



Short communication

Recombinant rabies virus glycoprotein synthesis in bioreactor by transfected *Drosophila melanogaster* S2 cells carrying a constitutive or an inducible promoter

Daniella Cristina Ventini^{a,d}, Renato Mancini Astray^a, Marcos Alexandre Nobre Lemos^a, Soraia Attie Calil Jorge^a, Camilo Calderón Riquelme^{b,c}, Claudio Alberto Torres Suazo^c, Aldo Tonso^d, Carlos Augusto Pereira^{a,d,*}

^a Laboratório de Imunologia Viral, Instituto Butantan, Avenida Vital Brasil 1500, São Paulo 05503-900, SP, Brazil

^b Departamento de Ciencias Químicas e Farmacéuticas, Universidad Arturo Prat, Av. Arturo Prat 2120, Iquique, Chile

^c Laboratório de Tecnologia de Cultivos Celulares, Departamento de Engenharia Química, Universidade Federal de São Carlos, Via Washington Luis, Km 235, 13565-905 São Carlos, SP, Brazil

^d Laboratório de Células Animais, Departamento de Engenharia Química, Escola Politécnica, Universidade de São Paulo, Av. Prof. Luciano Gualberto, trav. 3 n. 380, 05508-970 São Paulo, SP, Brazil

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ABSTRACT

S2 cell populations (S2AcRVGP2K and S2MtRVGP-Hy) were selected after transfection of gene expression vectors carrying the cDNA encoding the rabies virus glycoprotein (RVGP) gene under the control of the constitutive (actin) or inducible (metallothionein) promoters. These cell populations were cultivated in a 1 L bioreactor mimicking a large scale bioprocess. Cell cultures were carried out at 90 rpm and monitored/controlled for temperature (28 °C) and dissolved oxygen (10 or 50% air saturation). Cell growth attained $\sim 1.5\text{--}3 \times 10^7$ cells/mL after 3–4 days of cultivation. The constitutive synthesis of RVGP in S2AcRVGP2K cells led to values of 0.76 $\mu\text{g}/10^7$ cells at day 4 of culture. The RVGP synthesis in S2MtRVGP-Hy cell fraction increased upon CuSO_4 induction attaining specific productivities of 1.5–2 $\mu\text{g}/10^7$ cells at days 4–5. RVGP values in supernatant as a result of cell lysis were always very low ($<0.2 \mu\text{g}/\text{mL}$) indicating good integrity of cells in culture. Overall the RVGP productivity was of 1.5–3 mg/L. Our data showed an important influence of dissolved oxygen on RVGP synthesis allowing a higher and sustained productivity by S2MtRVGP-Hy cells when cultivated with a DO of 10% air saturation. The RVGP productivity in bioreactors shown here mirrors those previously observed for T-flasks and shaker bottles and allow the preparation of the large RVGP quantities required for studies of structure and function.

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Drosophila melanogaster Schneider 2 (S2) cells have become increasingly utilized over the past few years for the expression of heterologous proteins (see special issue on Heterologous Gene Expression in *Drosophila melanogaster* cells: Cytotechnology, 2008 volume 57). High levels of protein expression with pharmacological and biotechnological interest can be achieved using *Drosophila* Expression System procedure. Insect cell cultures are easier to handle than mammalian cells, being capable of multiplying in monolayers or in suspension at temperatures ranging from 22 to 30 °C. The recombinant rabies virus glycoprotein (RVGP) is an interesting biotechnological product as it can be used for the induction of protective immune response against rabies infection (Jallet et al., 1999; Perrin et al., 1985; Wiktor et al., 1984). RVGP is also a valuable model for the study of complex recombinant glycoprotein

expression since it is a viral membrane protein showing a biologically dependent oligomerization (Gaudin et al., 1992; Gaudin, 1997; Maillard and Gaudin, 2002; Sissioff et al., 2005). So, its expression, purification and stabilization represent a bioprocess challenge and its production at high quality in large scale is a welcome model for the expression of many other viral membrane glycoproteins.

In previous publications we have described the establishment of S2 cell lines expressing the RVGP and metabolic characteristics of these cells in culture (Lemos et al., 2009; Yokomizo et al., 2007; Bovo et al., 2008; Galesi et al., 2008). The *Drosophila* Expression System (DES) was developed in such a way allowing us a constitutive or an inducible gene expression, based respectively on the use of actin or metallothionein promoters (Invitrogen, Carlsbad, CA). We have modified the standard DES by constructing a single plasmid bearing cDNAs coding for the expression gene (RVGP) and the selection gene (*hygromycin*), allowing so a simple transfection procedure instead of a recommended standard co-transfection procedure (Lemos et al., 2009). In these cell population, RVGP levels as high as 5.5 $\mu\text{g}/10^7$ cells could be achieved upon cultivation in 100 mL shake flasks (Lemos et al., 2009). Preliminary data have

* Corresponding author at: Laboratório de Imunologia Viral, Instituto Butantan, Avenida Vital Brasil 1500, São Paulo 05503-900, SP, Brazil. Tel.: +55 11 37267222; fax: +55 11 37261505.

E-mail address: grugel@butantan.gov.br (C.A. Pereira).

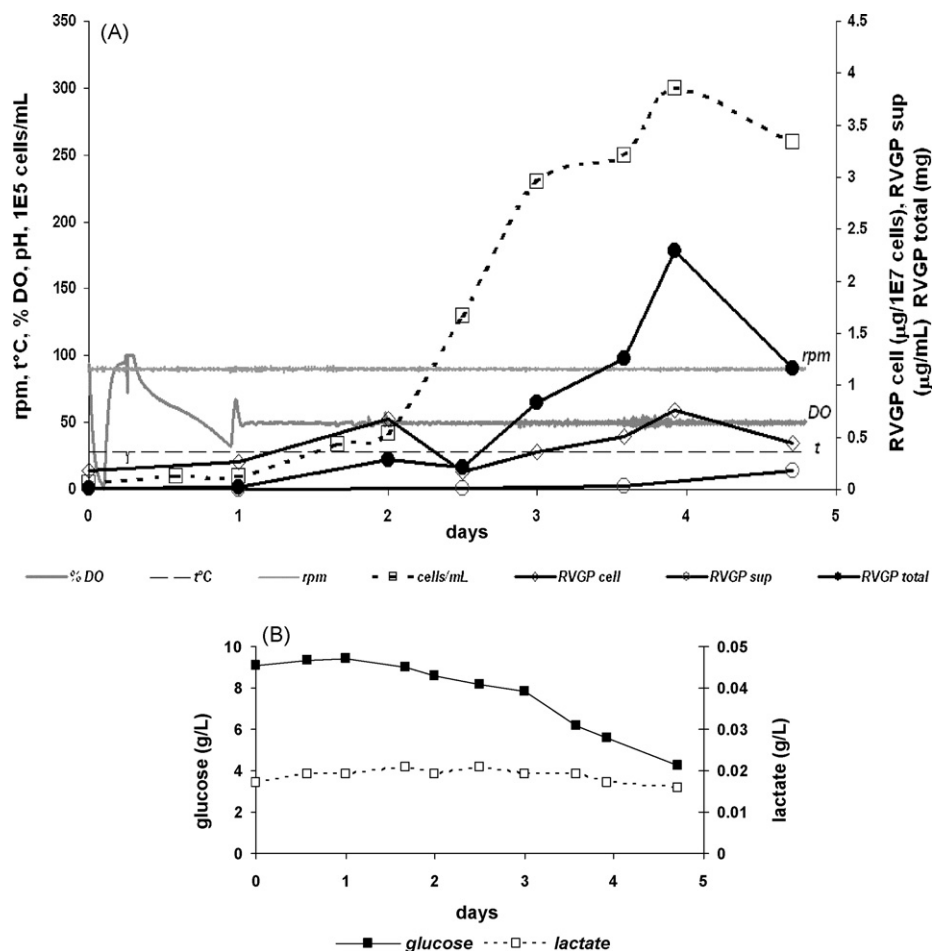


Fig. 1. Kinetic of S2AcRVGP2K cell growth, metabolism and byproduct synthesis for a bioreactor run. (A) Cell growth, dissolved oxygen (DO), agitation (rpm), temperature (t) and RVGP synthesis as indicated by its cell content (RVGP cell), supernatant detection (RVGP sup) and total synthesis (RVGP total). (B) Glucose consumption and lactate production. Cell cultures were performed in a 1 L BioFlo 110 bioreactor with SF900II medium. Data shown are representative of several runs performed in these conditions.

shown that recombinant RVGP produced has a preserved oligomerization and proper glycosylation allowing its biological activity of inducing a protective immune response in animals (Astray et al., 2008; Yokomizo et al., 2007).

The aim of the work presented here was the development of a bioprocess using a bioreactor to produce large quantities of RVGP in order to carry out studies of its purification and structure/function.

The two S2 cell populations used in this work were previously described (Lemos et al., 2009; Yokomizo et al., 2007). They have the RVGP gene expression under the control of inducible metallothionein (S2MtRVGP-Hy cells) or constitutive actin promoters (S2AcRVGP2K cells) and were obtained by, respectively, transfection or co-transfection procedures. The cells were grown in a 1 L BioFlo 110 bioreactor using SF900II medium with control of agitation (90 rpm), temperature (28 °C) and dissolved oxygen (DO). Cell culture runs were started with a cell seeding of 5×10^5 cells/mL. When the concentration reached $4\text{--}5 \times 10^6$ cells/mL, RVGP expression by S2MtRVGP-Hy cells was induced with 700 μM of CuSO₄. Kinetic studies were made by analyzing cell growth and metabolism (glucose and amino acids consumption and lactate and amino acids production). Glucose and lactate were measured in a YSI 2700 (Yellow Spring Instrument Incorporated, Ohio) biochemistry analyzer and amino acids were analyzed by the Pico-tag system, using a reverse-phase HPLC column (Waters Sweden, Sollentuna, Sweden). The RVGP expression was measured by ELISA, as previously described (Astray et al., 2008), and expressed in the cell fraction by its specific (RVGP/10⁷

cells) or total (RVGP/total number of cells in culture) synthesis or in the supernatant by its volumetric value (RVGP/mL) (Augusto et al., 2009).

The S2AcRVGP2K cell growth attained a maximal cell concentration of $\sim 3 \times 10^7$ cells/mL (Fig. 1A) in contrast to S2MtRVGP-Hy cells (Figs. 2A and 3A) where the maximal cell concentration was of $1.5\text{--}1.8 \times 10^7$ cells/mL. The glucose consumption by both cell populations showed similar kinetics (Figs. 1B, 2B and 3B). Glucose reached low levels after day 5, when the culture entered a irreversible death/decline phase. As expected for insect cell cultures, lactate was not synthesized (Figs. 1B, 2B and 3B) during the culture which was confirmed by the absence of pH shift during the culture (data not shown). Both, glucose and lactate seemed to be not limiting factors for S2 cell growth. Amino acids consumption/synthesis as evaluated in detail for S2MtRVGP-Hy cell cultures (Fig. 3C), showed an overall consumption during the culture, some of them possibly limiting the cell growth. Also the proper synthesis of the RVGP could be hindered by unavailability of amino acids which were well consumed during cell growth (leucine, serine, valine, threonine, proline) or present in low concentration in the media formulation (glycine) and enters importantly in the RVGP composition (Tordo et al., 1986) (Fig. 3C and D). Alanine, an end product of glutamine metabolism, was shown to be synthesized during S2 cell culture. As described for Lepidoptera (Sf9) insect cells, glucose derived pyruvate would be the carbon precursor for alanine and the glutamine would provide the nitrogen (Amable and Butler, 2008; Bettger and McKeehan, 1986). As far as investigated

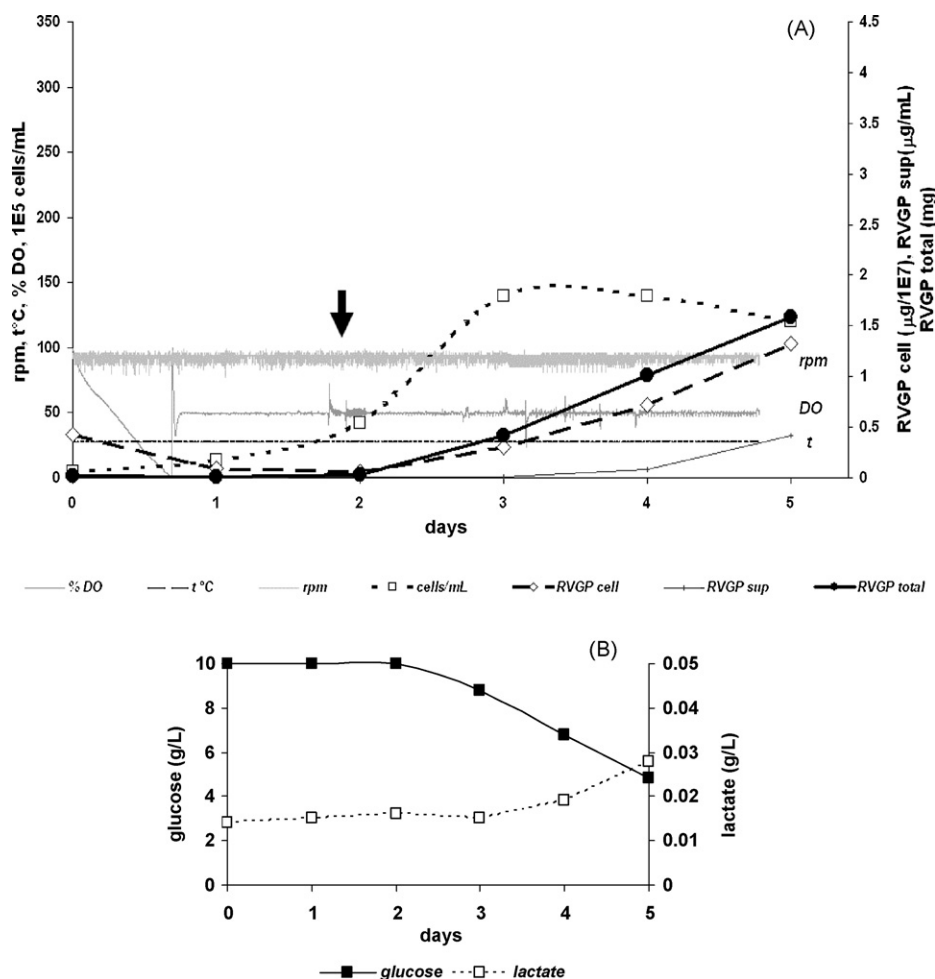


Fig. 2. Kinetic of S2MtRVGP-Hy cell growth, metabolism and byproduct synthesis for a bioreactor run. (A) Cell growth, dissolved oxygen (DO), agitation (rpm), temperature (t) and RVGP synthesis as indicated by its cell content (RVGP cell), supernatant detection (RVGP sup) and total synthesis (RVGP total). (B) Glucose consumption and lactate production. Cell culture was performed in a 1 L BioFlo 110 bioreactor with SF900II medium. Arrow indicates time of cell culture induction with 700 μM of CuSO₄. Data shown are representative of several runs performed in these conditions.

in this study, the S2MtRVGP-Hy cell growth and metabolism was shown to be not affected by the DO of 50 or 10% used for cultivation (Figs. 2 and 3). Nevertheless S2MtRVGP-Hy cell culture performed with a DO of 10% air saturation (Fig. 3A) allowed a higher and sustained RVGP synthesis when compared to cell cultures performed with a DO of 50% air saturation (Fig. 2A). Significant differences of RVGP productivity were observed from the CuSO₄ induction (day 2) throughout these assays until day 5 ending with an overall productivity of 3 and 1.6 mg of RVGP, for respectively, cultures at DO of 10% and 50% (Figs. 2A and 3A).

Accordingly to bioreactor runs with different cell constructions and conditions, the best results of RVGP expression were obtained with inducible S2MtRVGP-Hy cells in the following culture conditions: at 28 °C, 90 rpm agitation, pitched blade impellers, DO controlled at 10% of air saturation and 4 gas sparging aeration.

As compared to our previous data of RVGP synthesis by S2MtRVGP-Hy cells cultivated in shake flasks (Lemos et al., 2009), the present data in a well controlled environment provided by the bioreactor (Figs. 2A and 3A) showed that the cells were able to attain a higher concentration (1.8×10^7 cells/mL instead of 5.2×10^6 cells/mL in shake flasks) but the specific RVGP synthesis was lower ($1.5\text{--}2 \mu\text{g}/10^7$ cells instead of $5.5 \mu\text{g}/10^7$ cells). This observation suggested us that: (1) the optimized cell growth provided by the bioreactor conditions did not favor the specific RVGP production. Data showed also that S2 cells are capable of storing a

higher RVGP amount than observed in bioreactor conditions. Since specific RVGP synthesis did not parallel cell growth a further effort has to be made in order to optimize specific RVGP synthesis independently of the cell growth and (2) specific cell culture media for production of a given membrane protein has to be formulated in order to provide the necessary amino acids for both the cell growth and the recombinant protein synthesis.

Bioreactors are basically conceived for optimizing the cell growth and expecting that this leads to optimized product synthesis. Nevertheless, our evidence that lower specific recombinant protein synthesis was achieved in such a system reinforces the hypothesis that we can still optimize the production of RVGP by the S2MtRVGP-Hy cells upon proper cultivation in a well controlled bioreactor environment favoring product accumulation and not cell multiplication.

The bioreactor cultivation of S2 cells expressing RVGP is more than never a very promising way to obtain the essential high quantities of the synthesized protein. Although the specific RVGP synthesis was not as high as what we could obtain in small shake flasks, the total protein that can be obtained (3-mg/L) (Fig. 3A and data not shown) is more than suitable for the purposes envisaged.

In conclusion, beside its contribution to future optimization approaches of productivity, the present study already provides the bioprocess conditions for further essential studies of stable glycoprotein extraction from recombinant S2 cells that can now be

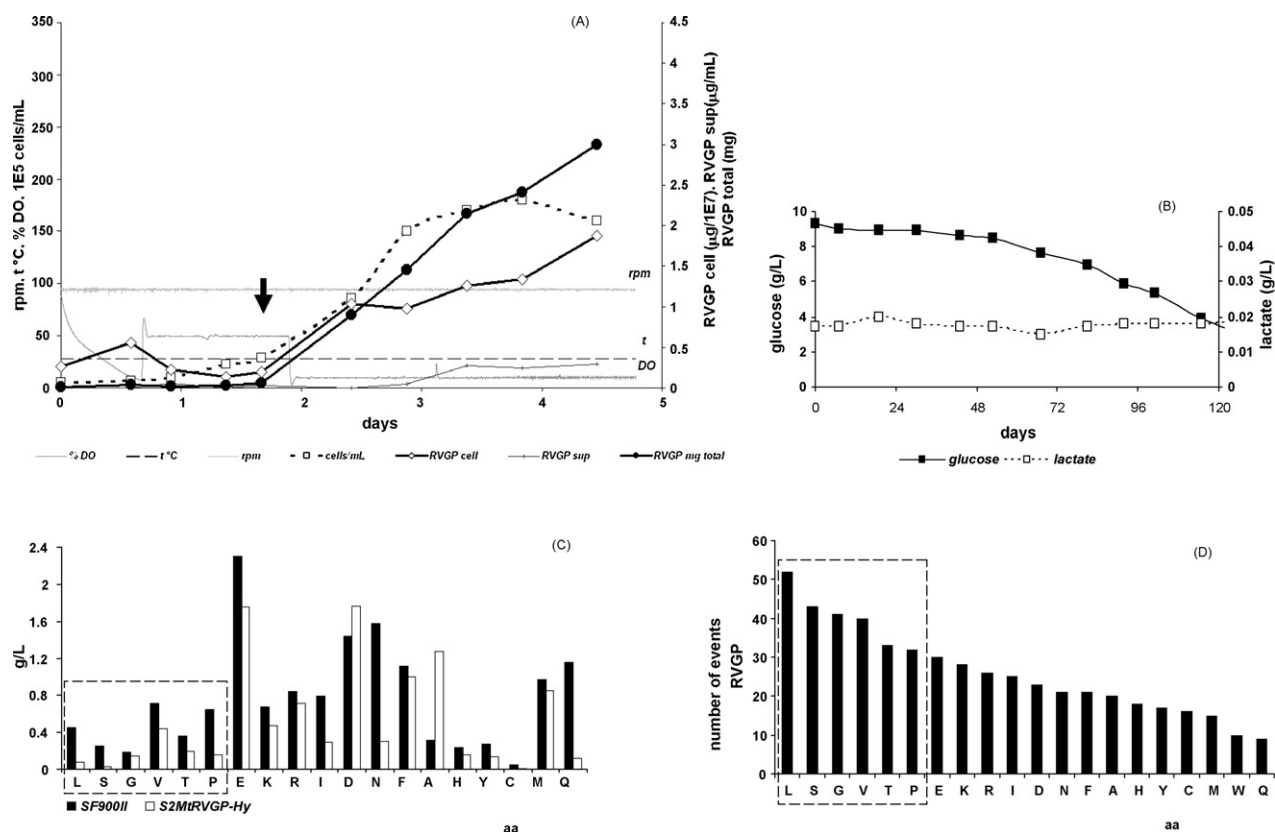


Fig. 3. Kinetic of S2MtRVGP-Hy cell growth, metabolism and byproduct synthesis for a bioreactor run. (A) Cell growth, dissolved oxygen (DO), agitation (rpm), temperature (t) and RVGP synthesis as indicated by its cell content (RVGP cell), supernatant detection (RVGP sup) and total synthesis (RVGP total). (B) Glucose consumption and lactate production. (C) Amino acids consumption/synthesis at day 4 of culture. (D) Proportional amino acid content of RVGP (Tordo et al., 1986), with more abundant amino indicated in dashed box. Cell culture was performed in a 1 L BioFlo 110 bioreactor with SF900II medium. Arrow indicates time of cell culture induction with 700 μM of CuSO₄. Data shown are representative of several runs performed in these conditions.

properly performed for development of efficient purification procedures preceding studies of structure and function.

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