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Bioprocess optimization for cell culture based influenza vaccine production

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ABSTRACT

Uncertainties and shortcomings associated with the current influenza vaccine production processes demand attention and exploration of new vaccine manufacture technologies. Based on a newly developed mammalian cell culture-based production process we investigated selected process parameters and describe three factors that are shown to impact productivity, process robustness and development time. They are time of infection, harvest time and virus input, or multiplicity of infection (MOI). By defining the time of infection as 4–5 days post cell seeding and harvest time as 2–3 days post-infection and comparing their effect on virus production, MOI is subsequently identified as the most impactful process parameter for live attenuated influenza vaccine (LAIV) manufacture. Infection at very low MOI (between 10^{-4} and 10^{-6} FFU/cell) resulted in high titer virus production (up to 30-fold productivity improvement) compared to higher MOI infections (10^{-3} to 10^{-2} FFU/cell). Application of these findings has allowed us to develop a platform process that can reduce the development time to approximately three weeks for an influenza vaccine manufacture process for new strains.

1. Introduction

Influenza or flu is a major cause of acute respiratory illness worldwide, particularly among children and the elderly population. Each year infection by flu viruses results in 36,000 deaths and more than 200,000 hospitalizations in the United States alone [1]. Although the traditional influenza vaccines have been used to provide protection against seasonal flu epidemics, alternative production strategies and different types of vaccines are actively being pursued to overcome the limitations inherent to these vaccines, particularly those that may become more evident during a pandemic flu outbreak [2–5].

The seasonal flu vaccines are used to vaccinate the general population and consist of three different virus strains (type A/H1N1, type A/H3N2 and type B). These strains are selected by the World Health Organization and US Food and Drug Administration based on surveillance data of flu virus strains circulating in the human population prior to the flu season and recommendations are made to the flu vaccine manufacturers early in the year, typically February for the Northern Hemisphere. The virus strains are used to generate a vaccine that is typically targeted for pre-season distribution beginning in July to September and initial immunization shortly thereafter. Thus, the vaccine manufacturers are challenged with a tight production schedule and have very limited time to develop or optimize the production conditions to improve process yields [6].

Flu vaccines have traditionally been manufactured using embryonated hens' eggs. In recent years cell culture based production technology has been explored and demonstrated to be a viable alternative to egg-based technology [7–9]. In contrast to the conventional egg-based production process, cell-based production technology promises shorter production cycles, greater surge capacity, greater process control and more reliable and well characterized production substrates [10,11]. Furthermore, cell culture based manufacturing processes are capable of providing larger quantities of vaccine within a shorter period of time which is especially important during a pandemic outbreak when the egg supply from specific pathogen free flocks is limited. Following the successful launch of a cold-adapted (*ca*) Live Attenuated Influenza Vaccine (LAIV) approved for use in 2–49 year old individuals [12] and produced using conventional egg-based production technology, MedImmune initiated a cell culture based flu vaccine development program. This new vaccine manufacture process makes use of the adherent Madin–Darby Canine Kidney (MDCK) cells as a production cell substrate, and grows and infects the cells on microcarrier beads under a controlled environment in bioreactors [9].

The first generation LAIV cell culture flu vaccine manufacturing process produced vaccine bulk at high titers (>8 $\log_{10}$ FFU/ml) for 3 selected virus strains [9]. However, when the process was evaluated using additional flu strains, large variations in productivity were observed among different viruses, ranging from 7 to 9 $\log_{10}$ FFU/ml. This variability creates a high degree of uncertainty with respect to expected process yields, particularly for novel virus strains that

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lack sufficient manufacturing history. The result is significant risk to the timely production and supply of flu vaccine. Since flu vaccine strains change frequently and process optimization time is limited, it would be advantageous to develop a robust platform process that is relatively insensitive to virus strain changes and provides consistency in productivity for most seasonal and even future unknown pandemic flu viruses. To achieve this goal we pursued a strategy of identifying critical process parameters that meet the following three criteria: (a) broad impact, affecting virus productivity of multiple virus strains; (b) minimum change requirement, i.e. modification of these parameters does not result in major alterations of the platform process such as change of production substrate, production medium, use of additional process equipment; (c) short optimization time (less than 2–3 weeks). In this communication we describe our efforts at identifying three critical process parameters that meet the above criteria (time of infection, harvest time and multiplicity of infection) and demonstrate their utility in developing the platform vaccine manufacture process. Using 25 different *ca* influenza vaccine strains that belong to multiple types or subtypes of flu viruses (Type B, A/H1N1, A/H2N2, A/H3N2, A/H5N1, A/H7N3, and A/H9N2 influenza viruses) we show that up to a 30-fold improvement in virus productivity can be achieved by optimizing only a few selected process parameters. In addition, we can also reduce the variability in productivity among different virus strains and thus develop a more robust manufacturing process for vaccine production.

2. Materials and methods

2.1. Cell line

Anchorage dependent Madin Darby Canine Kidney (MDCK, ATCC CCL-34) cells were originally obtained from American Type Culture Collection (Manassas, VA), cloned by limit dilution and adapted to serum-free growth [11]. One of the MDCK cell clones, 9B9-1E4, was tested for susceptibility for *ca* reassortant influenza virus infection and banked for this study [10]. The cells were cultured in T-flasks or roller bottles before being seeded in bioreactors as described below.

2.2. Virus strains

Wild type (*wt*) influenza viruses used in this study were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). The corresponding *ca* reassortant viruses (listed in Table 1) were made by reassorting *wt* virus with LAIV master donor strains, A/Ann Arbor/6/60 or B/Ann Arbor/01/64 following the classical reassortment procedure that was established previously [13]. In the work presented here, only the *ca* reassortant viruses were used to infect MDCK cells.

2.3. Static culture conditions (cell thaw and expansion)

Frozen stock of MDCK cells was recovered by thawing the cells directly in a T75 flask (Corning, Lowell, MA) containing pre-warmed serum free proprietary growth medium and culturing them for 3–4 days. For further cell expansion, cells were subcultured in T-225 flasks and roller bottles (Corning, Lowell, MA) in the same growth medium every 3–4 days. Cultivation was carried out at 37 °C in a 5% CO_2 incubator. For passaging, MDCK cells were trypsinized using TrypLE™ Select (Gibco, Carlsbad, CA) for 15 min in T-75 and T-225 flasks or for 20 min in roller bottles. The trypsinization was stopped by addition of lima bean trypsin inhibitor solution (Worthington, Lakewood, NJ) as recommended by the supplier. Initial cell seeding density of 5×10^4 cells/mL and 6.7×10^4 cells/mL was used for T-225 (100 mL) flasks and roller bottles (300 mL/850 cm^2), respectively. After culturing for 3–4 days, cells were harvested from roller bottles and used to inoculate 3 L bioreactors.

2.4. Bioreactor culture conditions

MDCK cells were grown on Cytodex 3 microcarriers (GE Healthcare, Piscataway, NJ) in a proprietary serum-free growth medium, in fully controlled stirred 3 L glass bioreactors (Applikon Biotechnology, Foster City, CA). A cell seeding density of 9×10^4 cells/mL and microcarrier concentration of 2 g/L was used. pH was controlled at 7.4 by sparging CO_2 and addition of 1 M NaOH as needed. Temperature was maintained at 37 °C until virus infection. Dissolved oxygen was allowed to decline from 100% during the early culture and was maintained at 50% of air saturation by sparging pure oxygen during later culture times. The agitation rate was kept constant at 90 rpm.

2.5. Virus infection

To determine the optimal time of infection, MDCK cells were seeded in a 3 L bioreactor and then infected daily from 1 day post cell seeding (dps) up to 5 dps. At the time of infection, MDCK cell culture was removed from the 3 L bioreactor mother culture and divided in to 30 mL aliquots to set up daughter cultures in 125 mL shake flasks. Prior to infection, medium exchange with fresh medium was performed followed by addition of TrypLE™. Select to a final concentration of 3% (volume/volume). The daughter cultures were then infected by a *ca* virus strain at specific virus inputs, or multiplicity of infection (MOI). After infection, all shake flasks were incubated at 33 °C and 100 rpm in a 5% CO_2 incubator for up to 4 days. The cell culture fluid was collected daily post infection from one or 2 different infections depending on the virus strain and the virus titer was determined by Fluorescent Focus Assay (FFA), as previously described [14].

2.6. Analytical methods

Daily samples were taken from the 3 L bioreactor cultures for viable cell density and metabolite analysis until daughter cultures were set up in shake flasks for infection. After infection, the shake flask cultures were sampled for 4 more days for infectious virus titer measurement. Cell number and viability were determined using a NucleoCounter (New Brunswick Scientific Co. Inc, New Brunswick, NJ). Offline pH, nutrient and metabolite concentrations were obtained using a Bioprofile 400 (Nova Biomedical, Waltham, MA). Infectious virus titer was measured by infecting MDCK cell monolayers with culture supernatants using FFA [14].

3. Results

3.1. Effect of time of infection on virus replication kinetics

To investigate the effect of time of infection on virus productivity, MDCK cell cultures were seeded on microcarriers and grown for different lengths of time, varying between 1 and 5 days, before infection with *ca* A/Wisconsin/67/05 under four different conditions: three different MOIs, i.e., 0.01, 0.001 and 0.0001 FFU/cell and a constant virus input of 2000 FFU/ml based on the cell culture volume. The constant volume-based virus input was chosen to determine if a more operation-friendly process was feasible and eliminate the need for calculating the amount of inoculum virus based on viable cell density (VCD) before each infection, Fig. 1 shows a typical virus production time course in cell cultures that were infected at a low MOI (0.001 FFU/cell). The upward trend of