



Production of yellow fever virus in microcarrier-based Vero cell cultures

Marta Cristina O. Souza^{a,b}, Marcos S. Freire^{a,*}, Erica A. Schulze^{a,b}, Luciane P. Gaspar^a, Leda R. Castilho^b

^a Oswaldo Cruz Foundation (FIOCRUZ), Bio-Manguinhos, Viral Vaccine Program, Avenida Brasil 4365, 21045-900 Rio de Janeiro/RJ, Brazil

^b Federal University of Rio de Janeiro, COPPE, Cell Culture Engineering Laboratory, Cx. Postal 68502, 21941-972 Rio de Janeiro/RJ, Brazil

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ABSTRACT

In this work, the propagation of the 17DD yellow fever virus in Vero cells grown on Cytodex-1 microcarriers was evaluated. After verifying that upon infection the virus adsorption step could be performed under continuous agitation, experiments were carried out in spinners and sparged lab-scale stirred-tank bioreactor to evaluate the use of a commercial serum-free medium (VP-SFM) and to investigate the effects of multiplicity of infection (MOI) and time of infection (TOI) on virus production. Virus titers as high as 8.4×10^8 pfu/mL were obtained upon infection with MOI of 0.02 and TOI of 3 days, using the serum-free medium in the sparged bioreactor.

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1. Introduction

Yellow fever (YF) is an endemic disease in some areas of tropical South America and sub-Saharan Africa. It causes hemorrhagic fever and has a high case-fatality rate (20–50%) [1]. The YF virus belongs to the *Flavivirus* genus of the *Flaviviridae* family. It is an arthropod-borne agent, transmitted by mosquitoes mainly of the genera *Aedes* and *Haemagogus*.

The commercially available vaccines against YF are live-attenuated and based on the virus substrains 17DD and 17D-204 [2]. Used for prevention of the disease since 1936, over 400 million doses of the vaccine have already been administered worldwide [3,4]. It confers long-lasting immunity and has an extensive safety and efficacy record [3]. However, since the late 1990s, several cases of serious adverse effects have been registered worldwide following administration of vaccine doses manufactured in the United States, France, Brazil and China [5].

The current vaccine is produced by the different manufacturers in embryonated eggs, based on a technology that has remained almost unchanged since the late 1930s [4]. Egg-based technology poses some drawbacks, such as the need for high amounts of specific pathogen-free eggs, high labor-intensity, and presence of large amounts of chicken embryo proteins in the final product (as high as 250 µg/dose) [4]. Moreover, the process is slow and difficult to scale-up, so large strategic stocks must be kept to respond in cases of epidemics.

Based on these observations, the development of a new inactivated vaccine seems interesting to be used as an alternative either for the vaccination of travelers who will stay for a short period

in endemic areas or as a prime-boost vaccination strategy. For the introduction of a new YF vaccine, the development of a new technology based on current standards for human vaccines is needed.

Vero is a continuous cell line derived from African green monkey kidney [6]. After extensive research to evaluate safety aspects, Vero cells have been accepted for the production of human viral vaccines [7,8] and are adopted e.g. for the commercial production of human polio and rabies vaccines [9].

In the present work, the production of YF virus in Vero cells grown on microcarriers was studied. The influence of factors such as agitation, culture medium and infection conditions (multiplicity of infection and time of infection) was investigated, resulting in a promising protocol for producing high amounts of virus in a serum-free process.

2. Materials and methods

2.1. Cell cultivation and culture media

Vero cell line CCL 81 was obtained from the American Type Culture Collection (ATCC, VA, USA). Culture media were either high-glucose (4.5 g/L) DMEM (Gibco, Invitrogen Corp., CA, USA) supplemented with 2 mM glutamine (Gibco, Invitrogen Corp., CA, USA) and 5% (v/v) fetal bovine serum (FBS; Cultilab, Campinas/SP, Brazil), or the serum-free medium VP-SFM (Gibco, Invitrogen Corp., CA, USA) supplemented with 4 mM glutamine.

Cells originally growing in serum-containing DMEM were adapted to VP-SFM according to the manufacturer's instructions. A stock of adapted cells was frozen and kept at -196°C .

In order to prepare inocula for microcarrier-based cultivations, cells were propagated in monolayer in culture flasks at 37°C under 5% CO_2 atmosphere. After reaching confluence, cells were trypsinized, counted and used to inoculate spinner flasks or the

* Corresponding author. Tel.: +55 21 3882 9446; fax: +55 21 2260 4727.
E-mail address: freire@bio.fiocruz.br (M.S. Freire).

bioreactor at a ratio of 16 cells per microcarrier bead (corresponding to 70 cells/ μg of microcarrier).

Spinner flask experiments were carried out with a working volume of 100 mL, containing 3 g/L of Cytodex-1 microcarriers (GE Healthcare, Uppsala, Sweden), at 37 °C and 40 rpm under 5% CO_2 atmosphere. Microcarriers were prepared according to the manufacturer's instructions.

Bioreactor experiments were carried out in a BioFlo 110 system (New Brunswick Scientific, model M1273-0054), fitted with a 1.3-L vessel (0.4–1.0 L working volume). Temperature was controlled at 37 °C, pH at 7.2, dissolved oxygen at 50% air saturation, and agitation at 60 rpm. Cytodex-1 concentration was 3 g/L, and the working volume used was 0.4 L. To protect cells from sparging and agitation, 0.1% (m/v) Pluronic F68 (Sigma–Aldrich Co., MO, USA) was added to the medium in all bioreactor experiments.

Samples from spinners or the bioreactor were taken daily for evaluation of cell growth, virus titer, glucose, ammonia and lactate.

2.2. Infection conditions

Prior to infection, agitation was stopped, microcarriers were allowed to settle down and 70% of the culture supernatant was removed. Then agitation was restarted, and 1 mL of a viral suspension containing the 17DD yellow fever virus, previously diluted according to the desired final culture volume and multiplicity of infection (MOI), was added to the spinner or to the bioreactor. After a period of 1 h for virus adsorption onto the cells, the volume was completed with fresh medium to 100%.

In order to evaluate the effects of agitation during the viral adsorption step, after removal of culture supernatant and addition of virus seed, a continuous agitation at 40 rpm for 1 h was compared to an intermittent agitation regime, which consisted of stirring the cultures at 40 rpm for 1 min, then allowing them to rest for 15 min, and so successively for a total adsorption time of 1 h. In this set of experiments, the culture medium used was DMEM containing 5% FBS.

In most experiments, MOI was 0.002 and infection was carried out on the 5th day post-inoculation. In the experiments performed to evaluate the effects of MOI and time of infection (TOI) on virus production, a MOI of 0.02 (10-fold higher) was used and infection was carried out on the 3rd day post-inoculation.

2.3. Analytical methods

Samples (1 mL) were withdrawn daily to measure total cell count, glucose, ammonia, lactate and, after infection, virus titer.

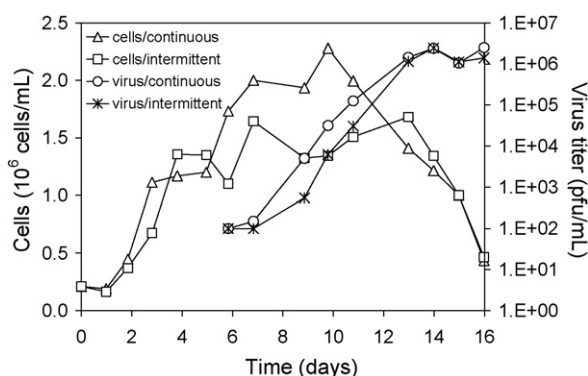


Fig. 1. Vero cell growth and YF virus production in DMEM supplemented with 5% FBS in spinner flasks. On the 5th day post-inoculation, viral infection was carried out at a MOI of 0.002. Following infection, during a 1-h virus adsorption step, agitation was either continuous or intermittent.

To measure the total cell concentration, nuclei staining with crystal violet was carried out according to Sanford et al. [10]. After centrifugation of samples at 100 $\times$ g for 15 min, the supernatant was removed and the same volume of a 0.1% (m/v) crystal violet solution was added. After homogenization, samples were maintained at 37 °C for 1 h. After the appropriate dilutions, stained cell nuclei were counted in a Neubauer chamber.

The concentrations of glucose and ammonia in the supernatants were determined using enzymatic tests commercialized by Roche/Boehringer Mannheim/R-Biopharm (Cat. No. 10139106035 for D-glucose and Cat. No. 11112732035 for ammonia). L-Lactate concentration was determined using a YSI 2700 Select analyzer (YSI Life Sciences, YSI Inc., OH, USA), fitted with a membrane containing immobilized L-lactate oxidase.

After infection, samples of supernatants were also assayed for virus titer by plaque titration on Vero cell monolayers, as described previously [11].

3. Results and discussion

3.1. Effects of agitation upon viral infection

Upon infection with a virus, aiming at improving virus adsorption onto the cells, many authors adopt intermittent agitation of cell culture systems [12,13]. In order to better understand cell–virus interactions upon infection of Vero cells with the YF virus, the use of both continuous and intermittent agitation during the virus adsorption step was compared. Considering that the practical procedure during infection may become an important issue when scaling up a virus production process, these experiments aimed at establishing the procedure to be adopted during the further steps of the work, both at spinner and bioreactor scale.

On the 5th day post-inoculation, 70% of the spinner volume was removed, cells were infected at a MOI of 0.002, and continuous agitation was compared to an intermittent 1-h agitation regime. According to the results shown in Fig. 1, intermittent agitation was deleterious to cell growth, since cell concentration maxima were approximately 25% higher for the continuously agitated culture. This might be due to oxygen transfer limitations that may have arisen during the adsorption step, affecting cell metabolism and further cell propagation.

However, virus production kinetics was equivalent for both agitation conditions, yielding peaks of approximately 2.4×10^6 pfu/mL on the 14th day post-inoculation (Fig. 1). Based on these results, all further experiments in this work were carried out performing the virus adsorption step under continuous agitation. Specially when shifting to bioreactors, the possibility of using continuous agita-

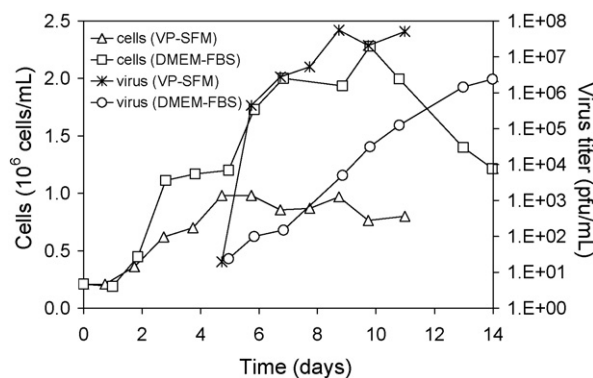


Fig. 2. Comparison of Vero cell growth and YF virus production in serum-containing medium (DMEM + 5% FBS) and serum-free medium (VP-SFM), in spinner flasks. Infection was carried out on the 5th day post-inoculation at a MOI of 0.002.

tion is an advantage, since an additional control system would be required to promote intermittent agitation.

3.2. Virus production in serum-containing and serum-free media

In order to investigate virus production in a commercial serum-free medium, experiments were carried out in spinners and bioreactors to evaluate cell growth and virus production kinetics in DMEM containing 5% FBS and in VP-SFM.

Fig. 2 shows the results obtained at spinner flask scale. Cell growth was significantly enhanced in the presence of serum, yielding a maximum cell concentration over 2-fold higher than that obtained in VP-SFM.

However, virus production was quicker and resulted in a higher maximum viral titer for the serum-free medium. While a titer of 5.5×10^7 pfu/mL was obtained for VP-SFM, an approximately 23-fold lower titer (2.4×10^6 pfu/mL) was observed for the serum-containing culture. The higher titer combined with the lower cell density obtained in VP-SFM means that cell-specific virus productivity was higher in the absence of serum.

In two other studies on serum-free virus production carried out in spinner flasks [9,14], higher cell-specific productivities were also observed for the serum-free media as compared to serum-supplemented media. Studying the production of reovirus, Butler et al. [9] observed higher viral titers for the serum-free medium, although Vero cell growth was similar in both serum-free and serum-containing media. Liu et al. [14], investigating the production of enterovirus in Vero cell culture, reported a higher cell death rate after infection in the serum-free culture, but obtained a similar viral titer and a similar immunogenicity in both media.

Quesney et al. [15] compared Vero cell growth and death in the presence and absence of serum. Although they observed higher viable cell densities for the serum-containing medium, cell cycle analysis showed that cell division was more active for the cells grown in the serum-free medium. The lower viable cell densities found by the authors in the absence of serum was shown to be due to a higher cell death rate, which was accompanied by detachment of dead cells from the microcarrier beads. Since among the viable cells, which kept attached to the microcarriers, the fractions of cells in S + G2-M phases were larger for the serum-free medium, the higher cellular activity of these cells in serum-free medium could be an explanation for the higher cell-specific virus production observed in the present work and in the other above-mentioned reports.

3.3. Virus production in stirred bioreactors using serum-free medium

Given the promising results obtained with VP-SFM in spinner flasks, Vero cell culture and YF virus production were further investigated using a stirred-tank lab-scale bioreactor, with bubble aeration and automated control of temperature, pH and dissolved oxygen. As can be observed in Fig. 3A, a maximum cell density of 1.6×10^6 cells/mL and a maximum virus plateau of approximately 1.0×10^8 pfu/mL were obtained 9 days post-inoculation and 4 days post-infection, respectively. Virus titer stopped increasing on the 9th day post-inoculation, exactly when cell density started to decrease, and both phenomena may be related to glucose exhaustion or ammonia and lactate accumulation in the culture (Fig. 3B).

From both data (cell concentration and virus titer) it becomes evident that in the bioreactor the better controlled environmental conditions and/or the enhanced oxygen transfer achieved by direct sparging provided favorable conditions that enabled a 60% increase in cell density and a 100% increase in virus titer, indicating that microcarrier-based virus production in stirred-tank bioreactors with bubble aeration is a feasible alternative for obtaining large amounts of YF virus.

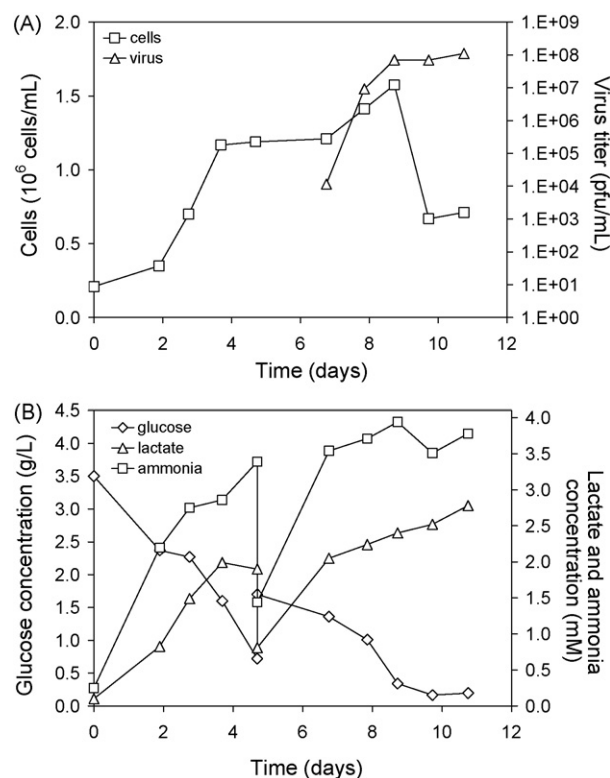


Fig. 3. (A) Vero cell growth and virus production, and (B) glucose, ammonia and lactate concentrations along cultivation in VP-SFM medium in a 1-L sparged bioreactor, operated with 400 mL working volume. Infection was carried out on day 5 post-inoculation at a MOI of 0.002.

Using a similar biological system (Vero cells grown on Cytodex-1 microcarriers in VP-SFM) for the production of rabies virus, Rourou et al. [16] found a 30% increase in cell density and a 20% increase in titer when moving from spinner flasks to a 2-L bioreactor. However, differently from the present findings, Rourou et al. [16] could only carry out the process when exclusively using surface aeration in the bioreactor, since sparging resulted in no cell growth.

3.4. Evaluation of MOI and TOI values to increase virus production

According to Genzel et al. [17], the multiplicity of infection (MOI) influences virus growth dynamics but not final virus yield. Also Audsley and Tannock [18], who studied the propagation of influenza virus in Vero and MDCK cells, and Maranga et al. [19], who investigated the production of virus-like particles with a baculovirus insect cell system, have observed that in cells infected with higher MOIs maximum titers were attained earlier than in cultures infected at lower MOIs. However, according to Maranga et al. [19], at high MOIs the specific productivity decreased when cells were infected in a late growth phase.

Therefore, we decided to investigate the effects of MOI and TOI on yellow fever virus production by evaluating the use of a 10-fold higher MOI (0.02 instead of 0.002), but decreasing the time of infection (TOI) from 5 to 3 days. As shown in Fig. 4, this combination of MOI and TOI resulted beneficial for YF virus production, yielding a maximum virus titer of 8.4×10^8 pfu/mL.

FIOCRUZ (Brazil) currently produces 30 million doses of YF vaccine per year, processing approximately 4000 specific pathogen-free eggs per week, in campaigns that usually last about 20 weeks [4,20]. Freire et al. [4] proposed as an alternative the production of the 17DD YF virus in chicken embryo fibroblast (CEF) cell cultures, with viral titers ranging from 2.1×10^6 to 6.2×10^6 pfu/mL (6.32–6.79 log₁₀ pfu/mL).

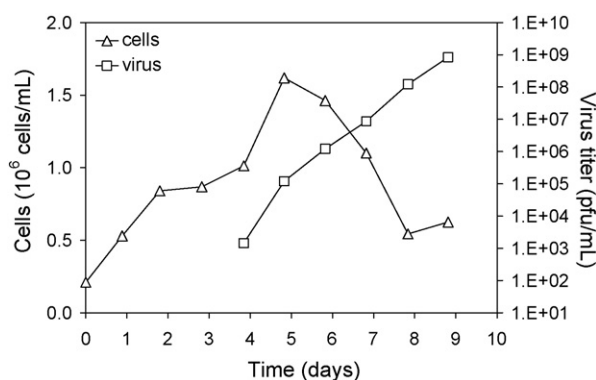


Fig. 4. Vero cell growth and virus production in VP-SFM medium in a 1-L sparged bioreactor, operated with 400 mL working volume. Infection was carried out on the 3rd day post-inoculation at a MOI of 0.02.

However, the higher virus titers obtained in the present work indicate that the use of the certified Vero cell line in a serum-free bioreactor-based process may be a very interesting alternative to the egg-based production platform. Considering the virus titers obtained in the present work, enough antigen for 30 million doses of the current attenuated vaccine would be contained in less than 5 L of serum-free culture.

4. Conclusions

In the present work, several parameters of interest for the establishment of a new platform for the production of yellow fever virus were investigated. It was verified that the adsorption step upon infection with the YF virus can be carried out under continuous agitation, facilitating process scale-up. The use of a commercial serum-free medium showed to successfully promote virus replication in Vero cells grown on Cytodex-1 microcarriers in stirred systems, yielding virus titers up to 5.5×10^7 pfu/mL in spinner flasks and 1.0×10^8 pfu/mL in a lab-scale stirred-tank bioreactor with bubble aeration. Further improvement of the virus production process was possible through manipulation of the multiplicity of infection (MOI) and time of infection (TOI). By increasing MOI 10-fold to 0.02 and decreasing TOI from 5 to 3 days, maximum virus titer was increased to 8.4×10^8 pfu/mL. These results thus represent a promising alternative for the production of yellow fever vaccine, specially regarding the large antigen amounts that may be needed for a new inactivated vaccine.

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