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Insect cell entrapment, growth and recovering using a single-use fixed-bed bioreactor. Scaling up and recombinant protein production

D. Ventini-Monteiro[a,b], S. Dubois[c,1], R.M. Astray[a,b], J. Castillo[c,1], C.A. Pereira[a,b,*]

[a] Instituto Butantan, Laboratório de Imunologia Viral, Av. Vital Brasil 1500, 05503-900 São Paulo, Brazil
[b] Universidade de São Paulo, Escola Politécnica, Departamento de Engenharia Química, Laboratório de Células Animais, Av. Prof. Luciano Gualberto 380, 05508-900 São Paulo, Brazil
[c] Pall Life Science/ATMI/Artelis, rue de Ransbeek 310, 1120 Brussels, Belgium

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ABSTRACT

Insect cells are largely used for industrial production of vaccines, viral vectors and recombinant proteins as well as in research and development as an important tool for biology and bioprocess studies. They grow in suspension and are semi-adherent cells. Among the cell culture systems enabling scalable bioprocess the single-use fixed-bed iCELLis® bioreactors offer great advantages. We have established the conditions for *Drosophila melanogaster* Schneider 2 (S2) and *Spodoptera frugiperda* (Sf9) cells entrapment into the fixed-bed, cell growth and recover from the fixed-bed once high cell densities were attained. Our established protocol allowed these cells, at a cell seeding of $2 \times 1E5$ cells/microfiber carriers (MC) ($3.5 \times 1E6$ cells/mL; $1.7 \times 1E4$ cells/cm^2), to grow inside a 4 m^2/200 mL fixed-bed attaining a concentration of $5.3 \times 1E6$ cells/MC ($9.5 \times 1E7$ cells/mL; $4.7 \times 1E5$ cells/cm^2) for S2 cells or $4.6 \times 1E6$ cells/MC ($8 \times 1E7$ cells/mL; $4.1 \times 1E5$ cells/cm^2) for Sf9 cells. By washing the fixed-bed, entrapped cells could then be recovered from the fixed-bed at a high rate (>85%) with high viability (>95%) by increasing the agitation to 1200/1500 rpm. Although the cell yields in the fixed-bed bioreactor were comparable to those obtained in a stirred tank (respectively, $1.3 \times 1E10$ and $2.5 \times 1E10$ total cells), S2 cells stably transfected with a cDNA coding for the rabies virus glycoprotein (RVGP) showed a 30% higher preserved rRVGP production (2.5 ± 0.1 and 1.9 ± 0.1 μg/1E7 cells), as evidenced by a conformational ELISA evaluation. These findings demonstrate not only the possibility to entrap, cultivate to high densities and recover insect cells using a single-use fixed-bed bioreactor, but also that this system provides suitable physiological conditions for the entrapped cells to produce a cell membrane associated recombinant protein with higher specific biological activity as compared to classical suspension cell cultures.

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1. Introduction

Bioprocesses for animal cell culture and production of cell products are of importance for large scale production and for preparation of high quality grade for further structure and function studies. Several large scale cell culture technologies are available for these purposes (Wang et al., 2005; Warnock and Al-Rubeai, 2006). The most current are represented by the traditional stirred tank vessels and more recent ones are represented by approaches such as fixed-bed, wave, cell factory, hollow fiber. In addition, recent technological developments propose single-use disposable equipment to replace the classical reusable glass or stainless based systems (Loeffelholz et al., 2014). Beside economic constraints, the choice of the animal cell culture based technology depends on the cell type to be used and the biological characteristics of the cell product. In this sense the main parameters to be considered to choose the most suitable cell culture system are the productivity, the degradation and the recovery for both the cells and the cell product. A single one, on the above mentioned cell culture technologies, hardly fulfill the requirements of a given industry or investigation laboratory, which are often diversified in terms of cell substrate and cell product.

Insect cells, such as Lepidoptera (*Spodoptera frugiperda*—Sf9) or Diptera (*Drosophila melanogaster* Schneider 2—S2) cells, are non-adherent and largely used for industrial production of vaccines, viral vectors and recombinant proteins as well as for investigation in research and development laboratories as an important tool for biology and bioprocess studies (Cox, 2012; Drugmann et al., 2012; Moraes et al., 2012; Jongh et al., 2013).

* Corresponding author at: Laboratório de Imunologia Viral, Instituto Butantan, Avenida Vital Brasil 1500, São Paulo 05503-900, SP, Brazil.
E-mail address: carlos.pereira@butantan.gov.br (C.A. Pereira).
[1] Present address: Univercells, Rue Auguste Piccard 48, 6041 Gosselies, Belgium.

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The PALL iCELLis single-use fixed-bed system is available in two versions, a nano one with a possibility of using fixed-beds with 40–200 mL, microfiber carriers (MC) with a compaction of 1 or 1.5 offering a surface area of 0.53–4 m^2, and a 500 one, with a fixed-bed of 5–25 L, compaction 1 or 1.5 offering a surface area of 66–500 m^2. The iCELLis bioreactors are flexible, scalable and disposable combining the advantages of single-use with fixed-bed. The fixed-bed is packed with MC which allows the cells retained on the carrier to grow to high cell densities. Medium flows through fixed-bed from bottom to top, then falls as a thin film down the outer wall where the O_2 transfer take place. A perfusion with a recirculation loop mode can be used and allows the regulation of the falling film height to adjust the dissolved oxygen (DO) transfer. The agitation speed and gas flow are continuously measured and regulated through its controller. The iCELLis bioreactors were initially conceived for cultivation of adherent cells for synthesis of secreted cell products taking the advantages of the single-use approach.

The main drawback of the fixed-bed cell culture technology, where the cells are retained in an area inside the bioreactor vessel (the fixed-bed) and the cell culture medium flows through this area is the difficulty to retain non-adherent cells, to recover adherent cells from the fixed-bed for scaling-up and for harvesting cell associated products.

Our aim in this study was to provide suitable conditions to circumvent the above described drawbacks of the fixed-bed technology. We have established cell culture conditions enabling the entrapment of S2 and Sf9 cells into the fixed-bed, the achievement of high cell densities, the recovering of cells from the fixed-bed, the harvesting of a recombinant rabies virus glycoprotein (rRVGP), a cell membrane associated product synthesized by stable transfected S2 cells.

The findings show that our insect cell culture protocol enabled the entrapment of both S2 and Sf9 cells into the fixed-bed single-use bioreactor. The cultures attained a cell density as high as those described for adherent cells and then could be recovered from the fixed-bed at high rates with high viability. Most important, such culture conditions in the fixed-bed bioreactor allowed the entrapped S2 cells to produce a membrane associated cell product at higher quality levels as compared to that obtained from suspension cultures in a classical stirred thank bioreactor.

Our established conditions for insect cell culture in the iCELLis fixed-bed bioreactor opens the possibility to cultivate non adherent cells in a fixed-bed and then recover them for scaling up or for harvesting cell associated products with high quality in terms of preserved protein structure and biological function.

2. Material and methods

2.1. Insect cell lines

The *D. melanogaster* Schneider 2 (S2) cell population used in this work was previously described in detail (Lemos et al., 2009; Ventini et al., 2010; Moraes et al., 2012). It has the RVGP gene expression under the control of $CuSO_4$ inducible metallothionein promoter and was obtained by transfection procedure. The *S. frugiperda* (Sf9) wild type cell population was kindly supplied by Dr. Paolo Zanotto (Universidade de São Paulo). Cells were grown and maintained in the serum free SF-900 III medium (Gibco, Life Tecnologies). Media for bioreactor cell culture were always supplemented with 1% of Antibiotic-Antimycotic (Life Technologies).

2.2. Configuration of the iCELLis nano fixed-bed bioreactor

The PALL Life Sciences iCELLis nano fixed-bed bioreactor (Fig. 1) was used for insect cell cultivation with a protocol specific developed for these cells (Table 1). Briefly, we used a custom fixed-bed of 4 m^2/200 mL filled with microfibers carriers (MC) with a 1.5 compaction (C 1.5). The working volume in the bioreactor was of 700 mL and 500 mL in the recirculation loop (RL). The medium falling film was adjusted to 6 cm and the agitation for cell entrapment during the first 24 h was of 755 rpm (1 cm/s) and during the cell growth of 610 rpm (0.5 cm/s). The recirculation loop worked with 50% medium change at the exponential cell growth phase and recirculation rate of 15 times per day.

2.3. Experimental approach for cell culture

S2 or Sf9 cells were initially cultivated with SF-900 III medium in shaker flasks as already described (Rodas et al., 2005; Moraes et al., 2012). As shown in Table 1, they were then inoculated into the iCELLis fixed-bed bioreactor at a cell density of 2 × 1E5 cells/MC (3.5 × 1E6 cells/mL of fixed-bed). The parameters of cell culture were adjusted to a dissolved oxygen (DO) value of 30% and temperature (t) of 28 °C. The O_2, DO, t and pH were recorded by a JUMO PCA and PCC program. The cell concentration in the fixed-bed bioreactor was determined by manually collecting a MC and nuclei counting using a cell lysis buffer. Images were taken using an Olympus CK2 microscope. S2 cells were induced, as indicated, with $CuSO_4$ for the rRVGP synthesis. The cells were also grown in a 1 L BioFlo 110 bioreactor (New Brunswick) using SF-900 III medium with control of agitation (90 rpm), temperature (28 °C) and dissolved oxygen (DO: 30%).

2.4. Cell recovering from iCELLis fixed-bed bioreactor

Once the cell cultures attained high cell densities, a cell recovering procedure was initiated. A fixed-bed washing with fresh medium was initiated by steps of 5 min. intermittent agitation (30 s paused after 2.5 min) with cells recovering and medium change in the bioreactor. Cell density was evaluated by cell counting in a hemocytometer and cell viability was evaluated by trypan blue exclusion. In each cell recovering step the cell number, percent and viability was recorded.

2.5. rRVGP quantification by ELISA

The rRVGP concentration in cell lysates was determined by ELISA as previously described (Astray et al., 2008). Microplates were sensitized (3 h at 37 °C) with conformational monoclonal D1 anti-RVGP antibodies, specific to the RVGP trimeric form (Fournier-Caruana et al., 2013; Jallet et al., 1999; Lafon et al., 1985). Samples of S2 cells were lysed with detergent and incubated in microplates. Bounded rRVGP was recognized by antibodies labeled with peroxidase. Absorbance was measured at 492 nm in an absorbance microplate reader (MultiskanTM EX, MJ-Labsystems). The rRVGP cell contents, expressed in μg/1E7 cells were calculated by comparison of the OD measured for samples with the OD measured for rRVGP standard-curve

3. Results

3.1. Kinetics of insect cell entrapment and growth in the fixed-bed bioreactor

A protocol designed for entrapment and growth of insect cells in the iCELLis fixed-bed bioreactor was developed (Table 1). The essential features of this protocol are represented by the microfibers carriers (MC) fixed-bed compaction of 1.5, the falling film of 6 cm and the agitation of 610 rpm (0.5 cm/s). These conditions allowed the insect cells (S2 and Sf9) to be properly entrapped

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