Molecular strategies to increase the levels of heterologous transcripts in *Komagataella phaffii* for protein production

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**ABSTRACT**

*Komagataella phaffii* (formerly *Pichia pastoris*) is a well-known fungal system for heterologous protein production in the context of modern biotechnology. To obtain higher protein titers in this system many researchers have sought to optimize gene expression by increasing the levels of transcription of the heterologous gene. This has been typically achieved by manipulating promoter sequences or by generating clones bearing multiple copies of the desired gene. The aim of this work is to describe how these different molecular strategies have been applied in *K. phaffii* presenting their advantages and drawbacks.

**KEYWORDS**

gene transcription; heterologous expression; *Komagataella phaffii*; multicity integration; promoter engineering

**ARTICLE HISTORY**

Received 10 February 2017
Accepted 10 February 2017

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**Introduction**

The methylotrophic yeast *Komagataella phaffii*, still mostly known by its old name, *Pichia pastoris*, is considered one of the most important platforms in modern biotechnology for the production of heterologous proteins. This is due to several features including its high volumetric productivity and ability to perform some posttranslational modifications in a manner similar to mammalians cells (for review see [1,2]). However, a clear limitation of this system is its low specific productivity which has prompted many researchers to pursue molecular strategies to improve protein production. The most popular approach to reach this goal is to offer to the translational machinery of the cell higher titers of a particular transcript. This is usually accomplished by driving the expression of the heterologous gene with a suitable promoter or by simply increasing the copy number of the target gene. [3] One should consider that gene overexpression may pose a metabolic burden to the physiology of the cell thus resulting in disappointing outputs. Nonetheless, most researchers still consider these approaches (summarized in Fig. 1) as a starting point in the endeavor of obtaining higher protein titers in *K. phaffii*.

**Inducible and constitutive promoters**

*K. phaffii* vectors are derived from a few integrative plasmids which can carry expression cassettes under the control of inducible or constitutive promoters. A detailed review on the promoters available for protein production in *K. phaffii* is provided elsewhere. [4] The first expression system for *K. phaffii* was based on the promoter of the alcohol oxidase gene (*P\textsubscript{AOX1}*) involved in the first step of methanol metabolism. This strong and methanol-induced promoter was partly responsible for the popularity of this yeast as a heterologous protein production platform since the 1980s. [5,6] *P\textsubscript{AOX1}* is repressed by glucose, glycerol and ethanol, therefore it represents a reliable tool when expressing toxic or growth-impairing proteins due to its tight regulation. [7] Several other promoters involved in the methanol utilization pathway have been isolated and classified as strong, intermediate or weak according to its expression levels in comparison to *P\textsubscript{AOX1}*. [8] Because induction by methanol represents a hazardous disadvantage for large-scale processes alternative inducible promoters have been considered. A novel regulated promoter has recently been identified from a high-affinity glucose transporter gene (*GTH1*) which is repressed by...
glycerol. Since \( P_{\text{GTH1}} \) is responsive to carbon source depletion it should represent an attractive and cheap alternative to other regulated systems that rely on the addition of an external source of the inducing agent. Recently, 2 inducible promoters from the \( K. \text{phaffii} \) rhamnose utilization pathway were proposed for the production of food-grade and therapeutically important recombinant proteins. Also, extensive transcriptome analysis has led to the identification of new promoters regulated by the carbon source which should represent a fine addition to the \( K. \text{phaffii} \) promoter toolbox.

Another alternative to methanol-induction is the use of constitutive expression systems which may be more advantageous when considering the fermentation strategy used. Whereas inducible expression requires a growth phase before the addition of methanol (production phase) in a constitutive system protein production is concomitant to cell growth, thus favoring continuous fermentation processes. The promoter derived from the glycolytic glyceraldehyde 3-phosphate dehydrogenase gene (\( P_{\text{GAP}} \)) is one of the most popular constitutive systems. Full transcription from \( P_{\text{GAP}} \) is achieved when grown in glucose as a carbon source. In addition to \( P_{\text{GAP}} \) other moderately strong constitutive systems may be used such as \( P_{\text{TEF1}} \) and \( P_{\text{PGK1}} \).

### Promoter engineering

A wide range of available promoters is an advantage for \( K. \text{phaffii} \) as a heterologous protein production platform since it allows the construction of fine-tuned systems and strains while also meeting requirements set by specific proteins. For protein production purposes, intermediate or weak promoters may be more advantageous when protein folding becomes an issue as a result of gene overexpression. Likewise, in metabolic engineering applications a diverse set of promoters with different strengths allows a more accurate tuning of the expression levels and metabolic flux. Transcription-factor binding sites (TFBS), upstream repression sites (URS) and upstream activation sequences (UAS) from different \( K. \text{phaffii} \) promoters may direct the design of new synthetic promoter sequences in the future. Promoter libraries have already been constructed using not only random mutagenesis methods but also with specific synthetic core promoter sequences derived from wild-type promoter alignments. The \( P_{\text{GAP}} \) sequence has also been manipulated through error-prone PCR for the construction of a \( \text{GAP} \)-promoter library with strengths varying from \(~0.6\%\) to 19.6-fold of that of the wild-type \( P_{\text{GAP}} \).

### Multi-copy number clones

One of the most common strategies to increase the levels of mRNA is by introducing several copies of the heterologous gene. In several cases this has led to a significant improvement in heterologous protein production, most notably when considering intracellular expression. However, in some cases involving secreted proteins, a higher gene copy number resulted in reduced heterologous protein production. For example, production of porcine insulin precursor (PIP) under inductive conditions was significantly affected in clones bearing \( >12 \) copies of the heterologous gene. This has been attributed to bottlenecks in the secretory pathway which may become overwhelmed by the effects of high titers of a particular heterologous protein. Strategies to overcome these barriers may include the use of new signal peptides based on the \( K. \text{phaffii} \) secretome and combinatorial engineering of secretion helper factors involved in protein folding and vesicle trafficking.

In \( K. \text{phaffii} \), expression vectors are typically integrated into the host chromosome by homologous
recombination and non-homologous end joining events. Multiple integration events may occur at low frequency (5–6%) by the integration of expression cassettes in tandem in a head-to-tail configuration. These rare integration events can be favored by using vectors with dominant markers based on antibiotic resistance genes which allow the screening of desired clones under drug selective pressure. The use of dominant markers is the basis of the “posttransformational vector amplification” method (PTVA) or its recently described variant “liquid PTVA”. In PTVA, cells transformed with one or few copies of a vector carrying the marker that confers resistance to zeocin or G418 can be selected in higher concentrations of the antibiotic in a stepwise manner resulting in isolation of multi-copy clones several days after the initial transformation.

Multi-copy integration events can be enhanced by targeting vector insertion to repetitive sequences in the genome such as the rDNA cluster (rDNA) which comprises a 7450-bp repeated sequence organized in tandem. When a specific sequence is targeted to this cluster, a low copy number of integrated vectors is initially obtained, then, a strong selective pressure is applied for vector amplification. The use of the non-transcribed spacer region (NTS) of the rDNA as an integration target in combination with the PTVA method has led to the successful isolation of multi-copy clones in K. phaffii.

In addition to the high costs of eukaryotic antibiotics, an important drawback in the use of dominant markers is that in several clones an increase in drug resistance does not necessarily reflect multi-copy integration or an improvement in protein production. Moreover, there are concerns that the accidental release of genetically modified organisms bearing drug-resistance markers may be horizontally transferred to environmental organisms bearing the expression cassettes, a laborious procedure which also requires the availability of strains with several genetic markers. However, the resulting strains may be further crossed to generate diploid cells thus increasing overall copy number of the desired sequence.

Recently, an episomal plasmid carrying the panARS sequence was tested for recombinant protein production in S. cerevisiae as a strategy to amplify expression plasmids. In K. phaffii, the use of defective ADE1 and ADE2 alleles as selection markers has favored multiple integration with the concomitant increase in heterologous protein production.

Although the use of defective markers for multi-copy integration in K. phaffii requires the development of new auxotrophic mutant strains and the use of specific media it presents some clear advantages. Most importantly, clones with different copies of the desired gene are easily obtained on a single transformation event without the need of laborious replica plating steps as required in PTVA. Also, it is a cheap alternative to drug selection and recombinant clones that carry auxotrophic marker do not pose any significant threat to the environment.

Other less frequently used methods to obtain multi-copy clones have been described. One involves the in vitro construction of multimers of the expression cassette. In this case the size of the expression cassette may hamper DNA manipulation. Another method involves the sequential transformation of the host strain with different vectors bearing the expression cassettes, a laborious procedure which also requires the availability of strains with several genetic markers. However, the resulting strains may be further crossed to generate diploid cells thus increasing overall copy number of the desired sequence.
the near future as a platform for heterologous protein production in _K. phaffii_.

**Conclusion**

It is clear that the improvement on protein production as a result of the use of any of the molecular strategies described in this work should be assessed on a case-by-case basis. In our experience, the use of a vector bearing a repetitive target sequence combined with an auxotrophic defective marker represents an interesting start point for the development of an expression platform in _K. phaffii_.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Funding**

This work was supported by grants from Fundação de Amparo à Pesquisa do Distrito Federal (FAP/DF) and CNPq, Brazil.

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