Journal of Biotechnology 161 (2012) 328–335

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

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

Nickeli Rossi [a], Bruna G. Silva [a], Renato Astray [b], Kamilla Swiech [a,1], Carlos A. Pereira [b], Claudio A.T. Suazo [a,*]

[a] *Departamento de Engenharia Química, Universidade Federal de São Carlos, Via Washington Luis, Km 235, CEP 13565-905, São Carlos-SP, Brazil*
[b] *Laboratório de Imunologia Viral, Instituto Butantan, Av. Vital Brasil, 1500 CEP 05503-900 São Paulo, Brazil*

ARTICLE INFO

Article history:
Received 24 December 2011
Received in revised form 2 May 2012
Accepted 8 May 2012
Available online 20 July 2012

Keywords:
Low temperature
Bioreactor
Insect cells
Drosophila melanogaster S2
Rabies virus glycoprotein
Recombinant protein production

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

* Corresponding author. Tel.: +55 16 33518048; fax: +55 16 33518266.
E-mail addresses: nickeli.r@hotmail.com (N. Rossi), bruna_gaby@yahoo.com.br (B.G. Silva), renatoastray@butantan.gov.br (R. Astray), kamilla@fcfrp.usp.br (K. Swiech), grugel@butantan.gov.br (C.A. Pereira), claudio@ufscar.br (C.A.T. Suazo).
[1] Present address: Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, Campus USP, 14040-903, Ribeirão Preto, SP, Brazil.

0168-1656/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jbiotec.2012.05.016

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