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Production, purification, and characterization of recombinant rabies virus glycoprotein expressed in PichiaPink™ yeast

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ABSTRACT

The commonly used host for industrial production of recombinant proteins *Pichia pastoris*, has been used in this work to produce the rabies virus glycoprotein (RABV-G). To allow a constitutive expression and the secretion of the expressed recombinant RABV-G, the PichiaPink™ commercialized expression vectors were modified to contain the constitutive GAP promoter and the α secretion signal sequences. Recombinant PichiaPink™ strains co-expressing the RABV-G and the protein chaperone PDI, have been then generated and screened for the best producer clone. The influence of seven carbon sources on the expression of the RABV-G, has been studied under different culture conditions in shake flask culture. An incubation temperature of 30°C under an agitation rate of 250 rpm in a filling volume of 10:1 flask/culture volume ratio were the optimal conditions for the RABV-G production in shake flask for all screened carbon sources. A bioreactor Fed batch culture has been then carried using glycerol and glucose as they were good carbon sources for cell growth and RABV-G production in shake flask scale. Cells were grown on glycerol during the batch phase then fed with glycerol or glucose defined solutions, a final RABV-G concentration of 2.7 μg/l was obtained with a specific product yield ($Y_{P/X}$) of 0.032 and 0.06 $\mu g/g_{(DCW)}$ respectively. The use of semi-defined feeding solution enhanced the production and the $Y_{P/X}$ to 12.9 μg/l and 0.135 $\mu g/g_{(DCW)}$ respectively. However, the high cell density favored by these carbon sources resulted in oxygen limitation which influenced the glycosylation pattern of the secreted RABV-G. Alternatively, the use of sucrose as substrate for RABV-G production in large scale culture, resulted in less biomass production and a $Y_{P/X}$ of 0.310 $\mu g/g_{(DCW)}$ was obtained. A cation exchange chromatography was then used for RABV-G purification as one step method. The purified protein was correctly folded and glycosylated and able to adopt trimeric conformation. The knowledges gained through this work offer a valuable insight into the bioprocess design of RABV-G production in *Pichia pastoris* to obtain a correctly folded protein which can be used during an immunization proposal for subunit Rabies vaccine development.

1. Introduction

The demand for recombinant proteins is rising steadily with the increased market requirements. Due to its prominent role, therapeutic proteins (vaccines, enzymes, hormones, monoclonal antibodies…) constitute a growing sector of the biopharmaceutical industry[1]. The major challenge in biopharmaceuticals production is to achieve the highest yield of the purest product within the shortest time and at the lowest cost. To overcome these trials, much interest must be taken first when choosing the protein expression host and then in the bioprocess design. Depending on the features of the protein to be produced, prokaryotic hosts, such as the widely used bacteria *Escherichia coli,* can be used as they show a rapid growth, high production capacity and cultivation simplicity. However, when the recombinant protein contains posttranslational modifications, eucaryotic systems, like mammalian cells or yeasts, are recommended to obtain a full functional protein [1, 2].

The methylotrophic yeast *Pichia pastoris (P. pastoris; syn. Komagataella phaffii)*, has been largely used as a successful expression system among yeasts hosts. It's generally recognized as safe (GRAS) organism and offer distinctive advantages such as its ability to grow fast to a high cell density and to produce and secrete to the culture medium

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appropriate folded recombinant proteins at high yields [3]. Because of its attractive features this heterologous expression system is used in this work to produce the Rabies virus glycoprotein (RABV-G). It's the only exposed protein on the outer surface of the rabies virus, and is the major antigen involved in the induction of neutralizing antibodies and the main stimulus of host immune response during infection and vaccination [4]. it's organized in an homotrimer in the viral envelope. Each monomer is a 505 amino acid type-1 transmembrane glycoprotein of about 65 kDa composed of three domains: a cytoplasmic domain, a hydrophobic transmembrane domain and an outer ectodomain containing four major antigenic sites, one minor antigenic site, three potential N-linked glycosylation sequons (Asn-X-Ser/Thr) and seven disulfide bridges essential for protein folding and immunogenic site formation [5–7]. Diverse heterologous expression systems have been used to produce this protein [8,9]. Since the correct conformation and oligomerization of the protein are critical for its immunogenicity, the development of an efficient expression and purification process leading to a high yield of native like recombinant RABV-G, was the main objective of different studies [10–13].

We describe here the influence of different culture conditions on the expression of a functional form of rabies virus glycoprotein in *Pichia pastoris* using the commercialized PichiaPink™ expression system, under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter (PGAP) as, it has been well established that protein expression, folding and secretion in *P. pastoris* expression system are directly influenced by culture condition (temperature, pH, carbon source, oxygen availability, specific growth rate…) [14–18]. Experiments were conducted first in shake flask scale, and then the optimized parameters were applied in a fed-batch bioreactor culture. The produced protein was then purified and characterized.

2. Materials and methods

2.1. Strains and plasmids

A chemically competent *Escherichia coli* TOP10 Bacterial strain (Invitrogen, Life Technologies Corporation AS, CA, USA) was used for propagation of recombinant vectors.

The PichiaPink Expression Strain Set (Invitrogen) was used in this work, containing 4 *Pichia pastoris* adenine auxotrophic strains three of which are Protease-deficient. The parental strain, Strain 1 from which the other strains are derived is of genotype **ura5Δ::ScSUC2 ade2Δ::lacZ-URA5-lacZ** hence, all strains carry the ade2 deletion.

The PichiaPink vectors pPINK-HC and pPINK-LC and the pGAPZαB (Invitrogen) were used for the construction of the expression vectors.

The plasmid Ppuzzle containing the PDI1 chaperone sequence available in our laboratory and used in a previous work [19], was used for the co-expression of folding-assisting factor. The plasmid contains a resistance gene for G418 to select transformants.

2.2. Construction of recombinant expression vectors

The pPINK-HC and pPINK-LC vectors express proteins intracellularly under the methanol-induced *AOX1* promoter by default. Both vectors were modified in order to introduce the sequence of the α-mating factor secretion signal and replace the AOX1 promoter by the GAP promoter. The vectors were double digested using BglII and KpNI to remove the AOX1 promoter sequence. The obtained pPINK-HC and pPINK-LC vectors backbone were ligated to the fragment containing the GAP promoter _α- factor secretion signal sequence resulted from restriction digestion of the pGAPZαB vector using BglII and EcoRI restriction enzymes.

The codon optimized RABV-G sequence (Rabies virus PV strain), based on the codon usage bias of *P. pastoris* for optimal expression, (Genbank, accession number **KT878717**, obtained from GeneCust (Dudelange, Luxembourg) was PCR amplified using primers with the following sequences containing respectively EcoRI and KpnI restriction sites indicated in bold.:

Forward: TACT**GAATTC**AAGTTTCCAATTTACACTATTCCA

Reverse: AGTA**GGTACC**TTACAAACCAGTTTCACCACCAGACTTGTA

The PCR product were double digested by the endonucleases EcoRI and KpnI and ligated in addition to the GAP promoter _α- factor secretion signal sequence to the pPINK-HC and pPINK-LC vectors backbone in a three-way ligation. To generate the recombinant expression vectors pPINK-HC::αGAP_RABV-G **(pHC-αG)** and pPINK-LC::αGAP_RABV-G **(pLC-αG)**.

2.3. Yeats transformation

The four Pichia Pink strains were made electrocompetent according to the manufacturer's instruction and each strain was transformed with SpeI linearized pHC-αG or pLC-αG and the AvrII linearized Ppuzzle, both at the same time.

After electroporation in a Gene Pulser Xcell System (BIORAD), YPDS media was added to electroporated cells and incubated for 2 h at 30 °C. The culture was then spread onto PAD (pichia adenine dropout) agar plate plus 500 μg/ml G418 and incubated at 30 °C until white distinct colonies are formed. The integration of the expression cassette in the genome of the obtained transformants, was verified by colony PCR with primers for the RABVG.

2.4. Culture media

The recombinant expression strains were grown on chemically defined media (CDM) contained per liter: Carbon source (20 g of Glucose, Glycerol, Sucrose, Lactose or Sorbitol, or 1%Methanol), 2 g citric acid monohydrate, 12.6 g (NH4)2HPO4, 0.5 g MgSO4•7H2O, 0.9 g KCl, 0.022 g CaCl2•2H2O, and 4.6 mL PTM trace salts stock solution, 2.5 ml Vitamins Solution.

PTM trace salts stock solution contained per liter: 6.0 g CuSO4•5H2O, 0.08 g NaI, 3.0 g MnSO4•H2O, 0.2 g Na2MoO4•2H2O, 0.02 g H3BO3, 0.5 g CoCl2, 20.0 g ZnCl2, 5.0 g FeSO4•7H2O, and 5.0 mL H2SO4.

Vitamins Solution: 0.8 g Calcium panthotenate, 8.0 g myo-inositol, 0.8 g Thiamine dichloride, 0.8 g Pyridoxine hydrochloride, 0.2 g Nicotinic acid, 0.8 g D (+)- biotine, 4.0 g K2HPO4

The feed solution contained per liter: 400 g Glycerol or glucose or Sucrose and 12 ml PTM1 trace salts stock solution

The semi-defined feed solution contained per liter: 400 g Glucose, 15 g casamino acid and 12 ml PTM1 trace salts stock solution

2.5. Culture conditions

2.5.1. Shake flask

The recombinant clone was cultured in 5 mL YPD medium in a 50 mL conical flask overnight in a shaker incubator at 30 °C, 250 rpm agitation rate. Cells were collected by centrifugation (2500 g, 3 min) and resuspended in 250 ml baffled Erlenmeyer baffled flasks containing 25 ml of CDM media, unless otherwise stated, supplemented with the appropriate carbon source. Each culture was initiated at one unit OD 600 nm and incubated for 72 h in a shaker at 30 °C and 180 rpm, unless otherwise stated. Samples of each culture were collected periodically each 24 h and analyzed for cell density and RABV-G production. Cultures were supplemented with 2% carbon source repeatedly every 24 h.

2.5.2. Bioreactor

Scale-up expression was performed in a 5-L bioreactor (Infors, Bottmingen, Switzerland). For the inoculum preparation, yeasts cells were pre-grown in a shaker incubator at 30 °C, 250 rpm agitation rate in a 2 L Erlenmeyer baffled flask filled with 200 mL of CDM medium containing 2% (w/v) glycerol. The overnight culture was then used to inoculate the initial batch culture of 2 L CDM working volume

containing 40 g/l (w/v) of carbon source. The fermentation parameters were set as follow: growth temperature = 30 °C, pH controlled at 7 by addition of 5 M KOH, stirring rate = 600 rpm, dissolved oxygen was set at 40% of air saturation by 1 vvm aeration, stirring and enrichment of the inlet air with pure oxygen. The fed-batch growth phase was started when the initial carbon source was exhausted after about 24 h.The feed medium was fed according to a predetermined exponential feeding rate and with a controlled specific growth rate μ = μmax/2 using the following equation [20].

$$F(t) = \frac{\left(\mu V(t_0) X_0 e^{\Lambda(\mu(t-t_0))}\right)}{S Y_{\frac{x}{s}}}$$

where F is the feed rate (l/h); V is the culture volume when the medium feed started, (l); X is biomass dry cell weight (g /l); μ is the specific growth rate (h^{-1}); S is carbon source concentration in the feed medium (g/l); $Y_{X/S}$ is the cell yield on substrate (gX/gS); t and to are the run time and the run time at the start of the fed-batch production period respectively (h) and μmax is the maximum specific growth rate determined during the batch phase.

Samples were taken periodically and analyzed for biomass, recombinant protein production and residual carbon source detection.

3. Analytical methods

Cell density was determined by measuring the absorbance at 600 nm (OD.600). Correlation between OD 600 nm and dry cell weight was determined according to standard protocols. One unit of OD 600 was found to be equivalent to 0.27 g/l dry cell weight DCW.

Residual glycerol and glucose concentrations in the culture media were determined using Glycerol Assay Kit (Megazyme) and Glucose GOD-PAP kit (BIOLABO) respectively.

3.1. Protein purification

Tow liters of culture broth were centrifuged to remove yeast cells and debris at 13,000 rpm for 15 min at 4 °C. The culture supernatant was concentrated by ultrafiltration using Millipore Cogent μScale TFF System through an Ultracel membrane of 10 kDa molecular weight cut-off. During the TFF process a diafiltration step was applied for buffer exchange by adding the start buffer of the next step purification: Phosphate buffer (50 mM; pH 6.7). A final concentration was then applied and the retentate was used for further purification. One ml of the concentrated sample was loaded to a preequilibrated HiTrap SP HP 1 ml column connected to an AKTA purifier system. The bound proteins were eluted with a linear gradient of 1 M NaCl. The purity of the recombinant protein in the eluted fractions was analyzed by Silver stained SDS-PGE gels and the protein concentration was estimated by a Nanodrop spectrophotometer.

3.2. Polyacrylamide gel electrophoresis assay

Protein samples were separated on 10% polyacrylamide gel electrophoresis (PAGE) in the presence of the detergent sodium dodecyl sulfate (SDS) and under reducing conditions in presence of β-mercaptoéthanol and heat denaturing. While native PAGE, conducted to analyze the oligomerization state of the purified RABVG has been caried in absence of all denaturing and reducing reagents (SDS, β-mercaptoéthanol and heat denaturing). PAGE gels were then silver stained using ProteoSilver Silver Stain Kit (Sigma Aldrich) or transferred to a nitrocellulose membrane (GE Healthcare) for immunoblotting assay.

3.3. Immunoblotting assay

The polyacrylamide gel separated proteins were transferred to a nitrocellulose membrane (GE Healthcare) using a Bio-rad wet transfer system. The nitrocellulose membrane was blocked overnight at 4 °C in blocking buffer: PBS 1X, 0.02% Tween-20- 3% skimmed milk. After being washed (washing buffer: PBS 1X, 0.5% Tween-20) the membrane was incubated 1 h with a 1:2000 diluted in-house produced and purified polyclonal anti-rabies virus immunoglobulin produced in horse immunized with inactivated Rabies virus. The membrane was washed 3 times with the washing buffer and incubated 1 h with an anti-horse IgG-HRP conjugate produced in rabbit (Sigma Aldrich) and then washed again 3 times with washing buffer before adding the ECL solution (GE Healthcare) HRP substrate. The protein was detected on an X-ray film (GE Healthcare).

3.4. Deglycosylation of the purified protein

To assess whether the recombinant rabies virus glycoprotein was correctly N-glycosylated, 10 μg of the purified recombinant protein and the native rabies virus glycoprotein contained in the reference rabies vaccine were denatured at 100 °C in presence of the Glycoprotein Denaturing Buffer then treated by the PNGase F (Biolabs) according to the manufacturer instructions. The treated, and the untreated proteins were analyzed by western blotting to detect molecular weight shift resulting from the N-glycans removal.

3.5. Fluorescence inhibition test

A threefold serial dilution of the purified recombinant RABV-G was performed in a 96-well plate. A 50 μl of the Rabies virus strain Pasteur vaccine (PV) previously diluted at 1:80 a dose that causes infection in 100% of BHK-21. 50 μl of BHK21 cells was added to each well of the plate (6×10^4 cells/ well). BHK-21 cells were then added to each well and incubated for 22 h at 37 °C, 5% CO2. As positive inhibition control, only 100 μL of MEM culture media was added to the cells and as negative inhibition control the cells were incubated in presence of only the virus. The cells were washed and then fixed with acetone 80% for 30 min. The plate was stained with fluorescein-labeled anti-rabies nucleocapsid immunoglobulins (Sanofi Diagnostic Pasteur, Marnes la Coquette, France).

3.6. MTT assay

The cell viability assay was performed using Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma M5655). BHK-21 cells (5 × 104 cells/ well) were deposited in 96-well tissue culture microtiter plates with 50 μL of various protein concentrations of the RABVG/ the Rabies reference vaccin or 50 μl of MEM medium for the untreated Cell control. The plate was incubated 24 h at 37 °C, under a humidified 5% CO2 atmosphere. The medium was then removed and 50 μL of 1 mg/ mL MTT solution, prepared in MEM-10, was added to each well and the plates were incubated three hours at 37 °C, under a humidified 5% CO2 atmosphere. The MTT solution was removed and 100 μL of dimethyl sulfoxide (DMSO) were added to each well and the plate was gently shaked to solubilize the formazan crystals. The absorbances were measured at 560 nm using plate reader. The percentage of cell viability was determined as% viability = 100 x (sample absorbance/ untreated cells absorbance).

3.7. Enzyme-linked immunosorbent assay test (ELISA)

The quantification of secreted RABV-G in the fermentation culture broth was performed by an indirect ELISA test using as standard a Rabies reference vaccine (validation antigen Lot 09) reconstituted in 1 mL of distilled water corresponding to a concentration of 10 μg/mL of rabies virus glycoprotein (Rabies PV strain) obtained from Pasteur Institute of Paris. Culture supernatant samples of 100 μl each were coated, in duplicate, in a 96-well plate after being diluted in sample buffer containing Polyoxyethylene −10 tridecylether (Sigma P2393) 1% and Diethanolamine (BDH 103,934) 1.5% in PBS. After incubation for 2 h at

37 °C, the plate was washed 5 times with wash buffer (PBS 1X, 0.05% Tween-20) and 100 µl of the monoclonal antibody anti-glycoprotein TW1 (NIBSC, Hertfordshire, UK) diluted to 1/10,000 in blocking buffer (BSA 2% in PBS 1X, 0.2% Tween-20) was added to each well and incubated for 2 h at 37 °C. The plate was washed as before and 100 µl of anti-human IgG – Peroxidase conjugated antibody (Sigma Aldrich) was added and incubated 1 h at 37 °C. Finally, the plate was washed, and the peroxidase substrate, OPD (o-phenylenediamine dihydrochloride) (Sigma Aldrich) was added and incubated 30 min in the dark and after the incubation period the plate was read at 450 nm.

3.8. Determination of transcript levels by real-time PCR

Recombinant yeast cells were grown on different carbon sources for 24 h at 30 °C and 180 rpm. sample was taken from the different culture conditions and diluted to obtain 1 ml sample of one unit OD 600 nm for each condition. The cells were pelleted, and the total RNA was extracted using TRI Reagent solution (Sigma Aldrich) according to manufacturer's instructions. One µg of the total RNA extracted was used to synthetize the first strand cDNA using the SuperScript™ IV First-Strand Synthesis System (Invitrogen) according to manufacturer's provided protocol. The transcripts level was then determined by quantitative real-time PCR using SYBR Green I Master reagent and The LightCycler® 480 System (Roche). The expression levels of the RABV-G gene were then calculated by applying the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) using the transcript level of cell grown on glucose as calibrator.

The following primers of the RABV-G and the β-actin endogenous housekeeping gene were used in this experiment: **Actin-F:**

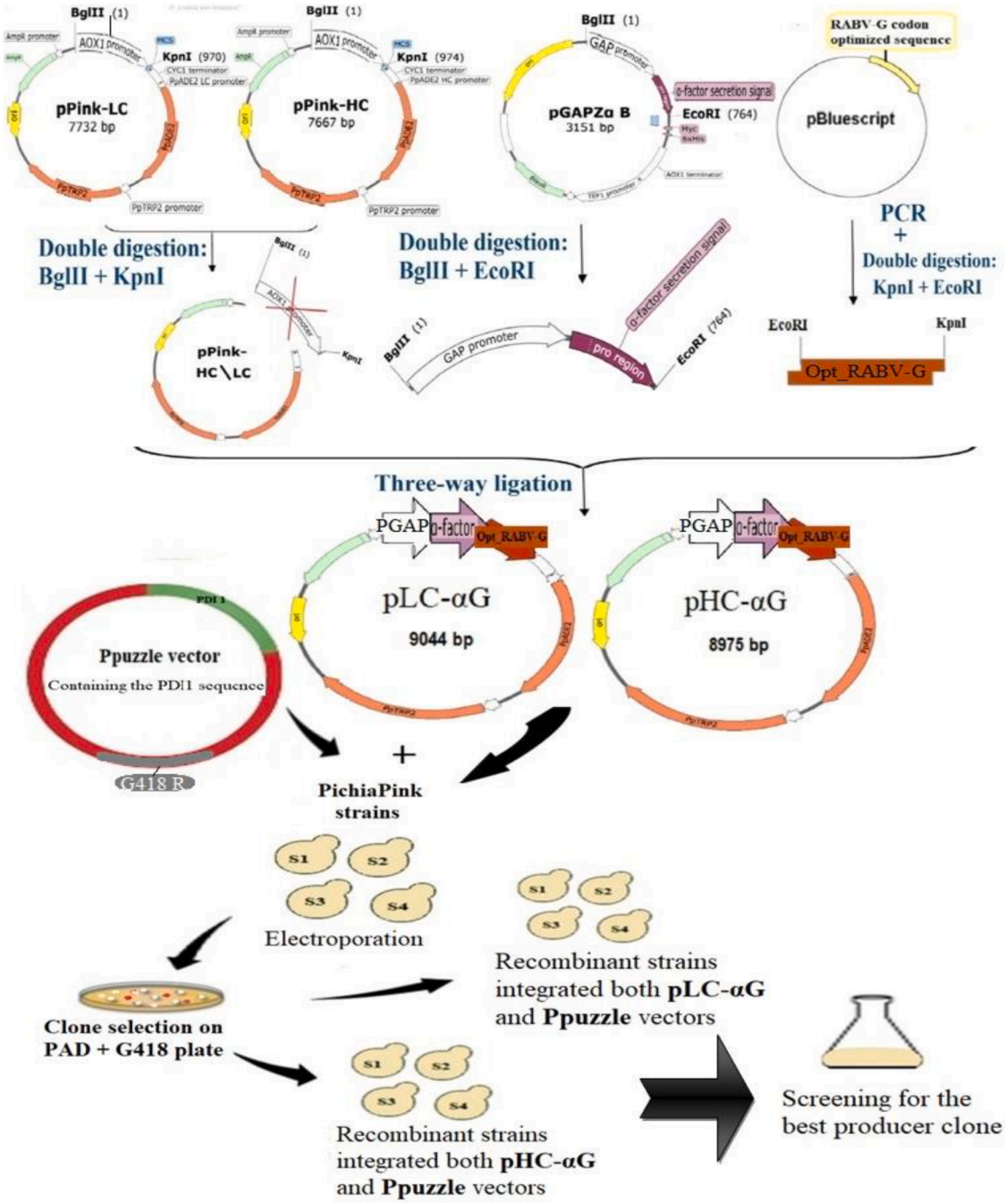

Fig. 1. Schematic representation of the expression vectors construction and expression Strains transformation.

CCTGAGGCTTTGTTCCACCCATCT. **Actin-R:** GGAACATAGTAGTAC CACCGGACATAACGA. **qRABV-G-F:** TCCATTGGCTGATCCATCTACTG. **qRABV-*G* – *R*:** CCAGTTTGGCAAACCCAGATC

4. Results

4.1. Construction of the expression vectors and recombinant strain selection

Protein expression in *P. pastoris* is mostly controlled by the strong inducible promoter AOX1 of the alcohol oxidase 1 encoding gene, the first enzyme of methanol metabolism pathway. Although the AOX1 is a strong and a tightly regulated promoter, the constitutive GAP promoter simplifies the cultivation strategy and help to avoid the need for using toxic methanol as inducible carbon source [21]. The commercialized vector set of the PichiaPink System are designed for inducible intracellular protein expression. Therefore, the pPINK-HC and pPINK-LC vectors were modified to replace the inducible promoter AOX1 contained by the constitutive GAP promoter and to integrate the α- factor secretion signal sequence in frame with the codon optimized RABV-G **(opt-RABV-G)** sequence. The construction of the recombinant expression vectors pPINK-HC::αGAP_RABV-G **(pHC-αG)** and pPINK-LC::αGAP_RABV-G **(pLC-αG)** was a three-way ligation (Fig. 1). The obtained recombinant vectors pHC-αG and pLC-αG were checked by sequencing before electroporation of competent Pichia pink cells.

It has been reported that co-expression of Protein Disulfide Isomerase 1(PDI1), one of the endoplasmic reticulum (ER) foldase recruited in disulfide bond processing in nascent protein, is a suitable approach to improve heterologous protein folding and secretion in different case study [22] and especially in the production the RABV-G [19]. Therefore each strain of the of the Pichia pink strains were transformed by both: one of the recombinant vectors pHC-αG and pLC-αG in addition to the Ppuzzle vector containing the PDI1 sequence [19,23]. The transformation of the four used Pichia Pink strains and the two different recombinant vectors combination led to eight different group of recombinant strains (Fig. 1).

Six white colonies were randomly selected from each transformation condition for RABV-G productivity screening by Western blotting. The RABV-G corresponding band, of the expected 65 kDa molecular weight was present in all analyzed culture supernatants (data not shown). The best producer clone, which have the most intense band, was an S4 strain containing the pLC-αG vector. This clone named S4LC-G, was retained for further culture condition optimization.

4.2. Shake flask culture conditions

Since the carbon source has an impact on the strength of the expression of PGAP and therefore on recombinant protein production [24], the influence of seven different carbon sources (Table 1) on RABV-G production under different culture conditions of temperature, agitation, and working volume was investigated.

4.2.1. Influence of temperature

The productivity of the selected RABV-G producer clone S4LC-G was screened in shake flask at different temperatures: 20, 25, 30 and 32 °C. The culture was conducted in chemically defined media in presence of different carbon sources and maintained for 72 h at 180 rpm, with periodic sampling every 24 h for growth and productivity profiling.

Table 1
Screened carbon source types.

Monosaccharide(Used at 2% W/V)	Disaccharide(Used at 2% W/V)	Alcohol(Used at 1% V/V)	Sugar alcohols (Used at 2% W/V)
Glucose Fructose	Sucrose Lactose	Methanol	Sorbitol Glycerol

All carbon sources maintained, approximately, their same impact on growth profile at all temperature tested. The highest growth rate was obtained in presence of glycerol followed by glucose and fructose whereas methanol, sorbitol and lactose did not support cell growth in all screened temperature conditions. Cell growth on sucrose was most important at 30 °C (Fig. 2). Incubation temperatures supported RABV-G production were 20 °C,30 °C and 32 °C. The optimal temperature to produce the RABV-G for all the investigated carbon sources was 30 °C. At 20 °C only glycerol, fructose and glucose supported the production of RABV-G.

It has been reported that growth temperatures above 32 °C could stop protein expression, induce ER stress, and cell death [25,26]. In this study, the effect of high temperature incubation was observed on the clone productivity rather than the growth profile. The intensity of the RABV-G band was less intense than the one obtained at 30 °C incubation temperature for all carbon sources.

The production of RABV-G under different conditions gave different Western blotting profiles. The analyzed supernatant of cultures conducted on glycerol, fructose and glucose as a carbon source shows the same western blot profile: in addition to the RABV-G of 65 kDa, extra bands at higher molecular weight are present at all temperatures. These bands become more intense along the incubation time in contrast to the band of 65 kDa which become less intense by 72 h of culture. These extra bands may correspond to oligomerized forms of the RABV-G. In fact, the native rabies virus glycoprotein is known to undergo pH dependent conformational changes and it was reporter that an acidic pH incubation result in the exposition of surface hydrophobic regions leads to self-aggregation of the glycoprotein and viral particles [27,28] which may explain the detection of these bands in cultures grown on carbon sources favoring high biomass production resulting in the acidification of the culture media.

Western blot profile of culture supernatants grown on lactose as the sole carbon source, shows a band which is more intense and of lower molecular weight than the expected RABV-G band, it seems to be a non-glycosylated form of RABV-G as it has the same molecular weight as the PNGase F treated RABV-G product (Fig. 6-c) and the computed molecular weight of the RABV-G amino acid sequence of 56,594.91 Da. Only this form was detected in 72 h sample of culture incubated on lactose at 20 °C and in addition to the 65 kDa band in 48 h and 72 h samples incubated at 30 °C and was not present in samples incubated at 32 °C.

The influence of each carbon source on the RABV-G transcription level under the optimal growth temperature 30 °C, led to RABV-G production on all screened carbon sources, was also examined. The mRNA of the RABV-G was quantified by qPCR relatively to β-actin gene in 24 h total RNA samples. the transcripts levels were then calibrated to the transcript level of RABV-G in cells grown on glucose the commonly used carbon source in PGAP controlled expression systems. The highest transcription level was detected in lactose, methanol then in sucrose grown cells. The transcription level on glycerol was 4-fold higher than glucose and fructose obtained transcription levels (Fig. 3).

4.2.2. Influence of oxygen availability

The dissolved oxygen content is a key parameter in process development for recombinant protein production because of its great impact on the host cell physiology and metabolism and so, on the productivity. *Pichia pastoris* is classified as Crabtree-negative yeast preferring to grow in a respiratory mode and it exhibits specific physiological responses in hypoxic condition such as an upregulation of the genes under the control of glycolytic promoters like the GAP promoter [29].

To investigate the influence of oxygen availability on the RABV-G production in *P. pastoris* together with the use of a variety of carbon sources, shake flask cultures were carried out at 30 °C, 180 rpm agitation and at 5:1 and 3:1 total volume/culture volume ratio. This incubation conditions could create a low oxygen environment as these filling volumes are considered above the fill volume, of 10:1 flask/culture volume

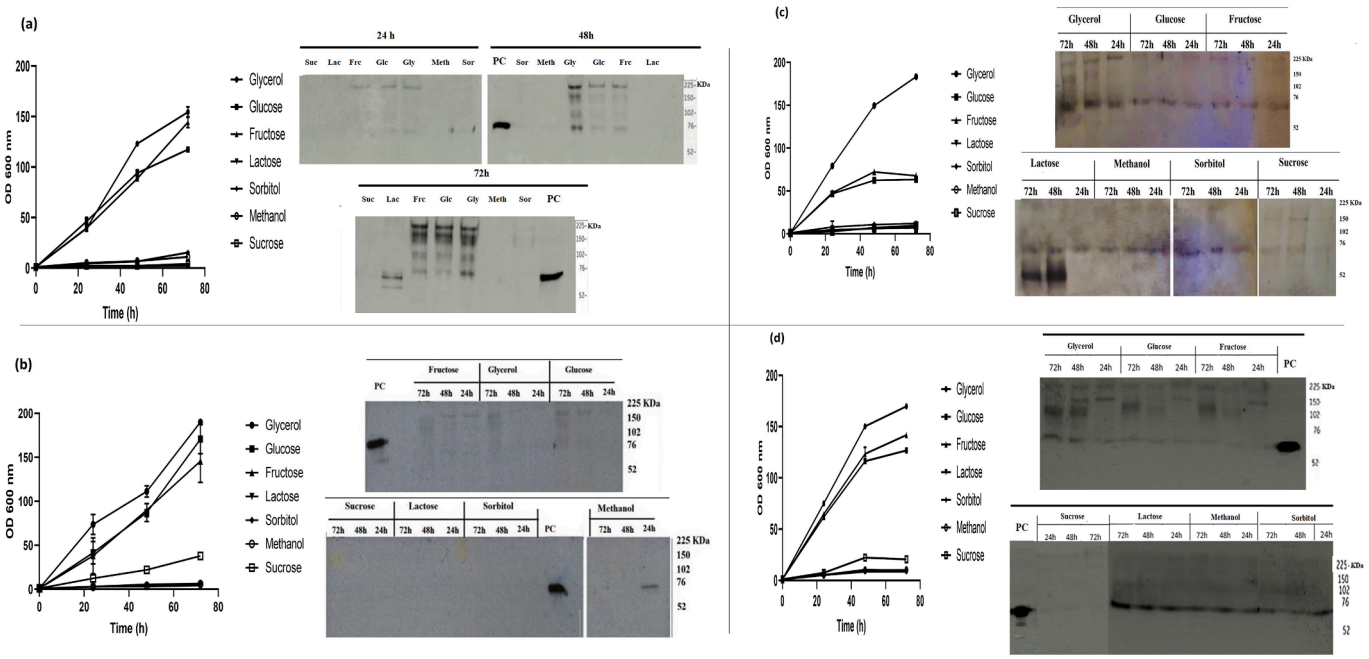

Fig. 2. Influence of growth temperature and carbon source on recombinant RABV-G production in shake flask culture. Growth profile (cell density measured at OD 600 nm) and Western blot analyses of the productivity of the recombinant clone S4LC cultured in shake flask using seven different carbon sources (Glucose (Glc), Glycerol (Gly), Fructose (Frc), Lactose (Lac), Sorbitol (Sor), Methanol (Meth)) at 180 rpm agitation rate, in filling volume of 10:1 flask/culture volume ratio and at incubation temperature of (a) 20 °C, (b) 25 °C, (c) 30 °C, and (d) 32 °C. the secretion of the recombinant RABV-G was investigated by western blot analyses of 20 µl of pelleted culture broth samples from different culture time-points (24 h, 48 h, and 72 h). PC (= positive control): native Rabies virus glycoprotein contained in Rabies reference vaccine consisting of purified inactivated rabies virus particles of PV vaccine strain.

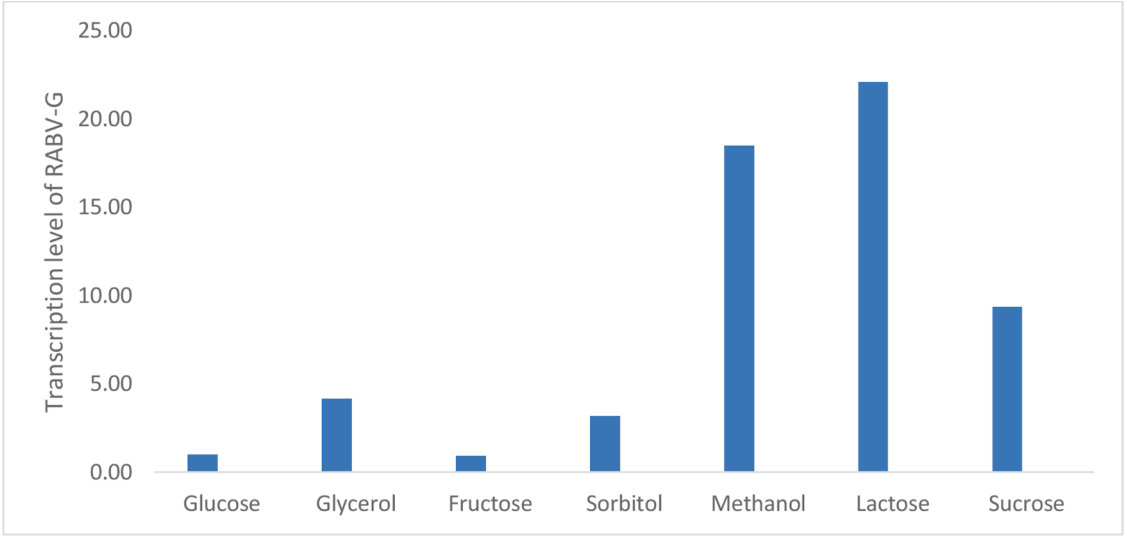

Fig. 3. Relative transcription level of gene encoding the recombinant RABV-G in diffrent carbon source growth condition at 30 °C and 180 rpm shake flask cultures. Yeast cells total RNA was extracted at the 24-h time point, and the mRNA level of RABV-G was quantifiedgene by real time PCR, relative to β-actin endogenous gene and then the transcript levels were normalized relative to cells grown on glucose the commonly used carbon source in GAP promoter expression systems .

ratio, producing better oxygen transfer [30].

These oxygen-limiting conditions were not of significant impact on the growth profile compared to the previous obtained results of incubation at 30 °C, 180 rpm agitation and in 10:1 flask/culture volume ratio, therefore the growth data is not presented here. However, RABV-G production was found to be greatly influenced by the reduction of oxygen availability. When cultures were carried in 5:1 flask/culture volume ratio, the productivity was dramatically reduced on glycerol, glucose, and fructose carbon sources according to western blot profile and no band was detected in methanol culture samples. The analyzed supernatants from cultures containing sucrose, lactose, or sorbitol as carbon source show only the “non glycosylated form” band which is lower of the RABV-G molecular weight and of high intensity (Fig. 4-a), remembering the same band detected when lactose was used as carbon source in the precedent experiments (Fig. 2).

When cell culture environment was becoming more hypoxic by increasing the filling volume up to 3:1 flask/culture volume ratio, RABV-G band of 65 KDa was no more detectable in any of the analyzed samples, only the “non glycosylated form” was present in all 24 h samples except glucose and sucrose and in all 48 h and 72 h samples except for

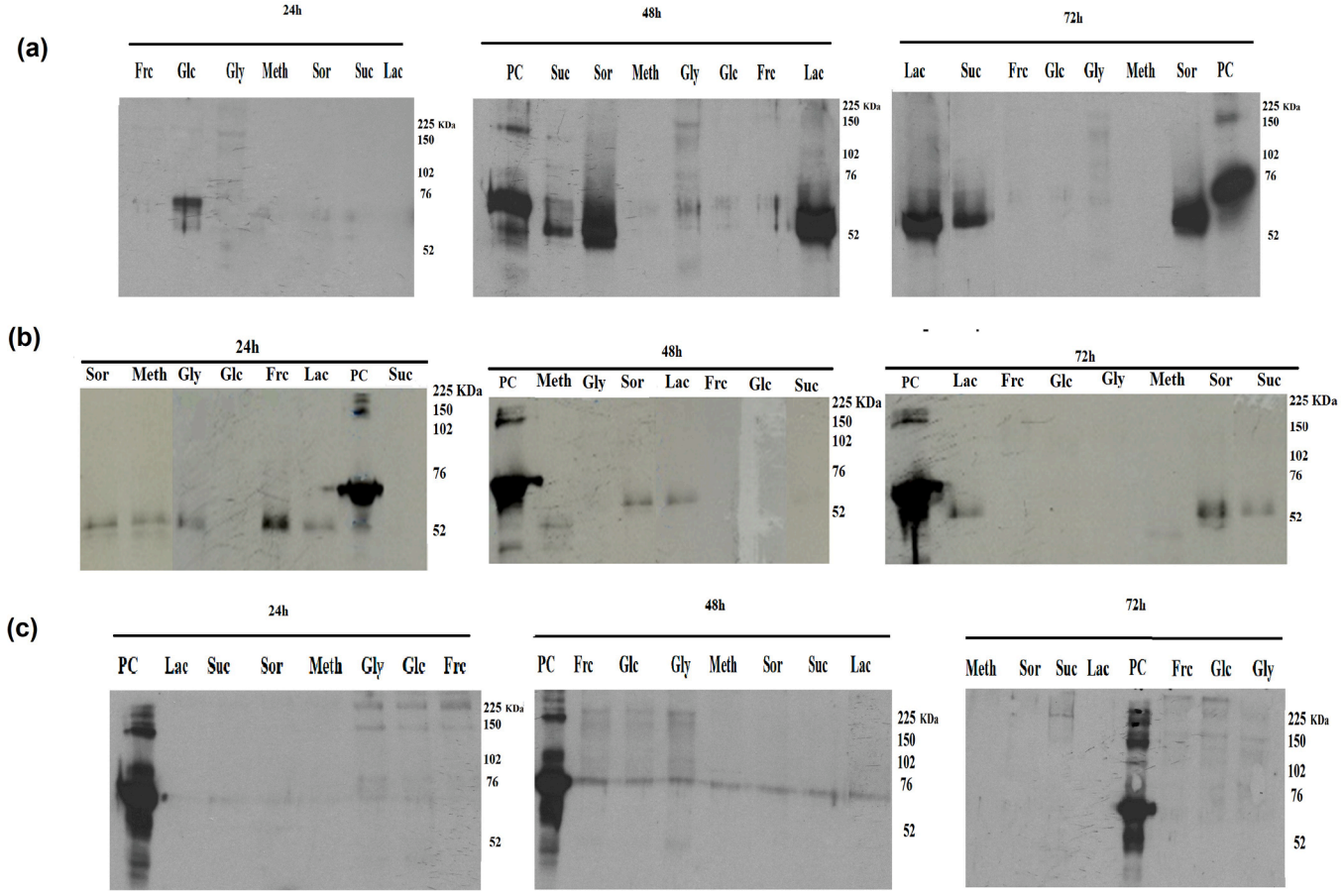

Fig. 4. Influence of oxygen availability and carbon source on RABV-G production in Shake flask culture. The production of RABV-G was investigated by western blot analysis of 20 µl of pelleted culture broth samples from different culture time-points (24 h, 48 h, and 72 h) of cultures grown on seven different carbon sources (Glucose (Glc), Glycerol (Gly), Fructose (Frc), Lactose (Lac), Sorbitol (Sor), Methanol (Meth)) in hypoxic condition at30 °C, 180 rpm in filling volume of (a) 5:1 and (b) 3:1 flask/culture volume ratio and cultures grown in ameliorated oxygen transfer condition (c) at 30 °C, 250 rpm and in filling volume of 10:1 flask/culture volume ratio. PC (= positive control): native Rabies virus glycoprotein contained in Rabies reference vaccine consisting of purified inactivated rabies virus particles of PV vaccine strain.

glycerol fructose and glucose (Fig. 2-b). According to these results reduction in oxygen availability favored the production of a "non-glycosylated form" of RABV-G. Also, the production of a non-glycosylated form of RABV-G or the total loss of production as a result of oxygen limitation, seem to be carbon source dependent and that may be because of the differences in their metabolic pathways, or they can be also growth dependent as the carbon sources with relatively low cell growth presented similar production profiles.

In order to enhance the oxygen transfer in the shake flask, the agitation rate was increased to 250 rpm in a filling volume of 10:1 flask/culture volume ratio. This incubation condition resulted in the disappearance of the "non-glycosylated form" even in culture conducted on lactose as carbon source. The RABV-G band was found only in 48 h samples of all carried cultures (Fig. 2-c).

4.3. High cell density fermentation

Recombinant rabies virus glycoprotein production was also investigated in a 5 L bioreactor. A fed-batch culture was performed at 30 °C, pH 7, 40% of dissolved oxygen (DO) and an agitation rate of 600 rpm. Glycerol was used in the batch phase, since it's a non-fermentable substrate mainly used as initial carbon source in *P. pastoris* fermentations to generate high cell density. A high biomass concentration, of about 40 g/l dry cell weight (DCW), was attained at the end of the batch stage (Fig. 5). However, the high cell density was not accompanied by a high RABV-G production as shown by the Western blot profile of analyzed samples from various time-points (Fig. 5).

The fed batch phase was started after total glycerol depletion, which is marked by a slight increase in the pH value. A predetermined exponential feeding strategy was applied using glycerol or glucose as carbon source. Biomass production was higher on glycerol than on glucose. The high accumulated biomass resulted in more oxygen demand leading to the decrease of the DO concentration below 10%. Therefore, the culture was stopped after 27 h when DO is near zero and the bioreactor oxygen transfer capacity become unable to provide the biomass oxygen need (Fig. 5-a). When glucose was used as fed carbon source the culture was kept longer, and the DO value did not fall to a critical level. However, RABV-G production was not better on glucose (Fig. 5-b). The use of semi-defined feeding solution, containing 1.5% casamino-acids in addition to glucose, enhanced the productivity 4.7-fold compared to cultures fed with glycerol or glucose defined solutions which led to a final secreted RABV-G concentration of about 2.7 µg/l as determined by indirect ELISA test. The addition of casamino-acids to the feeding solution has also boosted the biomass production which resulted in a sharp decrease of DO level. Even by the increase of the agitation rate up to 1000 rpm, the dissolved oxygen concentration was continuously decreasing as biomass increased (Fig. 5-c).

The western blot profile of various time points samples analysis shows the presence of extra bands of lower and higher molecular weight in addition to the RABV-G band of 65 kDa (Fig. 5). According to the

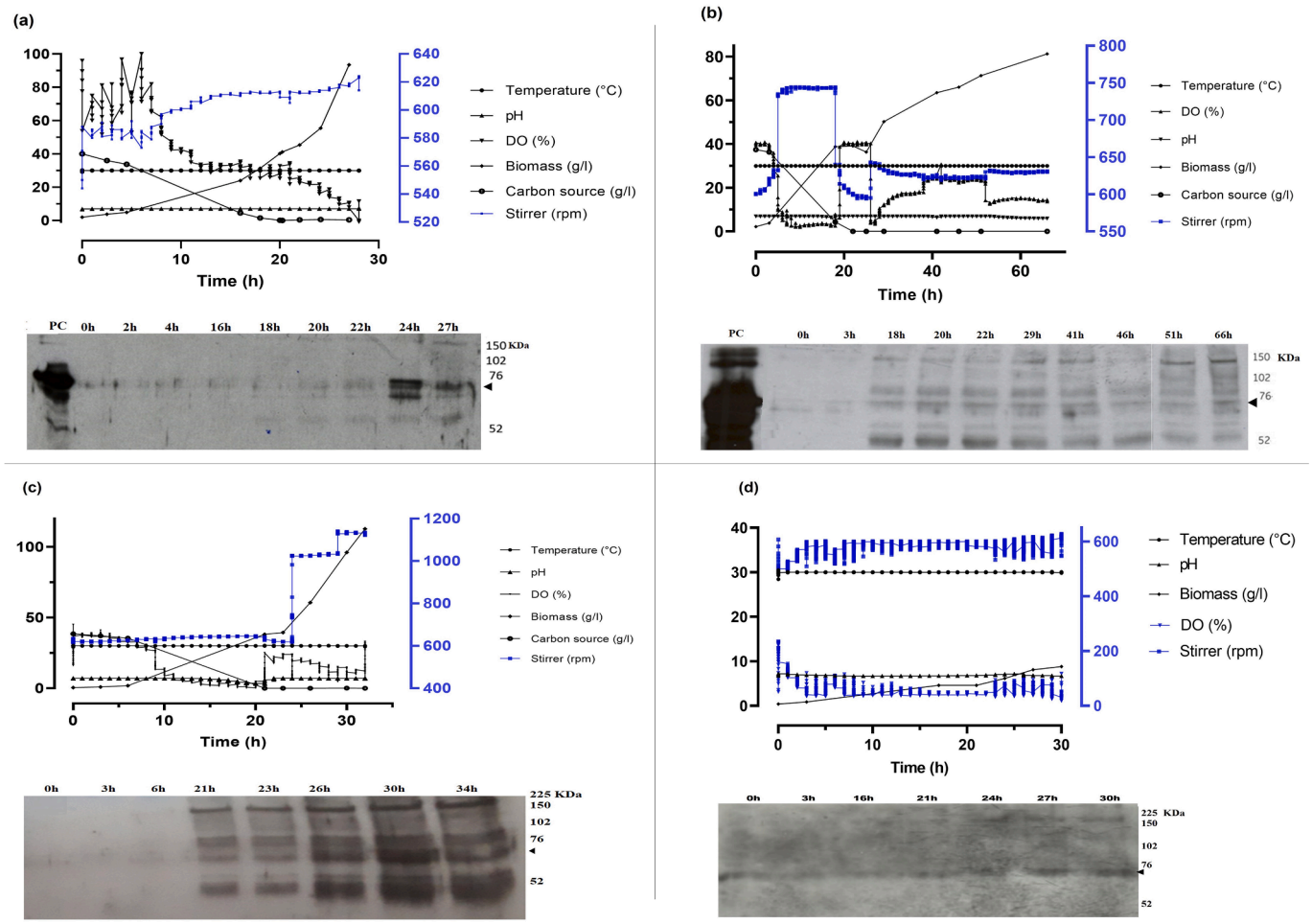

Fig. 5. Time courses of fed-batch fermentation production of the recombinant RABV-G in 5 l bioreactor. Curves (upper part) of Biomass production (Dry cell weight g/l), Residual Substrate (carbon source g/l), Dissolved oxygen level (DO%), Stirrer rate (rpm), Temperature (°C) and pH variation in addition to western blot analysis (lower part) of time-course secretion of the produced RABV-G during cultures grown, in the batch phase, on glycerol as carbon source and fed with glycerol (a) or glucose (b) or glucose + casamino acid (c) in the fed batch phase; and culture grown on sucrose as carbon source in both the batch and fed batch phases (d).The secretion of the recombinant RABV-G was investigated by western blot analyses of 20 µl of pelleted culture broth samples from different culture time-points. The arrow on western blot figures indicates the monomeric correctly glycosylated form of RABV-G and the higher bands represent aggregated and oligomerized forms while the lower bands are non-glycosylated forms. PC (= positive control): native Rabies virus glycoprotein contained in Rabies reference vaccine consisting of purified inactivated rabies virus particles of PV vaccine strain.

previously obtained results in the shake flask cultures, the bands below 65 KDa may be the non-glycosylated form of RABV-G as these bands are becoming more intense when dissolved oxygen concentration decreased. The superior bands could be the aggregated forms of RABV-G supposed to be formed as a result of culture medium acidification. The culture pH was set at 7, but because the dead-band was adjusted to 0.5 there was a slight decrease in the pH which led to the aggregation of the RABV-G (Fig. 5).

The impact of sucrose, as alternative carbon source, on the RABV-G production was also investigated. As in shake flask culture, this carbon source gave a relatively low cell density compared to glucose and glycerol which helped in a way to avoid oxygen limitation condition and thus the secretion of the RABV-G non-glycosylated form (Fig. 5-d).The ELISA determined concentration of recombinant RABV-G obtained at the end of the culture was 2.6 µg/l, with a biomass concentration of 8.802 g-DCW/l, corresponding to a specific product yield ($Y_{P/X}$) of 0.310 µg/g DCW which is higher than yields obtained on cultures grown on glycerol during the batch phase and fed with glycerol or glucose defined media of 0.032 and 0.06 $\mu g/g_{(DCW)}$ respectively and 2.3 fold higher than yield obtained on culture fed with semi defined feeding solution, during a process time course of about 30 h.

4.4. Protein purification and characterization

Two liters of culture broth containing the secreted RABV-G was centrifuged to eliminate yeast cells and debris. The obtained supernatant was then concentrated, and buffer exchanged on an Ultracel membrane, of 10 kDa molecular weight cut-off, using a Cogent µScale Tangential Flow Filtration (TFF) System. The recovered retentate, containing the recombinant RABV-G, was loaded to a 1 ml HiTrap SP HP cation exchange chromatography column connected to an AKTA purifier chromatography system. The analysis of amino acid sequence of the recombinant RABV-G (web.expasy.org/protparam/) show a theoretical isoelectric pH (pHi) of 7.25. The RABV-G contained in the loading buffer, Phosphate buffer 50 mM, of pH 6.7 will carry a positive charge allowing it to bind to the cation exchanger. Not all RABV-G molecules present in the sample were retained, there was some losses in the flow-through and wash fractions as shown by SDS-PAGE and western blot analysis of collected fractions (Fig. 6-a). The bound proteins were then eluted by linear gradient of NaCl. The analysis, by western blot and SDS-PAGE, of the collected fractions revealed that the elution of RABV-G extends mostly over the fractions E6-E13, at around 600 mM NaCl, and almost only the RABV-G band of 65 KDa was present. The total concentration of these fractions was 1.174 mg/ml as estimated by

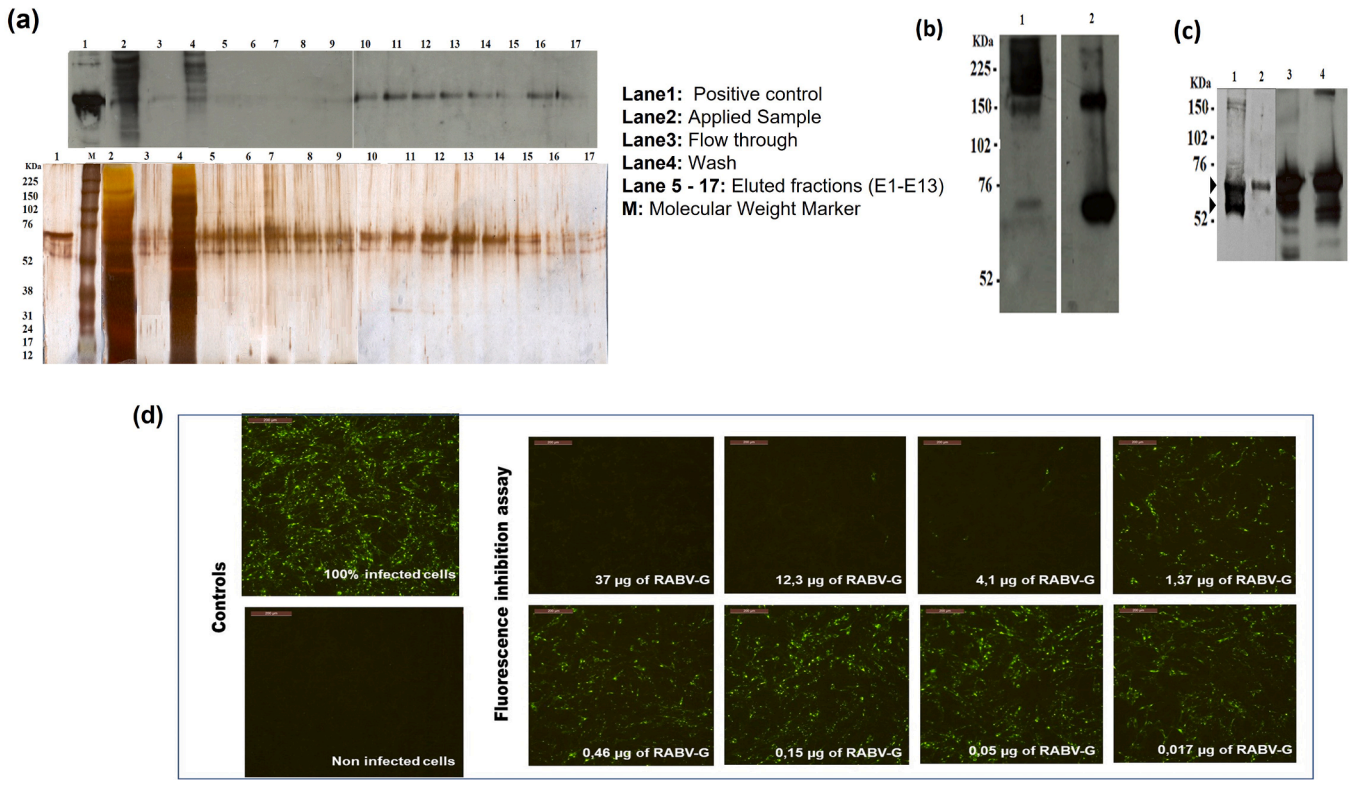

Fig. 6. Purification and characterization of recombinant RABV-G (a) 10% SDS-PAGE silver-stained gel (lower part) and western blotting (upper part) analysis of collected fraction during recombinant RABV-G purification using SP Sepharose cation exchange chromatography column. (b) western blotting analysis of (1) purified recombinant RABV-G and (2) native Rabies virus glycoprotein trimerization state under native PAGE condition. (c) western blot analysis of PNGase treated (1), (3) and untreated (2), (4) purified recombinant RABV-G and native Rabies virus glycoprotein respectively, to characterize the glycosylation pattern of the purified RABV-G compared to the native Rabies virus glycoprotein. (d) competitive inhibition of Rabies virus cell binding in presence of varying concentrations of purified recombinant RABV-G as demonstrated by Fluorescence inhibition assay compared to controls: cell control with no fluorescence and virus control with typical fluorescence of 100% infectivity. Scale bar 200 µm.

Nanodrop spectrophotometer. The collected fractions containing only the RABV-G were pooled, concentrated, and desalted by centricon for further characterization.

The purified protein was treated by the PNGase F, which remove N-linked carbohydrates, to investigate its glycosylation profile. The deglycosylation of the recombinant RABV-G induced a band shift as demonstrated by the western blot profile, indicating that the glycoprotein was N-glycosylated and has the same carbohydrate amount as compared to the deglycosylated Rabies virus native glycoprotein which exhibited the same molecular weight shift after PNGase F treatment (Fig. 6-c). Furthermore, the oligomeric state of the purified protein was characterized by performing an electrophoretic migration under non-denaturing conditions for both the purified recombinant RABV-G and the Rabies virus native glycoprotein. The western blot profile of the analyzed samples shows the presence of dimer and trimer bands of about 130 KDa and 195 KDa respectively (Fig. 6-b), thus the purified recombinant RABV-G is able to mediate correct conformation as the Rabies virus native glycoprotein.

The purified recombinant RABV-G was also able to interact with cell surface receptors and inhibiting, by a competitive mechanism, rabies virus infection of BHK-21 cells as demonstrated by Fluorescence inhibition test. A dose-response effect was observed and 37 µg of RABV-G was able to inhibit 100% of the infection (Fig. 6-d). The fluorescence inhibition test was similarly performed for the reference Rabies vaccine (Fig. S1) and the cytotoxicity effect of both the purified RABV-G and the reference rabies vaccine on BHK-21 cells viability was assessed by microscopic analysis of cell morphology changes and MTT assay (Fig. S2). The obtained MTT results, presented as ratio of untreated cells, show a 20% reduction of cell viability in presence of 37 µg dose of purified RABV-G.

5. Discussion

The rabies virus glycoprotein, a type-1 transmembrane protein, is the main antigen of rabies virus essential for its infectivity, pathogenicity, immunogenicity and involved in the host cell receptor recognition and binding [4,31,32]. Herein, we report the expression, purification, and characterization of the RABV-G in the methylotrophic yeast *Pichia pastoris*. The expression of RABV-G was under the control of the PGAP which, even though is a constitutive promoter, its expression strength varies under certain growth conditions such as carbon source, oxygen supply, substrate depletion and osmotic stress [24,29,33]. In this context, the influence of seven different carbon sources on the RABV-G expression has been investigated in distinct shake flask incubation conditions and the optimized parameters have been then applied for high cell density process. The most commonly used carbon sources in PGAP driven expression systems are glycerol and glucose and their efficiency as substrate for recombinant protein production have been discussed in several studies [24,34]. In this work glycerol, glucose and fructose were good carbon sources for both cell growth and RABV-G production. The RABV-G transcript level was higher on glycerol relative to glucose grown cells. The other screened carbon sources (sorbitol, methanol, sucrose, and lactose), which gave the highest RABV-G transcript level, have supported the recombinant protein production but not biomass proliferation. Thus, growing cells on carbon sources conferring low growth rate, seems to be of a positive impact on the production of RABV-G, and this may be by increasing the specific production of this difficult to express protein. Indeed, it has been reported that recombinant protein synthesis, folding and secretion are greatly influenced by the yeast cell growth rate, however no standard rule exists for the best correlation between the growth rate and the recombinant protein

specific production [17,35,36]. Moreover, in this work, maintaining a low cell density, by using sucrose instead of glycerol and glucose in bioreactor culture, was a good approach to overcome limiting oxygen condition which have been found influencing the produced RABV-G glycosylation pattern in both shake flask and bioreactor cultures. Several transcriptomic and proteomic studies have described the specific response of the Crabtree-negative yeast *P. pastoris* toward hypoxia [14, 29,30]. This adaptive response to reduced oxygen revealed general stress response as well as an upregulation of key pathways such as glycolysis and pentose phosphate pathways in addition to alterations in lipid metabolism. These mentioned physiological changes had beneficial effects on the production and secretion rate, in hypoxic conditions, of a recombinant antibody Fab fragment under the control of the glycolytic GAP promoter [14,29]. Whereas this effect has not been observed in our case and the hypoxic condition led to the production of a non-glycosylated form of the RABV-G. Unfortunately, there is only limited information concerning the direct impact of environmental stress regulations on recombinant proteins expression and folding in *P. pastoris*. Compared to the Crabtree-positive yeast *Saccharomyces cerevisiae*, the yeast *P. pastoris* shows differences in its adaptive regulation of core metabolism at low oxygen availability [29]. However, an impairment of glycosylation pathway precursors affecting the cell wall integrity of the yeast *Saccharomyces cerevisiae* because of oxygen limitation, has been described during the scale up of a high-cell density fed-batch process [37]. Exometabolomics and cell wall integrity analysis suggested that there was a partial inhibition of glycosylation pathway due to low supply of phosphorylated sugars and a low level of mannose-6-phosphate [37]. The modulation of proteins glycosylation patterns was also attributed to changes in glycan cycle and the increased glucose uptake because of the upregulation of glycolysis pathway in addition to the reduction in intracellular adenosine triphosphate (ATP) levels observed during pathologic hypoxic condition in mammalian cells [38,39].

The RABV-G non-glycosylated form was also detected, in the current study, in culture grown on lactose as carbon source. Indeed, *P. pastoris* is not able to grow on lactose not on sucrose except for strains transformed with *Saccharomyces cerevisiae* invertase gene SUC2, like the PichiaPink strains used in this work, can efficiently grow on sucrose [40]. As such, cells grown on lactose as carbon source were facing a starvation stress which explain the very low growth profile they had (Fig. 2). Under carbon limiting condition, *P. pastoris* exhibits an increased accumulation of storage carbohydrate and a physiological adjustment triggering a downregulation of cell cycle genes in addition to a metabolic reprogramming as well as an upregulation of stress-related genes [17,41]. Furthermore, it has been reported that in *P. pastoris* carbon limiting condition decreases global translation [42] and the low growth rate affects recombinant protein N-linked glycosylation [36] which may explain our obtained result. However, we observed that when the incubation temperature and the agitation rate of cultures grown on lactose as carbon source, was increased up to 32 °C (Fig. 2-d) and 250 rpm (Fig. 4-c) respectively, the produced RABV-G was correctly glycosylated and that may be because of the attenuation of the causative effect by the changed incubation parameters. In fact, yeast cells undergoing starvation induce the expression of heat and oxidative stress response genes [42,43]. The enhancement of the oxygen supply by the increase of the agitation rate, has probably reduced the oxidative stress in the starving yeast cells which have been found expressing aerobic respiration genes and consuming more oxygen to ensure longer survival [43]. Similarly, the high temperature incubation was found increasing the oxidative stress, and the overexpression of antioxidant enzyme genes conferring thereby a protection against oxidative stress [44]. Additionally, several studies talked about the generation of mitochondrial reactive oxygen species (ROS) in mammals and yeast cells as an adaptive response to hypoxic condition, leading to an oxidative stress [45,46]. Taken together, these observations tend to suggest, but do not prove, that a causative effect behind the alteration of RABV-G glycosylation may be also the production of ROS and the resulted oxidative stress, which are characteristic of all culture conditions led to the production of a non-glycosylated RABV-G in this study. Hence, different stress adaptive responses can result in the alteration of the recombinant RABV-G N-glycosylation pattern, a molecular approach is then needed for better characterize the mechanism led to these results.

The N-Linked glycosylation pattern of the rabies virus glycoprotein influences its folding and intracellular transport as well as its cell surface expression and Immunogenicity [47–50]. Only one or two of the three potential N-linked oligosaccharide acceptor sites (Asn37, Asn247, and Asn319) present on the amino acid sequence of the Rabies virus glycoprotein, are glycosylated [5,51]. Although the non-glycosylated RABV-G is not efficiently transported to the cell surface, only single oligosaccharide can be sufficient for cell surface expression of this glycoprotein and it was found that core glycosylation at Asn37, which is considered inefficient and biochemically undetectable, still sufficient to support a biological function and cell surface expression [47,49]. Thus, the obtained non-glycosylated RABV-G in our work must be at least core-glycosylated to be secreted and not retained by the sophisticated ER quality control system of *P. pastoris* cell.

During our study we observed that the produced recombinant RABV-G quality is influenced also by the pH of the culture and a slight acidification of the medium led to the aggregation of the secreted protein. In fact, the pH can influence the RABV-G structure and three different pH-dependent states can be undertaken by the Rabies virus glycoprotein: a native state detected at the viral surface above pH 7, an activated hydrophobic state involved in the first steps of the virus fusion process, and the third fusion-inactive conformation [27,52]. Another conformational state, characterized as a transient state, has been detected at acidic pH, responsible of viral aggregation because of exposition of surface hydrophobic region on the protein resulting in its acquisition of distinct biochemical, structural, and antigenic properties [27,28]. The rate and reversibility of this aggregation phenomenon was found pH, temperature, and time dependent [28]. In this regard, considerations must be taken about the incubation's pH of the RABV-G in order to maintain the homogeneity of the final product structure.

During the purification of the recombinant RABV-G, we also tried to keep the pH value close to neutrality to maintain a native conformation of the RABV-G during and after the purification process. The purification was performed using cation exchange chromatography on SP Sepharose column. We observed that the eluted fractions contained mainly the recombinant RABV-G. Indeed, *P. pastoris* presents the advantage to secrete low level of endogenous proteins even during high cell density and its secretome have low isoelectric points determined to be less than six [53,54]. Thus, the chosen buffer pH conditions in our work seem to favor the binding of the recombinant RABV-G, which has an isoelectric point of 7.25, rather than *P. pastoris* contaminant proteins. Consequently, this procedure can be used as single step purification method of the recombinant RABV-G produced in *P. pastoris*. Although different approaches have been applied for efficient purification of the Rabies virus glycoprotein, maintaining the correct conformation and the immunological property of the protein remain the main challenge [10, 55]. In our study, the purified RABV-G has been found glycosylated and able to oligomerize in trimer as same as the Rabies virus native glycoprotein, and it was well recognized by the purified horse anti-rabies immunoglobulin used in all immunoblotting essays conducted during this work. Further evidence of the correct confirmation of the purified recombinant RABV-G was its ability to interact with BHK-21 Rabies virus entry receptors in a competitive binding mechanism displayed as an inhibitory effect of cell infection by the Rabies virus. Similar competition binding assays conducted to measure the binding affinity of Rabies virus with host cell receptors, indicated that only suitable conformation of isolated rabies virus glycoprotein is able to bind to host cell entry receptor and inhibit the virus attachment [56–58]. These results indicate that the recombinant production of RABV-G in *Pichia pastoris* and the applied downstream protein purification method

allowed to have a properly folded and glycosylated native-like protein which can be an interesting candidate for Rabies subunit vaccine development.

6. Conclusion

During this work we studied the expression of the recombinant rabies virus glycoprotein in the methylotrophic yeast *Pichia pastoris* under different growth conditions. Our results indicated that a variety of carbon sources can be used to produce the recombinant RABV-G under the control of the GAP constitutive promoter. We found that high oxygen supply, neutral pH, slow growth rate and 30 °C incubation temperature were the optimal incubation conditions for RABV-G production in shake flask and bioreactor scales. In addition to the simplicity of the upstream design cultivation, the use of *P. pastoris* as heterologous expression system for RABV-G production also offered the ease of the downstream purification of our protein while maintaining the rabies virus glycoprotein native characteristics. This work provides new insights and more flexibility in terms of bioprocess design to produce a correctly folded RABV-G which can be used during an immunization proposal for subunit Rabies vaccine development.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2022.e00736.

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