modify its N-glycosylation machinery, with the goal of creating more human-like polysaccharide structures (18,19).

With the advent of the omics era and improved and comprehensively annotated genome sequences available, advanced strain engineering and systems biology approaches are being increasingly embraced by researchers to further improve the *Pichia* system. Recently developed tools for rapid and efficient genome engineering, like the CRISPR/Cas9 system, will undoubtedly boost these developments further.

Long-Term Prospects

Regulatory bodies, such as the FDA or EMA, are likely to approve more *Pichia* produced recombinant protein therapeutics in the future. Users of *P. pastoris* appreciate its high productivity and low complexity downstream processes; the eliminated risk of endotoxin or viral contamination; the straightforward and fast generation of stable cell lines; the robust expression and simplified cultivation requirements; and the excellent scalability of up to approximately 200,000 litres. New technologies to increase efficiency and product quality, the availability of glycoengineered strains creating heterologous proteins predominantly as single glycoforms and modern cell line engineering tools will further push *Pichia* towards being a competitive alternative to *E. coli*, CHO and other production systems.

Note:

*The organism known as *Pichia pastoris* has been re-classified as *Komagataella phaffii*. As common among most biotechnologists, the name *Pichia pastoris* will still be used for this expression system.

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