

Influence of Culture Conditions on Vero Cell Propagation on Non-Porous Microcarriers

Marta Cristina de Oliveira Souza¹, Marcos da Silva Freire² and Leda dos Reis Castilho^{1*}

¹Programa de Engenharia Química -COPPE; Universidade Federal do Rio de Janeiro; C. P. 68502; Ilha do Fundão; leda@peq.coppe.ufrj.br; 21941-970; Rio de Janeiro - RJ - Brasil. ²Instituto de Tecnologia em Imunobiológicos (Bio-Manguinhos) -FIOCRUZ; Av. Brasil, 4365; freire@bio.fiocruz.br; 21045-900; Rio de Janeiro - RJ - Brasil

ABSTRACT

Animal cell cultures are widely employed for the production of viral vaccines and for recombinant protein expression. The cell line Vero is a continuous, adherent cell line, which has been recommended by the World Health Organization for the production of human vaccines. For the large-scale production of vaccines, microcarriers, which are microspheres that serve as support for the cells, are being increasingly used. The use of microcarriers in stirred bioreactors allows high cell densities and, consequently, high virus titres to be achieved. With the aim of selecting appropriate culture conditions for the cultivation of Vero cells at high cell densities, in this work the influence of several variables (agitation rate, ratio of inoculated cells to microcarrier mass and fetal bovine serum concentration) on cell growth on Cytodex 1 microcarriers was studied. Under the best conditions determined, a comparison with Vero cell cultivation on Cytodex 3 microcarriers was carried out.

Key words: Vero cells, microcarriers, Cytodex 1, Cytodex 3, high-cell-density cultivation

INTRODUCTION

Animal cell technology is largely employed in the manufacture of viral vaccines and recombinant therapeutic proteins (Doyle and Griffiths, 1998). Many of the cell lines employed are anchorage dependent, and their proliferation relies on their adhesion to surfaces (Cruz et al., 1999; Butler et al., 2000). For large-scale cultivation of adherent cells, systems using microcarriers are advantageous, since they provide a large surface area in a relatively reduced culture volume and allow cells to grow in a homogeneous environment, where parameters such as temperature, dissolved oxygen and pH can be

monitored and controlled (Kadouri, 1994; Spier and Kadouri, 1997).

Vero cell line propagation on microcarriers is being increasingly employed in the commercial production of vaccines such as rabies and poliomyelitis (Butler et al., 2000; Frazzati-Gallina et al., 2001). When using microcarriers, an approach to further increase cell density and virus production is to operate bioreactors in perfusion mode (Mendonça and Pereira, 1998; Mendonça and Pereira, 1995).

Microcarriers are small spheres, either porous or non-porous, with diameters in the range of 100 - 500 µm and densities in the range of 1.00 - 1.06 g mL⁻¹. There are more than 20 different microcarrier types commercially available, made

* Author for correspondence

of several different materials, such as dextran, polystyrene, collagen, gelatin, cellulose and glass (Kadouri, 1994). The initial adhesion of cells onto microcarriers, which is essential for further cell proliferation, is influenced by different factors. One of them is the ratio of inoculated cells to microcarriers. According to Forestell et al. (1992), although inoculum density does not interfere with environmental conditions controlling cell adhesion, the cell-to-carrier ratio is an important parameter since it influences the distribution of cells on the spheres and determines the proportion of microcarriers that do not become occupied with cells (Ng et al., 1996). According to literature (Amersham Pharmacia Biotech, 1999), the key to obtain high cell yields in microcarrier cell cultures is to assure that all or almost all microcarriers are inoculated with cells, since bead-to-bead transfer does not frequently occur along cultivation.

Agitation is another variable that significantly influences cell proliferation on microcarriers, whereby it presents two contradictory effects on cell adhesion. According to Sun et al. (2000), a high agitation rate increases the collision frequency between cells and microcarriers, favouring the initial adhesion of cells onto the carriers. However, on the other hand, a minimum cell-carrier contact time is needed to ensure that cells remain adhered and proliferate. Since high agitation rates shorten cell-carrier contact time, a decrease in adhesion efficiency may occur. Furthermore, high stirring velocities may damage the cells membrane, decreasing cell adhesion.

Another variable that considerably influences adhesion and, consequently, cell proliferation, is the type and concentration of proteins in the culture medium (Mukhopadhyay et al., 1993; Ng et al., 1996). Additionally, microcarrier type and surface properties, culture medium composition and pH, and physiological state of cells are

reported in literature to influence culture performance when using microcarriers.

Thus, the aim of the present work was to study the influence of several variables - microcarrier type, serum concentration in the medium, agitation rate and cell-to-carrier ratio - on the growth of Vero cells on non-porous microcarriers. Statistical experimental design was used as a tool to evaluate the effect of these different variables.

MATERIALS AND METHODS

Cell line and culture medium

The cell line Vero, established from African green monkey (*Cercopithecus aethiops*) kidney cells by Yasumara and Kawakita (1963) was used. Vero cell line CCL 81 was obtained from American Type Culture Collection (ATCC). This cell line is considered adequate for the production and standardization of vaccines for human use and does not produce interferons due to a chromosomal deletion (Desmyter et al., 1968). The cells were cultivated under 5% CO₂ atmosphere in high-glucose (4.5 g L⁻¹) DMEM medium, supplemented with glutamine and fetal bovine serum (FBS).

Cell cultivation in spinner flasks

Stirred flasks ("spinners") were used for tests with Cytodex 1 and Cytodex 3 microcarriers (Amersham/GE Healthcare). The inocula for all experiments were prepared by propagating cells in T flasks. After trypsinization, a cell suspension was prepared and used as inoculum. In each run, 300 mg carriers were suspended in 100 mL culture medium, resulting in a total available surface area of 1320 and 810 cm² for Cytodex 1 and Cytodex 3, respectively. After inoculation, spinner flasks were maintained under agitation at 37°C and 5% CO₂.

Table 1 - Experimental conditions employed for mammalian cell growth on Cytodex 1 carriers.

Experiment	Agitation rate (rpm)	Cell-to-carrier ratio (cell/µg)	FBS concentration (% v/v)
1	40	10	1
2	70	70	1
3	70	10	5
4	40	70	5
5	55	40	3
6	55	40	3
7	55	40	3

Experimental design

A 2^{3-1} fractional factorial design was employed to investigate the influence of agitation rate, cell-to-carrier ratio and FBS concentration on cell growth. Experimental conditions are shown in Table 1.

To evaluate the results, the values of the variables shown in Table 1 were normalized according to Equation (1), in order to allow comparing the order of magnitude of the effects of the different variables.

$$y_i^* = 2 \cdot (y_i - \bar{y}) / (y_{max} - y_{min}) \quad (1)$$

where y_i is the absolute value of the variable, y_{min} and y_{max} are the extreme values in the investigated variable range, \bar{y} is the mean variable value, and y_i^* is the normalized value.

Sampling and analytical methods

In agitated flasks, 1 mL samples were withdrawn daily. After centrifugation at 1100 rpm for 15 minutes, the supernatant was removed and 1 mL 0.1% crystal violet solution was added. After homogenization, the samples were maintained at 37°C for 1 hour. After the appropriate dilutions, stained cell nuclei were counted in a Neubauer chamber.

RESULTS AND DISCUSSION

Effects of culture conditions on Vero cell propagation on Cytodex 1 microcarriers

Since various factors affect mammalian cell growth on microcarriers (Sun et al., 2000; Murkhopadhyay et al., 1993; Amersham Pharmacia Biotech, 1999; Forestell et al., 1992; Ng et al., 1996), a fractional factorial experimental design was carried out to help identifying the most appropriate conditions for obtaining high cell densities on Cytodex 1 microcarriers. The experimental conditions tested (expressed as normalized values), as well as the maximum cell densities obtained in the different runs, are shown in Table 2. The lowest cell density (1.3×10^6 cells mL^{-1}) was obtained in experiment 1, when all variables were at their lowest limit (serum concentration: 1%; agitation: 40 rpm; cell-to-carrier ratio: 10 cells/ μg carrier). On the other hand, cell density was highest (3.5×10^6 cells mL^{-1}) in experiment 4, when agitation remained low (40 rpm), but serum concentration and cell-to-carrier ratio were at their highest levels (5% and 70 cells/ μg carrier, respectively).

Through statistical analysis of the experimental results, it was verified that all the three variables tested were significant at a 95% confidence level. A linear model (Equation 2) was obtained to describe the experimental data ($R = 0.974$).

Table 2 - Maximum cell density obtained in the 2^{3-1} experimental design using Cytodex 1.

Run	Normalized variables			Max. cell density (10^6 cells mL^{-1})
	Agitation rate (A)	Cell-to-carrier ratio (B)	FBS concentration (C)	
1	-1	-1	-1	1.3
2	+1	+1	-1	1.9
3	+1	-1	+1	1.5
4	-1	+1	+1	3.5
5	0	0	0	1.8
6	0	0	0	1.7
7	0	0	0	1.8

$$X = 19.2 - 3.5 A + 6.5 B + 4.5 C \quad (2)$$

where X is cell concentration (given in 10^5 cells mL^{-1}), A is the normalized agitation rate, B is the normalized cell-to-carrier ratio, and C is the

normalized concentration of fetal bovine serum in the culture medium.

As shown in Fig. 1, the empirical model given by Equation (2) was able to adequately describe the experimental data. As explicated by Equation (2), all three variables exerted effects of equal order of magnitude on maximum cell concentration,

although the effect was negative (-3.5) for the agitation rate and positive for the SFB concentration (+4.5) and the cell-to-carrier ratio (+6.5). This was in agreement with the fact that the

highest cell density was obtained for a minimum agitation rate and maximum cell-to-carrier ratio and serum concentration (Table 2).

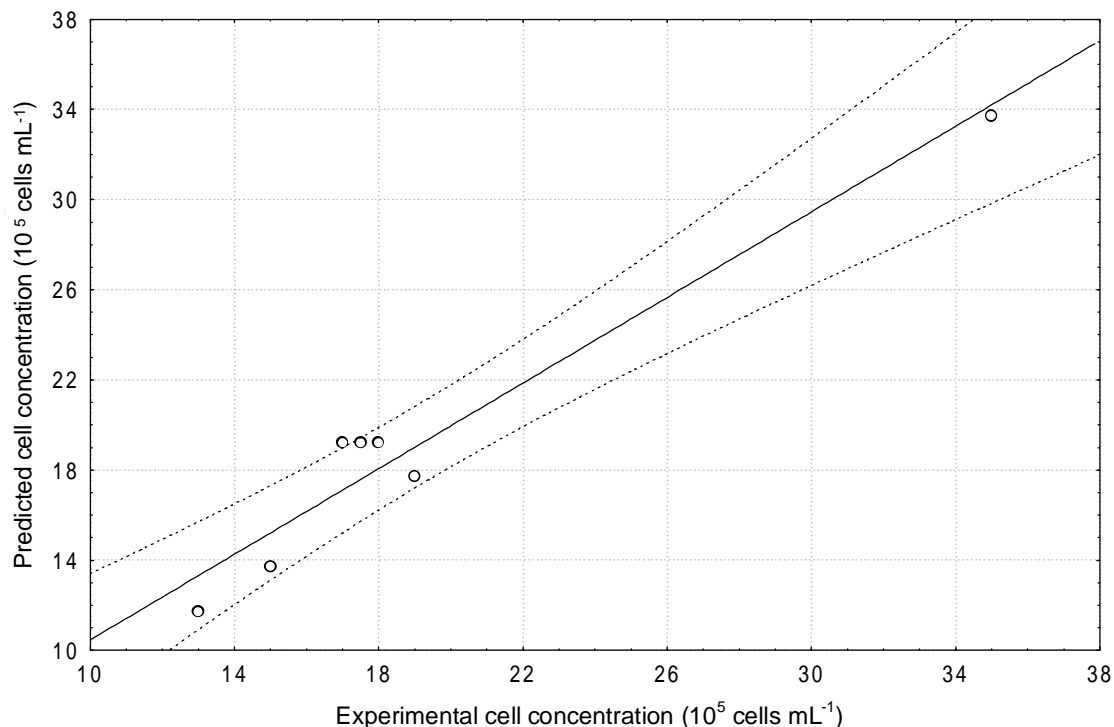


Figure 1 -Comparison of experimentally observed values and those predicted by Equation (2)

When compared to Vero cell propagation in T flasks (data not shown), the cell yield per medium volume obtained in experiment 4 was 3 times higher. Sun et al. (2000) studied the influence of the agitation rate in the range of 20 to 45 rpm on Vero cell adhesion onto Cytodex 3 microcarriers. They observed that low stirring velocities (20 rpm) favoured cell adhesion, but led to a non-homogeneous cell distribution on microcarriers. At 45 rpm, cell adhesion rate was significantly decreased. They concluded that optimal agitation rate was 30 rpm, while in the present work using Cytodex 1 40 rpm was the most adequate stirring velocity.

Mukhopadhyay et al. (1993) studied the kinetics of cell adhesion on Cytodex 1, 2 and 3 microcarriers. They found the highest adhesion rate at a cell-to-carrier ratio of 15 cells per bead. Considering that (according to the microcarriers manufacturer) one gram of Cytodex 1 contained 4.37×10^6 beads, the range studied in the present work was 2.3-16.3

cells per bead. Thus, the best results in the present work were obtained at 16.3 cells per bead, which were quite similar to those found by Mukhopadhyay et al. (1993).

The influence of serum concentration on Vero cell adhesion and growth kinetics was studied by Ng et al. (1996). These authors observed that the cell adhesion rate decreased considerably when serum concentration was increased to 10%. They justified this behaviour by explaining that adsorption of serum proteins could be blocking microcarrier adhesion sites. On the other hand, they noted that as serum concentration was increased from 1 to 5%, the lag phase was reduced from 48 h to 0 h and the maximum cell density was increased from 8.7×10^5 to $14.8 \times 10^5 \text{ cells mL}^{-1}$. In the present work also higher serum concentrations resulted in higher cell concentrations.

Comparison of Vero cell growth on Cytodex 1 and Cytodex 3 microcarriers

Due to the different surface properties of different microcarriers, and due to the effects that these properties can have on growth kinetics of cell on microcarriers, in this work a comparison of Vero cell propagation on Cytodex 1 and Cytodex 3 was carried out. Cytodex 1 was formed by substituting a cross-linked dextran matrix with positively charged DEAE (N,N-diethylaminoethyl) groups. Cytodex 3 was also formed by a cross-linked dextran matrix, but was coated with a thin layer of denatured collagen, being indicated by the

manufacturer for cells with an epithelial-like morphology.

Vero cell propagation on these two microcarriers is shown in Fig. 2. For both microcarriers, under the conditions employed (agitation rate: 40 rpm; cell-to-carrier ratio: 70 cells per μg carrier; FBS concentration: 5%), a lag phase of approximately 20 h was observed. Maximum cell concentration for Cytodex 3 (2.7×10^6 cells mL^{-1}) was reached on day 6. For Cytodex 1, although maximum cell density (3.5×10^6 cells mL^{-1}) was reached only on day 8, it was approximately 30% higher than for Cytodex 3.

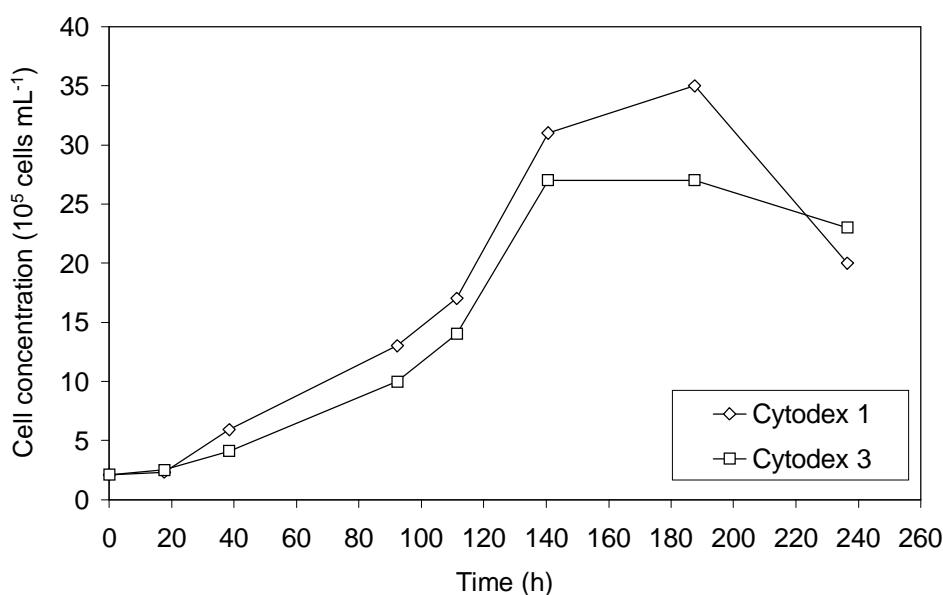


Figure 2 - Total Vero cell concentration in spinner flasks with 3 mg mL^{-1} Cytodex 1 and Cytodex 3 microcarriers (agitation rate: 40 rpm; cell-to-carrier ratio: 70 cells per μg carrier; FBS concentration: 5%)

Although the bead mass was the same in both experiments, Cytodex 1 had a surface area of $4400 \text{ cm}^2 \text{ g}^{-1}$, while Cytodex 3 had $2700 \text{ cm}^2 \text{ g}^{-1}$. In order to evaluate if the earlier stationary phase and lower cell density obtained with Cytodex 3 was due to its lower surface area per gram microcarrier, the specific growth rate (μ) and duplication time (t_D) of cells in both the carriers during the exponential growth phase were calculated. For Cytodex 1, the growth rate was 0.0194 h^{-1} and the duplication time was 35.7 h, while for Cytodex 3, μ was slightly reduced (0.0179 h^{-1}), and t_D was 39.8 h. These results indicated that cells on Cytodex 3 carriers may

have entered the stationary phase earlier due to the lower surface area available for growth.

Ng et al. (1996), who also grew Vero cells on Cytodex 1 microcarriers at a serum concentration of 5% and an agitation rate of 40 rpm, observed a specific growth rate of 0.0307 h^{-1} , which was quite higher than the one obtained in the present work. However, these authors used calf serum instead of FBS. According to Mukhopadhyay et al. (1993), Vero cell adhesion in Cytodex 2 carriers varied considerably depending on the type of serum used in the culture medium, and this could be due to the competition of serum proteins with cells for adhesion sites on the beads.

CONCLUSIONS

It was shown that agitation rate in spinner flasks, FBS concentration in the culture medium and the ratio of inoculated cells to microcarriers exerted statistically significant effects on Vero cell growth on Cytodex 1 microcarriers. Within the ranges studied in this work, it was shown that a high cell-to-carrier ratio combined with a high FBS concentration and a low agitation rate resulted in high cell densities. Under such culture conditions, when compared to Cytodex 3, Cytodex 1 allowed a 30%-higher cell concentration to be obtained, which was probably related to the lower surface area per bead mass of Cytodex 3 microcarriers.

ACKNOWLEDGEMENTS

Thanks are due to Capes, CNPq, FAPERJ and Bio-Manguinhos/FIOCRUZ for the financial support.

RESUMO

Cultivos de células animais são amplamente utilizados para a produção de vacinas virais e para a expressão de proteínas recombinantes. A linhagem celular Vero é uma linhagem contínua, dependente de ancoragem, recomendada pela Organização Mundial de Saúde para a produção de vacinas de uso humano. Para a produção de vacinas virais em larga escala, vêm sendo cada vez mais empregados microcarregadores, que são microesferas que servem de suporte para as células. O emprego de microcarregadores em biorreatores agitados permite a obtenção de altas densidades celulares e, conseqüentemente, de altos títulos de antígenos virais. Com o objetivo de selecionar condições de cultivo adequadas, estudou-se, neste trabalho, o efeito das variáveis agitação, razão de células inoculadas por microcarregador e concentração de soro fetal bovino sobre o crescimento de células Vero em microcarregadores Cytodex 1. Nas melhores condições selecionadas, o desempenho dos microcarregadores Cytodex 1 e Cytodex 3 foi comparado.

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Received: September 29, 2004;
Revised: February 25, 2005;
Accepted: March 25, 2005.