Maximising Protein Yields with Pichia pastoris

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The market launch of insulin approximately 30 years ago is generally regarded as the commercial advent of recombinant DNA technology. Since then the biotechnology drugs market has been growing at remarkable rates and the biologics industry is now generating annual revenues of more than $100bn, corresponding to over 15% of the total pharmaceutical market. Today, monoclonal antibodies (mAbs) are the dominating class of proteins on the biologics market. Besides mAbs, other proteins, such as for example cytokines, hormones, blood factors, fusion proteins or therapeutic enzymes, have found pharmaceutical application. As a matter of fact, the diversity of protein molecules developed or marketed by the pharmaceutical industry also reflects certain varieties of molecular architectures and properties. Given the fact that so far the one universal expression host meeting all scientific and commercial requirements for manufacturing of recombinant proteins has not been established, one would conclude that the demand for a considerable variety of protein molecules has to be met by numerous different expression hosts. Nevertheless, from the group of mammalian expression systems Chinese hamster ovary (CHO) cells are the dominating technology, and on the microbial side, this role is occupied by Escherichia coli. These two expression systems have truly become the main workhorses of the industry, and are much appreciated by their well established performance and safety profiles. Roughly 30 to 40% of all biologics are produced in microbial organisms, and besides E. coli, yeasts such as Saccharomyces cerevisiae, Hansenula polymorpha and Pichia pastoris have found some application.

Pichia in the Biologics Market

Biopharmaceuticals manufactured in Pichia pastoris which have been approved in different markets are human serum albumin (Mitsubishi Tanabe Pharma Corporation; product was withdrawn recently), hepatitis-B surface antigen, interferon alpha 2b (Shantha Biotechnics, now part of Sanofi-aventis Group) and Insulin (Biocon Ltd). Notably, in late 2009 ecallantide (marketed as KalbitorTM, Dyax Corp.), a plasma kallikrein inhibitor for the treatment of acute attacks of hereditary angioedema, has been granted market approval by the US Food and Drug Administration, and is currently undergoing approval process in the European Union. Judging from its current status and the recent approval of a Pichia-derived product in a major market, Pichia pastoris is gaining ground as a validated expression system for biopharmaceutical manufacturing.

Pichia pastoris is one of the few yeast species classified as methylotrophic, due to its capability of metabolising methanol as the sole source of carbon and energy. In this context, the AOX1 promoter regulating the expression of the first enzyme involved in the yeast’s methanol catabolism – namely alcohol oxidase 1 – is one of the strongest promoters from the microbial kingdom known to date, and its application has proven a very good means to achieve high expression levels of recombinant proteins. From this perspective, it is not surprising that this regulatory element is the focus in one of the strategies to maximise protein yields with Pichia pastoris as discussed below.

A wealth of reports has been published describing the production of hundreds of different proteins in Pichia pastoris, and in many cases product titers of several g/L - sometimes even exceeding the 10 g/L margin - have been achieved. In addition, it is generally appreciated that this organism is also very effective in secreting a protein of interest into the culture medium, obviating tedious raw product recovery. For example, human serum albumin was obtained in the >15 g/L range by secretion into the culture medium (VTU Technology GmbH) and intracellular expression of a hydroxynitrilase enzyme furnished >20 g/L of product (Graz University of Technology).

Historically, Phillips Petroleum Company used Pichia pastoris in an industrial setting in the 1970s for the generation of single cell protein for production of high-protein animal feed. The company took advantage of the organism’s methylotrophic nature and fed the cultures with (at that time) cheap methanol. However, with the breakout of the oil crisis and rising methanol prices, the process never became competitive. In the 1980s researchers at SIBIA (Salk Institute Biotechnology/Industrial Associates Inc.) developed Pichia as a
system for foreign protein expression, and in the 1990s Research Corporation Technologies Inc. acquired the rights from Phillips Petroleum, and the components of the system became commercially available through Invitrogen (now Life Technologies) fuelling worldwide research and development with Pichia.

Nowadays, more and more companies are entering the biopharmaceuticals arena, and as a consequence an increasing number of players will be competing for market share in the different therapeutic areas. From that point of view, the economics of biopharmaceutical manufacturing are very likely to become more challenging. Secondly, with several biologics coming off patent soon, a price competition will be brought about by the launch of follow-on biologics. Today these products account for roughly 1% of revenues in the biopharmaceutical industry, but annual growth rates are projected to be approximately 50% over the next five years, and 30% over the next ten years. In light of that situation, biologics developers and manufacturers may have to devote increased effort to cost of goods reduction. Of course, there can be no doubt that apart from manufacturing costs there are many more significant cost factors and drivers to biologics pricing, but manufacturing costs become increasingly detrimental, and increasing cell line titers will at least lay the foundation for a healthy cost structure.

Maximising Yields

For the purpose of introducing a generalised view on different options to maximise protein yields with Pichia pastoris, it may be useful to discuss aspects concerning gene design, generation of expression cell line and genetic manipulation of the organism, as well as cultivation. Codon optimisation of the gene encoding a protein of interest should be taken into consideration in order to avoid transcriptional and/or translational bottlenecks. Many of the commercial suppliers of synthetic DNA have elaborated effective procedures to design and optimise genes for Pichia expression. As stated above, efficient secretion of recombinant proteins controlled by signal sequences is a very useful feature of Pichia pastoris, and a number of different signal sequences having different effects on expression efficiency have been tested, among them Saccharomyces cerevisiae MF (alpha-mating factor prepro leader sequence), PHO1 (signal sequence of Pichia acid phosphatase 1) or human serum albumin signal sequence, to name a few. In some cases, the native signal sequence of a target protein also turned out to be beneficial. In general, MF has been shown to be most useful in terms of efficiency and versatile applicability, making it the number one choice for this purpose.

The proper choice of promoters is a very important success factor in recombinant protein expression. Several different promoters have been applied with Pichia, of which GAP (Pichia glyceraldehyde dehydrogenase promoter) and AOX1 (Pichia alcohol oxidase 1 promoter) are the most important ones. GAP is a relatively strong constitutive promoter, and in some rare cases expression yields above 1 g/L range can be achieved. In addition, it is a useful alternative if the use of methanol which needs to be present for AOX1 induction needs to be avoided.
In terms of expression strength and protein yield the AOX1 promoter is much more powerful, allowing for titers of more than 10 g/L of secreted target protein. The gene product of AOX1 is alcohol oxidase 1, one of two alcohol oxidases – the other one being alcohol oxidase 2 – catalysing the initial step of the yeast’s methanol metabolism. Although alcohol oxidase 1 accounts for more than 90% of the activity in this catabolic step, it is a relatively weak enzyme due to its poor affinity to molecular oxygen (the co-substrate in this reaction). As a consequence, huge amounts of alcohol oxidase 1 have to be produced by the cell once methanol is present, which explains the exceptional strength of this promoter. The AOX1 promoter is tightly controlled depending on the carbon source, and works by a repression/de-repression – induction mechanism. It is strongly repressed in the presence of conventional carbon sources such as glucose or glycerol, displays some activity at low concentrations of these substrates (de-repression), and unfolds its entire potential upon induction with methanol. In connection with recombinant protein manufacturing this tight control regime allows for a rapid accumulation of biomass - which is important for high protein yields - during the initial cultivation phase, without overburdening the organism’s metabolism prior to methanol addition.

Recently, researchers at VTU Technology GmbH have developed a panel of genetically modified AOX1 variants, and it was found that some of these variants exhibit even greater expression strength than the native promoter, resulting in higher protein yields.

A gene encoding a certain target protein, or to be more precise an entire expression cassette, is usually integrated into the genome of Pichia pastoris upon transformation of the cells, thereby allowing for a different frequency of integration events, resulting in clones with different numbers of gene copies. It is well accepted that copy number variations influence expression levels, and it has been shown several times that higher copy numbers lead to higher expression levels although the yield increase with copy number is not linear. In this context, James M. Cregg (Keck Graduate Institute of Applied Life Sciences, Claremont, CA) has developed a method termed posttransformational vector amplification (PTVA) by which high copy number clones can be generated after the transformation event by exposing the cells to high concentration of selection marker. However, this interconnection of copy numbers and product yield is not as universal as it may seem, and one should keep in mind that for some proteins – for example toxic proteins or proteins requiring demanding posttranslational modifications – low copy numbers in the genome lead to better results. In other words, in some cases milder gene dosage will ultimately lead to higher space-time yields.

The process of protein synthesis inside a cell from gene to functional polypeptide comprises multiple steps and mechanisms, and a plethora of different proteins are involved. Recombinant overexpression of a foreign protein at high levels may face bottlenecks, as the cellular machinery may not be able to cope with this overflow. This problem has been successfully addressed by co-expression of several proteins, mainly those involved in protein folding, disulfide bridge formation, proteolytic processing as e.g. signal sequence cleavage, stress response or transport across organelles. Numerous protein factors influencing protein maturation and secretion have been identified e.g. by applying transcriptomics, and beneficial effects on target protein yields by co-overexpression have been demonstrated. In VTU’s labs this principle has been applied along with AOX1 promoter variants. These AOX1 variants not only show different expression strengths, but also exhibit different regulatory patterns during cultivation, mostly with respect to their de-repression behaviour. More specifically, in the phase of carbon source change from glycerol to methanol – i.e. the change of promoter status from de-repression to induction - selected members of that protein library show elevated activity in the de-repression phase compared to native AOX1, and therefore allow for a pre-formation of one or more yield enhancing (helper) factors. This operation principle ensures that elevated amounts of crucial factors are already present at the time when methanol induces the strong expression of the target protein. This can help to alleviate the metabolic burden of the cells during heterologous protein production. By selecting the most efficient combination of promoter variants and helper factors aligned with the specific requirement of a given target protein, a tremendous boost of expression levels and multiplication of protein yields can be achieved.

**Screening is Key**

The quest for the optimal clone for the production of a desired protein will only be successful if as many as possible of the aspects described above are tuned properly. However, a priori there is no patent remedy as different target proteins with different sequences and properties will require different combinations of the described aspects. As a consequence, there is a huge diversity caused by different copy numbers and the choice of tools such as promoters, signal sequences and helper factors, and this diversity has to be addressed in the course of expression strain generation. Since it is not possible to control all important aspects influencing transcription, translation, maturation and potentially secretion of a foreign protein of interest in a rational fashion, the screening of clones resulting from transformation is attributed a pivotal role. Firstly, this implicates that it might not be sufficient to analyse just a handful of transformants. Usually, when variable components such as genetic elements or different strains are included in the expression strategy, the number of clones to be screened for identifying high expressors should be several thousand, and methods for including multiple selection markers should be available. This is of great importance, especially for the expression of hetero-multimeric proteins and/or with concomitant co-expression of yield enhancing factors in order to minimise the appearance of false positives. Secondly, for strain selection purposes, efficient and reliable protocols for cultivation of the transformants in micro scale are indispensable. This is to make sure that out of thousands, the best expressers will be identified with very high fidelity in a high throughput screening fashion. Indeed, the stage of expression strain generation and clone selection – when performed properly – holds great potential to create a firm base for further development in a time-saving manner. A well balanced interplay of yield enhancing aspects identified in high throughput screening is a key to success, and paired with rapid and reliable scale-up can substantially increase speed-to-clinic.

**Cultivation of Pichia**

After having generated a productive cell line, its potential will need to be exploited by
cultivation on a larger scale in bioreactors, and there are several parameters to be considered. In general, the optimal cultivation procedure very much depends on the individual production strain and the target protein to be expressed, and therefore only the most important yield enhancing aspects will be discussed.

Pichia pastoris has the ability to grow to very high cell densities, amounting to more than 150 g/L of cell dry weight, and large amounts of cells will produce large amounts of recombinant protein. On the other hand maximum yields can only be achieved if the cells are kept vital throughout the entire cultivation process, not only to maintain maximum productivity, but also to prevent cell lysis accompanied by the release of endogenous proteases, resulting in sometimes rapid product degradation. For maintaining high productivity it may on some occasions be beneficial to perform the production phase at lower temperatures (20°C or below) in cases where expression is controlled by the AOX1 promoter and cultivation is divided into initial biomass generation followed by induction/production.

It has been shown several times that production controlled by the AOX1 promoter can also be performed efficiently by using methanol/glycerol or methanol/sorbitol mixtures in the induction phase, fully maintaining productivity as long as the levels of repressing carbon source such as glycerol are carefully controlled and kept low. This strategy will help to reduce the amount of hazardous methanol to be used in the process (although industrial Pichia cultivations applying methanol as the sole carbon source have been performed at a several 10,000 L scale).

Of course an alternative which completely avoids the necessity for methanol is to apply GAP-controlled expression, feeding the culture with glycerol or glucose alone. In this context, a subset of VTU’s AOX1 promoter library shows highly appealing features. Some of the variants have been found to elicit high productivities already during the derepression phase before the initiation of methanol dosing (in this phase wild type AOX1 is relatively weak). Maintaining these culture conditions, expression titers of several g/L of secreted proteins have been obtained. This means that these AOX1 variants are the strongest and most effective promoters known to date for recombinant protein production in Pichia pastoris for both methanol induction and methanol-free expression.

Furthermore, Pichia pastoris is distinguished by several additional advantageous features for the production of recombinant proteins. The stable integration of foreign DNA into the yeast’s genome leads to high mitotic stability of expression cell lines, which is a very important factor for batch-to-batch reproducibility. Besides, the application of strong promoter variants (e.g. AOX1 variants) can furnish highly expressing low copy integrants, and mitotic stability will be enhanced with a decreasing number of copies of expression cassettes. Pichia is able to perform posttranslational modifications such as proteolytic/signal sequence cleavage, folding or disulfide bond formation. Interestingly, despite the apparently well-functioning secretory pathway, Pichia releases only few endogenous proteins into the culture medium, and therefore foreign proteins can be obtained with purities of up to 80% post-fermentation. This tremendously simplifies downstream processing – yet another means of COG reduction. Cultivations are performed in chemically-defined minimal media, devoid of any antibiotic selection markers. Pichia is also very convenient in terms of product safety, since viral or endotoxin contamination will not be an issue.

Apart from yield enhancing efforts, Pichia-related research has seen fascinating developments in the field of genetic engineering. Protease deficient strains have been available for quite some time now, and recently glyco-engineered strains conferring mammalian-like glycosylation patterns on recombinant glycoproteins have been developed by Research Corporation Technologies Inc. (in collaboration with Ghent University) and GlycoFi Inc. (a subsidiary of Merck & Co. Inc.).

In summary, Pichia pastoris is a good choice to be selected as host for protein manufacturing – including biologics – as with this organism high yields of quality products can be obtained, and with the recent approval of a Pichia-derived biopharmaceutical product by the FDA the system has gained validation in the biologics industry. The availability of the entire Pichia genome will further fuel research and improvements to the system, and will very likely act as a catalyst for the discovery of many more yield enhancing aspects.