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Behavior of Wild-type and Transfected S2 Cells Cultured in Two Different Media

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Abstract An animal protein-free medium composed of IPL-41 containing 6 g L^{-1} yeastolate ultrafiltrate, 10 g L^{-1} glucose, 2 g L^{-1} lactose, 5 g L^{-1} glutamine, 1% lipid emulsion, and 0.1% Pluronic F-68 was used for producing recombinant proteins in batch mode employing two cell lines, S2AcRVGP2k expressing the G glycoprotein from rabies virus (RVGP) and S2AcHBsAgHy-9C expressing the surface antigen of hepatitis B virus (HBsAg), both obtained from *Drosophila melanogaster* S2 cells. Growth of wild-type S2 cells was also evaluated in the same medium. Cell behavior in the tested medium was compared to that verified in Sf900 II®. The results show that in shake flasks, S2AcRVGP2k and S2AcHBsAgHy-9C cells reached around 2×10^7 cells mL^{-1} in both media. In supplemented IPL-41 and Sf900 II® media, S2AcRVGP2k cells produced 367 ng RVGP mL^{-1} and 638 ng RVGP mL^{-1}, respectively, while S2AcHBsAgHy-9C cells correspondently produced 573 ng HBsAg mL^{-1} and 322 ng HBsAg mL^{-1} in the mentioned media. In stirred tanks, S2AcRVGP2k cells reached 3×10^7 cells mL^{-1} and produced up to 758 ng RVGP mL^{-1}. In general, glucose was consumed by cells, while lactate and ammonia were produced.

Keywords S2 cells · Metabolism · RVGP · HBsAg · Animal protein-free medium

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Introduction

Insect cells are used to produce a large number of recombinant proteins because they provide the expression of high levels of proteins and can present high growth rates [1]. In the last decade, several insect cell lines such as *Spodoptera frugiperda* (Sf9), *Trichoplusia ni* (T-ni), and *Drosophila melanogaster* S2 cells have been used for recombinant protein production [2–13].

Several proteins have been successfully produced using the *D. melanogaster* cells expression system. These include human plasminogen [14] and mouse endostatin [15]. Non-secreted and secreted recombinant proteins such as the G glycoprotein from rabies virus (RVGP) and the surface antigen of hepatitis B virus (HBsAg), respectively, can be expressed in *Drosophila* Expression System (DES), both showing to be appropriately processed and biologically active [13, 16–23]. Furthermore, S2AcRVGP2 cells cultivated in IPL-41 medium supplemented with 10 g L^{-1} glucose, 0.5 g L^{-1} fructose, 2 g L^{-1} lactose, 3.5 g L^{-1} glutamine, 0.6 g L^{-1} tyrosine, 1.48 g L^{-1} methionine, 6 g L^{-1} yeastolate ultrafiltrate, 1% chemically defined lipid concentrate, and 0.1% Pluronic F-68 are able to produce up to 31 ng RVGP L^{-1} in batch culture provided that dissolved oxygen and pH are controlled at 40% and 6.3, respectively [24].

In *Drosophila* cells, the plasmid-based non-lytic expression system is used and high copy numbers of recombinant plasmid vectors may be inserted into the host cell genome, with the advantage that foreign proteins are stably expressed without reduction in the number of protein-producing cells [11]. The *Drosophila* expression system has been available for several years, since these cells are able to synthesize proteins with adequate post-translational modifications [25]. The culture of *Drosophila* cells can be performed in several media, but serum-free medium is recommended, due to the cost, lot-to-lot variability and downstream processing difficulties associated to the use of serum-supplemented media [19, 26–29]. Moreover, regulatory agencies such as the European Medicine Evaluation Agency and the Food and Drug Administration (USA) encourage biological compounds manufacturers to reduce or eliminate the use of substances of animal origin [30]. Serum-free medium can be successfully formulated by supplementing basal media with yeastolate and a lipid emulsion (containing cholesterol, alfa-tocopherol acetate, fatty acids, Tween 80, and Pluronic F-68), among other components [5, 20].

In this study, IPL-41 supplemented with 10 g L^{-1} glucose, 6 g L^{-1} yeastolate ultrafiltrate, 1% chemically defined lipid concentrate, 2 g L^{-1} lactose, 5 g L^{-1} glutamine, and 0.1% Pluronic F-68 and SF900 II® were used, aiming to obtain high RVGP and HBsAg concentrations from transfected S2 cells. In addition, different cell culture systems were evaluated (shake flasks and a stirred tank bioreactor).

Regarding the products aimed in this work, Yokomizo et al. [13] observed that RVGP synthesized by S2AcRVGP2 cells was capable of inducing neutralizing anti-rabies antibodies production in vivo, protecting the animals against experimental rabies virus challenge. In addition, HBsAg is an approved component for human vaccines against hepatitis B, inducing both cellular and humoral responses [31], while transfected S2 cells (S2AcHBsAgHy cells) produce efficiently HBsAg [17] in Insect Xpress medium (13.5 μg/10^7 cells). Hence, RVGP and HBsAg are promising candidates as vaccine components and also for diagnostic purposes.

Materials and Methods

Cell Lines and Media

S2AcRVGP2k cells, a subclone of S2AcRVGP2 cells, producing the G glycoprotein from rabies virus RVGP, and S2AcHBsAgHy-9C cells producing HBsAg were used in this study.

Further details on cell modifications are provided in the literature [13, 17]. Wild-type *D. melanogaster* S2 cells obtained from Invitrogen Co. were also used. After establishing a working cell bank, the cells were subcultured in the commercial serum-free medium Sf900 II® (Invitrogen Co.) and in supplemented IPL-41 medium (Invitrogen Co.) containing 10 g L^{-1} glucose, 6 g L^{-1} yeastolate ultrafiltrate, 1% chemically defined lipid concentrate from Invitrogen Co., 2 g L^{-1} lactose, 5 g L^{-1} glutamine, and 0.1% m/v Pluronic F-68 from Sigma Chemical Co.

Insect Cell Culture

Frozen cells, initially adapted to Sf900 II® and to supplemented IPL-41 media by subsequent passages in each media in 25 cm^2 flasks (Nunc) at 28 °C, were used. After thawing, around 1 mL of cell suspension was transferred to 10 mL of fresh medium. The cells were centrifuged at 800×*g* for 5 min and then the pellet was re-suspended in the tested fresh medium. After that, the cells were cultivated in 75 cm^2 flasks (Nunc) for 72 h at 28 °C. To obtain the inoculum, the cells were subcultured in 100 mL shake flasks (Duran), with working volumes of 20 mL, for 96 h at 28 °C. After this period, the cells were recovered by centrifugation at 800×*g* for 5 min and re-suspended in fresh medium to attain concentrations sufficient to initiate all experiments at a viable cell concentration of 7.5×10^5 cells mL^{-1}.

Cell Cultivation in Shake Flasks

Cell cultivation in Sf900 II® and supplemented IPL-41 media was performed in batch mode using 100 mL shake flasks (with working volumes of 20 mL). All tests were performed in at least duplicate. The cells were incubated at 28 °C in a rotary shaker (New Brunswick Scientific/Innova 4230) at 100 rpm. Samples were periodically taken for cell count (90 μL), as well as for quantification of nutrients and metabolites (from 25 to 500 μL) and also for recombinant protein concentration (1,000 μL).

Cell Cultivation in Stirred Tank Bioreactor

S2AcRVGP2k cell culture was also conducted in a 2 L stirred tank bioreactor (Biostat B, B. Braun Biotech International, Germany), filled with 1.2 L of the previously described supplemented IPL-41. The bioreactor was equipped with a pitched blade impeller (B. Braun) and the agitation rate was maintained at 100 rpm. Temperature was controlled at 28 °C and dissolved oxygen concentration at 40% through a bubble-free aerating system consisting of 5 m silicon tubing. The gas flow varied from 1 up to 1.5 standard liter per minute of gas mixture (N_2 and O_2). Also in this case, samples were taken daily for cell count, residual nutrients and metabolite analysis, as well as for RVGP measurement.

Analytical Methodology

Cell concentration and viability were determined by trypan blue exclusion [32] using a hemocytometer (improved Neubauer, Brand).

Glucose, glutamine, glutamate, and lactate concentrations were analyzed using the YSI 2700 analyzer (Yellow Spring Instruments, USA).

Ammonium content was determined through a 95-12 Orion probe analyzer coupled to a 225 Mettler, potentiometer.

HBsAg was recovered from the supernatant of S2AcHBsAgHy-9C cells by centrifugation at 180×*g* for 10 min. HBsAg concentration was measured using the hepatitis B virus surface antigen enzyme immunoassay kit from Biomerieux (France), as indicated by the manufacturer.

The recombinant RVGP concentration was determined both in cell mass and in culture spent medium [33] by ELISA (Institut Pasteur, Paris). S2AcRVGP2k cells were centrifuged at 714×*g* for 10 min and washed with phosphate-buffered saline containing sucrose (320 mM), while the supernatant was stored at −20 °C. After washing, the phosphate-buffered saline (PBS) was eliminated by a new centrifugation step and the cells were re-suspended in lysis buffer (25 mM Tris, 25 mM NaCl, 5 mM $MgCl_2$, 0.2% Nonidet P-40 at pH 7.4) in an ice bath [34]. After 1 h at 4 °C with homogenization, with 15 min intervals, the supernatant was recovered after centrifugation at 714×*g* at 4 °C for 5 min and tested for the target protein. Both the RVGP recovered by cell lysis and the portion obtained from the supernatant of S2AcRVGP2k cells in culture were analyzed.

To evaluate RVGP expression by immunofluorescence, samples of wild-type S2 cells and S2AcRVGP2k cells were placed on glass slides and washed with PBS after cell adhesion. The cells were treated with cold acetone (80%) for 10 min in ice bath. The acetone was removed and the immunofluorescence reaction was performed with IgGD1-FITC-labeled monoclonal antibody anti-rabies glycoprotein, directed to antigenic site III, diluted in 0.02% of Evans Blue counterstain (1:400) for 60 min at 37 °C. After that, the samples were washed twice with PBS and observed through a fluorescence microscope (Olympus BX51) and META LSM 510 laser scanning confocal microscope.

Determination of Maximum Specific Growth Rate

The maximum specific growth rate (μ_{max}) was calculated through the following equation:

$$\mu_{\max} = \frac{1}{X}\frac{\mathrm{d}X}{\mathrm{d}t} \tag{1}$$

where, X is the viable cell concentration and t is the time.

Determination of Glucose-specific Uptake Rate

The glucose specific uptake rate (q_{Glc}) was determined during the exponential phase, being calculated according to the following equation [35]:

$$q_{\mathrm{Glc}} = -\left(\frac{\mathrm{d}S/dt}{\mathrm{d}X/dt}\right)\mu_{\max} \tag{2}$$

where, S refers to glucose concentration, X to the viable cell concentration, μ_{max} to the maximum specific growth rate, and t to the culture time.

Determination of Maximum Cell Productivity

Maximum cell productivity (P_{max}) was determined during the exponential phase using the following equation [36]:

$$P_{\max} = \frac{X_f}{\frac{1}{\mu_{\max}}\ln\frac{X_f}{X_o} + t_{\mathrm{lag}}} \tag{3}$$

where, X_f and X_o refer to final and initial cell concentration, μ_{max} is the maximum specific growth rate and t_{lag} is the length of the *lag* phase.

Results and Discussion

Growth and Metabolism of Wild-type S2 Cells in Shake Flasks

The influence of supplemented IPL-41 and Sf900 II® media on maximum viable cell concentration (X_{max}) and metabolism observed during the same cultivation period were evaluated in the first step of this study (Fig. 1, Table 1). X_{max} was similar for wild-type S2 cells cultured in supplemented IPL-41 and Sf900 II® media, equal to 2.6 and 2.4×10^7 cells mL^{-1}, respectively.

Figure 1 also shows that wild-type S2 cells showed to be better adapted to Sf900 II® medium, since their lag phase was longer in supplemented IPL-41 medium.

Glucose was exhausted in wild-type S2 cell cultures, suggesting that the glycolytic pathway is intense in dipterous cells. Galesi et al. [19] observed that recombinant S2 cells strongly consumed glucose until the sixth day of culture in Sf900II® medium, when this sugar was exhausted. In insects, as in most animals, glucose is the main source of energy. For *D. melanogaster* flies, for instance, glucose is the most efficient sugar for rapidly restoring the capacity for continuous flight [37].

Still regarding glucose metabolism, S2 cells showed lower values of q_{Glc} in supplemented IPL-41 medium in comparison to in Sf900 II® (4.7 and 8.2×10^{-12} g $cell^{-1}$ h^{-1}, respectively).

Lactate was not strongly produced by wild-type S2 cells, reaching at most 0.06 g L^{-1} in Sf900 II® medium. It was previously reported that transfected S2 cells cultured in Sf900

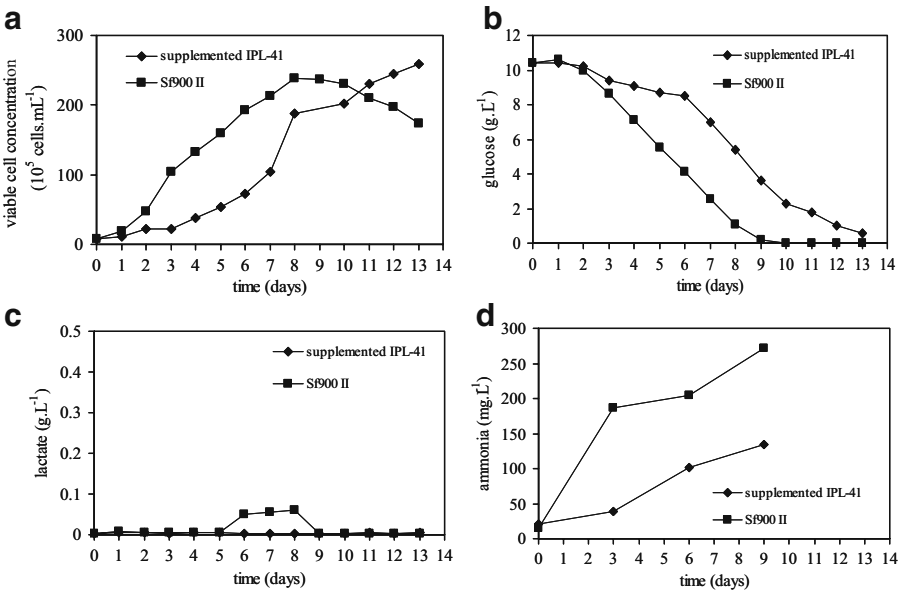

Fig. 1 Time course of cell (**a**), glucose (**b**), lactate (**c**), and ammonia (**d**) concentration for wild-type S2 cells cultured in 100 mL (20 mL working volumes) shake flasks at 100 rpm and 28 °C

Table 1 Kinetic variables of wild-type S2 cells cultured in supplemented IPL-41 and Sf900 II® media. Cells were maintained in shake flasks at 100 rpm and 28 °C.

Medium	X_{max} (10^5 cells mL^{-1})	μ_{max} (h^{-1})	P_{max} (10^5 cells mL^{-1} h^{-1})	q_{Glc} (10^{-12} g $cell^{-1}$ h^{-1})	Final to initial glutamine ratio (%)	Final to initial glutamate ratio (%)
Wild-type S2 cells						
Supplemented IPL-41	259	0.016	0.06	4.7	86.9	81.1
Sf900 II®	238	0.036	1.42	8.2	76.3	95.9

X_{max} maximum viable cell concentration, μ_{max} maximum specific cell growth rate, P_{max} maximum cellular productivity, q_{Glc} glucose-specific uptake rate.

II® medium, in shake flasks, produce lactate at low levels, reaching only around 0.057 g L^{-1} [22]. On the other hand, it was also observed that lactate is produced by *Drosophila* cells after complete depletion of glutamine at the end of the exponential phase [18].

Ammonia concentration increased during wild-type S2 cells cultivation, reaching 135 and 271 mg L^{-1} in supplemented IPL-41 and Sf900 II® media, respectively. However, insect cells are not as sensitive as mammalian cells to ammonia presence.

Growth, Metabolism, and Product Formation by Recombinant S2 Cells

In the last years, several studies were performed focusing on HBsAg and RVGP production using the DES. Vaccines against hepatitis B virus (HBV) were manufactured initially from HBsAg isolated from the plasma of asymptomatic carriers [38]. Because of the inherent risk of HBV transmission and of contamination with other infectious agents, the plasma-derived vaccine was replaced almost completely by recombinant material produced in vitro. Nowadays, HBsAg can be produced by mammalian cells [38], *Drosophila* S2 insect cells [16, 17], yeasts [39] and even by plant cells [40].

S2AcHBsAgHy-9C cells producing HBsAg were cultured in supplemented IPL-41 and in Sf900 II® media, in shake flasks (Fig. 2). It was observed that the cultures presented similar performance in supplemented IPL-41 and Sf900 II® media, growing to maximum concentrations of 1.7 and 2.1×10^7 cells mL^{-1}, respectively. Glucose was not exhausted and its residual concentration was around 2 g L^{-1} in both media. Figure 2 also shows that lactate was consumed simultaneously to glucose until day 5, when S2AcHBsAgHy-9c cells growth was interrupted. After that, lactate was produced by cells in Sf900 II® medium, reaching around 0.12 g L^{-1}, while maximum ammonia concentration reached 627 mg mL^{-1} in the same medium.

To find out which of the tested media was more effective in stimulating recombinant protein production, their effects on HBsAg production were investigated. As seen from Fig. 2, both media had a promoting effect on HBsAg production, but the supplemented IPL-41 medium showed to be the most effective. In this medium, HBsAg concentration was around 600 ng mL^{-1} at day 7. Besides containing high concentrations of glucose (10 g L^{-1}) and glutamine (5 g L^{-1}), supplemented IPL-41 medium is also a source of vitamins, lipids, and carbohydrates such as lactose and maltose.

Figure 3 shows equivalent results obtained for S2AcRVGP2k cells cultivated in the same media in shake flasks, and in Table 2 the kinetic parameters of the two recombinant cell lines cultured in these conditions are summarized.

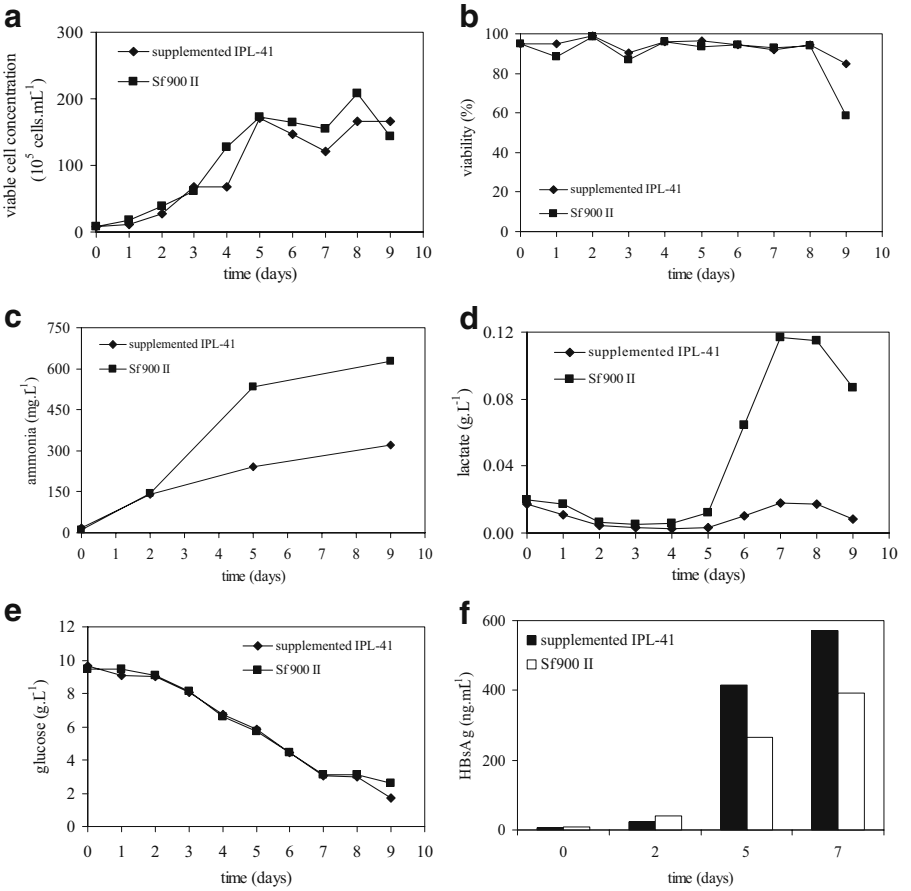

Fig. 2 Time course of cell concentration (**a**), viability (**b**), ammonia (**c**), lactate (**d**), glucose (**e**), and HBsAg (**f**) concentrations for S2AcHBsAgHy-9C cells cultured in 100 mL (20 mL working volumes) shake flasks at 100 rpm and 28ºC in different media

Similar to S2AcHBsAgHy-9C cells, also for the S2AcRVGP2k cell line, no significant differences were observed in $X_{max.}$ In supplemented IPL-41 medium, 2.0×10^7 cells mL^{-1} were attained, while 1.9×10^7 cells mL^{-1} were achieved in Sf900 II®. Glucose practically was exhausted in both media after 7 days (Fig. 3c). When compared to the recombinant S2AcRVGP2k and S2AcHBsAgHy-9C cells (Tables 1 and 2), the tested wild-type S2 cells consumed less glutamine. Changes in cell metabolism may indeed occur after gene alteration procedures and have been previously reported, for instance, for BHK-21A cells [41]. Many vector copies may have been incorporated in the S2 cells genome, which in turn may have intensified protein synthesis, causing the increase in glutamine consumption, since this compound is a protein precursor.

Lactate was produced by cells from days 3-6, probably due to oxygen limitation or glutamine limitation [18], since high cell concentrations were observed in this period. However, lactate concentration remained below 0.3 g L^{-1}.

Similar results were observed for ammonia production in both media. The cells maintained in Sf900 II® produced up to 545 mg L^{-1} of ammonia, while in supplemented

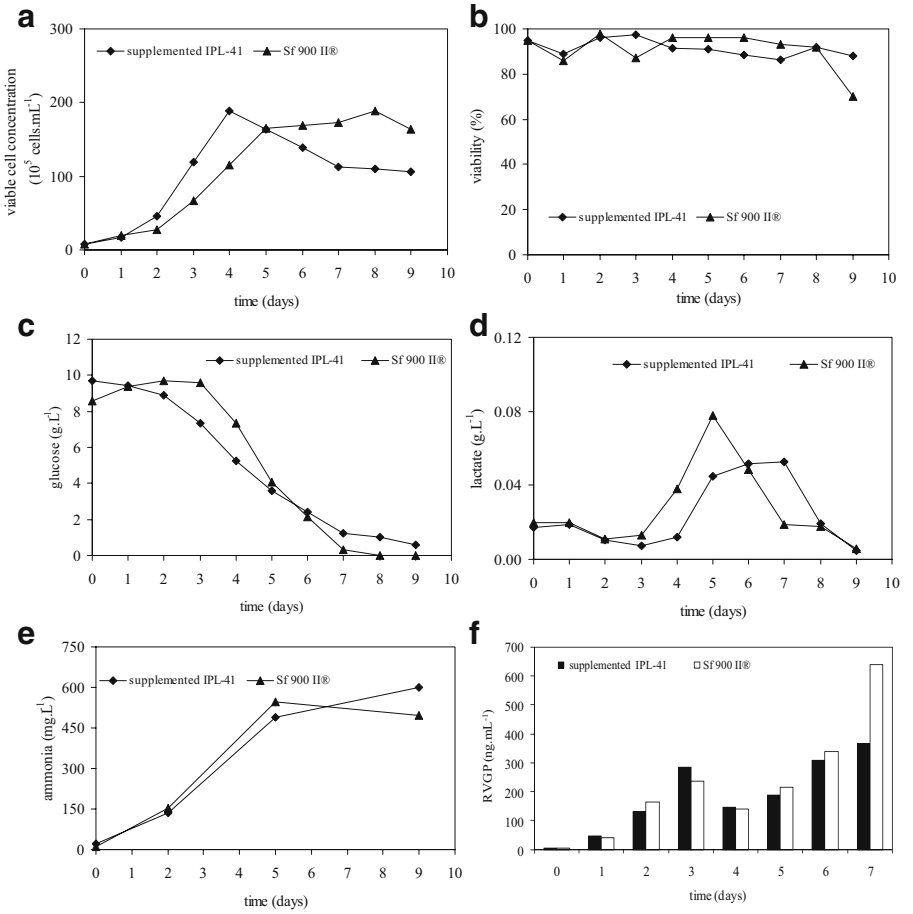

Fig. 3 Time course of cell concentration (**a**), viability (**b**), glucose (**c**), lactate (**d**), ammonia (**e**) and RVGP (**f**) concentrations for S2AcRVGP2k cells cultured in 100 mL (20 mL working volumes) shake flasks at 100 rpm and 28 °C

IPL-41 medium the production reached 600 mg L^{-1}. Previous studies show that transfected S2 cells are not strongly affected by high ammonia concentration in the medium, what supports the general observation that they consist on a robust cell expression system [20].

As shown in Table 2, μ_{max} values were 0.038 h^{-1} and 0.030 h^{-1} for S2AcRVGP2k cells cultured in supplemented IPL-41 and Sf900 II® media, respectively. In supplemented IPL-41 medium, the cells reached 1.64×10^5 cells mL^{-1} h^{-1}, while in Sf900 II® medium the maximum cellular productivity was 0.92×10^5 cells mL^{-1} h^{-1}. In contrast, RVGP content obtained in Sf900 II® medium at day 7 was higher than that observed in supplemented IPL-41 medium.

Regarding S2AcRVGP2k cells culture in a stirred tank bioreactor (results shown in Fig. 4), the highest RVGP concentration (1,143 ng mL^{-1}) was obtained for cells cultivated in supplemented IPL-41 medium (Fig. 4f), being 90% higher than that achieved in shake flasks. In addition, the viability at the end of exponential phase was above 90% (Fig. 4b). Besides, S2AcRVGP2k cells reached a specific growth rate of 0.047 h^{-1} during the

Table 2 Kinetic variables and protein concentration of S2AcRVGP2k and S2AcHBsAgHy-9C cells cultured in supplemented IPL-41 and Sf900 II® media.

Medium	X_{max} (10^5 cells mL^{-1})	μ_{max} (h^{-1})	P_{max} (10^5 cells mL^{-1} h^{-1})	q_{Glc} (10^{-12} g $cell^{-1}$ h^{-1})	Final to initial glutamine ratio (%)	Final to initial glutamate ratio (%)	$RVGP_{max}$ (ng mL^{-1})	$HBsAg_{max}$ (ng mL^{-1})
S2AcHBsAgHy-9C cells								
Sup. IPL-41	167	0.038	0.82	6.6	11.1	28.5	–	573
Sf900 II®	207	0.023	0.62	4.7	3.7	25.9	–	322
S2AcRVGP2k cells								
Sup. IPL-41	189	0.038	1.64	7.9	4.25	48.9	367	–
Sf900 II®	199	0.030	0.92	13.5	3.88	66.1	638	–

X_{max} maximum viable cell concentration, μ_{max} maximum specific cell growth rate, P_{max} maximum cellular productivity, q_{Glc} glucose-specific uptake rate, $RVGP_{max}$ RVGP concentration in the lysate, $HBsAg_{max}$ HBsAg concentration.

exponential phase, and the highest cellular productivity (1.8×10^5 cells mL^{-1} h^{-1}) was attained in the same conditions. These results demonstrate the superiority of the oxygenated system with submerged membranes when compared to cell culture in shake flasks.

Glucose was strongly consumed by cells during cell growth (Fig. 4c) in the bioreactor. This fact suggests that glucose is a limiting substrate in this culture condition, since cell growth stopped at day 6, when this nutrient concentration was quite low in the supplemented IPL-41 medium. The highest q_{Glc} was obtained in the bioreactor, equal to 27×10^{-12} g $cell^{-1}$ h^{-1}. Interestingly, this high uptake rate has not promoted overflow metabolism and formation of by-products such as lactate and ammonia (Fig. 4d and e, respectively). The cells consumed strongly glutamine, but not glutamate (data not shown).

Figure 4 also shows variations in both pH and dissolved oxygen concentration during S2AcRVGP2k cell culture in the stirred tank bioreactor. The pH remained around 6.2 (Fig. 4g), being, therefore, around the value recommended for insect cells culture [42]. The dissolved oxygen concentration remained at 40% during most of the culture period (value adjusted as the set point in the bioreactor control system) as shown in Fig. 4h. However, on day 5, the air supply was accidentally interrupted and remained in this way for 8 h. Despite this long disturbance, the cells did not die immediately, what is not surprising, since insect cells can tolerate anoxia better than mammalian cells [43]. However, cell growth decreased and ceased afterwards.

According to Fig. 4h, RVGP production increased in the lysate until day 5, after that, its concentration decreased. The concentration of RVGP in the supernatant was low during all cell culture period, what is consistent with the facts that this protein occurs associated with the cell membrane and also that low cell lysis levels were observed along the cultivation. A decrease in RVGP concentration was noticed after day 5. This behavior was already observed previously [24], and may be attributed to the fact that protein denaturation or aggregation can occur during recovery of transmembrane protein in samples with high cell concentration.

One of the main goals of cell cultivation for the production of biologicals is to achieve in a short period, high concentrations of metabolically active cells capable of rapidly synthesizing the target product in high quantities. S2AcRVGP2k cells collected at the exponential growth phase from shake flasks and expressing the RVGP on their membrane were assayed through immunofluorescence labeling (Fig. 5). The cells were maintained in

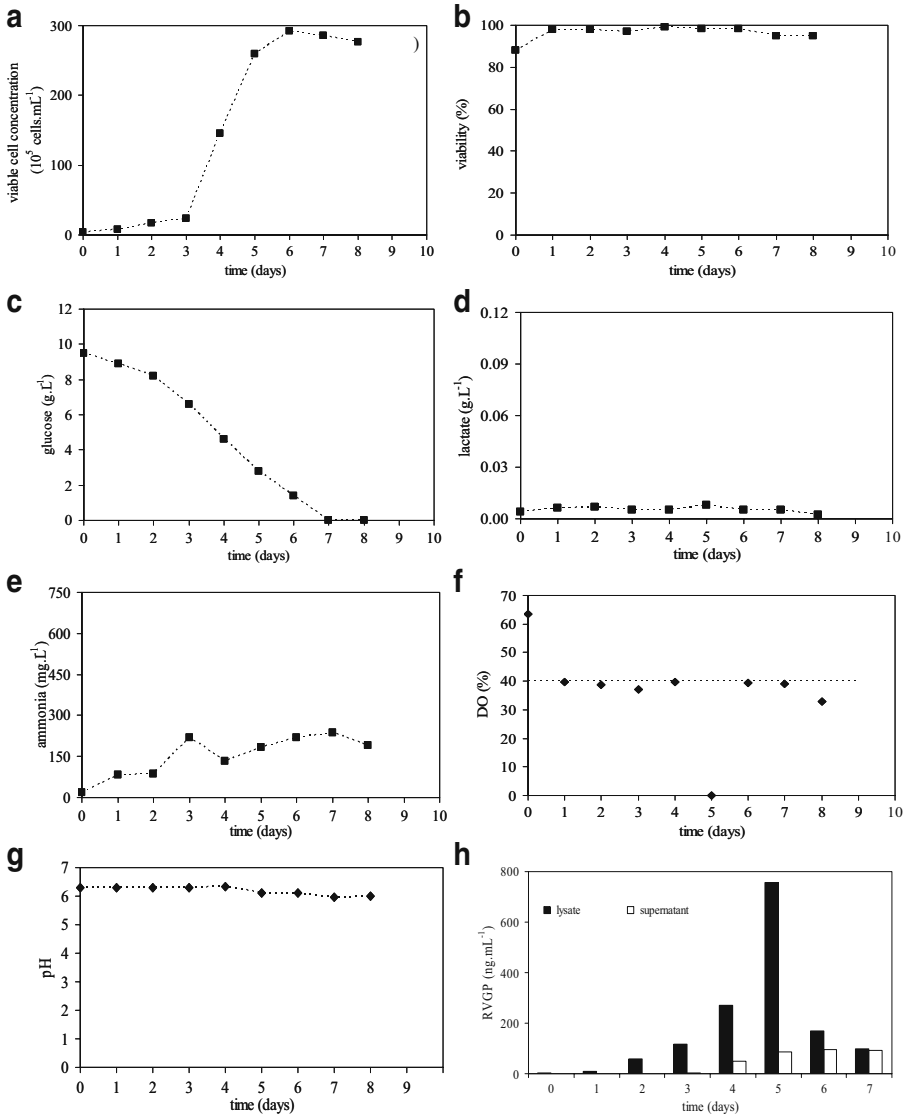

Fig. 4 Time course of cell concentration (**a**), viability (**b**), glucose (**c**), lactate (**d**), ammonia (**e**) and RVGP (**f**) concentrations for S2AcRVGP2k cells cultured in 2 L (1.2 L working volumes) bioreactor at 100 rpm and 28 °C. pH (**g**) and DO (**h**) were monitored, trial points (♦) and set point (−). Supplemented IPL-41 medium was used

supplemented IPL-41 medium and in Sf900 II® medium before treatment with anti-RVGP IgGD1-FITC labeled antibody (a and c, respectively) and similarly cultured wild-type S2 cells were used as controls (b and d, respectively).

The results show that not only the supplemented IPL-41 medium was able to increase maximum viable cell concentration, but also to provide adequate RVGP expression by recombinant S2 cells. Around 83% of the cells in the population were fluorescent in supplemented IPL-41 (Fig. 5a), while in Sf900 II® medium (Fig. 5c), this proportion was

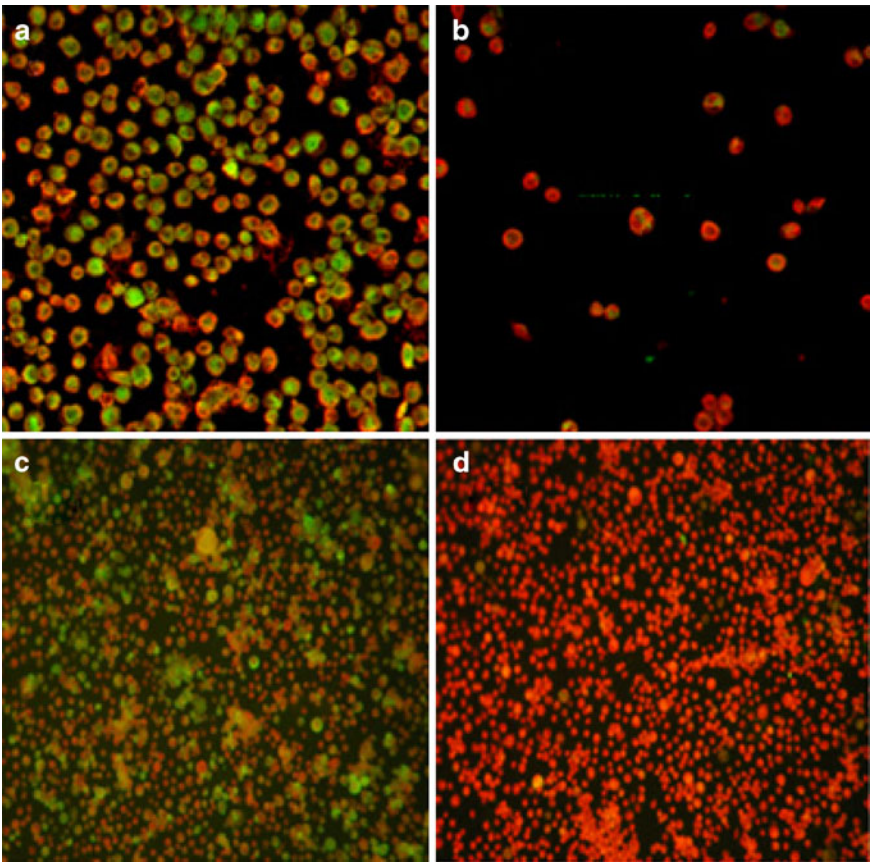

Fig. 5 S2AcRVGP2k (**a**) and S2 (**b**) cells cultured in supplemented IPL-41 medium (40X). S2AcRVGP2k (**c**) and S2 (**d**) cells in Sf900 II® medium (20X). A treatment with anti-RVGP IgGD1 FITC labeled antibody diluted in Evans blue reagent (1:300) for 60 min at 37 °C was performed. Cells stained in green present a marker for RVGP. Pictures represent fields of two independent experiments

only around 60%. However, after cell disruption to recover the target protein, it was verified that the highest RVGP concentration was achieved in cultures maintained in Sf900 II® medium (Table 2). These results indicate that the employed RVGP recovery process seems to be more efficient in Sf900 II® medium. In addition, the Sf900 II® medium formulation may contain protease inhibitors, helping to preserve protein activity, which were not added in the supplemented IPL-41 medium.

Conclusion

Culture experiments with transfected S2 cells showed that supplemented IPL-41 is a good alternative to stimulate the production of both tested recombinant proteins, RVGP and HBsAg, being, however, better for HBSAg. Unlike for mammalian cell cultures, lactate and ammonia apparently are not involved in growth inhibition. In addition, lactate did not accumulate under aerobic conditions. Besides, yield in bioreactor expressed as a function of the medium employed was higher than in shake flasks.

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References

1. Butler, M. (2006). *Cytotechnology, 50*, 57–76.
2. Maiorella, B., Inlow, D., Shauger, A., & Harano, D. (1998). *Nature Biotechnology, 6*, 1406–1410.
3. Akhnoukh, R., Kretzmer, G., & Schügert, K. (1996). *Enzyme and Microbial Technology, 18*, 220–228.
4. Benting, J., Lecat, S., Zacchetti, D., & Simons, K. (2000). *Analytical Biochemistry, 278*, 59–68.
5. Taticek, R. A., Choi, C., Phan, S.-E., Palomares, L. A., & Shuler, M. L. (2001). *Biotechnology Progress, 17*, 676–684.
6. Ikonomou, L., Bastin, G., Schneider, Y. J., & Agathos, S. N. (2001). *In Vitro Cellular & Developmental Biology, Animal, 37*, 549–559.
7. Valle, M. A., Kester, M. B., Burns, A. L., Marx, S. J., Spiegel, A. M., & Shiloach, J. (2001). *Cytotechnology, 35*, 127–135.
8. Elias, C., Carpentier, B., Durocher, E., Bisson, Y., Wagner, L., & Kamen, R. (2003). *Biotechnology Progress, 19*, 90–97.
9. Shin, H., & Cha, H. J. (2002). *Biotechnology Progress, 18*, 1187–1194.
10. Shin, H. S., & Cha, H. J. (2003). *Protein Expression and Purification, 28*, 331–339.
11. Cha, H. J., Shin, H. S., Lim, H. J., Cho, H. S., Dalal, N. N., Pham, M. Q., et al. (2005). *Biochemical Engineering Journal, 24*, 225–233.
12. Lim, H. J., & Cha, H. J. (2006). *Enzyme and Microbial Technology, 39*, 208–214.
13. Yokomizo, A. Y., Jorge, S. A. C., Astray, R. M., Fernandes, I., Ribeiro, O. G., Horton, D. S. P. Q., et al. (2007). *Journal of Biotechnology, 2*, 102–109.
14. Nilsen, S. A., & Castellino, F. J. (1999). *Protein Expression and Purification, 16*, 136–143.
15. Park, J. H., Kim, H. Y., Han, K. H., & Chung, I. S. (1999). *Enzyme and Microbial Technology, 25*, 558–563.
16. Deml, L., Wolf, H., & Wagner, R. (1999). *Journal of Virological Methods, 79*, 191–203.
17. Jorge, S. A. C., Santos, A. S., Spina, A., & Pereira, C. A. (2008). *Cytotechnology, 57*, 51–59.
18. Bovo, R., Galesi, A. L. L., Jorge, S. A. C., Piccoli, R. A. M., Moraes, A. M., Pereira, C. A., et al. (2008). *Cytotechnology, 57*, 23–35.
19. Galesi, A. L. L., Pereira, C. A., & Moraes, A. M. (2007). *Journal of Biotechnology, 2*, 1399–1407.
20. Batista, F. R. X., Pereira, C. A., Mendonça, R. Z., & Moraes, A. M. (2008). *Cytotechnology, 57*, 11–22.
21. Galesi, A. L. L., Aguiar, M. A., Astray, R. M., Augusto, A. F. P., & Moraes, A. M. (2008). *Cytotechnology, 57*, 73–81.
22. Swiech, K., Silva, C. S., Arantes, M. K., Yokomizo, A. Y., Astray, R. M., Pereira, C. A., et al. (2008). *Biotechnology and Applied Biochemistry, 49*, 41–49.
23. Swiech, K., Rossi, N., Silva, B. G., Jorge, S. A. C., Astray, R. M., & Suazo, C. A. T. (2008). *Cytotechnology, 57*, 61–66.
24. Batista, F. R. X., Moraes, A. M., Büntemeyer, H., & Noll, T. (2009). *Biologicals, 37*, 108–118.
25. Hansen, B. E., Andersson, E. C., Madsen, L. S., Engberg, J., Sondergaard, L., Svejgaard, A., et al. (1998). *Tissue Antigens, 51*, 119–128.
26. Hink, W. F. (1991). *In Vitro Cellular & Developmental Biology, Animal, 27*, 397–401.
27. Schlaeger, E. J., Foggetta, M., Vonach, J. M., & Christensen, K. (1993). *Biotechnology Techniques, 7*, 183–188.
28. Batista, F. R. X., Pereira, C. A., Mendonça, R. Z., & Moraes, A. M. (2005). *Cytotechnology, 49*, 1–9.
29. Batista, F. R. X., Pereira, C. A., Mendonça, R. Z., & Moraes, A. M. (2006). *Electronic Journal of Biotechnology, 9*, 522–532.
30. Castle, P., & Robertson, J. S. (1999). *Developments in Biological Standardization, 99*, 191–196.
31. Zhao, L. S., Qin, S., Zhou, T. Y., Tang, H., Liu, L., & Lei, B. J. (2000). *World Journal of Gastroenterology, 6*, 239–243.
32. Freshney, R. I. (1994). *Culture of animal cells: a manual of basic technique* (3rd ed.). New York: Wiley.
33. Perrin, P., Lafon, M., & Sureau, P. (1996). In F. X. Meslin, M. M. Kaplan, & H. Koprowaski (Eds.), *Laboratory techniques in rabies* (pp. 383–388). Geneva: WHO.

34. Astray, R. M., Augusto, E., Yokomizo, A. Y., & Pereira, C. A. (2008). *Journal of Biotechnology, 3*, 98–103.
35. Mendonça, R. Z., Palomares, L. A., & Ramírez, O. T. (1999). *Journal of Biotechnology, 72*, 61–75.
36. Wang DI, Cooney CL, Demain AL, Dunnill P, Humphrey AE, Lilly MD (1979) in Fermentation and enzyme technology. John Wiley and Sons, New York, pp. 374
37. Echalier G (1997) in *Drosophila* Cells in culture. Academic Press, New York, pp. 1–67
38. Holzer, J. W., Mayrhofer, J., Leitner, J., Blum, M., Webersinke, G., Heuritsch, S., et al. (2003). *Protein Expression and Purification, 29*, 58–69.
39. Hardy, E., Martínez, E., Diago, D., Díaz, R., González, D., & Herrera, L. (2000). *Journal of Biotechnology, 77*, 157–167.
40. Sunil Kumar, G. B., Ganapathi, T. R., Revathi, C. J., Prasad, K. S. N., & Bapat, V. A. (2003). *Protein Expression and Purification, 32*, 10–17.
41. Carvalhal, A. C., Coroadinha, A. S., Alves, P. A., Moreira, J. L., Hauser, H., & Carrondo, M. J. T. (2002). *Enzyme and Microbial Technology, 30*, 95–109.
42. Mitsuhashi, J. (1989). In J. Mitsuhashi (Ed.), *Invertebrate cell system applications* (pp. 3–20). Tokio: CRC Press.
43. Rhiel, M. A., & Murhammer, D. W. (1995). *Biotechnology and Bioengineering, 47*, 640–650.