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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

DROSOPHILA S2 cell culture in a WAVE Bioreactor: potential for scaling up the production of the recombinant rabies virus glycoprotein

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Abstract

The transmembrane rabies virus glycoprotein (RVGP) is the main antigen of vaccine formulations used around the world to prevent rabies, the most lethal preventable infectious disease known. The objective of this work was to evaluate the potential of a bioreactor using wave-induced agitation in the initial steps of scaling up the rRVGP production process by a *Drosophila melanogaster* S2 cell line to produce rRVGP in sufficient quantities for immunization and characterization studies. Taking advantage of some remarkable features recognized in *Drosophila* S2 cells for scaling the culture process, a robust recombinant lineage (S2MtRVGPH-His) engineered by our group for the expression of rRVGP using a copper-inducible promoter was used in the bioreactor cultures. The WAVE Bioreactor was chosen because it represents an innovative approach to the cultivation of animal cells using single-use technology. For that purpose, we firstly established a procedure for culturing the S2MtRVGPH-His lineage in 100 mL Schott flasks. Using an inoculum of 5×10^5 cells/mL in culture medium (Sf900-III) induced with solution of $CuSO_4$ (0.7 mM) and a convenient pH range (6.2–7.0), optimal parameter values such as time of induction (72 h) and temperature (28 °C) to increase rRVGP production could be defined. This procedure was reproduced in culture experiments conducted in a WAVE Bioreactor™ 2/10 using a 2 L Cellbag. The results in Schott flasks and in WAVE Bioreactor™ were very similar, yielding a maximum titer of rRVGP above of 1 $mg.L^{-1}$. The immunization study showed that the rRVGP produced in the bioreactor was of high immunogenic quality.

Keywords Rabies virus glycoprotein · Rabies vaccine · *Drosophila melanogaster* S2 · WAVE Bioreactor · Scale-up · Recombinant protein production

Introduction

The *Drosophila* S2 cell line has been effectively used as a recombinant protein production platform (Geisler et al. 2015) expressing high levels of several proteins (Moraes et al. 2012) and supporting the development of new vaccine candidates such as the dengue virus (Clements et al. 2011), HIV-1 virus (Yang et al. 2012), and rabies (Astray et al. 2013). Rabies is the most lethal preventable infectious disease known. The transmembrane rabies virus glycoprotein (RVGP) is the main antigen of human or veterinary vaccine formulations, inducing virus-neutralizing antibodies which can prevent the onset of the disease (Perrin et al. 1985; Ertl 2009; Astray et al. 2017).

The S2 cell line has been evaluated for transmembrane recombinant RVGP (rRVGP) expression using metal-inducible promoters (Lemos et al. 2009; Astray et al. 2013). Extensive characterizations related to cell growth, rRVGP production, cell culture parameters, and metabolic aspects have been published elsewhere (Bovo et al. 2008; Galesi et al. 2008; Swiech et al. 2008a; Swiech et al. 2008b; Ventini et al. 2010; Moraes et al. 2012; Rossi et al. 2012). More recently, a robust cell line (S2MtRVGPH-His) has been developed with a list of features designed for high expression in

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suspended culture and high-efficiency purification. The next move is to develop the means to produce sufficient quantities of high quality rRVGP for immunization, characterization, and safety studies.

In order to achieve rRVGP production with high titer using the S2MtRVGPH-His cell line, it is imperative to assess the ability to maintain their growth and glycoprotein expression characteristics throughout increasing scales of cultivation. In this context, the WAVE Bioreactor™, first described by Singh (1999), is a successful and extensively used approach for the cultivation of animal cells, mainly used in small or medium scale cell cultures to produce high-added value pharmaceuticals, as it offers high process flexibility, as well as cost and time savings (Loffelhotlz et al. 2013).

The WAVE Bioreactor also has advantages such as a Food and Drug Administration (FDA) approved disposable chamber, the fact that there is no need of a sterilization step and stainless steel construction, and a rocking motion agitation that promotes shear damage reduction by eliminating mechanical mixers. Additionally, the increased wavy liquid-air interface provides a high-efficiency mass transfer surface to nutrients, oxygen, and metabolites, especially when the process reaches high cell densities (Singh 1999; Fries et al. 2005; Wang et al. 2012)

Taking all these attributes of the WAVE Bioreactor into account, the single-use rocking motion bioreactor is a strong candidate to use in the initial stages of bioprocess scale-up or non-clinical and preclinical trial assessments (Loffelhotlz et al. 2013). Although the WAVE Bioreactor has been successfully used in the production of recombinant cytosolic proteins with the S2 cell system (Fries et al. 2005; Wang et al. 2012), to the best of our knowledge, there are no case records of the production of recombinant transmembrane glycoproteins, such as RVGP. This kind of bioproduct normally exhibit minor expression levels when compared to soluble proteins (Santos et al. 2016) and usually comprises more complex stages of development in molecular biology, as well as in culture and purification processes.

Thus, the main objective of this work was to evaluate the potential use of a WAVE Bioreactor in the initial steps of scaling up the production process of rRVGP by the S2MtRVGPH-His cell line. Kinetic features of cell growth and rRVGP production, as well as metabolic parameters, were also studied.

Materials and methods

Culture of S2MtRVGPH-His cells in Schott flasks

The recombinant S2MtRVGPH-His cell line used in this work was obtained by transfection with a modified pMT/V5-HisA (Life Technologies, Carlsbad, CA) vector. The selection marker for hygromycin was previously introduced in this vector with the RVGP gene (Lemos et al. 2009). In this paper, the polyhistidine tag was introduced by direct cloning of its corresponding sequence at the RVGP C terminal, using the *Not*I restriction enzyme. S2 cells were then transfected with the new construction and lipofectamine (Thermo Fisher Scientific, Waltham, CA) and selected with hygromycin (Life Technologies, Grand Island, NY). These cells can express rRVGP by using the metallothionein promoter upon induction with $CuSO_4$. The polyhistidine tag enables the purification of rRVGP by immobilized metal affinity chromatography (IMAC). Finally, the S2MtRVGPH-His was expanded to obtain the master and work cell banks.

A population of 1×10^7 S2MtRVGPH-His cells stored in liquid nitrogen was thawed in a water bath at 28 °C and selected by three subcultures in Sf-900 III medium (Life Technologies, Carlsbad, CA) containing 50 mg.mL^{-1} of hygromycin B in T25-cm^2 cell culture flasks (Corning®, Corning, NY). Then, the cells in suspension were inoculated in 20 mL of Sf-900 III culture medium in 100 mL tightly closed Schott flasks at a concentration of 5×10^5 cells.mL^{-1}. The final culture was performed at 28 °C and 100 rpm in a rotatory incubator, G-25KC model (New Brunswick Scientific, Edison, NJ) for 192 h. The initial pH was 6.3, which increased during the culture to a final value around 7.0.

Parameters of growth and rRVGP production were investigated in different culture conditions: induction of rRVGP expression at 0, 24, 48, or 72 h of culture with solution of $CuSO_4$, 0.7 mM (Sigma-Aldrich, St. Louis, MO) and temperatures of 22, 25, or 28 °C. The induction of rRVGP was performed at 72 h of culture at 25 and 28 °C. At 22 °C, it was performed at 96 h because of the low number of cells identified 72 h. Samples were withdrawn every 24 until 192 h of culture. After determining the best combination of temperature and induction time for the rRVGP expression, an experiment in this best condition was carried out in triplicate ($n = 3$). The procedure used is shown in detail in Fig. 1.

Culture of S2MtRVGPH-His cells in the WAVE Bioreactor

Following the same procedure described above, a suspension of S2MtRVGPH-His cells in a concentration of 5×10^5 cells.mL^{-1} was expanded in four 20 mL Schott flasks to achieve an inoculum of 3.3×10^8 total viable cells in the mid-exponential growth phase. These cells were then transferred to a disposable 2 L Cellbag (GE Healthcare, Chicago, IL) containing approximately 650 mL of Sf-900 III culture medium and immediately accommodated over the WAVE platform. The culture was carried out in batch mode in the WAVE Bioreactor 2/10 system (GE Healthcare, Chicago, IL). This equipment was provided with a temperature controller, O_2/air mix adjustment device and two optical electrodes

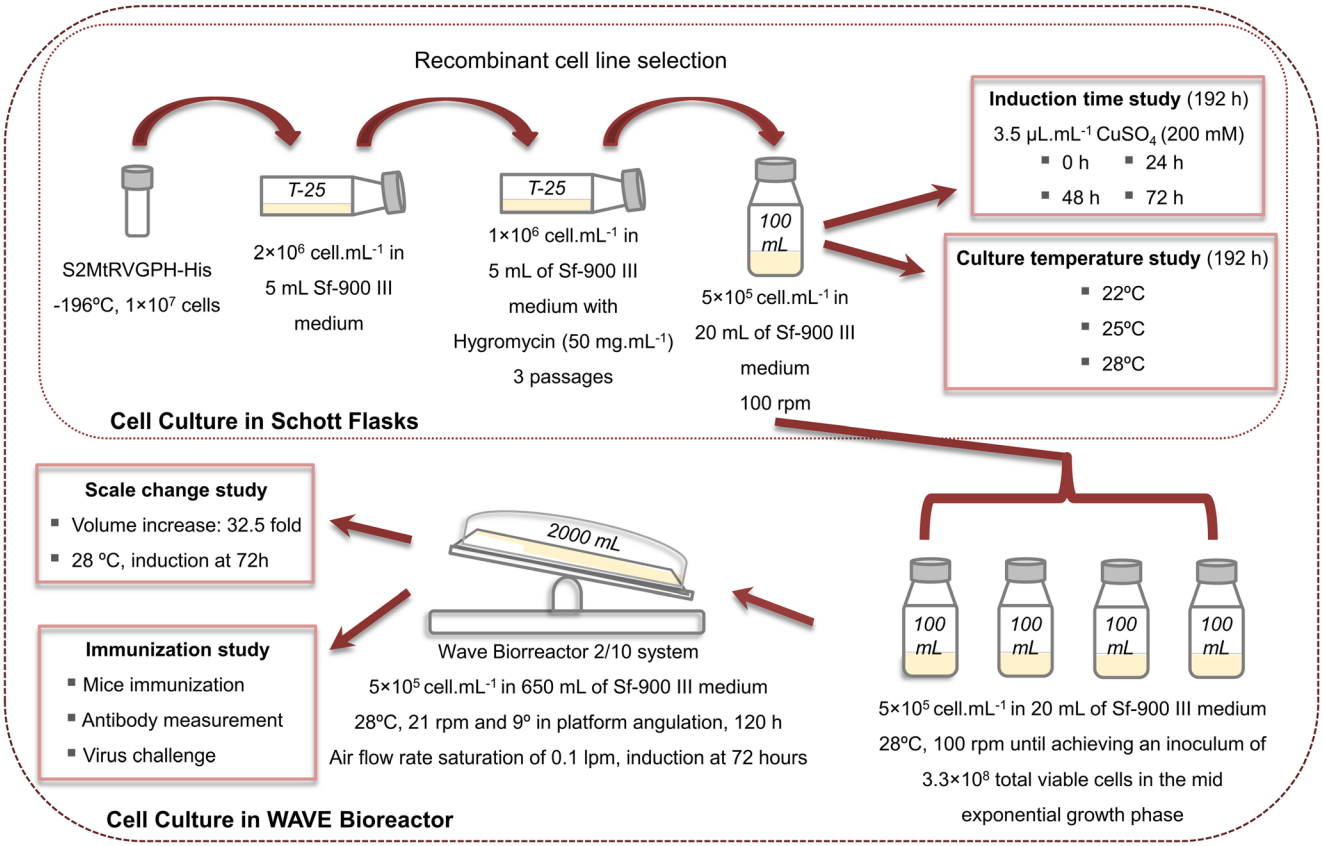

Fig. 1 Schematic representation of *Drosophila melanogaster* S2MtRVGPH-His cell culture in Sf-900 III. In Schott flasks, batch cultures were carried out for 192 h with an induction time at 0, 24, and 48 or 72 h (induction time study) and at 22, 25, or 28 °C (culture temperature study). In the WAVE Bioreactor, batch cultures induced at 72 h and 28 °C lasted 120 h (scale change study). The quality of the rRVGP produced by the S2MtRVGPH-His cells taken at 120 h of culture from the WAVE Bioreactor was evaluated (immunization study)

for monitoring dissolved oxygen (DO) and pH. The temperature was controlled at 28 °C and the agitation by rocking motion was set at a speed of 21 rpm and an angle of 9°, based on the study carried out by Wang et al. (2012). For aeration, air sterilized through the Cellbag inlet filter was injected at a flow rate of 0.10 L per minute. The DO concentration in the liquid medium was monitored by the optical electrode from 26 to 120 h.

Similarly to the cultures in Schott flasks, the pH was only monitored, not controlled, starting with a value of 6.3 and increasing during the culture to a convenient final value around 7.0. The rRVGP expression was induced at 72 h of culture with solution of $CuSO_4$, 0.7 mM (Sigma-Aldrich, St. Louis, MO). Samples from cell cultures were withdrawn every 24 h, until 120 h of culture. Two independent runs ($n = 2$) were performed in the conditions previously optimized for rRVGP production in Schott flasks. The procedure followed is described in Fig. 1.

Analytical methods

Aliquots of the samples taken from the S2MtRVGPH-His culture experiments every 24 h were analyzed for viable cell concentration and viability using hemacytometer (LO-Laboroptik, Lancing, UK) by the trypan blue 0.04% (Life Technologies, Grand Island, NY) exclusion method (Doyle and Griffths 2007). Culture samples were centrifuged at 600 g for 5 min and a cell pellet was used to quantify the rRVGP expression. The cell culture supernatant was used to quantify the metabolites glucose, lactate, ammonia, and amino acids.

For rRVGP quantification, 10^6 cells were centrifuged at 1000 g for 3 min, the pellet was treated with a lysis buffer comprising 25 mM NaCl, 5 mM KCl, 2 mM Tris, and 0.2% Igepal (Sigma-Aldrich, St. Louis, MO), analyzed using an ELISA kit (Rabies Glycoprotein Enzyme Immunoassay, Pasteur Institute, Paris, France). The rRVGP concentration was calculated from a standard curve prepared using inactivated virus particle rabies glycoprotein, as described previously (Perrin et al. 1996; Astray et al. 2008).

The glucose and lactate quantifications were performed by enzymatic analysis in a YSI 7100-06A multiparameter bioanalytical system (YSI, Yellow Springs, OH) and the ammonia, by a selective ion electrode (Orion 710A, Thermo Fisher Scientific, Waltham, MA), which determined the potential (mV) in the sample mixed with an ionic strength adjustment solution (Thermo Fisher Scientific, Waltham,

MAA). The mV reading was converted to ammonia concentration through a standard curve.

Amino acids (Amino Acid Standard, AccQ-Tag, Waters Corporation, Milford, MA) were quantified by high-performance liquid chromatography (HPLC). The samples were centrifuged at 10000*g* for 10 min and filtrated with Amicon Ultra, 100 K (Millipore, Billerica, MA). The analysis was performed using the methodology described by Heinrikson and Meredith (1984) with some modifications. Briefly, samples were dried in nitrogen stream and hydrated with a solution containing ethanol, ultrapure water and triethylamine (2:2:1), and dried in nitrogen stream. After that, samples were derivatized with ethanol, ultrapure water, triethylamine, and phenylisothiocyanate, PITC reagent (7:1:1:1), and again dried in nitrogen stream. Before the HPLC injection, dried samples were ressuspended in a buffer diluent solution containing Na_2HPO_4 and acetonitrile (95:5) at pH 7.0 (Waters Corporation, Milford, MA). The method used analyzed 19 amino acids.

The HPLC equipment consisted of two W515 pumps, a W717 refrigerated autosampler and a UV W486 detector at 254 nm (Waters Corporation, Milford, MA). Chromatographic separation was achieved using a Pico-tag (300 × 3.9 mm) amino acid analysis column (Waters Corporation, Milford, MA) at 36 °C. The mobile phase used consisted of two eluents (A and B). Eluent A was a mixture of 940 mL of solution 1 (0.14 M sodium acetate trihydrate (Panreac, Barcelona, Spain) with 0.05% (*v*/*v*) trimethylamine (Sigma-Aldrich, St. Louis, MO) and 0.02% (*v*/*v*) ethylenediaminetetraacetic acid, EDTA (Sigma-Aldrich, St. Louis, MO) 1 $g.L^{-1}$), pH adjusted to 6.4 with acetic acid (Sigma-Aldrich, St. Louis, MO) and 60 mL of acetonitrile (J. T. Baker, Center Valley, PA). Eluent B was composed of acetonitrile (60%), ultrapure water (40%) with 0.02% (*v*/*v*) EDTA (1 $g\,L^{-1}$). A gradient elution program was used with an increasing gradient of eluent B, as described by Waters (White et al. 1986). An eluent flow rate of 1 mL min^{-1} was utilized.

Calculation of parameters related to growth and production of rRVGP

To determine the specific growth rate (μ, h^{-1}) of the cells in an interval of 24 h, the approximate expression of Eq. 1 was used.

$$\mu = \frac{1}{X}\frac{dX}{dt} \sim \frac{1}{X}\frac{\Delta X}{\Delta t} \quad (1)$$

where ΔX and Δt are the concentration differences of cells ($cells.mL^{-1}$) and time interval (24 h) between two experimental measurements, respectively. X is the mean viable cell concentration of the four withdrawn samples ($cell.mL^{-1}$).

The mean specific rRVGP production (γ, $ng.cell^{-1}.h^{-1}$) was evaluated by calculating the mean specific rRVGP production after induction until 120 h of culture for comparison between the experiments. The rRVGP specific production for each day was determined by the approximate expression in Eq. 2.

$$\gamma = \frac{1}{X}\frac{dP}{dt} \sim \frac{1}{X}\frac{\Delta P}{\Delta t} \quad (2)$$

where ΔP and Δt are the concentration differences of rRVGP ($ng.mL^{-1}$) and time interval (24 h) between two experimental measurements, respectively. X is the mean viable cell concentration of the four withdrawn samples ($cell.mL^{-1}$).

Mice immunization, antibody measurement and virus challenge

Aliquots of the S2MtRVGPH-His cells taken at 120 h of culture from the WAVE Bioreactor were frozen at − 80 °C in a cryopreservation buffer containing glycerol (Sigma-Aldrich, Steinheim, Germany) until the immunization experiment. For mice immunization, cell samples were thawed at 37 °C and centrifuged at 4000*g* for 6 min for buffer removal. Then, cells were re-suspended in PBS with phenylmethylsulfonyl fluoride, PMSF 1 mM (Sigma-Aldrich, Steinheim, Germany) and lysed in a homogenizer (Yason, Guangdong, China) at maximum speed for 5 min. Cell lysates were ultracentrifuged at 120000*g* for 30 min and the pellet, corresponding to membrane and insoluble cell structures, was suspended in PBS and frozen at − 80 °C. The same procedure was followed for S2 non-transfected cells as a negative control. The trimeric rRVGP content was determined by ELISA from a thawed membrane preparation, following the procedures described above for cell pellets. The membrane preparation containing rRVGP is a partially purified preparation from the recombinant cells. Previous assays were performed in order to check the immunogenicity of these preparations using other recombinant S2 cells expressing the rRVGP (unpublished). This preparation can be considered good enough to evaluate if the produced rRVGP has the desired antigenic properties, prior to further experiments using the fully purified rRVGP. At the moment of immunization, the membrane preparations were thawed and formulated diluting the samples with PBS to achieve 1.0 μg rRVGP/dose (300 μl).

Four week-old Balb/C mice were divided into groups of five animals each and immunized with the membrane preparations by intraperitoneal route. The vaccination schedule consisted of prime and two booster doses at 7 and 14 days after the first dose. Two negative group controls (saline solution and membranes from non-transfected S2 cells) and a positive group control (commercial vaccine-Defensor®/ Pfizer, White Hall, USA) were added to a total of 4 groups. One week after the last boost, mice were challenged with 30 μl of 10^5 DL50 of the fixed rabies virus sample Challenge Virus Standard (CVS) by intracranial inoculation and observed for

30 days for development of symptoms. Animals with symptomatology were euthanized. Serum samples were collected by the retro-orbital route immediately before and after the virus challenge and the seroconversion was evaluated by titration of anti-RVGP antibodies using Platelia® (Bio-Rad, Marnes-la-Coquette, France). All the procedures using animals were approved by the local Ethics Committee on Animal Use.

Statistical analysis

Statistical testing to identify the significant similarity between data points was performed using the *t* test ($p \leq 0.05$). The standard error was presented as bars in all graphics and by numerical values in all tables. Cell counting was carried out on four 1 mm^2 grids, considered as one event, and the viable cell concentration was calculated as the mean ± standard error of four events.

Results

Culture of S2MtRVGPH-His cells in Schott flasks

The purpose of these preliminary experiments was to identify an ideal time of induction for rRVGP production and an optimal temperature of growth for S2MtRVGPH-His cells.

The ideal time to initiate the induction of the rRVGP expression is an important factor in reaching the end of the culture by reconciling two conflicting objectives: large amounts of cell and recombinant glycoprotein. Aiming to investigate the optimal time to induce the rRVGP expression under the control of metallothionein, culture experiments in Schott flasks with $CuSO_4$ induction at 0, 24, 48, and 72 h were performed (Fig. 2a, b).

In Fig. 2a, b, it can be observed that the induction time exerts a very strong influence on both the rRVGP expression and cell growth, respectively. The addition of $CuSO_4$ solution prior to 72 h caused strong inhibition of cell growth and a decrease in cell viability, leading to low levels of rRVGP expression. On the other hand, induction at 72 h allowed high cell concentration, with the highest specific growth rate (Table 1) and rRVGP production.

Insect cells have the capacity to grow at temperatures in the range of 20 to 28 °C (Galesi et al. 2008; Swiech et al. 2008b; Ventini et al. 2010; Moraes et al. 2012; Rossi et al. 2012). Thus, the influence of temperature on growth kinetics and on the rRVGP expression by S2MtRVGPH-His was investigated in culture experiments at 22, 25, and 28 °C (Fig. 2c, d). The results of cell growth at 25 and 28 °C were very similar, possibly because they were within a narrow interval of maximum cell growth. The growth at 22 °C was much lower, despite the increase seen at 24 h after $CuSO_4$ induction. Cultures at the three temperatures reached a maximum concentration just before 168 h. After reaching the peak, the concentration tends to oscillatory behavior by alternating oxygen depletion and cell lysis. On the other hand, up to 168 h the three cultures showed increasing concentrations of rRVGP and, thereafter, degradation of the glycoprotein, which can be attributed to the action of proteases released in the medium by the lysed cells. This behavior of growth and rRVGP expression with recombinant S2 cells has also been observed in precedent studies (Swiech et al. 2008a; Rossi et al. 2012). The culture parameters at the three temperatures in Table 1 confirm the almost identical specific growth rates at temperatures of 25 and 28 °C, as well as the lowest value at 22 °C. It can also be concluded that the value of $\overline{\gamma}$ increases with the temperature.

During all the experiments performed in Schott flasks in a rotatory shaker, the pH increased during 192 h of culture (started at 6.2 and ended close to 7.2).

Culture of S2MtRVGPH-His cells in the WAVE Bioreactor and scale change study

Two cultures were performed in the WAVE Bioreactor at 28 °C for 120 h, with an expression induction time at 72 h, according to the best conditions defined in the previous experiments in Schott flasks. Aiming to prioritize the production of high quality rRVGP in the growth phase, avoiding the limitation of protein synthesis by amino acid depletion, the two replicate cultures were stopped at 120 h. The S2MtRVGPH-His cell growth and rRVGP production profiles (Fig. 3e, d), respectively, show graphically the similarities of the culture processes in the bioreactor. To verify the reproducibility of the culture experiments in the WAVE Bioreactor, Table 2 was made to compare the cell growth and rRVGP production data from batch cultures 1 and 2. The results of this analysis and the standard error associated enable us to infer that the two experiments were similar in terms of cell growth and glycoprotein production.

The analysis of critical substrate consumption in Fig. 3a–c shows that although glucose, glutamine, and proline were the most consumed substrates in the WAVE Bioreactor cultivation, there was no depletion of substrates. The glutamine and proline uptake were high as both were consumed more than 80%, and the consumption of glucose was 30%. In Fig. 3a–c, it is possible to follow the consumption of the substrates (glucose, glutamine, and proline) considered as most limiting in the cultivation of this cell and the respective end metabolites of their degradation (lactate, ammonia, and alanine). Lactate was present in very low concentrations (0.001–0.01 g/L) in all experiments and ammonia and alanine were produced in significant amounts. Ammonia reached 45.8 and 38.7 $mmol.L^{-1}$ in batch cultures 1 and 2, respectively and alanine reached 18.96 and 15.53 $mmol.L^{-1}$ in batch cultures 1 and 2, respectively.

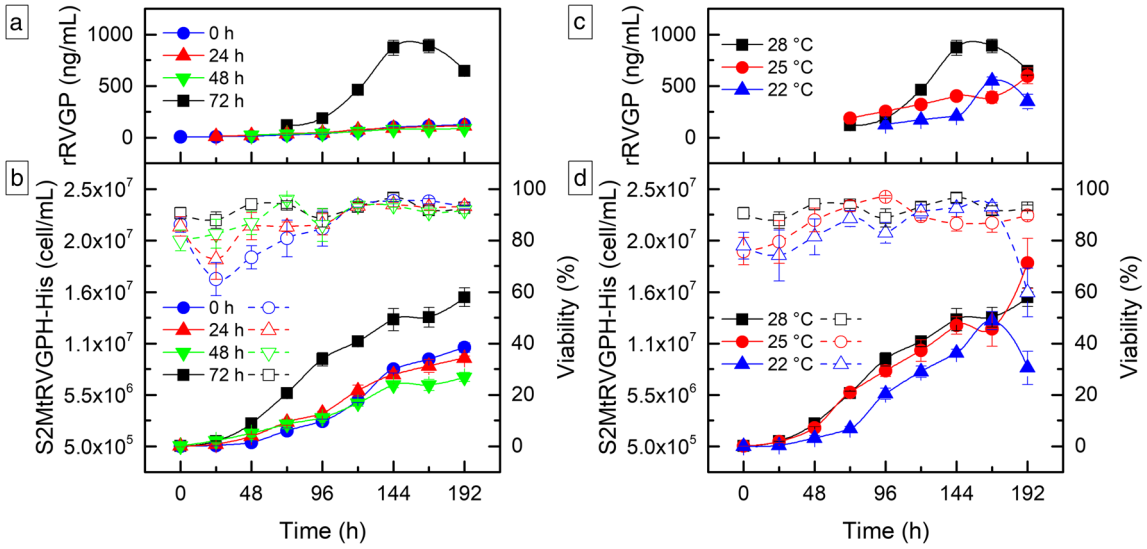

Fig. 2 Batch cultures in Schott flasks with S2MtRVGPH-His cells in Sf-900 III culture medium. Influence of induction time at 28 °C on: **a** Concentration of rRVGP. **b** Concentration of viable cells (solid line) and viability (dashed line). Temperature influence induced with $CuSO_4$ at 72 h on **c** concentration of rRVGP and **d** viable cell concentration (solid line) and viability (dashed line)

In order to evaluate the effects caused by the change in the culture process from Schott flasks to the WAVE Bioreactor for rRVGP production, a comparison of the results obtained in the experiments is presented in Fig. 3f, g and Table 2. The cell density obtained at 120 h (X_{max}), specif growth rate (μ) and specific rRVGP production (γ) in both studies did not present a significant difference (*t* test, $p \leq 0.05$).

Immunization studies

The quality of the rRVGP produced by the S2MtRVGPH-His cells was evaluated by immunization of mice (Table 3).

The required titer to protect an animal against rabies is considered to be 0.5 EU.mL^{-1} (EU, equivalents to international units), therefore the results indicate that the rRVGP presented to the immune system of the vaccinated mice was able to produce high levels of anti-RVGP antibodies, statistically not different from the levels induced by the commercial vaccine ($p > 0.05$). Mice immunized with rRVGP also survived the rabies virus challenge, whereas mice immunized with saline or membrane preparation from the wild-type S2 did not.

Discussion

The ideal time to induction of the rRVGP expression is an important factor in reaching the end of the culture by reconciling two conflicting objectives: large amounts of cell and recombinant glycoprotein. As reported by Norgate et al.

Table 1 Influence of induction time and temperature on the growth rate of S2MtRVGPH-His and rRVGP specific production in Sf-900 III culture medium in Schott flasks

Conditions	$\overline{\mu}_{bi}$ [a] (h^{-1})	$\overline{\mu}_{ai}$ [a,d] (h^{-1})	$\overline{\gamma}$ [d] ($\times 10^{-7}$ ng.cell^{-1} h^{-1})
Induction time (h)[b]			
0	–	0.019 ± 0.005	2.29 ± 0.60
24	0.014 ± 0.005	0.019 ± 0.006	2.34 ± 0.67
48	0.025 ± 0.010	0.013 ± 0.004	1.65 ± 0.47
72	0.032 ± 0.007	0.011 ± 0.002	10.02 ± 1.76
Temperature (°C)[c]			
28	0.032 ± 0.007	0.011 ± 0.002	10.02 ± 1.76
25	0.032 ± 0.007	0.011 ± 0.002	3.44 ± 0.77
22	0.024 ± 0.010	0.011 ± 0.002	2.41 ± 0.45

All values are reported as mean values ± standard error

[a] The specific growth rate before and after the induction are represented by μ_{bi} and μ_{ai}, respectively

[b] Induction time study was performed at 28 °C

[c] The temperature study the induction was in 72 h for the cultures at 25 and 28 °C and in 96 h for the culture at 22 °C

[d] Calculated until 120 h

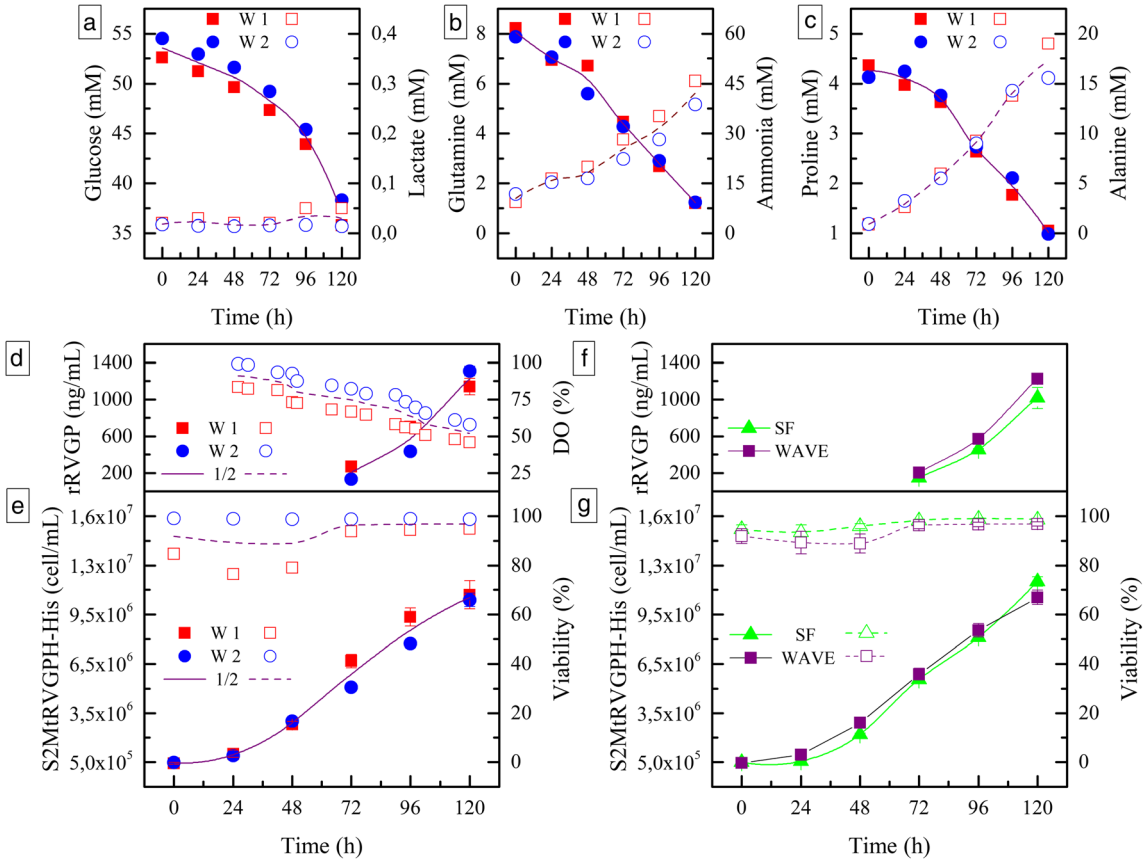

Fig. 3 Batch cultures in WAVE Bioreactor with S2MtRVGPH-His cells at 28 °C in Sf-900 III culture medium induced at 72 h. Consumption of main substrates and synthesis of main by-products during both batch cultures (W1 and W2): **a** glucose (solid line) and lactate (dashed line); **b** glutamine (solid line) and ammonia (dashed line), and **c** proline (solid line) and alanine (dashed line). Comparing the duplicates of cultures in the WAVE Bioreactor **d** rRVGP production (solid line) and dissolved oxygen, DO (dashed line). **e** Concentration of viable S2 cell (solid line) and viability (dashed line). The experimental points represent the values obtained in batch cultures (1 and 2). The middle line describes the average values of the two batch cultures. The influence of change in the culture process, Schott flask ($n = 3$) and the WAVE Bioreactor ($n = 2$), on **f** concentration of rRVGP and **g** viable cell concentration (solid line) and viability (dashed line)

(2010), high levels of copper in solution could be toxic to *Drosophila* cells. Then, premature addition of the $CuSO_4$ solution can inhibit cell growth and generate a small population of rRVGP producing cells. Late addition allows for high cell growth, however, with little remaining time to explore rRVGP expression under adverse conditions due to nutrient exhaustion and proteolytic degradation.

The behavior of the S2MtRVGPH-His cell line with respect to the induction time was similar to that observed in the cultivation of a recombinant S2 cell line for expression and secretion of transferrin (Lim and Cha 2006), an important glycoprotein in the transport and release of Fe^{3+} in the human organism. In addition to the characterization of the production behavior not associated with cell growth, these researchers were able to predict (using a mathematical model) that the best strategy to reach high yields of recombinant protein is to maximize cell densities before the induction of expression. According to the model prediction, the optimal induction time to reach maximum recombinant protein yield was 80 h. This is similar to what was experimentally determined in this study as the best induction time. However, it is important to note that the culture media, the transfection protocols, and the concentrations of both inoculum and $CuSO_4$, used by Lim's group, were different. Therefore, the cultivation process for the production of optimized amounts of glycoprotein is highly dependent on the induction time.

The behavior of the S2MtRVGPH-His cell line related to the capacity to grow at temperatures in the range of 20 to 28 °C has been investigated. Ventini et al. (2010) performed a productive S2 cell culture in a bioreactor at 28 °C using a recombinant S2 cell line (S2MtRVGPHy) similar to the one used in this study, in which the RVGP gene (without histidine tag) expression was also controlled by the metallothionein promoter. Other studies, such as (Galesi et al. 2008; Swiech et al. 2008a), both using S2 cells expressing RVGP under the control of a constitutive actin promoter (S2AcRVGP cells), attained moderate RVGP expression levels at 28 °C. On the other hand, Rossi et al. (2012), who used the constitutive actin promoter, demonstrated that the best rRVGP production was achieved at 20 °C. In the present study, it can also be

Table 2 Parameters of batch cultures performed with S2MtRVGPH-His in Sf-900 III medium in Schott flasks and WAVE Bioreactor at 28 °C and induction with $CuSO_4$ at 72 h

Type of culture	$\overline{\mu}_{bi}$ [a] (h^{-1})	$\overline{\mu}_{ai}$ [a] (h^{-1})	$\overline{\gamma}$ ($\times 10^{-7}$ ng.cell^{-1}.h^{-1})	X_{max} [b] ($\times 10^7$cell.mL^{-1})	Maximum rRVGP[c] (ng/mL)
Reproducibility					
Batch[d] culture 1	0.036 ± 0.012	0.010 ± 0.002	20.32 ± 3.78	1.07 ± 0.09	1138 ± 91
Batch[d] culture 2	0.030 ± 0.008	0.015 ± 0.001	29.83 ± 2.34	1.04 ± 0.04	1308 ± 51
Scale change					
Experiments in Schott flasks[e]	0.030 ± 0.006	0.015 ± 0.001	21.32 ± 3.02	1.15 ± 0.03	1016 ± 116
Experiments in bioreactor[f]	0.033 ± 0.007	0.012 ± 0.002	24.89 ± 4.89	1.05 ± 0.04	1223 ± 58

[a] The specific growth rate before and after the induction are represented by $\overline{\mu}_{bi}$ and $\overline{\mu}_{ai}$, respectively

[b] X_{max} reached at 120 h

[c] Concentration of rRVGP by volume of culture media used in bioreactor or Schott flasks at 120 h

[d] Experiments performed in WAVE Bioreactor independently

[e] Values are average of three independent experiments ($n = 3$)

[f] Values are average of two independent experiments ($n = 2$)

concluded that the mean specific rRVGP production ($\bar{y}$) increases with the temperature.

The increase in pH during all the experiments performed in Schott flasks may be seen as an advantage in the development of a cultivation process for the production of rRVGP. The pH started at 6.2, optimal for growth (Moraes et al. 2012), and generally ended very close to 7.2, which is considered the ideal pH for RVGP stability (Roche and Gaudin 2002). For this reason, in the experiments carried out in the WAVE Bioreactor, the pH was monitored but not controlled.

Then, in the previous experiments in Schott flasks, a convenient pH range and parameter values such as time of induction (72 h) and temperature (28 °C) to optimize rRVGP production could be defined.

The S2MtRVGPH-His cell growth and rRVGP production profiles (Fig. 3e, d) show the similarities of the culture processes in the WAVE Bioreactor. Observing the cell concentration, the cultures were stopped at 120 h because when approaching the stationary phase, the lack of some amino acids causes a deficient formation of proteins that are later degraded by the proteolytic action triggered by cellular mechanisms of control. The degradation of rRVGP under these conditions was previously reported after depletion of some amino acids in bioreactor cultures of a recombinant S2 lineage (Swiech et al. 2008a; Rossi et al. 2012). On the other hand, observing the results of the rRVGP concentration at the end of the cultures in the WAVE Bioreactor, it can be seen that the slope of the curve shows a behavior of increasing the biosynthesis rate. This may represent an interesting practical potential of developing a fed-batch strategy for rRVGP production, avoiding nutrient depletion at advanced culture times, allowing the extension of the most productive phase of rRVGP.

The adoption of a culture time of 120 h seems to be a suitable strategy for the production of high quality rRVGP as there was no depletion of substrates, cell death and rRVGP degradation. Glucose and glutamine are known to be energy sources and precursors for biomass synthesis (Gòdia and Cairo 2006; Swiech et al. 2008a; Moraes et al. 2012). Proline is also used as an energy source once it is known to be consumed in muscle for flight in *Diptera* insects (Scaraffia and Wells 2003; Gòdia and Cairo 2006). The glutamine and proline uptake were high (both more than 80%) and this behavior may explain the relatively low consumption of glucose (30%) as an energy source.

Lactate, ammonia, and alanine metabolites were detected throughout the culture following a pattern similar to that

Table 3 Seroconversion and survival after virus challenge of immunized mice

Sample	Before challenge	After challenge	
	Antibody titer (EU.mL^{-1})	Antibody titer (EU.mL^{-1})	Survival (%)
rRVGP	2.92 ± 0.61	3.54 ± 1.59	100
Rabies vaccine	3.40 ± 1.59	4.90 ± 0.42	100
S2 wild type	0	0	0
Saline	0	0	0

All the values are reported as mean values ± standard deviation

described by Swiech et al. (2008a), Ventini et al. (2010) and Rossi et al. (2012) with other recombinant S2 cell lines targeting rRVGP production. Lactate was present at very low concentrations (0.001–0.01 g/L) in all experiments and ammonia and alanine were produced in significant amounts. Ammonia reached 45.8 and 38.7 mmol.L^{-1} (batch cultures 1 and 2, respectively). In the experiments carried out by Swiech et al. (2008a), the amount of ammonia reached the level of 62 mmol.L^{-1}, concentration that had not previously been reported in animal cell culture. Alanine reached 18.96 and 15.53 mmol.L^{-1} (batch cultures 1 and 2, respectively). The production of alanine was described as an important strategy to prevent the accumulation of ammonia in solution, avoiding reaching toxic levels (Bathia et al. 1997; Macaranga et al. 2002).

It is also important to take into account the observation made by Borash et al. (2000) that *Drosophila melanogaster* can develop a high genetic tolerance to toxic compounds, such as ammonia and urea. However, monitoring ammonia levels during the cultivation process is important because this metabolite significantly affects the product quality (Portner and Schafer 1996) and the distribution of glycan structures (Butler 2005). Although this mechanism has not been fully elucidated, it may influence the glycoprotein glycosylation patterns, affecting the quality of the final product obtained.

The scaling up of the Schott flask S2MtRVGPH-His cell culture to the WAVE Bioreactor should cause a drastic modification of the microenvironment around the cell, which may have harmful effects on cell growth and rRVGP production. Since there is little published information regarding insect cell culture in bioreactors, especially of cells that show attractive attributes to use in large-scale bioprocesses such as S2 cells, it is of the utmost importance to assess the potential use of these cells at larger scales. To this end, culture under optimized conditions in Schott flasks with a volume of 20 mL were reproduced in a 650 mL bioreactor, which has aroused great interest in the production of large-scale recombinant proteins because of the innovative technological concepts mentioned before. In the present study, the cell density obtained at 120 h (X_{max}), μ, and γ in both studies did not present a significant difference (t test, $p \leq 0.05$), yielding a maximum titer of rRVGP above of 1 mg/L in WAVE Bioreactor. Thus, an efficient scale-up process was obtained as a 32.5-fold increase volume was performed (20 mL in Schott flasks to 650 mL in WAVE Bioreactor) with a smooth transition, showing a great perspective to be industrially scaled.

Analyzing the quality of rRVGP produced in bioreactor, there is much evidence that a good quality antigen is mandatory for mice survival to a rabies virus challenge (Ertl 2009). The immunization study shows a small, not statistically significant, increase in the antibody titers after the challenge. Thus, indicating the effective rapid neutralization of the rabies virus in a context of high levels of neutralizing antibodies, also demonstrating that the rRVGP primed immune cells secreted antibodies which recognized the identity of the rRVGP present in the virus. These facts confirmed that the rRVGP produced by the recombinant S2 cells presents characteristics of a good antigen.

Finally, a general observation that deserves to be highlighted in relation to the results obtained in this work is the verification of the inverse relation of cell growth to the production of rRVGP. Generally, following $CuSO_4$ induction, the observed behavior was always a decrease in μ while increasing the γ. This trend was also observed by Barnes et al. (2001), Lim and Cha (2006) and Hayter et al. (1992) and attributed to a metabolic shift in recombinant cells to prioritize the production of foreign proteins at the expense of cell growth (Glick 1995). This kinetic feature of the S2MtRVGPH-His cell culture may be very important for defining more rational rRVGP production strategies in the WAVE Bioreactor.

Overall, the rRVGP production with a robust recombinant cell line (S2MtRVGPH-His) using the WAVE Bioreactor as a modern and economical alternative to conventional equipment enables efficient scale-up of the production with high quality immunoactive glycoprotein. This bioprocess may be promising in terms of obtaining rRVGP in the near future in the order of grams for use in immunological, preclinical or clinical assessments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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