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Statistical optimization of influenza H1N1 production from batch cultures of suspension Vero cells (sVero)

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ABSTRACT

Efficient vaccine production requires the growth of large quantities of virus produced with high yield from a safe host system. Human influenza vaccines are produced in embryonated chicken eggs. However, over the last decade many efforts have allowed the establishment of cell culture-derived vaccines.

We generated a Vero cell line adapted to grow in suspension (sVero) in a serum-free medium and evaluated it for its potential as host cell for influenza vaccine production. Initially we studied the capacity of sVero cells to grow in the presence of incremental concentrations of trypsin. In comparison with adherent Vero cells (aVero), we found that sVero cells maintain their growth kinetics even with a three-fold increase in trypsin concentration.

The influence of the conditions of infection on the yield of H1N1 produced in serum-free suspension cultures of sVero cells was investigated by a 2^2 full factorial experiment with center point. Each experiment tested the influence of the multiplicity of infection (m.o.i.) and trypsin concentration, on production yields at two levels, in four possible combinations of levels and conditions, plus a further combination in which each condition was set in the middle of its extreme levels.

On the basis of software analysis, a combination of m.o.i. of 0.0066 $TCID_{50\%}$/cell and trypsin concentration of 5 μg/1.0 $\times$ 10^6 cells with a desirability of 0.737 was selected as the optimized condition for H1N1 production in sVero cells.

Our results show the importance of proper selection of infection conditions for H1N1 production on sVero cells in serum-free medium.

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1. Introduction

Vaccination remains the most effective strategy for controlling and preventing the disease caused by influenza viruses. In the United States, commercial trivalent inactivated viral vaccines are produced in chicken embrionated eggs. This technology faces serious difficulties associated with the manufacturing process scale up, such as requiring 6–12 months to produce chicken flocks capable of producing embrionated eggs for production, the need for extensive manufacturing controls to address the inherent microbial burden in eggs, and the potential shortage of production substrates due to environmental factors that impacts on eggs supply for vaccine production [1–3].

Several cell lines including Vero, MDCK, PER.C6 and HEK-293 have been proposed as production substrates for influenza vaccine production [4–9]. The use of suspension cell lines can simplify the production at industrial scale by eliminating complex processing steps associated with microcarriers during start-up, expansion and cell monitoring. Recently, several groups have focused their efforts in the identification of suspension cell-based production platforms avoiding the use of microcarriers or roller bottles [2,8].

In a previous report, we demonstrated the use of Vero cells successfully adapted to grow in suspension mode, in serum free media and in the absence of disaggregating agents for highly efficient production of different viruses [10]. This cell line, named sVero, offers the advantage of being easily grown in suspension at high cell densities (up to 1.9 $\times$ 10^7 cells/mL in perfused cultures).

The strategy of virus production in batch cultures is determined mostly by the selection of the infection conditions, such as m.o.i., physiological state of the cellular biomass and, in the case

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of influenza, trypsin concentration. The addition of proteases to the cell medium culture is essential for influenza virus production since it has been reported that infection of most cell lines can only occur if hemagglutinin glycoproteins on the surface of viral particles are first cleaved into HA1 and HA2 in order to interact with the host cell line [7,11–13]. These factors interact with each other and can be manipulated to optimize virus yields. The optimal combination of these conditions must be established for every system because it depends on both, the characteristics of the cell line and the nutritional capability of the culture medium employed in a given production process [14].

The aim of this study was to investigate the effect of several parameters on the production of H1N1 virus in suspension cultures of sVero cells in a serum-free medium. Initially, we studied the impact of cell density and cell culture physiological stage at the time of infection on influenza propagation. Based on this initial condition, a 2^2 full factorial design with center point was carried out in order to compare the performance of cultures infected in two different ranges of m.o.i. and trypsin concentration.

2. Materials and methods

2.1. Cell lines

The sVero cell line [10] is a subpopulation adapted to grow in suspension of the Vero E6 cell line (ATCC CRL-15869), taking into account its ability to grow in agitated suspension cultures in serum-free medium as isolated cells. Adherent Vero E6 cells were grown in T-flasks in MEM (Gibco, USA) supplemented with 10% Foetal Calf Serum (FCS, Bioser, Argentina), at 37 °C, 5% CO_2.

2.2. Suspension cultures of sVero cells

Suspension cultures were maintained at 37 °C and 5% CO_2 in 125 mL spinner flasks (Bellco, USA) agitated at 40 rpm in serum-free medium. Subcultures were grown every 2 days, starting from an initial cell density of 4.0×10^5 viable cells/mL. Cell density was determined using a Neubauer haemocytometer. Viability was assayed by the Trypan Blue dye exclusion method. Each sample was processed by duplicate counting of total and viable cells.

2.3. Virus and virus quantification

H1N1 virus A/Salomon Islands/3/06 was obtained from the National Institute of Respiratory Diseases "Emilio Coni", Santa Fe, Argentina.

Hemagglutination (HA) titers were determined in 96 microwell plates using human red blood cells based on standard techniques [15]. Infectious virus titers were measured using the 50% tissue culture infectious doses ($TCID_{50\%}$) assay, based on the cytopathic effect [16] in Vero cells. A positive control with pre-specified acceptable titers was included for conducting HA and $TCID_{50\%}$ titrations.

2.4. Replication of H1N1 virus in aVero and sVero cells

aVero and sVero cells were infected at an m.o.i. of 0.1 $TCID_{50\%}$/cell. aVero cells were seeded using MEM supplemented with 10% FCS, and after 24 h media was exchanged and cells were infected in serum-free medium in order to avoid trypsin inactivation due to FCS factors.

Trypsin (Gibco) was added daily at a concentration of 6 μg/1.0×10^6 cells. During infection all cultures were maintained at 37 °C and 5% CO_2. $TCID_{50\%}$ assays were performed to quantify infectious virus and compare replication kinetics of H1N1 in aVero and sVero cells.

2.5. Impact of initial cell density and physiological state

The impact of cell density and physiological state at the time of infection on H1N1 production was investigated. Three batch cultures were run by seeding sVero cells at 0.4×10^6 cells/mL in serum-free medium. After adding 6 μg trypsin/1.0×10^6 cells, they were infected with H1N1 virus at m.o.i. = 0.01 $TCID_{50\%}$/cell and without medium exchange. The first culture was infected after seeding (0.4×10^6 cells/mL), and the other two, when cultures reached densities of 0.76×10^6 and 1.70×10^6 cells/mL, respectively. After infection, incubation was performed at 37 °C and 5% CO_2. Samples were taken 24 and 48 h post infection and trypsin was added after each sampling. Supernatants were stored at −80 °C, until further analysis.

2.6. Experimental design and data analysis. Viral production optimization

The influence of the infection conditions on H1N1 virus production in serum-free suspension cultures of sVero cells was investigated by a 2^2 full factorial assay with center point. The design of this experiment consists of two factors, each one in two levels, in which all possible combinations of factors and levels are assayed in four runs, plus one additional experimental run with each factor set up at the middle of this range [17]. In this study the two factors were m.o.i. and trypsin concentration. The design allowed us to evaluate the individual effects of each of these factors as well as the interaction between them. The responses evaluated in every run were maximum density of viable cells, infectious virus titers (calculated as $TCID_{50\%}$/mL), and total virus particles (calculated from HA assays). Each experiment was performed in duplicate and the center point in triplicate. In order to estimate the adequacy of the regression models, the analysis of variance (ANOVA) and the Fischer's *F*-test were used. Experimental design and statistical analysis were performed by the Design Expert® software.

3. Results and discussion

3.1. Replication of H1N1 virus in aVero and sVero cells

As a growth control assay, Vero cells were grown in serum-free medium with the addition of a maximum of 10 μg trypsin/1×10^6 cells and in the absence of viral infection, showing no alterations in their growth patterns. Also sVero cells showed an increased tolerance to trypsin of up to 30 μg trypsin/1.0×10^6 cells without altering their growth kinetics (data not shown). Maximum infectious virus titer was found to be comparable for both aVero and sVero cells. Viral productivity was 5.62×10^7 $TCID_{50\%}$/mL for sVero cells and 3.16×10^7 $TCID_{50\%}$/mL for aVero cells. Results demonstrate that sVero cells are highly permissive to H1N1 virus infections and are able to generate high virus titers in comparison to aVero cells.

3.2. Impact of initial cell density and physiological state

Production dependence of H1N1 virus with sVero cells on the initial cell density and the physiological state of the cells during infection was investigated. Maximum titers were obtained infecting cells during the intermediate phase of exponential growth (Table 1). No significant improvement in virus titers was obtained from the batch infected 48 h after seeding at 1.70×10^6 cells/mL when compared to the one infected 24 h after seeding at 0.76×10^6 cells/mL. Therefore, the latter condition was chosen for optimization experiments.

Table 1
Maximum infectious titers at different cell densities.

Trial	Time after seeding (h)	Cell density at infection (cells/mL)	Max viral titer ($TCID_{50\%}$/mL)
1	0	0.40×10^6	5.62×10^4
2	24	0.76×10^6	3.16×10^6
3	48	1.70×10^6	3.16×10^6

Table 2
Experimental design used to study the effects of m.o.i. and trypsin concentration on viral production.

Condition	Trials	m.o.i ($TCID_{50\%}$/mL)		Trypsin (μg/1.0×10^6 cells)	
		Coded	Uncoded	Coded	Uncoded
1	1–2	−1	0.00010	−1	5
2	3–4	−1	0.00010	1	15
3	5–6	1	0.01000	−1	5
4	7–8	1	0.01000	1	15
5	9–11	0	0.00505	0	10

3.3. Experimental design and data analysis. Viral production optimization

In order to study the combined effect of m.o.i. and trypsin concentration on H1N1 production using sVero cells, experiments were performed according to statistical design requirements. Table 2 shows the levels at which these factors were combined according to a 2^2 full factorial design with center point. Table 3 shows the values calculated for *F*-value and *P*-values (*P*-value is the probability of the null hypothesis). The *P*-value is used to estimate whether *F* is high enough to indicate statistical significance.

We established low m.o.i. experimental points since infection at high m.o.i. demands large amounts of viral stock when working at industrial scale. Also at high m.o.i. values (m.o.i. > 1) the number of viable virus-producing cells is reduced shortly after infection and often results in lower virus yields [8]. At the lowest m.o.i. value assayed, post-infection proliferation levels were expected to be higher. Nevertheless, maximum cell density was statistically independent of m.o.i. during the experiments (Table 3). In addition, m.o.i. had no significant effect on infectious and HA titers at least in the range assayed.

When working with asynchronic infections, serial passages of viral replication are often required in order to achieve optimal virus yields. This is only possible if infectious particles are formed at each replication step [18]. Even tough the addition of trypsin to the culture medium is not necessary for virus assembly and release, the activation of influenza virus particles depends on the cleavage of hemaglutinin glycoproteins after each replication step. Therefore, the addition of trypsin is required at the moment of infection and subsequently at least every 24 h, since Vero cells are known to release protease inactivating factors into the culture medium [19]. Our results showed that total and active virus titers were statistically dependent on trypsin concentration. Similar results have been published for other suspension cell lines although in some cases the influence of trypsin concentration is related to its effect on cell growth kinetics [7,8].

Post-infection cell death and viral yields kinetics were analyzed for each experiment. Data from central point runs (trypsin 10 μg/1.0×10^6 cells; m.o.i. 0.00505 $TCID_{50\%}$/cell) showed that infectious virus yield reached a maximum average of 3.12×10^8 $TCID_{50\%}$/mL at 36 hpi when viability was around 50%, and remained over 1.0×10^8 $TCID_{50\%}$/mL until all cells were already dead (Fig. 1). After that, infectious titer decreased exponentially. HA titer peaked at 80 hpi and remained stable over time.

Variations in m.o.i. showed no significant influence on maximum viral titers, but at higher m.o.i. values cell viability declined to less than 50% before 36 hpi (Fig. 2A and B), and total and infectious titers raised earlier. Variations of trypsin concentration did not affect viral production kinetics or cell death kinetics. Low m.o.i. and low trypsin concentration resulted in sharper infectious virus production peaks, but increasing trypsin concentration at this condition did not seem to affect cell death kinetics (Fig. 2C and D).

In all the experiments HA titers reached a plateau after 55 hpi, and in some cases a later increase in HA titer occurred even tough cell viability had already reached 0%, indicating that some viral budding may occur after complete cell death (Fig. 3). These findings are consistent with results reported elsewhere describing cells that may release viral particles into the culture medium even at later apoptotic stages [20].

For optimization purposes and when more than one response is being analyzed, it is more efficient to model each elementary response separately and then apply a multi-criteria decision-making procedure such as the Derringer and Suich desirability function [21]. This method proposes a desirability function which includes the researcher priorities and desires to build the optimization procedure. The procedure involves creating a function for each individual response d_i and finally obtaining a global function *D* that should be maximized choosing the best conditions of the designed variables. The function *D* varies from 0 (totally undesirable value) to 1 (all responses are maximized simultaneously), and it is defined by the Eq. (1):

$$D = \left(\prod_{i=1}^{n} d_i^{\,r_i} \right)^{1/\sum r_i} \qquad r_i = 1, 2, 3, 4, 5 \tag{1}$$

Table 3
Main effects and interaction effects of m.o.i. and trypsin concentration on maximum cell density, maximum infectious titer and maximum HA titer.

Factor	Regression coefficient	Standard error	*F* value	Prob > *F*
Maximum viable cell density				
Model			0.9520	0.4731
m.o.i.	2.31×10^4	7.40×10^4	0.0977	0.7652
Trypsin	-8.69×10^4	7.40×10^4	1.3800	0.2847
Interaction	-8.69×10^4	7.40×10^4	1.3800	0.2847
Maximum infectious titer				
Model			5.8798	0.0322*
m.o.i.	−0.0568	0.1080	0.2768	0.6177
Trypsin	−0.4193	0.1080	15.0505	0.0082*
Interaction	0.1643	0.1080	2.3121	0.1792
Maximum HA titer				
Model			9.3476	0.0112*
m.o.i.	2.5000	2.5400	0.9656	0.3637
Trypsin	−13.0000	2.5400	26.1115	0.0022*
Interaction	−2.5000	2.5400	0.9656	0.3637

* Significant factors ($P < 0.05$).

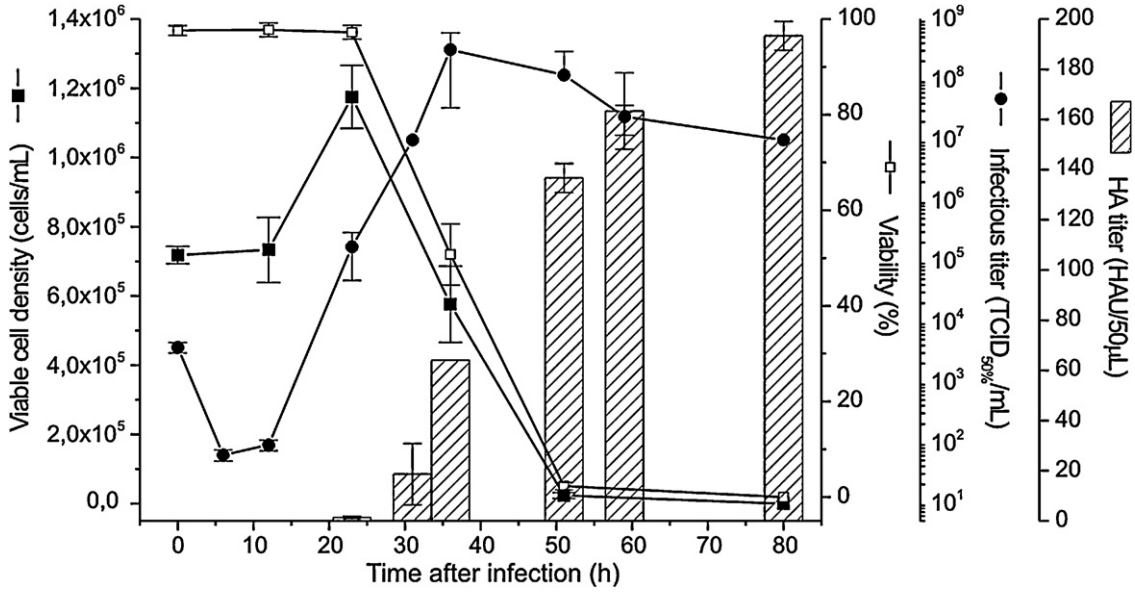

Fig. 1. Cell growth, viability, total and infectious virus production kinetic for center point conditions (m.o.i = 0.00505; trypsin concentration = 10 μg/1.0 × 10^6 cells).

where d_i is the partial desirability function for each response i and r_i is the relative importance assigned to this response. Relative importance r_i is a comparative scale for putting emphasis to each d_i in the expression of the function D.

Our objective was to maximize the overall desirability function, and therefore it was necessary to calculate a partial desirability function for each response in order to obtain from these results an overall desirability. A linear partial desirability function was selected for each response. For infectious titer and HA titer a maximization of d was imposed. As this optimization has the objective of being applied to a productive vaccine process, the importance of the partial desirability function of hemagglutinating particles titer was the highest possible value (5), because influenza vaccines usually consist of inactivated virus material and so, the main characteristic for product concentration in vaccine production is the concentration of total virus particles. For the infectious titer, a weight of 4

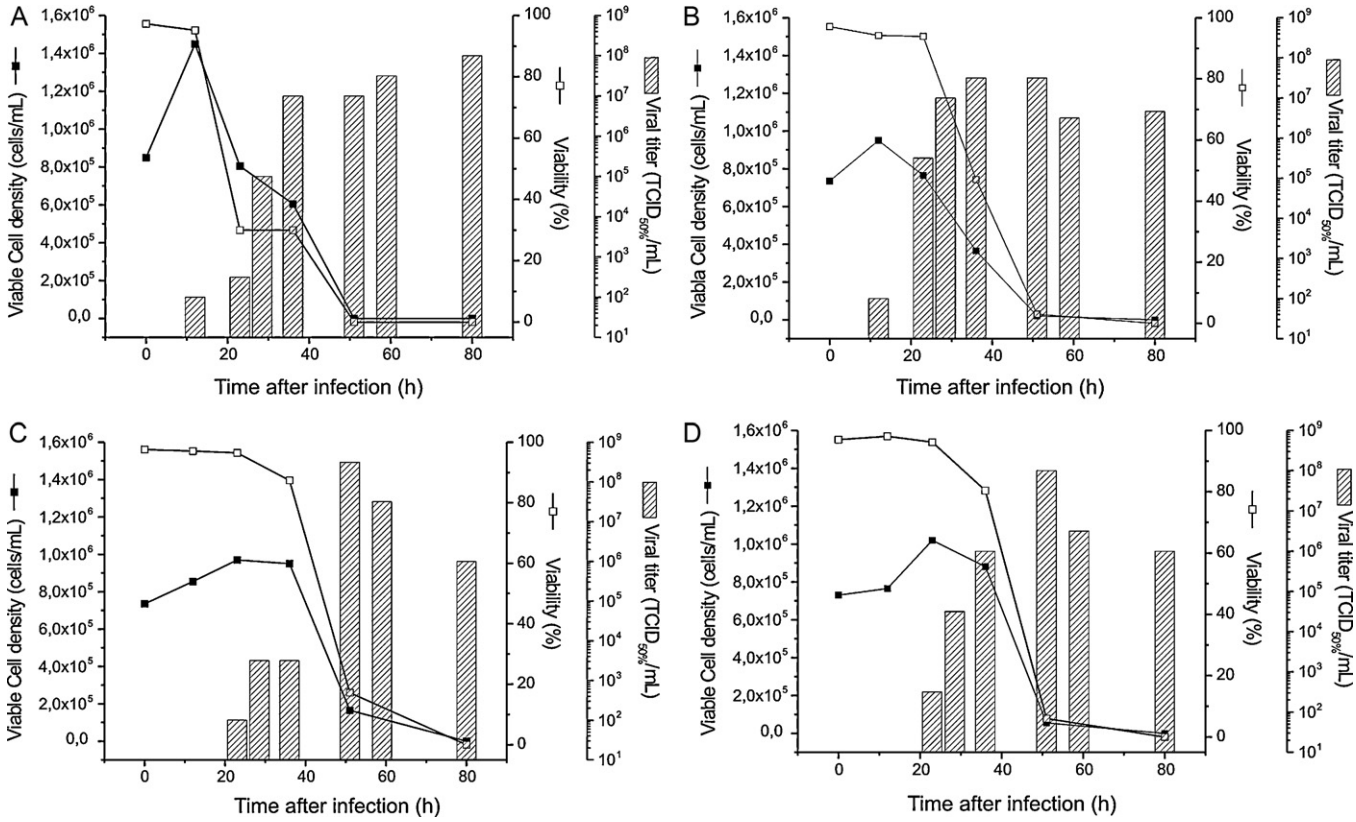

Fig. 2. Cell growth, viability and infectious virus production kinetics for peripheral conditions. (A) m.o.i. = 0.01 $TCID_{50\%}$/cell; trypsin concentration = 5 μg/1.0 × 10^6 cells. (B) m.o.i. = 0.01 $TCID_{50\%}$/cell; trypsin concentration = 15 μg/1.0 × 10^6 cells. (C) m.o.i. = 0.0001 $TCID_{50\%}$/cell; trypsin concentration = 5 μg/1.0 × 10^6 cells. (D) m.o.i. = 0.0001 $TCID_{50\%}$/cell; trypsin concentration = 15 μg/1.0 × 10^6 cells.

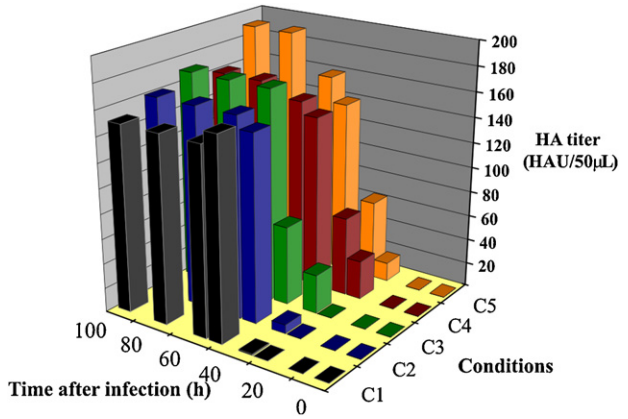

Fig. 3. HA titers for single representative runs of each experimental conditions. C1 (−1; −1); C2 (−1; 1); C3 (1; −1); C4 (1; 1); C5 (0; 0). *Coded and uncoded values in* Table 2.

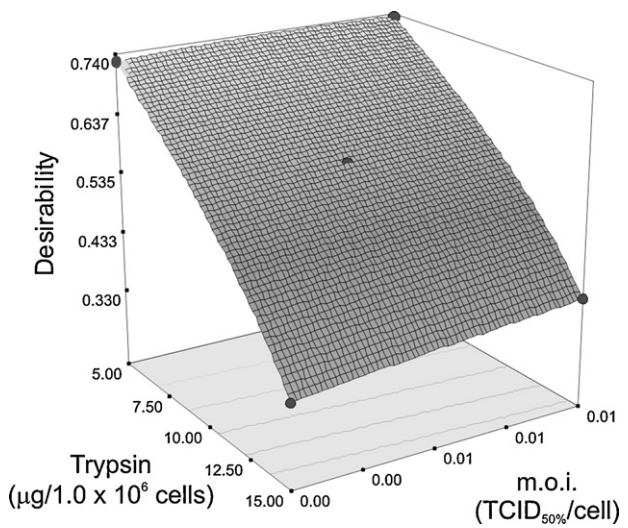

Fig. 4. 3D response surface diagram showing the effects of m.o.i. and trypsin concentration on the global desirability function. Dots (•) indicate the experimental points.

was given in response to the need of high infectious viral yields for stock formulation.

Global desirability represents the global quality of the compromise solution; this was calculated by combining single desirability functions. The three-dimensional plot of the global desirability obtained is shown in Fig. 4. This statistical optimization of the infection parameters performed using Design Expert® showed that overall desirability of response decreases when trypsin concentration is increased over 5 μg/1.0 × 10^6 cells. This means that even tough we achieved higher viral titers (live and total) at the center point condition, optimal titers should be achieved by working with lower trypsin concentrations.

On the basis of the calculation steps defined for the optimization algorithm, the optimal values of the test variables were an m.o.i. of 0.0066 $TCID_{50\%}$/cell and trypsin concentration of 5 μg/1 × 10^6 cells with the corresponding *D* of 0.737. These results are consistent for other cell lines such as MDCK and PER.C6, which showed optimal production yields at the lowest non-zero concentration assayed. For MDCK cells this may be related to the fact that cell growth is strongly affected by trypsin [7,8].

4. Conclusions

Mammalian cell lines should be well defined, grown in suspension to high densities in serum free media and support cost effective replication of viruses in order to be of industrial interest. sVero cells constitute a potential candidate for viral vaccine production since they can be easily scaled up for production in stirred tank bioreactors. They are also fully adapted to grow in low cost serum-free medium [10].

Results in this study demonstrate that sVero cells are susceptible to influenza H1N1 infection and are able to support effective production, delivering high viral yields in batch cultures.

For most cell lines, influenza vaccine production is still poorly understood and process optimization is often empirical. Metabolic profiles for MDCK-based influenza production have been recently published [22], but little information is available when it comes to the interaction between influenza viruses and Vero cells.

Optimization assays were performed in order to empirically establish optimal conditions for the production of infectious and total viral particles. This responds to the need to attain high yields for both, inactivated vaccine production and stock development. Best results were obtained maintaining low concentrations of trypsin throughout the experience. This contributes to lower costs and reduces operational volumes associated to large scale production. Results demonstrate the importance of an adequate selection of infection conditions to obtain high viral yields. They could also be applied to the development of a large-scale process for H1N1 production in serum-free medium.

Infection at lower m.o.i. values did not have a significant effect on production yields; hence making it possible to work at higher scales without the need of large volumes of viral stocks.

Previous findings showed that sVero cells retained the susceptibility to viral infection after being adapted to single cell suspension cultures. They are able to reach high cell densities and attain optimal virus yields with many viruses known to infect Vero cells.

Results from the present work show that sVero cells may represent a valuable tool for industrial production of influenza vaccines. Further optimization of production parameters and scale up to stirred tank bioreactors in perfusion mode should result in higher reproducible yields.

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