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Effect of hypothermic temperatures on production of rabies virus glycoprotein by recombinant *Drosophila melanogaster* S2 cells cultured in suspension

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ABSTRACT

Aiming at maximizing the production of transmembrane rabies virus glycoprotein (rRVGP), the influence of hypothermic temperature on a recombinant *Drosophila melanogaster* S2 cell culture in Sf-900II medium was investigated. Cell growth and rRVGP production were assessed at 4 culture temperatures in Schott flasks: 16, 20, 24 and 28 °C. The maximum specific growth rates μ_{max} were, respectively: 0.009, 0.019, 0.038 and 0.035 h^{-1}, while the maximum rRVGP levels C_{max}^{rRVGP} were: 0.075, 2.973, 0.480 and 1.404 mg L^{-1}. The best production temperature (20 °C) was then tested in a bioreactor with control of pH and dissolved oxygen in batch and fed-batch modes. In the batch culture, μ_{max} and C_{max}^{rRVGP} were 0.060 h^{-1} and 0.149 mg L^{-1} at 28 °C and 0.026 h^{-1} and 0.354 mg L^{-1} at 20 °C, respectively. One batch-culture experiment was carried out with adaptation of the cells by the temperature falling in steps from 20 °C to 16 °C, so that μ_{max} fell from 0.023 to 0.013 h^{-1}, while C_{max}^{rRVGP} was improved to 0.567 mg L^{-1}. In the fed-batch mode at 20 °C, μ_{max} was 0.025 h^{-1} and C_{max}^{rRVGP} was 1.155 mg L^{-1}. Taken together, these results indicate that the best strategy for optimized rRVGP production is the culture at hypothermic temperature of 20 °C, when μ_{max} is kept low and with feeding of limitant aminoacids.

1. Introduction

Mammalian cells, such as CHO (Chinese hamster ovary) and BHK (Baby hamster kidney) have been extensively studied and are widely used in industry to produce recombinant proteins (Sunley and Butler, 2010). Insect cells have recently emerged as a new and powerful alternative, since they require milder culture conditions than mammalian cells, such as lower temperatures and no CO_2 addition to the gas phase and can grow easily in serum-free suspension cultures at high cell densities (Moraes et al., 2012). Cell lines such as *Spodoptera frugiperda* (Sf) and *Trichoplusia ni* (BTI-TN-5BI-4 or High-Five™) have been used mainly to produce heterologous proteins upon recombinant baculovirus infection (Ikonomou et al., 2003). More recently, several studies conducted with the insect cell line *Drosophila melanogaster* S2, have shown that these cells can be stably transfected and grown in suspension serum-free cultures reaching cell concentrations as high as 5×10^7 cells mL^{-1} (Pamboukian et al., 2008).

S2 cells have been considered a promising platform for recombinant protein production (Bernard et al., 1994; Kim et al., 2008; Moraes et al., 2012). Recombinant proteins such as human dopamine β-hydroxylase (Li et al., 1996), human β-secretase (Chang et al., 2005), human plasminogen (Nilsen and Castellino, 1999), site-specific biotinylated human myeloid differentiation factor 88 (MyD88) (Basile et al., 2007), the E glycoprotein of the Japanese Encephalitis Virus (JEV) (Zhang et al., 2007), fish gonadotropin (GtHs) subunits of luteinizing hormone (LH), follicle stimulating hormone (FSH) (Zmora et al., 2007) and active tetravalent dengue virus glycoprotein (Clements et al., 2010) have been successfully expressed and secreted by recombinant S2 cells.

The researchers in the biotechnological field are putting a great deal of effort into maximizing recombinant protein expression in transfected cell lines. Highly productive cells are essential to the technical and economical viability of the bioprocess when it is scaled up for industrial production. A number of strategies to improve expression have been used with mammalian cell lines, resulting in significant rises in the yield of recombinant protein; two of these may be highlighted due to its easy implementation: (a) hypothermal growth conditions – i.e. culturing cells at temperatures lower than optimal for growth (Kumar et al., 2007; Li et al., 2006) and (b) addition of substances that promote the expression

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of the recombinant protein, such as dimethyl sulfoxide, sodium butyrate, rapamycin, AMP and glycerol (Kumar et al., 2007; Li et al., 2006). Hypothermal cell culture is beneficial in several ways, despite the drop in specific growth rate (μ). The following are some of the most interesting advantages of this strategy: high cell viability is maintained for a longer time (Furukawa and Ohsuye, 1998), specific productivity is raised (Furukawa and Ohsuye, 1999; Hendrick et al., 2001); proteolytic activity is reduced (Yoon et al., 2003), glycosylation is improved (Bollati-Fogolin et al., 2005; Yoon et al., 2003) and sensitivity to apoptotic agents is reduced (Sakurai et al., 2005). Hypothermal culture has proved very efficient in raising the amount of heterologous protein expressed (Kumar et al., 2007) and can thus be described as a simple, cheap and productive strategy to achieve such improvement.

Even though the response of mammalian cells to low temperature and the biological mechanisms involved are not yet fully understood, significant progress has been made towards elucidating them in recent research (Roobol et al., 2009; Gammell et al., 2007; Underhill and Smales, 2007; Baik et al., 2006). Success in the use of low temperature conditions to boost recombinant protein expression in cell culture depends on the type of cell used, CHO cells being the best studied system and the one that has given the best results in practical terms (Al-Fageeh et al., 2006). In contrast, hybridoma cells showed little or no improvement in specific production of monoclonal antibodies when the culture temperature was lowered (Chong et al., 2008). Insect cell lines, on the other hand, having appeared more recently on the scene of applied biotechnology, remain little investigated and nothing has, as yet, being published on the potential optimization of their productive capacity at low temperatures.

Seminal work on the use of transfected *D. melanogaster* S2 cells (Swiech et al., 2008a; Yokomizo et al., 2007) showed them to possess very interesting characteristics when cultured in suspension: (a) a remarkable growth capacity and; (b) a high potential for the expression of a recombinant membrane-bound protein, the rabies virus glycoprotein (rRVGP). In recent works (Batista et al., 2009; Galesi et al., 2008), researchers have succeeded in improving the rate of growth of these cells by altering the culture medium and operating conditions in the bioreactor, but as far as glycoprotein expression rates are concerned, further research effort will be needed to identify the development strategies that may lead to useful increases in the concentration of glycoprotein. In a previous study (Swiech et al., 2008b) a comparison was accomplished of the two strategies highlighted above with the purpose of increasing the rRVGP production – addition of substances (DMSO and glycerol) and use of hypothermic temperature. The results revealed the high potential of the hypothermic growth for increased expression of rRVGP.

In this context, we decided to assess in this work the influence of mild hypothermic culture temperature on the production of transmembrane recombinant rabies virus glycoprotein by suspended *D. melanogaster S2* cells, in preliminary small-scale studies in shaken flasks and in conditions nearer to the production scale in a bioreactor. The results demonstrate further the potential usefulness of these cells in a bioreactor, both in terms of their growth characteristics and their notably enhanced rate of expression of recombinant protein.

2. Materials and methods

2.1. Materials

2.1.1. Cells and culture conditions

The cells employed in the study were of the *D. melanogaster* S2 recombinant line S2AcGPV2K, expressing the rabies virus glycoprotein with immunogenic activity, as described previously by Yokomizo et al. (2007). The stock cells were conserved frozen in liquid N_2 at −196 °C. After activation and expansion at 28 °C, these cells were cultured in 250 mL Schott flasks in a rotary shaker, and in a 2 L tank bioreactor with gentle stirring, in Sf-900 II medium (GIBCO BRL) supplemented with amino acids previously identified as stoichiometrically limitants (Swiech et al., 2008c): 1.944 g L^{-1} proline, 1.350 g L^{-1} glutamine, 0.0067 g L^{-1} cysteine and 0.066 g L^{-1} serine (Sigma–Aldrich). The experimental work was carried out in two stages. The first consisted of small-scale tests in the 250 mL Schott flasks with 30 mL of medium, inoculated with 5×10^5 cells mL^{-1} and shaken at 100 rpm. In these experiments, four culture temperatures were assessed: 16°, 20°, 24° and 28 °C (control). The temperatures at which rRVGP production was most promising in Schott flasks were then reproduced in the bioreactor cultures, in the second stage.

The large-scale tests were run in a 2 L Bioflo 110 bioreactor (New Brunswick Scientific, NJ, USA), with a working volume of 1 L, stirred by a marine (three) blade impeller rotating at 150 rpm and held at constant pH (6.2) and dissolved oxygen (50% of air saturation). The pH was adjusted by adding 0.5 M NaOH and 8% (v/v) H_2SO_4, while oxygenation was effected by gas diffusion through a tubular silicone membrane (7.4 m long, i.d. 1.6 mm). The initial cell density was 5×10^5 cells mL^{-1}, the inoculum being taken from a mid-exponential phase culture raised at 28 °C in a 1 L spinner flask (Wheaton) rotated at 100 rpm. The best temperature for rRVGP production (20 °C) identified in Schott-flask cultures was tested in bioreactor cultures operated in batch and fed-batch modes; in the latter, the initial working volume was 850 mL and feed stream flowed at 3 mL h^{-1}. With the aim of testing the effect of a gradually reduced temperature, as opposed to the usual "cold-shock" approach (step change to lower temperature), on the production of rRVGP, a batch run was carried out with the temperature falling in stages (20 → 18 → 16 °C). Owing to an observation in the Schott flask cultures at 20 °C that serine, glutamine, cysteine and asparagine ran out during the test, it was decided to raise the concentrations of these amino acids in the medium used for bioreactor culture at this temperature.

2.2. Analytical methods

2.2.1. Monitoring of cell cultures experiments

Samples from the culture were collected periodically and alliquoted for cell density and viability determination. The remaining volume was centrifuged at 1000 rpm for 10 min and the supernatant was stored at −20 °C for subsequent analysis of substrates metabolites and rRVGP.

2.2.2. Cells density and viability

Total cell density (C_{xt}) was measured under an Olympus BX 40 optical microscope, in a hemocytometer, while exclusion of the stain trypan blue was used to distinguish and count the viable cells (C_{xv}) as described in Doyle and Griffiths (1998). Both measurements were made in quadruplicate and the result expressed as cells mL^{-1}.

The maximum specific cell growth rate (μ_{max}) in batch and fed-batch cultures was determined from the slope of the linear range of the curve $\ln(VC_x^v) = f(t)$ and expressed in units of h^{-1}, V being the volume (mL) of culture medium in the bioreactor. The cell productivity (P_x) of the culture for both operating modes was calculated by the expression $(C_x^{max} - C_x^0)/t_{cul}$, where t_{cul} (h) is the time taken to the cell density to vary from the initial C_x^0 to the maximum C_x^{max} value of the culture.

2.2.3. Analysis of substrates and metabolites

High-performance liquid chromatography (HPLC) was performed in a Aminex HPX-87H (Bio-Rad) resin column, with 5 mM

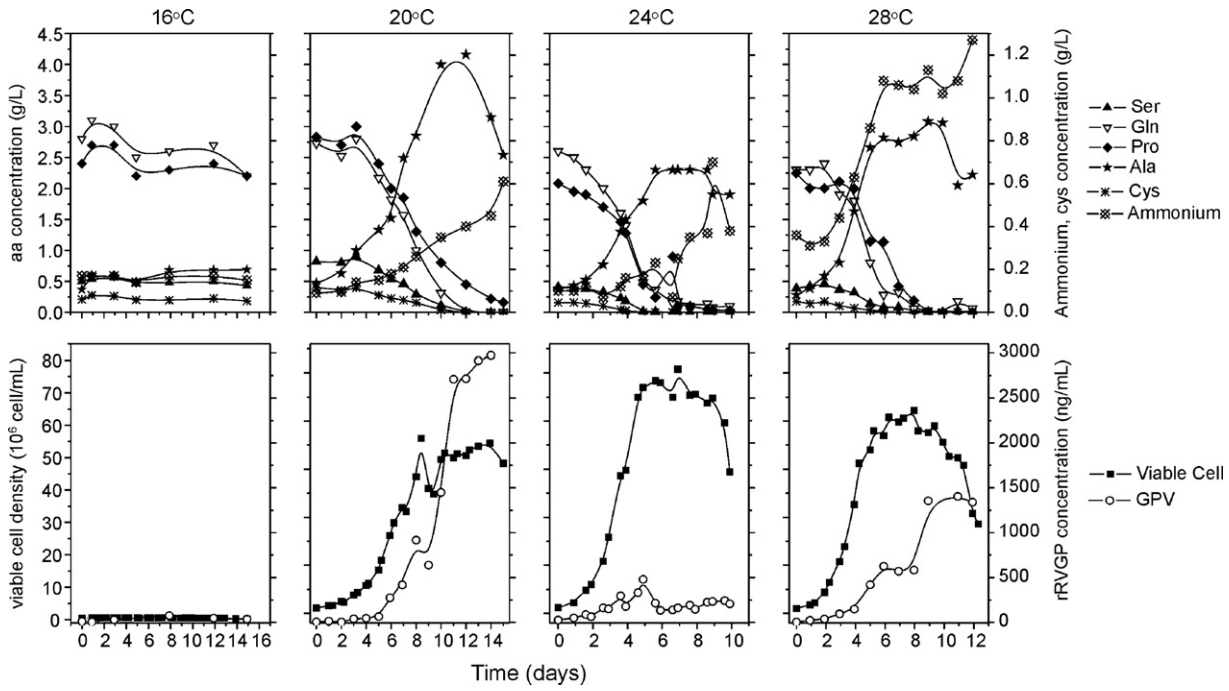

Fig. 1. Comparison of growth, rRVGP production and metabolism of S2AcGPV K cells at 16, 20 and 28 °C, when cultured in Sf-900 II medium, supplemented with 1.944 g L^{-1} proline, 1.350 g L^{-1} glutamine, 0.007 g L^{-1} cysteine and 0.066 g L^{-1} serine, in 250 mL Schott flasks (30 mL working volume) at initial pH 6.2 in shaken at 100 rpm.

H_2SO_4 as the mobile phase, flowing at 0.6 mL min^{-1}, in a Waters chromatograph fitted with a refractive index detector (W 410, waters) for carbohydrates and a UV detector (210 nm) for lactic acid. Amino acid concentrations were also determined by HPLC, in a Waters reversed-phase column, employing the pico-tag system and UV detection at 254 nm. Ammonium ions were analyzed by an Orion 710A ion-selective electrode (Thermo-Scientific). Taken into account the dilutions, the final value of aminoacids concentration was expressed in g L^{-1}.

In the small scale experiments carried on Schott flasks there was no analysis of glucose and lactic acid as the consumption of the high glucose levels present in Sf-900 II culture medium (10 g L^{-1}) was far from exhaustion during the aerobic phase of the culture. In the experiments in bioreactor with dissolved oxygen controlled at 50% of air saturation the glucose and lactic acid concentration were monitored during the whole experiment.

2.2.4. Analysis of rRVGP

The rabies virus glycoprotein was analyzed by an ELISA (enzyme linked inmunosorbent assay) method, in which the wells of a microplate were first sensitized with purified polyclonal anti-RVGP antibody. After this, rabies glycoprotein released from the cells, as established by Astray et al. (2008), was bound by the antibody, washed and then exposed to anti-RVGP, conjugated to a peroxidase molecule. This enzyme marker was used to catalyze the production of a colored compound, so that the amount of rRVGP bound in each well could be assayed by reading the absorbance of that well in a spectrophotometric microplate reader and comparing it with the absorbances generated by a series of dilutions of the reference antigen, in nearby wells, as described by Astray et al. (2008).

The concentration of rRVGP is represented by C^{max}_{rRVGP} expressed as ng mL^{-1}.The mean specific production rate ($\bar{q}_{rRVGP}$) in batch and fed-batch cultures was determined by using the mean value of the calculations made with the expression $q_{rRVGP} = ((V_{i+1}C^{i+1}_{rRVGP} - V_iC^i_{rRVGP})/\Delta t)/\bar{X}_v\bar{V}$) for pairs of experimental values considered during the whole time period of exponential cellular growth and expressed in units of ng (10^6 cells)$^{-1}$ h^{-1}. The term ($V_{i+1}C^{i+1}_{rRVGP} - V_iC^i_{rRVGP}$) is the variation of rRVGP mass during the time interval Δt, $\overline{X_vV}$ is the average cell number for that time interval and V is the volume of culture medium in the bioreactor. Note that V is constant for batch mode and variable for fed-batch mode. The rRVGP productivity (P_{rRVGP}) of the culture was calculated with the expression $(C^{max}_{rRVGP} - C^0_{rRVGP})/t_{cul}$, where t_{cul}(h) is the time taken to the glycoprotein varying from the initial C^0_{rRVGP} to the maximum C^{max}_{rRVGP} value during the culture.

3. Results and discussion

3.1. Small-scale experiments in Schott flasks

The purpose of these preliminary tests was to identify an optimal temperature range for production of rRVGP in S2AcGPV2K cells, which would act as a basis for the design of bioreactor culture experiments. The results for the cell growth, metabolism and production of rRVGP in the Schott-flask cultures are shown in Fig. 1, while Table 1 summarizes the kinetic parameters for growth and rRVGP production at the tested temperatures.

3.1.1. Culture at 16 °C

As can be seen in Fig. 1, at 16 °C the insect cells grew very little in the first few days and then fell into decline until the end of the run. This could be due to the severe cold shock to which the cells were exposed when the temperature was suddenly dropped from 28 to 16 °C. The cell growth and rRVGP production kinetics parameters are shown in Table 1. The maximum rRVGP content in the culture (C^{max}_{rRVGP}) was just 75.15 ng mL^{-1}, very low in comparison with the 1404.9 ng mL^{-1} produced in the control (28 °C) experiment. The maximum productivity (P^{max}_{rRVGP}) was also very low, at 0.40 ng mL^{-1} h^{-1}. With so little growth and recombinant protein synthesis, there was practically no consumption of substrates or production of metabolites. Evidently, the cold shock from 28 to 16 °C is not a good strategy for raising the production of this recombinant protein as, even though the yield per cell was high, there were so few cells that the productivity was negligible.

Table 1
Kinetics parameters for growth and rRVGP production of S2AcGPV2K cells at 16, 20, 24 and 28 °C, cultured in Sf-900 II medium supplemented with 1.994 g L^{-1} proline, 1.350 g L^{-1} glutamine, 0.007 g L^{-1} cysteine and 0.066 g L^{-1} serine, in 250 mL Schott flasks (30 mL working volume) at initial pH 6.2, shaken at 100 rpm.

Growth			
Temperature (°C)	μ_{max} (h^{-1})	C_x^{max} (10^6 cells mL^{-1})	P_x^{max} (10^6 cells mL^{-1} h^{-1})
16	0.009 ± 0.0002	0.67	0.02
20	0.019 ± 0.0005	18.70	0.10
24	0.038 ± 0.0001	25.40	0.15
28 Control	0.035 ± 0.0001	21.30	0.11
rRVGP production			
Temperature (°C)	$\bar{q}_{rRVGP}$ ng (10^6 cells)$^{-1}$ h^{-1}	C_{rRVGP}^{max} (ng mL^{-1})	P_{rRVGP}^{max} (ng mL^{-1} h^{-1})
16	0.70	75.15	0.40
20	0.97	2973.06	8.30
24	0.75	480.34	4.08
28	0.62	1404.90	5.36

3.1.2. Culture at 20 °C

When the culture temperature was dropped from 28 °C to 20 °C, the maximum specific growth rate fell from 0.036 h^{-1} to 0.020 h^{-1} and the maximum cell density from 21.3×10^6 cell mL^{-1} to 18.7×10^6 cell mL^{-1}, as may be seen in Table 1, while the period of high cell viability was extended appreciably. The total production and mean specific production of rRVGP were considerably higher at 20 °C than in the control run at 28 °C (Table 1). Moreover, in the 20 °C culture there was a significant rise in the rRVGP productivity compared with the control because of high values of both q_{rRVGP} and C_x^{max} leading to a high accumulated value of the glycoprotein productivity (P_x) at the end of the growing phase. In fact, it was in this experiment that the highest specific production and highest productivity of the recombinant protein were achieved in Schott flask cultures. In addition, no decline in the concentration nor any indication of degradation of rRVGP were observed once the depletion of nutrients started later (day 11) than the experiments at 24 °C and 28° C. A feature of this culture that is worth mentioning is the sharp rise in the rate of production of rRVGP and growth almost null, seen between days 9 and 11 of the experiment. The only correlation observed, which could plausibly explain this phenomenon, is the low dissolved oxygen concentration that occurred at this time in a parallel test culture carried out in a Schott flask under similar conditions (data not shown). Assuming this conjecture to be correct, it may be supposed that low levels of dissolved oxygen reduced the rate of cell proliferation, which favored a high rate of rRVGP synthesis. This inverse relationship between cellular growth and synthesis of rRVGP has been consistently reproduced in other work with S2 cell lineages (Swiech et al., 2008b).

The amino acids that limited the cell growth rate on being exhausted prematurely were cysteine, serine and glutamine (Fig. 1). On day 11, rRVGP production decelerated as the supply of cysteine ran out. This is the amino acid with the lowest concentration in Sf-900 II medium and it carries out very important functions in protein stabilization (Freedman, 1984) and in the metabolism of other amino acids (Doverskog et al., 1998). The production of alanine was very high, but it started to be consumed as the glutamine was exhausted. Ammonium was produced in large amounts; however, it seemed to have little effect on cell growth.

3.1.3. Culture at 24 °C

Reduction of the culture temperature from 28 °C to 24 °C had a positive effect on cell growth, increasing both μ_{max} (0.038 h^{-1} against 0.035 h^{-1} in the control) and the maximum cell density (25.4×10^6 cells mL^{-1} against 21.3×10^6 cells mL^{-1}). It can be inferred from this result that the optimal temperature of growth of S2AcGPV2K lineage is around 26 °C and not 28 °C as suggested by the commercial supplier (http://products.invitrogen.com/ivgn/product/R69007, 2011). However, the total production and maximum productivity of rRVGP fell significantly at 24 °C. Furthermore, the mean specific production of rRVGP was reduced at 24 °C in relation to that at 28 °C (see Table 1). The production of rRVGP may have been limited by the high consumption and sequential shortage of cysteine, serine, proline and glutamine that started very early in the experiment – at day 4. A high quantity of alanine was produced, but it was consumed once proline and glutamine fell to low concentrations. In Fig. 1, a high production of ammonium can be seen which reached 0.699 g L^{-1} in the medium. The deleterious effect of this metabolite is difficult to evaluate due to the parallel effect caused by the shortage of aminoacids.

3.1.4. Culture at 28 °C

It can be seen in Fig. 1 that in the control culture, at 28 °C, the S2AcGPV2K cells had a very similar growth curve to that at 24 °C in the initial growth phase. Thus, the maximum growth rates (μ_{max}, see Table 1) were 0.035 and 0.038 h^{-1}, respectively. The maximum concentration of recombinant protein (C_{rRVGP}^{max}) was 1404.9 ng nL^{-1}, less than half the value reached at 20 °C (2973.06 ng mL^{-1}). The maximum productivity (P_{rRVGP}^{max}) was about 5.36 ng mL^{-1} h^{-1}, appreciably less than at 8.30 ng mL^{-1} at 20 °C. Thus, the cultures at 20 °C were the most productive in terms of C_{rRVGP}^{max} and P_{rRVGP}^{max}. From day 5 of the control culture, cysteine, serine, glutamine and proline were sequentially exhausted, resulting in reductions of both the production of recombinant protein and cell growth. Ammonia production reached the highest levels in Schott flasks experiments, 1.2 g L^{-1}. Research carried out regarding the accumulation of ammonia in insect cell culture has shown that the concentrations observed here can reach toxic levels, especially when glucose were near exhaustion (Bedard et al., 1997; Ohman et al., 1996). Tn-5 cells can accumulate high levels of ammonium by the end of the culture, exceeding 0.341 g L^{-1}, and yet exhibit a metabolism similar to that of normal mammalian cells (Benslimane et al., 2005). So, it can be seen that a temperature of 28 °C, considered until now as the best temperature for the growth of S2AcGPV2K cells, proved far from ideal for the production of rRVGP.

Summarizing the results from cultures in Schott flasks, the strategy that enabled the best cell growth, with the highest values of both the maximum specific rate of cell growth (μ_{max}) and the maximum cell density (C_x^{max}), was to drop the temperature of the medium from 28 °C to 24 °C. This also minimized the duration of the lag phase. In contrast, reducing the temperature still further, to 20 °C, diminished rate of growth, production of ammonium and the maximum cell density to below the control values and lengthened

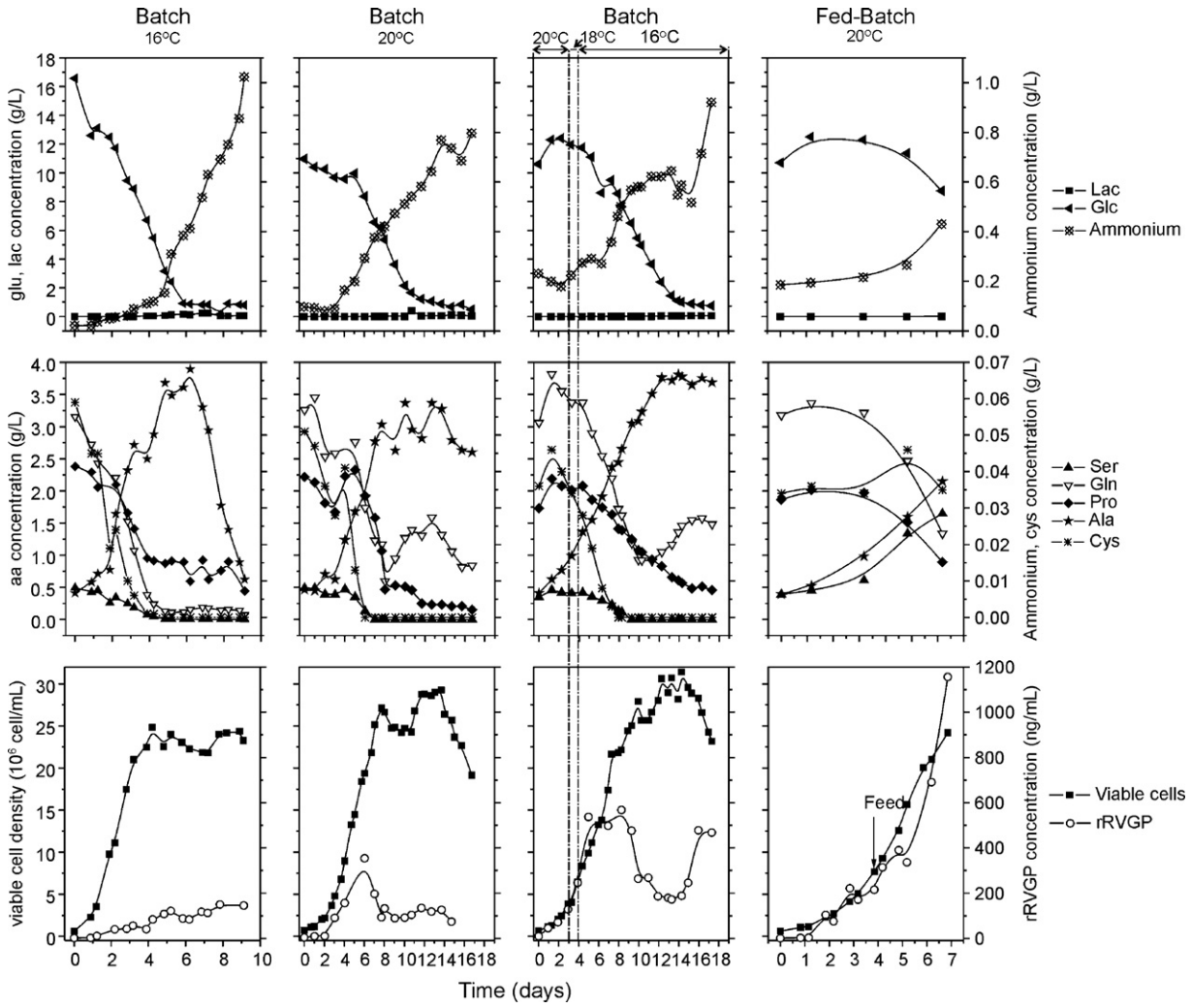

Fig. 2. Comparison of growth, rRVGP production and metabolism of S2AcGPV2K cells at various temperatures, when cultured in a Bioflo 110 in 1L of Sf-900 II medium, supplemented with 1.944 g L^{-1} proline, 2.350 g L^{-1} glutamine, 0.013 g L^{-1} cysteine, 0.132 g L^{-1} serine and 0.066 g L^{-1} asparagine, at pH 6.2, dissolved 0_2 50%, stirred at 150 rpm.

the lag phase considerably. On the other hand, production of rRVGP was strongly enhanced at 20 °C, with very significant rises in the kinetic parameters of production. Meanwhile, the attempt to culture the cells at 16 °C gave very poor results both for cell growth and for rRVGP production and was clearly not a good strategy for these purposes. Therefore, with the aim of boosting the production of the recombinant rabies glycoprotein, the culture strategy chosen for testing in the bioreactor, where the conditions (pH and dissolved oxygen) could be controlled better, was to drop the temperature to 20 °C.

3.2. Bioreactor experiments

The profiles of cell growth, substrate consumption and rRVGP production in the 1-L cultures carried out in the Bioflo 110 bioreactor are shown in Fig. 2, while Table 2 lists the kinetic parameters for cell growth and rRVGP production at each of the temperatures tested in these cultures.

3.2.1. Batch cultures at 28 °C

The maximum specific rate of growth of the S2AcGPV2K cells in this culture was 0.060 h^{-1}, about twice the rate observed in Schott flasks at 28 °C, and the maximum cell density (25.4×10^6 cell mL^{-1}) was also higher than the value of C_x^{max} in the small-scale culture (21.3×10^6 cell mL^{-1}). The sudden end of the exponential growth phase and a drop in rRVGP production at around 4 days coincided with the exhaustion of cysteine in the medium, as can be seen in Fig. 2. Radford et al. (1997) cultured Sf 9 cells in Sf-900II medium in bioreactor and found that the only amino acid completely consumed by the cell culture was cysteine, and the authors attributed the end of exponential growth to this factor. It could also be seen that the consumption of other amino acids ended when there was no more cysteine; in other words, it is likely that the exhaustion of exogenous cysteine disables cell metabolism. Moreover, those researchers observed that the concentration of that amino acid was seen to limit the production of recombinant protein, since cysteine was exhausted 60 h after infection with baculovirus and this event was associated with a reduction in the yield of recombinant protein.

In this work, the alanine, by-product produced by the cells began to be consumed and ammonia production accelerated at the moment when the level of glucose fell below 2 g L^{-1}. This behavior is in agreement with Ohman et al. (1995), who found that glucose limitation in Sf9 cultures induces a radical change in cell metabolism and alanine is consumed as a carbon source, causing the release of ammonium. On the other hand, the low levels of lactate, by-product of the glycolysis is easily explained, once it is not normally produced by insect cells growing aerobically (Rhiel and Murhammer, 1995).

Table 2
Kinetic parameters for growth and rRVGP production in 1 L cultures of S2AcGPV2K cells in a Bioflo 110 bioreactor, at various temperatures with controlled pH (6.2) and dissolved O_2 (50% air saturation) stirred at 150 rpm. Sf-900 II medium was supplemented with 1.944 g L^{-1} proline, 2.35 g L^{-1} glutamine, 0.013 g L^{-1} cysteine, 0.132 g L^{-1} serine and 0.066 g L^{-1} asparagine, to raise rRVGP production.

	Growth				
	Temperature (°C)	t_{lag} (h)	μ_{max} (h^{-1})	C_x^{max} (10^6 cells mL^{-1})	P_x^{max} (10^6 cells mL^{-1} h^{-1})
Bio 1	28	21.1	0.060 ± 0.0002	25.7	0.25
Bio 2	20	49.0	0.026 ± 0.0006	29.7	0.15
Bio 3	20	70.5	0.023 ± 0.0008	31.0	0.12
	18		0.028 ± 0.0002		
	16		0.013 ± 0.0008		
Bio 4[c]	20	52.3	0.028 ± 0.0001[a]	24.3	0.15
			0.025 ± 0.0004[b]		
	rRVGP production				
	Temperature (°C)	$\bar{q}_{rRVGP}$ ng (10^6 cells)$^{-1}$ h^{-1}	C_{rRVGP}^{max} (ng mL^{-1})	P_{rRVGP}^{max} (ng mL^{-1} h^{-1})	
Bio 1	28	0.16	149.98	0.80	
Bio 2	20	0.43	354.14	2.44	
Bio 3	20 → 18 → 16	0.98	567.38	2.86	
Bio 4[c]	20	0.82	1155.94	7.03	

[c] Fed-batch culture; b – before feed; a – after feed.

3.2.2. Batch culture at 20 °C

In view of the total exhaustion of glutamine, serine, asparagine and cysteine observed in the Schott-flask test at 20 °C, these amino acids were added to the bioreactor medium in greater, non inhibitory amounts. Exceedingly higher amounts of the most critical aminoacids cysteine and serine were found inhibitory of cell growth. The Sf-900II medium was thus supplemented with 1.944 g L^{-1} proline, 2.35 g L^{-1} glutamine, 0.013 g L^{-1} cysteine, 0.0132 g L^{-1} serine and 0.066 g L^{-1} asparagine. Overgaard et al. (2007) make it clear that the metabolic profile in the fruitfly (*D. melanogaster)* changes sharply as the temperature fluctuates and that some of these metabolic changes may be associated with cell cryoprotection. Lowering the temperature from 28 °C to 20 °C led to an approximately three-fold reduction of μ_{max} which fell from 0.060 h^{-1} to 0.026 h^{-1}, although the maximum cell density rose from 25.7×10^6 cell mL^{-1} to 29.7×10^6 cell mL^{-1} (Table 2), with no significant fall in the high cell viability throughout the culture period. In contrast, all the kinetic parameters related to recombinant protein production were improved at 20 °C, as may be seen in Table 2.

Nevertheless, the results achieved in the Schott flask experiment were not equaled in the bioreactor; thus, the total rRVGP production per unit volume was about nine-fold higher in the Schott flask culture. A plausible explanation for this result is the difference between the oxygen levels in the flask and bioreactor. In the flask, the oxygen concentration falls monotonically until the end of the culture, when it approaches zero. In the bioreactor, dissolved oxygen is maintained at around 50% of air saturation, so that the growth rate remains high for longer periods in this culture than in the Schott flask and less metabolic energy is left over from the degradation of carbohydrates and amino acids, for the synthesis of recombinant glycoprotein. Thus, amino acids that are limiting in this synthesis, such as serine and cysteine, are exhausted earlier in the bioreactor culture, explaining the higher degradation of the protein. These two amino acids play very important parts in the folding and internal crosslinking of the protein. On one hand, serine participates strongly in the post-processing of the protein by side-chain glycosylation, which in turn enables better folding and helps protect the protein from digestion by proteases (Jentoft, 1990). On the other hand, cysteine is well known for its ability to form —S—S— covalent bridges within the protein molecule, a crucial part of the stabilization of tertiary structure (Freedman, 1984).

3.2.3. Batch culture at three temperatures, 20 → 18 → 16 °C

Since some of the above results suggested that a reduction in the rate of cell growth might be responsible for the observed increase in protein production, one experiment was carried out in the bioreactor in which the temperature was lowered in stages from 20 °C to 16 °C, with the aim of assessing in more detail the capacity of lower temperatures to raise rRVGP production. It was this culture that achieved the highest maximum cell density of the study, reaching 31×10^6 cell mL^{-1}, which until now is a record in the literature for batch cultures of S2 cells. The maximum specific growth rate at 20 °C was about three times lower than that of the control (at 28 °C). Next, the cells showed an accelerated growth at 18 °C, but μ_{max} fell back about half the rate at 20 °C, when the temperature was lowered to 16 °C, as shown in Table 2. Once again, the strategy of slowing the growth rate of the cells promoted the production of recombinant protein: the total production was increased to four times the control value (at 28 °C).

However, the amino acids serine and cysteine were again exhausted (at about the 8th and 4th day, respectively, simultaneously with downward changes in the rate of growth and rRVGP production, reinforcing their importance for the biosynthesis and stability of this glycoprotein. The other amino acids were also consumed, but without being completely exhausted. The glucose concentration fell steadily, reaching a low value at the end of the run, while alanine and ammonium rose to high concentrations, although it is believed that these levels were insufficient to inhibit cell growth. At the start of culture, there was a small rise in the level of glucose and in some aminoacids, apparently due to the metabolic change induced by the switch to lower temperature. Thus, Overgaard et al. (2007) observed, in an experiment on characterization of the physiology of *D. melanogaster*, an increase in the concentrations of glucose and alanine, immediately after the fruit flies had been subjected to a cold shock or a treatment of fast adaptation to cold, when they noted higher sugar levels, especially of glucose and trehalose.

3.2.4. Fed-batch culture at 20 °C

This experiment was carried out with a feed of Sf-900 II medium supplemented with 0.86 g L^{-1} cysteine and 7.4 g L^{-1} serine, since it was demonstrated in the previous culture experiments that these two amino acids are critical to cell growth and biosynthesis of rRVGP, such that rRVGP was degraded when they fell to low levels or were completely exhausted. As can be observed in Fig. 2, the objective of maintaining the levels of the two amino acids

constant until the end of the culture was partially achieved in the case of cysteine, even though at the cost of reaching the threshold of exhaustion that began at the fourth day of the experiment, where all the fed aminoacid was consumed. On the other hand, the serine concentration showed a little increasing trend that would be more balanced if the feeding contained a smaller amount of this amino acid. Such conditions enabled the cell growth to be maintained, while avoiding both the loss of rRVGP by degradation and the accumulation of excess ammonium. The fed-batch culture at 20 °C showed the best results for recombinant rabies virus protein production, reaching the highest concentration in the culture and the greatest rRVGP yield per cell of all the bioreactor experiments (see Table 2).

Taking a global picture of the results obtained in the bioreactor cultures there are some interesting features that deserve to be highlighted, as the beneficial metabolic effects got with the hypothermic temperature. Under this condition it was observed low production of lactate, which reached a maximum value of 0.243 g L^{-1} at 28 °C versus 0.062 g L^{-1} at 16 °C and 0.012 g L^{-1} at 20 °C in the fed-batch experiment. On the other hand, the maximum concentration of ammonia was 1.02 g L^{-1} at 28 °C versus 0.92 g L^{-1} at 16 °C and 0.43 g L^{-1} at 20 °C with fed-batch. Another interesting feature is the lower necessity of glutamine by the cells, once it was synthesized in the experiments at 20 °C and 16 °C. Lastly, the depletion of nutrients caused adverse effects on cell growth that were not evident by the trypan blue viability. In some experiments, like in the decreasing in steps (20 → 18 → 16 °C) for example, analysis of cytosolic lactate dehydrogenase activity revealed significant increasing values of cell lysis (data not shown) after the exhaustion of cysteine and serine. In contrast, the fed-batch at 20 °C experiment showed negligible cell lysis.

Comparing all the bioreactor results, it can be reported that culture performed at three different temperatures (20,18 and 16 °C) was the one that showed the lowest maximum specific rate of growth (μ_{max}), but the highest maximum cell density (C_x^{max}). Lowering the culture temperature from 28 °C to 20 °C resulted in a reduced rate of growth, lower ammonium concentration, lower maximum cell productivity and longer lag phase. The best rRVGP production was achieved in the fed-batch culture at 20 °C, which gave under conditions of no depletion of nutrients, a total production and maximum productivity around seven and eight times higher than those observed in the control experiment (28 °C), respectively.

The biphasic strategy suggested by Sunley and Butler (2010), namely, an initial growth phase at physiological temperature followed by a shift to sub-physiological temperature to induce protein synthesis, is not applicable to the production of rRVGP by S2 cells because the synthesis of this membrane glycoprotein and the cell proliferation occur at the same time. Then the culture has to be well balanced to conciliate the growth arrest with the enhanced protein synthesis in a tunable culture. This means that the growth cannot be severely limited or strongly stimulated by the hypothermic temperature because it can adversely affect the total protein yield.

Finally, the following step for the optimization of the bioprocess should be the assessment of the potential of the fed-batch at 20 °C at both, constant or decreasing temperature during the culture, in order to establish policies that allow the reduction of growth and the achievement of high productivity and high quality of the rRVGP to be produced.

4. Conclusions

The strategy that yielded the best growth of the recombinant S2 cells in Schott flasks was to culture them at 24 °C. However, production of rRVGP was not so high at this temperature. Conversely, when the culture temperature was dropped to 20 °C, the growth rate and cell density fell considerably, but the amount of recombinant protein synthesized was greatly enhanced, attaining the highest concentration seen in any culture in this study, 2973 ng mL^{-1}. The other strategies tested here in Schott flasks, where cells were cultured at 16 °C and 28 °C, proved unsuitable for high production of this glycoprotein. Thus, batch and fed-batch fermentations in the bioreactor were carried out at 20 °C, with a control at 28 °C.

In the bioreactor, by far the most efficient experiment for rRVGP production was the fed-batch culture at 20 °C. This showed that, when there was no shortage of nutrients in the culture medium, no degradation of the protein product was observed, corroborating the importance of these amino acids in synthesis and stabilization of rRVGP.

The strategy of controlling the cell growth by maintaining the culture at a sub-optimal temperature, to induce greater protein production, proved highly effective, as the expression of rRVGP at 20 °C was much greater than at 28 °C allowing to validate the principle of mild hypothermic temperature to *D. melanogaster* S2 cells as it was done to mammalian cells. In production cultures of CHO cells, the use of low temperatures has been found to be the most efficient and practical strategy to control the proliferation of the cells and enhance survival and productivity (Kumar et al., 2007).

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