



## Regular article

# Rabies virus glycoprotein enhanced expression in *Pichia pastoris* using the constitutive GAP promoter



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## ABSTRACT

Rabies virus glycoprotein (RABV-G) is responsible for inducing neutralizing antibodies synthesis, which are a key element for the protection against rabies infection. There is an urgent need to develop cost effective vaccines despite the availability of commercial products. In a previous study, we described the expression of RABV-G in the yeast *Pichia pastoris* under the control of AOX1 promoter and  $\alpha$ -factor mating factor from *Saccharomyces cerevisiae* to target the protein towards secretion. We showed that the expressed RABV-G was recognized by rabies virus neutralizing antibodies; nevertheless the secretion level remained low; being around 128 ng/mL. Such a low yield will preclude the use of this system for a biotechnological application. In an attempt to improve the secretion level of RABV-G in *Pichia pastoris*, we investigated in the current study the impact of the constitutive GAP promoter on the expression of the target protein. The expression level was slightly increased to 150 ng/mL and the produced RABV-G showed strong antigenicity based on the RFFIT test. Interestingly, co-expression of *Pichia pastoris* endogenous genes encoding for five factors involved in oxidative protein folding (*PDI1*, *GPX1*, *ERO1*, *GLR1* and *YAP1*) had a beneficial impact on RABV-G expression, which was enhanced to 2261 ng/mL.

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## 1. Introduction

The ability of an expression system to secrete a heterologous protein in its native conformation is one the most critical parameters to take into account while selecting an expression system. Generally, yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, etc. are considered as an effective system for protein secretion.

However efficient secretion of recombinant proteins can be challenging; some proteins are only secreted at low amounts [1]. Various factors affect the expression yield of foreign proteins in yeasts; the choice of promoter is among the most important ones. The latter is a major element for the transcription of the heterologous gene and therefore is a key part of an effective expression system of recombinant proteins. The folding process involving disulfide bonds formation and other post-translational modifications are required for functional heterologous protein production; the expression of complex heterologous proteins in a yeast host often leads to unfolded and misfolded product which triggers

the activation of the unfolded protein response (UPR) and ER-associated degradation (ERAD) resulting in low product yields [2].

Promoters are generally classified as constitutive or inducible. Various constitutive promoters have been used to express heterologous proteins in *Pichia pastoris* (*P.pastoris*); among them the strong GAP promoter (glyceraldehyde-3-phosphate dehydrogenase) [3]. With this promoter, the heterologous protein will be expressed along with cell growth if the protein is not toxic to the cell. Therefore, it can easily be suitable to continuous culture that theoretically enables unlimited production of the recombinant protein [4].

Heterologous protein expression in *P. pastoris* can be also driven by inducible promoters such as  $P_{AOX1}$  (alcohol oxidase 1),  $P_{ADH1}$  (alcohol dehydrogenase 1),  $P_{DAS}$  (dihydroxyacetone synthase),  $P_{MOX}$  (Methanol oxidase),  $P_{FLD1}$  (formaldehyde dehydrogenase), etc. These promoters are controlled by the cellular physiological state, such as intracellular ion concentrations, lack or accumulation of essential amino acids, and may be sensitive to catabolite repression [5]. The use of a promoter that is controlled by the carbon source simplifies the fermentation process, since it has the advantage to allow the separation of the cell growth phase and the protein production phase, leading to a significant biomass buildup before inducing the expression of the recombinant protein. One advantage of the  $P_{AOX1}$  regulatory system is that expression of het-

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erologous genes whose products are toxic to cells can be performed by growing the yeast under repressing conditions [6].

Most of the reported foreign proteins expressed in *P. pastoris* are controlled either by AOX1 or GAP expression systems [7]. The combined use of AOX1 and GAP promoters to produce a foreign protein was described in the study of N-glycan engineering of glycoproteins [8]. Combined use of these two promoters has also been reported to express and individually collect two different recombinant proteins from the same recombinant clone of *P. pastoris* [4].

The secretion of a correctly folded protein is one of the major advantages for selecting yeasts for foreign protein expression. However, not all heterologous proteins can be successfully secreted in yeasts, and endoplasmic reticulum (ER) accumulation of highly expressed proteins is still a challenge [9].

Retention of recombinant proteins in the ER can induce the UPR pathway through the synthesis of chaperones and foldases such as BIP/KAR2 and PDI1 by transcriptional activation in the nucleus [10]. It has been reported in different studies that the secretion process can be improved by overexpression of proteins which favor the folding and processing of others [11,12]. Some of these supporting factors, like protein disulfide isomerase (PDI1), have an effect on protein folding while others prevent them from aggregation (BIP) [12]. Overexpression of the UPR-regulating transcription factor HAC1, which induces the synthesis of chaperone genes involved in the secretion process [12] can also be applied to improve the production level of recombinant proteins.

We demonstrated in a previous work that high-level expression of RABV-G in *P. pastoris* showed several limitations. The expression of RABV-G under the control of AOX1 promoter was low comparatively to other proteins produced in this host. Co-overexpression of factors involved in oxidative protein folding such as protein disulfide isomerase (PDI1) and ER oxido-reductin (ERO1), and other protein factors which are not directly involved in protein folding, like Glutathione peroxidase (GPX1), enhanced RABV-G secretion in *P. pastoris*. The secreted amount of the expressed protein was increased from 128 ng/mL to 1230 ng/mL [13]. Nevertheless such level remains low for a biotechnological application.

In this study, we first investigated the expression of RABV-G in *P. pastoris* under the control of GAP promoter to improve the expression of this protein with special attention to the occurrence of proteins related to the UPR and ERAD pathways. Our analysis revealed that RABV-G expression under the control of GAP promoter was improved when compared to AOX1 promoter. However, increased UPR and ERAD related proteins in response to secretory RABV-G production were noticed. To increase RABV-G production level, we co-expressed five factors involved in oxidative protein folding (PDI1, ERO1, GPX1, GLR1, YAP1) in the recombinant clone harboring seven copy of RABV-G gene; the effect of each protein on the expression level of the heterologous protein was investigated.

## 2. Materials and methods

### 2.1. Strains, plasmids, and media

*Escherichia coli* DH10B was used to produce recombinant vectors. *P. pastoris* KM71H strain was used as a host strain to express and secrete the recombinant protein.

The expression vector pGAPZαB was purchased from Invitrogen (CA, USA). The plasmid pPUZZLE [14] was used for cloning and expression of the folding assisting factors (PDI1, GPX1, ERO1, GLR1 and YAP1). Primers were supplied by Metabion International AG (Germany). Fast SYBR Green Master Mix was obtained from Roche (Mannheim, Germany).

Zeocin was supplied by Invitrogen (CA, USA) and G418 was purchased from Life Science Technology (NY, USA).

YPD (1% yeast extract, 2% tryptone, 2% dextrose), BMGY (2% tryptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base,  $4 \times 10^{-5}$  biotin, and 1% glycerol), and BGY (2% peptone, 1% yeast extract, 100 mM potassium phosphate, pH 6.0, 1.34% yeast nitrogen base,  $4 \times 10^{-5}$  biotin, and 1% glucose monohydrate) were used to grow *P. pastoris* strains. YPD agar plates (1% yeast extract, 2% tryptone, 2% dextrose, 2% agar) were also used to grow *P. pastoris* KM71H cells and YPD plates containing Zeocin (100 μg/mL) were used for the selection of positive *P. pastoris* transformants.

### 2.2. Construction of expression vectors and transformation of *P. pastoris*

RABV-G gene was amplified from the plasmid pUC19 (GeneCust, Luxembourg) using the primers G –F and G-R (Table 1), the purified PCR product was ligated into pGAPZαB vector to generate pGAPZαB-RABV-G.

PDI1, ERO1, GPX1, GLR1 and YAP1 fragments were isolated from pPUZZLE.Zeocin by SfiI and SbfI enzymes and ligated into the similarly digested pPUZZLE.Kanamicyne. The final recombinant plasmids were validated by DNA sequencing to check the sequences and the orientation of genes.

For construction of single-copy or multi-copy *P. pastoris* clone, the expression vector (pGAPZαB-RABV-G linearized with BstXI or pPUZZLE-helper factor gene linearized with AvrII) was introduced into *P. pastoris* KM71H according to the Easy Select™ *Pichia* Expression Kit protocol, by using Micropulser Electroporator (Bio-Rad, CA, USA). After electroporation, cells transformed with pGAPZαB-RABV-G were plated on YPD agar plates containing Zeocin (100 μg/mL). While for clones co-expressing helper factor genes, cells were plated on YPD agar plates containing both Zeocin (100 μg/mL) and Geneticin (0.5 mg/mL).

### 2.3. Expression of RABV-G protein and cell lysis

The expression of RABV-G in *P. pastoris* clones was performed as follows: positive transformants were selected on YPD agar plates containing Zeocin (100 μg/mL) and grown at 30 °C for 3 days. A single colony was inoculated in 2 mL of BMGY medium in deep well plates, and then grown at 30 °C with shaking at 250 rpm till  $OD_{600} = 2-6$ . Cells were then collected by centrifugation at 13,000g for 5 min at 4 °C and resuspended in 2 mL of BGY medium and grown at 30 °C. Cells were harvested after 72 h by centrifugation at 13,000g for 5 min. Cell pellets were resuspended in yeast breaking buffer (50 mM sodium phosphate, 1 mM PMSF, 1 mM EDTA, and 5% (v/v) glycerol). Aliquots of 1 mL of resuspended cell pellet were added to 0.5 mL of acid-washed glass beads (Sigma-Aldrich, St. Louis, USA) and lysed with Vortex for 30 s by vigorous mixing followed by 30 s incubation in ice. This mixing/freezing cycle was repeated 10 times. Samples were collected by centrifugation at 16,000g for 20 min at 4 °C and the supernatant containing the soluble proteins was collected. The pellet containing the membrane proteins was further treated with 400 μL yeast breaking buffer plus 2% (w/v) SDS. After centrifugation at 4000g for 5 min at 4 °C, supernatants containing the membrane proteins were collected and analyzed by western blot.

### 2.4. Western blot

Protein samples were separated on 12% SDS-polyacrylamide gel electrophoresis using BioRad cell system (CA, USA) then transferred to a nitrocellulose membrane (GE Healthcare, Uppsala, Sweden). After transfer, the membrane was incubated overnight at 4 °C in 3% BSA to block the non specific binding. The membrane was washed with the washing buffer (PBS 1X, 0.05% Tween-20), and then probed

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