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Development and optimisation of a procedure for the production of *Parapoxvirus ovis* by large-scale microcarrier cell culture in a non-animal, non-human and non-plant-derived medium

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Summary For the production of a chemically inactivated *Parapoxvirus ovis* (PPVO), an adherent bovine kidney cell line was cultivated on Cytodex®-3 microcarriers in suspension culture. The inactivated and purified virus particles have shown immune modulatory activity in several animal models. PPVO was produced by a biphasic batch process at the 3.5 and 10 L scale. Aeration was realised by bubble-free membrane oxygenation via a tube stator with a central two-blade anchor impeller. In order to increase efficiency, process robustness and safety, the established process was optimised. The cell line was adapted to a protein-free medium (except recombinant insulin) in order to increase biosafety. A scale up to a 50 L pilot plant with direct cell expansion was performed successfully. In parallel, the biphasic batch process was optimised with special emphasis on different operating conditions (cell number, Multiplicity of Infection (MOI), etc.) and process management (fed-batch, dialysis, etc.). The quality and concentration

Abbreviations: BHK, baby hamster kidney; *BK KL 3A*, *Bovine Kidney Klon 3A*; bp, base pairs; CHO, Chinese Hamster Ovaria Cells; CPE, cytopathic effect; EMEM, Eagle's Minimal Essential Medium; IGF, insulin-like growth factor; IU, International units; HBV, hepatitis-B-virus; HEK, human embryo kidney; HCP, host cell protein; HPLC, high performance liquid chromatography; HPPF, humane plasma protein fraction; MDBK, Madin Darby Bovine Kidney; MDCK, Madin Darby Canine Kidney; MOI, multiplicity of infection; PBS, phosphate buffered saline; PPVO, *Parapoxvirus ovis*; qEM, quantitative electron microscopy; $TCID_{50}$, tissue culture infective dose 50%; VEF-batch, Volume Expanded Fed-batch.

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of the purified virus particles was assessed by quantitative electron microscopy, residual host cell protein and DNA-content and, finally, biologic activity in a transgenic mouse model. This integrated approach led to a new, safe, robust and highly productive large-scale production process, called ''Volume-Expanded-Fed'' Batch with cell densities up to 6—7e06 cells/mL. By subsequent dilution of infected cells into the next process scale, an increase in total productivity by a factor of 40 (related to an established biphasic batch process) was achieved.

Nomenclature

V	volume (L)
X	viable cell number (mL^{-1})
Greek letters	
μ	overall specific growth rate (d^{-1})

Introduction

The production of recombinant proteins and vaccines in animal cells for therapeutic applications is of increasing interest. Recombinant DNA technology and increasing knowledge of complex interactions (for example immune response caused by infectious diseases) opened the gates for the treatment of these diseases. Due to their ability to properly fold and glycosylate such proteins, animal cell culture is often the ideal expression system [1—3]. In general, there are two types of animal cells: suspension cell lines and adherent cell lines, with the latter requiring a surface for attachment and spreading. Vaccines, for example, are commonly produced by adherent cell lines. Relative to stationary culture systems (T-flasks, cell factories, etc.) specialized bioreactors such as fluidized bed reactors or hollow fibre reactors are established, but are challenging to scale up and are both cost-intensive as well as staff-intensive [4]. Another method to cultivate adherent cell lines is via microcarrier technology. The use of microcarrier-based production systems has been described for several processes up to a production scale of 1 m^3 [5—11]. However, cultivation of mammalian cells typically requires complex media composition, specialized bioreactor design and stringent environmental controls [12]. These factors can lead to elongated development timelines for highly productive large-scale production processes.

Media design affects growth, cell density and productivity of the cells. Animal cells do not have the ability to synthesise all essential compounds. Therefore, a complex media formulation is necessary [13,14]. To support animal cell cultures, serum of animal origin, protein fractions of human or animal origin and plant or animal hydrolysates are often used to support growth and production [11,15,16]. The utilisation of these raw materials increases the risk of biological contamination (virus, bacteria, prions, DNA, etc.), as well as batch-to-batch variability and leads to unfavourably high protein content for product purification [17]. Therefore, the adaptation from serum-containing media to serum- and/or protein-free media in animal cell culture is a significant issue. This is even more so an issue for virus production [18].

To increase biological safety and process robustness, serum-free media have been developed in recent years, and serum-free processes for virus production have been described, for example, in the case of influenza virus production on MDCK cells [11,18—21]. Also, adaptation techniques for various cells have been provided [13,14,17,22,23]. The basis of the adaptation strategy is to ''force'' the cells to grow and produce without essential compounds provided by supplements of serum. In general, the adaptation to serum and/or protein-free media is also the first step to adapt anchorage-dependent cell lines to suspension conditions that are more advantageous for culture handling, process robustness and scale-up. However, the commercially available media have not been optimised for a specific cell line, process and product. Even with comparable growth rates, a high product yield or virus titre cannot necessarily be consistently achieved. A further media development (avoiding inhibitions or limitations) and an optimisation of the production process in terms of factors such as operation mode (batch, fed-batch, perfusion) or multiplicity of infection, has to be performed in most cases.

Compared to microorganisms, animal cells are more shear sensitive [24] and exhibit lower growth and production rates. Also, animal cells are more sensitive to pO_2, pCO_2, temperature and pH fluctuations. Substrate limitations and the concentrations of low molecular weight waste products such as NH_4^+ or lactate are of importance. Their inhibitory effects on growth and (virus) production of several cell lines have been described in the literature, varying over a wide range of concentrations from 2 to 10 mM [25—27]. In addition, osmolality has been described to influence virus production. To address these environmental control challenges, several cultivation systems and operation modes (batch, fed-batch, dialysis, perfusion, etc.) have been developed [4,28]. Fed-batch processes and perfusion processes, for example, require a detailed knowledge of specific consumption and production rates and the identification of limiting and inhibiting compounds. Such data are suitable to develop a sophisticated stoichiometric model that provides the basis for the design of fed-batch processes, as previously described for the production of antibodies [1,29—34]. Also, complex perfusion processes for virus production have been reported [35]. A detailed knowledge of the metabolism of the cell is necessary. Most publications providing a stoichiometric model are based on cell lines currently used in industry for recombinant protein and antibody production (CHO, BHK, etc.). Compared to these cell lines, comparatively little is known about the metabolism during the growth and virus production phase of *Vero* cells or MDCK cells [10], the typical virus production cell lines of choice. The generation of these important data and the identification of limiting and inhibiting compounds in virus production